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INFLUENCE OF SOCIAL STRESS ON THE REWARDING EFFECTS OF COCAINE

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TESIS DOCTORAL

Presentada per:

SANDRA MONTAGUD ROMERO

Dirigida per:

Dra. MARTA RODRÍGUEZ ARIAS

Dr. JOSÉ MIÑARRO LÓPEZ

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El Doctor José Miñarro López, Catedrático de Psicobiología y la Doctora Marta Rodríguez Arias, Profesora Titular de Psicobiología de la Unidad de Investigación Psicobiología de las Drogodependencias, del Departamento de Psicobiología, de la Facultad de Psicología de la Universitat de Valencia:

CERTIFICAN

Que la Tesis Doctoral presentada por Sandra Montagud Romero con el título "Influence of social stress on the rewarding effects of cocaine" ha sido realizada bajo su dirección. Tras haberla examinado hacen constar su autorización para que se realicen los trámites conducentes a su defensa.

Y para que conste a los efectos oportunos, firman el presente certificado en Valencia a 26 de Septiembre de 2016.

Fdo. José Miñarro López

Fdo. Marta Rodríguez Arias

Als meus pares, Federico i Esperanza.

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La realització de la present Tesis Doctoral ha sigut possible gràcies a les següents ajudes:

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RESUM

Les relacions socials són un procés crucial en el desenvolupament de l'ésser humà, i en altres espècies socials que viuen en jerarquies socials complexes. L'entorn i l'ambient en el qual es desenvolupa un individu és fonamental, ja que tindrà un gran impacte sobre el seu benestar, el manteniment de la salut i la seua supervivència (Kessler i cols., 2010). La resposta a l'estrés és un factor clau en el manteniment de l'homeòstasi i la salut de l'individu. Aquesta dependrà de la interacció amb l'entorn i l'activitat de certs sistemes regulatoris de l'organisme, com són el sistema nerviós simpàtic i l'eix hipotàlem-hipofisari-adrenal (HPA) (Stratakis i Chrousos, 1995). Diferents tipus d'estrés (psicològic o físic) poden provocar patrons de comportament i respostes fisiològiques qualitativament diferents. En els últims anys, s'han començat a realitzar estudis centrats en la comprensió del fenomen de l'estrés psicològic o social, així com de les seves conseqüències (per exemple, Miczek i cols., 2008; Rodríguez-Arias i cols., 2013). Avui dia, totes les persones experimentem estrés social diàriament en els diferents entorns per on ens movem, produint conseqüències psicològiques i comportamentals negatives, tant immediates com a llarg termini.

Podem destacar l'estrés entre els principals factors de risc implicats en el consum de substàncies. Aquest, no només té un paper fonamental en la recaiguda al consum de les drogues (Koob, 2010; Koob i Volkow, 2010), sinó que també, a l'inici, l'escalada i al manteniment del patró de consum (Sinha, 2008; Koob, 2010; Logrip i cols. , 2011; Sinha i cols., 2011; Logrip i cols., 2012; Rodríguez-Arias i cols., 2013). Donada l'estreta relació entre els sistemes cerebrals implicats en l'addicció i l'estrés, els estressors ambientals poden provocar canvis a llarg termini en la funcionalitat del sistema cerebral de recompensa, afavorint fenòmens com el de la recaiguda. L'activació del sistema d'estrés sembla un element crucial en l'estat emocional negatiu produït per la dependència i pot conduir a la cerca de la substància a través del reforç negatiu (Koob, 2010).

Destacar l'alta prevalença del consum de cocaïna arreu del món, sent la segona droga il·legal més consumida a Europa després del cànnabis, i el psicoestimulant més consumit, tant és així que suposa un problema de salut dins l'àmbit de les drogodependències amb greus conseqüències socials i econòmiques (EMCDDA., 2016). Es tracta d'un problema que no només afecta als drogodependents, sinó al seu entorn tant familiar com social. El coneixement de les bases neurobiològiques de l'addicció permet millorar les estratègies de prevenció i tractament. En canvi, raons ètiques impedeixen dur a terme molts d'aquests estudis en éssers humans. Per tant, és important utilitzar models animals, els quals ens permeten un major grau de control experimental.

Els estudis sobre estrés en models animals avaluen les conseqüències neurobiològiques i fisiològiques a curt o mitjà termini, i pocs d'ells es centren en les conseqüències a llarg termini, així com en la influència d'aquestes neuroadaptacions en el posterior consum de drogues (Burke i Miczek, 2014). Els estressors emocionals i socials són els principals activadors de la resposta d'estrés en els éssers humans, la qual cosa explica la importància translacional d'investigar aquests aspectes en animals. En els procediments amb animals, l'estrés per derrota social és un model naturalista que implica un encontre agonístic entre congèneres, el qual representa un factor d'estrés de validesa ecològica i etològica en ratolins (Tornatzky i Miczek, 1993).

En rosegadors, després d'haver sigut vençuts (amb aquest paradigma d'estrés per derrota social), s'han observat canvis profunds a nivell fisiològic i de comportament (de Groot i cols., 1999; Lumley i cols., 1999; Keeney i cols., 2001; Griebel i cols., 2002; García-Pardo i cols., 2015). A més a més, s'ha demostrat en repetides ocasions, que l'exposició a diferents procediments de derrota social augmenta els efectes reforçants de diferents tipus de drogues psicoestimulants, com la cocaïna i l'amfetamina, utilitzant principalment el paradigma de l'autoadministració intravenosa (AA) (Miczek i cols., 2008; Neisewander i cols., 2012). La majoria dels estudis es centren en l'edat adulta, mentre que l'efecte de la derrota amb adolescents no està tant estudiada (Burke i cols., 2011). L'adolescència és un període de vulnerabilitat

per al consum de drogues, com a conseqüència de la manca de maduració cerebral (Rodríguez-Arias i Aguilar, 2012). El consum de drogues i l'exposició a diferents condicions ambientals negatives són especialment nocives en aquesta etapa de la vida.

Com s'ha comentat anteriorment, la majoria dels estudis que avaluen els efectes de l'estrés social sobre els efectes reforçants de la cocaïna han emprat el procediment de l'AA. Cal recordar que les claus ambientals són un factor a destacar tant en l'ús com en la recaiguda del consum. El paradigma destacat, i no menys important, per a mesurar el poder de les claus ambientals associades al reforç de les drogues és el condicionament de preferència de lloc (CPL) (Aguilar i cols., 2013). Aquest model ha sigut poc utilitzat en els estudis d'estrés social, raó per la qual serà un dels paradigmes centrals d'aquest treball (McLaughlin i cols., 2006). La AA junt amb el CPL són els models animals que millor representen el procés addictiu en animals, ja que podem mesurar la motivació conjuntament amb la rellevància de les claus ambientals.

El principal objectiu d'aquesta tesi doctoral serà determinar la influència de l'estrés social (a curt i llarg termini) sobre els efectes gratificants de la cocaïna, i millorar el coneixement dels substrats neurobiològics d'aquests efectes. Per aquesta raó, el primer que es va estudiar fou l'efecte de l'estrés, utilitzant la derrota social aguda (DSA) sobre els efectes reforçants de la cocaïna utilitzant el procediment del CPL. També avaluarem si l'efecte de la DSA seria diferent si l'experiència de l'encontre agonístic es produïa durant l'adolescència o en l'edat adulta.

A continuació, es van estudiar els efectes a llarg termini de l'experiència de l'estrés per derrota social repetida (DSR) durant l'adolescència sobre els efectes reforçants de la cocaïna, utilitzant els procediments de l'AA i el CPP. A més a més, també vam avaluar els efectes a llarg termini de la DSR en períodes d'edat distints.

Un cop caracteritzats els efectes de la derrota social, DSA i DSR, sobre els efectes gratificants de la cocaïna, ens vam centrar en la detecció de factors

de vulnerabilitat que podrien estar influint en aquests efectes. En un estudi realitzat a la Universitat de Tufts (EE.UU), es va determinar la influència d'un tret de la personalitat, com és la impulsivitat. Aquest estudi es va realitzar al Departament de Psicologia durant el període de 6 mesos, en una estada d'investigació, en el prestigiós laboratori dirigit pel professor K. A. Miczek. A més, durant aquest període es va analitzar la influència del factor alliberador de corticotropina (CRF) en la resposta a l'estrés i la seva influència en la cerca de la cocaïna després d'un període d'abstinència forçada. Seguidament, en un altre estudi es van avaluar les diferències genètiques en la conducta agressiva, i com l'experiència podia modificar la resposta agonística, en funció de la soca dels ratolins.

En l'etapa final d'aquest treball, vam tractar de desvetllar alguns dels possibles mecanismes subjacents als efectes de la derrota social sobre la recompensa de la cocaïna. En primer lloc, es va determinar la rellevància de la neurotransmissió dopaminèrgica (DA) en els efectes a llarg termini de la DSR, sobre la inducció dels efectes reforçants de la cocaïna. Utilitzarem eines farmacològiques, així com l'anàlisi dels nivells dels receptors D1R i D2R, en l'escorça cerebral i l'hipocamp. Una vegada determinants els seus nivells, en un segon estudi, ens vam focalitzar en com la DSR modifica el control sobre la via DA. Per aquesta raó, es van avaluar factors dopaminèrgics de transcripció com el Nurr i el Pitx3, i la importància del factor neurotròfic derivat del cervell (BDNF), els quals poden modificar la funció de les neurones de DA.

Tot seguit, i com a segon mecanisme, vam tractar d'ampliar el coneixement sobre la implicació dels canvis epigenètics que la derrota social indueix, i com aquests es relacionen amb l'increment de la recompensa a la cocaïna. Després de mesurar els canvis que la RSD va induir en l'acetilació d'histones, es van analitzar els efectes dels inhibidors dels enzims histona a deacetilasa (HDAC) i histona acetil transferasa (HAT), els quals es van administrar abans de la RSD.

En els últims anys, molts estudis han associat el procés neuroinflamatori amb la base dels trastorns mentals, com són la depressió o

l'esquizofrènia. No obstant això, pocs coneixements es tenen sobre el procés neuroinflamatori en resposta a l'estrés. Com a estudi pioner en aquesta àrea d'investigació, es va avaluar com l'exposició durant l'adolescència a RSD altera la barrera hematoencefàlica (BBB), la qual es veu afectada per les respostes neuroinflamatòries.

En general, els nostres resultats augmenten els coneixements dels efectes de la derrota social sobre els efectes reforçants de la cocaïna. Els efectes de l'estrés sobre el CPL induït per cocaïna varien depenent del procediment utilitzat (DSA o DSR) i de l'edat dels animals quan l'experimenten. No obstant això, la DSR incrementa els efectes gratificants de la cocaïna, independentment de les edats. Aquests efectes poden ser modulats per la genètica, la qual pot influir en la resposta a la derrota, i també pels trets de personalitat, com és el cas de la impulsivitat.

Hem ampliat el coneixement sobre la funció de la neurotransmissió DA en els efectes reforçants de la cocaïna induïts per estrés. On els receptors D1R i D2R estan involucrats en aquests efectes, encara que el D1R té una implicació major. La derrota social també modifica els factors de transcripció que regulen l'expressió del gen de la DA, destacant l'adolescència com un període sensible. L'alteració de la neurotransmissió DA pot modular l'augment de l'expressió del BDNF via ERK/CREB, la qual al mateix temps pot produir canvis neoplàstics en àrees del cervell relacionades amb la recompensa.

Tanmateix, els nostres resultats mostren per primera vegada en aquesta àrea que ratolins exposats a DSR en l'adolescència sofreixen canvis significatius en l'estructura de la BHE, la qual cosa indica que la derrota social augmenta la permeabilitat de la BHE, probablement mitjançant d'alteració en les proteïnes estructurals. Tot seguit, utilitzant el procediment de la DSR, vam posar de manifest per primera vegada que els canvis epigenètics induïts per l'estrés social s'associen amb un increment dels efectes gratificants dels psicoestimulants, restaurant al mateix temps el paradigma del CPL amb una dosi llindar de la cocaïna, la qual podria ser bloquejada per la inhibició de l'enzim HAT.

Per concloure, els avanços en el coneixement dels substrats neurobiològics implicats en els resultats que l'estrés social indueix sobre els efectes gratificants de la cocaïna, poden contribuir al desenvolupament d'estratègies farmacològiques i conductuals per al tractament de l'addicció a les drogues.

ABSTRACT

Social relations are crucial not only for human development, but also for other species that live according to social hierarchies. The environment in which individuals develop is critical due to its impact on wellness, health maintenance and survival (Kessler et al., 2010). Another key factor in maintaining human homeostasis and health is the stress response. This response depends on the interaction between the environment and the activity of some regulatory body systems, such as the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis (HPA) (Stratakis and Chrousos, 1995). Different types of stress (physical or psychological) can induce qualitatively varied behavioral patterns and physiological responses. In recent years, several studies have focused on the phenomenon of social and psychological stress and its consequences (i.e. Miczek et al., 2008; Rodríguez-Arias et al., 2013). Nowadays, people experience stress daily due to the social environments in which they operate, which has negative behavioral and psychological consequences, both immediate and long-term.

Stress is one of the main risk factors involved in substance abuse and addiction. Addiction is a chronic multifactorial relapsing disorder resulting from an interaction of biological and environmental aspects (Ellenbroek et al., 2005; Enoch, 2006). Research has demonstrated that stress is a risk factor for the initiation, maintenance and escalation of drug consumption (Logrip et al., 2011; Sinha et al., 2011; Koob, 2012). There is a close association between brain systems involved in addiction and stress, as environmental stressors can cause long-term changes in the brain's rewarding system function, inducing phenomena such as relapse. Activation of the stress system seems to be crucial for the negative emotional state induced by dependence, which drives drug-seeking through negative reinforcement mechanisms (Koob, 2010).

It is important to emphasize the high prevalence of cocaine consumption worldwide, as it is the second most consumed illegal drug in

Europe, after cannabis. Cocaine use is a health problem with serious social and economic consequences (EMCDDA, 2015) affecting not only drug addicts, but also their families and social environment. Knowledge of the neurobiological basis of addiction allows prevention and treatment strategies to be improved. As ethical reasons rule out performing studies in human subjects, animal models are a vital tool for experimental research.

To date, studies performed in animal models have focused on evaluating the immediate or short-term neurobiological and physiological consequences of stress. However, only a few works have studied the long-term consequences or the influence of these neuroadaptations in subsequent drug intake (e.g. Burke and Miczek, 2014). Social and emotional stressors are the main triggers of the stress response in humans, which explains the translational importance of this research in animals. In animal procedures, social defeat stress is a naturalistic model of stress that involves an agonistic encounter between conspecifics and is thought to represent a stressor of ecological and ethological validity in mice (Tornatzky and Miczek, 1993). Rodents expose to social defeat stress show physiological and behavioral changes (de Groot et al., 1999; Lumley et al., 1999; Keeney et al., 2001; Griebel et al., 2002; García-Pardo et al., 2015). Moreover, it has been repeatedly demonstrated that exposure to different procedures of social defeat increases the reinforcing effects of different types of psychostimulant drugs, including cocaine and amphetamine. Most studies have used the intravenous self-administration (SA) paradigm (Miczek et al., 2008; Neisewander et al., 2012), and almost all have employed only adult rodents, with few reports having addressed the issue in adolescents (e.g. Burke et al., 2011). Adolescence is a period of enhanced vulnerability to drug abuse because the brain has not yet matured (Rodríguez-Arias and Aguilar, 2012). Consumption of drugs of abuse and exposure to different negative environmental conditions are especially harmful at this stage of life.

As commented on above, most of the studies that have evaluated the

effects of social stress on cocaine reward have employed the SA procedure. Conditioned place preference (CPP) is the most widely used paradigm to measure the association between environmental cues and drug reinforcement (Aguilar et al., 2013). The SA and CPP models provide a complete evaluation of the rewarding effects of drug of abuse, as they allow us to measure the role of both motivation and environmental cues. CPP, on the other hand, has rarely been used in studies of social stress (McLaughlin et al., 2006).

The principal aim of the experiments presented in this doctoral thesis was to characterize how social stress exposure modifies the rewarding effects of cocaine and to further our knowledge of the neurobiological substrates of these effects. To do this, we studied the effect of acute social defeat (ASD) stress on the rewarding effects of cocaine using the CPP procedure. We also evaluated if the effect of ASD differed when experienced during adolescence rather than in adulthood.

In a second phase, we used the CPP and SA procedures to assess the long-lasting effects of repeated social defeat (RSD) stress during adolescence on the rewarding effects of cocaine. Again, we explored if these long-lasting effects differed when social stress was experienced in adolescence versus adulthood.

Once we had characterized the effects of ASD and RSD on cocaine reward, we set out to detect the vulnerability factors affecting these effects. We determined the influence of a personality trait – impulsivity - in a study performed at the Department of Psychology of Tufts University (USA), during a 6-month stay in the prestigious laboratory directed by Professor K.A. Miczek. Furthermore, during my stay, we analyzed the role of corticotrophin releasing factor (CRF) in the response to stress and its influence on cocaine-seeking after a period of forced abstinence. In another study, we also evaluated genetic differences with respect to aggression, and how experience modifies the agonistic response depending on the strain of the mice used.

In a final phase of the study, we aimed to unveil some of the mechanisms underlying the effects of social defeat on cocaine reward. Firstly, we determined the role of dopamine (DA) neurotransmission in the long-lasting effects of RSD on the rewarding effects of cocaine. We used pharmacological tools as well as measured D1R and D2R levels in the cortex and hippocampus. Once this role had been determined, we performed a further study to explore how social defeat modifies control of the DA pathway. To do this, we assessed DA transcription factors, such as Nurr and Pitx3, and the role of (brain-derived neurotrophic factor) BDNF, which can in turn modify the function of DA neurons.

As a second putative mechanism, we explored the implication of the epigenetic changes induced by social defeat and how they are related to an increase in cocaine reward. After measuring alterations of histone acetylation provoked by RSD, we analyzed the effects of the histone acetyltransferase (HAT) and histone deacetylase (HDAC) inhibitors, administered before RSD, on cocaine-induced CPP.

In the last few years, many reports have highlighted neuroinflammatory processes as constituting the basis of mental disorders such as depression or schizophrenia. However, little is known regarding the neuroinflammatory process in response to stress. As a first and pioneering study in this field, we evaluated how exposure to RSD during adolescence alters the blood-brain barrier (BBB), which is highly affected by the neuroinflammation response.

Overall, our results expand our knowledge about the influence of social defeat on cocaine effects. The effects of social defeat stress on the CPP induced by cocaine vary depending on the social stress procedure used (ASD or RSD) and the age of the animals when it is experienced. However, RSD augments the rewarding effects of cocaine, independently of whether stress is suffered during adolescence or adulthood. These effects can be modulated by genetics, which modify the response to defeat and are influenced by

personality traits such as impulsivity.

We also throw new light on the role of DA neurotransmission in the effects of RSD on cocaine reward. Both D1R and D2R are involved in these effects, although D1R seems to be more implicated. Social defeat also modifies the transcription factors that regulate DA gene expression, thus highlighting adolescence as a more sensitive period. Alterations of DA neurotransmission could modulate the increased expression in BDNF via ERK/CREB or other pathways, which in turn would mediate neuroplastic changes in brain areas related to reward.

In addition, our results show for the first time that adolescent mice exposed to RSD undergo significant changes in BBB structure, indicating that social defeat increases BBB permeability, probably through alterations in structural proteins. Furthermore, using the RSD paradigm, we show, also for the first time, that the epigenetic changes induced by social stress are associated with an increase in the rewarding and reinstating effects of a threshold dose of cocaine in the CPP paradigm that can be blocked by the inhibition of HAT enzyme.

Advances in knowledge surrounding the neurobiological substrates implicated in the effects of social stress on the rewarding effects of cocaine are likely to contribute to the development of pharmacological and behavioral strategies for the treatment of drug addiction.

ÍNDEX

ABREVIATURES

3,4 metilendioximetamfetamina (MDMA)

Accidents cerebrovasculars (ACV)

Àcid gamma- aminobutíric (GABA)

Adenosín monofosfat cíclic (AMPc)

Amígdala basolateral (BLA)

AMPc que respon a l'element vinculant en proteïnes (CREB)

Àrea Tegmental Ventral (ATV)

Autoadministració (AA)

Barrera hematoencefàlica (BHE)

Catecol-O-metiltransferasa (COMT)

Condicionament de preferència de lloc (CPL)

Derrota Social (DS)

Derrota Social Aguda, paradigma de l'encontre agonístic en un entorn neutral (DSA)

Derrota Social Repetida, paradigma resident/intrús (DSR)

Dies postnatals (DPN)

Dopamina (DA)

Eix hipotàlem-hipofisari-adrenal (HPA)

Eix simpàtic-adreno-medul·lar (SAM)

Enquesta domiciliària sobre alcohol i drogues en Espanya (EDADES)

Enquesta Estatal sobre l'Ús de Drogues en Estudiants d'Ensenyança Secundària (ESTUDES)

Escorça del Nucli Accumbens (NAccSh)

Escorça Prefrontal (CPF)

Escorça Prefrontal Medial (CPFm)

Factor alliberador de corticotropina (CRF)

Factor neurotròfic derivat del cervell (BDNF)

Gir dentat (DG)

Histona acetil transferasa (HAT)

Histona deacetilasa (HDAC)

Hormona adrenocorticotropa (ACTH)

Institut Nacional de les Drogues d'Abús (NIDA)

Locus Coeruleus (LC)

Metal·loproteïnases (MMP)

Monoamino oxidasa (MAO-A)

Neurones dopaminèrgiques del mesencèfal (mdDA)

N-metil-D-aspartat (NMDA)

Noradrenalina (NA)

Nucli Accumbens (NAcc)

Nucli de l'estria terminal (BNST)

Nucli paraventricular de l'hipotàlem (NPV)

Potenciació a llarg termini (LTP)

Proopiomelanocortina (POMC)

Proteïna kinasa A (PKA)

Ràtio fixa (RF)

Ràtio progressiva (RP)

Senyal extracel·lular regulada per kinasa (ERK)

Síndrome General d'Adaptació (SGA)

Sistema Nerviós Central (SNC)

Tirosina hidroxilasa (TH)

Transportador de dopamina (DAT)

Transportador vesicular de monoamina 2 (TVMA2)

ABBREVIATIONS

Activated Leukocyte Cell Adhesion Molecule (ALCAM)

Acute social defeat (ASD)

Anterior ventral tegmental area (aVTA)

Blood–brain barrier (BBB)

Brain-derived neurotropic factor (BDNF)

Conditioned place preference (CPP)

Conditiones Stimulus (CS)

Corticotropin-releasing factor (CRF)

Dopamine (DA)

Extracellular Signal Regulated Kinase (ERK/MAPK)

Hipotalamic pituitary adrenal axis (HPA)

Histone acetyltransferase (HAT)

Histone deacetylase (HDAC)

Junctional Adhesion Molecules (JAM)

Mitogen-activated protein kinases (MAPKs)

Posterior ventral tegmental area (pVTA)

Repeated social defeat (RSD)

Self-administration (SA)

Social Defeat (SD)

Tropomyosin receptor kinase B (TrkB)

Unconditioned Stimulus (US)

Ventral Tegmental Area (VTA)

1. INTRODUCCIÓ

1. Introducció General

1. Introducció general

Les relacions socials són un procés crucial en el desenvolupament de l'ésser humà, i en altres espècies socials que viuen en jerarquies socials complexes. L'entorn i l'ambient en el qual es desenvolupa un individu és fonamental, ja que tindrà un gran impacte sobre el seu benestar, el manteniment de la salut i la seua supervivència (Kessler i cols., 2010). La resposta a l'estrés és un factor clau en el manteniment de l'homeòstasi i la salut de l'individu. Aquesta dependrà de la interacció amb l'entorn i l'activitat de certs sistemes regulatoris de l'organisme, com són el sistema nerviós simpàtic i l'eix hipotàlem-hipofisari-adrenal (HPA) (Stratakis i Chrousos, 1995). Diferents tipus d'estrés (psicològic o físic) poden provocar patrons de comportament i respostes fisiològiques qualitativament diferents. En els últims anys, s'han començat a realitzar estudis centrats en la comprensió del fenomen de l'estrés psicològic o social, així com de les seves conseqüències (per exemple, Miczek i cols., 2008; Rodríguez-Arias i cols., 2013). Avui dia, totes les persones experimentem estrés social diàriament en els diferents entorns per on ens movem, produint conseqüències psicològiques i comportamentals negatives, tant immediates com a llarg termini.

Podem destacar l'estrés entre els principals factors de risc implicats en el consum de substàncies. Aquest, no només té un paper fonamental en la recaiguda al consum de les drogues (Koob, 2010; Koob i Volkow, 2010), sinó que també, a l'inici, l'escalada i al manteniment del patró de consum (Sinha, 2008; Koob, 2010; Logrip i cols., 2011; Sinha i cols., 2011; Logrip i cols., 2012; Rodríguez-Arias i cols., 2013). Donada l'estreta relació entre els sistemes cerebrals implicats en l'addicció i l'estrés, els estressors ambientals poden provocar canvis a llarg termini en la funcionalitat del sistema cerebral de recompensa, afavorint fenòmens com el de la recaiguda. L'activació del sistema d'estrés sembla un element crucial en l'estat emocional negatiu produït per la dependència i pot conduir a la cerca de la substància a través del reforç negatiu (Koob, 2009).

El sistema neurobiològic involucrat en la resposta a l'estrés s'ha relacionat amb el sistema neurobiològic involucrat amb l'addicció, el nexe dels quals és l'anomenada amígdala estesa (Koob, 2009; Rodríguez-Arias i cols., 2013). Molts dels efectes motivacionals de les drogues podrien estar implicats en un circuit comú, formant una entitat separada dintre del prosencèfal basal (Alheid i Heimer, 1988). Tanmateix, s'ha demostrat que el circuit de l'amígdala extensa s'engloba des de l'escorça del nucli accumbens (NAcc), fins al nucli de l'estria terminal (BNST), i el nucli central de l'amígdala (Alheid i Heimer, 1988; de Olmos i Heimer, 1999; Koob, 2009), on neurotransmissors, com el factor alliberador de corticotropina (CRF), la noradrenalina (NA) i la dopamina (DA), interactuen.

Els estudis sobre estrés en models animals avaluen les conseqüències neurobiològiques i fisiològiques a curt o mitjà termini, i pocs d'ells es centren en les conseqüències a llarg termini, així com en la influència d'aquestes neuroadaptacions en el posterior consum de drogues (Burke i Miczek, 2014). Els estressors emocionals i socials són els principals activadors de la resposta d'estrés en éssers humans, la qual cosa explica la importància traslacional al seu estudi en animals. Dintre d'aquests models, existeixen diferents tipus d'estressors com per exemple, estressors farmacològics, físics, emocionals o socials. Pel que fa als models socials d'estrés es representen per la derrota social, separació maternal o aïllament social. Així mateix, aquells estressors produïts per derrota o subordinació es consideren estressors amb rellevància etològica que imiten les situacions de la vida real (Tornatzky i Miczek, 1993). En aquest treball utilitzarem l'exposició a diferents protocols de derrota social, ja que s'ha observat que poden produir canvis i augmentar la vulnerabilitat a desenvolupar psicopatologies (Björkqvist, 2001; Miczek i cols., 2008; García-Pardo i cols., 2014).

En rosegadors, després d'haver sigut vençuts (amb aquest paradigma d'estrés per derrota social), s'han observat canvis profunds a nivell fisiològic i de comportament (de Groot i cols., 1999; Lumley i cols., 1999;

Keeney i cols., 2001; Griebel i cols., 2002; García-Pardo i cols., 2015). A més a més, s'ha demostrat en repetides ocasions, que l'exposició a diferents procediments de derrota social augmenta els efectes reforçants de diferents tipus de drogues psicoestimulants, com la cocaïna i l'amfetamina, utilitzant principalment el paradigma de l'autoadministració intravenosa (AA) (Miczek i cols., 2008; Neisewander i cols., 2012). La majoria dels estudis es centren en l'edat adulta, mentre que l'efecte de la derrota amb adolescents no està tant estudiada (Burke i cols., 2011). Cal recordar que les claus ambientals són un factor a destacar tant en l'ús com en la recaiguda del consum. El paradigma destacat, i no menys important, per a mesurar el poder de les claus ambientals associades al reforç de les drogues és la preferència de lloc condicionat o condicionament de preferència de lloc (CPL) (Aguilar i cols., 2013). Aquest model ha sigut poc utilitzat en els estudis de l'estrés social, raó per la qual serà un dels paradigmes centrals d'aquest treball (McLaughlin i cols., 2006). La AA junt amb el CPL són els models animals que millor representen el procés addictiu, ja que podem mesurar la motivació conjuntament amb el paper de les claus ambientals.

El principal objectiu d'aquest treball serà determinar la influència de l'estrés social (a curt i llarg termini) sobre els efectes del consum de cocaïna, utilitzant principalment el paradigma del CPL, encara que altres paradigmes com la AA han sigut utilitzats. Al mateix temps, investigar els mecanismes implicats amb eixa resposta d'estrés, els quals a la vegada poder estar modulant eixos efectes reforçants als psicoestimulants. A més a més, hem de destacar la utilització d'animals adults i adolescents, ja que també hem estudiat com la variable edat pot modificar els efectes de la derrota social.

2. Derrota social (DS): paradigma d'estrés

2. Derrota Social (DS): paradigma d'estrés

L'estrés és una condició comú en la vida diària de les persones que pot alterar la biologia dels organismes i, per tant, influir en el manteniment d'un estat saludable i el desenvolupament de malalties (Kudielka i Kirschbaum, 2005). Aquest terme fou definit per primera vegada en 1975 per Hans Selye, com una resposta no específica de l'organisme davant qualsevol situació de demanda, caracteritzada per l'acció dels sistemes neurobiològics, com és el cas de la secreció dels glucocorticoides. No obstant, encara que la resposta d'estrés en un primer moment siga adaptativa i intente mantenir l'equilibri homeostàtic, por acabar danyant l'organisme si es manté a llarg termini. Aquest fenomen fou demostrat pel mateix Selye (1975), el qual va postular el Síndrome General d'Adaptació (SAG). Aquest síndrome consta de tres fases: reacció d'alarma, resistència i fase d'esgotament. La reacció d'alarma és l'activació de diferents sistemes de regulació que impliquen canvis en l'organisme i busquen la capacitat del subjecte per adaptar-se o fer front als reptes del medi. Quant a la fase de resistència la situació estressant no s'atura, per tant, la lluita i la inversió de recursos orgànics continua. Per últim, a la fase d'esgotament es poden donar alteracions com a conseqüència de la debilitació dels recursos biològics de l'individu i fins i tot es pot donar la mort (Selye, 1975) (Figura 1).

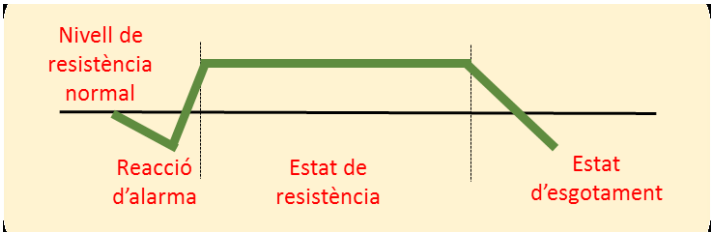

Figura 1. Síndrome General d'Adaptació, Seyle 1975.

Actualment no existeix un concepte establert d'aquest terme, ja que cada investigador ha posat èmfasi en un aspecte diferent, ja siga la cognició, la motivació, l'emoció, el comportament o els aspectes biològics. No obstant això, les diferents definicions coincideixen amb que la resposta a

l'estrés, activa diferents sistemes de regulació del cos per adaptar l'organisme als reptes interns o externs. Altrament, es posa en marxa el sistema HPA, implicat en la resposta a l'estrés, amb l'alliberació d'hormones tals com el CRF, l'hormona adrenocorticotropa (ACTH) i els glucocorticoides; o el sistema simpàtic-adrenal, amb l'alliberació de catecolamines (adrenalina i NA) (Kupfermann, 1991). Per últim, també està involucrat en aquest tipus de resposta el sistema immunològic (Costa-Pinto i Palermo-Neto, 2010; Capuron i Miller, 2011).

L'estrés és una construcció complexa (Cannon, 1935; Selye, 1956) on s'exposa de manera forçada a esdeveniments o condicions que normalment s'eviten per un animal (Piazza i LeMoal, 1998). Els diferents tipus de factors estressants utilitzats com a metodologia en models animals (Lu i cols., 2003; Aguilar i cols., 2009) els podem classificar en quatre categories: farmacològics, físics, emocionals i socials. Donat que el nostre treball es centra en l'estrés social, a continuació detallarem cadascun dels diversos procediments que es poden utilitzar per a generar en models animals aquest tipus d'estrés.

2.1. Tipus d'estrés social

Focalitzant-se en les experiències o condicions socials que indueixen estrés en els animals trobem: separació maternal, derrota social, amuntegament (*crowding*) i aïllament social (Lu i cols., 2003; Shaham i cols., 2003; Miczek i cols., 2008; Ribeiro Do Couto i cols., 2009).

- Separació maternal: implica la separació de les cries respecte les seues mares durant diversos minuts o hores, en un o diferents dies (Martini i Valverde, 2012). Restaurar la cerca de la substància (psicoestimulants) pot ser modulada per aquest tipus d'estressor (Lynch i cols .,2005).

- Aïllament social: es privarà a l'animal dels beneficis que comporta la interacció social. Aquest es troba modulat per l'edat i duració de l'exposició, però en general els rosegadors exposats a l'aïllament són

més sensibles als efectes de les substàncies d'abús (Kosten i cols., 2000; Ding i cols., 2005; Lynch i cols., 2005; Zhang i cols., 2005; Ribeiro Do Couto i cols., 2009).

- Amuntegament: és duplicarà el nombre de rosegadors per caixa (normalment habiten quatre junts, aleshores habitaran huit). Aquest tipus d'estressor es relaciona amb un augment dels efectes de la cocaïna al paradigma de reinstauració de CPL en animals adults (Ribeiro Do Couto i cols., 2009).

- Derrota social: induirem estrés mitjançant l'exposició a un encontre agonístic amb altre animal. Existeixen dues variants d'aquest model: paradigma resident/intrús i encontre agonístic en àrea neutral.

    - Resident/intrús o derrota social repetida (DSR): en aquest paradigma s'utilitza la diada per la qual s'estableix un subjecte dominant i un subjecte subordinat. L'animal experimental s'exposa a un oponent agressiu en la pròpia gàbia d'aquest últim, on l'animal experimental és considerat intrús (Miczek i cols., 2008). Consta de tres fases: la primera on s'introdueix l'animal experimental a la caixa on habita l'oponent agressiu o resident, el qual està protegit dels atacs per una reixa durant 10 minuts; en la segona fase es treu la reixa durant 5 minuts, donant pas al contacte físic i a la pròpia confrontació; i finalment en la tercera fase, es col·loca la reixa de nou durant 10 min. Aquest procediment es va realitzar un total de quatre vegades, a intervals de 72 hores. El resident presenta conductes tant d'amenaça com d'atac, mentre que l'intrús davant l'experiència repetida d'aquestes trobades mostra conductes de fugida, defensa o submissió. Com a conclusió, els animals socials desenvolupen jerarquies basades en la dominància a través d'aquest tipus d'interaccions agonístiques (Huntingford i Turner, 1987). Aquest estrés es realitza de forma repetida i intermitent

i s'ha descrit que incrementa els efectes reforçants de la cocaïna principalment en la AA (Covington i Miczek, 2005). En el nostre treball, la DSR es realitzarà en els dies postnatals (DPN) següents:

	DPN	1DSR	2DSR	3DSR	4DSR
Adolescents	21	27	30	33	36
Adults	42	47	50	53	56

- Encontre agonístic en un entorn neutral o derrota social aguda (DSA): l'animal s'enfronta a un oponent agressiu equiparat amb edat i pes (Ribeiro do Couto i cols., 2006) en una zona neutra per a ambdós durant un període de 10 minuts, amb prèvia habituació a l'espai d'un minut. Considerarem que un animal ha sigut derrotat o vençut quan adopta la postura de submissió vertical (Rodríguez-Arias i cols., 1998). Aquest tipus d'encontre també augmenta els efectes reforçants de la cocaïna, així com la recaiguda a dosis priming (Ribeiro do Couto i cols., 2006). En el nostre treball, la DSA es realitzarà en els DPN següents:

	DPN	DSA/CPL (fase condicionament)
Adolescents	21	29-30-31-32
Adults	42	50-51-52-53

2.2. Particularitats de la DS durant l'adolescència.

L'adolescència és un període de transició entre la infantesa i l'edat adulta. En aquesta etapa es produeixen canvis fisiològics i neuroendocrins associats a la maduresa sexual (pubertat), així com canvis psicològics i socials que condueixen a la independència parental (Spear, 2000; Sisk i Foster, 2004). En humans s'associa aquest període a les edats compreses entre 9-18 anys, encara que alguns autors la consideren fins als 25 anys, emmarcant aquests últims com una adolescència tardana (Baumrind, 1987).

Aquesta transició a una etapa més madura, amb els respectius

aspectes psicobiològics canviants, es pot identificar en moltes espècies de mamífers, de manera que es possible estudiar alguns dels seus canvis en models animals, com els canvis hormonals, l'emergència de patrons conductuals orientats sexualment, la preferència i cerca de la novetat, l'assumpció de riscos o els elevats nivells de conducta afiliativa i de joc (Spear, 2000; Laviola i cols., 2003). En els rosegadors existeixen diferents criteris a l'hora de definir l'etapa que comprén l'adolescència, encara que aquesta es sol considerar el període dels 21 als 60 dies d'edat, des del deslletament fins al començament del període adult, dividint-se aquest període en adolescència primerenca o juvenil (21-34 DPN), adolescència intermèdia (34-46 DPN) i adolescència tardana (46-59 DPN) (Laviola i cols., 2003).

La transició de la infantesa a l'edat adulta inclou el desenvolupament i reorganització de l'encèfal (McCormick, 2010). Durant la pubertat, la maduració cerebral (la qual comença en edats prenatals) es completa, potenciant i expressant el comportament adult (Schneider, 2008). En relació amb els canvis cerebrals, existeixen nombroses alteracions que es donaran en aquest període com la maduració de processos en l'escorça prefrontal medial (CPFm) i en regions límbiques, les quals es caracteritzen per canvis progressius com puguen ser la mielinització o l'eliminació sinàptica competitiva (Spear, 2000; Powell, 2006). Aquests canvis maduratius afecten també a regions com l'hipocamp (Wolfer i Lipp, 1995) i l'estriat, on s'observa una disminució de la substància gris i un augment de la substància blanca (Sowell i cols., 2001; Rodríguez-Arias i Aguilar, 2012). La falta de maduresa dels circuits de l'escorça frontal observats en els estudis de neuroimatge suggereix que els adolescents són més sensibles a la recompensa i a avaluar les bones experiències més positivament en comparació a l'atribució negativa (Crews i Boettiger, 2009; Geier i Luna, 2009). El cervell adolescent opera en un estat promotivational com a conseqüència d'una limitada capacitat inhibitòria, mal control regulador, una hiperactivitat dopaminèrgica en el nucli accumbens (NAcc) quan es processen estímuls apetitius, i una hiperactivitat de l'amígdala (Rodríguez-Arias i Aguilar, 2012) (Figura 2).

Addicionalment, en aquesta etapa maduren els sistemes de

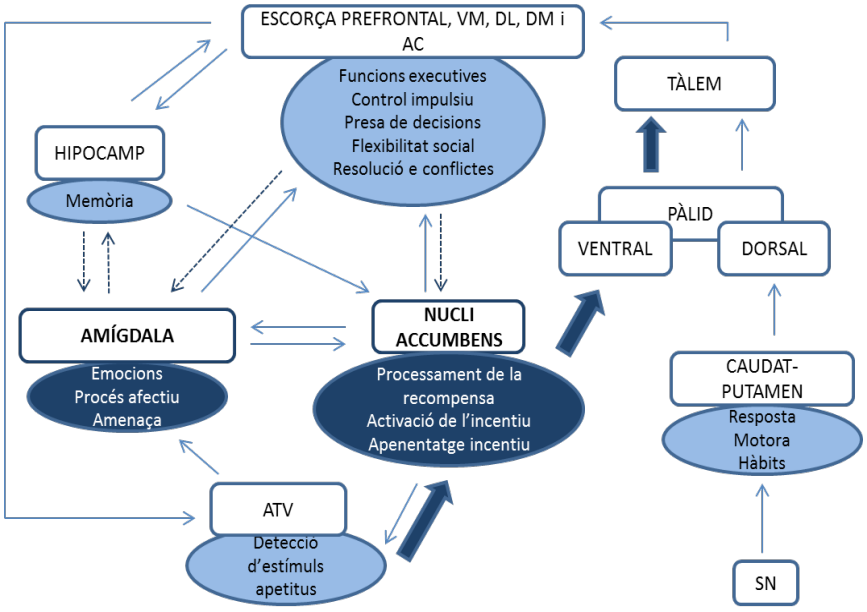

Figura 2. Circuit neural involucrat en el comportament motivacional. Les línies gruixudes i el color blau oscur representen àrees i connexions hiperactives en l'encèfal; tant les linies fines com les intermitents (blau clar) representen àrees cerebrals hipoactives en els adolescents, en comparació amb els adults. Imatge modificada de Rodríguez-Arias i Aguilar, 2013.

neurotransmissió com és el cas de sistema glutamatèrgic, dopaminèrgic i el sistema cannabinoide endogen (Rodríguez de Fonseca i cols., 1993; Spear, 2000; Crews i cols., 2007). Els nivells de DA basals són més baixos, encara que els adolescents mostren un augment en l'alliberament de DA induït per substàncies d'abús (Laviola i cols., 2001; Badanich i cols., 2006). D'acord amb Bjork i cols (2010), els nuclis relacionats amb les emocions i la recompensa es troben molt actius. Per exemple, l'amígdala i el NAcc en els adolescents mostren major activitat que en els adults (Ernst i cols., 2011). Per tant, nombrosos comportaments descrits com típics en l'adolescència (impulsivitat, consum de drogues, desinhibició, control cognitiu immadur...) tenen una base biològica, la qual s'explica per la immaduresa del processament neural en l'escorça prefrontal (CPF) i altres regions corticals i subcorticals

involucrades en la presa de decisions. Els adolescents, doncs, expressaran un comportament esbiaixat i dirigit cap a l'assumpció de riscos i a la reactivitat emocional durant aquest període (Yurgelun-Todd, 2007; Casey i cols., 2011; Sturman i Moghaddad, 2011; Rodríguez-Arias i Aguilar, 2012).

Els comportaments típics de l'adolescència en humans, com l'assumpció de riscos, autonomia, major responsabilitat, influència entre iguals, impulsivitat, egocentrisme, curts períodes de son, major interés i un major conflicte amb els pares, estarien relacionats amb el consum de drogues (Spear, 2011; Sturman i Moghaddad, 2011). Tots aquests comportaments poden desenvolupar conductes disruptives a l'escola, conduir begut, tindre conductes sexuals insegures, comportaments antisocials, i per descomptat, consum de drogues legals o il·legals (Doremus-Fitzwater i cols., 2010; Spear, 2011; Eaton i cols., 2012). Per tant, l'exposició a substàncies tòxiques en l'adolescència, pot alterar aquests processos de maduració afectant a una nova organització sinàptica, obstaculitzant així un desenvolupament psicològic adequat (Andersen, 2003; Crew i cols., 2007; Schramm-Sapyta i cols., 2009) i incrementant el risc a desenvolupar dependència en edats futures (Vega i cols., 2002; Dawson i cols., 2012; Conway i cols., 2016; Meier i Hatsukami, 2016; Strong i cols., 2016).

El sistema nerviós adolescent està en contacte continu amb factors ambientals que desencadenen estrés (McCormick, 2010). La literatura clínica mostra que, de la mateixa manera que ocorre amb els adults, l'estrés en l'adolescència incrementa el risc per al consum de substàncies (Hoffman i cols., 2000; King i Chassin, 2008). L'estrés produeix canvis en l'estructura i funcionament cerebral on involucrarà l'eix HPA, el qual s'activarà i augmentarà les concentracions de glucocorticoides i cortisol així com potenciarà la funcionalitat dels receptors corresponents distribuïts en regions límbiques i frontals (McCormick, 2010).

Entre els estressors socials que es sofreixen durant l'adolescència trobem el *bullying* escolar. L'assetjament escolar (o *bullying*) és un problema crucial a dia d'avui tant al nostre país com a la resta de països europeus, on

el nombre de casos s'incrementa cada dia (Brunstein Klomek i cols., 2016). El *bullying* és una forma d'assetjament i violència entre iguals que es dóna amb freqüència a l'àmbit escolar, sent una o més persones aquelles que exerceixen una conducta nociva, de manera intencionada i recurrent contra un o diversos individus. Es caracteritza principalment per l'abús sistemàtic del poder i per la presència d'atacs de tipus tant físic com psicològic, verbal i social (Valdebenito i cols., 2015). Aquells subjectes afectats presenten una baixa autoestima, sentiments de solitud, i pot incrementar el risc de desenvolupar trastorns psicològics com la depressió o l'ansietat en l'edat adulta, i augmentar la vulnerabilitat pel consum de drogues (Ttofi i cols., 2016). Tant l'abús de drogues com el *bullying* són problemes freqüents durant els anys escolars ocasionant conseqüències a llarg termini (Luk i cols., 2012).

Tot i que l'adolescència és una etapa on s'és més vulnerable a la victimització (Frisén i cols., 2007), actualment hi ha escassos estudis amb rosegadors adolescents que hagen avaluat els efectes conductuals de l'estrés social (Watt i cols., 2009; Burke i cols., 2011; Kovalenko i cols., 2014). Alguns estudis apunten que els adolescents responen conductualment de forma diferent als adults, per la qual cosa investigar les seves conseqüències resulta de gran importància (Bingham i cols., 2011; Buwalda i cols., 2013).

En resposta a l'estrés social agut, els rosegadors adolescents mostren una menor resposta fisiològica, amb uns nivells de corticosterona que només s'eleven de forma significativa després de derrotes repetides, a diferència del que s'observa en animals adults (García-Pardo i cols., 2014). En rosegadors adolescents, el joc agressiu es confon amb les derrotes, no experimentant aquestes com el que realment són. En aquesta etapa l'organisme i les hormones encara estan en ple desenvolupament, i l'agressió entre mascles no s'experimenta de la mateixa manera que durant la maduresa, la qual cosa els porta a una menor competitivitat per la territorialitat (García-Pardo i cols., 2014).

Un estudi recent ha demostrat mitjançant el paradigma de la AA, que aquells adolescents derrotats socialment de manera intermitent,

consumeixen més cocaïna durant períodes il·limitats de 24h en l'adultesa (Burke i Miczek, 2015; Burke i cols., 2016), independentment del tipus d'hàbitat, és a dir, si el rosegador habita de manera aïllada o bé conjuntament amb un altre mascle (Burke i Miczek, 2015). A més a més, la latència davant la postura de defensa després de repetits episodis de derrota social és altament predictiva del consum de cocaïna quan aquests animals són adults, tant en el programa fixe com en el progressiu (Burke i cols., 2016). El que indica, que el comportament submís durant l'adolescència està d'alguna manera relacionat amb el consum de drogues en l'edat adulta.

En canvi, respecte al CPL, un estudi realitzat per Burke i cols. (2011), va demostrar que aquells rosegadors que havien sigut derrotats en l'adolescència augmentaven la preferència per l'amfetamina a l'edat adulta, el que suggereix que l'estrés social té un gran impacte en el consum de drogues en la seva vida posterior. Aquest efecte també l'observem amb altres drogues com la 3,4 metilendioximetamfetamina (MDMA), on la DSR indueix un augment a llarg termini dels efectes de la substància en el CPL (García-Pardo i cols., 2015).

2.3. Modulació genètica i ambiental de la derrota social

L'agressió és un comportament social complex present en la majoria de les espècies animals, com insectes, peixos i gairebé tots els mamífers, incloent els éssers humans. Aquesta, s'ha definit com ''el comportament que inflingeix dany i perjudici o amenaça al fer-lo'' (Berkowitz, 1993) o com ''qualsevol forma de comportament dirigit cap a l'objectiu de danyar o ferir a un altre ésser viu que està motivat per evitar aquest tipus de tracte'' (Baron i Richardson, 1994). Exhibeix diferents dimensions en termes d'orígens, motivacions, expressions i funcionalitat, així com inclou diferents patrons de comportament (Miczek i cols., 2007). Aquest tipus de comportament és beneficiós, ja que es sol utilitzar per obtenir aliments, aigua i altres recursos, per mantenir la parella amb presència d'altres mascles, per defensar el seu territori, els descendents o el rang social. No obstant això, els patrons de conducta agressiva comporten una sèrie de riscos associats a lesions o

fins i tot a la mort, així com una elevada despesa d'energia (Smith i Price, 1973; Haller, 1995). Mentre que l'agressió és un tipus de comportament que presenten la majoria de les espècies, la violència únicament la porta a terme l'ésser humà, on es defineix com "una acció exercida per una o diverses persones on es sotmet de manera intencionada al maltractament, pressió o sofriment, manipulació o una altra acció que atempte contra la integritat tant física com psicològica i moral de qualsevol persona o grup de persones" (Platt, 1992).

La investigació sobre l'agressió utilitzant models animals ha estudiat la implicació etològica del comportament, com ara la seva funcionalitat en la supervivència i la reproducció dels animals, així com el seu desenvolupament filogenètic i ontogenètic (Miczek i Meyer-Lindenberg, 2014). La conducta agressiva ofensiva en rosegadors (entre congèneres) s'ha organitzat de forma ritual, on existeixen comportaments de persecució, moviments característics de la cua (especialment en ratolins), amenaces, postures defensives en

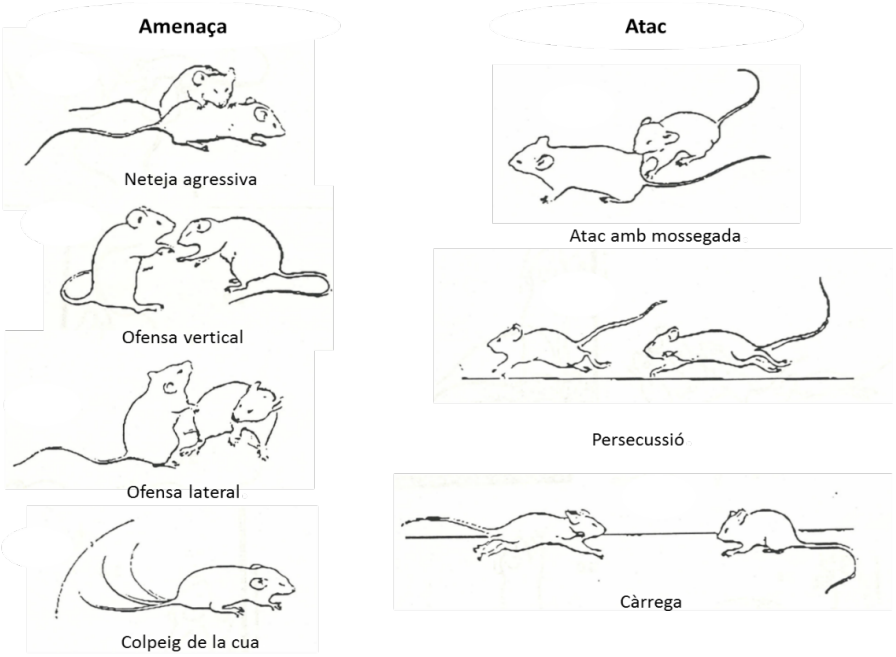

Figura 3. Conductes agonístiques (Martínez i cols., 1991).

posició vertical, i les mossegades d'atac (Miczek i O'Donnell, 1978)(Figura 3). Àrees del cos com l'esquena i els flancs de l'adversari són aquelles on l'atac es dirigeix (Blanchard i Blanchard, 1977; Blanchard i cols., 1979, 2001).

D'altra banda, en els rosegadors mascles, comportaments defensius

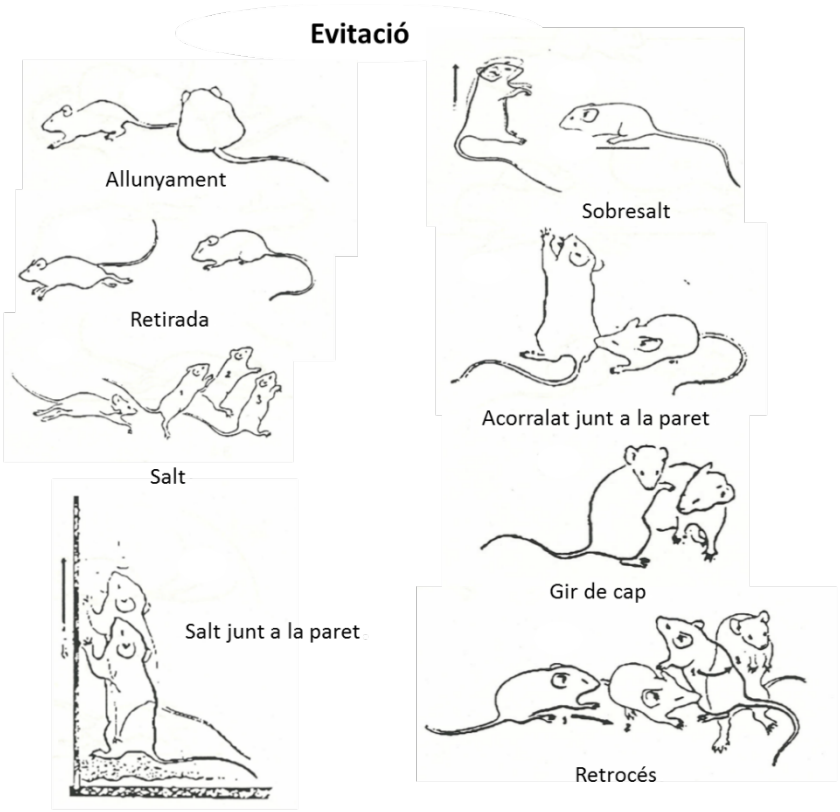

Figura 4. Conductes d'evitació (Martínez i cols., 1991).

específics inclouen evitació/fugida, congelació, postures defensives, i les amenaces o els atacs defensius dirigits als musells dels depredadors o dels seus congèneres (Blanchard i Blanchard, 2003) (Figura 4 i 5). En particular, l'estrés per subordinació social combina diversos nivells d'estrés físic i psicològic, que poden resultar en anhedonisme i evitació social, així com alteracions metabòliques en el cas dels animals derrotats (Kudryavtseva i col., 1991; Berton i cols., 2006; Krishnan i cols., 2007; Bondar i cols., 2009).

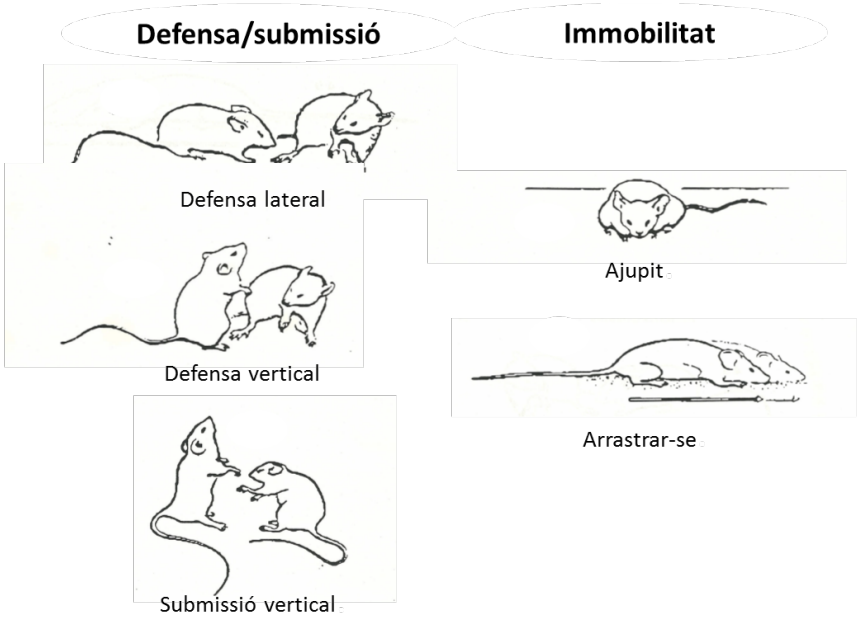

Figura 5. Conductes de defensa/submissió i immobilitat (Martínez i cols., 1991).

L'evitació social és un comportament natural, adaptatiu i complex que permet a l'animal retirar-se deliberadament de situacions desagradables (Blanchard i cols., 2005). Quan aquesta és exagerada i sostinguda en el temps es considera un símptoma patològic (Charney i Manji, 2004; Southwick i cols., 2005), ja que reflecteix tant un "estat depressiu" com un "estat d'ansietat" associat amb la por (Steimer, 2011; Toth i cols., 2012; Toth i Neumann, 2013).

En els últims anys, l'objectiu d'analitzar els organismes genètics s'ha centrat en entendre les identitats, els rols i les relacions de diferents gens els quals podrien determinar el comportament dels individus (Voikar i cols., 2001). No sols la genètica, sinó que la investigació sobre els efectes de la regulació socioambiental en ratolins ha demostrat tenir efectes sobre el comportament, els quals podrien influir també en els patrons de comportament agressiu.

Respecte als factors ambientals, s'ha observat que l'habitatge en un ambient enriquit té efectes beneficiosos sobre el benestar i el funcionament cognitiu dels animals; en canvi, en algunes soques de ratolins (com DBA/2J

o CFLP) s'ha vist l'efecte contrari on aquest tipus d'ambient augmentaria l'agressió i promouria en l'organisme la resposta d'estrés (McGregor i Ayling, 1990; Marashi i cols., 2003; Abou-Ismail i cols., 2011; McQuaid i cols., 2012). D'altra banda, en aquells rosegadors que són exposats a temperatures elevades (Moshkin i cols., 1993) o es troben allotjats de manera individual, és a dir, habiten aïlladament, s'incrementa el patró d'agressivitat (Rodríguez-Arias i cols., 1998; García-Pardo i cols., 2015), modulant també el comportament agonístic al cohabitar amb la femella (Han i cols., 2015; Holly i cols., 2015). La restricció d'aliments també s'ha utilitzat com a model d'estrés etològic, ja que existeix una relació estreta entre la privació d'aliments i els increments d'agressivitat (Nakamura i cols., 2008). Conjuntament amb tots aquests factors, l'efecte de l'experiència a ser exposats a derrotes socials positives, també augmenta eixes conductes agonístiques en ratolins (Kudryavtseva i cols., 2014); mentre que si s'exposa a rates a aquest estressor en l'adolescència, no s'observen canvis en l'edat adulta en els comportaments agressius (Coppens i cols., 2014).

Actualment, existeix una gran evidència de les diferències comportamentals entre soques. Fenotips conductuals per a les soques C57BL/6 i 129S6/SvEv han mostrat diferències en l'ansietat, l'activitat locomotora i la interacció social independentment de les condicions d'habitatge pre-experimentals (Abramov i cols., 2008); així com també s'han observat diferències indivisuals en les soques CBA/Lac i C57BL/6J (Avgustinovich i cols., 2007). Concretament els ratolins C57BL/6 són més reactius i més exploradors quan es comparen amb la soca 129S6/SvEv, els quals es mostren inactius i més ansiosos (Crabbe i cols., 1999; Tarantino i cols., 2000; Contet i cols., 2001). Les proves que estimen el comportament de tipus depressiu mostren als 129S6/SvEv més vulnerables cap a aquest tret (Liu i Gershenfeld, 2001; 2003). D'altra banda, la soca C57BL/6J mostra majors nivells d'activitat exploratòria i motora quan es compara amb la soca CBA/Lac, activitat que es manté encara que els animals s'hagen exposat a un estressor físic agut (Avgustinovich i cols., 2007).

Tanmateix, s'han trobat diferències significatives en els nivells de

dominància entre soques utilitzant el model etològic de jerarquia social (Osadchuk i cols., 2009). La soca CD-1 és coneguda per tenir una de les taxes més altes d'atac entre mascles (Kudryavtseva i cols., 2002), però els ratolins mascle BKW (Barnard i Luo, 2002) en comparació amb els CD-1 realitzen major nombre de conductes agressives (Fitchett i cols., 2005). Així mateix, quan interactuen diferents soques en un mateix ambient per tal d'estudiar les respostes conductuals, observem que la soca no consanguínia ICR(CD-1) i la consanguínia BALB/c són més agressives que les soques C57BL/6, CBA/Ca i DBA/2. Aquestes exhibeixen nivells d'agressió més baixos, com sol ocórrer amb la majoria de soques consanguínies (Nevison i cols., 1999; Kudryavtseva i cols., 2006), essent la soca DBA/2 aquella que mostra menors nivells d'ansietat (Kudryavtseva i cols., 2002; Kudryavtseva, 2006; Vishnivetskaia i cols., 2013).

L'exposició a derrota social crònica durant 21 dies en rosegadors de les soques C57BL/6J i CBA/Lac incrementa l'ansietat (Kudryavtseva i Avgustinovich, 1998; Kudryavtseva i cols., 2006; Kovalenko i Kudryavtseva, 2015), mentre que la resposta a comportaments depressius no es mostra tant clara (Kudryavtseva i Avgustinovich, 1998; Berton i cols., 2006; Krishnan i cols., 2007; Golden i cols., 2011). En canvi, davant 10 dies de derrota social crònica, la soca C57BL/6J disminueix la conducta d'interacció social (Berton i cols., 2006 i Tsankova i cols., 2006) sense mostrar evidència de dominància social (Lockwood i Turney, 1981). Els mascles de la soca 129SvEv són altament vulnerables a l'estrés crònic social, mostrant alteracions fisiològiques i de comportament, com ara augment de pes, augment de corticosterona plasmàtica, trastorns psicomotors, augment de la por, ansietat i evitació social (Dadomo i cols., 2011).

3. Cocaïna i reforç: efectes de la derrota social

3. Cocaïna i reforç: efectes de la derrota social

3.1. Introducció i consum

La cocaïna és una droga estimulant del sistema nerviós central (SNC), la qual actua específicament sobre el sistema dopaminèrgic (Pascual Pastor, 2001). Un alcaloide natural que s'extreu de la fulla de coca, planta originària de Sud-Amèrica, la qual és usada pels indígenes dels Andes per inhibir la fam, la set i el cansament (Balcells Oliveró, 2001). Segons l'Institut Nacional de les Drogues d'Abús (NIDA), la cocaïna es considera un potent estimulant addictiu que afecta directament el cervell. Encara que fou denominada la droga dels 80 i els 90 per la seva gran popularitat, és una de les substàncies psicoactives més antigues conegudes fins al moment. Les fulles de la coca, origen de la cocaïna, es masteguen i ingereixen des de fa milers d'anys, i la substància química purificada, el hidroclorur de cocaïna, ha estat una substància d'abús durant més de 100 anys. Per exemple, al principi del segle XX la cocaïna purificada va ser el principal ingredient actiu en la majoria de tònics i elixirs desenvolupats per tractar una gran varietat de malalties.

Avui dia, la cocaïna es considera una droga psicoanalèptica, la qual produeix una activació general del SNC i queda registrada en la llista II de la legislació dels Estats Units, que comprén totes les substàncies a les quals únicament s'accedeix sota prescripció mèdica. Els efectes del consum de la cocaïna es perceben als 2-3 minuts (en el cas d'administrar-se per via nasal), als 8-10 segons (via fumada) i als 30-45 segons (via intravenosa), arribant al pic màxim als 15-30 minuts, 5-10 minuts i 5-20 minuts respectivament, desapareixent els efectes al poc de temps (Rodríguez i Franco, 2000; Gold i Jacobs, 2005). La rapidesa en l'inici i finalització dels seus efectes li concedeix una gran capacitat reforçant que pot conduir a un consum compulsiu, pel que és considerada una droga altament addictiva (Cadet i cols., 2014).

Destacar l'alta prevalença del consum de cocaïna arreu del món, sent la segona droga il·legal més consumida a Europa després del cànnabis, i el psicoestimulant més consumit, tant és així que suposa un problema de salut dins l'àmbit de les drogodependències amb greus conseqüències socials i

econòmiques (EMCDDA, 2016). Es tracta d'un problema que no només afecta els drogodependents, sinó a tot el seu entorn tant familiar com social. La majoria dels consumidors, el 90%, es concentren en un nombre restringit de països com són Espanya, Regne Unit, França i Països Baixos, entre altres (Figura 6).

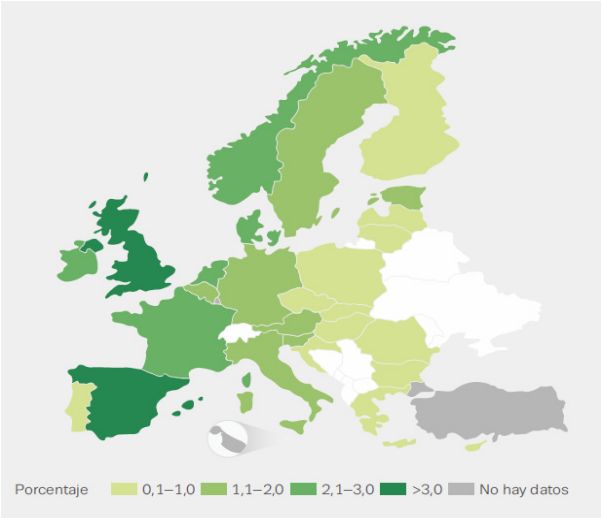

Figura 6. Prevalença del consum de cocaïna durant l'últim any en joves entre 15-34 anys. Informe Europeu sobre Drogues, 2016 (EMCDDA, 2016).

S'estima que uns 2,4 milions d'adults joves europeus, de 15 a 34 anys (el 1,9% d'aquest grup d'edat) van consumir cocaïna en l'últim any. Molts consumidors de cocaïna prenen aquesta droga en contextos recreatius, augmentant el consum durant els caps de setmana i les vacances (EMCDDA, 2016).

A nivell Nacional, les dades de l'Enquesta Domiciliària sobre Alcohol i Drogues en Espanya, 1995-2013 (EDADES, 2015), mostren que el 2,2% de la població entre 18 i 64 anys ha consumit cocaïna en els últims 12 mesos, mantenint-se el consum d'aquesta substància en nivells baixos i estables. L'edat mitjana del primer consum (21,3 cocaïna en pols; 23,2 cocaïna base) és superior a l'edat d'inici d'altres substàncies com el tabac, l'alcohol o el cànnabis. En relació a les prevalences per sexe, en totes les enquestes

realitzades es troben indicadors de consum més elevats en el cas dels homes (3,5 vegades major que en les dones). Per segments d'edat, s'observen indicadors majors per al grup de entre 15-34 anys comparat amb el grup de 35-64 anys.

Segons l'Enquesta Estatal sobre l'ús de Drogues en Estudiants d'Ensenyança Secundària, 1994-2014 (ESTUDES, 2016), un 3,6% dels estudiants espanyols entre 14-18 anys, han consumit cocaïna (pols/base) alguna vegada en la seua vida, 2,5% en l'últim any, i un 1,5% en l'últim mes, essent la proporció de consumidors masculins molt superior a la de les consumidores. Així el 4,8% dels xics van manifestar haver realitzat consum ocasional o experimental (alguna vegada en la vida), el que suposa una proporció quasi del doble de la registrada entre les xiques. L'edat d'inici en Espanya es troba en 15,5 anys i no ha variat de manera significativa en els últims anys. La forma de consum més prevalent continua sent la cocaïna en pols, les proporcions de consum se situen en 2,9%, 2% i 1,1% (respectivament per als consums d'alguna vegada a la vida, últim any i últim mes).

Des de 2004 s'ha anat observant un descens en el consum de cocaïna entre els estudiants d'aquest grup d'edat (14 a 18 anys) i es pot afirmar que, al 2012, la tendència mostra una clara estabilització, sobretot per als consums no purament experimentals, és a dir, els corresponents a l'últim any i últim mes, què són molt similars als registrats en l'any 1996, abans d'iniciar-se l'ascens de les prevalences de cocaïna. El consum de tipus experimental (alguna vegada a la vida) continua baixant encara més discretament i, principalment, com ja s'ha assenyalat, com a conseqüència d'un descens en la proporció de dones que proven aquesta substància.

L'addicció a la cocaïna es caracteritza per una persistent i alta susceptibilitat a la recaiguda. A més, el seu ús compulsiu s'associa amb múltiples trastorns cardiovasculars, neurològics i psiquiàtrics (essent habitual la comorbiditat). Un 73,4% dels addictes a la cocaïna tenen un diagnòstic dual (conjuntament amb una malaltia mental) i aquest està associat a una major gravetat del problema, una major discapacitat per al subjecte i un pitjor curs

en la seva evolució. La combinació de la psicoteràpia i la farmacoteràpia és essencial per al tractament dels trastorns per abús de substàncies, encara que cap d'ells sembla ser totalment eficaç en el tractament de la dependència de les drogues i més específicament a la cocaïna (EMCDDA, 2016).

Per tot això, la prevenció del consum de drogues es considera un repte social prioritari en la lluita contra l'addicció. D'aquesta manera, l'obtenció de marcadors psicofisiològics que ens permetin detectar els subjectes més vulnerables a desenvolupar un trastorn per consum de cocaïna, ens facilitaria la realització de campanyes de prevenció més eficaces dirigides a la població amb major risc a mostrar aquest ús compulsiu de la droga.

3.2. Mecanisme d'acció

A nivell farmacològic la cocaïna pot actuar com a anestèsic local i com a estimulant del SNC, mitjançant el sistema simpàtic. Com anestèsic local, bloquejarà els canals de sodi a la membrana neuronal, inhibint la despolarització i bloquejant tant la iniciació com la conducció dels impulsos nerviosos (Catterall i Mackie, 2006; Goldstein i cols., 2009). A més a més, al SNC interfereix en la recaptació de neurotransmissors en les terminals nervioses com l'adrenalina i la NA, de manera que la persistència d'aquests en l'espai sinàptic justificaria els exagerats efectes simpaticomimètics de la substància (Hollander i cols., 1998). També influeix sobre la serotonina (Shanti i Lucas, 2003; Hoffman, 2006), l'increment de l'activitat de la qual pot produir problemes cardíacs i sembla estar implicada també en l'addicció i l'efecte de la recompensa de la cocaïna (O'Dell i cols., 2000; Lason, 2001; Knuepfer, 2003; Shanti i Lucas, 2003). No obstant això, l'excés d'activitat dopaminèrgica sembla ser el causant de gran part dels símptomes produïts en el SNC (Goldstein i cols., 2009).

Al sistema mesolímbic (component clau en l'avaluació de la recompensa) la cocaïna dificulta el mecanisme de control dopaminèrgic, ja que bloqueja el transportador de la DA i com a resultat, les molècules, que havien de ser recaptades, romanen en acció, augmentant i sobre-activant les cèl·lules receptores, el que explica la resposta eufòrica de la droga (Franco

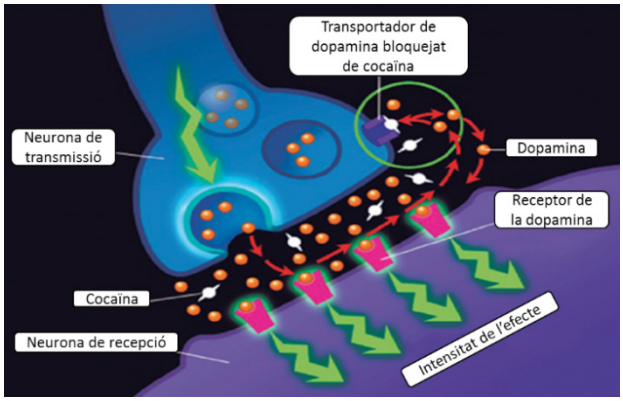

Figura 7. Mecanisme d'acció de la cocaïna. La cocaïna bloqueja el procés de reciclatge provocant una acumulació de DA en la sinapsis. Aquest augment de la DA s'associa als efectes reforçants provocats per la cocaïna. Imatge modificada del National Institute of Drug Abuse (NIDA).

i cols., 2004)(Figura 7). Per tant, existeix un augment de la transmissió dopaminèrgica de les neurones que projecten des del mesencèfal ventral (ATV) al prosencèfal (cervell anterior), incloent l'CPFm i el NAcc. Aquest sistema acaba estenent-se i influint sobre àrees com l'estriat ventral, l'amígdala, el BNST, l'àrea septal lateral i l'hipotàlem lateral (Adinoff, 2004; Cachope i Cheer, 2014). Essent aquest sistema l'objectiu farmacològic del reforç i l'estimulació locomotriu produïda per la cocaïna (Dackis i O'Brien, 2001).

La cocaïna produeix una gran toxicitat tant en el SNC com en el sistema cardiovascular. Pel que fa a la toxicitat produïda al sistema cardiovascular, la cocaïna inhibeix la recaptació de les amines biògenes, produint un efecte simpaticomimètic intens. Igual que les drogues que bloquegen els canals de sodi, la cocaïna es classifica com un agent antidisrítmic de tipus I (Shanti i Lucas, 2003; Hoffman, 2006) i com a conseqüència, poden aparèixer arítmies després del seu consum. Així mateix, pot produir vasoconstricció mitjançant l'increment de la NA neuronal, un efecte directe de la benzoilecgonina en els vasos sanguinis (intervingut pel calci), increment dels nivells de l'endotelin-1 (un poderós vasoconstrictor) i una reducció de la producció d'òxid nítric (un vasodilatador), la qual cosa facilita l'aparició de la hipertensió, dels accidents

cerebrovasculars (ACV), de les isquèmies cardíaques i infarts en els teixits (Tella i cols., 1993; McCord i cols., 2008).

3.3. Efectes reforçants de la cocaïna

L'avaluació de les propietats reforçants de la cocaïna s'ha convertit en una àrea principal d'investigació en psicofarmacologia. El reforç es compara algunes vegades amb l'experiència subjectiva del plaer, però per apropar-nos a aquest concepte, hem d'estudiar els efectes organitzadors que té en el comportament. S'han identificat dos efectes organitzadors principals del reforç. El més fonamental és l'estímul reforçat (estímul incentiu) el qual té la capacitat d'elicitar respostes d'aproximació i manteniment del contacte amb l'estímul. L'altre efecte organitzador és la capacitat d'incrementar la probabilitat que les respostes que el precedeixen es tornen a donar (Carr i cols., 1988).

3.3.1. Autoadministració

Centrant-se en la noció operant del reforç introduïda per Skinner (1938), l'efecte reforçant d'una droga és, per definició, un augment en la probabilitat de que l'animal s'administre de nou la substància (Lu i cols.,

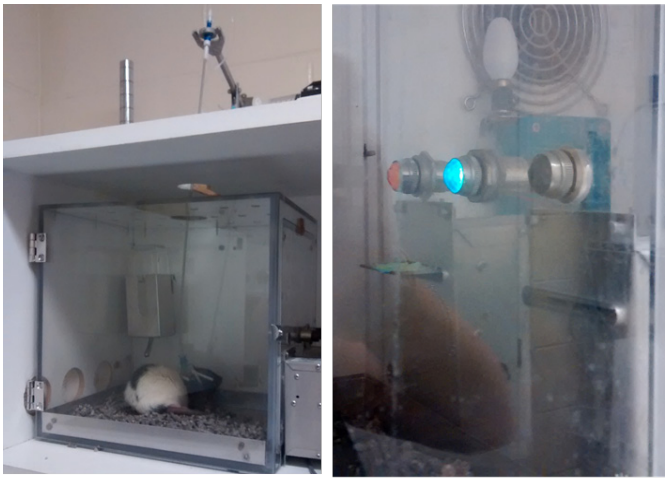

Figura 8. Imatge del procediment de l'autoadministració intravenosa en rata. Universitat de Tufts (Boston).

2003). En rosegadors, concretament rates, l'efecte reforçant de la cocaïna fou caracteritzat per primera vegada per Pickens i Thompson (1968), els quals van demostrar que els animals podien aprendre a pressionar una palanca per a rebre una injecció intravenosa de cocaïna. Al substituir la droga per una solució salina, la conducta de pressionar la palanca va desaparèixer, demostrant-se així que la cocaïna presentava reforç positiu (Figura 8).

Per avaluar el valor del reforç d'una droga, els procediments operants empren un programa de ràtio fixa (RF), on l'animal ha de fer un nombre determinat de respostes no variables al llarg del procés, per així aconseguir una infusió de droga. D'aquesta manera, el nombre de respostes vàlides de l'animal per aconseguir una infusió, solen ser majors com més potents siguen les propietats reforçants d'una droga (Moser i cols., 2010). A més dels programes de ràtio fixa, també s'han desenvolupat els programes de ràtio progressiva (RP), on es va augmentant de manera gradual el nombre de respostes necessàries perquè l'animal obtinga la infusió de droga desitjada. D'aquesta manera, es pot avaluar la motivació que l'animal té per consumir la droga (Depoortere i cols., 1993; Tabakoff i Hoffman, 2000). Un altre índex de la motivació i de l'esforç que l'animal té per aconseguir la droga s'obté amb el denominat *breaking point* o punt de tall, que determina el punt on l'animal arriba al màxim de respostes per obtenir una dosi, utilitzant un programa de RP, on l'administració de la droga requereix respostes cada vegada majors (Yap i Miczek, 2008).

Molts són els estudis descrits que posen de manifest el poder addictiu de la cocaïna en diferents espècies. Els resultats mostren en el cas dels ratolins, que l'adquisició de l'AA en programes de RF es donen en dosis des de 0.25 mg/kg fins a 4mg/kg (Deroche i cols., 1997; Rocha i cols., 1997,1998; Kuzmin i cols., 2000), incrementant l'efecte reforçant de la cocaïna dosis-dependent (Ruiz-Durántez i cols., 2006; Schramm-Sapyta i cols., 2006). A més a més, davant programes de RP s'observa un augment del *breaking point*, és a dir, un augment per la motivació de l'animal per aconseguir la droga (Soria i cols., 2005; Olsen i Winder, 2006), el qual es veu potenciat quan els animals són pretractats amb amfetamina (Yap i Miczek, 2007). En canvi, quan s'administra

un tractament continuat d'amfetamina, els animals mostren una disminució del reforç induït per cocaïna (Chiodo i cols., 2008). Mentre que en ratolins no s'observen diferències de sexe per adquirir l'AA (Griffin i cols., 2007), en el cas de les rates, les femelles mostren una major sensibilització als efectes reforçants de la cocaïna (Lynch i Carroll, 1999; Carroll i cols., 2002; Lynch i Taylor; 2004; Jackson i cols., 2006; Lynch, 2008).

Patrons conductuals com l'alta resposta a la novetat o alts nivells d'exploració provoquen una adquisició més ràpida de l'AA de cocaïna tant en programes de RF com en RP (Bush i Vaccarino, 2007; Davis i cols., 2008), essent els nivells alts d'impulsivitat aquells que més correlacionen amb majors nivells de recaiguda (Perry i cols., 2008). Aquests són trets característics de l'adolescència, pel que s'ha observat, que en comparació amb els animals adults, aquells adolescents exposats a aquest procediment adquireixen més ràpidament l'AA a la cocaïna (Leslie i cols., 2004; Higuera-Matas i cols., 2008)

3.3.2. Condicionament de Preferència de Lloc

El CPL és un model basat en l'aprenentatge clàssic o Pavlovià, per avaluar la recompensa condicionada induïda per diferents estímuls (Bardo i Bevins, 2000; Tzschentke, 2007). Ha estat àmpliament utilitzat per estudiar els efectes de recompensa de les drogues addictives condicionades (Aguilar i cols., 2009), ja que els estímuls contextuals poden adquirir propietats apetitives secundàries (efectes gratificants condicionats) quan es combinen amb un reforçador primari (Tzschentke, 2007). Els animals són entrenats per a associar un ambient específic neutre amb l'efecte de la droga administrada, i l'altre compartiment diferent amb solució salina (Bardo i Bevins, 2000; Lu i cols., 2003; Sanchis-Segura i Spanagel, 2006) (Figura 9). De forma general, l'administració de cocaïna estableix robustos CPL tant en rates com en ratolins (Mueller i Steward, 2000). El que demostra, que la cocaïna també és reforçant quan s'utilitza aquest paradigma.

Entre les diferents dosis que estableixen el CPL trobem que mentre que en la dosis de 3mg/kg els rosegadors adquireixen el CPL (Estelles i cols., 2006), és a partir de la dosis de 6mg/kg de cocaïna quan a més de desenvolupar

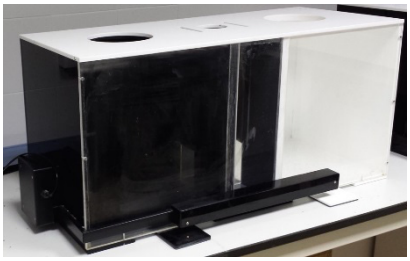

Figura 9. Caixa de CPL utilitzada en els diferents experiments. Facultat de Psicologia, València.

aquest procediment, reinstauren de nou la conducta de cerca de la substància (Laviola i cols., 1992; Cunningha i cols., 1999) una vegada extingit el CPL (Busse i Riley, 2004; Maldonado i cols., 2006; Aguilar i Rodríguez-Arias, 2009); essent una dosis de 25mg/kg una dosis el suficientment efectiva que cursa en múltiples recaigudes (Maldonado i cols., 2006; Aguilar i cols., 2009; Rodríguez-Arias i cols., 2009). Considenrant-se la inducció del CPL per cocaïna dosis-dependent (Maldonado i cols., 2006; Diller i cols., 2007). En canvi, s'ha observat que la dosis d'1 mg/kg és una dosis subumbral, l'administració de la qual no comporta l'adquisició del CPL, llevat que es manipulen altres variables com l'estrés o trets del comportament (Vidal-Infer i cols., 2012; Arenas i cols., 2014; Montagud-Romero i cols., 2014).

Animals preexposats a la mateixa cocaïna o a altres psicoestimulants, desenvolupen una major preferència de lloc induïda per la substància, suggerint que el abús previ podria conduir a un increment de la sensibilitat en els animals davant les accions reforçants d'aquesta substància (Shippenberg i cols., 1996; Itzhak i Martin, 2002). En canvi, l'exposició prenatal a la cocaïna deteriora el desenvolupament del CPL en l'edat adulta (Malanga i cols., 2007), sobretot quan s'administren les dosis més elevades (25mg/kg) (Estellés i cols., 2006).

Respecte al sexe no s'han observat diferències, i utilitzant dosis efectives, tant mascles com femelles, adquireixen el CPL (Campbell i cols., 2000; Russo i cols., 2003; Nazarian i cols., 2004). No obstant això, els patrons conductuals com l'alta resposta a la novetat incrementa els efectes reforçants

condicionats de la cocaïna a dosis subllindars en l'adultesa, quan aquests animals són pretractats amb cocaïna en l'adolescència (Mateos-García i cols., 2015).

3.3.3. Síndrome d'Abstinència

El síndrome d'abstinència fa referència a tota eixa sèrie de símptomes tant físics com psicològics que apareixen quan es deixa de consumir una substància (amb potencial addictiu), el que crea un intens malestar en l'organisme i pot provocar que la persona consumisca de nou. Durant el període d'abstinència a la cocaïna, el reforçament negatiu associat amb la seua dependència, expressat generalment per l'ansietat, contribueix a la recaiguda i al manteniment del consum d'aquesta substància (Markou i cols, 1993). En aquest sentit, està àmpliament acceptada la correlació entre l'ansietat i l'addicció a la cocaïna, on els alts nivells d'ansietat són un dels factors destacats que contribueixen a la recaiguda (Lejuez i cols, 2008; Valzachi i cols., 2013). D'acord amb els informes clínics s'ha demostrat que els addictes comunament experimenten ansietat en la fase primerenca de l'abstinència, però no davant períodes prolongats (Gawin i Kleber, 1986). L'abstinència en humans desenvolupa símptomes psiquiàtrics diferents a l'ansietat, com la depressió, irritabilitat i inatenció (Rogerio i Takahashi, 1992; Basso i cols., 1999).

En rosegadors, trobem diferents símptomes psiquiàtrics que s'experimenten davant l'abstinència a la cocaïna com són: ansietat, psicosi, depressió, així com també alteracions cognitives (Dalley i cols., 2005; Perrine i cols., 2008; Tang i cols., 2014; Craige i cols., 2015). En rates, la cocaïna pot induir comportaments similars a l'ansietat (Blanchard i Blanchard, 1999), i efectes semblants a la depressió quan aquesta s'ha administrat tant prenatalment (Overstreet i cols., 2000; Sobrian i cols., 2003) com en edats posteriors (Mutschler i Miczek, 1998; Magalhaes i cols., 2002; Perrine i cols., 2008). L'estat d'anhedonisme en rates, induït per l'abstinència a la cocaïna,

és proporcional a la quantitat de cocaïna consumida (Markou i Koob, 1991; Koob i Le Moal, 1997). A més a més, també es veu deteriorat el rendiment atencional visual per l'abstinència aguda de la substància (Dalley i cols., 2005).

Cal assenyalar que els estudis preclínics no mostren canvis en quant a l'ansietat, si l'abstinència es dóna durant un període prolongat (Erb, 2010). Un fenotip ansiós després d'un llarg període d'abstinència és evident, només en condicions en què l'animal està exposat a un factor estressant o se li administra l'hormona de l'estrés, el factor alliberador de corticotropina (Mantsch i cols., 2008), o s'exposa a senyals contextuals prèviament associades amb la cocaïna (Erb i cols., 2006).

3.4. Efectes de la derrota social en el condicionament de la preferència de lloc induït per cocaïna

Com hem comentat prèviament, l'estrés és un factor de risc relacionat en el desenvolupament de l'addicció i en la vulnerabilitat a la recaiguda (Sinha, 2008). Nombrosos estudis, troben en models animals una associació entre l'estrés agut i l'estrés crònic, i l'augment de la motivació per iniciar l'ús al consum de substàncies addictives (Sinha, 2001; Sinha i cols., 2006; Koob i Kreek, 2007). La recaiguda durant períodes d'abstinència, que constitueix el principal problema en el tractament de l'addicció a les drogues, sovint s'associa amb l'exposició a l'estrés, el que pot provocar un estat subjectiu de necessitat per obtenir la droga. En models animals l'ànsia per la droga i la reinstauració del consum després de períodes prolongats d'abstinència, s'activen de manera fiable per l'exposició a esdeveniments estressants (Shaham i cols., 2000; Sinha, 2001; Yahyavi-Firouz-Abadi i See., 2009).

El paradigma del CPL ha estat utilitzat en múltiples ocasions amb rosegadors de laboratori, per tal d'estudiar els canvis fisiològics, conductuals o neurobiològics causats per experiències socials d'estrés, tant agudes com cròniques (McLaughlin i cols., 2006; Hymel i cols., 2014; García-Pardo i cols., 2015). No obstant això, pocs estudis han avaluat els efectes de l'estrés per derrota social sobre el CPL induït per cocaïna (Taula 1).

De forma general, els estudis demostren que la derrota social de caràcter agut o crònic incrementa o potència els efectes reforçants condicionats a la cocaïna (a dosis de 1, 3, 10, 15 i 25 mg/kg) en ratolins mascles adults (McLaughlin i cols., 2006; Land i cols., 2009; Hymel i cols., 2014; Reguilón i cols., sota revisió), ja que aquestos rosegadors augmenten el temps que romanen en el compartiment aparellat amb la substància (McLaughlin i cols., 2006; Hymel i cols., 2014). Adquirit el CPL de cocaïna i una vegada extingida la conducta de cerca de la substància, s'ha observat que un encontre agonístic en una zona neutral restableix el CPL induït per cocaïna en rosegadors adults (Land i cols., 2009; Titomanlio i cols., 2013), augmentant també la susceptibilitat de la reinstauració induïda per una dosis priming de cocaïna (Ribeiro do Couto i cols., 2009).

En el cas de l'adolescència, els resultats mostren que rates exposades a derrota social crònica, durant cinc dies, adquireixen en CPL d'amfetamina (a dosis d'1 mg/kg) quan aquestes són adultes (Burke i cols., 2011).

Els resultats dels diferents estudis són consistents i assenyalen que l'estrés social en general incrementa eixos efectes reforçants condicionats als psicoestimulants tant en dosis efectives, com en aquelles dosis establertes com a subllindars (Maldonado i cols., 2006; Vidal-Infer i cols., 2012).

En canvi, davant la resposta a altres substàncies s'observa que la DSA disminueix la sensibilitat dels ratolins adults als efectes gratificants de la MDMA en el paradigma del CPL (García-Pardo i cols., 2014); mentre que l'exposició a un estrés agut incontrolable previ a l'administració de la droga, potència els efectes reforçants condicionats del CPL induït per morfina (Will i cols., 1998).

3.5. Efectes de la derrota social en l'autoadministració de cocaïna

En models animals, la relació entre l'estrés i l'addicció a psicoestimulants s'ha avaluat principalment per mitjà del paradigma de l'AA (Taula 2).

Els resultats que observem en l'AA van en la línia d'aquells obtinguts

Animal	Soca	Sexe	Estrés per DS	Intèrval última DS	Droga	Dosi	Resultat	Referènca
Ratolí	C57Bl/6	Mascle	Crònic (3 dies)	Immediatament	Cocaïna (s.c)	15mg/kg	Increment CPL	McLaughlin i cols., 2006
Ratolí	C57Bl/6	Mascle	Agut	Immediatament	Cocaïna (s.c)	10mg/kg	Reinstauració del CPL induïda per DS	Land i cols., 2009
Ratolí	OF1	Mascle	Agut	Immediatament	Cocaïna (i.p)	50mg/kg	Reinstauració del CPL induïda per dosis priming	Ribeiro do Couto i cols., 2009
Rata adolescent	Sprague-Dawley	Mascle	Crònic (5 dies)	16 dies	Amfetamina (i.p)	1mg/kg	Increment CPL	Burke i cols., 2011
Ratolí	OF1	Mascle	Agut	Immediatament	Cocaïna (i.p)	25mg/kg	Reinstauració del CPL induïda per DS	Titomanlio i cols., 2013
Ratolí	C57Bl/6	Mascle	Crònic (6 derrotes en 3 dies)	Immediatament	Cocaïna (s.c)	10mg/kg	Increment CPL	Hymel i cols., 2014
Ratolí	OF1	Mascle	Agut	Immediatament	Cocaïna (i.p)	1mg/kg	Increment CPL	Reguilon i cols., 2016
						3mg/kg	Increment CPL	
						25mg/kg	Adquisició CPL i increment de la reinstauració	

Taula 1. Estudis que han utilitzat el paradigma del CPL per avaluar els efectes reforçants dels psicoestimulants (cocaïna i amfetamina) en rosegadors prèviament derrotats.

amb el procediment del CPL. Concretament, en l'AA s'ha observat que quatre episodis breus d'estrés per derrota social sobre el curs d'una o dues setmanes han demostrat un increment en l'adquisició de l'AA a una dosis baixa de cocaïna (Haney i cols., 1995; Tidey i Miczek, 1997), així com un increment del *breaking point* en el programa de reforç de la RP en rosegadors (Covington i Miczek, 2005; Covington i cols., 2008; Quadros i Miczek, 2009; Boyson i cols., 2011; Holly i cols., 2012; Boyson i cols., 2014; Burke i Miczek., 2015; Wang i cols., 2016); aquest increment també s'observa davant la derrota social crònica (Han i cols., 2015).

Un cop establerta l'AA de cocaïna, breus episodis d'estrés social, abans de cada sessió experimental poden augmentar significativament la taxa de consum de drogues, especialment a dosis baixes (Miczek i Mutschler, 1996).

En condicions d'accés il·limitat a la cocaïna durant 24 h (binge), els rosegadors socialment derrotats s'autoadministren cocaïna amb intervals interinfusió més curts i en majors quantitats (Nikulina i cols., 2004; Covington i Miczek, 2005; Covington i cols., 2008; Boyson i cols., 2011; Miczek i cols., 2011; Boyson i cols., 2014; Holly i cols., 2015; Yap i cols., 2015). Mentre que la derrota abans de les sessions de condicionament operant augmenta el consum de cocaïna, el retard entre l'exposició a la derrota social i l'autoadministració de cocaïna dissipa els efectes de l'estrés sobre l'adquisició (Covington i cols., 2005; Yap i Miczek., 2007; Covington i cols., 2008; Cruz i cols., 2011; Burke i Miczek, 2015).

Davant les diferències de sexe, observem que tant els mascles com les femelles derrotades durant quatre períodes intermitents, s'autoadministren més cocaïna que el grup control (Haney i cols., 1995; Holly i cols., 2012) sent les femelles qui realitzen major nombre d'infusions davant un *binge* de cocaïna de 24h (Holly i cols., 2012). En canvi, quan la derrota és crònica i es volen veure els efectes a llarg termini d'aquesta, no existeixen diferències entre aquelles que han sigut derrotades i aquelles que no quan s'exposen a l'autoadministració (Shimamoto i cols.,2015).

Animal	Soca	Sexe	Estrés DS	Intèrval última DS	Droga	Dosi	Resultats	Referències
Rata	Sprague-Dawley	Mascle i femella	4 DS (1 setmana)	6 dies	cocaïna	0,32mg/kg FR	Increment de les injeccions en la SA (ambdós sexes)	Haney i cols., 1995
Rata	Long-Evans	Mascle	Recurrents	Inmmediatament	cocaïna	0,25- 0,125- 0,063- 0.031 FR i FR5	Increment de les injeccions en la SA	Miczek i Mutschler, 1996
Rata	Long-Evans	Mascle	Crònic (4 dies)	Inmmediatament	cocaïna	0,75mg/kg FR	Adquireixen la SA	Tidey i Miczek, 1997
Rata	Long-Evans	Mascle	4 DS / 72h	10-15 dies	cocaïna	0,75mg/kg FR	Incrementa la escalada en el binge 24h	Covington i Miczek, 2001
Rata	Sprague-Dawley	Mascle	4DS	3 dies	cocaïna	0,25 mg/kg FR1	HS tarden més en adquirir SA que els controls LS adquireixen el SA més ràpidament que els control	Kabbaj i cols., 2001
Rata	Long-Evans	Mascle	4 DS / 72h	10 dies	cocaïna	0,75 mg/kg FR	Adquireixen més ràpid la SA	Covington i Miczek, 2005
						0,3 mg/kg PR	Major "breaking point" i més infusions en el binge 24h	
Rata	Long-Evans	Mascle	4 DS / 72h	15 dies	cocaïna	0,75mg/kg FR i FR5	No diferències	Covington i cols 2005
						0,3mg/kg FR	Més infusions en el binge de 24h	
Ratolí	CFW	Mascle	Crònic (10 dies)	10 dies	cocaïna	1mg/kg FR2	No hi ha diferències	Yap i Miczek, 2007
						0,3mg/kg PR	No hi ha diferències	
Rata	Long-Evans	Mascle	4 DS / 72h	15 dies	cocaïna	0,75mg/kg FR i FR5	-	Covington i cols 2008
						0,3mg/kg PR	Major breaking point	
						0.2, 0.4, 0.8 FR	Més infusions en el binge de 24h	
							Antagonista NMDA ic VTA redueix la SA	
Rata	Long-Evans	Mascle	4 DS / 72h	10 dies	cocaïna	0,75mg/kg FR i FR5	Major nombre d'infusions	Quadros i Miczek., 2009
						0,3mg/kg PR	Major breaking point	
						0,3mg/kg FR5	Major consum en binge de 24h	

Taula 2(1). Estudis que utilitzen el paradigma de l'AA per a avaluar els efectes reforçants dels psicoestimulants (cocaïna) en rosegadors derrotats socialment.

Animal	Soca	Sexe	Estrés DS	Intèrval última DS	Droga	Dosi	Resultats	Referències
Rata	Long-Evans	Mascle	4 DS / 72h	11-14 dies	cocaïna	0,75mg/kg FR i FR5	No diferències	Cruz i cols., 2011
						0,3mg/kg PR		
						0,3mg/kg FR5	Major consum en escalada en binge de 24h	
Rata	Long-Evans	Mascle	4 DS / 72h	15 dies	cocaïna	0,75 mg/kg FR	No diferències	Boyson i cols., 2011
						0,3 mg/kg FR5	Major "breaking point" i més infusions en el binge 24h	
							Antagonista CRFR1 ip o ic VTA frena el consum en escalada	
Rata	Long-Evans	Mascle i femella	4 DS / 72h	10 dies	cocaïna	0,75 mg/kg FR	No diferències	Holly i cols., 2012
						0,3 mg/kg FR	Més infusions en el binge 24h, les femelles majors infusions que els mascles	
Rata	Long-Evans	Mascle	4 DS / 72h	10 dies	cocaïna	0,75 mg/kg FR 0,3 mg/kg PR	No diferències	Boyson i cols., 2014
						0,3 mg/kg FR5	Major "breaking point" i més infusions en el binge 24h	
							Antagonista CRFR1 i R2 ic VTA frena el consum en escalada	
Rata	Long-Evans	Mascle adolescent	4 DS / 72h	PD 65	cocaïna	0,75mg/kg FR i FR5	No diferències	Burke i Miczek., 2015
						0,3mg/kg PR	Major nombre d'infusions "Pair housed"	
						0,3mg/kg FR5	Consum en escalada binge 24h "Pair housed"	
Rata	Long-Evans	Mascle	4 DS / 72h	10 dies	cocaïna	0,75mg/kg FR i FR5	-	Yang i cols., 2015
						0,3mg/kg PR	No diferències	
						0,3mg/kg FR5	Consum en escalada 24h	
							Inhibidor MAPK/ERK ic VTA frena l'escalada	

Taula 2(2). Estudis que utilitzen el paradigma de l'AA per a avaluar els efectes reforçants dels psicoestimulants (cocaïna) en rosegadors derrotats socialment.

Animal	Soca	Sexe	Estrés DS	Intèrval última DS	Droga	Dosi	Resultats	Referències
Rata	Long-Evans	Femella	Crònic (2 al dia/21 dies)	17 dies	cocaïna	0,75 mg/kg FR 0,3 mg/kg PR	No diferències	Shimamoto i cols., 2015
						0,3 mg/kg FR5	Les femelles "stress resistent" més infusions en el binge 24h	
Ratolí	Swiss Webster	Mascle	Crònic (10 dies)	6 dies	cocaïna	cocaïna	0.3, 0.6 mg/kg incrementa el nombre "nosepoke" SA	Han i cols., 2015
Rata	Long-Evans	Mascle adolescent	4 DS / 72h	15 dies	cocaïna	0,75mg/kg FR i FR5	No diferències	Burke i cols., 2016
						0,3mg/kg PR		
						0,3mg/kg FR5	Consum en escalada 24h	
							Antagonista CRFR1 ic VTA frena el consum en escalada	
Rata	Sprague-Dawley	Mascle	4 DS / 72h	1 semana	cocaïna	0,75mg/kg FR i FR5	No diferències	Wang i cols., 2016
						0,3mg/kg PR	Major "breaking point" i corresponent nombre d'infusions	
						0,375mg/kg FR5	Consum en escalada 12h	
							BDNF ic VTA potència l'escalada en el consum	

Taula 2(3). Estudis que utilitzen el paradigma de l'AA per a avaluar els efectes reforçants dels psicoestimulants (cocaïna) en rosegadors derrotats socialment.

Durant l'adolescència, l'exposició a DSR també incrementa el *breaking point* en el programa de reforç de la RP en rosegadors, així com les infusions davant un *binge* de cocaïna de 24h, quan aquests animals són adults, fent-los més sensibles al reforç de la substància (Burke i Miczek., 2015; Burke i cols., 2016).

3.6. Efectes de la derrota social en la sensibilització motora a la cocaïna

La sensibilització conductual es defineix com un augment de la resposta comportamental davant l'administració aguda d'una substància (generalment psicoestimulants), com a resultat de neuroadaptacions produïdes per l'administració intermitent d'agents sensibilitzants (Vanderschuren i Kalivas, 2000). Més comunament, la sensibilització és induïda per l'administració repetida d'estimulants psicomotors com l'amfetamina o cocaïna (Taula 3) (Karler i cols., 1989; Kalivas, 1995). No obstant això, l'administració repetida d'opiacis, nicotina, cafeïna i alcohol comparteixen efectes de sensibilització conductual i neuroquímica amb els dels estimulants psicomotors (Shuster i cols.,1975; Meliska i cols., 1990; Phillips i cols., 1994; Itzhak, 1999).

La derrota social episòdica indueix sensibilització creuada als estimulants psicomotors en forma d'activitat locomotora augmentada (Miczek i cols., 1999; Covington i Miczek, 2001; Nikulina i cols., 2004; Miczek i cols., 2011). Quatre episodis intermitents breus d'estrés per derrota social produeixen sensibilització conductual, la qual es fa evident per una resposta locomotora incrementada després d'una administració aguda d'amfetamina (generalment 1mg/kg) o cocaïna (7,5 i 10 mg/kg) (Boyson i cols., 2011; Cruz i cols., 2011; Miczek i cols., 2011; Nikulina i cols., 2012; Boyson i cols; 2014; Wang i cols., 2014), sense induir estereotípies (Kuczenski i Segal, 1989). Aquesta evidència també la podem observar quan s'exposa a l'animal a una DSA i a una dosis "desafio" de 40 mg/kg de cocaïna i de 0,25 o 1 mg/kg de D-amfetamina (Nikulina i cols., 1998; Miczek i cols., 1999; de Jon i cols., 2005; Wang i cols., 2013); i davant l'exposició crònica a la derrota social (Miczek i cols., 1999; Yap i cols., 2005; Dietz i cols., 2008; Han i cols., 2015).

Les femelles, en concret, mostren davant l'exposició prèvia a episodis

intermitents agonístics, una major resposta locomotora a l'administració aguda de cocaïna en comparació amb les controls, i una major magnitud en la resposta en comparació amb els mascles estressats (Holly i cols., 2012); mentre que aquests resultats no s'observen quan la derrota social és crònica (Shimamoto i cols., 2015).

L'increment de la resposta locomotora, a una dosis aguda de cocaïna (10mg/kg) o D-amfetamina (1mg/kg), també s'observa en rosegadors adults, quan aquests han sigut exposats a la derrota social, tant de forma intermitent com crònica, durant el període de l'adolescència (Burke i cols., 2013; Burke i Miczek, 2015).

Animal	Soca	Sexe	Estrés DS	Dosis inducció sensib	Intèrval última DS	Droga	Dosi	Resultats	Referències
Ratolí	CFW	Mascle	1 DS	salino	inmediatament o 7 dies	Cocaïna	40mg/kg	Sensibilització locomotora (SL)	Nikulina i cols., 1998
Rata	Long-Evans	Mascle	Crònic (5 dies)	D-amfetamina 1mg/kg	Inmediatament	D-amfetamina	0,6mg/kg	Sensibilització locomotora	Miczek i cols., 1999
Ratolí	CFW	Mascle	1 DS	salino	Inmediatament, 3,5, 7 o 9 dies	Cocaïna	40mg/kg	Sensibilització locomotora (inmediat, 5 i 7 dies)	Miczek i cols., 1999b
Rata	Long-Evans	Mascle	4DS/72h	saline	10 dies	D-amfetamina	1mg/kg	Sensibilització locomotora	Covington i Miczek, 2001
					10 i 15 dies	Cocaïna	7.5 i 10 mg/kg	Sensibilització locomotora (10mg/kg)	
Rata	Long-Evans	Mascle	4 DS / 72h	salino	17 i 70 dies	D-amfetamina	1mg/kg	Sensibilització locomotora	Nikulina i cols., 2004
Rata	Long-Evans	Mascle	4 DS / 72h	salino	10 dies	Cocaïna	10mg/kg	Sensibilització locomotora	Covington i cols., 2005
					60 dies	D-amfetamina	1mg/kg	Sensibilització locomotora	
Rata	Wistar	Mascle	1 DS	-	3, 14 i 21 dies	D-amfetamina	0.25 or 1.0 mg/kg	Sensibilització locomotora (3 dies)	de Jong i cols., 2005
Ratolí	CFW	Mascle	Crònic (10 dies)	salino	10 dies	D-amfetamina	1,5	Sensibilització locomotora	Yap i cols., 2005
				D-amfetamina 1,5mg/kg (10 dies)			1mg/kg		
Rata	Long-Evans	Mascle	4DS/72h	-	10 dies	amfetamina	1mg/kg	Sensibilització locomotora	Covington i Miczek, 2005
Ratolí	CFW	Mascle	4 DS / 72h	salino	10 dies	D-amfetamina	1,5mg/kg	Sensibilització locomotora	Yap i Miczek, 2007
							Acumulatiu 1-1,8-3mg/kg	Sensibilització locomotora (1.8 i 3mg/kg)	
Rata	Long-Evans	Mascle	4 DS / 72h	salino	10 dies	Cocaïna	10mg/kg	Sensibilització locomotora	Covington i cols., 2008
								Antagonista NMDA (AP-5 a dosis 5 nmol/side) bloqueja SL	
Rata	Sprague-Dawley	Mascle	Crònic (4 dies)	salino	14 dies	D-amfetamina	0.5, 1, 1.5, mg/kg	No apareix sensibilitat motora (la DS elimina las dif. LR i HR)	Dietz i cols., 2008
Rata	Long-Evans	Mascle	4 DS / 72h	-	10 dies	Cocaïna	10mg/kg	Sensibilització locomotora	Quadros i Miczek, 2009

Taula 3(1). Estudis que realitzen sensibilització locomotora als psicoestimulants amb prèvia derrota social en rosegadors.

Animal	Soca	Sexe	Estrés DS	Dosis inducció sensib	Intèrval última DS	Droga	Dosi	Resultats	Referències
Rata	Long-Evans	Mascle	4 DS / 72h	salino	11 dies	Cocaïna	10mg/kg	Sensibilització locomotora	Miczek i cols., 2011
			Crònic (36 dies)		12 dies			No sensibilitazació motora	
Rata	Long-Evans	Mascle	4 DS / 72h	3 injeccions de salino	11 dies	Cocaïna	10mg/kg	Sensibilització locomotora	Boyson i cols., 2011
								Antagonista del receptor CRF-R1 bloqueja SL	
Rata	Long-Evans	Mascle	4 DS / 72h	salino	10 dies	Cocaïna	10mg/kg	Sensibilització locomotora	Cruz i cols., 2011
Rata	Long-Evans	Mascle i femella	4 DS / 72h	3 injeccions de salino	10 dies	Cocaïna	10mg/kg	Sensibilització locomotora (major femelles)	Holly i cols., 2012
								Increment dels nivells de DA en NAcSh	
Rata	Sprague-Dawley	Mascle	4 DS / 72h	salino	10 dies	D-amfetamina	1mg/kg	Sensibilització locomotora	Nikulina i cols., 2012
Rata	Sprague-Dawley	Mascle adolescent	Crònic (5 dies)	salino	15 dies	D-amfetamina	1mg/kg	Sensibilització locomotora	Burke i cols., 2013
				amfetamina 1mg/kg 5 dies				Sensibilització locomotora	
Rata	Sprague-Dawley	Mascle	1 DS	salino	3 i 14 dias	D-amfetamina	1mg/kg	Sensibilitzaciólocomotora (3 dies)	Wang i cols., 2013
Rata	Long-Evans	Mascle	4 DS / 72h	3 injeccions de salino	10 dies	Cocaïna	10mg/kg	Sensibilització locomotora	Boyson i cols., 2014
Rata	Sprague-Dawley	Mascle	4 DS / 72h	salino	10 dies	D-amfetamina	1mg/kg	Sensibilització locomotora	Wang i cols., 2014
								KO rNAc TrkB prevenen la SL	
Rata	Long-Evans	Mascle	4 DS / 72h	-	10 dies	Cocaïna	10mg/kg	Sensibilització locomotora	Yap i cols., 2015

Taula 3(2). Estudis que realitzen sensibilització locomotora als psicoestimulants amb prèvia derrota social en rosegadors.

Animal	Soca	Sexe	Estrés DS	Dosis inducció sensib	Intèrval última DS	Droga	Dosi	Resultats	Referències
Ratolí	Swiss Webster	Mascle	Crònic (10 dies)	-	6 dies	D-amfetamina	1,5mg/kg	Increment dels nivells de DA en NAcSh	Han i cols., 2015
Rata	Long-Evans	Femella	Crònic (2 al dia/21 dies)	salino	10 dies	Cocaïna	10mg/kg	No efectes	Shimamoto i cols., 2015
Rata	Sprague-Dawley	Mascle	4 DS / 72h	salino	10 dies	D-amfetamina	1mg/kg	Sensibilització locomotora	Johnston i cols., 2015
Rata	Long-Evans	Mascle adolescent	4 DS/ 72h	salino	15 dies	Cocaïna	10mg/kg	Sensibilització locomotora	Burke i Miczek, 2015

Taula 3(3). Estudis que realitzen sensibilització locomotora als psicoestimulants amb prèvia derrota social en rosegadors.

4. Mecanismes a través dels quals la DS altera el reforç induït per la cocaïna

4. Mecanismes a través dels quals la DS altera el reforç induït per la cocaïna

4.1. Sistema de resposta a l'estrés: CRF

Davant la resposta d'estrés, s'activen diferents mecanismes hormonals que impliquen l'activació de l'eix HPA i l'eix simpàtic-adreno-medul·lar (SAM). L'eix HPA realitza la seva funció mitjançant hormones com el CRF, l'ACTH i els glucocorticoides. El SAM ho fa mitjançant l'alliberament de catecolamines, és a dir, adrenalina i NA.

Davant una situació d'estrés l'eix HPA s'activa per la secreció del CRF des del nucli paraventricular de l'hipotàlem (NPV) (Goeders, 2002). Aquestes neurones alliberen el CRF al sistema portal circulatori, arribant a l'adenohipòfisi, on la interacció del CRF amb els receptors CRF-R1 de la hipòfisi anterior, indueix la síntesi de proopiomelanocortina (POMC), un precursor de proteïnes que produeix altres menors com l'ACTH i les β-endorfines. L'ACTH derivada del POMC circula per l'organisme fins a arribar a les glàndules adrenals, estimulant la síntesi i alliberament d'adrenocorticoides (cortisol en el cas dels humans i corticosterona en rosegadors) per l'escorça adrenal (Goeders i Clampitt, 2002) induint una mobilització de les reserves energètiques amb accions sobre la glucogènesis i el metabolisme dels greixos, proteïnes i carbohidrats.

El funcionament d'aquest eix està regulat per un feedback negatiu, de manera que la detecció de glucocorticoides indueix una disminució de l'alliberament del CRF en l'hipotàlem i de POMC en la hipòfisi anterior (Kudielka i Kirschbaum, 2005).

D'altra banda l'activació del SAM s'inicia en el tronc de l'encèfal, concretament, al Locus Coeruleus (LC) on es produeix una descàrrega de NA. Els nervis del sistema simpàtic es projecten a la medul·la adrenal mitjançant sinapsis colinèrgiques, que després de l'estimulació de les cèl·lules endocrines, secreten adrenalina i NA que tenen efecte a nivell perifèric produint canvis que preparen l'organisme per superar l'estressor (Chrousos i Gold, 1992;

Goeders, 2003).

L'estrés, com ja hem comentat, és un factor involucrat en el consum de cocaïna, on concretament l'acció del CRF i els respectius receptors podrien estar implicats (Vale i cols., 1981). El CRF modula moltes respostes fisiològiques i de comportament relacionades amb l'estrés i el consum de drogues (Koob, 1999; Weiss i cols., 2001; Koob i Zorrilla, 2010). A més d'activar l'eix HPA, els axons del CRF projecten a zones extrahipotalàmiques, incloent l'amígdala, el BNST, i l'ATV (Swanson i cols., 1983; Sawchenko i cols., 1993; Bale i Val, 2004). L'alliberament del CRF a l'ATV causa neuroadaptacions sinàptiques de les neurones DA en la via mesolímbica (Saal i cols., 2003; Ungless i cols., 2003; Borgland i cols., 2004). El CRF incrementa l'alliberament de DA de les neurores de l'ATV mitjançant el receptor CRF-R1 (Wanat i cols., 2008), mentre que l'activació dels receptors postsinàptics CRF-R2 també incrementen l'exitabilitat neuronal DA en l'ATV mitjançant la potenciació dels receptors ionotròpics i metabotròpics del glutamat (Fiorillo i Williams, 1998; Ungless i cols., 2003). Conjuntament, l'activació dels receptors CRF-R1 i CRF-R2 en l'ATV condueixen a un increment de l'activitat neuronal DA, la qual pot causar canvis neuronals així com adaptacions conductuals a llarg termini.

Diferents estudis, mostren que l'activació del CRF-R1 i CRF-R2 en l'ATV poden intervindre en el consum en escalada de la cocaïna, mitjançant el paradigma de l'AA, així com en la reinstauració d'eixe consum induït per estrés (Wang i cols., 2005; Blacktop i cols., 2011; Boyson i col., 2011). L'antagonisme del CRF-R1, abans de la derrota social, pot previndre el desenvolupament de la sensibilització locomotora (Boyson i cols., 2011) mentre que l'antagonistme, tant dels receptors CRF-R1 i CRF-R2, prevenen la sensibilització dopaminèrgica, així com l'escalada del consum de cocaïna, durant períodes il·limitats de 24 h (Lodge i Greace, 2005; Specio i cols., 2008; Boyson i cols., 2014). A més, els dos subtipus de receptors també estarien implicats en la reinstauració del consum de cocaïna induït per estrés (Sarnyai i cols., 2001; Wang i cols., 2005; Blacktop i cols., 2011).

4.2. Sistema Dopaminèrgic

La DA és un neurotransmissor implicat en una sèrie de funcions com el control del moviment voluntari, l'estat d'ànim, l'atenció, la motivació, la son, la memòria de treball, l'aprenentatge, així com l'experiència del càstig i la recompensa (Pierce i Kalivas, 1997; Polter i Kauer, 2014).

Aquest neurotransmissor es sintetitza mitjançant la hidroxilació i descarboxilació de la L-tirosina en els neurones corresponents. El seu alliberament està regulat per autorreceptors pertinents a la família del D2 i heterorreceptors als terminals DA com els receptors glutamatèrgics N-metil-D-aspartat (NMDA), els de l'àcid gamma-aminobutíric (GABA) i els colinèrgics.

Existeixen cinc subtipus de receptors DA mitjançant els quals intervindrà a nivell cerebral, i es poden agrupar en dues famílies: la família D1 i la del D2 (Callier i cols., 2003; Smythies, 2005; Tritsch i Sabatini, 2012). La família de receptors D1 comprén els receptors D1 i D5, actuant sobre la proteïna G i activant l'adenilat ciclasa, augmentant així, la producció d'adenosin monofosfat cíclic (AMPc) (Lee i cols, 2000; Vallone i cols, 2000; Tritsch i Sabatini, 2012; Baik, 2013). Al SNC la major densitat de receptors D1 la trobem a les vies nigroestriada, mesolímbica i mesocortical, concretament als nuclis del caudat-putamen (estriat), NAcc, substància negra, bulb olfactori, amígdala i escorça frontal, i en menor densitat a l'hipocamp, cerebel, hipotàlem i àrees talàmiques (Beaulieu i Gainetdinov, 2011; Keeler i cols., 2014). La família del receptor D2 comprén els receptors D2, D3 i D4. Els receptors D2 actuen a través de proteïnes G, inhibint l'adenilat ciclasa i disminuint l'activitat de l'AMPc (Lee i cols., 2000; Beaulieu i Gainetdinov, 2011; Keeler i cols., 2014). Els receptors D2 es troben en gran densitat al cos estriat, al NAcc, l'ATV i al tubercle olfactori, i en menor densitat, a l'hipocamp, l'amígdala, l'hipotàlem i regions corticals (Beaulieu i Gainetdinov, 2011; Keeler i cols., 2014).

L'acció de la DA a la sinapsi conclou principalment amb la recaptació d'aquesta per la membrana presinàptica a través del transportador de

dopamina (DAT) (Ritz i Kuhar, 1993). A més, la DA s'elimina parcialment per oxidació amb la monoamino oxidasa (MAO-A) i la catecol-O-metiltransferasa (COMT) en l'espai sinàptic.

El sistema DA es divideix en quatre vies principals: mesocortical (des de l'ATV a l'CPF), mesolímbic (des de l'ATV al NAcc), nigroestriada (des de la substància negra a l'estriat), i tuberoinfundibular (des de l'hipotàlem a la

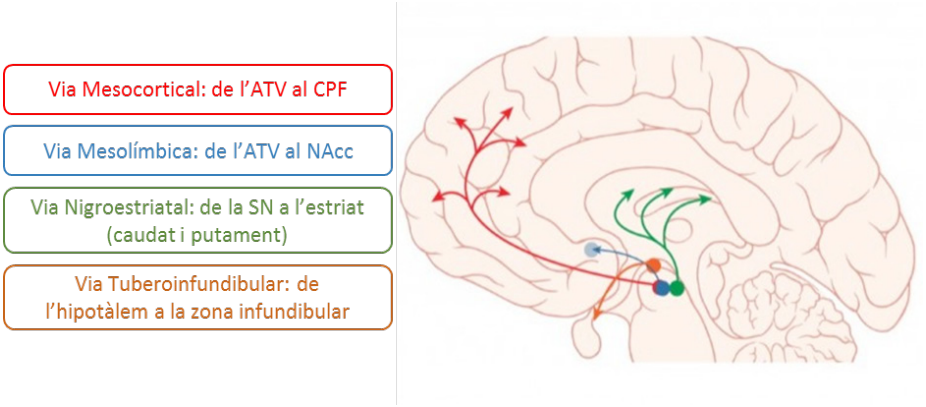

Figura 10. Vies Dopaminèrgiques (modificat de Netter, 2011).

glàndula pituïtària). La via mesocorticolímbica es reconeix actualment com la via clau en l'addicció a les drogues (Hou i cols., 2014).

La via mesolímbica, clau en l'avaluació de la recompensa, està formada pels cossos cel·lulars de la DA a l'ATV (Olds i Milner, 1954). Estos axons projecten DA al NAcc, a l'estriat ventral, a l'amígdala, a la BNST, a l'àrea septal lateral, i a l'hipotàlem lateral. A l'ATV també es troben cèl·lules del GABA, aquestes neurones proporcionen aferències inhibitòries a les neurones DA. L'ATV rep les principals aferències glutamatèrgiques exciatòries i colinèrgiques des de l'escorça prefrontal ventromedial (prelímbic ventral, infralímbic i escorça peduncle dorsal), subicle ventral, nucli subtalàmic, nucli parabraquial, nucli tegmental pedunculoponti i nucli tegmental lateral dorsal (Cachope i Cheer, 2014).

4.2.1. Receptors dopaminèrgics i estrés

Les experiències estressants modifiquen l'activitat d'àrees cerebrals implicades amb els efectes reforçants dels psicoestimulants (Koob, 2008; Sinha, 2008; Belujon i Grace, 2011). Sabem que les neurones dopaminèrgiques del mesencèfal i les seves estructures diana estan críticament involucrades en les modificacions dels circuits neuronals subjacents a una varietat de canvis d'adaptació i comportaments patològics, incloent els trastorns mentals i el desenvolupament i manteniment de l'addicció (Hyman i cols., 2006; Wolf, 2010).

Estudis amb CPL mostren que l'administració intraaccumbens o intraperitoneal (i.p.) d'agonistes dopaminèrgics D1 i D2 afavoreixen el desenvolupament del CPL (White i cols., 1991; Mallet i Beninger, 1994; Abrahams i cols., 1998; Khroyan i cols., 1998). Altrament, si l'administració sistemàtica o intraaccumbens d'antagonistes D1 bloquegen el CPL induït per la cocaïna (Cervo i Samanin, 1995; Pruitt i cols., 1995; Nazarian i cols., 2004; Liao, 2008), hi poden existir resultats controvertits, pel que fa al bloqueig dels receptors D2. Alguns estudis han trobat que els antagonistes D2 no afecten al CPL induït per cocaïna (Cervo i Samanin, 1995; Pruitt i cols., 1995; Baker i cols., 1996; Nazarian i cols., 2004), mentre que altres han demostrat que els receptors D2 estan involucrats en la reinstauració del CPL induït per cocaïna (Badanich i Kirstein, 2012).

Diferents estudis han observat que l'estrés per derrota social altera la neurotransmissió DA (Tidey i Miczek, 1996; Cabib i cols., 2000; Isovich i cols., 2001; Razzoli i cols., 2011; Shimamoto i cols., 2015). Aquest tipus d'estrés s'ha relacionat amb l'augment d'alliberament de DA extracel·lular de l'escorça del nucli accumbens (NAccSh)(Tidey i Miczek, 1997; Piazza i Le Moal 1998; Han i cols., 2015; Holly i cols., 2015), en resposta a la cocaïna (Miczek i cols., 2011; Boyson i cols., 2014) o a la D-amfetamina (Han i cols., 2015). Pocs estudis han investigat els efectes de l'estrés per derrota social sobre els canvis en els receptors de la DA, on s'han obtingut resultats discrepants. No obstant això, s'ha trobat que la derrota social en l'adolescència bloqueja la disminució

de receptors D2 del NAcc induïda per amfetamina (Burke i cols., 2011), però incrementa els nivells dels receptors D1 en els nuclis caudat i putamen quan els animals són adults (Novick i cols., 2011). A més a més, s'ha observat en l'escorça prefrontal i l'amígdala, una disminució de l'expressió del receptor D1 (en ratolins derrotats en l'adultesa) (Huang i cols., 2016). En canvi, no s'han trobat diferències en els nivells de receptors D1 i D2 entre animals derrotats i control a l'escorça prefrontal, l'hipocamp i l'amígdala després una derrota crònica de 10 dies (Jin i cols., 2015). Així mateix, la infusió intraCPFm d'un antagonista del receptor dopaminèrgic D2 abans de cada episodi de derrota durant l'adolescència prevé les reduccions del *turnover* de DA a l'CPFm induït per la derrota en l'edat adulta primerenca (Watt i cols ., 2014). Sabem que l'estrés social agut, incrementa els efectes gratificants condicionat a la cocaïna i que l'administració d'un antagonista dels receptors dopaminèrgics D2 (raclopride) abans de l'experiència d'estrés, bloqueja este efecte sense afectar les propietats gratificants de la cocaïna en el CPL (Reguilon i cols., sota revisió).

4.2.2. Factors de Transcripció del Sistema DA

Les cèl·lules necessiten adaptar-se constantment a canvis ambientals, modificant els patrons de l'expressió gènica. Els mateixos gens estan presents en totes les cèl·lules d'una espècie, però no tots estan actius al mateix temps. En la modulació de l'expressió gènica és necessària la presència de seqüències reguladores i proteïnes capaces d'adreçar l'expressió dels gens. La regulació de l'expressió gènica és un dels esdeveniments més importants en el control del desenvolupament i de les respostes a canvis ambientals. Les proteïnes encarregades de la regulació de l'expressió gènica són conegudes com a factors de transcripció.

Els factors de transcripció són proteïnes que s'uneixen a l'ADN per controlar l'expressió de gens. Aquestes proteïnes reguladores estimulen o reprimeixen la taxa transcripcional dels seus gens diana a l'unir-se a regions promotores específiques. Açò activarà o desactivarà cascades de senyalització de gens. Els factors de transcripció tenen funcions fonamentals en gairebé

tots els processos biològics (desenvolupament, creixement i respostes a factors ambientals) i s'assumeix que tenen un paper preponderant en l'evolució de les espècies. Entre els factors de transcripció destacats trobem l'AMPc que respon a l'element vinculant en proteïnes (CREB) i al cFosB.

Nurr 1 forma part dels factors de transcripció que són necessaris per al desenvolupament i el manteniment del fenotip dopaminèrgic al llarg de la vida d'un individu (Smits i Smidt, 2006; Kadkhodaei i cols., 2009; Bissonette i Roesch, 2015). Alguns estudis han observat que Pitx és un factor essencial per a Nurr1, el qual actuaria com a potenciador, ja que intervindria en la modulació transcripcional d'alguns gens diana, com el TH (l'enzim tirosina hidroxilasa, implicant en la síntesi de catecolamines), el receptor dopaminèrgic D2, el DAT i el TVMA2 (transportador vesicular de monoamina 2) (Jacobs i cols., 2009; Reddy i cols., 2012; Bissonette i Roesch, 2015). Nurr 1 també funciona com un gen intermediari en el SNC, sent induït en resposta a una lesió focal (Honkaniemi i cols., 1995), i és expressat majoritàriament en regions del mesencèfal (Zetterström i cols., 1996; Saucedo-Cardenas i cols., 1998).

Aquells rosegadors que no expressen Nurr1 (−/−knockout), presenten una disminució en el desenvolupament de les neurones dopaminèrgiques del mesencèfal (mdDA), amb una reducció del 98% de la DA estriatal i una reducció del 30% en la NA (Zetterström i cols., 1996; Le i cols., 1999). Mentre que en humans s'ha observat que l'expressió de Nurr1 en les neurones de DA disminueix en els consumidors de cocaïna crònics (Bannon i cols., 2002).

La influència de Pitx3 en el desenvolupament de les neurones mdDA localitzades al SNC també s'ha observat en rosegadors (Smidt i cols., 1997; Burbach i cols., 2003; Hwang i cols., 2003; Nunes i cols., 2003; van den Munckhof i cols., 2003; Smidt i cols., 2004; Zhao i cols., 2004; Smits i cols., 2005). A més, s'ha suggerit mitjançant l'anàlisi d'una deficiència molecular i cel·lular de Pitx3d en el sistema mdDA, que els mecanismes adaptatius causen hiperestimulació del NAcc (Smits i cols., 2005). Aquells animals que sobreexpressen Pitx3-GFP (knock-in) mostren que durant el període de

desenvolupament, i en l'edat adulta hi ha una superposició gairebé del 100% en l'expressió de TH i Pitx3 dins de les neurones del mdDA (Zhao i cols., 2004; Maxwell i cols., 2005). Aquesta estreta relació indica un paper destacat per a Pitx3 en la diferenciació i manteniment de les neurones mdDA (Smidt i cols., 1997).

La via ERK/MAPK (*Extracellular Signal Regulated Kinase)* és una ruta de senyalització cel·lular central que connecta les nombroses senyals extracel·lulars als receptors de membrana i mitjançant l'activació de cascades de senyalització modula factors de transcripció, on finalment controla la regulació de gens (Sweatt, 2001; Kelleher i cols., 2004; Thomas i Huganir, 2004). L'ERK/ MAPK és una de les vies més conservades al llarg de l'evolució (Xia i cols., 1995; Schaeffer i Weber, 1999) i es caracteritza per ser activada en resposta a una àmplia varietat d'estímuls extracel·lulars, a més dels factors de creixement, com són el sèrum, les citoquines, el calci, les hormones i els neurotransmissors (Peyssonnaux i Eychène, 2001). En general, s'associa aquesta via amb el control de diferents processos cel·lulars fonamentals, incloent la proliferació cel·lular, supervivència, diferenciació i metabolisme (O'Neill i Kolch, 2004; Wellbrock i cols., 2004). A nivell molecular la senyalització d'aquesta via es regula mitjançant la fosforilació i desfosforilació per quinases i fosfatases (Johnson i Lapadat, 2002).

La via de l'ERK, comprén la fosforilació d'ERK1/2, els qual són unes proteïnes kinases que regulen la senyal extracel·lular. Aquest és un sistema que activa una cascada intracel·lular, la qual dota al sistema d'una gran ampliació de la senyal inicial. Una proporció notable d'ERK1/2 s'acumula en el nucli de la cèl·lula on regula la transcripció de diversos gens, donant una resposta immediata (Chen i cols., 1992; Gonzalez i cols., 1993; Lenormand i cols., 1993). També s'ha observat que c-Fos i CREB són substrats fosforilables per la via ERK/MPAK. La fosforilació del CREB (pCREB) incrementa la interacció d'aquest amb la maquinària transcripcional, regulant per tant la transcripció de gens, com és el cas del factor neurotròfic derivat del cervell (BDNF) (Davis, 1995; Grewal i cols., 1999; Vanhoutte i cols., 1999)(Figura 9).

El BDNF, una neurotrofina important per a la plasticitat sinàptica, és un dels candidats moleculars subjacents al desenvolupament de l'adaptació neoplàstica permanent a estressors de tipus social o d'altres tipus (Vasconcelos i cols., 2015). El BDNF modula les respostes cel·lulars i de plasticitat

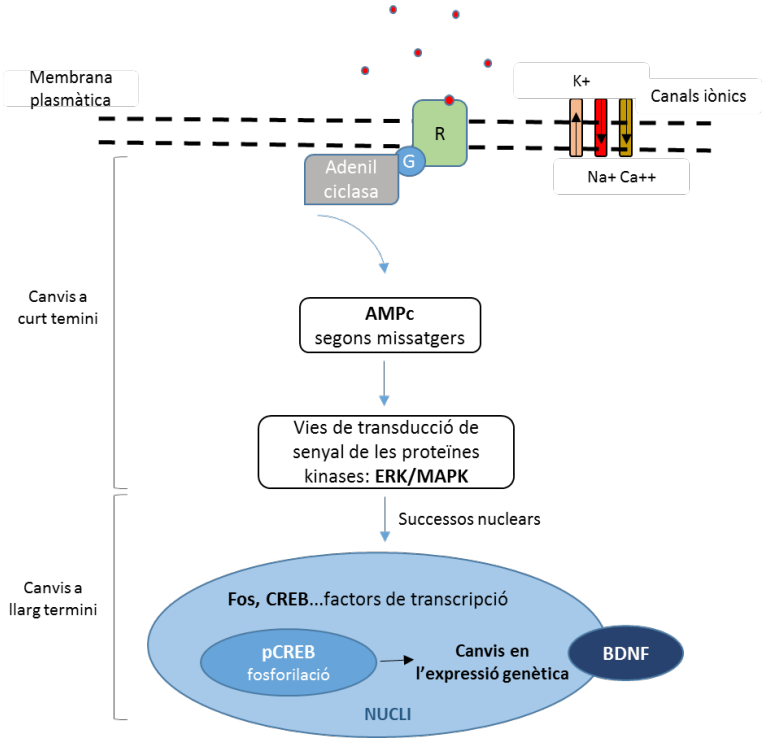

Figura 11. Canvis en l'expressió gènica. Cascades de senyalització que impliquen l'activació de factors de transcripció.(Carlezon i cols., 2005).

sinàptica a l'estrés i a l'exposició a drogues d'abús. L'estrés indueix canvis duraders en la senyalització del BDNF a les regions mesocorticolímbiques, les quals poden regular el circuit de la recompensa (Nikulina i cols., 2012).

Concretament, la derrota social indueix canvis en l'expressió del BDNF (Tsankova i cols, 2006; Krishnan i cols, 2007), degut a modulacions prèvies de la senyal extracel·lular regulada per la quinasa (ERK) i com a conseqüència en CREB (Wilkinson i cols., 2009). Aquesta neurotrofina té rols oposats: els rosegadors que experimenten la derrota social episòdica i aquells que mostren un comportament per subordinació crònic, augmenten

i disminueixen, respectivament, la resposta al BDNF (Miczek i cols., 2011). La disminució observada en aquells rosegadors amb un comportament de subordinació crònica, s'ha relacionat amb la supressió persistent de la recompensa a la cocaïna i la sacarina, les quals es poden relacionar amb l'anhedonisme (Duman i Monteggia, 2006), provocat pel deteriorament dels processos de gratificació (Miczek i cols., 2011).

A més, s'ha demostrat en regions com ara, l'hipocamp i l'amígdala basolateral (BLA), un paper funcional per al BDNF. En l'hipocamp, la regulació de l'expressió del BDNF per l'estrés crònic social no és clara, s'informa tant de reducció, d'augment com de la no expressió de canvis (Pizarro i cols., 2004, Tsankova i cols., 2006; Lagace i cols., 2010; Coppens i cols., 2011; Duclot i Kabbaj, 2013). A més, el BDNF a l'amígdala estimula l'adquisició del condicionament per derrota social en hàmsters (Taylor i cols., 2011). En l'exposició a encontres agonístics, es va trobar que els guanyadors tenien menors nivells del BDNF ARNm en la BLA, i nivells menors del BDNF ARNm en el gir dentat (DG) (Tsankova i cols., 2006; Yang i cols., 2016). No obstant això, els ratolins derrotats socialment, van mostrar un augment de l'expressió del BDNF sense cap canvi en pCREB o ERK. S'ha descrit que la derrota social activa BLA per mitjà de la PKA (proteïna kinasa A) (Yang i cols., 2016), la qual activa CREB per a la transcripció del BDNF, que a la vegada està involucrat en la potenciació a llarg termini (LTP) a través de la formació de noves sinapsis (Markham i cols., 2014). En general, aquests resultats indiquen que la senyalització del BDNF és probable que tinga efectes diferents en distintes àrees del cervell després de la derrota social.

Els psicoestimulants com ara la cocaïna interfereixen amb la senyalització dopaminèrgica mitjançant l'elevació dels nivells de DA en la sinapsi (Venton i cols., 2006; Seger, 2010). L'administració repetida de psicoestimulants pot causar canvis permanents en els nivells de DA, així com alteracions transitòries o permanents en l'expressió del BDNF i de la TH. D'aquesta manera, implicarà al BDNF i a la DA, en les neuroadaptacions a llarg termini induïdes per les substàncies d'abús (Pettit i cols., 1990; Fumagalli i cols, 2007; Schroeder i cols., 2008; McGinty i cols., 2010). El BDNF s'expressa

amb força en la via mesolímbica, essent aquesta via el substrat anatòmic per a les interaccions íntimes entre la cocaïna, la DA, i el BDNF (McCarthy i cols., 2012).

Les neurones piramidals de l'CPF són la principal font del BDNF en el caudat-putamen i NAcc (Altar i cols., 1997). El BDNF potència l'alliberament de DA en el NAcc a través de l'activació dels receptors TrkB (*tropomyosin receptor kinase B*) en les neurones dopaminèrgiques de l'ATV (Goggi i cols., 2003). La plasticitat sinàptica en l'ATV juga un paper essencial en les respostes de comportament primerenquess, així com en les adaptacions a llarg termini de l'exposició de drogues (Kauer, 2004). Els estudis mostren que l'ARNm i proteínic del BDNF estan regulats diferencialment en les diferents fases de l'addicció. Per exemple, l'exposició aguda a la cocaïna produeix un augment de l'expressió del BDNF en l'CPF, l'ATV, el cos estriat, i el NAcc com a resultat d'un augment transitori en pCREB (Le Foll i cols., 2005; Berglind i cols., 2007; Fumagalli, 2007; Graham i cols., 2007). Aquests canvis van acompanyats pels corresponents canvis epigenètics específics de la regió en el gen del BDNF. L'augment de l'expressió del BDNF després de l'exposició aguda a la cocaïna s'associa amb canvis induïts per la cocaïna en l'associació de pCREB amb el promotor del BDNF exó IV i modificacions transitòries de les histones que permeten la transcripció del gen del BDNF (LaPlant i Nestler, 2011). Aquests canvis aguts en l'expressió del BDNF poden representar les etapes inicials de la plasticitat, o la formació d'una "memòria a drogues", que propose les bases per a posteriors alteracions induïdes per les drogues en el comportament (Maze i Nestler, 2001).

4.3. Canvis Epigenètics

Les evidències experimentals han demostrat amb contundència que no únicament la genètica o aquells factors heretables porten a l'individu a presentar diferents comportaments, diferents respostes davant tractaments farmacològics i diferent susceptibilitat davant una patologia determinada (per exemple, l'addicció a drogues) (Figura 10). L'ambient també és un factor a destacar, aquest influeix en el genoma, i de manera conjunta contribuiran

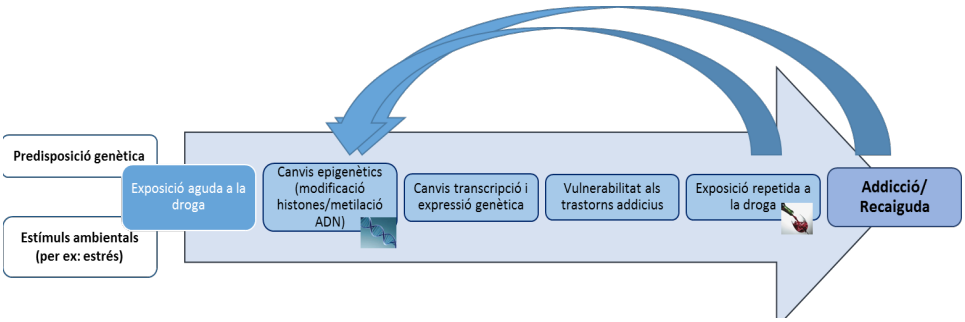

Figura 12. Relació entre genética, factors ambientals, exposició a substàncies d'abús i vulnerabilitat a desenvolupar addicció. Implicació dels canvis epigenètics. Imatge modificada de Robison i Nestler, 2011.

al desenvolupament d'un individu (Cloninger i cols., 1981).

Els factors epigenètics poden proporcionar el vincle entre els estímuls ambientals i l'heretabilitat genètica, és a dir, produeixen canvis estables en l'expressió gènica que són heretables, però no inclouran modificacions en la seqüència de l'ADN (Bird, 2007; Siegmund i cols. 2007; Tsankova i cols. 2007). Els mecanismes epigenètics tradueixen els estímuls ambientals per a promoure alteracions estables en l'estructura de la cromatina, la qual funciona per a activar o reprimir la transcripció de gens (Jaenisch i Bird, 2003).

Els canvis epigenètics es donen remodelant la cromatina, és a dir, modificant l'ADN, les histones i/o proteïnes independents a les histones. Una de les modificacions més destacada és l'acetilació de les histones (Peixoto i Abel, 2013), una modificació post-translacional dels residus de lisines a les cues amino terminals de les histones (Levenson i Sweatt, 2005; Sananbenesi i Fischer, 2009; Morris i cols., 2010). Aquest tipus de modificació s'associa amb un augment en els nivells de transcripció genètica (Chuang i cols., 2009; Sananbenesi i Fischer, 2009; Morris i cols., 2010; Lubin i cols., 2011; Trollope i cols., 2012), mentre que la hipoacetilació tindrà l'efecte oposat (Forsberg i Bresnick, 2001; Ito i Adcock, 2002). L'acetilació d'histones està controlada per dos tipus d'enzims: l'enzim histona acetiltransferasa (HAT), la qual facilita l'activació transcripcional (Bannister i Kouzarides, 1996; Ogryzko i cols., 1996;

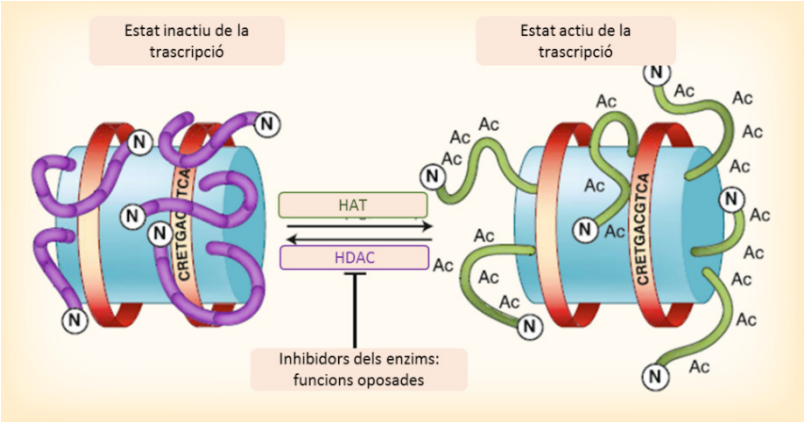

Figura 13. Activitat dels enzims acetiltrasnferasa (HAT) i la deacetilasa (HDAC). Funsions oposades dels inhibidors. Imatge modificada de McQuown i Wood, 2010.

veure revisió Roth i cols., 2001), i la histona deacetilasa (HDAC), la qual amb una acció oposada, augmenta la càrrega positiva i l'afinitat de les histones per adherir-se a l'ADN. L'ADN estarà carregat negativament, i per tant, silenciarà la transcripció genètica (Tsankova i cols., 2006) (Figura 11). D'altra banda, la inhibició de la HDAC permet l'expressió de gens. Diversos estudis han implicat aquesta inhibició en la formació de records associats al context de les drogues, records per descàrrega elèctrica i aprenentatge (Vecsey i cols., 2007; Malvaez i cols., 2010; McQuown i Wood, 2010) (Figura 12).

En el cas de les substàncies addictives, la cocaïna incrementa els nivells de H3K9/14ac o H4K5/8/12/16ac en els promotors del gen Fos, FosB, BDNF II, i CDK5 al cos estriat, gens implicats en la transcripció i regulats per la cocaïna (Kumar i cols., 2005). Les histones H3K9/14ac i H4K5/8/12/16ac són marcadors d'activació transcripcional (Kouzarides, 2007), implicant així l'acetilació d'histones en la regulació mediada per la cocaïna d'aquests gens (Rogge i cols., 2013). A més a més, s'ha demostrat en ratolins, que la inhibició de l'enzim HDAC (classe I), en el NAcc, bloqueja la plasticitat induïda per la cocaïna, alterant les adaptacions comportamentals que desencadena la cocaïna (Kennedy i cols., 2013). Altres enzims HDACs vinculats a l'addicció a la cocaïna són la HDAC2 i la HDAC11, els quals augmenten de manera significativa en l'encèfal després de l'autoadministració de cocaïna, en rates

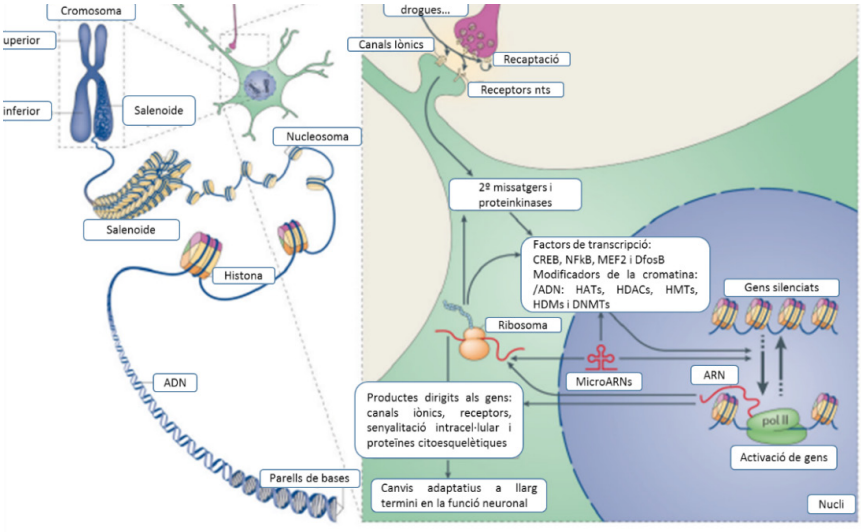

Figura 14. Mecanismes de regulació genètica i transcripcional davant l'exposició a diferents estímuls, com puguen ser les drogues o l'estrés. Imatge modificada de Wong i cols., 2011.

(Host i cols., 2011).

Són diversos els fàrmacs que s'utilitzen per a inhibir els enzims relacionats amb l'acetilació d'histones. Els inhibidors no específics de la HDAC (butirat de sodi o tricostatina A) administrats prèviament al CPL induït per cocaïna potencien els efectes conductuals d'aquest CPL (Kumar i cols., 2005; Renthal i Nestler, 2008; Hui i cols., 2010; Itzhak i cols., 2013; Raybuck i cols., 2013). En canvi, l'administració prèvia de curcumin (inhibidor de la HAT) 30 min abans del condicionament, inhibeix eixe CPL induït per cocaïna (Hui i cols., 2010). Aquests resultats suggereixen que el bloqueig mitjançant la inhibició de l'enzim HAT, impedeix els efectes conductuals, mentre que la inhibició de l'HDAC (el contrari) augmenta l'acetilació, promovent així canvis més profunds de comportament.

Un altre mecanisme que provoca canvis a nivell epigenètic és la metilació. Concretament, s'ha observat que la trimetilació de la histona H3 lisina 4 (H3K4Me3) està estretament associada amb la iniciació de la

transcripció, i sovint, es correlaciona la metilació de la histona H3 (K4) i acetilació d'histones, amb la competència transcripcional (Rice i Allis, 2001; Bernstein i cols., 2005).

Els mecanismes epigenètics són una causa subjacent de nombrosos estats de malaltia psiquiàtrica i poden intervindre en l'impacte de l'estrés sobre la funció dels circuits neuronals (Tsankova i cols., 2006 ; Sananbenesi i Fischer, 2009; Nelson i Monteggia, 2011). Si bé s'estan estudiant, recentment, les respostes a llarg termini del comportament, després de la derrota social (Hammels i cols., 2015; García-Pardo i cols., 2015), s'ha centrat poca atenció als canvis epigenètics induïts per aquest tipus d'estrés. La modificació epigenètica induïda per la derrota social crònica, s'ha abordat en diversos estudis. Els augments en l'acetilació de l'histona H3 ha estat la troballa més freqüent. Per exemple, després de 30 min, 24 hores o 10 dies d'estar exposat a estrés per derrota social crònica, l'augment de l'acetilació de H3/K14 s'ha descrit en el NAcc, CPFm, rafe dorsal o hipocamp (Covington i cols., 2009; Hollis i cols., 2010, Hinwood i cols., 2011; Hollis i cols., 2011; Kenworthy i cols, 2014). Aquests canvis semblen estar influenciats per les variacions interidividuals (com puga ser la resposta a la novetat), ja que l'augment de l'acetilació de l'H3 després de la derrota social només s'ha observat en aquells rosegadors que responen amb més intensitat (Hollis i cols., 2011). Rosegadors menys resistents a l'estrés també van mostrar majors nivells d'acetilació de la histona H3 (Kenworthy i cols., 2014).

No obstant això, s'han trobat resultats controvertits respecte la histona H4, encara que la majoria dels estudis no han observat canvis en aquesta proteïna després de la derrota social, altres han trobat una major acetilació de la histona H4/K12 en aquelles rates menys resistents a l'estressor (Tsankova i cols, 2006; Hollis i cols, 2010, 2011 ; Kenworthy i cols, 2014). Altres canvis epigenètics s'han descrit després de la derrota social crònica, com disminucions dels nivells globals de la dimetilació d'H3K9 (H3K9me2) en el NAcc només en ratolins susceptibles (Covington i cols., 2011). No obstant això, H3-K27me2 s'incrementa un mes després de la cessació de l'estrés crònic per derrota social, en els promotors del BDNF en l'hipocamp (Tsankova

i cols., 2006).

Diferents enzims que controlen aquests processos també van ser alterats després de la derrota social. Una disminució en els nivells de l'enzim HDAC en NAcc es va observar 24 hores després de l'última derrota, i la infusió continuada de MS-275 (100 M) o Saha (100 M) al NAcc van revertir l'evitació social induïda per l'estrés en ratolins derrotats i reinstaurà la quantitat de temps que els animals dedicaven a interactuar socialment (Covington i cols., 2009; Covington i cols., 2011).

4.4. Neuroinflamació

El terme neuroinflamació fa referència generalment a la cascada de successos que genera modificacions cel·lulars (a nivell de la micròglia, astroglia i cèl·lules immunes infiltrades) i moleculars (citoquines proinflamatòries, quimioquines) en forma de resposta immune dins del SNC.

El SNC és el més sensible de l'organisme. La senyalització química i elèctrica entre les neurones requereix d'un microambient controlat per a una adequada funcionalitat. Per això, per a mantindre la homeòstasis, existeixen barreres que separen el fluid intersticial cerebral de la sang i regulen de manera precisa el pas de les substàncies (Abbot i cols., 2010).

Actualment sabem que en la majoria dels vertebrats, les barreres selectives que separen la sang del teixit nerviós i dels fluid intersticial es localitzen en el SNC, totes elles restringeixen el pas de substàncies entre la sang i el teixit on es troben (Banks, 1995). De totes elles, la barrera hematoencefàlica (BHE) establerta a nivell de l'endoteli vascular, és aquella que constitueix la major i més extensa superfície de control dels fluïts moleculars directament entre la sang i les cèl·lules del SNC (Abbott, 2013). Aquesta barrera està present en totes les regions cerebrals a excepció d'aquelles regulades pel sistema nerviós autònom i les glàndules endocrines (Cardoso i cols., 2010).

La BHE permet regular el flux de substàncies entre la sang i el parènquima cerebral al limitar el transport paracel·lular, establint el transport

transcel·lular com la via de pas majoritària. Aquesta disposa d'un mecanisme de transport especialitzat que permet l'entrada de nutrients requerits i afavoreix l'expulsió al torrent sanguini de metabòlits o substàncies tòxiques (Abbott, 2013).

La BHE que existeix a nivell de l'endoteli especialitzat pels vasos sanguinis cerebrals constitueix una barrera física de difusió selectiva. A més de l'endoteli, diversos tipus de teixit cel·lular, i no cel·lular, participen en el

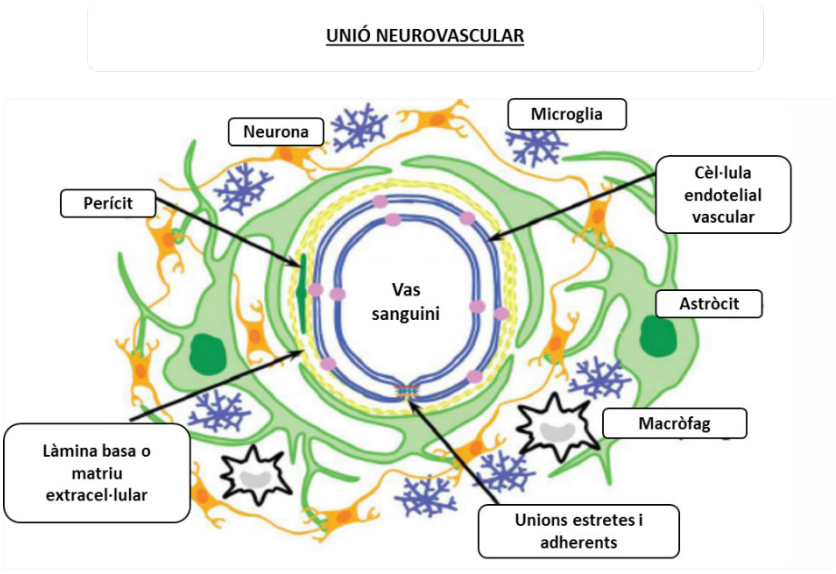

Figura 15. Esquema de la unitat neurovascular amb els components cel·lulars principals. Imatge modificada de Willis i cols., 2011.

manteniment i integritat de la barrera formant una segona línia de defensa. La comunicació existent entre grups de neurones i cèl·lules glials amb l'endoteli vascular en relació amb la regulació del fluid cerebral, s'anomena unió vascular, imprescindible per a la formació de la BHE (Figura 13) (Hawkins i Davis, 2005; Verkman, 2005; Abbott i cols., 2006; Obermier cols., 2013).

Les cèl·lules endotelials del sistema nerviós presenten unions estretes i adherents que uneixen espais entre les cèl·lules adjacents i seran les responsables d'actuar com a barrera per a conferir una baixa permeabilitat

paracel·lular per a les substàncies polars i alta resistència elèctrica als vasos, a més de restringir el transport únicament a vies de pas específiques (Gloor i cols., 2001; Hawkins i Davis, 2005; Daneman, 2012). Les unions estretes estan formades per proteïnes integrals i adaptadores citosòliques. Les proteïnes integrals formaran els contactes íntims entre les cèl·lules, on trobem, molècules d'adhesió (JAM, Junctional Adhesion Molecules), claudina, la qual és imprescindible per a la formació de les unions estretes, sent la més expressada en l'endoteli la claudina-5, i ocludina (Hawkins i Davis, 2005; Abbott i cols., 2006; Luissint i cols., 2012). Les proteïnes adaptadores citosòliques són essencials per a la formació d'un complex multimol·lecular a l'ancorar les proteïnes integrals amb l'actina del citoesquelet endotelial (Gloor i cols., 2001; Abbott i cols., 2006; Daneman, 2012). La baixa expressió de la claudina-5 es relaciona amb la ruptura de la BHE i amb un increment de la permeabilitat (Hawkins i Davis, 2005; Cardoso i cols., 2010 Luissint i cols., 2012). En canvi, davant la pèrdua d'ocludina, la resta de proteïnes de les unions estretes poden mantindré la funcionalitat de la BHE (Cardoso i cols., 2010). Diversos mecanismes, com l'estrés oxidatiu, mediadors inflamatoris (TNF-α, IL-1β) i agents infecciosos (VIH) entre altres, poden desencadenar mecanismes de senyalització que permeten incidir sobre les proteïnes de les unions estretes, induint la dissociació i incrementant la permeabilitat de la BHE (Persidsky i cols., 2006; Cardoso i cols., 2010).

La làmina basal és el nom que rep la matriu extracel·lular especialitzada, íntimament associada a l'epiteli. Dintre de la unió neurovascular, la làmina basal constitueix l'únic component no cel·lular de la BHE (Obermeier i cols., 2013). Aquesta làmina és secretada per les cèl·lules endotelials, astròcits i perícits a qui proporciona suport físic (Hallmann i cols., 2005; Hawkins i Davis, 2005). A més de la seua funció estructural, la làmina basal intervé en la regulació del comportament de les cèl·lules que es secreten i contribueix a la baixa permeabilitat de la BHE a l'impedir el moviment de molècules i la migració de leucòcits i cèl·lules tumorals. Es compon principalment per proteïnes estructurals com la laminina i col·lagen IV, els qual generaran una xarxa en forma de làmina que envolta el vas sanguini (Hohenester i Yurchenco,

2013). L'alteració de les proteïnes que componen la làmina basal tenen com a conseqüència l'alteració de les unions estretes i de la integritat de la BHE (Kalluri, 2003; Hallmann i cols., 2005).

Existeixen altres elements que formaran part d'aquesta barrera tan complexa com són els astròcits, els quals secreten components en la làmina basal i participen en la inducció i manteniment de les unions estretes, així com modulen els sistemes de transport endotelial i el to vascular amb la finalitat d'afavorir l'adquisició de substrats energètics. Així mateix, també hi formen part els perícits, els quals dintre de la BHE, participen en la síntesis de la làmina basal i indueixen la distribució localitzada dels astròcits al voltant dels vasos sanguinis; essencials per a la formació, maduració i manteniment de la BHE. Les neurones, micròglia perivascular i leucòcits circulants, aquests últims, modularan la BHE i poden interaccionar amb les cèl·lules endotelials i contribuir a les propietats de la barrera (Cardoso i cols., 2010). En general, qualsevol alteració en l'expressió i/o funcionalitat dels components de les unions estretes i la làmina basal, o de les cèl·lules que constitueixen la unió neurovascular, poden comprometre la integritat de la barrera incrementant la permeabilitat (Pun i cols., 2009; Daneman, 2012).

Recentment, la interacció entre les drogues d'abús i la funció cerebrovascular ha rebut un interés significatiu (Ho i cols., 2009; Egleton i Abbruscato, 2014; O'Shea i cols., 2014), ja que s'han relacionat amb la neuropatologia. L'evidència general suggereix que l'abús de psicoestimulants té influència a llarg termini sobre la reologia i el dinamisme vascular cerebral (Polesskaya i cols., 2011). Concretament, la cocaïna exhibeix interaccions específiques cel·lulars a l'endoteli, el sistema immune i el sistema neuroendocrí, les quals convergeixen amb la disfuncionalitat de la BHE (Fiala, 1998; Zhang, 1998). La major conseqüència a la resposta inflamatòria produïda per la cocaïna i la qual disminueix la integritat de la BHE, és un augment de la filtració de cèl·lules immunes des de la circulació sanguínia (Dhillon, 2008; Gandhi, 2010; Yao, 2011). La cocaïna indueix l'augment de mediadors inflamatoris, així com l'expressió de les molècules d'adhesió ICAM-1 (intercel·lulars) i VCAM-1 (vasculars) (Gan i cols., 1999), modulant

al mateix temps la inducció transcripcional i translacional de la proteïna de membrana ALCAM (*Activated Leukocyte Cell Adhesion Molecule)* en cultius de cèl·lules endotelials microvasculars del cervell humà (Yao i cols., 2011). A més, la cocaïna augmenta l'adhesió dels leucòcits a les cèl·lules endotelials i, posteriorment, augmenta la transmigració de leucòcits a través de la paret dels vasos sanguinis cerebrals, en particular, en condicions inflamatòries (Gan i cols., 1999; Yao i cols., 2011). En conjunt, aquests resultats constitueixen una evidència on la cocaïna afectaria la integritat de la BHE.

Encara que la permeabilitat de la BHE s'ha associat a diverses alteracions neurològiques, fins al moment no hi ha resultats sobre els efectes de l'estrés social en la permeabilitat d'aquesta barrera. En canvi, existeix l'evidència de com la DSR modifica l'expressió d'enzims antioxidants i dels nivells de marcadors d'estrés oxidatiu (Patki i cols., 2014) i indueix neuroinflamació (Wohleb i cols., 2011; Hanke i cols., 2012; Wohleb i cols., 2013). En diferents models, s'ha comprovat un augment dels nivells proinflamatoris de citoquines (Shaftel i cols., 2007) o de la formació de radicals lliures (Gasche i cols., 2001). Per aquesta raó, la integritat de la BHE es pot veure alterada com a conseqüència de la fosforilació de les vies de senyalització de les proteïnes quinases activades per mitogen (MAPKs, *Mitogen-Activated Protein kinases*). A més, les metal·loproteïnases (MMP) implicades en tots aquells processos fisiològics que requereixen la modificació de la matriu extracel·lular i remodelació tissular (l'angiogènesis, migració cel·lular, regeneració axonal, mielinització i plasticitat sinàptica) es poden veure activades (Rosenberg, 2009; Tian i Kyriakides, 2009; Katsu i cols., 2010).

2. AIMS and HYPOTHESIS

2. AIMS and HYPOTHESIS

As we have seen in the previous section, further research is essential to understand the role of stress in addictive processes. The main objective of the present Doctoral Thesis is to identify the neurobiological substrates that lie behind the increase in drug seeking (for example, cocaine) induced by social stress. To pursue this objective we used as the principal methodology ASD/RSD and its effects on the acquisition, expression and reinstatement of CPP, together with SA. The knowledge acquired will undoubtedly contribute to the development of new pharmacotherapies for reducing the rates of relapse, one of the most important challenges to current research into drug addiction.

Study 1: The aim of the study was to evaluate the influence of acute social defeat on the conditioned rewarding and reinstating effects of different doses of cocaine using the CPP paradigm. We studied the effects of ASD on the acquisition, extinction and reinstatement of cocaine-induced CPP in adolescent and adult mice.

Hypothesis:

- Animals exposed to the ASD (adolescents and adults) will experience the reinforcing effects of a subthreshold cocaine dose (1 mg / kg) and develop CPP.
- Animals exposed to the ASD (adolescents and adults) will need more sessions for the CPP induced by 1mg/kg of cocaine to be extinguished.
- Animals exposed to the ASD (adolescents and adults) will show higher levels of corticosterone than non-defeated mice.
- When conditioned with 25 mg/kg of cocaine, CPP will be reinstated in animals exposed to ASD (adolescents and adults) with lower priming

doses of cocaine than non-stressed mice.

- Adolescent mice exposed to ASD will exhibit different corticosterone levels to adult defeated animals.
- Adolescent mice exposed to ASD will be more sensitive to the rewarding effects of cocaine than defeated adult animals.

Study 2: This study aimed to determine the long-term effects of RSD experienced during adolescence on the rewarding effects of cocaine using the CPP and SA procedures. As a second aim, we studied whether RSD induces a neuroinflammation response, evaluating the structure and function of the BBB in the NAcc and hippocampus.

Hypothesis:

- Animals exposed to the RSD will acquire CPP induced by a subthreshold cocaine dose (1 mg / kg).
- When conditioned with 25 mg/kg, animals exposed to the RSD will display a reinstated CPP with lower priming doses of cocaine than non-stressed mice.
- Animals exposed to the RSD will acquire cocaine-SA faster than those in the exploration group.
- Animals exposed to the RSD will be more motivated to seek cocaine in the SA (will discriminate the active hole faster).
- Animals exposed to RSD will display a disruption of the BBB structure and permeability in the NAcc and hippocampus.

Study 3: The aim of the present study was to evaluate the influence of DA receptors (DR1 and DR2) on the long-term effects of RSD on the conditioned rewarding and reinstating effects of cocaine using the CPP

procedure.

Hypothesis:

- Pretreatment with a D1R antagonist (SCH 23390) prior to RSD will block the development of the CPP induced by a subthreshold dose of cocaine.
- Pretreated with a D2R antagonist (raclopride) prior to RSD will block the development of the CPL induced by a subthreshold dose of cocaine.
- Exposure to RSD will alter the levels of D1R and D2R for up to three weeks after the last social defeat.

Study 4: The aim of this study was to demonstrate that the long-term effects of RSD on the conditioned rewarding effects of cocaine are mediated by epigenetic modifications.

Hypothesis:

- Animals exposed to RSD will show an increase in acethylation levels in histone H3(K9), H4(K12) and H3K4me3.
- Animals exposed to RSD will show changes in their HAT and HDAC enzyme levels.
- Animals pretreated with the HAT enzyme inhibitor (Curcumin) before each RSD will not develop a CPP induced by a subthreshold cocaine dose.
- Animals pretreated with the HDAC enzyme inhibitor (Valproic acid) prior to each RSD will display an enhanced CPP induced by a subthreshold dose of cocaine.

Study 5: The aim of this study was to evaluate if RSD during adolescence or adulthood induces long-term modifications of DA transcription factors and the BDNF.

Hypothesis:

- Exposure to RSD during adolescence or adulthood will change Nurr1 and Pitx3 levels in the VTA three weeks after the last social defeat.
- Exposure to RSD during adolescence or adulthood will modify ERK, CREB and BDNF protein levels in the DG and BLA.

Study 6: The aim of this research was to assess how social dominance and patterns of agonistic behavior are determined by genetics but also influenced by experience. Four different strains of mice were compared using the same procedure employed to produce RSD.

Hypothesis:

- All the intruder mice, irrelevant of their strain, will be defeated and will show submissive behaviors during the agonistic encounters.
- All the resident mice, irrelevant of their strain, will display threat and attack behaviors during the agonistic encounter.
- Depending on the strain, there will be different levels of aggression among the resident mice.
- Submissive and avoidance behaviors exhibited by intruder animals will correlate with the aggression shown by resident mice.
- The experience of being exposed to repeated agonistic encounters will mediate aggression and submissive/avoidance behaviors depending on the strain of mice.

Study 7 (Tufts University): This experiment focused on the role of CRF and its receptors in the VTA on the effects of social defeat on cocaine seeking after forced abstinence. This model has been proposed as a translational model of relapse.

Hypothesis:

- Animals exposed to RSD will receive more cocaine infusions during the SA procedure.
- Animals exposed to RSD after a period of forced abstinence will be more motivated to seek cocaine.
- Animals exposed to RSD but pretreated with a CRF-R1 or a CRF-R2 antagonist (administrated into the VTA) before being reintroduced to the context previously associated with cocaine will show less cocaine seeking.

Study 8 (Tufts University): This study aimed to determine whether individual differences in stress-escalated cocaine SA can be explained by differences in the physiological response to social defeat (measuring plasma corticosterone).

Hypothesis:

- Animals exposed to RSD and considered as fast rats (shorter latency to enter the threatening environment) will express higher levels of corticosterone after each social defeat in comparison to slow and control rats.
- Animals not exposed to stress will need more time to acquire SA.
- Animals exposed to RSD and considered fast rats will acquire SA more rapidly than slow rats.

3. RESULTS

STUDY 1

Acute social defeat stress increases the conditioned rewarding effects of cocaine in adult but not in adolescent mice

S. Montagud-Romero, M.A. Aguilar, C. Maldonado, C. Manzanedo, J. Miñarro, M. Rodríguez-Arias

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Acute social defeat stress increases the conditioned rewarding effects of cocaine in adult but not in adolescent mice

S. Montagud-Romero, M.A. Aguilar, C. Maldonado, C. Manzanedo, J. Miñarro, M. Rodríguez-Arias *

Unidad de Investigación Psicobiología de las Drogodependencias, Departamento de Psicobiología, Facultad de Psicología, Universidad de Valencia, Spain

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ABSTRACT

Stressful experiences modify activity in areas of the brain involved in the rewarding effects of psychostimulants. In the present study we evaluated the influence of acute social defeat (ASD) on the conditioned rewarding effects of cocaine in adolescent (PND 29–32) and adult (PND 50–53) male mice in the conditioned place preference (CPP) paradigm. Experimental mice were exposed to social defeat in an agonistic encounter before each session of conditioning with 1 mg/kg or 25 mg/kg of cocaine. The effects of social defeat on corticosterone levels were also evaluated. Adult mice exposed to ASD showed an increase in the conditioned reinforcing effects of cocaine. Only these mice developed cocaine-induced CPP with the subthreshold dose of cocaine, and they needed a higher number of extinction sessions for the 25 mg/kg cocaine-induced CPP to be extinguished. In adolescent mice, on the other hand, ASD reduced the conditioned reinforcing effects of cocaine, since CPP was not produced with the lower dose of cocaine and was extinguished faster when they were conditioned with 25 mg/kg. Adult mice exposed to social defeat displayed higher levels of corticosterone than their controls and adolescent mice. Our results confirm that the effect of social defeat stress on the acquisition and reinstatement of the CPP induced by cocaine varies depending on the age at which this stress is experienced.

1. Introduction

Addiction is a chronic multifactorial relapsing disorder that is a result of an interaction of biological and environmental factors (Ellenbroek et al., 2005; Enoch, 2006). Research has demonstrated that stress is a risk factor for the initiation, maintenance and escalation of drug consumption and for relapse after periods of detoxification (Koob, 2012; Logrip et al., 2011; Sinha et al., 2011). In fact, stressful experiences modify the activity of brain areas involved in the rewarding effects of psychostimulants (Belujon and Grace, 2011; Koob, 2010; Sinha, 2008). In addition, activation of brain stress systems seems to be a key element of the negative emotional state produced by dependence, which drives drug-seeking through negative reinforcement mechanisms (Koob, 2010).

Similar to repeated administration of psychostimulants and other drugs of abuse, repeated exposure to stress can heighten sensitivity to drug-induced psychomotor stimulation. In some cases, this sensitized behavioral response correlates with enhanced drug-induced DA and glutamate responses in the nucleus accumbens (NAcc) and increased cellular activation of reward-associated brain regions (Deroche et al., 1995; Miczek et al., 2004; Nikulina et al., 2004; Pacchioni et al., 2007). For example, exposure to a brief, intermittent episode of social defeat stress activates mesolimbic DA pathways that project from the ventral tegmental area (VTA) to the NAcc and medial prefrontal cortex (PFC) (Anstrom et al., 2009; Di Chiara and Imperato, 1988; Tidey and Miczek, 1997). This suggests that environmental stressors produce long-term alterations in the function of brain reward pathways in the same way as drugs of abuse do (Quadros and Miczek, 2009).

Stress can be produced through forced exposure to events or conditions that are normally avoided by an animal (Piazza and Le Moal, 1998). Different types of stressors have been used in studies exploring the role of stress in drug addiction in animal models (Lu et al., 2003; Aguilar et al., 2009). Typically, physical stressors consist of exposing subjects to an aversive environmental event, such as foot shock, restraint or tail pinch (Rodríguez-Arias et al., 2013). On the other hand, there are different social experiences that induce social stress in animals, such as maternal deprivation, social isolation, crowding or social defeat (Lu et al., 2003; Miczek et al., 2008; Ribeiro Do Couto et al., 2009; Shaham et al., 2003). All these stimuli trigger nervous and hormonal mechanisms of stress that lead to neurochemical and behavioral adaptations, making animals more prone to drug-seeking (Goeders, 2002; Logrip et al., 2012; Marinelli and Piazza, 2002; Miczek et al., 2008; Moffett et al., 2007). Social defeat stress is a naturalistic model of stress that involves an agonistic encounter between conspecifics and is thought to represent a stressor of ecological and ethological validity in mice (Tornatzky and Miczek, 1993). It induces robust physiological, behavioral and endocrine responses, including circadian rhythm

* Corresponding author at: Departamento de Psicobiología, Facultad de Psicología, Universitat de Valencia, Avda. Blasco Ibáñez, 21, 46010 Valencia, Spain. Tel.: +34 96 386 46 37; fax: +34 96 386 46 68.
E-mail address: marta.rodriguez@uv.es (M. Rodríguez-Arias).

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disturbances, avoidance behavior and elevated levels of corticosterone (Meerlo et al., 2002; Lumley et al., 2000). In these circumstances, social animals develop dominance-based social hierarchies based on agonistic interactions (Huntingford and Turner, 1987). Experimentally, the effect of social stress is often studied using dyads through which one dominant individual and one subordinate individual are established. When animals are defeated, they exhibit physiological and behavioral changes such as submissive behavior, ACTH and increased corticosterone levels (Martí-Carbonell et al., 1992), as well as an increase of dopamine (DA) release in the NAcc and the PFC (Tidey and Miczek, 1996).

In animal models, the relation between stress and psychostimulant addiction has been evaluated mainly by means of the self-administration (SA) paradigm. Four brief episodes of social defeat stress over the course of one week have been shown to increase the acquisition of cocaine SA at a low dose (Tidey and Miczek, 1997; Haney et al., 1995). Under conditions of unlimited access to cocaine for 24 h (binge), socially defeated rats self-administer cocaine with shorter inter-infusion intervals and in greater quantities (Covington and Miczek, 2005). Once cocaine SA is established, brief episodes of social defeat stress prior to each experimental session can significantly increase the rate of drug intake, particularly at lower unit doses (Miczek and Mutschler, 1996). While defeat immediately prior to operant sessions enhances cocaine SA, increasing the delay between exposure to social defeat and cocaine self-administration training dissipates the effects of stress on acquisition (Covington and Miczek, 2001, 2005). More recently, it has been demonstrated that intermittent experience of defeat increases cocaine-taking and response rates during binges (Covington et al., 2008).

The conditioned place preference (CPP) paradigm offers a simple method of assessing the conditioned reward induced by different stimuli (Bardo and Bevins, 2000; Tzschentke, 2007). It has been widely used to study the conditioned rewarding effects of addictive drugs (Aguilar et al., 2009), although only one study has evaluated the effects of social defeat stress on cocaine-induced CPP. In the report in question, social defeat stress-exposed mice (12–16 weeks old) conditioned with cocaine exhibited significant potentiation of CPP for the drug-paired chamber with respect to unstressed mice (McLaughlin et al., 2006). A more recent study investigated the effects of social defeat inflicted on male rats during adolescence on the conditioned place preference induced by amphetamine in adulthood (Burke et al., 2011). Adolescent social defeat increases preference for amphetamine-paired cues in adulthood, suggesting that social stress has a great impact on drug behavior in later life.

Age at the time of drug exposure and/or stressful experiences may be factors involved in cocaine dependence. Adolescence is a highly vulnerable developmental period concerning exposure to drugs of abuse (Schneider, 2008; Rodriguez-Arias and Aguilar, 2012). The risk-taking behavior of adolescents is thought to be related to the fact that their decision-making capacity is more vulnerable to disruption by stress (Sturman and Moghaddam, 2011). Periadolescent rodent (PND 30–45) exhibits a unique psychopharmacological profile, showing hyporesponsivity to the acute effects of psychostimulant agents and enhanced behavioral sensitization to repeated and intermittent drug exposure (Adriani and Laviola, 2000; Bolanos et al., 1998; Laviola et al., 1995). Behavioral experiments in laboratory animals have revealed that drugs of abuse are generally more rewarding and less aversive for adolescents than for adults (for review see Schramm-Sapyta et al., 2009), and that the former age group is more vulnerable to stressors than younger or older counterparts (Stone and Quartermain, 1997; Vazquez, 1998; Buwalda et al., 2011). Social stress during adolescence appears to reduce mesocortical DA levels, thus undermining maturation of cortical DA through D2 dopamine receptor regulation of DA synthesis or glucocorticoid-facilitated pruning of cortical DA fibers (Burke and Miczek, 2014).

The aim of the present study was to evaluate the influence of social defeat on the conditioned rewarding and reinstating effects of different doses of cocaine in the CPP paradigm. We studied the effects of acute social defeat (ASD) on the acquisition, extinction and reinstatement of cocaine-induced CPP in adolescent and adult mice. To date, no study has systematically evaluated the influence of age on the effects that social defeat exerts on the conditioned rewarding effects of cocaine. There are two main variations of this model according to the paradigm of aggression used: resident/intruder encounter or agonistic encounter in a neutral environment. In the latter model, which we have used in the present study, the experimental animal suffers social defeat in an aggressive social encounter with a conspecific of equal age and body weight during a 10-minute period (Ribeiro Do Couto et al., 2006), immediately before being placed in the CPP apparatus. Aggressive opponents are housed individually for a month prior to the encounter, since isolation heightens aggression in mice (Rodríguez-Arias et al., 1998). In all experiments, after the initial reinstatement of CPP, mice underwent extinction of CPP once more. The priming effect of progressively lower doses of cocaine was tested in order to evaluate the sensitivity of animals to the reinstating effects of this drug when associated with a specific context.

2. Material and methods

2.1. Animals

Male OF1 mice (Charles River, Barcelona, Spain) arrived at our laboratory at 21 (n = 90) or 42 (n = 86) days of age. All mice (except those used as aggressive opponents) were housed in groups of four in plastic cages (25 × 25 × 14.5 cm) for 8 days before the experiments began. To reduce their stress levels in response to experimental manipulations, mice were handled for 5 min per day on each of the 3 days prior to initiation of the CPP. Aggressive opponents were individually housed in plastic cages (23 × 13.5 × 13 cm) for a month prior to experiments in order to heighten aggression (Rodríguez-Arias et al., 1998) (30 adult mice). All mice were housed under the following conditions: constant temperature; a reversed light schedule (white lights on 19:30–07:30 h); and food and water available ad libitum, except during behavioral tests. All procedures were conducted in compliance with the guidelines of the European Council Directive 2010/63/UE regulating animal research and were approved by the local ethic's committees.

2.2. Drugs

Animals were injected intraperitoneally with 1 or 25 mg/kg of cocaine hydrochloride (Laboratorios Alcaliber, Madrid, Spain) in a volume of 0.01 ml/g of weight. Control groups were injected with physiological saline (NaCl 0.9%), which was also used to dissolve the drugs. The doses of cocaine were selected on the basis of previous studies showing that 1 mg/kg is a threshold dose (Vidal-Infer et al., 2012; Arenas et al., 2014; Montagud-Romero et al., 2014) and that 25 mg/kg induces a strong CPP that is reinstated after priming with 12.5 mg/kg of cocaine (Rodríguez-Arias et al., 2009).

2.3. Apparatus

For place conditioning, we employed eight identical Plexiglas boxes with two equally-sized compartments (30.7 cm long × 31.5 cm wide × 34.5 cm high) separated by a gray central area (13.8 cm long × 31.5 cm wide × 34.5 cm high). The compartments had different colored walls (black vs white) and distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed the recording of the position of the animals and their crossings from one compartment to the other. The equipment was

controlled by three IBM PC computers using MONPRE 2Z software (CIBERTEC, SA, Spain).

2.4. Conditioned place preference procedure

2.4.1. Acquisition

Place conditioning, which consisted of three phases, was carried out during the dark cycle following a procedure that was unbiased in terms of initial spontaneous preference (Manzanedo et al., 2001). During the first phase—or preconditioning (Pre-C)—mice were allowed access to both compartments of the apparatus for 900 s per day on 3 consecutive days. On day 3, the time spent in each compartment was recorded. Animals showing a strong unconditioned aversion (less than 33% of session time; i.e. 250 s) or preference (more than 67% of the session time; i.e. 650 s) for any compartment were discarded from the rest of the study. In each group, half of the animals received the drug or vehicle in one compartment while the other half received it in the other compartment. ANOVA showed that there were no significant differences between the time spent in the drug-paired and the vehicle-paired compartments during the Pre-C phase. In the second phase (conditioning), which lasted 4 days, animals were conditioned with cocaine or saline. An injection of physiological saline was administered before confining the mice to the vehicle-paired compartment for 30 min. After an interval of 4 h, the animals received cocaine immediately prior to confinement to the drug-paired compartment for a further 30 min. The central area was made inaccessible by guillotine doors during conditioning. In the third phase—or postconditioning (Post-C)—which took place on day 8, the guillotine doors separating the two compartments were removed, and the time spent in each compartment by the untreated mice was recorded during a 900-s observation period. The difference in seconds between the time spent in the drug-paired compartment during Post-C and Pre-C tests is a measure of the degree of conditioning induced by the drug. If this difference is positive, then the drug has induced a preference for the drug-paired compartment, while the opposite indicates an aversion.

2.4.2. Extinction of CPP

All groups in which CPP was confirmed were subsequently exposed to the extinction procedure. Animals underwent two extinction sessions per week in which they were placed in the apparatus for 900 s until the time spent in the drug-paired compartment was similar to that of the Pre-C phase. CPP is considered to be extinguished when there is no significant difference between the time spent in the drug-paired compartment in the extinction session and that spent in the same compartment during Pre-C (Student's t test).

2.4.3. Reinstatement of CPP

The reinstatement tests were the same as for Post-C (free ambulation for 900 s) and were performed only in the groups that showed CPP. In the reinstatement phase, half the dose received during the conditioning phase (0.5 or 12.5 mg/kg) was administered in a different room to that of the conditioning sessions, 15 min before the test. The aim of this procedure was to administer the drug in a non-contingent way with respect to conditioning, so that the animal did not associate the contextual cues of the experimental room with the drug.

After this first reinstatement test, the groups that demonstrated reinstatement—i.e. a positive significant difference between the time spent in the drug-paired compartment in the reinstatement and the last extinction tests (confirmed with a Student's t test)—were re-tested until a new extinction was confirmed. The following day, the effects of the priming (a quarter of the dose used for conditioning) on reinstatement of place preference were evaluated following the procedure described previously. This procedure was repeated with progressively lower priming doses until a non-effective priming injection was determined.

2.5. Procedure of acute social defeat encounters

To induce social defeat stress, the animals underwent an agonistic encounter for 10 min immediately before being confined to the drug-paired compartment. These encounters took place in a transparent plastic cage (23 × 13.5 × 13 cm) that was neutral to both animals. The aggressive opponent had previous fighting experience and had screened positive for a high level of aggressive behavior. Mice in the social defeat (SD) groups exhibited avoidance/flee and defensive/submissive behaviors after suffering aggression (threat and attack) from an opponent, as observed in previous studies (Ribeiro Do Couto et al., 2006, 2009). The criterion used to define an animal as defeated was the adoption of a specific posture signifying defeat, characterized by an upright submissive position, limp forepaws, upwardly angled head, and retracted ears (Miczek et al., 1982; Rodríguez-Arias et al., 1998). All agonistic encounters were videotaped and evaluated using a computerized system by an observer who was blind to the treatment (Brain et al., 1989). This custom-developed program allows estimation of the time engaged in different broad functional categories of behavior—threat, attack, avoidance/flee and submission—each of which is characterized by a series of different postures and elements (Rodríguez-Arias et al., 1998). Adolescent and adult mice underwent the encounters when they were 29–30–31–32 PND and 50–51–52–53 PND, respectively. Exploration groups (EXP) did not suffer social defeat, but instead explored the neutral transparent plastic cages for 10 min without having any contact with an opponent. Control animals did not suffer any manipulation before the CPP procedure. The experimental procedure is outlined in Table 1.

2.6. Procedure of corticosterone measurements (ELISA)

Blood sampling for corticosterone determination was performed by the tail-nick procedure, in which the animal is wrapped in a cloth and a 2-mm incision is made at the end of the tail artery. The tail is then massaged until 50 μl of blood is collected in an ice-cold Microvette® CB 300 capillary tube (Sarstedt, Germany). Blood samples were kept on ice, and plasma was separated from whole blood by centrifugation (5 min, 5000 *g*) and transferred to sterile 2 ml microcentrifuge tubes. Plasma samples were stored at −80 °C until determination of corticosterone. All blood samples were taken between 10 am and 1 pm. On the day of the assay, samples were diluted (in a proportion of ~1:40) in the Steroid Displacement Reagent mix provided with the kit. Corticosterone levels in diluted plasma were then analyzed using a corticosterone EIA kit (Enzo® Life Sciences, Catalog No. ADI-900-097, 96 Well kit), according to the manufacturer's instructions, and an iMark microplate reader (Bio-Rad) and Microplate Manager 6.2. software. The optical density was read at 405 nm, with 590 nm correction.

To evaluate the effect of social defeat on corticosterone levels in adolescent and adult animals, several mice used in the CPP study were employed (n = 6 or 8 in each group). Blood samples were taken from two groups for each age immediately after the first and fourth agonistic encounters (on PND 29 and 32 for adolescent and PND 50 and 53 for adult mice). Four more groups experienced social defeat on the same days and were conditioned with 1 or 25 mg/kg of cocaine, respectively. They were then confined to one compartment of the CPP apparatus for 30 min, and blood samples were taken after the first and last agonistic encounters. Two more groups underwent social defeat on the same days, but were treated with saline. They were then confined to a compartment of the CPP apparatus for 30 min and blood samples were taken after the first and last agonistic encounters. Eight groups underwent the same procedure, with the exception that the mice were submitted to exploration instead of social defeat.

2.7. Statistical analyses

For the CPP induced by 1 mg/kg of cocaine, the time spent in the drug-paired compartment during Pre- and Post-C tests was analyzed

Table 1
Experimental design.

Experimental design				
Groups	(n=)	Group	Conditioning	Reinstatement
Adolescent mice			PND 26–33	
Control	14	A-Control-C1	No manipulation + 1 mg/kg cocaine	0.5 mg/kg
	15	A-Control-C25	No manipulation + 25 mg/kg cocaine	12.5 and 6.25 mg/kg
EXP	15	A-EXP-C1	Exploration without cospecific + 1 mg/kg cocaine	0.5 mg/kg
	17	A-EXP-C25	Exploration without cospecific + 25 mg/kg cocaine	12.5 and 6.25 mg/kg
ASD	13	A-ASD-C1	Social defeat + 1 mg/kg cocaine	
	16	A-ASD-C25	Social defeat + 25 mg/kg cocaine	12.5 and 6.25 mg/kg
Adult mice			PND 47–54	
Control	14	Y-Control-C1	No manipulation + 1 mg/kg cocaine	0.5 mg/kg
	18	Y-Control-C25	No manipulation + 25 mg/kg cocaine	12.5, 6.25, 3.125 and 1.56 mg/kg
EXP	12	Y-EXP-C1	Exploration without cospecific + 1 mg/kg cocaine	0.5 mg/kg
	15	Y-EXP-C25	Exploration without cospecific + 25 mg/kg cocaine	12.5, 6.25, 3.125 and 1.56 mg/kg
ASD	12	Y-ASD-C1	Social defeat + 1 mg/kg cocaine	0.5 and 0.25 mg/kg
	15	Y-ASD-C25	Social defeat + 25 mg/kg cocaine	12.5, 6.25, 3.125 and 1.56 mg/kg

with a mixed three-way ANOVA, with two between-subjects variables—Age, with two levels (adolescents and adults), and Treatment, with three levels (control, ASD and EXP)—and a within-subjects variable—Days, with two levels (Pre-C and Post-C). In the groups showing CPP, extinction and reinstatement values were analyzed by means of an ANOVA with the same variables, with the exception that Days had four levels (Pre-C, Post-C, extinctions and reinstatements). For the CPP induced by 25 mg/kg of cocaine, the time spent in the drug-paired compartment was analyzed with an ANOVA with the same variables, although, in this case, Days had six levels (Pre-C, Post-C, two extinctions and two reinstatements). In all cases, post hoc comparisons were performed with Bonferroni tests. In addition, extinction and reinstatement values were analyzed with Student t tests. The time required for the preference to be extinguished in each animal was analyzed by means of the Kaplan–Meier test with Breslow (generalized Wilcoxon) comparisons when appropriate (Daza-Losada et al., 2009). Although the mean of the group as a whole determined the day on which extinction was considered to have been achieved, preference was confirmed to have been extinguished when a mouse spent 380 s or less in the drug-paired compartment on two consecutive days. We chose this time based on the values of all the Pre-C tests performed in the study (mean = 370 s).

Corticosterone levels at minute 0 (immediately after social encounter) or at minute 30 (after pharmacological treatment) were analyzed with a mixed ANOVA with two between-subjects variables—Age, with two levels (adolescent and adults), and Treatment, with two levels (ASD and EXP)—and a within-subjects variable—Days, with two levels (first and fourth encounters). A further ANOVA compared corticosterone values at minute zero with those after administration of saline, C1 or C25, with two between-subjects variables—Age and Time, with four levels (zero, saline, C1 and C25)—and a within-subjects variable—Days. Post hoc comparisons were performed with Bonferroni tests.

A mixed ANOVA with one between variable—Age, with two levels (adolescent and adults)—and a within-subjects variable—Days, with four levels (first, second, third and fourth encounters)—was employed to evaluate each of the behaviors during the social encounter.

3. Results

3.1. Behavioral characterization of social defeat in adolescent and adult mice

The times engaged by the opponent mice in aggressive behaviors are shown in Table 2. In the aggressive opponent mice, the ANOVA revealed a significant effect of the variable Age for the time spent in threat [$F(1,21) = 32.630$; $p < 0.001$], and attack [$F(1,21) = 20.526$; $p = 0.001$]. When confronted with adults, these mice spent more time in threat ($p < 0.001$) and attack ($p < 0.001$) than when confronted with adolescent mice.

In defeated mice, the ANOVA revealed a significant effect of the interaction Days × Age on defense/submissive [$F(3,63) = 3.341$; $p = 0.02$] and avoidance and flee [$F(3,63) = 2.811$; $p = 0.05$] behaviors. With respect to adults, adolescent mice spent less time in submissive/defensive and avoidance and flee behaviors during the first encounter ($p < 0.01$), and spent more time in submissive behaviors during the second, third and fourth encounters than in the first encounter ($p < 0.02$).

3.2. Effects of acute social defeat on acquisition and reinstatement of CPP

3.2.1. 1 mg/kg cocaine-induced CPP

The ANOVA revealed a significant effect of the variable Days [$F(1,74) = 38.789$; $p < 0.001$] and the interaction Days × Age [$F(1,74) = 4.046$; $p < 0.05$] (see Fig. 1a and b). A further ANOVA performed separately in adolescent and adult mice revealed that all the groups except for the social defeat adolescent group (A-ASD-C1) developed CPP, as they spent more time in the drug-paired compartment in the Post-C test than in the Pre-C test ($p < 0.001$). Moreover, adult mice spent more time in the drug-paired compartment than adolescents ($p < 0.05$). In the groups showing CPP, the ANOVA revealed an effect of the variable Days [$F(3,186) = 14.563$; $p < 0.001$] and the interaction Days × Treatment [$F(3,186) = 2.771$; $p < 0.01$]. In the reinstatement test, socially defeated mice showed significantly higher scores than those belonging to the control or exploration groups ($p < 0.05$ and $p < 0.01$, respectively).

Extinction of preference was achieved after two extinction sessions in all groups. Administration of a priming dose of 0.5 mg/kg of cocaine induced reinstatement of the preference only in the social defeated adult group (Y-ASD-C1) ($p < 0.01$). This preference was extinguished after two sessions. A new priming dose of 0.25 mg/kg of cocaine did not reinstate the preference.

3.2.2. 25 mg/kg cocaine-induced CPP

The ANOVA revealed a significant effect of the variable Days [$F(5,450) = 43.302$; $p < 0.001$]. All the groups developed CPP, as they all spent more time in the drug-paired compartment in the Post-C test than in the Pre-C test ($p < 0.001$) and showed reinstatement of the preference after 12.5 and 6.25 mg/kg of cocaine ($p < 0.001$ in all cases) (see Fig. 2a and b). The interaction Day × Age also had a significant effect [$F(5,450) = 3.458$; $p < 0.01$]. The time spent in the drug-paired compartment during the second reinstatement test was significantly higher in adult animals ($p < 0.001$).

The Kaplan–Meier analysis of the data recorded following the Post-C test revealed that more time was required to achieve extinction in the A-EXP-C25 (4) and A-Control-C25 (3) groups than in the A-ASD-C25

Table 2
Behavior of mice during agonistic encounters. Mean cumulative times (±SEM) spent engaging in different behavioral categories (threat, attack, avoidance/flee, defense/submission) and latency to initiate these behaviors during the agonistic encounter by experimental mice (adolescent n = 12 and adult n = 10/11) and by aggressive opponents. ***p < 0.001, significant difference with respect to adolescent mice. **p < 0.01 with respect adult mice; *p < 0.02, significant difference with respect to the first social encounter.

		Behavior of mice during agonistic encounters			
Experimental mice		1st social defeat	2nd social defeat	3rd social defeat	4th social defeat
Avoidance	Adolescent	9 ± 4 **	14 ± 3	9 ± 3	9 ± 2
	Adult	25 ± 5	13 ± 4	13 ± 4	13 ± 3
Defence/submissive	Adolescent	19 ± 9 **	125 ± 23 *	129 ± 30 *	207 ± 28 *
	Adult	162 ± 31	211 ± 40	174 ± 35	196 ± 31
Aggressive opponents		1st social defeat	2nd social defeat	3rd social defeat	4th social defeat
Threat	Adolescent	10 ± 3	18 ± 4	16 ± 4	22 ± 4
	Adult	57 ± 8	57 ±12	50 ± 12	51 ±12
				***	
Attack	Adolescent	3 ± 1	12 ± 3	8 ± 3	9 ± 2
	Adult	34 ± 6	28 ± 7	22 ± 6	21 ± 6
				***	

group (1) ($\chi^2 = 19.253$; $p < 0.001$) (see Fig. 3a). Once the preference had been extinguished, it was reinstated in all the adolescent groups with a priming dose of 12.5 mg/kg of cocaine ($p < 0.01$). This new preference was extinguished after one session in all the adolescent groups. A new priming dose of 6.25 mg/kg of cocaine did not reinstate the preference in any of the adolescent groups.

In the case of the adult mice (see Fig. 3b), the Kaplan–Meier analysis of the data recorded following the Post-C test revealed that more extinction sessions were required in the A-EXP-C25 (2) and A-Control-C25 (2) groups than in the A-ASD-C25 (1) group ($\chi^2 = 13.235$; $p < 0.01$). Preference was reinstated in all the groups with a priming dose of 12.5 mg/kg of cocaine ($p < 0.01$). The Kaplan–Meier analysis also showed that the number of sessions required to extinguish the preference was significantly higher in the Y-ASD-C25 group (9) than in the Y-Control-C25 (1) and Y-EXP-C25 (3) groups ($\chi^2 = 10.295$; $p < 0.01$). A new priming dose of 6.25 mg/kg of cocaine reinstated the preference in all the groups (Y-Control-C25 $p < 0.05$; Y-EXP-C25 $p < 0.01$; Y-ASD-C25 $p < 0.001$). Again, the Kaplan–Meier analysis showed that the number of sessions required to extinguish the preference was significantly higher in the Y-ASD-C25 group (12) than in the Y-Control-C25 (1) and Y-EXP-C25 (1) groups ($\chi^2 = 9.093$; $p < 0.01$). A new priming dose of 3.125 mg/kg reinstated the preference in all the adult groups (Y-Control-C25 $p < 0.01$, Y-EXP-C25 $p < 0.001$, Y-ASD-C25 $p < 0.05$). No differences were observed in the time needed to extinguish this preference among the three groups (1 for the Y-Control-C25, 2 for the Y-EXP-C25 and 3 for Y-ASD-C25 group). No further reinstatements were observed.

3.3. Effect of acute social stress on corticosterone levels

Blood concentrations of corticosterone (pg/ml) are shown in Fig. 4a and b. The ANOVA of the data obtained at minute 0 revealed a significant effect of the variables Age [$F(1,28) = 22.596$; $p < 0.001$] and Treatment [$F(1,28) = 20.806$; $p < 0.001$], and the interaction Age × Treatment [$F(1,28) = 33.280$; $p < 0.001$]. Post-hoc comparisons showed that socially defeated adult mice had higher corticosterone levels than controls ($p < 0.001$) and socially defeated adolescents ($p < 0.001$).

The ANOVA of the data obtained at minute 30 after saline treatment revealed a significant effect of the interaction Age × Treatment [$F(1,24) = 4.141$; $p < 0.05$]. Socially defeated adult mice displayed higher corticosterone levels than non-stressed animals ($p < 0.01$). On the other hand, non-stressed adolescent mice had higher corticosterone levels than non-stressed adults ($p < 0.001$). No differences were observed after administration of 1 mg/kg of cocaine. After treatment with 25 mg/kg of cocaine the interaction Age × Treatment [$F(1,24) = 8.111$; $p < 0.001$] showed an effect. Corticosterone levels in ASD adult and adolescent mice were higher than in non-stressed animals ($p < 0.001$ in all cases). However, adult stressed mice displayed higher corticosterone levels than adolescents ($p < 0.001$).

The ANOVA comparing the values at minute zero with those after administration of saline, C1 or C25 showed an effect of the interaction Age × Time [$F(3,47) = 10.193$; $p < 0.001$]. In socially defeated adult mice, corticosterone levels after 25 mg/kg of cocaine were significantly higher than at minute zero or after receiving saline or 1 mg/kg of cocaine ($p < 0.01$ in all cases). In socially defeated adolescent mice, administration of saline or any of the cocaine doses significantly increased corticosterone levels with respect to minute zero ($p < 0.01$ for Sal and C1 and $p < 0.05$ for C25). In the same mice, saline administration increased corticosterone more than any of the doses of cocaine ($p < 0.01$ in both cases). Non-stressed adult or adolescent mice receiving saline or 1 mg/kg of cocaine exhibited higher corticosterone levels with respect to minute zero ($p < 0.01$, in both cases). Moreover, in non-stressed adolescent mice saline induced a higher increase than any of the cocaine doses ($p < 0.001$), and the lower dose induced a higher increase than the highest dose ($p < 0.01$).

4. Discussion

Research employing animal models to explore drugs of abuse in adolescence has increased in the past decade, but the relationship between drug abuse, adolescence and stress has remains poorly understood (Burke and Miczek, 2014). In the present study we have observed

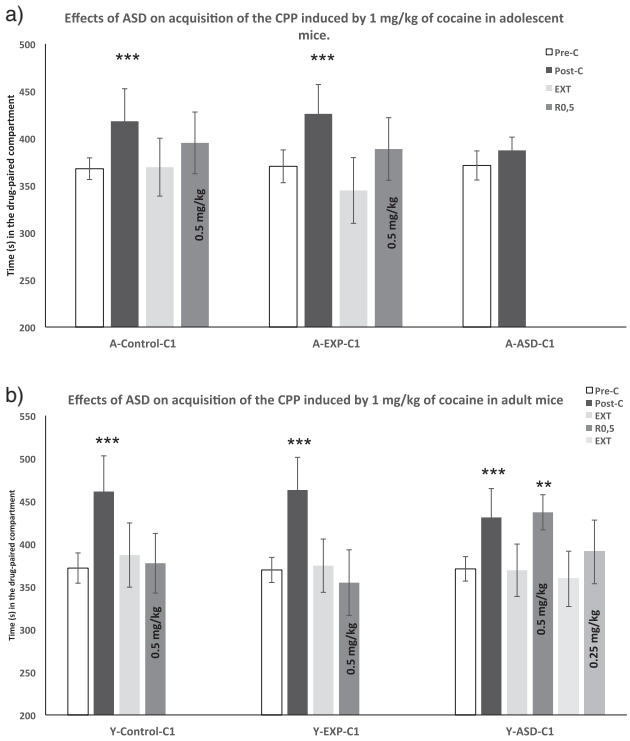

Fig. 1. Effects of social defeat on acquisition of the CPP induced by 1 mg/kg of cocaine in adolescent (a) and adult (b) mice. During the conditioning phase, animals were divided into the following three treatment groups: Control-C1 (adolescent n = 14; adult n = 14), conditioned with 1 mg/kg of cocaine; EXP-C1 (adolescent n = 15; adult n = 12), exposed to a new cage and allowed them to explore before each session of conditioning with 1 mg/kg of cocaine; ASD-C1 (adolescent n = 13; adult n = 12), exposed to social defeat during an agonistic encounter before each session of conditioning with 1 mg/kg of cocaine. The bars represent the time (in seconds) spent in the drug-paired compartment before conditioning sessions in the pre-conditioning test (white bars), after conditioning sessions in the post-conditioning test (black bars), in the last extinction session (light gray bars) and during the reinstatement test (dark gray bars). ***$p < 0.001$, significant difference in the time spent in the drug-paired compartment vs pre-conditioning test; **$p < 0.01$ significant difference in the time spent in the drug-paired compartment vs extinction test.

that the effects of social defeat stress on the acquisition and reinstatement of the CPP induced by cocaine (1 mg/kg or 25 mg/kg) vary depending on the age of the animals. Rodent adolescence is separated into early (P21–34), mid- (P34–46) and late-adolescence (P46–59) (Laviola et al., 2003; Tirelli et al., 2003). In the present study we have evaluated the effect of acute social defeat on early (P29 to 32) (referred to as "adolescent" throughout the manuscript) and late (P50 to 53) (referred to as "adult") adolescence. Adult mice exposed to ASD showed an increase in the conditioned reinforcing effects of cocaine. These mice developed CPP after being conditioned with 1 mg/kg of cocaine, as occurred in the rest of the groups, but the preference was reinstated after a priming dose of 0.5 mg/kg of cocaine only in ASD mice. In addition, although the response to 25 mg/kg of cocaine was similar to that observed in control and exploration groups, ASD adult mice needed a higher number of extinction sessions for the preference to be extinguished after priming-induced reinstatement. On the other hand, ASD reduced the conditioned reinforcing effects of cocaine in adolescent mice. The lower dose of cocaine induced CPP in control and exploration groups, but did not produce any effect in adolescent ASD mice. In addition, socially defeated adolescent mice developed CPP after conditioning with 25 mg/kg of cocaine, and reinstated this behavior after a priming dose of 12.5 mg/kg, but the extinction occurred in less sessions than in the control and exploration groups.

Our results in adult mice are in line with those previously reported. The CPP paradigm is assumed to reflect the secondary motivational properties of drugs and their potential for abuse (Tzschentke, 2007), and can also be used to evaluate the reinstatement of drug-seeking after extinction (Aguilar et al., 2009). According to previous research, different types of stress have different effects on the acquisition, extinction and reinstatement of CPP induced by cocaine. Chronic unpredictable stress enhances the place conditioning effects of cocaine (Haile et al., 2001), while exposure to a single inescapable tailshock enhances the subsequent CPP response to oxycodone, but not to cocaine (Der-Avakian Et al., 2007). Several studies in mice have shown that administration of footshocks (Redila and Chavkin, 2008), immobilization stress (Sanchez et al., 2003) or forced swim (Ross et al., 2012; Vaughn

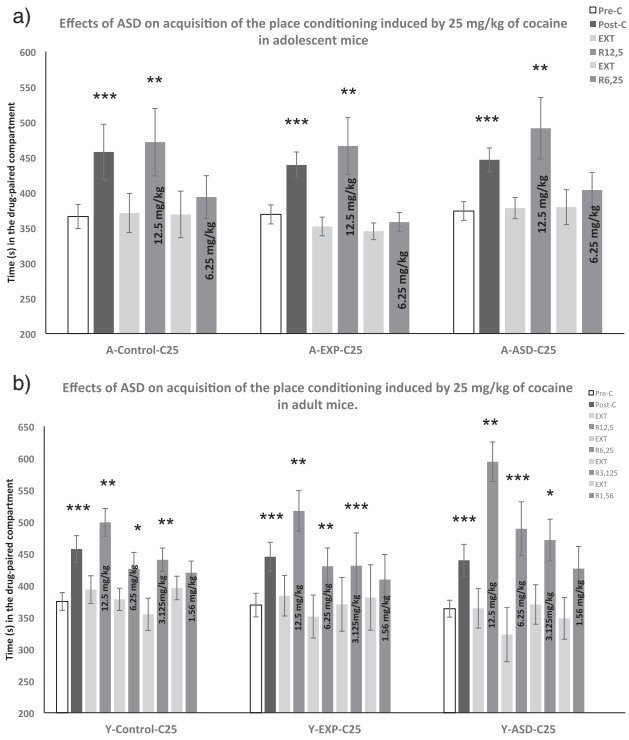

Fig. 2. Effects of social defeat on acquisition of the CPP induced by 25 mg/kg of cocaine in adolescent (a) and adult (b) mice. During the conditioning phase, animals were divided into the following three treatment groups: Control-C25 (adolescent n = 15; adult n = 18), conditioned with 25 mg/kg of cocaine; EXP-C25 (adolescent n = 17; adult n = 15), exposed to a new cage and allowed them to explore it before each session of conditioning with 25 mg/kg of cocaine; ASD-C25 (adolescent n = 16; adult n = 15), exposed to social defeat during an agonistic encounter before each session of conditioning with 25 mg/kg of cocaine. The bars represent the time (in seconds) spent in the drug-paired compartment before conditioning sessions in the pre-conditioning test (white bars), after conditioning sessions in the post-conditioning test (black bars), in the last extinction session (light gray bars), and during the reinstatement test (dark gray bars). ***$p < 0.001$, significant difference in the time spent in the drug-paired compartment vs pre-conditioning or extinction tests; **$p < 0.01$ and *$p < 0.05$ significant difference in the time spent in the drug-paired compartment vs extinction tests.

et al., 2012; Vranjkovic et al., 2012) prior to CPP testing reinstates preference for the previously cocaine-paired chamber. Acute exposure to restraint stress also reinstates amphetamine-induced CPP when tests are performed during adolescence, but not when animals are tested in adulthood (Cruz et al., 2010).

Few studies have evaluated the response of social defeat-stressed animals to cocaine-induced CPP. In accordance with the results reported herein, adult mice exposed to social defeat stress (using the intruder-resident model) have been shown to exhibit significantly stronger CPP for the cocaine-paired chamber than unstressed mice (McLaughlin et al., 2006). Furthermore, social defeat during an agonistic encounter in a neutral area prior to a reinstatement test has been shown to reinstate cocaine CPP in adult mice (Land et al., 2009; Titomanlio et al., 2013) and to increase susceptibility to cocaine-induced reinstatement of CPP (Ribeiro Do Couto et al., 2009). These results are in line with those of numerous studies which have demonstrated that social defeat increases vulnerability to cocaine self-administration (Haney et al., 1995; Miczek and Mutschler, 1996; Tidey and Miczek, 1997; Covington and Miczek, 2001, 2005; Covington et al., 2005; Yap and Miczek, 2007; Covington et al., 2008; Quadros and Miczek, 2009; Boyson et al., 2011; Cruz et al., 2011).

In contrast to that observed in adults, socially defeated adolescent mice showed a decreased response to cocaine-induced CPP. Although adolescent rodents can exhibit place conditioning, their sensitivity to drugs and the intensity of their psychopharmacological response vary dramatically during ontogenesis. Psychostimulants are considerably more effective in producing place conditioning between PND 15 and 22 (Laviola et al., 1992, 1994), and much less so as animals grow up and enter adolescence. For instance, amphetamine-induced place conditioning is weaker (or even absent) in adolescent subjects when compared to adults (Adriani and Laviola, 2003). The establishment of a contextual place conditioning is known to depend on activation of D2 receptors (Beninger et al., 1989; White et al., 1991), and, although dopaminergic receptors are overexpressed in the dorsal and ventral striatum during adolescence, this peak is much more pronounced for D1 than for D2 receptors (Gelbard et al., 1989; Teicher et al., 1995). Our

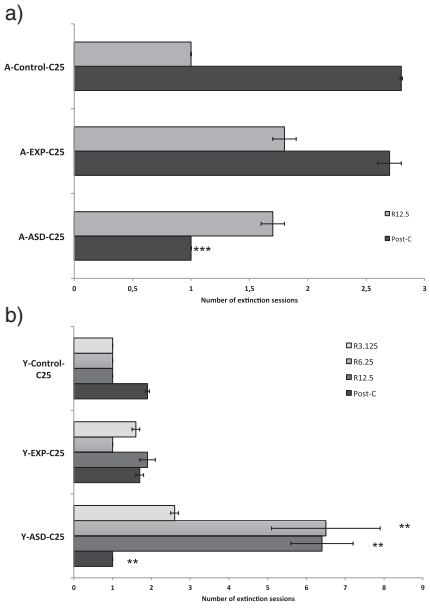

Fig. 3. a) Effects of social defeat on extinction sessions of the place conditioning induced by 25 mg/kg of cocaine in adolescent mice. During the conditioning phase, animals were divided into the following three treatment groups: Control-C25 (n = 15), conditioned with 25 mg/kg of cocaine; EXP-C25 (n = 17), exposure to a new cage and allowed to explore it before each session of conditioning with 25 mg/kg of cocaine; ASD-C25 (n = 16), exposure to social defeat during an agonistic encounter before each session of conditioning with 25 mg/kg of cocaine. The bars represent the number of sessions needed to extinguish the preference after the Post-C test (dark gray) or after the first reinstatement test (light gray) in the drug-paired compartment during conditioning sessions. b) Effects of social defeat on extinction sessions of the place conditioning induced by 25 mg/kg of cocaine in adult mice. During the conditioning phase, animals were divided into the following three treatment groups: Control-C25 (n = 18), conditioned with 25 mg/kg of cocaine; EXP-C25 (n = 15), exposure to a new cage and allowed to explore it before each session of conditioning with 25 mg/kg of cocaine; ASD-C25 (n = 15), exposure to social defeat during an agonistic encounter before each session of conditioning with 25 mg/kg of cocaine. The bars represent the number of sessions needed to extinguish the preference after the Post-C test (black) or after the first (dark gray), second (light gray), and third (lighter gray) reinstatement tests in the drug-paired compartment during conditioning sessions.

non-stressed adolescent (PND 29–32) mice displayed a steady CPP, with social defeat at this age proving to undermine the effects of place conditioning with cocaine. This result contrasts with the increase in amphetamine-induced CPP in socially defeated adolescent rats reported by Burke et al. (2011). However, there are several differences between the two studies that could explain the lack of concordance. In addition to employing adolescent rats, Burke et al.'s animals were exposed to a more intense social defeat; they were in contact with the aggressive resident (separated by a mesh barrier) for 35 min following the social defeat, which took place on 5 consecutive days. In addition, social defeat was experienced by older adolescent mice (PND 35 to 40) than those used in our study (PND 29–32), and this difference would have a critical bearing in terms of rising testosterone levels in males (Hofford et al., 2011). Finally, we performed CPP in adolescent mice, while Burke et al. initiated CPP on PND 56.

This decrease in cocaine-induced CPP in adolescent defeated mice could have been related with a less intense agonistic encounter. Although aggressive opponent mice displayed threat and attack behavior when confronted with either adult or adolescent intruder mice, they spent significantly less time engaging in aggressive behaviors and presented longer latencies to threat when confronted with the younger mice. Consequently, avoidance and defensive behavior were observed in both age groups, although adolescents spent less time engaging in these behaviors during the first social encounter. In this way, aggressive opponent mice exhibited aggression and experimental mice showed avoidance and submissive behaviors in both adult and adolescent age groups.

In line with this, adolescent mice did not show increases in corticosterone levels after social defeat. However, as expected, significant increases in corticosterone levels were observed in ASD adult mice immediately after social defeat. It could be argued that immaturity of the HPA axis was the cause of this lack of response; however, corticosterone levels were higher in all adolescent groups when they received an injection of saline and were confined to the CPP apparatus, irrespective of whether or not there was social defeat or exploration, thus confirming previous results obtained in our laboratory (García-Pardo et al., 2014). These results demonstrate that early adolescent mice possess a functional HPA axis. In fact, the increase in corticosterone observed after saline injection in the exploration group of early adolescents was higher than in adult mice, which suggests that the adolescents were characterized by enhanced reactivity. These results are in accordance with those of a previous study in which we observed that saline injection or exposure to the CPP apparatus enhanced corticosterone levels in adult mice, though not in a significant way (Do Couto et al., 2011). In this context, the HPA axis has been shown to be hyper-responsive during adolescence (for review see Klein and Romeo, 2013; McCormick, 2010).

Another possible explanation for the lack of corticosterone response to social defeat is that adolescent mice do not perceive social defeat as a stressful situation. Periadolescent rodents are generally hyperactive and particularly involved in affiliative and playful behaviors, which are important for the establishment of adult-like social relationships (Cirulli et al., 1996; Terranova et al., 1998). Social housing conditions are known to provide animals with mild daily stressors that come about through interactions between individuals (Haller et al., 2000). In particular, during periadolescence, rodents are exposed to intense and playful social interactions, which progressively shift towards an adult-like competitive pattern as they grow older (Terranova et al., 1998). Play-fighting begins to rise as early as P18 (Bolles and Woods, 1964) and peaks between P30 and 40 (Meaney and Stewart, 1981; Panksepp, 1981; Panksepp et al., 1984; Pellis and Pellis, 1990). Thus, it is possible that, when exposed to such encounters, adolescent mice experience it as play-fighting rather than social defeat, especially as the body contact targets in play-fighting are often the same as those in real fighting (Aldis, 1975). Therefore, it may be difficult to distinguish intense play-fighting from low-intensity real fighting (Hole and Einon, 1984). Behavioral observations in mice subjected to repeated agonistic interactions suggest that fighting is rewarding for the winner (Kudryavtseva et al., 1991). If this is the case, adolescent mice are likely to be less responsive to cocaine. In accordance with this hypothesis, we have previously observed that adolescent mice living in crowded conditions do not present reinstatement after a cocaine priming, while adult mice housed in the same conditions show a significantly increased response to cocaine (Ribeiro do Couto et al., 2009). The fact that most playful attacks in adolescent mice elicited defensive reactions limited to evasion undermines this argument; in the present study, socially defeated adolescent mice displayed submissive behavior similar to that observed in adults (Pellis and Pasztor, 1999).

Corticosterone response to cocaine administration also varied between adolescent and adult mice. In adolescents, both cocaine doses decreased corticosterone levels significantly in non-stressed and stressed individuals. Conversely, no changes were observed in adult

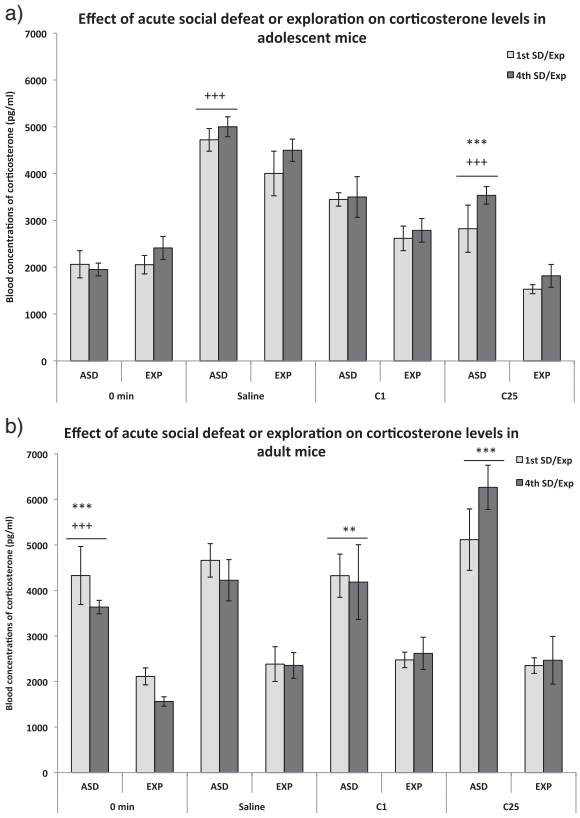

Fig. 4. Effect of acute social defeat or exploration on corticosterone levels. (a) Adolescent or (b) adult mice were divided into the following treatment groups: baseline conditions (EXP, 0 min; adolescent n = 7; adult n = 7), immediately after social defeat exposure (ASD, 0 min; adolescent n = 8; adult n = 10), 30 min after social defeat or exploration and saline treatment (ASD saline 30 min; adolescent n = 7; adult n = 7; EXP saline 30 min; adolescent n = 7; adult n = 7), 30 min after social defeat or exploration and treatment with 1 mg/kg of cocaine (ASD C1 30 min; adolescent n = 7; adult n = 7; EXP C1 30 min; adolescent n = 6; adult n = 7), and 30 min after social defeat or exploration and treatment with 25 mg/kg of cocaine (ASD C25 30 min; adolescent n = 7; adult n = 7; EXP C25 30 min; adolescent n = 7; adult n = 7). ***$p < 0.001$, **$p < 0.01$ significant difference from the corresponding EXP group. +++$p < 0.001$ significant difference from the corresponding adolescent/adult group.

controls, while the highest cocaine dose significantly increased corticosterone levels in adult ASD mice. Previous reports have shown that acute cocaine administration induces increases in corticosterone levels in mice (Moldow and Fischman, 1987; Budziszewska et al., 1998) and that stress, and consequently corticosterone, can increase DA neuronal firing and levels (Thierry et al., 1976; Piazza et al., 1996). In humans, elevated cortisol levels increase subjective feelings of stress, which result in a greater propensity to relapse to cocaine use (Back et al., 2010). Our results highlight that adult mice are more sensitive to cocaine-induced increases in corticosterone, an effect that may be related to resistance to the extinction of cocaine-induced CPP. Among the neural systems that mature during adolescence in mice, DA neurons in the VTA that project to the mPFC and NAcc are perhaps the most critical for processing salient events, including responses to psychostimulants (for review see Everitt and Wolf, 2002; Schultz, 2002; Wise, 1996). Evidence suggests that early NAc DA maturation and delayed mPFC DA maturation are responses to stress. Lyss et al. (1999) found that neuronal activation occurred mostly in the NAc when stress was experienced in early adolescence, but in the mPFC when experienced in late adolescence. These developmental differences could be one of the reasons for the contrasting responses to cocaine observed in adult and adolescent mice.

Moreover, response to psychostimulants in stressed adolescent animals seems to depend on several variables, such as the type of stressor employed or the dose administered and number of exposures. Several studies have demonstrated that certain types of stress (i.e., social defeat) sensitize, while others protect (i.e., single

restraint) against psychostimulant-induced CPP. For example, a single restraint or forced wheel-running session—arguably less intense stressors than repeated social defeat—have been shown to reduce CPP for amphetamine in adulthood or for cocaine when tested 1 day later (Richtand et al., 2012; Thanos et al., 2010). Therefore, it could be hypothesized that, if adolescent mice experience social defeat as a less intense stressor than adults, this experience can protect them against cocaine-induced CPP. Indeed, rats experiencing social defeat in adolescence have been shown to exhibit increased locomotion in response to an acute dose of amphetamine in adulthood, although this response was absent after chronic amphetamine treatment (Burke et al., 2013). However, at higher doses of psychostimulants, adolescent socially defeated animals exhibit lower levels of drug-stimulated locomotion than controls (Burke et al., 2010; Trzcinska et al., 2002). Therefore, another possible explanation for the undermining of the conditioned rewarding effects of cocaine by social defeat is that this type of stress impairs the reinforcing effect of the drug, as the CPP procedure implies four consecutive cocaine administrations.

In line with the present results, we have previously observed that adolescent rats exposed to maternal separation do not acquire MDMA-induced CPP (Llorente-Berzal et al., 2013). Similarly, after experiencing social defeat, young adult mice have been reported to be less sensitive to the rewarding effects of MDMA, while no effects have been observed in adolescent mice (García-Pardo et al., 2014). This body of evidence supports the hypothesis that exposure to either type of social stress, during early life or late adolescence, reduces the sensitivity of animals to the rewarding effects of MDMA. Although these results cannot be compared with those presented here, they are relevant examples of a diminished response to psychostimulants after other types of stress.

5. Conclusions

Basic research seeks to model the link between stress and drug abuse. It is quite clear that many rodent models of adverse adult experiences can cross-sensitize with the behavioral effects of abused drugs, particularly psychostimulants. Traumatic social interactions, such as bullying or physical attack, are known to be risk factors for substance abuse. Brief agonistic confrontations between a rodent and an aggressive conspecific are an ethologically relevant animal model of such experiences (Koolhaas et al., 1997). Our results confirm that adult experience of social defeat increases the response to cocaine-induced CPP. However, experience of social defeat during adolescence produces completely contrasting results, with these animals being less sensitive to cocaine-induced CPP. Impairment of reward processes can have significant consequences in terms of drug use and subsequent development of dependence, since subjects that experience reduced levels of reward after drug use may increase their consumption in order to achieve rewarding effects (Bruijnzeel et al., 2004; Leventhal et al., 2010). Moreover, greater drug consumption has been repeatedly associated with enhanced vulnerability to the development of drug addiction (Chambers et al., 2003; Crews et al., 2007). Therefore, adverse experiences during adolescence would seem to predict an increased use of illicit substances.

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STUDY 2

Effects of repeated social defeat on adolescent mice on cocaine-induced CPP and self-administration in adulthood: integrity of the blood–brain barrier

M. Rodríguez-Arias, **S. Montagud-Romero**, A. Rubio-Araiz, M.A. Aguilar, E. Martín-García, R. Cabrera, R. Maldonado, F. Porcu, M.I. Colado, J. Miñarro

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Effects of repeated social defeat on adolescent mice on cocaine-induced CPP and self-administration in adulthood: integrity of the blood–brain barrier

Marta Rodríguez-Arias[1,4], Sandra Montagud-Romero[1,4], Ana Rubio-Araiz[3,4], María A. Aguilar[1,4], Elena Martín-García[2,4], Roberto Cabrera[2,4], Rafael Maldonado[2,4], Francesca Porcu[3,4], María Isabel Colado[3,4] & José Miñarro[1,4]

Unidad de Investigación Psicobiología de las Drogodependencias, Departamento de Psicobiología, Facultad de Psicología, Universitat de València, Spain[1], Laboratory of Neuropharmacology, Departament de Ciencies Experimentals i de la Salut, Universitat Pompeu Fabra, Spain[2], Departamento de Farmacología, Facultad de Medicina, Universidad Complutense, Instituto de Investigación Sanitaria Hospital, Spain[3] and Red de Trastornos Adictivos del Instituto de Salud Carlos III, Spain[4]

ABSTRACT

Social stress in adulthood enhances cocaine self-administration, an effect that has been related with an increase in extracellular signal-regulated kinase and p38α mitogen-activated protein kinase phosphorylation. A detrimental effect of cocaine on blood–brain barrier (BBB) integrity has also been reported. This study evaluates the effects of repeated social defeat (RSD) during adolescence on the reinforcing and motivational effects of cocaine in adult mice and the changes induced by RSD on BBB permeability. Cocaine self-administration, conditioned place preference and quantitative analysis of claudin-5, laminin, collagen-IV and IgG immunoreactivity took place 3 weeks after RSD. Mice socially defeated during adolescence developed conditioned place preference and exhibited reinstated preference with a non-effective dose of cocaine (1 mg/kg). RSD mice needed significantly more sessions than control animals for the preference induced by 25 mg/kg of cocaine to be extinguished. However, acquisition of cocaine self-administration (0.5 mg/kg per injection) was delayed in the RSD group. Mice exposed to RSD displayed significant changes in BBB structure in adulthood, with a marked reduction in expression of the tight junction protein claudin-5 and an increase in basal laminin degradation (reflected by a decrease in laminin and collagen-IV expression) in the nucleus accumbens and hippocampus. The detrimental effect induced by cocaine (25 mg/kg) on collagen-IV expression in the hippocampus was more pronounced in RSD mice. In summary, our findings suggest that stress and cocaine can increase the long-term vulnerability of the brain to subsequent environmental insults as a consequence of a sustained disruption of the BBB.

Keywords Blood–brain barrier, cocaine, conditioned place preference, self-administration, social defeat.

Correspondence to: Marta Rodriguez-Arias, Unidad de Investigación Psicobiología de las Drogodependencias, Departamento de Psicobiología, Facultad de Psicología, Universitat de València, Avda. Blasco Ibáñez, 21, 46010 Valencia, Spain. E-mail: marta.rodriguez@uv.es

INTRODUCTION

Social environment constitutes a key factor of survival and maintenance of health in most animal species (Dunbar, 2010; Weidt *et al.*, 2012). In humans, experience of social stress, especially during childhood or adolescence, increases the risk of suffering mental disorders (Kessler *et al.*, 2010). Appropriate animal models are of great use when exploring the mechanisms by which social stress affects health. The social defeat paradigm has been successfully employed in laboratory rodents to throw light on the neurobiological, physiological and behavioral changes caused by acute or chronic social defeat experience (Tornatzky & Miczek, 1993; Buwalda *et al.*, 1999). The resident/intruder paradigm, in which the intruder is repeatedly exposed to attacks and threats from a dominant rodent, is considered the best model of bullying in laboratory rodents (Vidal *et al.*, 2007; Watt *et al.*, 2009). Stressful events disrupt the extensive re-organization of limbic monoamine systems such as the mesocorticoaccumbal

dopamine (DA) system. DA activity in the accumbens and other subcortical DA terminal regions seems to be less pronounced in adolescents than in adults (Andersen & Gazzara, 1993). Basal levels of synaptic DA are lower during this phase of development, although drug-induced DA release is greater and increases faster in adolescents (Badanich *et al.*, 2006; Laviola *et al.*, 2001). Stressful experiences in early life have a great impact on the subcortical (Andersen, 2003) and mesolimbic DA systems, altering their development (Andersen and Teicher, 2009). Specifically, exposure to stress increases DA content and decreases serotonin turnover in the nucleus accumbens (NAc) and produces neural adaptations in the ventral tegmental area (Andersen *et al.*, 1999; Hall *et al.*, 1998; Andersen & Teicher, 2009).

Adult rats defeated during adolescence show lower baseline DA content and attenuation of amphetamine-induced DA increases in the medial prefrontal cortex (Watt *et al.*, 2009), as well as reduced neurogenesis in the dentate gyrus (Kovalenko *et al.*, 2014), while amphetamine-induced DA release has been shown to be enhanced in their NAc core (Burke *et al.*, 2010).

Few reports have addressed the issue of how adolescent exposure to social defeat can increase the probability of compulsive drug taking later in life, as has previously been shown to occur in animals (Ding *et al.*, 2005; Howes *et al.*, 2000). To date, no studies have been performed to evaluate cocaine self-administration or cocaine-induced conditioned place preference (CPP) in animals socially defeated during adolescence. It has, however, been shown that rats deprived of social interaction during adolescence self-administer more cocaine at low unit doses than non-isolated subjects (Ding *et al.*, 2005), but less at high unit doses (Howes *et al.*, 2000).

Although there are no data available on the effect of social stress on blood–brain barrier (BBB) permeability, there is substantial evidence to show that repeated social defeat (RSD) modifies the expression of antioxidant enzymes and levels of oxidative stress markers (Patki *et al.*, 2014) and induces neuroinflammation (Wohleb *et al.*, 2011, 2013; Hanke *et al.*, 2012). In several models, a rise in proinflammatory cytokine levels (Shaftel *et al.*, 2007) or free radical formation (Gasche *et al.*, 2001) has been shown to disrupt the integrity of the BBB through an increased phosphorylation of the downstream signaling pathways mitogen-activated protein kinases (MAPKs) and matrix metalloproteinase (MMP) activation (Tian & Kyriakides, 2009; Katsu *et al.*, 2010). The primary anatomical substrate of the BBB is the cerebral microvascular endothelium, which, together with pericytes, astrocytes, neurons and the basal lamina, constitutes a neurovascular unit (Hawkins & Davis, 2005). The endothelial cells of the BBB are characterized by the presence of cell-to-cell tight junctions formed by transmembrane molecules such as claudins (claudin-5 appears to be the most abundant), occludins and junction adhesion molecules. Endothelial cells and pericytes are surrounded by a basement membrane made up of extracellular matrix molecules, including collagens, laminins and heparan sulfate proteoglycans (Persidsky *et al.*, 2006). Laminin and collagen-IV have been identified as substrates for several MMPs (Lee *et al.*, 2009).

Cocaine induces expression of the adhesion molecules ICAM-1 and VCAM-1 (Gan *et al.*, 1999) and mediates transcriptional and translational induction of ALCAM in microvascular endothelial cell cultures of the human brain (Yao *et al.*, 2011). In addition, cocaine enhances leucocyte adhesion to endothelial cells and subsequently increases leucocyte transmigration across the cerebral vessel wall, in particular under inflammatory conditions (Gan *et al.*, 1999; Yao *et al.*, 2011). Together, these findings constitute evidence that cocaine impairs BBB integrity.

The present study was aimed to determine the effect of RSD on (1) acquisition and reinstatement of cocaine-induced CPP; (2) acquisition and motivation for operant self-administration of cocaine; and (3) BBB structure and permeability in the NAc and hippocampus and their regulation by the subsequent administration of cocaine.

MATERIAL AND METHODS

Animals

Male OF1 ($n = 124$) or CD1 ($n = 60$) (Charles River, Barcelona, Spain) mice arrived at our laboratory at 21 days of age. CD1 mice were used for self-administration studies owing to their greater sensitivity to this technique. All animals (except those used as aggressive opponents) were housed in groups of four in plastic cages ($25 \times 25 \times 14.5$ cm) for 8 days before the experiments began. Aggressive opponents were housed individually in plastic cages ($23 \times 13.5 \times 13$ cm) for a month prior to experiments in order to heighten aggression (Rodríguez-Arias *et al.*, 1998) (30 OF1 and 10 CD1 adult mice). After the RSD procedure, CD1 mice were moved to the CEEA-PRBB (ethical committee for animal experimentation of the Center of Biomedicine Research of Barcelona) for the self-administration procedure. Mice were housed individually in controlled laboratory conditions at a constant temperature of 21 ± 1°C and 55 ± 10 percent humidity. All experiments were conducted under a reversed cycle (lights off at 08:00 hours and on at 20:00 hours), and mice were tested during the first hours of the dark phase. Food and water were available *ad libitum* to mice in the cocaine experiment. All procedures were conducted in compliance with the guidelines of the European Council

Directive 2010/63/UE regulating animal research and were approved by the local ethics committees (CEEA-PRBB and University of Valencia).

Drugs

For the CPP study, animals were injected intraperitoneally with 1 or 25 mg/kg of cocaine hydrochloride (Laboratorios Alcaliber, Madrid, Spain) in a volume of 10 ml/kg of weight. Physiological saline (NaCl 0.9 percent) was used to dissolve the drug. The doses of cocaine were selected on the basis of previous studies (Rodríguez-Arias *et al.*, 2009; Vidal-Infer *et al.*, 2012; Arenas *et al.*, 2014; Montagud-Romero *et al.*, 2014).

For the self-administration study, cocaine hydrochloride was dissolved in sterile 0.9 percent physiological saline. Ketamine hydrochloride (100 mg/kg) (Imalgene 1000; Rhone Merieux, Lyon, France) and xylazine hydrochloride (20 mg/kg) (Sigma, Madrid, Spain) were mixed and dissolved in ethanol (5 percent) and distilled water 95 percent. This anesthetic mixture was administered intraperitoneally in an injection volume of 20 ml/kg of body weight. Thiopental sodium (5 mg/ml) (Braun Medical S. A., Barcelona, Spain) was dissolved in distilled water and delivered by infusion of 0.1 ml through the intravenous catheter.

For intravenous catheter surgery, the mice were anesthetized with a ketamine/xylazine mixture (20 ml/kg of body weight) and implanted with indwelling intravenous silastic catheters, as previously described (Soria *et al*, Neuropsychopharmacology, 29: 1122-1133, 2005). In brief, a 6-cm length of silastic tubing (0.3 mm inner diameter, 0.6 mm outer diameter) (Silastic®, Dow Corning, Houdeng-Goegnies, Belgium) was fitted to a 22-gauge steel cannula (Semat, Herts, England) that was bent at a right angle and then embedded in a cement disk (Dentalon Plus, Heraeus Kulzer, Germany) with an underlying nylon mesh. The catheter tubing was inserted 1.3 cm into the right jugular vein and anchored with suture. The remaining tubing ran subcutaneously to the cannula, which emerged from the midscapular region. All incisions were sutured and coated with antibiotic ointment (Bactroban, GlaxoSmithKline, Madrid, Spain).

Procedure and apparatus

Repeated social defeat encounters

Animals in the corresponding group were exposed to four episodes of social defeat lasting 25 minutes each on postnatal days 27, 30, 33 and 36. Each episode consisted of three phases and began by placing the experimental animal or intruder in the home cage of the aggressive opponent or resident for 10 minutes. During this initial phase, the intruder was protected from attack by a wire mesh wall that permitted social interaction and species-typical threats from the male aggressive resident (Covington & Miczek, 2001). In the second phase, the wire mesh was removed, and a 5-minute period of confrontation began. In the third phase, the wire mesh was replaced for a further 10 minutes to allow social threats from the resident. The exploration group underwent the same protocol, but without the presence of a 'resident' mouse in the cage. Following this last phase, animals were kept in the vivarium for 3 weeks, after which the behavioral tests began. The second phase of each social defeat protocol was video recorded and ethologically analyzed. Threat and attack behaviors were scored in resident mice, and avoidance/flee and defensive/submissive behaviors were evaluated in intruder mice.

Three different sets of mice were employed in this study. A detailed description of the experimental procedure is provided in Table 1.

Conditioned place preference

Details of the apparatus and the procedure of cocaine CPP are described in the Supporting Information and follow the protocol described previously (Rodríguez-Arias *et al.*, 2009; Vidal-Infer *et al.*, 2012).

Intravenous cocaine self-administration

Cocaine self-administration sessions were performed in accordance with the previously described protocols (Soria *et al.*, 2005). Details of the apparatus, surgery and the cocaine self-administration procedure are included in the Supporting Information.

Corticosterone measurements (enzyme-linked immunosorbent assay)

Corticosterone determination was performed as previously described (García-Pardo *et al.*, 2014), and a detailed description is included in the Supporting Information.

Immunohistochemistry

Mice were anesthetized with sodium pentobarbital and perfused transcardially through the left ventricle with 100 ml of phosphate-buffered saline (0.1 M PBS, pH = 7.4) followed by 100 ml of 4 percent paraformaldehyde–PBS. Brains were removed, postfixed in the same solution for 4 hour at room temperature and cryoprotected by immersion in 30 percent sucrose–PBS at 4°C. The brains were sliced at 30 μm in the coronal plane and stored in cryoprotectant solution. They were then frozen and stored at −20°C. Immunohistochemical studies of the NAc and hippocampus were performed, and the

Table 1 Experimental procedure

		Social defeat							
Groups	*n*	*1st*	*2nd*	*3rd*	*4th*	*3 weeks*	*Experimental procedure*		*Reinstatement*
PND		27	30	33	36		58–64	66	
	17						CPP: 1 mg/kg cocaine	Post-C test	
	15						CPP: 25 mg/kg Cocaine		12.5 and 6.25 mg/kg
Control	5	Exploration without cospecific					1 mg/kg cocaine	Brain samples	
	5						25 mg/kg cocaine		
	25						Cocaine self-administration		
	18						CPP: 1 mg/kg cocaine	Post-C test	0.5 mg/kg
	14						CPP: 25 mg/kg cocaine		12.5 and 6.25 mg/kg
RSD	5	Social defeat					1 mg/kg cocaine	Brain samples	
	5						25 mg/kg cocaine		
	25						Cocaine self-administration		

CPP = conditioned place preference; PND = postnatal day; RSD = repeated social defeat.

sections were localized using a mouse brain stereotaxic atlas (Franklin & Paxinos, 1997).

For labeling studies, cerebral free-floating sections were blocked by incubation with 0.5 percent BSA, 10 percent normal goat serum and 0.1 percent Triton X-100 for 1 hour and incubated at 4°C with the appropriate primary antibodies (Claudin-5, Life Technologies (Carlsbad, CA, Estados Unidos), 1:500; Laminin, Sigma, 1:1000; Collagen IV, Abcam, 1:500 (Cambridge, Reino Unido)) followed by the secondary antibody Alexa Fluor™ 488 donkey antimouse IgG (1:1000) and Alexa Fluor™ 594 donkey antirabbit IgG (1:1000). They were then mounted in ProLong®Gold with DAPI (Life Technologies).

IgG leakage from serum into the brain was assessed as a marker of vasculature damage. After three washes with 0.1 M PBS, sections were blocked by incubation with 0.5 percent BSA, 10 percent goat serum and 0.1 percent Triton X-100 for 60 minutes and then incubated at 4°C overnight with the antibody Alexa Fluor™ 594 donkey antimouse IgG (1:1000) and covered with ProLong®Gold (Life Technologies). Images were acquired with a Zeiss Axio Imager A1 (Jena, Alemania) microscope with eight fields of 40× magnification per animal and condition. All images were converted to gray scale, and blood vessels were outlined to provide an integrated gray scale value for image analysis with IMAGEJ software (version 1.43; NIH, New York, NY, USA).

Figure representative images were acquired sequentially using a Leica TCS-SP2AOBS confocal microscope (Leica Microsystems, Heidelberg, Germany) for each fluorophore in order to avoid any cross-signal between them. Control experiments were performed in which sections were stained with each of the secondary antibodies or with a combination of them to rule out the possibility of reaction between them, and images were taken using the same settings for each antibody staining.

Statistical analyses

For the CPP data corresponding to each cocaine dose, the time spent in the drug-paired compartment during pre-C and post-C tests was analyzed with a mixed two-way ANOVA, with one between-subjects variable [Stress, with two levels (RSD and Control)] and a within-subjects variable [Days, with two levels (pre-C and post-C)]. In all cases, *post hoc* comparisons were performed with Bonferroni tests. In the groups showing CPP, extinction and reinstatement values were analyzed with Student's *t* tests. The time required for the preference to be extinguished in each animal was analyzed by means of the Kaplan–Meier test, with Breslow (generalized Wilcoxon) comparisons when appropriate (Daza-Losada *et al.*, 2009). Although the mean of the group as a whole determined the day on which extinction was considered to have been achieved, preference was confirmed to have been extinguished when a mouse spent 380 seconds or less in the drug-paired compartment on two consecutive days. We chose this time based on the values of all the pre-C tests performed in the study (mean = 370 seconds).

Corticosterone levels at minute 0 (immediately after) and 30 of the first and fourth social defeat and 3 weeks after the last social defeat encounter were analyzed with a mixed ANOVA with one between-subjects variable [Stress, with two levels (RSD and Control)] and a within-subjects variable (Time, with five levels). *Post hoc* comparisons were performed with Bonferroni tests.

An ANOVA with one within-subjects variable [Days, with two levels (first and fourth encounter)] was employed to evaluate each of the behaviors during the social encounter.

Data obtained within the different experimental groups of cocaine self-administration were compared using a three-way ANOVA with a between variable [Stress, with two levels (RSD and Control)] and repeated measures (day and hole as within group factors) followed by subsequent one-way ANOVA (hole as within group factor) when a main effect was revealed. Statistical significance criterion was $P < 0.05$. Data are expressed as number of reinforcers (mean + SEM) during each daily self-administration session for FR1 and FR3, and value of the breaking point (mean + SEM) obtained in the PR schedule.

Data of IgG extravasation and claudin-5, laminin and collagen-IV expression were analyzed using two-way ANOVA followed by a Bonferroni multiple comparison test (GRAPHPAD PRISM 5.0, GraphPad Software Inc., San Diego, CA, USA) with two between-subjects variables [Stress, with two levels (RSD and Control), and Treatment, with three levels (saline, 1 and 25 mg/kg)]. Differences were considered significant when $P < 0.05$. GRAPHPAD PRISM 5.0 and SPSS (Armonk, NY, Estados Unidos) version 19 software were used.

RESULTS

Behavioral characterization of social defeat in adolescent mice

The times opponent mice spent engaged in aggressive behaviors are shown in Supporting Information Table 2. In aggressive opponent mice, ANOVA revealed a significant effect of the variable Days for the time spent in attack [$F(1, 7) = 9.497$; $P < 0.01$], and the latency of threat [$F(1, 7) = 7.450$; $P < 0.05$] and attack [$F(1, 7) = 15.511$; $P = 0.01$]. During the fourth social defeat encounter, aggressive opponent mice threatened and attacked earlier and spent more time in attack behavior than during the first social defeat.

In defeated mice, the ANOVA revealed a significant effect of the variable Days on defense/submission [$F(1, 7) = 24.828$; $P = 0.01$] and latency of defense/submission [$F(1, 7) = 66.741$; $P = 0.001$] and avoidance [$F(1, 7) = 6.028$; $P = 0.05$]. Defeated mice spent more time engaged in submissive behavior and exhibited defensive/submissive and avoidance behaviors sooner during the fourth encounter than in the first encounter.

Effect of repeated social defeat on corticosterone levels

Blood concentrations of corticosterone are shown in the Supporting Information (Table S1).

Effects of repeated social defeat on the acquisition and reinstatement of cocaine-induced CPP

Conditioned place preference induced by 1 mg/kg of cocaine

The ANOVA revealed a significant effect of the Interaction Days × Stress [$F(1, 30) = 6.458$; $P < 0.01$] (Fig. 1a). The RSD-C1 group spent more time in the drug-paired compartment in the post-C test than in the pre-C test ($P < 0.01$). Extinction of preference was achieved after five extinction sessions and administration of a priming dose of 0.5 mg/kg of cocaine-induced reinstatement of the preference ($P < 0.05$). This preference was extinguished after four more sessions, and a further priming dose of 0.25 mg/kg of cocaine did not reinstate the preference.

Conditioned place preference induced by 25 mg/kg of cocaine

The ANOVA revealed a significant effect of the variable Days [$F(1, 27) = 62.215$; $P < 0.001$]. Both groups developed CPP, as they spent more time in the drug-paired compartment in the post-C test than in the pre-C test ($P < 0.001$) (Fig. 1b).

Kaplan–Meier analysis of the data recorded during the extinction test revealed that more time was required to achieve extinction in the RSD-C25 group (four sessions) than in the Control-C25 group (one session) ($\chi^2 = 27.000$; $P < 0.001$) (Fig. 1c). Once the preference had been extinguished, it was reinstated in all the adolescent groups with a priming dose of 12.5 mg/kg of cocaine ($P < 0.001$ in both cases). This new preference was extinguished after one session in both groups. A further priming dose of 6.25 mg/kg of cocaine reinstated once again the preference in both groups ($P < 0.01$ for Control-C25 and $P < 0.05$ for RSD-C25).

Effects of repeated social defeat on cocaine self-administration

The reinforcing properties of cocaine were also evaluated in mice that underwent RSD using an operant self-administration paradigm. Similar percentages of the mice exposed to social defeat (27 percent in 8.40 ± 0.51 days) and control animals (35 percent in 8.00 ± 0.37 days) reached the acquisition criteria of the operant responding during FR1 training. A significant discrimination between the active and inactive holes was revealed in social defeat (days 6, 8 and 9) and control (from day 3 to 9) mice during FR1 (Fig. 2a) and FR3 (days 1, 2 and 3 in both groups) training periods. A progressive increase in the number of active nose-poking responses was observed in both groups during FR1 training. No significant differences in the number of active nose pokes were detected between social defeat and

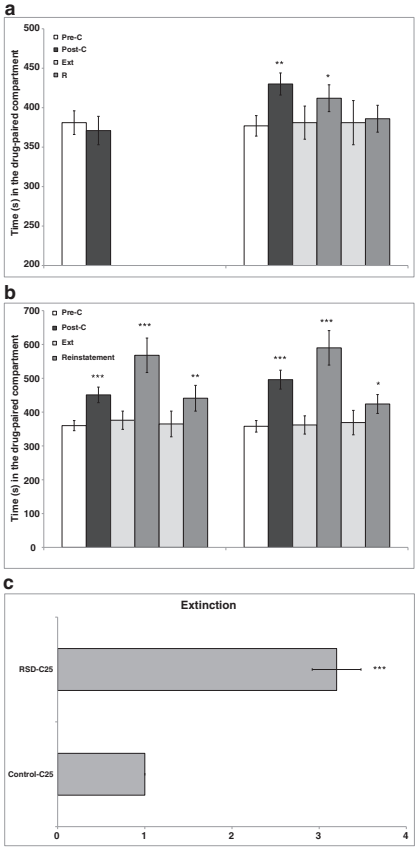

Figure 1 Effects of social defeat on acquisition of the CPP induced by cocaine. Bars represent the time (in seconds) spent in the drug-paired compartment before conditioning sessions in the pre-conditioning test (white bars), after conditioning sessions in the post-conditioning test (black bars), in the last extinction session (light gray bars) and during the reinstatement test (dark gray bars). (a) CPP induced by 1 mg/kg of cocaine. $^{**}P < 0.01$, $^{*}P < 0.05$ significant difference in the time spent in the drug-paired compartment versus pre-conditioning test or versus extinction test. (b) CPP induced by 25 mg/kg of cocaine. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ significant difference in the time spent in the drug-paired compartment versus pre-conditioning test or versus extinction test. (c) Bars represent the number of sessions needed to extinguish the preference after the post-C test. $^{***}P < 0.001$, significant difference with respect to control group

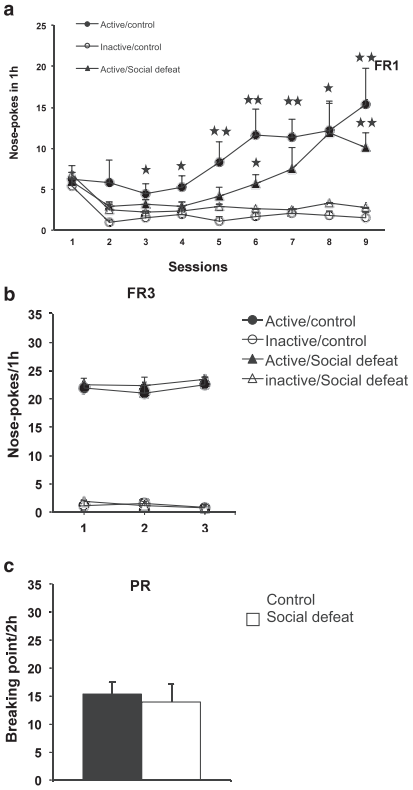

Figure 2 Acquisition of cocaine self-administration and motivation for cocaine in RSD and control mice. Mean number of active and inactive nose pokes during FR1 (a) or (b) FR3 schedule of reinforcement to obtain cocaine at the dose of 0.5 mg/kg/infusion i.v. in daily 1-hour sessions over 9 days of FR1 and 3 days of FR3; (c) mean breaking point in a single progressive ratio session that lasted 2 hours. Data are expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$ comparison between groups (Student–Newman–Keuls)

control groups during FR1 and FR3. In the PR schedule, the breaking points achieved by both groups were also similar (Fig. 2b and c). More information is presented in Tables S3 to S5.

Changes induced by social defeat in the expression of claudin-5 and basal laminin proteins and in IgG extravasation in the NAc and effect of cocaine

Quantitative analysis of claudin-5, laminin, collagen-IV and IgG immunoreactivity by two-way ANOVA (Fig. 3a–d) revealed a significant effect of Stress and Treatment, but not Interaction, indicating that stress had a similar effect in saline-treated and cocaine-treated mice (Table S6). Mice exposed to social defeat showed a decrease in claudin-5, laminin and collagen-IV expression and an increase in IgG immunoreactivity with respect to the control group, effects that were not

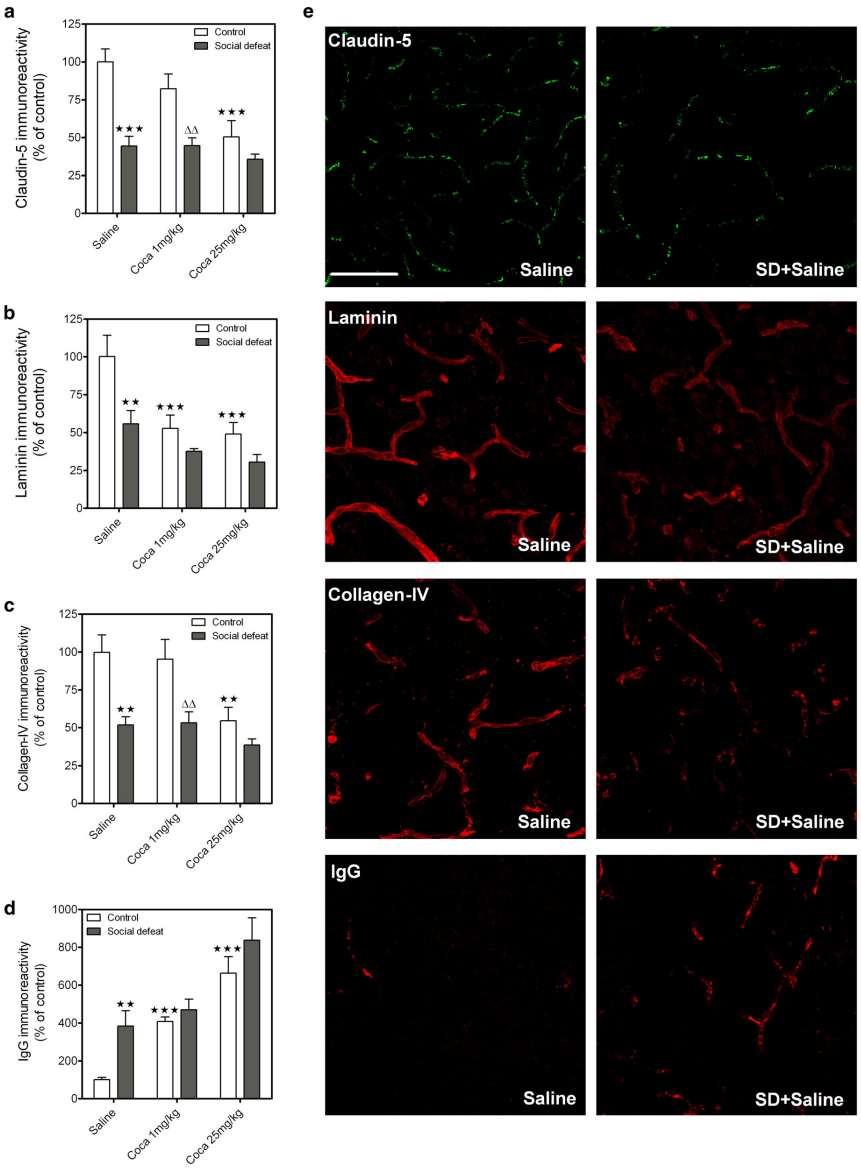

Figure 3 Claudin-5 (a), laminin (b), collagen-IV (c) and IgG (d) immunoreactivity in the nucleus accumbens of mice exposed to social defeat and treated with cocaine (1 and 25 mg/kg, i.p.). (e) Fluorescence images (40×) of representative claudin-5, laminin, collagen-IV and IgG immunostained sections of nucleus accumbens. Scale bar = 100 μm. Results shown as mean ± SEM (n = 4–8). Difference from control saline group: $^{**}P < 0.01$, $^{***}P < 0.001$. Different from mice treated with cocaine (1 mg/kg) but not exposed to social defeat: $^{\Delta\Delta}P < 0.01$

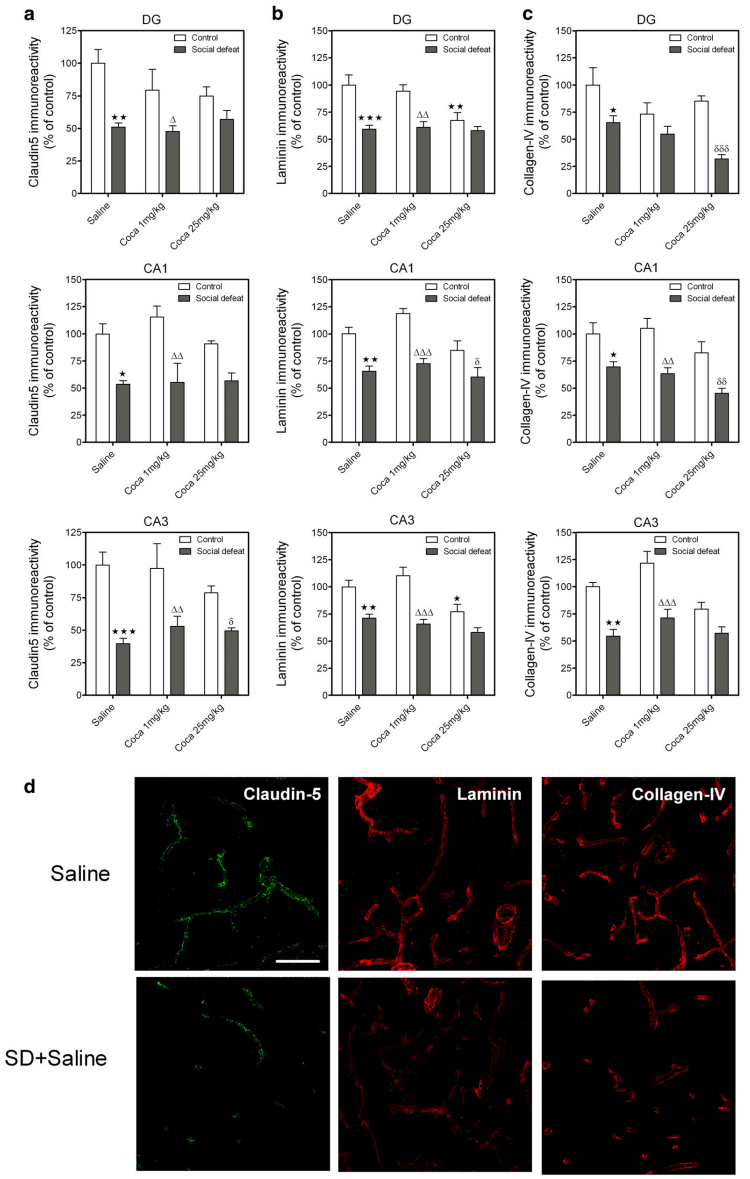

Figure 4 Claudin-5 (a), laminin (b) and collagen-IV (c) immunoreactivity in dentate gyrus (upper panel), CA1 (middle panel) and CA3 (lower panel) of mice exposed to social defeat and treated with cocaine (1 and 25 mg/kg, i.p.). (d) Fluorescence images (40×) of representative claudin-5, laminin and collagen-IV immunostained sections of CA1. Scale bar = 100 μm. Results shown as mean ± SEM ($n = 3$–7). Difference from control saline group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Difference from mice treated with cocaine (1 mg/kg) but not exposed to social defeat: $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$, $^{\Delta\Delta\Delta}P < 0.001$. Difference from mice treated with cocaine (25 mg/kg) but not exposed to social defeat: $^{\delta}P < 0.05$, $^{\delta\delta}P < 0.01$, $^{\delta\delta\delta}P < 0.001$

modified by cocaine administration. Cocaine at the dose of 25 mg/kg significantly reduced claudin-5, laminin and collagen-IV and increased IgG immunostaining compared with the saline group. At the dose of 1 mg/kg, cocaine induced a reduction only of laminin and an increase of IgG immunostaining with respect to the saline group. Mice exposed to social defeat and receiving cocaine 1 mg/kg exhibited a decrease in claudin-5 and collagen-IV immunoreactivity compared with the corresponding cocaine control group.

Figure 3e shows fluorescence images (40×) of representative claudin-5, laminin and collagen-IV immunostained sections of NAc.

Changes induced by social defeat on the expression of claudin-5 and basal laminin proteins in the hippocampus and effect of cocaine

Quantitative analysis of claudin-5, laminin and collagen-IV in the dentate gyrus, CA1 and CA3 by two-way ANOVA (Fig. 4a–d) revealed a significant effect of Stress and Treatment, but not Interaction, indicating that stress exerted a similar effect in saline-treated and cocaine-treated mice (for more details, see Supporting Information, Table S7). Mice exposed to social defeat showed a decrease in claudin-5, laminin and collagen-IV expression in the three hippocampal areas studied compared with the control group. Cocaine at the dose of 25 mg/kg significantly reduced laminin in the dentate gyrus and CA3 with respect to saline treatment. Mice exposed to social defeat and given cocaine 1 mg/kg showed a decrease in claudin-5 and collagen-IV immunoreactivity in the three areas studied (except for collagen-IV in the dentate gyrus) compared with their cocaine-treated counterparts. Social defeat plus 25 mg/kg of cocaine reduced claudin-5 in the CA3, laminin in the CA1, and collagen-IV in the dentate gyrus and CA1 with respect to the corresponding cocaine control group.

Figure 4d shows fluorescence images (40×) of representative claudin-5, laminin and collagen-IV immunostained sections of CA1.

DISCUSSION

This study provides the first evidence that RSD during adolescence impairs the structure and permeability of the BBB and modifies the rewarding effects of cocaine in adulthood. The novelty of our results lies in the evaluation of the long-lasting effects of RSD during adolescence on (1) the conditioned reinforcing effects of cocaine during adulthood and (2) BBB integrity by means of the quantification of claudin-5 (a tight junction protein), laminin and collagen-IV (main proteins forming basal lamina) immunoreactivity and IgG extravasation in the NAc and hippocampus.

Conditioned place preference and self-administration

Mice that experienced social defeat during adolescence developed CPP with a dose of cocaine (1 mg/kg) that was not effective in control animals. In addition, a priming dose of 0.5 mg/kg of cocaine reinstated the extinguished preference in RSD mice. An effective dose of cocaine (25 mg/kg) induced CPP in all groups, but RSD mice needed significantly more sessions than control animals for the preference to be distinguished. Only one study has previously evaluated the effect of chronic social defeat stress during adolescence on the CPP induced by amphetamine. In accordance with our results, Burke *et al.* (2011) observed that RSD during adolescence increased preference for amphetamine-paired cues in adulthood.

Although numerous studies show that social defeat in adulthood increases cocaine self-administration (e.g. Miczek *et al.*, 2004), to date, no study has evaluated the effects of social defeat when experienced during adolescence. Social stressors experienced in adulthood can significantly shorten the latency to acquire cocaine self-administration and to maintain this behavior at low unit doses (Kabbaj *et al.*, 2001; Yap *et al.*, 2015; Han *et al.*, 2015). Studies in this line have been performed mainly in rats, and cocaine self-administration has been evaluated immediately after the stressful experience or after a maximum of 10 days. Our results show that RSD experienced during adolescence delays the acquisition of cocaine self-administration when an effective dose is administered in adulthood. Control animals identified the inactive hole in the third session, while defeated mice required six sessions to discriminate the active hole. We cannot rule out that this delay may have been due to deficits in learning or memory processes. Although several studies have shown that RSD during adolescence does not affect aversive memory in the passive avoidance task or spatial learning (Buwalda *et al.*, 2005; Rodriguez-Arias *et al.*, 2015), previous findings have shown that social stress in adolescence results in deficits in hippocampal-based spatial memory (McCormick *et al.*, 2012; Sterlemann *et al.*, 2010). In line with this, a recent report by Novick *et al.* (2013) showed that rats defeated during adolescence displayed long-lasting deficits in spatial working memory performance. In our study, once self-administration was established, adolescent RSD did not seem to affect performance in a progressive schedule of reinforcement, suggesting that it does not contribute to the motivational properties of cocaine. Exposure during adolescence to other types of stress, such as early social isolation, resulted in heightened acquisition of cocaine

self-administration at low doses in adulthood, while a recent dose–response analysis has revealed that sensitivity to cocaine reinforcement is not altered (Baarendse *et al.*, 2014). Moreover, a single episode of early maternal deprivation has been shown to impair motivation for cocaine in adolescent mice; deprived mice require more time to achieve acquisition criteria, and the maximal effort required to obtain cocaine infusion is also significantly reduced (Martini & Valverde, 2012).

One possible explanation for the increased preference observed in our CPP study and the slow acquisition of self-administration is that RSD increased the sensitivity of the mice to the conditioned rewarding effects of cocaine. The low dose of cocaine administered in the CPP may have been experienced more intensely by defeated mice, which would have been more sensitive than their non-defeated counterparts. Consequently, these mice would have acquired CPP with a non-effective dose. However, as we employed an effective dose in the self-administration procedure, defeated mice would have experienced a stronger subjective effect, which would have induced them to self-administer this cocaine dose at a lower rate. In support of this hypothesis, previous studies have reported that although amphetamine-induced DA increases in the medial prefrontal cortex are attenuated in adult rats socially defeated in adolescence, the NAc core DA response to amphetamine is more pronounced than in non-defeated controls (Burke *et al.*, 2010). Moreover, it should be taken into consideration that social defeat during adolescence can differ to that observed in adult animals. In addition to the fact that adolescent rodents display lower physiological responses to social stressors (Adriani *et al.*, 1998), the first social encounter was less intense than the fourth, with aggressive opponents exhibiting less aggression. In line with previous reports (García-Pardo *et al.*, 2014), the corticosterone levels of the mice defeated in adolescence did not rise after the first social defeat encounter; in fact, the increase was significant only after the fourth social defeat. Defeated mice also exhibited higher levels of defense and submission in the last encounter. Importantly, each procedure was performed in a different strain of mice, which could be responsible for the different results obtained. Finally, the inconsistencies in the effects of RSD in the CPP and self-administration procedures (increased CPP and slower acquisition of nose-poking response) may also be due to the fact that these paradigms evaluate different aspects of reward. Self-administration models drug-taking behavior and evaluates the primary rewarding properties of drugs, while CPP assesses the incentive value of drug-associated cues for maintaining addictive behavior. We have found that exposure to RSD during adolescence, although not modifying the primary hedonic properties of cocaine in adult mice, increases the sensitivity of these animals to the conditioned rewarding effects of the drug, thus enhancing the ability of drug-related cues to maintain drug-seeking behavior.

Blood–brain barrier integrity

The current study shows for the first time that mice exposed to RSD undergo significant changes in BBB structure. RSD during adolescence induces a marked reduction in expression of the tight junction protein claudin-5 and an increase in basal laminin degradation (reflected by a decrease in laminin and collagen-IV expression) in the NAc in adulthood. Concomitantly, there is an increase in IgG extravasation, indicating that social defeat increases BBB permeability, probably through alterations in structural proteins. It is worth noting that the effect of stress on the disruption of BBB in our experimental animals was not brain region specific, as similar results were obtained when the subfields of the hippocampus, dentate gyrus, CA1 and CA3 were analyzed. In addition, social defeat induced the same alterations independent of previous cocaine exposure. Although there is a lack of studies on the effect of RSD on BBB integrity, there is abundant information regarding the effect of cocaine on BBB permeability. Our results confirm the detrimental effect of cocaine on the integrity of the BBB. Four administrations of the higher dose of cocaine on the post-C test day also affected BBB integrity in the NAc and increased the effect of social defeat on the hippocampus.

Although the mechanisms underlying these effects have not been evaluated in the current study, we believe that the effect of social defeat on BBB could have been produced by an increase in the activation of MMPs, specifically gelatinases such as MMP-9 and MMP-2. An increased gelatinolytic activity may disrupt BBB through proteolytic activity in the tight junctions of endothelial cells and basal lamina. In fact, laminin, collagen-IV and fibronectine are substrates of gelatinases. Numerous stimuli are reported to be involved in increased MMP activity and the basal laminin degradation that occurs as a consequence. Release of proinflammatory cytokines, particularly IL-1β, could be involved in augmented MMP-9 activity (Gottschall & Deb, 1996; Vecil *et al.*, 2000). Previous studies have shown that social defeat influences inflammatory immune processes, including concentrations and mRNA expression of the proinflammatory cytokines IL-6, IL-1β and TNF-α in both plasma and brain (Bartolomucci *et al.*, 2003; Audet *et al.*, 2011; McQuaid *et al.*, 2013), which may induce an increment in MMP expression, as observed in other conditions.

In addition to BBB disruption, mice socially defeated in adolescence take longer to acquire cocaine self-administration and are more sensitive to the rewarding properties of cocaine conditioning in adulthood. Recent

studies performed in socially defeated adult animals have demonstrated that this stress enhances DA release from the NAc shell in response to an acute dose of D-amphetamine or cocaine (Han *et al.*, 2015; Shimamoto *et al.*, 2015). This effect may be attributable to a high concentration of D-amphetamine or cocaine in the NAc owing to the increased BBB permeability induced by social defeat. Recently, it has been shown that RSD increases extracellular signal-regulated kinase phosphorylation in the ventral tegmental area and that inhibition of extracellular signal-regulated kinase activation prior to each social defeat attenuates the development of stress-induced sensitization and prevents stress-induced enhancement of cocaine self-administration during a continuous access binge (Yap *et al.*, 2015). Social defeat also induces phosphorylation of p38α MAPK in the dorsal raphe nucleus, and p38α MAPK deletion in serotonergic neurons prevents stress-induced reinstatement of cocaine seeking (Bruchas *et al.*, 2011). It is well known that MAPKs—downstream signaling pathways of proinflammatory cytokines—regulate MMP-9 expression and activity (Kim & Choi, 2010; Urrutia *et al.*, 2013) in such a way that an increase in MAPK phosphorylation increases MMP activity and facilitates BBB disruption. Inhibition of MAPK activity could prevent the effects induced by social defeat on BBB permeability and on the rewarding properties of drugs of abuse.

Together, these findings suggest that stress and cocaine can increase the long-term vulnerability of the brain to subsequent environmental insults as a consequence of sustained disruption of the BBB. This interaction between RSD and cocaine is particularly relevant, as chronic stress is a common contributing factor to the high rate of comorbidity between cocaine abuse and mental disorders.

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Disclosure/Conflict of Interest

None.

Authors Contribution

All named authors have made an active contribution to the conception, design, analysis and drafting of the article. MRA, SMR, MAA and JM performed the social defeat protocol and evaluated social, emotional and cognitive behavioral parameters; furthermore, they performed and evaluated the CPP paradigm and the corticosterone measurements. ARA, FP and MIC were responsible for the immunohistochemistry analysis. EMG, RC and RM were responsible for the cocaine self-administration experiments. All authors critically reviewed the content and approved the final version for publication. All authors have contributed equally to the overall coordination of the whole study.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Effect of acute social stress on corticosterone levels

Table S2. Behavior of mice during agonistic encounters

Table S3. Operant response of Control mice maintained by cocaine during acquisition

Table S4. Operant response of DSR mice maintained by cocaine during acquisition

Table S5. Operant response of Control and RSD mice maintained by cocaine during acquisition

Table S6. Statistical analysis (two-way ANOVA) of claudin-5, laminin, collagen-IV and IgG expression in the NAc of socially defeated mice treated with cocaine

Table S7. Statistical analysis (two-way ANOVA) of claudin-5, laminin and collagen-IV expression in the hippocampus of socially defeated mice treated with cocaine

STUDY 3

Role of dopamine neurotransmission in the long-term effects of repeated social defeat on the conditioned rewarding effects of cocaine

S. Montagud-Romero, M.D. Reguilon, C. Roger-Sanchez, M. Pascual, M.A. Aguilar, C. Guerri, J. Miñarro, M. Rodríguez-Arias

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Role of dopamine neurotransmission in the long-term effects of repeated social defeat on the conditioned rewarding effects of cocaine

S. Montagud-Romero [a], M.D. Reguilon [a], C. Roger-Sanchez [a], M. Pascual [b], M.A. Aguilar [a], C. Guerri [b], J. Miñarro [a], M. Rodríguez-Arias [a,*]

[a] Department of Psychobiology, Facultad de Psicología, Universitat de Valencia, Avda. Blasco Ibáñez 21, 46010 Valencia, Spain
[b] Department of Molecular and Cellular Pathology of Alcohol, Príncipe Felipe Research Center, C/Eduardo Primo Yúfera 3, 46012 Valencia, Spain

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ABSTRACT

Numerous studies report that social defeat stress alters dopamine (DA) neurotransmission in several areas of the brain. Alterations of the mesolimbic dopaminergic pathway are believed to be responsible for the increased vulnerability to drug use observed as a result of social stress. In the present study, we evaluated the influence of DA receptors on the long-term effect of repeated social defeat (RSD) on the conditioned rewarding and reinstating effects of cocaine. For this purpose, the D1R antagonist SCH 23390 and the D1R antagonist raclopride were administered 30 min before each social defeat and a cocaine-induced CPP procedure was initiated three weeks later. The expression of the D1R and D2R was also measured in the cortex and hippocampus throughout the entire procedure. Mice exposed to RSD showed an increase in the conditioned rewarding effects of cocaine that was blocked by both DA receptors antagonists when a subthreshold dose of cocaine was employed. However, while the vulnerability to reinstatement of the preference induced by 25 mg/kg cocaine-induced CPP was abolished by the D1R antagonist, it was practically unaffected by raclopride. Increases in D2R receptor levels were observed in the cortex of defeated animals after the first and fourth social defeats and in the hippocampus 3 weeks later. Nevertheless, D1R receptor levels in the hippocampus decreased only after the last social defeat. Our results confirm that RSD enhances the conditioned rewarding effects of cocaine and that both DA receptors are involved in this enduring effect of social stress.

1. Introduction

Stressful experiences modify the activity of brain areas involved in the rewarding effects of psychostimulants (Belujon and Grace, 2011; Koob, 2008; Sinha, 2008). A positive association between stress and increased drug intake and relapse to drug use has been described (Sinha et al., 2011). Preclinical studies report a significant association between acute and chronic stress and an increase in motivation to initiate use and augment the consumption of addictive substances (Sinha, 2001; Sinha et al., 2006; Koob and Kreek, 2007; Miczek et al., 2008). Furthermore, repeated exposure to stressors results in a long-term enhancement of dopamine (DA) release in the mesoaccumbens pathway in response to psychostimulant challenge (Sorg and Kalivas, 1991; Wilcox et al., 1986).

Different studies found that social defeat stress alters DA neurotransmission (Tidey and Miczek, 1996; Cabib et al., 2000; Isovich et al., 2001; Razzoli et al., 2011; Shimamoto et al., 2015). Social stressors have been related with increases in extracellular DA release in the shell of the nucleus accumbens (NAcc) (Holly et al., 2015; Han et al., 2015; Piazza and Le Moal, 1998; Tidey and Miczek, 1997), and also in response to cocaine (Miczek et al., 2011) or D-amphetamine (Han et al., 2015). Few studies have investigated the effects of social defeat stress on DA receptors and those to have done it have obtained discrepant results. Increases, decreases or no changes in the levels of D1R and D2R have been found after being exposed to social defeat (Lucas et al., 2004; Rasheed et al., 2010; Bagalkot et al., 2015; Huang et al., 2016; Jin et al., 2015).

In experimental models, acute exposure to different stressful experiences can promote psychostimulant use and increase the escalation of consumption (Shaham et al., 2000, 2003; Sanchez et al., 2003). Social defeat in an agonistic encounter increases vulnerability to acquiring and maintaining cocaine self-administration (Tidey and Miczek, 1997; Covington et al., 2005; Covington and Miczek, 2005), prompts an escalation of cocaine-seeking behavior (Burke and Miczek, 2015; Boyson et al., 2011; Covington et al., 2005; Miczek et al., 2011), and increases the conditioned rewarding effects of cocaine and MDMA (Rodríguez-Arias et al., 2015; García-Pardo et al., 2015; Montagud-Romero et al., 2015). The conditioned place preference (CPP) paradigm has been widely used to study the conditioned rewarding effects of addictive drugs, since contextual stimuli can acquire secondary appetitive properties (conditioned rewarding effects) when paired with a primary reinforcer, thereby highlighting the liability of

* Corresponding author.
E-mail address: marta.rodriguez@uv.es (M. Rodríguez-Arias).

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abuse (Tzschentke, 2007). While D1R antagonists block cocaine-induced CPP (Cervo and Samanin, 1995; Pruitt et al., 1995; Baker et al., 1998; Liao, 2008; Nazarian et al., 2004), there is controversial evidence regarding the blockade of D2R; some studies have reported that D2R antagonists do not affect cocaine-induced CPP (Cervo and Samanin, 1995; Pruitt et al., 1995; Baker et al., 1996; Nazarian et al., 2004), while others have shown that they play a role in the reinstatement of cocaine-induced CPP (Badanich and Kirstein, 2012).

The relationship between stress and DA receptors has been demonstrated repeatedly. Physical acute stress can increase the activity of D2R in the NAcc, and induces CPP that can be blocked by either D1R or D2R antagonists (Shen et al., 2010). Moreover, changes in D1R have been observed following social encounters during adolescence or adulthood (Avgustinovich and Alekseyenko, 2010; Novick et al., 2011). While the experience of acute social defeat increases the conditioned rewarding effects of cocaine immediately after the stress (Montagud-Romero et al., 2015), the administration of a D1R antagonist (raclopride) before the stress experience blocks the increase in the rewarding effects of cocaine induced by social stress without affecting the rewarding properties of cocaine in the CPP (Reguilón et al., under review). Equally, intra-mPFC infusion of a D2R antagonist prior to each defeat episode during adolescence has been shown to prevent defeat-induced reductions in mPFC DA turnover in early adulthood (Watt et al., 2014). On the other hand, pretreatment with SCH23390 (D1R antagonist), but not raclopride (D1R antagonist), blocks CRF-induced reinstatement of cocaine-seeking (Brown et al., 2012). Similar dissociation of the selective effects of D1R and D2R antagonists on the reinstatement of cocaine-seeking by central injections of other stress-related neuropeptides (Lopak and Erb, 2005) or intra-VTA injections of a Substance P analogue (Placenza et al., 2004) has also been reported.

The aim of the present study was to evaluate the influence of DA receptors on the long-term effect of repeated social defeat (RSD) on the conditioned rewarding and reinstating effects of cocaine. For this purpose, the D1R antagonist SCH 23390 and the D2R antagonist raclopride were administered 30 min before each social defeat and three weeks later cocaine-induced CPP procedure was initiated. Expression of the D1R and D2R receptors was also measured in the whole cortex and hippocampus brain structures throughout the entire procedure.

2. Material and methods

This study was designed to evaluate the role of DA receptors in the long lasting effects that social defeat induced in the CPP induced by cocaine. Adult mice were exposed to four social defeats and the rewarding effects of cocaine in the CPP were evaluated three weeks later. In the control groups, the D1R antagonist SCH 23390 or the D2R antagonist raclopride was administered before each social defeat or exploration. Three weeks after the last social defeat, cocaine (1 or 25 mg/kg)-induced CPP was initiated. Another set of mice was employed to obtain brain samples and determine levels of D1and D2R after the first and fourth social defeat, and also three weeks later.

2.1. Animals

A total of 289 male OF1 (Charles River, France) arrived at our laboratory at 42 days of age. All mice (except those used as aggressive opponents n = 30) were housed in groups of four in plastic cages (25 × 25 × 14.5 cm) for 8 days before the experiments began. Aggressive opponents were individually housed in plastic cages (23 × 13.5 × 13 cm) for a month prior to experiments in order to heighten aggression (Rodríguez-Arias et al., 1998). While 211 animals were tested in the CPP experiment, an independent set of 48 mice were used in the Western Blot procedure. Mice were housed in controlled laboratory conditions with the temperature maintained at 21 ± 1 °C and humidity at 55 ± 10%. All tests took place during the first few hours of the dark phase of a reversed light/dark cycle (lights off at 08:00 h and on at 20:00 h). Food and water were available ad libitum to all the mice used in this study. All procedures were conducted in compliance with the guidelines of the European Council Directive 2010/63/UE regulating animal research and were approved by the local ethics committees (University of Valencia).

2.2. Drugs

Animals were injected i.p. with 1 or 25 mg/kg of cocaine hydrochloride (Laboratorios Alcaliber, Madrid, Spain), 0.125 and 0.250 mg/kg of SCH 23390 (Research Biochemical International, Natick, USA), and 0.3 and 0.6 mg/kg of Raclopride (RACL) (Astra Laboratory, Sodertalje, Sweden) in a volume of 0.01 ml/g of weight. Control groups were injected with physiological saline (NaCl 0.9%), which was also used to dissolve the drugs. These doses were selected on the basis of previous studies (Manzanedo et al., 2001; Vidal-Infer et al., 2012; Arenas et al., 2014; Montagud-Romero et al., 2014).

2.3. Procedure and apparatus

The experimental design is depicted in Table 1.

2.3.1. Repeated social defeat encounters

Animals in the stress/defeated groups were exposed to 4 episodes of social defeat lasting 25 min each on postnatal days (PND) 47, 50, 53 and 56. Each episode consisted of three phases, which began by placing the experimental animal or intruder in the home cage of the aggressive opponent or resident for 10 min. During this initial phase, the intruder was protected from attack by a wire mesh wall that permitted social interaction and species-typical threats from the aggressive resident (Covington and Miczek, 2001). In the second phase, the wire mesh was removed from the cage and a 5-min period of confrontation began. The second phase of each social defeat protocol was video-recorded and ethologically analyzed. Threat and attack behaviors were scored in resident mice and avoidance/flee and defensive/submissive behaviors were evaluated in intruder mice. In the third phase, the wire mesh was replaced for a further 10 min to allow social threats from the resident. The non-stressed exploration groups underwent the same protocol, but without the presence of a "resident" mouse in the cage. Following this last phase, animals were kept in the vivarium for three weeks, after which the behavioral tests began.

In the corresponding groups, physiological saline, SCH 23390 or Raclopride were administered 30 min before each social encounter. Control groups received saline or the DA antagonist 30 min before exploration.

2.3.2. Conditioned place preference

2.3.2.1. Apparatus. For place conditioning, we employed eight identical Plexiglas boxes with two equally-sized compartments (30.7 cm long × 31.5 cm wide × 34.5 cm high) separated by a gray central area (13.8 cm long × 31.5 cm wide × 34.5 cm high). The compartments had different colored walls (black vs white) and distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed the recording of the position of the animals and their crossings from one compartment to the other. The equipment was controlled by three IBM PC computers using MONPRE 2Z software (CIBERTEC, SA, Spain).

2.3.2.2. Procedure of the CPP

2.3.2.2.1. Acquisition. Place conditioning, which consisted of three phases, was carried out during the dark cycle following a procedure that was unbiased in terms of initial spontaneous preference (Manzanedo et al., 2001). During the first phase - or preconditioning (Pre-C) - mice were allowed access to both compartments of the

Table 1
Experimental procedure.

Postnatal days		47	50	53	56	57–76	77	78–80	81–84	85	106	>108
Repeated social defeat/exploration		1st	2nd	3th	4th		Brain samples					
Western blotting	n = 48	Brain samples			Brain samples							
Conditioned place preference induced by	30 min before											
1 mg/kg cocaine	Exploration											
	n = 14	Saline	Saline	Saline	Saline							
	n = 14	SCH 0.250	SCH 0.250	SCH 0.250	SCH 0.250							
	n = 11	Rac 0.6	Rac 0.6	Rac 0.6	Rac 0.6							
	n = 13	Saline	Saline	Saline	Saline							
RSD	n = 15	SCH 0.250	SCH 0.250	SCH 0.250	SCH 0.250							
	n = 12	SCH 0.125	SCH 0.125	SCH 0.125	SCH 0.125	3 weeks						
	n = 19	Rac 0.6	Rac 0.6	Rac 0.6	Rac 0.6			Pre-C	Conditioning	Post-C	Extinction	Reinstatement
	n = 16	Rac 0.3	Rac 0.3	Rac 0.3	Rac 0.3							
Conditioned place preference induced by	30 min before											
25 mg/kg cocaine	Exploration											
	n = 14	Saline	Saline	Saline	Saline							
	n = 15	SCH 0.250	SCH 0.250	SCH 0.250	SCH 0.250							
	n = 12	Rac 0.6	Rac 0.6	Rac 0.6	Rac 0.6							
	n = 13	Saline	Saline	Saline	Saline							
RSD	n = 16	SCH 0.250	SCH 0.250	SCH 0.250	SCH 0.250							
	n = 18	Rac 0.6	Rac 0.6	Rac 0.6	Rac 0.6							

apparatus for 900 s per day on 3 consecutive days. On day 3, the time spent in each compartment was recorded. Animals showing a strong unconditioned aversion (<33% of session time; i.e. 250 s) or preference (>67% of the session time; i.e. 650 s) for any compartment were discarded from the rest of the study. In each group, during the conditioning phase, half of the animals received the drug or vehicle in one compartment while the other half received it in the other compartment. ANOVA showed no significant differences between the time spent in the drug-paired and the vehicle-paired compartments during the Pre-C phase. In the second phase (conditioning), which lasted 4 days, animals were conditioned with cocaine or saline. An injection of physiological saline was administered before confining the mice to the vehicle-paired compartment for 30 min. After an interval of 4 h, the animals received cocaine immediately prior to confinement to the drug-paired compartment for a further 30 min. The central area was made inaccessible by guillotine doors during conditioning. Two different doses of cocaine were used during conditioning phase: a subthreshold dose (1 mg/kg, proved not to be effective in controls) in order to evaluate increased sensitivity to the conditioned rewarding effects of cocaine; and an effective dose (25 mg/kg, that induces a strong preference and reinstatement induced by priming doses) in order to evaluate sensitivity to conditioned reinstatement of the preference. In the third phase—or postconditioning (Post-C)—which took place on day 8, the guillotine doors separating the two compartments were removed, and the time spent in each compartment by the untreated mice was recorded during a 900-s observation period. The difference in seconds between the time spent in the drug-paired compartment during Post-C and Pre-C tests is a measure of the degree of conditioning induced by the drug. If this difference is positive, then the drug has induced a preference for the drug-paired compartment, while the opposite indicates an aversion.

2.3.2.2.2. Extinction of CPP. All groups in which CPP was confirmed were subsequently exposed to the extinction procedure. Extinction consisted of placing animals in the apparatus (without the guillotine doors separating the compartments) for 900 s until the time spent in the drug-paired compartment by each group was similar to that of the Pre-C test and different from that of the Post-C test (Student's *t*-test). In this way, all the animals in each group were submitted to the same number of extinction sessions, independently of their individual scores. Extinction was always confirmed in a subsequent session 24 h later (confirmation session). For the subthreshold cocaine dose, the first extinction session took place three days after the Post-C test. However, the first extinction session of the CPP induced by 25 mg/kg of cocaine took place three weeks after the Post-C test.

2.3.2.2.3. Reinstatement of CPP. Twenty-four hours after the confirmation session, the effects of a priming dose of cocaine (half of the dose used during conditioning; 0.5 or 12.5 mg/kg) were evaluated. The reinstatement tests were identical to those in the Post-C procedure (free ambulation for 15 min), except that animals were tested 15 min after the administration of cocaine. Priming injections were administered in the vivarium, which constituted a noncontingent place to that of the previous conditioning procedure. After this first reinstatement test, the groups that demonstrated reinstatement—i.e. a positive significant difference between the time spent in the drug-paired compartment in the reinstatement and last extinction tests (confirmed with a Student's *t*-test) — were re-tested until a new extinction had been confirmed. After each reinstatement of CPP —i.e. a positive significant difference between the time spent in the drug-paired compartment in the reinstatement and last extinction tests (confirmed with a Student's *t*-test) — the animals were subjected to two weekly extinction sessions until the CPP had been completely extinguished. This procedure of extinction/reinstatement was repeated with decreasing doses (half the previous dose) until a priming dose was confirmed to be ineffective.

2.3.3. Tissue sampling

Animals were sacrificed by cervical fracture on PND 47-56-77. Within 2 min their brains were removed and placed on an ice-cold plate. The

entire brain structures, cortex and hippocampus were dissected following the procedure described by Heffner et al. (1980), and were then frozen on dry ice and stored at −80 °C.

2.3.4. Western blot analysis for D1R and D2R

Brain tissue from the cortex and hippocampus was homogenized in lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 8, 4 mM sodium chloride, 40 mM sodium fluoride and protease inhibitors) for 30 min on ice. An equal amount of cell lysate of each sample (40 μg of protein/lane) was loaded onto sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and was then blotted onto polyvinylidene fluoride membranes. Membranes were blocked with 5% BSA (bovine serum albumin) in TBS containing 0.1% Tween-20 (TBS/T), and were then incubated overnight with the following primary antibodies: anti-D1R (1:1000 Santa Cruz Biotechnology, Madrid, Spain) and anti-D2R (1:1000 Santa Cruz Biotechnology, Madrid, Spain). After washing with TBS/T, blots were incubated with HRP-conjugated antibodies. Blots were developed using the ECL system (ECL Plus; Thermo Scientific, Illinois, USA). All the membranes were stripped in sodium dodecyl sulfate (SDS) solution (0.4% SDS and 200 mM glycine, pH 2.5) during a 30-min period and were washed and incubated with anti-GAPDH mAb (Chemicon, California, USA) for 2 h as a loading control. The intensity of the bands was quantified with the image analysis software ImageJ 1.44p (National Institutes of Health, USA). The densitometry analysis is shown in arbitrary units normalized to the GAPDH loading control.

2.4. Statistical analyses

For the CPP data for each cocaine dose, the time spent in the drug-paired compartment during Pre- and Post-C tests was analyzed with a mixed three-way ANOVA, with two between-subjects variable – Stress (RSD and explore) – and -Treatment (Saline, SCH or Raclopride) - and a within-subjects variable – Days (Pre-C and Post-C). For the CPP data of the subthreshold dose of cocaine, two ANOVAs were performed: Stress x Treatment x Days, employing only the highest dose of the DA antagonists; and Treatment x Dose x Days, using only the groups exposed to RSD. Post hoc comparisons were performed with Bonferroni tests. In addition, extinction and reinstatement values were analyzed with Student's t tests. The time required for the preference to be extinguished in each animal was analyzed by means of the Kaplan–Meier test, with Breslow (generalized Wilcoxon) comparisons when appropriate. Although the mean of the group as a whole determined the day on which extinction was considered to have been achieved, preference was confirmed to have been extinguished when a mouse spent 380 s or less in the drug-paired compartment on two consecutive days. We chose this time based on the values of all the Pre-C tests performed in the study (mean = 370 s).

Statistical significance for D1R and D2R expression was determined by a one-way ANOVA, with one between-subjects variable - pretreatment, with four levels (Control, 1RSD, 4RSD, 3 W) -followed by a Bonferroni's post-hoc test. The results are reported as mean ± S.E.M.

3. Results

3.1. Effect of D1R and D2R antagonists on RSD provoked-increase of CPP induced by 1 mg/kg cocaine

The ANOVA (see Fig. 1) showed an effect of the interaction Days × Stress × Treatment [$F(2,82) = 3865$; $p < 0.025$]. Socially defeated mice treated with saline (RSD-Sal) developed CPP, spending more time in the drug-paired compartment during the Post-C test than in the Pre-C test ($p < 0.001$). Simple effects showed that none of the groups treated during social defeat with any of the SCH 23390 or raclopride doses developed 1 mg/kg cocaine-induced CPP. An ANOVA showed no differences between the effects induced by the different antagonist doses used.

In conclusion, both DA antagonists blocked the increase in the conditioned rewarding effects of cocaine induced by RSD when administered before each social defeat.

3.2. Effect of D1R and D2R antagonists on RSD provoked-increased of CPP induced by 25 mg/kg cocaine

The ANOVA of the effects of SCH 23390 on the cocaine-induced CPP (see Fig. 2) in control and socially defeated mice showed an effect of the variable Days [$F(1,82) = 81{,}736$; $p < 0.001$]. All the groups spent more time in the cocaine-paired compartment during the Post-C test than in the Pre-C test, thus developing cocaine-induced CPP ($p < 0.001$). There were no differences among the groups in the number of sessions needed to extinguish the preference of the Post-C test (1 to 5 sessions). Equally, preference was reinstated in all the groups after a priming injection of 12.5 mg/kg of cocaine ($p < 0.05$ for the group Exp-Rac 0.6 and $p < 0.001$ for the rest of the groups). Again, a priming dose of 6.25 mg/kg of cocaine reinstated the preference in the RSD-Sal ($p < 0.05$), Exp-Sal, Exp-SCH 0.250 and RSD-Rac 0.6 groups ($p < 0.01$ in all cases). Reinstatement of the preference after a priming dose of 3.125 was achieved only in the RSD-Sal and RSD-Rac 0.6 groups ($p < 0.01$). A further priming dose of 1.56 mg/kg of cocaine reinstated the preference only in the RSD-Sal groups ($p < 0.05$). No further reinstatement was obtained. There were also no differences in the number of sessions needed to extinguish the preference after any of the reinstatement tests (1 to 3 sessions).

In conclusion, when administered before each social defeat, both DA antagonists diminished sensitivity to reinstatement of the preference that developed after a cocaine priming dose, although a stronger effect was observed after blockade of D1R.

3.3. Effect of RSD on the expression of D1R and D2R in the cortex and hippocampus of adult mice

Repeated social defeat modified the protein expression of the D1R receptor in the hippocampus [$F(3, 20) = 3.957$; $p < 0.05$], decreasing the levels of D1R after the fourth encounter when compared with the control group ($p < 0.05$), without significant changes being detected in the cortex. (see Fig. 3a and b).

Repeated social defeat induces changes in the protein expression of the D2R receptor in the hippocampus [$F(3, 20) = 6.251$; $p < 0.01$] and cortex [$F(3, 20) = 5.179$; $p < 0.01$]. Social defeat induced increases in D2R levels in the hippocampus three weeks after the last defeat when compared with the control group ($p = 0.05$) and the fourth social defeat ($p < 0.01$) (see Fig. 4b). Equally, D2R levels in the cortex were increased after the first and fourth social defeat with respect to controls ($p < 0.01$ in both cases) (see Fig. 4a).

In conclusion, D2R increased in the cortex and hippocampus after RSD, but D1R did not show clear modifications.

4. Discussion

Our results confirm that RSD increases the conditioned rewarding effects of a subthreshold dose of cocaine and that both DA receptors are involved in this long-lasting effect of social stress. Blockade of D1R or D2R during social defeat completely abolished the long-lasting increase in the CPP induced by 1 mg/kg of cocaine. The augmented vulnerability to reinstatement of cocaine-induced CPP after extinction induced by social stress was impeded by the D1R antagonist but was practically unaffected by D2R receptor blockade. Increases in D2R levels were observed in the cortex of our animals in response to social defeat and in the hippocampus 3 weeks after the last social defeat, when the CPP procedure was initiated. However, D1R levels in the hippocampus decreased only after the last social defeat.

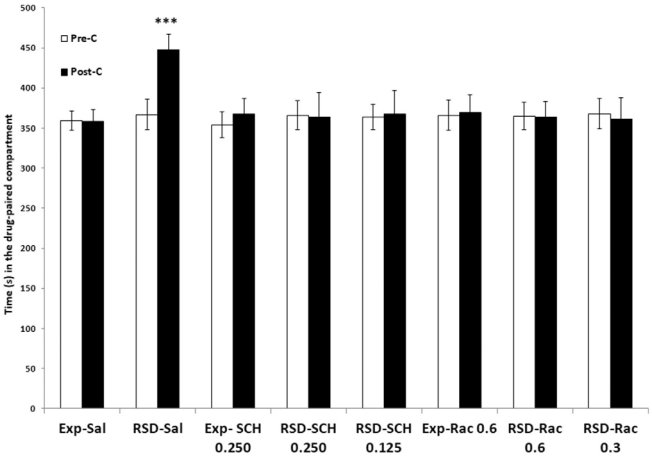

Fig. 1. Effect of D1R and D2R antagonists on RSD-provoked increase of CPP induced by 1 mg/kg cocaine. Animals were divided into the following eight groups: EXP-Sal group, allowed to explore a new cage and pretreated with saline (n = 14); 0.250 mg/kg of SCH23390 (Exp-SCH 0.250; n = 14); or 0.6 mg/kg of raclopride (Exp-Rac 0.3; n = 11). RSD-Sal group, exposed to the agonistic encounter and pretreated with saline (n = 13); 0.125 mg/kg of SCH23390 (RSD-SCH 0.125; n = 12); 0.250 mg/kg of SCH23390 (RSD-SCH 0.250; n = 15); 0.3 mg/kg of raclopride (RSD-Rac 0.3; n = 16); or 0.6 mg/kg of raclopride (RSD-Rac 0.6; n = 19). Bars represent the time (in seconds) spent in the drug-paired compartment before conditioning sessions in the pre-conditioning test (white bars), and after conditioning sessions in the post-conditioning test (black bars). ***$p < 0.001$, significant difference in the time spent in the drug-paired compartment vs pre-conditioning test.

4.1. RSD induces an increase in the rewarding and reinstating effects of CPP

The CPP paradigm reflects the secondary motivational properties of drugs and their potential for abuse (Tzschentke, 2007), and is commonly used to evaluate the reinstatement of drug-seeking after extinction (Aguilar et al., 2009). In numerous studies we have demonstrated that 1 mg/kg of cocaine is a subthreshold dose for the development of CPP in young and adult mice (Rodríguez-Arias et al., 2015; Mateos-García et al., 2015; Montagud-Romero et al., 2015; Arenas et al., 2016). Here we report that experience of social defeat during adulthood increases the conditioned rewarding effects of this low dose of cocaine 3 weeks after the last social defeat. This is the first report showing this long-lasting effect of RSD in adult subjects, although two previous studies have reported that RSD during adolescence increases preference for amphetamine- and cocaine-paired cues in adulthood (Burke et al., 2011; Rodríguez-Arias et al., 2015). There have been reports that acute social defeat stress also induces this effect in adult rodents, but when cocaine conditioning takes place immediately (McLaughlin et al., 2006; Montagud-Romero et al., 2015) or 24 h after social defeat stress (Hymel et al., 2014). On the other hand, all the groups (exploration and RSD) showed preference for an effective (25 mg/kg) dose of cocaine in the CPP and reinstatement of this preference with successive smaller doses of cocaine, as we have previously reported (Rodríguez-Arias et al., 2009; Maldonado et al., 2006). However, only defeated mice displayed reinstatement of preference after receiving a priming dose of 3.12 and 1.5 mg/kg of cocaine, therefore showing higher vulnerability to reinstatement of said preference. Furthermore, social defeat during an agonistic encounter in a neutral area prior to a reinstatement test has been shown to reinstate cocaine CPP in adult mice (Land et al., 2009; Titomanlio et al., 2013) and to increase susceptibility to cocaine-induced reinstatement of CPP (Ribeiro Do Couto et al., 2009). These results are in line with those of numerous studies which have demonstrated that social defeat increases vulnerability to cocaine self-administration (e.g. Boyson et al., 2011; Yap et al., 2015; Burke and Miczek, 2015).

4.2. Effects of D1R antagonist

Administration of a DA antagonist 30 min before each defeat experience and performance of the cocaine-induced CPP three weeks later allowed us to explore the effects of dopaminergic blockade without the interference of the acute effect of DA antagonists on cocaine reward. Blockade of the D1R with SCH 23390 produced a specific effect in the RSD group, while negligible changes were observed in control groups exposed to this DA antagonist. SCH 23390 administered before each social defeat completely impeded the development of preference in response to a subthreshold dose of cocaine in defeated mice. In addition, in stressed mice conditioned with 25 mg/kg of cocaine, blockade of D1R receptors during social defeat efficiently decreased vulnerability to reinstatement of the preference induced by a priming dose of cocaine. Although preference was reinstated in non-treated defeated mice with as low a dose as 1.56 mg/kg of cocaine, those treated with SCH 23390 only showed reinstatement after receiving a priming dose of 12.5 mg/kg of cocaine. This response is similar to that observed in non-stressed control mice. Therefore, blockade of D1R during the experience of stress inhibits the development of sensitivity to the acquiring of cocaine-induced CPP and its reinstatement.

It has been repeatedly shown that SCH 23390 fully blocks cocaine-induced CPP (Baker et al., 1996, 1998; Cervo and Samanin, 1995; Nazarian et al., 2004), and that higher doses of SCH 23390 are even capable of inducing place aversion (Manzanedo et al., 2001: Vidal-Infer et al., 2012). Taking into account this known effect, the present experimental design allows us to avoid this potent effect of D1R blockade and to explore only the role of these receptors on the long-lasting effects of social stress on cocaine reward.

4.3. Effects of D2R antagonist

With respect to D2R, our results do not show a consistent role of these receptors in the enduring effects of stress. Blockade of these

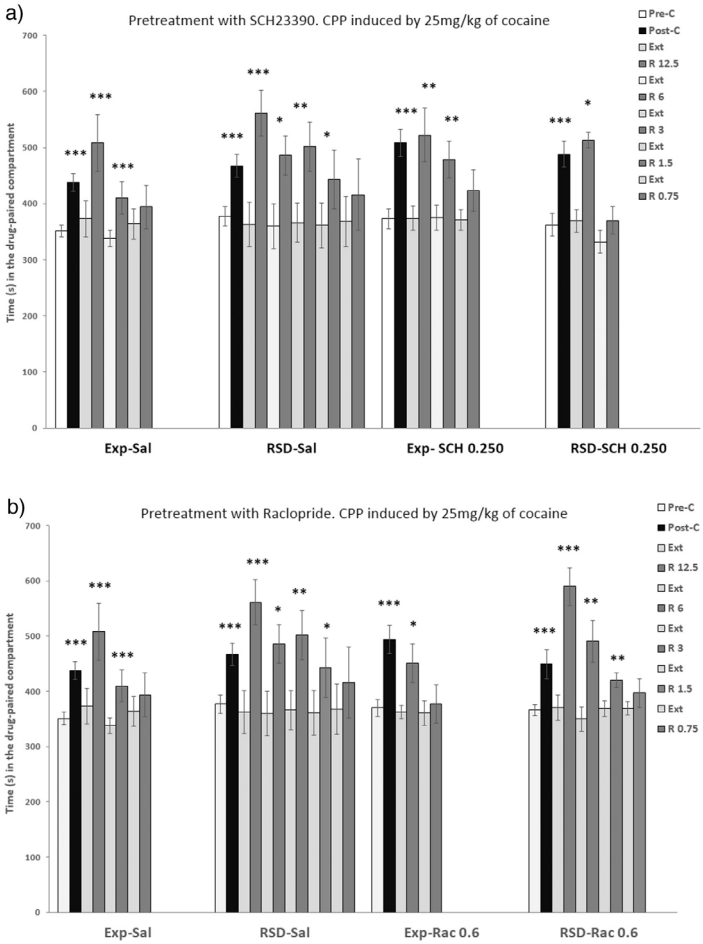

Fig. 2. Effects of the D1R and D2R antagonists SCH 23390 (a) and raclopride (b) on reinstatement of the CPP induced by 25 mg/kg of cocaine in control and socially defeated adult mice. Animals were divided into the following six groups: EXP-Sal group, allowed to explore a new cage and pretreated with saline (n = 14); 0.250 mg/kg of SCH23390 (Exp-SCH 0.250; n = 15); 0.6 mg/kg of raclopride (Exp-Rac 0.3; n = 12). RSD-Sal group, were exposed to the agonistic encounter and pretreated with saline (n = 13); 0.250 mg/kg of SCH23390 (RSD-SCH 0.250; n = 15); or 0.6 mg/kg of raclopride (RSD-Rac 0.6; n = 18). Bars represent the time (in seconds) spent in the drug-paired compartment before conditioning sessions in the pre-conditioning test (white bars), after conditioning sessions in the post-conditioning test (black bars), in the last extinction session (light gray bars) and during the reinstatement test (dark gray bars). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ significant difference in the time spent in the drug-paired compartment vs pre-conditioning or extinction test.

receptors with raclopride during social encounters completely abolished the CPP induced by a subthreshold cocaine dose, with no effect being observed in control mice. The effect on the CPP induced by an effective dose of cocaine was, however, less pronounced. Practically without affecting the response of the control animals, socially defeated mice treated with raclopride showed the same vulnerability to reinstatement of the preference with progressive smaller doses of cocaine as those defeated and treated with saline. In contrast to that found with D1R antagonists, previous reports have shown that this D2R antagonist does not exert any motivational effect on the CPP (Manzanedo et

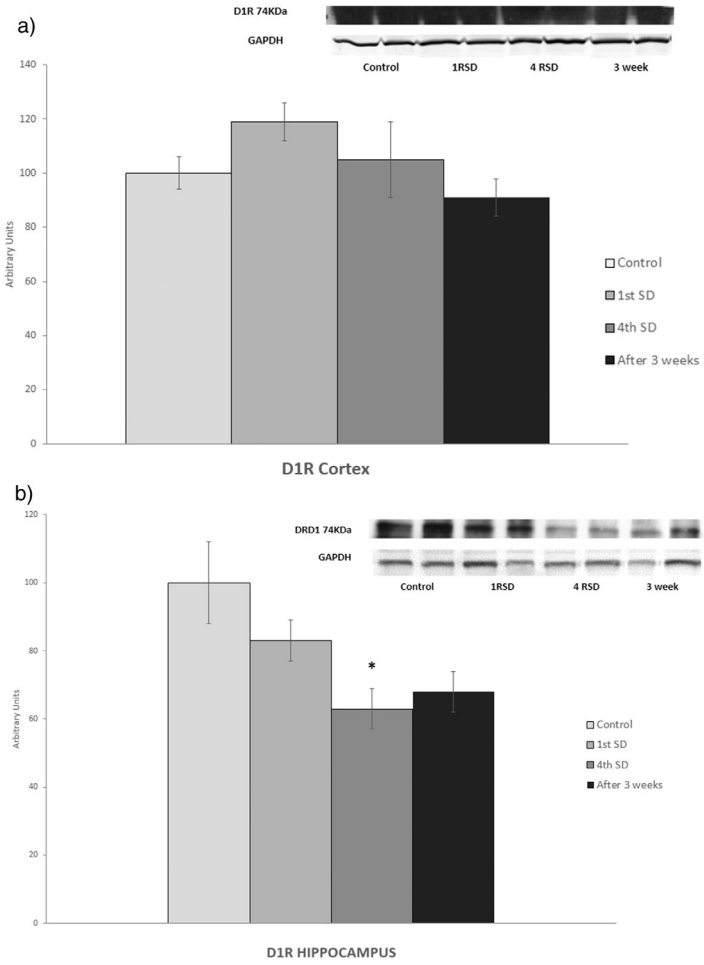

Fig. 3. Effect of RSD on D1R in the cortex and hippocampus of adult mice. Bars represent the relative levels of D1R in adult mice immediately after the first and fourth defeat and three weeks after the last encounter in the cortex (a) and hippocampus (b). Data are mean ± SEM (n = 6) of four independent groups. *p < 0.05, significant difference with respect to the control group.

al., 2001; Vidal-Infer et al., 2012) and that D2R antagonists have no effect on cocaine-induced CPP (Baker et al., 1996; Cervo and Samanin, 1995; Nazarian et al., 2004).

A previous work by Capriles and Cancela (1999) has shown that stimulation of both dopamine D1R and D2R is necessary for the development of single restraint stress-induced enhancement of D-amphetamine-induced CPP. The study in question employed physical stress, and the short term rewarding effects of d-amphetamine were measured after stress administering a mixed D1R/D2R antagonist. Despite differences with the present work, both studies suggest that the increased rewarding effects of cocaine in the CPP as a result of social defeat are dependent on DA receptor function.

4.4. Changes in D1 and D2 dopamine receptors

Using pharmacological tools, we found that DA receptors play a critical role in the long-lasting effects of RSD. We also found that RSD altered DA receptors in the cortex and hippocampus. Studies regarding

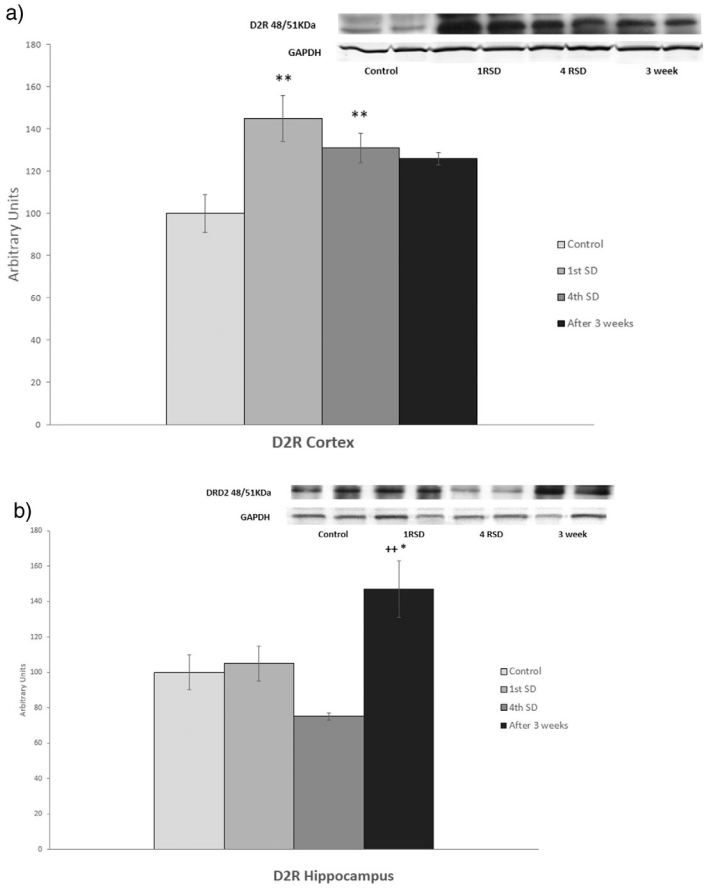

Fig. 4. Effect of RSD on D2R on the cortex and hippocampus of adult mice. Bars represent the relative levels of D2R of adult mice immediately after the first and fourth defeat and three weeks after the last encounter in the cortex (a) and hippocampus (b). Data are mean ± SEM (n = 6) of four independent groups. *p < 0.05, **p < 0.01 significant difference with respect to the control group. $^{++}$p < 0.01 significant difference with respect to the fourth social defeat.

the changes that social defeat induces in these receptors have been inconsistent. In the present study, we have observed increases of D2R in the cortex after the first and fourth social defeat, and three weeks later we detected an increase of D2R in the hippocampus and a tendency towards an increase in the cortex. A long-lasting increase of D2R in several brain regions has previously been described after social defeat (Lucas et al., 2004), as has an increase in the expression of D2R dimers in the prefrontal cortex (Bagalkot et al., 2015). However, in a recent study, Huang et al. (2016) did not observe differences in levels of D2R in several brain areas after 10-day exposure to chronic social defeat. Methodological differences and the brain areas studied could explain these divergent results.

Conversely, we did not find alterations of D1R in the cortex, but a decrease was detected in the hippocampus after the last social defeat, and was maintained three weeks later, though without reaching statistical significance. The literature regarding D1Rs is heterogeneous. Several binding and pharmacological studies have shown a decrease of these receptors in the frontal cortex induced by an agonistic behavior pattern (Avgustinovich and Alekseyenko, 2010; Kudryavtseva et al., 2008; Huang et al., 2016). However, other studies have observed no significant difference (Lucas et al., 2004; Burke et al., 2011; Jin et al., 2015), while one has even reported an increase in the number of D1-like receptors in the hippocampus 7 days after chronic unpredictable stress (Rasheed et al., 2010).

As mechanisms, both D1R and D2R receptors have been a major focus of addiction research. For example, D2R in the striatum and NAc have been inversely correlated with vulnerability to addiction in humans and rats (Hooks et al., 1994; Volkow et al., 2004). Moreover, increased D1R protein levels have been observed in the NAcc following repeated injections of cocaine (Unterwald et al., 2001). Gray et al. (2015) demonstrated that DA in the N Acc modulates both the acquisition and expression of social stress-induced behavioral changes. Chronic administration of psychostimulants and exposure to RSD stress induce robust structural plasticity of medium spiny neurons (MSNs) in the NAcc, which reflects functional alterations in synaptic strength at individual MSN synapses (Khibnik et al., 2015). A recent study has demonstrated that activation of D1-MSNs induces resilience to stress, whereas activation of D2-MSNs promotes susceptibility (Francis et al., 2015).

The mesolimbic dopaminergic system may be a critical neural link between aversive stress experiences and rewarding drug-taking (Han et al., 2015). Several studies have shown an increase of DA release due to enhanced phasic DA signaling in the mesolimbic pathway of socially-defeated rodents (Anstrom et al., 2009; Berton et al., 2006; Tidey and Miczek, 1996). It is possible that corticosterone released during social defeat stress influences the activity of DA neurons and ultimately contributes to the stress-induced escalation of cocaine intake (Marinelli and Piazza, 2002; Piazza and Le Moal, 1997). We have previously demonstrated that defeated mice display an increase in corticosterone after any social defeat (García-Pardo et al., 2015; Rodríguez-Arias et al., 2015). This DA increase might represent a crucial factor of associative learning as a response that prepares the organism for an appropriate reaction (Beeler et al., 2010) and which appears to be mediated by the release of corticosterone (Piazza et al., 1996).

In the present study, although social defeat induced more pronounced alterations of D2R than of D1R, pharmacological blockade of D1R with SCH 23390 induced a stronger impairment of social stress-induced effects on the rewarding effects of cocaine. One possible explanation is that the highest raclopride dose was not high enough to totally block all the D2R receptors, especially in the cortex, where these receptors are significantly increased after social defeat. On the other hand, the level of D1R remained unchanged or even decreased in the same conditions, suggesting that it is easier to completely block D1R.

Social defeat experiences can cross-sensitize with the behavioral effects of drugs of abuse, such as psychostimulants (Nikulina et al., 2004; Yap et al., 2005). Our results confirm that adult experience of RSD increases the response to cocaine-induced CPP, resulting in a heightened sensitivity to the acquisition and reinstatement of that CPP. In the present study we confirm that the mesolimbic dopaminergic system may be a critical neural link between aversive stress experiences and rewarding drug-taking (Han et al., 2015; Koob and Le Moal, 2001; Wise and Koob, 2014; Hammels et al., 2015). We demonstrate a role for DA receptors in the long-term effect of RSD on the conditioned rewarding and reinstating effects of cocaine. Pharmacological manipulation of DA receptors block the long-lasting effects of RSD on the rewarding effects of cocaine. Moreover, social stress alters the expression of D1R and D2R in the hippocampus and cortex. We have a relatively poor understanding of the mechanisms whereby social stress produces changes in the brain and behavior. An improved understanding of these mechanisms is critical for finding new treatment options for these debilitating conditions.

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STUDY 4

Up-regulation of histone acetylation induced by social defeat mediates the conditioned rewarding effects of cocaine

S. Montagud-Romero, J. Montesinos, M. Pascual, M.A. Aguilar, C. Roger-Sanchez, C. Guerri, J. Miñarro, M. Rodríguez-Arias

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`Up-regulation of histone acetylation induced by social defeat mediates the conditioned rewarding effects of cocaine

S. Montagud-Romero [a], J. Montesinos [b], M. Pascual [b], M.A. Aguilar [a], C. Roger-Sanchez [a], C. Guerri [b], J. Miñarro [a], M. Rodríguez-Arias [a,*]

[a] Department of Psychobiology, Facultad de Psicología, Universitat de Valencia, Avda. Blasco Ibáñez, 21, 46010 Valencia, Spain
[b] Department of Molecular and Cellular Pathology of Alcohol, Príncipe Felipe Research Center, C/Eduardo Primo Yúfera, 3, 46012 Valencia, Spain

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ABSTRACT

Social defeat (SD) induces a long-lasting increase in the rewarding effects of psychostimulants measured using the self-administration and conditioned place procedures (CPP). However, little is known about the epigenetic changes induced by social stress and about their role in the increased response to the rewarding effects of psychostimulants. Considering that histone acetylation regulates transcriptional activity and contributes to drug-induced behavioral changes, we addressed the hypothesis that SD induces transcriptional changes by histone modifications associated with the acquisition of place conditioning. After a fourth defeat, H3(K9) acetylation was decreased in the hippocampus, while there was an increase of HAT and a decrease of HDAC levels in the cortex. Three weeks after the last defeat, mice displayed an increase in histone H4(*K*12) acetylation and an upregulation of histone acetyl transferase (HAT) activity in the hippocampus. In addition, H3(K4)me3, which is closely associated with transcriptional initiation, was also augmented in the hippocampus three weeks after the last defeat. Inhibition of HAT by curcumin (100 mg/kg) before each SD blocked the increase in the conditioned reinforcing effects of 1 mg/kg of cocaine, while inhibition of HDAC by valproic acid (500 mg/kg) before social stress potentiated cocaine-induced CPP. Preference was reinstated when animals received a priming dose of 0.5 mg/kg of cocaine, an effect that was absent in untreated defeated mice. These results suggest that the experience of SD induces chromatin remodeling, alters histone acetylation and methylation, and modifies the effects of cocaine on place conditioning. They also point to epigenetic mechanisms as potential avenues leading to new treatments for the long-term effects of social stress on drug addiction.

1. Introduction

Stressful experiences in life cause physiological and behavioral impairments, including depression and anxiety-like behaviors, as well as memory deficits (Basta et al., 2007; Kessler, 1997; Post, 1992). Nowadays, social influences on the development of drug dependence and relapse is a topic of increasing interest among neuroscience fieldworkers. Since the nineties, several studies have highlighted stress as an important trigger of drug consumption, maintenance and relapse after detoxification periods (Miczek and Mutschler, 1996; Tidey and Miczek, 1997; for a revision see Miczek et al., 2008 or Burke and Miczek, 2015).

Among the different types of stressors, SD stress is a naturalistic paradigm consisting of an agonistic encounter between conspecifics (Tornatzky and Miczek, 1993) that generates emotional stress. In these circumstances, social animals develop dominance-based social hierarchies based on agonistic interactions (Huntingford and Turner, 1987). Experimentally, the effect of social stress is often studied using agonistic encounters through which a dominant rat or mouse (the resident or an aggressive individual) is confronted with a subordinate animal (intruder) in its home cage. The resident-intruder model has several advantages, including ecological and ethological validity, as well as avoiding habituation to stress through repeated exposure (Tidey and Miczek, 1997; Miczek et al., 2008). After a brief encounter with an aggressive individual, the defeated animal exhibits elevated glucocorticoid activity (increased corticosterone and ACTH levels) (Martí-Carbonell et al., 1992; García-Pardo et al., 2014, 2015; Montagud-Romero et al., 2015; Rodríguez-Arias et al., in press), tachycardia and hyperthermia for several hours (Schurman, 1980; Tornatzky and Miczek, 1993). Long-term changes, such as decreased aggression and sexual behaviors (Meerlo et al., 1996; García-Pardo et al., 2015), locomotor activity (Koolhaas et al., 1997; Meerlo et al., 1996), anhedonia (Rygula et al., 2005), heightened defensive/submissive behaviors, anxiety and impaired learning (Ruis et al., 1999; García-Pardo et al., 2015) have also been described. SD during adolescence also impairs the structure and permeability of the BBB (Rodríguez-Arias et al., in press). An increase of dopamine (DA) release in the nucleus accumbens (N Acc) and the prefrontal cortex (PFC) has been observed in defeated

* Corresponding author.
E-mail address: marta.rodriguez@uv.es (M. Rodríguez-Arias).

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animals (Tidey and Miczek, 1996; Anstrom et al., 2009; Watt et al., 2014). An increase in the rewarding effects of psychostimulants has also been reported in defeated rodents using self-administration and the conditioned place preference (CPP) procedures (Boyson et al., 2011, 2014; Cruz et al., 2011; Han et al., 2015; Miczek et al., 2008, 2011; Quadros and Miczek, 2009; Yap et al., 2015; Rodríguez-Arias et al., in press; Montagud-Romero et al., 2015).

The heritability of characteristics that make a particular human more susceptible to drug addiction cannot be explained simply by genetic factors (Schuckit et al., 1972; Cloninger et al., 1981). Environment plays an important role in the development of addiction, and both genetics and environment contribute to the individual's vulnerability to addiction (Bierut, 2011). Epigenetic factors may provide the missing link between environmental stimuli and genetic heritability. However, while long-term behavioral responses following SD have been studied in depth, little attention has been focused on epigenetic changes induced by this kind of stress. Epigenetic changes remodel chromatin, modifying DNA, histones and/ or non-histone proteins. One of the most important of these modifications is the acetylation of histones (Peixoto and Abel, 2013), an alteration of lysine residues on the histone amino terminal tails (Levenson and Sweatt, 2005; Sananbenesi and Fischer, 2009; Morris et al., 2010). This modification is closely related with a rise in levels of gene transcription (Chuang et al., 2009; Sananbenesi and Fischer, 2009; Morris et al., 2010; Lubin et al., 2011; Trollope et al., 2012), while hypoacetylation has the opposite effect (Forsberg and Bresnick, 2001; Ito and Adcock, 2002). Histone acetylation is controlled by the enzyme histone acetyl trasferase (HAT), which facilitates transcriptional activation (Bannister and Kouzarides, 1996; Ogryzko et al., 1996; for review see Roth et al., 2001). On the other hand, the histone deacetylase (HDAC) increases the net positive charge and the affinity of histones for the negatively charged DNA through a reverse action (Tsankova et al., 2006).

Epigenetic mechanisms are a relevant underlying cause of numerous psychiatric disease states, and may mediate the impact of stress on the function of neural circuits (Tsankova et al., 2006; Sananbenesi and Fischer, 2009; Nelson and Monteggia, 2011). Epigenetic modification induced by chronic SD has been addressed in several studies, with increases in histone H3 acetylation constituting the most frequent finding. For instance, after 30 min, 24 h or 10 days of exposure to chronic SD stress, increases in H3/K14 acetylation have been described in the N Acc, medial prefrontal cortex (mPFC), dorsal raphe or hippocampus (Covington et al., 2009; Hinwood et al., 2011; Hollis et al., 2010, 2011; Kenworthy et al., 2014). These changes seem to be mediated by inter-individual variances in the response to novelty, since an increase in H3 acetylation after SD is only observed in low-responding rats (Hollis et al., 2011). Rats that are less resilient to SD stress also display higher levels of histone H3 acetylation (Kenworthy et al., 2014). However, the results with respect to H4 are controversial; although most studies have observed no changes after SD, others have found increased acetylation in H4(K12) of less resilient rats (Tsankova et al., 2006; Hollis et al., 2010, 2011; Kenworthy et al., 2014). Other epigenetic changes have been described after chronic SD; for example, decreases in global levels of H3K9 dimetylation (H3K9me2) in the NAc were observed only in susceptible mice (Covington et al., 2011). However, H3(K27)me2 was increased in BDNF promoters in the hippocampus one month after cessation of chronic SD stress (Tsankova et al., 2006).

The different enzymes that control epigenetic processes are altered after SD. A decrease in HDAC 2 levels in the N Acc has been observed 24 h after the last defeat, and continuous infusion of either MS-275 (100 μM) or SAHA (100 μM) (both HDAC inhibitors) into the NAc was found to reverse stress-induced social avoidance in defeated mice and to restore the amount of time the animals spent interacting socially (Covington et al., 2009). Whereas HDAC inhibitors in the hippocampus reverse sucrose preference deficits, they reverse social avoidance only when administered to the amygdala and prefrontal cortex (Covington et al., 2011, 2015). Furthermore, a downregulation of HDAC6 (in raphe neurons) and HDAC5 (in the NAcc) has been reported 10 days after the last SD, and the HDAC inhibitor imipramine has been found to reverse HDAC5 levels (Espallergues et al., 2012; Renthal et al., 2007). Considered together, these results suggest that epigenetic changes are associated with the behavioral response to stress of socially defeated rodents.

Based on the aforementioned studies, which show that social stress produces histone acetylation in some brain structures and that these histone variations may be a mechanism underlying the long-lasting effects of SD, the aim of the present study was to characterize the effects of SD on histone acetylation and levels of HAT and HDAC enzymes. We studied alterations in histone acethylation and thrimethylation in the cortex and hippocampus, important brain regions for the regulation of behavioral and cognitive responses to stress, and which have been implicated in aggressive behavior (for review see Takahashi and Miczek, 2014). The importance of the PFC in the inhibitory control of aggression has been reported in primates, including humans (Nelson and Trainor, 2007). The hippocampus is essential for memory consolidation and storage, and plays important roles in neurogenesis and emotional mechanisms. In addition, it has been associated with escalated aggression (Takahashi and Miczek, 2014).

Since the aim of this study was to demonstrate that the long-term effects of social defeat on the conditioned rewarding effects of cocaine are mediated by histone modifications, we evaluated the effect of different chemical and social interventions 3 weeks later, as this is considered a time lapse in which the acute effects of such interventions (e.g. alcohol levels or corticosterone increases) completely disappear (Rodríguez-Arias et al., 2015, in press, 2016; Montesinos et al., 2015).

Since we found that changes in both histone and HAT/ HDAC enzyme levels were associated with SD, in a second study we assessed the effects of HAT (curcumin) and HDAC (valproic acid) inhibitors on the increase in the conditioned rewarding effects of cocaine induced by this social stress. HAT and HADAC inhibitors were administered prior to each defeat and the development of cocaine-induced conditioned place preference was evaluated three weeks later.

2. Material and methods

2.1. Animals

A total of 165 OF1 male mice (Charles River, Barcelona, Spain) of 42 days of age on arrival at our laboratory were employed as experimental subjects. All mice (except those used as aggressive opponents) were housed in groups of four in plastic cages (25 × 25 × 14.5 cm) for 8 days before the experiments began. Adult mice used as resident aggressive opponents ($n = 15$) were housed individually in plastic cages (21 × 32 × 20 cm) for a month prior to experiments in order to induce heightened aggression (Rodríguez-Arias et al., 1998). All mice were housed under the following conditions: constant temperature, a reversed light schedule (white lights on 19:30–07:30 h), and food and water available ad libitum, except during behavioral tests. Procedures involving mice and their care were conducted according to national, regional and local laws and regulations, which are in compliance with the Directive 2010/63/EU. Details of the number of animals and procedures are described in the supplementary material. Two different sets of mice were used in this study: the first ($n = 24$) was employed for the biochemical analyses (Western blot and Elisa tests); and the second ($n = 120$) was employed for the CPP procedure. Brain samples of the first set of mice were obtained after the 1st social defeat o exploration; after the 4th social defeat; and 3 weeks after the last defeat. All the mice belonging to the second set experienced four social defeats or explorations (controls). HAT or HDAC inhibitors were administered 30 min prior to each social defeat or exploration. Three weeks later, 1 mg/kg of cocaine-induced CPP was performed.

2.2. Drugs

Animals were injected with 1 mg/kg of cocaine hydrochloride (Laboratorios Alcaliber, Madrid, Spain). Physiological saline (NaCl 0.9%) was used to dissolve the drug. This dose of cocaine was selected on the basis of previous studies proving 1 mg/kg to be a threshold dose, which is not effective in control mice (Vidal-Infer et al., 2012; Arenas et al., 2014; Montagud-Romero et al., 2014). The HAT inhibitor *Curcuma longa* (*Turmeric*) (Sigma-Aldrich, Spain) (50 and 100 mg/kg) was dissolved in 20% dimethyl sulfoxide (DSMO) with 80% normal saline solution. Valproic acid (HDAC inhibitor) (Sigma- Aldrich, Spain) (250 and 500 mg/kg) was dissolved in physiological saline. All drugs were administered intraperitoneally (ip) in a volume of 0.01 ml/g of weight. The doses of HAT and HDAC inhibitors were chosen based on studies performed in other laboratories (Zhu et al., 2014; Hui et al., 2010; Bator et al., 2015; Takahashi et al., 2014).

2.3. Experimental design

To evaluate the acetylation effects of SD, four groups of animals were used. Three of them experienced SD between PND 47–56, but the control group of the same age only explored without meeting an opponent. All the animals were sacrificed by decapitation and their brains were collected immediately after the first ($n = 6$) and fourth ($n = 6$) SD, three weeks later ($n = 6$) the last SD, and after the first exploration in the case of the control group ($n = 6$). The hippocampus and cortex were dissected and stored at −80 °C until use.

To evaluate the effects of HAT and HDAC inhibitors on the place conditioning induced by 1 mg/kg of cocaine, eight groups were used ($n = 120$): the exploration group (EXP; $n = 15$) and the group exposed to repeated SD (RSD; $n = 15$), which received a saline injection before each defeat; the exploration groups, which received 100 mg/kg (EXP + C100; $n = 15$) of *C. longa* (a HAT inhibitor) or 500 mg/kg (EXP + VA500; $n = 15$) of *valproic acid* (a HDAC inhibitor) 30 min before each exploration; the defeated groups, which received 50 (RSD + C50; $n = 15$) or 100 mg/kg (RSD + C100; $n = 15$) of *C. longa* 30 min before each defeat; and two defeated groups, which received either 250 (RSD + VA250; $n = 15$) or 500 mg/kg (RSD + VA500; $n = 15$) of *valproic acid* 30 min before each defeat. CPP was assessed 3 weeks after the last SD. After the Post-C test, all groups underwent extinction sessions. When extinction had been confirmed, the animals received a priming dose of cocaine (half of the dose used during conditioning) and performed a reinstatement test. The experimental design is depicted in Table 1.

2.4. Repeated social defeat

Each SD episode consisted of three phases, which began by introducing the "intruder" (which lived with three other conspecific mice) to the home cage of the "resident" (the aggressive opponent) for 25 min. During the initial phase, which lasted 10 min, the intruder was protected from attacks, but the wire mesh walls of the cage allowed social interaction and species-typical threats from the male aggressive resident, thus serving as an instrument of instigation and provocation (Covington and Miczek, 2001; Fish et al., 1999). The wire mesh was then removed from the cage and the confrontation initiated, which lasted no >5 min. In the third and final phase, the wire mesh was returned to the cage for another 10 min to separate the two animals, but to, once again, allow social threats from the resident. Socially defeat-stressed animals were exposed to four episodes of SD, on days 1, 4, 7, and 10 (Tornatzky and Miczek, 1993). The control groups followed the same protocol but without the presence of "resident" mice. The animals were then housed in the vivarium for three weeks, after which acquisition of CPP initiated.

2.5. Apparatus

2.5.1. Conditioned place preference

For place conditioning, we employed eight identical Plexiglas boxes with two equal-sized compartments (30.7 cm long × 31.5 cm wide × 34.5 cm high) separated by a grey central area (13.8 cm long × 31.5 cm wide × 34.5 cm high). The compartments had different colored walls (black vs white) and distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed us to record the position of the animals and their crossings from one compartment to the other. The equipment was controlled by three IBM PC computers using MONPRE 2Z software (CIBERTEC, SA, Spain).

2.5.2. Procedure of the CPP

2.5.2.1. Acquisition of CPP. which consisted of three phases, was carried out during the dark cycle following a procedure that was unbiased in terms of initial spontaneous preference (Manzanedo et al., 2001). During the first phase - or preconditioning (Pre-C) - mice were allowed access to both compartments of the apparatus for 900 s per day on 3 consecutive days. On day 3, the time spent in each compartment was recorded. Animals showing a strong unconditioned aversion (<33% of session time; i.e. 250 s) or preference (>67% of the session time; i.e. 650 s) for any compartment were discarded from the rest of the study. In each group, half of the animals received the drug or vehicle in one compartment, while the other half received it in the other compartment. ANOVA

Table 1
Experimental design.

Postnatal days		*n*	47	50	53	56	57–76	77	78–80	81–84	85
Repeated social defeat/exploration			1st	2nd	3th	4th					
	Control	6	Brain								
	1st SD	6	samples								
Western blotting and Elisa test	4th SD	6				Brain samples					
	3 weeks	6						Brain samples			
				30 min before							
		15	Saline	Saline	Saline	Saline					
	Exploration	15	C100	C100	C100	C100					
		15	VA500	VA500	VA500	VA500					
		15	Saline	Saline	Saline	Saline					
		15	C50	C50	C50	C50					
	RSD	15	C100	C100	C100	C100					
Conditioned place preference induced by 1 mg/kg cocaine		15	VA250	VA250	VA250	VA250	3 weeks		Pre-C	Conditioning	Post-C
		15	VA500	VA500	VA500	VA500					

showed there were no significant differences between the time spent in the drug-paired and the vehicle-paired compartments during the Pre-C phase. In the second phase (conditioning), which lasted 4 days, animals were conditioned with cocaine or saline. An injection of physiological saline was administered before confining the mice to the vehicle-paired compartment for 30 min. After an interval of 4 h, the animals received cocaine immediately prior to being confined to the drug-paired compartment for a further 30 min. The central area was made inaccessible by guillotine doors during conditioning. In the third phase—or postconditioning (Post-C)-which took place on day 8, the guillotine doors separating the two compartments were removed, and the time spent in each compartment by the untreated mice was recorded during a 900-s observation period. The difference in seconds between the time spent in the drug-paired compartment during Post-C and Pre-C tests is a measure of the degree of conditioning induced by the drug. If this difference is positive, then the drug is considered to have induced a preference for the drug-paired compartment, while the opposite indicates an aversion.

2.5.2.2. Extinction of CPP. All groups in which CPP was confirmed were subsequently exposed to the extinction procedure. Animals underwent two extinction sessions per week, in which they were placed in the apparatus for 900 s until the time spent in the drug-paired compartment was similar to that of the Pre-C phase. CPP is considered to be extinguished when there is no significant difference between the time spent in the drug-paired compartment in the extinction session and that spent in the same compartment during Pre-C (Student's *t*-test).

2.5.2.3. Reinstatement of CPP. The reinstatement tests were the same as for Post-C (free locomotion for 900 s) and were performed only in the groups that showed CPP. In the reinstatement phase, 15 min before the test, half the dose received during the conditioning phase (0.5 mg/kg) was administered in a different room to that of the conditioning sessions (Aguilar et al., 2009). The aim of this procedure was to administer the drug in a non-contingent way with respect to conditioning, so that the animal did not associate the contextual cues of the experimental room with the drug.

2.6. Determination of HAT and HDAC activity

Total HAT and HDAC activity in the cortex and hippocampus of adult animals was measured after the first and the fourth SD and three weeks later of the last SD in the socially defeated animals and their controls (exploration animals). Nuclear fractions were isolated as previously described (Ishida et al., 2002). HAT and HDAC activity was determined with a colorimetric ELISA assay kit (Epigentek, Madrid, Spain). This assay measured the ratio between acetylated or undeacetylated histones, which was directly proportional to HAT and HDAC enzyme activity, respectively. Absorbance was determined using a spectrophotometer at 450 nm. The results were calculated using a standard curve following the manufacturer's instructions, and were expressed as ng/h/mg.

2.7. Western blot analysis

Brain tissue from the cortex and hippocampus was homogenized in lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 8, 4 mM sodium chloride, 40 mM sodium fluoride and protease inhibitors) on ice for 30 min. An equal amount of cell lysate of each sample (40 μg of protein/lane) was loaded onto sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and then blotted onto polyvinylidene fluoride membranes. Membranes were blocked with 5% BSA (bovine serum albumin) in TBS containing 0.1% Tween-20 (TBS/T), and were then incubated overnight with the following primary antibodies: *anti*-acetyl-histone H3 (K9), *anti*-acetyl-histone H4 (K12), and *anti*-trimethyl-histone H3 (K4) (Abcam, Cambridge, UK). After washing with TBS/T, blots were incubated with HRP-conjugated antibodies. Blots were developed using the ECL system (ECL Plus; Thermo Scientific, Illinois, USA). All the membranes were stripped for 30 min in sodium dodecyl sulfate (SDS) solution (0.4% SDS and 200 mM glycine, pH 2.5), and were washed and incubated with *anti*-GAPDH mAb (Chemicon, California, USA) for 2 h as a loading control. The intensity of the bands was quantified with the image analysis software ImageJ 1.44p (National Institutes of Health, USA), and the densitometry analysis is shown in arbitrary units normalized to the GAPDH loading control.

The following peptides, specifically modified for the histones studied, were employed as positive controls: acetylated Lysine 12 of H4 (ab154463), acetylated Lysine 9 of H3 (ab16635) and trimethylated Lysine 4 of H3 (ab92374) (obtained from Abcam, Cambridge, UK). The immunodetection of these peptides detects a single band of low molecular weight, determined by molecular weight markers. For the negative control, the blot was incubated only with the secondary antibody.

2.8. Statistics

To evaluate CPP acquisition, the times spent by animals in the drug-paired compartment were analyzed with a mixed ANOVA with one between-subjects variable - "pretreatment", with eight levels (EXP, EXP + C100, EXP + VA500, RSD, RSD + C50, RSD + C100, RSD + VA250 and RSD + VA500) - and one within subjects variable - "Days", with two levels (Pre-C and Post-C). Post-hoc comparisons were performed by means of Bonferroni tests. During extinction, differences between the time spent in the drug-paired compartment in Pre-C or Post-C and each extinction session were analyzed using Student's t-tests. To evaluate if priming doses induced reinstatement, the difference between the time spent in the drug-paired compartment in the reinstatement test and that spent in the last extinction session was analyzed using Student's t tests.

Statistical significance for H3 (K9), H4 (K12), trimethyl H3 (K4), HAT and HDAC activities was determined by a one-way ANOVA, with one between-subjects variable – "pretreatment", with four levels (Control, 1RSD, 4RSD, 3W) - followed by a Bonferroni's post-hoc test. The results are reported as mean ± S.E.M.

Normality tests (Kolmogorov-Smirnov test) for all the data allowed the use of parametric statistics.

3. Results

3.1. Effect of repeat social defeat stress on histone H3 (K9), histone H4 (K12) and trimethyl histone H3 (K4) activity in the cortex and hippocampus

SD modified H4 (K12) activity (see Fig. 1a) in the hippocampus [$F(3.20) = 7.845$; $p = 0.001$] and cortex [$F(3.20) = 8.399$; $p < 0.001$]. SD induced increases in H4 (K12) levels in the hippocampus three weeks after the last encounter with respect to controls ($p < 0.05$; effect-size 0.505) and the acetylation observed after the first and fourth defeats ($p < 0.001$; effect-size 0.688 and 0.620, respectively). However, H4 (K12) activity in the cortex was significantly decreased three weeks after the last encounter when compared with the first and fourth SDs ($p < 0.01$; effect-size 0.759 and 0.809, respectively).

SD induced less pronounced changes in H3(K9) acetylation (see Fig. 1b), with a decrease ($p < 0.05$; effect-size 0.695) observed in the fourth with respect the first SD in the hippocampus [$F(3.20) = 3.101$; $p < 0.05$].

Finally, SD also altered histone H3(K4) methylation levels (see Fig. 1c) in the hippocampus [$F(3.20) = 4.164$; $p < 0.01$] and cortex [$F(3.20) = 7.975$; $p < 0.001$]. SD induced increases in H3 (K4) methylation in the hippocampus three weeks after the last encounter with respect to controls ($p < 0.02$; effect-size 0.603). However, decreases after the fourth encounter and three weeks later were observed in the cortex when compared to the first SD ($p < 0.02$; effect-size 0.999 and 0.998, respectively).

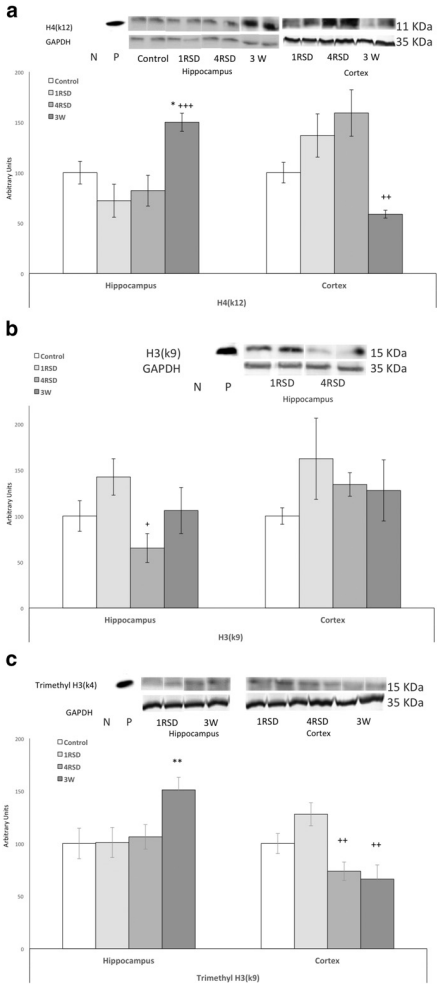

Fig. 1. Effects of repeated social defeat on H4(*k*12) acetylation (a), H3(k9) (b) acetylation and H3(K4) methylation (c) levels in the cortex and hippocampus of the animals. Nuclear extracts were isolated from the cortex and the hippocampus of adult mice immediately after the first exploration, or after the first and the fourth SD and three weeks later of the last SD in the socially defeated animals. Data are mean ± SEM ($n = 6$) of four independent groups. Positive (P) and negative control (N). a: $^{*}p < 0.05$, significant difference in relation to the control group, $++p < 0.01$ significant difference with respect to the first and fourth SD, $+++p < 0.001$ significant difference with respect to the first and fourth SD. b: $+p < 0.05$ significant difference with respect to the first SD. c: $^{**}p < 0.01$, significant difference in relation to the control group, $++p < 0.01$ significant difference with respect to the first SD.

3.2. Effect of repeated social defeat stress on HAT and HDAC activity in the cortex and hippocampus

Repeated SD stress modified HAT activity (see Fig. 2a) in the hippocampus [$F(3.19) = 3.098$; $p < 0.05$] and cortex [$F(3.19) = 6.748$; $p < 0.01$]. SD induced increases in HAT activity in the hippocampus, reaching statistical significance three weeks after the last encounter when compared to controls ($p < 0.05$; effect-size 0.529). HAT activity in the cortex was significantly increased after the fourth SD with respect to the control group ($p < 0.05$; effect-size 0.599) and the first SD ($p < 0.01$; effect-size 0.789).

On the other hand, HDAC activity (see Fig. 2b) was affected by SD only in the cortex [$F(3.19) = 32.607$; $p < 0.001$], showing a temporary increase after the first SD with respect to controls ($p < 0.01$; effect-size 0.674), but decreasing after the fourth defeat ($p < 0.001$; effect-size 0.821 and 0.838, respectively) and 3 weeks later ($p < 0.001$; effect-size 0.71 and 0.703, respectively) with respect to controls and the first SD.

3.3. HAT and HDAc inhibitors modify the effect of repeated social defeat stress on the conditioned place preference induced by 1 mg/kg of cocaine

The results regarding the effects of SD on 1 mg/kg of cocaine-induced CPP are presented in Fig. 3. ANOVA revealed a significant effect of the variable Days [$F(1.95) = 35.593$; $p < 0.001$], and the interaction Days × Treatment [$F(7.95) = 2.979$; $p < 0.01$]. The groups pretreated with saline, valproic acid or the low dose of curcumin (50 mg/kg) and exposed to SD developed CPP ($p < 0.01$ in all cases; effect-size 0.482; 0.325; 0.420; and 0.515, respectively). The group pretreated with the high dose of valproic acid (500 mg/kg) but without undergoing SD (EXP + VA500) also developed CPP ($p < 0.05$, effect-size 0.814). Two extinction sessions were required to extinguish the preference in the EXP + VA500, RSD, RSD + C50 and RSD + VA250 groups, and no reinstatement of the preference was obtained after a priming injection of 0.5 mg/kg of cocaine. However, the RSD + VA500 group needed 7 sessions for the preference to be extinguished and reinstatement was observed after the same priming dose of cocaine.

4. Discussion

Evidence reported over the last decade has revealed that, when exposed to SD experiences, the brain undergoes remodeling and functional modifications, which leads in turn to behavioral changes (for review see Hammels et al., 2015). Using the SD paradigm, the present study shows for the first time that the epigenetic changes induced by social stress are associated with an increase in the rewarding and reinstating effects of a threshold dose of cocaine in the CPP paradigm. We have seen how up-regulation of histone acetylation H4(K12) was accompanied by an increase in HAT activity in the hippocampus three weeks after the last social encounter. We have also observed that the increase in the conditioned rewarding effects of cocaine, which was observed only in defeated mice, was blocked by administration of the HAT inhibitor curcumin prior to each SD. Our results provide strong evidence of a role for epigenetic mechanisms in the long-term behavioral changes induced by SD.

A number of reports have shown that SD increases vulnerability to cocaine self-administration, whether the social stress is experienced during adolescence or adulthood (e.g. Burke and Miczek, 2015 or Yap et al., 2015). In agreement with these reports, we have previously reported that mice socially defeated during adolescence develop CPP with doses of cocaine that are non-effective in naïve mice (Rodríguez-Arias et al., in press). In the present study, we demonstrate that SD during adulthood also induces a long-lasting increase in the conditioned rewarding effects of cocaine. The enhanced sensitivity to cocaine in the CPP correlated with a progressive increase (with a medium effect-size), in the activity of hippocampal HAT, an enzyme that interacts with DNA and facilitates transcriptional activation (for review see

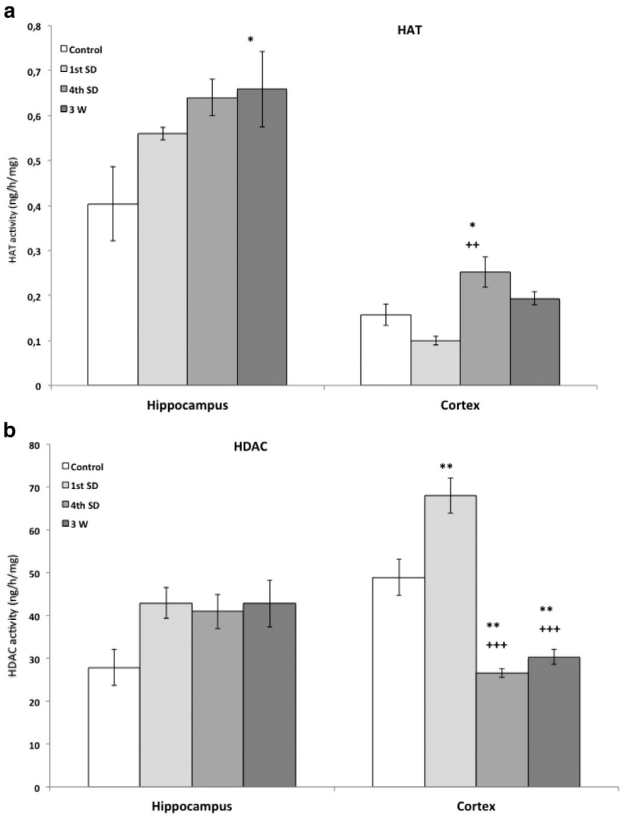

Fig. 2. Effects of repeated social defeat on HAT (a) and HDAC (b) activity in the cortex and hippocampus of adult animals. Nuclear extracts were isolated from the cortex and hippocampus of adult rats immediately after or after the first and the fourth SD and three weeks later of the last SD in the socially defeated animals. Data are mean ± SEM ($n = 6$) of the four independent groups. $^{*}p < 0.05$, $^{**}p < 0.01$, significant difference in relation to the control group, $++p < 0.01$, $+++p < 0.001$ significant difference with respect to the first SD.

Roth et al., 2001), which in turn increases acetylation of histone H4(K12) in the hippocampus (also with a medium effect-size). On the other hand, HAT activity in the cortex increased momentarily only after the fourth SD, and was not detected three weeks later. Therefore, there was a decrease (and not an increase) in H4(K12) acetylation in this structure. Blockade of HAT activity during each SD by administration of curcumin completely reverted cocaine-induced CPP in defeated mice, confirming that the alterations in histone acetylation correlated with the increase in the conditioned rewarding effects of cocaine. Previous reports have shown that inhibition of HAT activity with curcumin or through viral-mediated transfer and overexpression of specific HDACs in the NAc markedly inhibits cocaine-induced CPP (Renthal et al., 2007; Hui et al., 2010). However, in the present study, inhibition of HAT did not take place during conditioning, but several weeks earlier, prior to each SD, suggesting that epigenetic mechanisms induce plastic changes during SD.

Conversely, we did not observe long-term changes in the acetylation of histone H3(K9) in the cortex and hippocampus, with only a short-term decrease in H3(K9) acetylation being observed in the hippocampus after the fourth defeat, showing a small effect-size. On the other hand, the enzyme HDAC, which silences gene transcription (Tsankova et al., 2006), was altered only in the cortex, with an increase detected after the first SD and a decrease after the last defeat, with levels remaining low three weeks later. All of these changes were of a medium-to-large effects-size. Accordingly, administration of the HDAC inhibitor valproic acid before each SD potentiated the long-term effects of social stress. Defeated mice treated with valproic acid not only developed CPP with a subthreshold dose of cocaine, but also showed a reinstated preference after receiving a priming dose of 0.5 mg/kg of cocaine, an effect that was not observed in untreated defeated mice. Moreover, administration of valproic acid also induced the development of CPP in non-stressed mice. In line with our results, Covington et al. (2009)

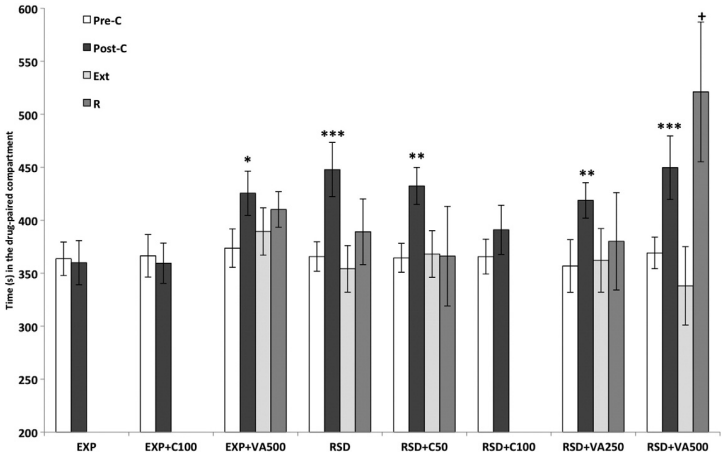

Fig. 3. Effects of social defeat on acquisition of the CPP induced by 1 mg/kg of cocaine in adult mice. During the conditioning phase, animals were divided into six groups depending on the treatment they received 30 min before each agonistic encounter: (EXP $n = 15$; RSD $n = 15$; RSD + C50 $n = 15$; RSD + C100 $n = 15$; RSD + VA250 $n = 15$; and RSD + VA500 $n = 15$). The bars represent the time (in seconds) spent in the drug-paired compartment before conditioning sessions in the pre-conditioning test (white bars), after conditioning sessions in the post-conditioning test (black bars), in the last extinction session (light grey bars), and during the reinstatement test (dark grey bars). $^{***}p < 0.001$, $^{**}p < 0.001$, $^{*}p < 0.05$ significant difference in the time spent in the drug-paired compartment vs pre-conditioning or extinction tests; $+p < 0.05$ significant difference in the time spent in the drug-paired compartment vs extinction tests.

observed a significant decrease in HDAC2 for up to 15 days after the last defeat episode. Conversely to that described for HAT inhibitors, administration of non-specific HDAC inhibitors (sodium butyrate or Trichostatin A) during conditioning has been shown to potentiate the behavioral effects of cocaine in the CPP (Kumar et al., 2005; Raybuck et al., 2013; Itzhak et al., 2013; Hui et al., 2010; Renthal and Nestler, 2008). It has been reported that HDAC inhibitors strengthen memory formation, thus increasing associative learning (Ploense et al., 2013). Human studies have also revealed that valproate is ineffective in reducing spontaneous and cue-induced cocaine craving (Reid and Thakkar, 2009). These results suggest that blockade of acetylation by inhibition of HAT impedes the behavioral effects of SD, while inhibition of HDAC increases acetylation, thus promoting deeper behavioral changes.

Trimethylation of H3 lysines 4 (H3K4me3) is closely associated with transcriptional initiation, and often correlates with increased levels of transcriptional activity, with a correlation between H3(K4) methylation, histone acetylation and transcriptional competency having been reported (Rice and Allis, 2001; Bernstein et al., 2005). Interestingly, acetylated isoforms of H3 and H4 are the preferential targets of histone methylation, suggesting that histone metyltranferases (HMTs) and HATs act synergistically to promote transcription by mechanisms that are yet to be determined (Annunziato et al., 1995). Interestingly, we have observed that H3(K4)me3 increased three weeks after the last defeat in the hippocampus but decreased in the cortex (in both cases with medium effect-size), changes that were comparable to those observed with respect to H4(K12) acetylation. In this way, our results are in the accordance with those of previous reports suggesting a synergistic action of HMT and HAT.

Few reports have addressed the epigenetic changes induced by SD, and their results are by no means consistent, mainly due to their use of non-comparable methodological procedures: variations in species or strains of animals, varying intensities of SD stress (e.g. the intruder rat placed in the cage of the resident for a total of 30 min per day for 7 consecutive days vs. 5 min per day for 10 days), changes observed only in less resilient rats, and the specific lysine under analysis are among the discrepancies that characterize the research carried out until now. Several studies have highlighted a delay in the expression of changes in H4(K12) acetylation induced by SD; for example, Kenworthy et al. (2014) observed an increase 7 days after chronic SD in the ventral hippocampus and dorsal raphe. In contrast, Hollis et al. (2010, 2011) did not observe changes in the hippocampus 30 min after the last defeat. Other studies have focused on alterations in acetylation of histone H3, and an increase in acetylation of H3(K9/14) has been reported in the hippocampus, medial prefrontal cortex (mPFC), dorsal raphe nucleus or NAc from 30 min after the last defeat to 10 days later (Covington et al., 2009; Hollis et al., 2010, 2011; Hinwood et al., 2011; Kenworthy et al., 2014). There are no previous reports about the effect of repeated SD on H3(K4)me3, but two studies have addressed changes in methylation after social stress. Ten days after the final defeat, global levels of H3(K9)me2 were found to be decreased in the NAc of susceptible animals only, indicating that increased repressive chromatin regulation contributes to pro-adaptive responses to stressful stimuli (Covington et al., 2011). Histone dimethylation at H3(K27)me2 is strongly enriched after chronic SD stress, and this modification is extremely long-lasting; it is present in the promoters of BDNF up to a month following cessation of the stress, suggesting that chronic stress creates a repressive state that is not easily reversed (Tsankova et al., 2006).

Changes in chromatin remodeling form part of many physiological and pathological processes, including carcinogenesis, brain development, synaptic plasticity and addiction (Levenson and Sweatt, 2005). Although the molecular mechanisms responsible for the rewarding effects of psychostimulants under social experiences remain unclear, differences in the type of stress remodeling the brain, as well as changes in specific histones and HAT/ HDAC enzymes, might explain the effects of cocaine in the CPP paradigm. Stress may increase cocaine-taking and -seeking through an action of the neuropeptide corticotropin-releasing factor (CRF) (Burke and Miczek, 2015). CRF released in the VTA can

increase the potential firing rate of VTA DA neurons (Wanat et al., 2008) and cause synaptic neuroadaptations of DA neurons within the mesolimbic pathway (Borgland et al., 2004). In addition, neuroinflammation mechanisms seem to play a role in stress plasticity and pathological outcomes. Adverse social experiences such as SD involve a dynamic process of immune cell migration to the brain and prime neuroimmune function (Deak et al., 2015), promoting brain region-specific activation of brain microglia, which leads in turn to prolonged behavior disturbances (Wohleb et al., 2011, 2014). Both stress and neuroinflammation have a clear impact on many types of molecular epigenetic mechanisms, from histone modifications to DNA methylation (Kaminska et al., 2016; McEwen et al., 2015). More research is needed to clarify the specific role of stress and neuroinflammatory processes in the epigenetic changes induced by SD.

To summarize, the present results provide evidence that repeated SD induces long-lasting epigenetic changes, up-regulating levels of histone acetylation H4(K12) and HAT activity in the hippocampus, which would seem to be responsible, at least partially, for variations in the effects of cocaine. Indeed, inhibition of HAT before each SD prevents the increase in the rewarding effects of cocaine that are otherwise observed. Notably, the increase in acetylation is accompanied by similar long-lasting increases in H3(K4)me3 in the hippocampus. Acetylation and methylation are complex processes that play a critical role in long-term memory storage and consolidation (Stafford and Lattal, 2011), and can mediate changes in cocaine-induced CPP. On the other hand, HDAC inhibition enables gene expression, and several studies have implicated it in the formation of context-drug associated memories, enhancement of context-shock associated memories and modulation of the extinction of context-drug association learning (Vecsey et al., 2007; Malvaez et al., 2010; McQuown and Wood, 2010). In line with this, we have seen how inhibition of HDAC before each SD induces the opposite effect, with a potentiation of cocaine-induced CPP and even reinstatement of the preference.

5. Conclusion

Our results imply that chromatin modification due to SD experiences increases gene transcription, which in turn modifies the rewarding effects of cocaine. Our findings support an important role for histone acetylation and methylation in the long-lasting effects of SD. Pharmacological manipulation of epigenetic changes, such as altered HAT function, deserves further investigation as a potential target in the management of SD-related psychiatric disorders.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.pnpbp.2016.04.016.

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STUDY 5

Repeated social defeat increases the rewarding effects of cocaine and the activity of the BDNF signalling pathway in memory-related brain areas

S. Montagud-Romero, C. Nuñez, M.C. Blanco-Gandia, E. Martínez-Laorden, M.A. Aguilar, J. Navarro-Zaragoza, P. Almela, M.V. Milanés, M.L. Laorden, J. Miñarro, M. Rodríguez-Arias

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Complete List of Authors:	Montagud-Romero, Sandra Nuñez, Cristina; Universidad de Murcia, 3Murcia Research Institute of Health Sciences (IMIB) and Faculty of Medicine Blanco-Gandia, M Carmen; University of Valencia, Psicobiologia Martinez-Laorden, Elena; Universidad de Murcia, 3Murcia Research Institute of Health Sciences (IMIB) and Faculty of Medicine Aguilar, Maria; University of Valencia, Psychobiology Navarro-Zaragoza, Javier; Universidad de Murcia, 3Murcia Research Institute of Health Sciences (IMIB) and Faculty of Medicine Almela, Pilar; Universidad de Murcia, 3Murcia Research Institute of Health Sciences (IMIB) and Faculty of Medicine Milanes, M Victoria; Universidad de Murcia, 3Murcia Research Institute of Health Sciences (IMIB) and Faculty of Medicine Laorden, M. Luisa Miñarro, Jose; University of Valencia, Psichobiology Rodriguez-Arias, Marta; University of Valencia, Psicobiologia
Keywords:	social defeat stress, adolescence, COCAINE, conditioned place preference, dopamine receptors, transcription factors, BDNF

Repeated social defeat increases the rewarding effects of cocaine and the activity of the BDNF signalling pathway in memory-related brain areas

Sandra Montagud-Romero[1], Cristina Nuñez [3], M Carmen Blanco-Gandia[1], Elena Martínez-Laorden[3], María A Aguilar[1,2], Javier Navarro-Zaragoza[3], Pilar Almela[3], Maria-Victoria Milanés[2,3], María-Luisa Laorden[2,3], José Miñarro[1,2], Marta Rodríguez-Arias[1,2]

[1]Department of Psychobiology, Facultad de Psicología, Universitat de Valencia, Avda. Blasco Ibáñez, 21, 46010 Valencia, Spain; [2]Red Temática de Investigación Cooperativa en Salud (RETICS-Trastornos Adictivos), Instituto de Salud Carlos III, MICINN and FEDER, Madrid, Spain. [3]Murcia Research Institute of Health Sciences (IMIB) and Faculty of Medicine, University of Murcia, Spain.

Correspondence: Dr. Marta Rodríguez-Arias
Departamento de Psicobiología,
Facultad de Psicología
Universitat de Valencia
Avda. Blasco Ibáñez, 21
46010 Valencia, Spain.
Tel.: +34 - 96 386 46 37
Fax: +34 - 96 386 46 68
E-mail: marta.rodriguez@uv.es

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Abstract

Rationale. Previous studies have demonstrated that social defeat stress alters the expression of BNDF (brain-derived neurotrophic factor) and modulates several transcription factors in different brain areas in adult animals.

Objective. The aim of the present study was to compare the long-term behavioral and neurochemical effects of social defeat in adult and adolescent mice.

Methods. Adolescent and young adult mice were exposed to four episodes of social defeat and were conditioned three weeks later with 1mg/kg of cocaine. Following the conditioned place preference (CPP) procedure, the expression of different proteins was measured in some brain structures.

Results. All mice exposed to RSD (repeated social defeat) showed an increase in the conditioned rewarding effects of a subthreshold dose of cocaine. Furthermore, only adolescent defeated mice displayed diminished levels of the transcription factors Pitx3 and Nurr1 in the ventral tegmental area (VTA), without changes in the expression of their target genes dopamine transporter (DAT) and D2 dopamine receptor (D2DR) in the NAc, though that of DAT and D2DR tended to be higher in socially defeated adult mice. Our results also revealed that expression of BDNF in the dentate gyrus (DG) and and basolateral amygdala (BLA) of defeated mice was enhanced independently of their age, and that this was accompanied by alterations in the cyclic AMP-responsive element-binding protein (CREB) in the dentate gyrus.

Conclusion. Our findings suggest that adolescence is a period of heightened sensitivity to the effects of RSD on behavioral and neuronal plasticity, thus implicating BDNF as an important biomarker.

Key words: social defeat stress, adolescence, cocaine, conditioned place preference, dopamine receptors, transcription factors, BDNF.

1 Introduction

Numerous studies have proved that stress is a risk factor for the initiation, maintenance and escalation of drug consumption and for relapse after periods of detoxification (Koob 2010; Sinha et al. 2011; Logrip et al. 2012). There is a close relationship between stress and brain systems involved in addiction (Belujon and Grace 2011; Rodríguez-Arias et al. 2013), since adverse life experiences increase the abuse of addictive substances (Caprioli et al. 2007; Miczek et al. 2008; Le Moal, 2009; Sinha et al. 2011). Repeated exposure to stress can heighten sensitivity to drug-induced psychomotor stimulation, with enhanced drug-induced dopamine (DA) and glutamate responses in the nucleus accumbens (NAc) and increased cellular activation of reward-associated brain regions (Deroche et al. 1995; Miczek et al. 2004; Nikulina et al. 2004; Pacchioni et al. 2007). These results suggest that environmental stressors produce long-term neuroadaptations in reward pathways, which can be similar to those induced by drugs of abuse (Quadros and Miczek 2009). Among the different rodent models of stress, social defeat stress is a naturalistic model that involves an agonistic encounter between conspecifics and is thought to represent a stressor of ecological and ethological validity in mice (Tornatzky and Miczek 1993).

Numerous studies have centred on the effect of social stress on psychostimulant addiction, although most of them have focused on socially defeated adult animals. Social defeat in adult rodents increases the acquisition of cocaine self-administration (Tidey and Miczek 1997; Haney et al. 1995), shortens inter-infusion intervals (Covington and Miczek 2005), increases cocaine-taking and response rates during binges (Covington et al. 2008), and potentiates the rewarding effect of cocaine in the conditioned place preference (CPP) (McLaughlin et al. 2006; Montagud-Romero et al. 2015). This sensitization to cocaine has been associated with sensitization of the mesocorticolimbic DA system (e.g., Garcia-Keller et al. 2013), a pathway critical for the formation of reward associations (Bromberg-Martin et al. 2010).

Recent investigations have shown the combined negative effects of adverse childhood experiences and substance dependence on the function of the HPA axis (Schäfer et al. 2010; Doan et al. 2014, Gerra et al. 2014). Adolescents are hypersensitive to stressful events, as illustrated by slower return to baseline levels of stress-stimulated plasma corticosterone release (Goldman et al. 1973; Romeo 2007). The transition from childhood to adulthood involves reorganization and neuronal maturation of the brain, and is considered a vulnerable period with respect to the consequences of exposure to

drugs of abuse (Schneider 2008). In animal models, adolescents show a greater and faster increase in drug-induced DA release, but lower basal levels of synaptic DA (Laviola et al. 2001; Badanich et al. 2006). In addition, the amygdala and the NAc of adolescents exhibit more activity that those of adults (Ernst et al. 2009). Social stress during adolescence appears to reduce mesocortical DA levels, thus undermining maturation of cortical DA through D2 dopamine receptor (D2DR) regulation of DA synthesis or glucocorticoid-facilitated pruning of cortical DA fibres (Burke and Miczek 2014). For the development and maintenance of the dopaminergic phenotype throughout the life of an organism several transcription factors, including Pitx3 and Nurr1, are vital (Smits and Smidt 2006; Kadkhodaei et al. 2009; Bissonette and Roesch 2015). Moreover, Pitx3 is thought to be essential for Nurr1-mediated transcription of its target genes, which include Th (tyrosine hydroxylase, the limiting enzyme of catecholamine synthesis), D2DR, Dat (dopamine transporter), and Vmat2 (Vesicular monoamine transporter 2) (Jacobs et al. 2009).

On the other hand, brain-derived neurotrophic factor (BDNF), an important neurotrophin for synaptic plasticity, is one of the molecular candidates underlying the development of persistent neuroplastic adaptation to social and other types of stress (for revision see Vasconcelo et al. 2015). Social defeat induces changes in the expression of BDNF (Tsankova et al. 2006; Krishnan et al. 2007), extracellular signal-regulated kinase (ERK) (Krishnan et al. 2007) and cyclic AMP-responsive element-binding protein (CREB) (Wilkinson et al. 2009). ERK phosphorylate CREB and active (phosphorylated) CREB stimulates the expression of target genes, including BDNF (Kandel et al. 2001; Barco et al. 2002; Bramham and Messaoudi 2005). Stress-induced long-lasting changes of BDNF signaling in mesocorticolimbic regions may regulate the reward circuit (Nikulina et al. 2012). This neurotrophin is capable of divergent neuroadaptations to social stress, as episodically or continuously defeated subordinated rats may show, respectively, an increased or suppressed BDNF response (Miczek et al. 2011). Furthermore, a functional role for BDNF has been demonstrated in regions outside the mesolimbic pathways, such as the hippocampus and basolateral amygdala (BLA) (Hall et al. 2000; Mizuno et al. 2000; Rattiner et al. 2004; Jasnow and Huhman 2001). In the hippocampus, the regulation of BDNF expression by chronic social stress is unclear, with reductions, increases or no changes having being reported (Pizarro et al. 2004; Tsankova et al. 2006; Lagace et al. 2010; Tailor et al. 2011; Coppens et al. 2011;

Duclot and Kabbaj 2013). Additionally, amygdalar BDNF is necessary for learning submission or subordinate social status in defeated hamsters (Taylor et al. 2011). In this paradigm, winners were found to have lower BDNF mRNA levels in the BLA and higher BDNF mRNA levels in the dentate gyrus (DG). Considered together, these results suggest that BDNF signalling has distinct effects in different brain areas following social defeat stress.

To date, our group is the only one to have studied the long-lasting effects of repeated social defeat (RSD) in adolescent animals. We have observed that mice socially defeated during adolescence show an increase in the conditioned rewarding effects of cocaine in the conditioned place preference (CPP) paradigm, although the same animals took longer to acquire cocaine self-administration with an effective dose (Rodríguez-Arias et al. 2015). In accordance with these results, rats deprived of social interaction during adolescence have been shown to self-administer less cocaine than non-isolated subjects at high unit doses (Howes et al. 2000).

The present study goes a step further and addresses the issue of how age modulates the long-lasting behavioral and neurochemical effects of social defeat. Adolescent or adult mice underwent repeated social defeat encounters and their response to cocaine was evaluated three weeks later in the CPP. The encounters were ethologically analyzed in order to compare the aggression exhibited by the residents to adolescent or adult intruders. Corticosterone response was also measured. In addition, we evaluated changes induced by social defeat in the dopaminergic mesocorticolimbic system that might influence the response of adolescent and adult mice to the rewarding properties of cocaine. To do this, the protein levels of Nurr1 and Pitx3 in the VTA and their target genes D2DR and DAT in the NAc were quantified. Since BDNF plays a fundamental contribution to plastic adaptations to life events and social defeat activates hypothalamic and limbic circuits, areas that underlie the processing of stress and reward, we also evaluated the expression of BDNF, CREB and ERK 1/2 in the DG and BLA in socially defeated adolescent and adult mice exposed to cocaine.

2. Material and methods

2.1. Animals

A total of 124 male OF1 (Charles River, France) arrived at our laboratory at 21 or 42 days of age. All mice (except those used as aggressive opponents n=30) were housed in groups of four in plastic cages (25×25×14.5 cm) for 8 days before the experiments began. Aggressive opponents were housed individually in plastic cages (23×13.5×13 cm) for a month prior to experiments in order to heighten aggression (Rodríguez-Arias et al. 1998). Mice were housed in controlled laboratory conditions with a constant temperature of 21±1 1C and humidity of 55±10%. Testing took place during the first hours of the dark phase of a reversed light/dark cycle (lights off at 08:00 h and on at 20:00 h). Food and water were made available ad libitum to the mice used in the cocaine experiments. All procedures were conducted in compliance with the guidelines of the European Council Directive 2010/63/UE regulating animal research and were approved by the local ethics committees (University of Valencia).

2.2. Drugs

Animals were injected intraperitoneally with 1 or 25 mg/kg of cocaine hydrochloride (Laboratorios Alcaliber, Madrid, Spain) in a volume of 0.01ml/g of weight. Control groups were injected with physiological saline (NaCl 0.9%), which was also used to dissolve the drugs. The doses of cocaine were selected on the basis of previous studies (Vidal-Infer et al. 2012; Arenas et al. 2014; Montagud-Romero et al. 2014, 2016).

2.3. Procedure and apparatus

2.3.1. Repeated Social Defeat encounters.

Animals in the corresponding group were exposed to 4 episodes of social defeat lasting 25 min each. Each episode consisted of three phases that began by placing the experimental animal or intruder in the home cage of the aggressive opponent or resident for 10 min. During this initial phase, the intruder was protected from attack by a wire mesh wall that permitted social interaction and species-typical threats from the male aggressive resident (Covington and Miczek 2001). In the second phase, the wire mesh was removed from the cage and a 5-min period of confrontation began. In the third phase, the wire mesh was replaced for a further 10 minutes to allow social threats from the resident. Adolescent mice were exposed to social defeat on postnatal day (PND) 27, 30, 33 and 36, while adult mice were exposed to defeat on PND 47, 50, 53 and 56. The exploration groups underwent the same protocol, but without the presence of a "resident" mouse in the cage. Following this last phase, animals were kept in the

vivarium for three weeks, after which the behavioral tests began. The second phase of each social defeat protocol was video-recorded and ethologically analysed. Threat and attack behaviors were scored in resident mice and avoidance/flee and defensive/submissive behaviors were evaluated in intruder mice.

Two different sets of mice were employed in this study. A more detailed description of the experimental procedure is provided in Table 1.

2.3.2. Conditioned place preference

Apparatus

For place conditioning, we employed eight identical Plexiglas boxes with two equal-sized compartments (30.7 cm long × 31.5 cm wide × 34.5 cm high) separated by a gray central area (13.8 cm long ×31.5 cm wide ×34.5 cm high). The compartments had different colored walls (black vs white) and distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed the recording of the position of the animals and their crossings from one compartment to the other. The equipment was controlled by three IBM PC computers using MONPRE 2Z software (CIBERTEC, SA, Spain).

Procedure of the CPP

Acquisition. Place conditioning, which consisted of three phases, was carried out during the dark cycle following a procedure that was unbiased in terms of initial spontaneous preference (Maldonado et al. 2006). During the first phase - or preconditioning (Pre-C) - mice were allowed access to both compartments of the apparatus for 900 s per day on 3 consecutive days. On day 3, the time spent in each compartment was recorded. Animals showing a strong unconditioned aversion (less than 33% of session time; i.e. 250 s) or preference (more than 67% of the session time; i.e. 650s) for any compartment were discarded from the rest of the study. In each group, half of the animals received the drug or vehicle in one compartment while the other half received it in the other compartment. ANOVA showed there were no significant differences between the time spent in the drug-paired and the vehicle-paired compartments during the Pre-C phase. In the second phase (conditioning), which lasted

4 days, animals were conditioned with cocaine or saline. An injection of physiological saline was administered before confining the mice to the vehicle-paired compartment for 30 min. After an interval of 4 h, the animals received cocaine immediately prior to confinement to the drug-paired compartment for a further 30 min. The central area was made inaccessible by guillotine doors during conditioning. In the third phase—or postconditioning (Post-C)—which took place on day 8, the guillotine doors separating the two compartments were removed, and the time spent in each compartment by the untreated mice was recorded during a 900-s observation period. The difference in seconds between the time spent in the drug-paired compartment during Post-C and Pre-C tests is a measure of the degree of conditioning induced by the drug. If this difference is positive, then the drug has induced a preference for the drug-paired compartment, while the opposite indicates an aversion.

Extinction of CPP. All groups in which CPP was confirmed were subsequently exposed to the extinction procedure. Animals underwent two extinction sessions per week in which they were placed in the apparatus for 900 s until the time spent in the drug-paired compartment was similar to that of the Pre-C phase. CPP is considered to be extinguished when there is no significant difference between the time spent in the drug-paired compartment in the extinction session and that spent in the same compartment during the Pre-C phase (Student´s t test).

Reinstatement of CPP. The reinstatement tests were the same as for Post-C (free ambulation for 900 s) and were performed only in the groups that showed CPP. In the reinstatement phase, half the dose received during the conditioning phase (0.5 or 12.5 mg/kg) was administered 15 min before the test in a different room to that of the conditioning sessions. The aim of this procedure was to administer the drug in a non-contingent way with respect to conditioning, so that the animal did not associate the contextual cues of the experimental room with the drug.

After this first reinstatement test, the groups that demonstrated reinstatement—i.e. a positive significant difference between the time spent in the drug-paired compartment in the reinstatement and last extinction tests (confirmed with a Student's t test) —were re-tested until a new extinction was confirmed. The following day, the effects of the priming (a quarter of the dose used for conditioning) on reinstatement of place

preference were evaluated following the procedure described previously. This procedure was repeated with progressively lower priming doses until a non-effective priming injection was determined.

2.3.3. Corticosterone measurements

Blood sampling for corticosterone determination was performed using the tail-nick procedure: the animal was wrapped in a cloth and a 2-mm incision was made at the end of the tail artery, and the tail was then massaged until 50 µl of blood was collected in an ice-cold Microvette CB 300 capillary tube (Sarstedt, Nümbrecht, Germany). To evaluate the effect of social defeat on corticosterone levels in adolescent animals, several of the mice used in the CPP study were employed (n=7 or n=8 in each group), but never in two consecutive measures. Blood samples were taken immediately or 30 min after the first and fourth agonistic encounters. A final sample was taken 3 weeks later, prior to the first pre-conditioning test. Blood samples were kept on ice, and plasma was separated from whole blood by centrifugation (5 min, 5000 g) and transferred to sterile, 2 mL microcentrifuge tubes. Plasma samples were stored at −80 °C until determination of corticosterone. All blood samples were taken between 10 am and 1 pm. On the day of the assay, samples were diluted (in a proportion of ~1:40) in the Steroid Displacement Reagent mix provided with the kit. Corticosterone levels in diluted plasma were then analyzed using a corticosterone EIA kit (Enzo® Life Sciences, Catalog No. ADI-900-097, 96 Well kit) according to the manufacturer's instructions, and an iMark microplate reader (Bio-Rad) and Microplate Manager 6.2. software. The optical density was read at 405nm, with 590nm correction.

2.4. Preparation of tissue extract

On day 65 (adolescents) or 85 (adults), mice were sacrificed by decapitation and the brains were rapidly removed and stored at -80 ºC for Western blot analyses. Brains were sliced on a cryostat and kept at −20°C until each region of interest comes into the cutting plane. Ventral tegmental area (VTA), NAc, dentate gyrus (DG) and basolateral amigdala (BLA) were micro-punched from frozen brain sections (500 µm) and sectioned using a cryostat according to the mice brain atlas of Frankin and Paxinos, 2008. Punches of the NAc (medial shell), VTA, DG and BLA were collected in Eppendorf tubes, according to the method of Leng, Feldon and Ferger (2004).

2.5. Electrophoresis and Western blotting

Punches from NAc, VTA, NAc, DG and BLA were placed in homogenization buffer. Samples were sonicated, vortexed and sonicated again prior to centrifugation. Each sample, which contained equal quantities of total proteins (20 µg), was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), which were blocked in 1% bovine serum albumin for 60 minutes at room temperature (RT). Incubations with the primary antibodies were made at 4 °C overnight: rabbit polyclonal anti-Nurr1 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit polyclonal anti-Pitx3 (1:750, Abcam, Cambridge, UK); rat monoclonal anti-DAT (1:2000, Millipore); rabbit polyclonal anti-DRD2 (1:500, Millipore);rabbit monoclonal anti-pERK ½ (1:750, Santa Cruz Biotechnology); rabbit polyclonal anti-pCREB (1:750, Millipore) and rabbit polyclonal anti-BDNF (1:250, Santa Cruz Biotechnology). Goat anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP)-linked (1:5000, Santa Cruz Biotechnology) or goat anti-rat IgG HRP-linked (1:5000, Santa Cruz Biotechnology) were used as secondary antibodies. After washing, immunoreactivity was detected with an enhanced chemiluminescent/chemifluorescent Western blot detection system (ECL Plus, GE Healthcare, LittleChalfont, Buckinghamshire, UK) and visualized by a ImageQuant LAS 500 imager (GE Healthcare). Blots were incubated with stripping buffer (glycine 25 mM, SDS 1%, pH 2) for 1 hour at 37 °C and subsequently reblocked and probed with rabbit polyclonal antiglyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5000; #2118, Cell Signaling Technology Inc.) or rabbit polyclonal anti α-tubulin (1:5000 Cell Signaling Technology Inc., Danvers, MA, USA), which were used as loading control. The ratios Nurr1/α-tubulin, Pitx3/α-tubulin, DAT/GAPDH, DRD2/GAPDH, pERK1/α-tubulin, pERK2/α-tubulin, pCREB/α-tubulin and BDNF/α-tubulin were plotted and analyzed.

2.6. Statistical analyses

For the CPP data, the time spent in the drug-paired compartment during Pre- and Post-C tests was analyzed with a mixed three-way ANOVA, with two between-subjects variables – Stress, with two levels (RSD and Control), and Age, with two levels (Adults and Adolescents) - and a within-subjects variable – Days, with two levels (Pre-C and Post-C). Post hoc comparisons were performed with Bonferroni tests.

Corticosterone levels at minute 0 (immediately after) and 30 of the first and fourth social defeat and 3 weeks after the last social defeat encounter were analyzed with a mixed ANOVA with two between-subjects variables - Stress, with two levels (RSD and Control), and Age, with two levels (Adult and Adolescent) - and a within-subjects variable – Time, with five levels. Post hoc comparisons were performed with Bonferroni tests.

To evaluate each of the behaviors during the social encounter, a mixed ANOVA with one within-subjects variable – Days, with two levels (first and fourth encounter), and a between-subject variable Age, with two levels (Adult and Adolescent) - was employed.

For the Western Blot data were analyzed by a two-way ANOVA with two variables - Stress, with two levels (RSD and Control), and Age, with two levels (Adult and Adolescent). Post hoc comparisons were performed with Newman Keuls tests.

3- **Results**

3.1. Behavioral characterization of repeated social defeat in adolescent and young adult mice

The times devoted to the behavioral categories by experimental and resident mice are shown in Table 2. In the resident mice, ANOVA revealed a significant effect of the interaction "Age X RSD" for the time spent in attack [$F(1,56) = 5.933$; $p < 0.05$], latency of attack [$F(1,56) = 4.416$; $p < 0.05$], and threat [$F(1,56) = 9.542$; $p < 0.01$]. When confronted with adolescents, resident mice showed a longer latency to threat ($p<0.01$) and attack ($p<0.001$), and spent less time in attack ($p<0.001$) than when confronted with an adult mice during the first social defeat. Moreover, resident animals threatened and attacked adolescent mice faster in the fourth social defeat than in the first ($p< 0.001$ in both cases).

The ANOVA showed an effect in the time spent in defensive/submissive behavior [$F(1,56) = 3.805$; $p < 0.05$] by the experimental mice. Adult mice spent more time engaged in these behaviors than adolescent in any of the social defeats ($p< 0.001$ in all cases). Adolescent mice exhibited more defence and submission during the fourth social defeat than in the first ($p< 0.01$).

3.2. Effect of repeated social defeat on corticosterone levels.

Blood concentrations of corticosterone (pg/ml) are shown in Table 3. ANOVA revealed a significant effect of Time × Age × Stress [F (4,108) = 5.379; p<0.001]. Posthoc comparisons showed higher corticosterone levels in socially defeated adult mice compared to controls after the 1st and the 4th social defeat (p< 0.05, 30 min after the 4th social defeat and ps< 0.001 for the rest). Defeated adolescents only showed this difference after the 4th social defeat (p< 0.001). Defeated adult mice exhibited higher levels of corticosterone than adolescents after all of the social encounters (ps< 0.001). However, three weeks later, control or defeated adult mice showed lower levels of corticosterone than adolescents (ps< 0.02).

3.3. Effect of social defeat on cocaine-induced CPP in adult and adolescent mice

ANOVA of the effects of RSD on cocaine-induced CPP in adult and adolescent mice (see Fig 1) showed an effect of the variables Days [F(1,52)=18.865; p<0.001] and Stress [F(1,52)=6.095; p<0.01] and the interaction Days x Stress [F(1,52)=12.848; p<0.001]. Socially defeated mice developed CPP regardless of their age (p< 0.001 between Pre- and Post-C test). In addition, RSD mice spent more time in the drug-paired compartment during the Post-C test than controls (p< 0.001). Defeated adult mice required 6 sessions for the preference to be extinguished, while defeated adolescent mice required 5 sessions. After extinction, a priming dose of 0.5 mg/kg of cocaine reinstated the preference in both groups (p< 0.05). No further extinctions were obtained.

3.4. Alterations in the expression of Pitx3 and Nurr1 in the VTA of adult and adolescent mice induced by social defeat

Two-way ANOVA for Pitx3 expression (Fig 2A) in the VTA revealed significant effects of the variable Age [F(1,18)=9.561; p=0.0063] and the interaction Age x Stress [F(1,18)=6.426; p=0.0207]. Newman-Keuls *post hoc* comparisons showed that Pitx3 significantly decreased in socially defeated adolescent mice compared with non-stressed adolescent animals (p<0.01) and with socially defeated adult mice (p<0.05).

Two-way ANOVA for Nurr1 showed no main effect of Age, Stress or the interaction Age x Stress. Nonetheless, a *post hoc* test revealed that socially defeated adolescent

mice exhibited significantly lower Nurr1 levels than non-stressed adolescent animals ($p<0.05$) (Fig 2B).

3.5. Effect of social defeat on dopaminergic markers in the NAc of adult and adolescent mice

Two-way ANOVA for D2DR and DAT expression revealed no main effects of the variables Age or Stress or of the interaction Age x Stress. Although Newman Keuls *post hoc* comparisons did not reveal significant changes in D2DR and DAT protein levels, a tendency to increase was observed in the expression of both proteins in adult socially defeated mice compared with their controls (Fig 2C and 2D).

3.6 Changes in the expression of pERK 1/2 , pCREB and BDNF in the DG and BLA.

Fig 3 and 4 show the effects of RSD on ERK ½, pCREB and BDNF expression in the DG and BLA nuclei of adult and adolescent mice.

In the DG two-way ANOVA for pERK1 and pERK2 expression revealed a significant interaction between the factors Age and Stress ($F(1,25)=9.24$; $p=0.0055$; $F(1,27)=11.80$; $P=0.0019$, respectively). Newman-Keuls post-hoc analysis revealed that pERK1 and PERK2 expression in the DG was increased ($p<0.05$) in socially defeated adolescent mice compared with their controls and defeated adult mice (Fig 3A and 3B). On the other hand, two-way ANOVA for pCREB and BDNF expression only showed a significant effect of the variable Stress ($F(1,20)=32.22$,$p<0.0001$; $F(1,20)=64.58$, $p<0.0001$, respectively). A Newman-Keuls post-hoc test revealed an increased expression of pCREB and BDNF in socially defeated adult and adolescent mice versus their respective controls (Fig 3C, D).

In the BLA two-way ANOVA for pERK 1, pERK 2 and pCREB showed no main effects for any of the variables or their interaction (Fig 4A, 4B and 4C). Two-way ANOVA for BDNF showed that only the variable Stress had a significant effect ($F(1,12)=17.47$, $p=0.0013$). Post-hoc test revealed an increased ($p<0.05$) expression of BDNF in adolescent and adult defeated mice versus their control groups (Fig 4D).

Discussion

The present study confirms that repeated social defeat during adolescence or adulthood induced long-lasting increases in sensitivity to the conditioned reinforcing effects of cocaine. Defeated mice developed CPP with a non-effective dose of cocaine and, after extinction, the preference was reinstated after receiving a priming dose of this drug. In addition, our study shows for the first time that only adolescent defeated mice receiving cocaine exhibited decreased levels of the transcription factors Pitx3 and Nurr1 in the VTA. However, there were no alterations in the expression of their target genes DAT and D2DR in the NAc, although the expression of DAT and D2DR tended to be higher in socially defeated adult mice**.** In parallel with the alterations in dopaminergic pathways, our results also revealed that defeated mice showed an increased expression of BDNF in the DG and BLA regardless of their age. Depending on the age of the mice and their anatomical structure, this increase was accompanied or not by changes in pERK or pCREB.

It has been previously reported that mice which are socially defeated during adolescence develop CPP with non-effective doses of cocaine (Rodriguez-Arias et al. 2015) or amphetamine (Burke et al. 2011). In the present study, we demonstrate for the first time that mice defeated during adulthood also show this long-lasting increase in sensitivity to cocaine. McLaughlin and co-workers (2006) reported significantly stronger CPP for the cocaine-paired chamber in mice acutely defeated during the conditioning phase. However, there are no reports of such as increase three weeks after the last exposure to stress. In line with the present report, social defeat has also been shown to increase vulnerability to cocaine self-administration during adolescence and adulthood (e.g. Burke and Miczek 2015; Yap et al. 2015). Although the response to cocaine in the CPP is similar in mice defeated during adolescence or adulthood, the study of social encounters between resident and intruder mice reveals significant differences depending on the age of the intruder. Resident mice were less aggressive with the adolescent intruder mice, needing more time to threaten or attack and spending less time in attack in comparison with the aggression shown when confronted with adult intruders. Consequently, adolescent mice showed weaker behavioral and hormonal responses. Adolescent intruder showed less avoidance/flee behaviors during the social encounters in comparison with adults. Moreover, in adolescent mice significant increases in corticosterone were only observed after the fourth social defeat, and, even

after this encounter, corticosterone levels were lower than those in adults. However, adult mice exposed to RSD presented higher corticosterone levels than controls at all the time points studied (0 and 30 min after the first and fourth episodes of defeat). This age effect is consistent with previous results observed in our laboratory (García-Pardo et al. 2014, 2015; Rodríguez-Arias et al. 2016). A number of studies have reported that the HPA axis is hyper-responsive during adolescence, probably due to an underdeveloped negative feedback (see Klein and Romeo 2013). For example, early adolescents exposed to repeated restraint secrete more corticosterone than adults exposed to the same procedure (Romeo et al. 2006). Therefore, the lower corticosterone response observed in adolescent mice could have been due to the fact that resident mice were less aggressive with the adolescent intruders and because adolescents did not experience defeat as a stressful event but rather as aggressive play. Despite this, adolescent defeated mice showed similar or even more profound behavioral and biochemical changes than adult defeated mice.

Chronic stress and drugs of abuse increase the activity of the VTA-NAc pathway, triggering common long-term cellular and molecular adaptations (Fitzgerald et al. 1996; Saal et al. 2003; Razzoli et al. 2011). Social defeat augments the firing of VTA DA neurons and increases BDNF in the NAc, thereby enhancing vulnerability to substance abuse (Krishnan et al. 2007; Anstrom et al. 2003; Watt et al. 2009, 2014; Burke et al. 2010, 2011). The maturation of DA neurons in the VTA that project to the medial prefrontal cortex (mPFC) and NAc during adolescence is perhaps the most critical neural system for the processing of salient events, including responses to psychostimulants (reviewed in Burke and Miczek 2014). Previous studies suggest that social defeat has more profound effects on molecular alterations in the mesocorticolimbic system and on mesocorticolimbic system-mediated addiction-related behavior when experienced in adolescence rather than adulthood. Stress during adolescence sensitizes NAc DA neurons (Cruz et al. 2012), and this effect persists into early adulthood (Burke et al. 2010). Moreover, DA activity (measured as a DOPAC/DA ratio) in the mPFC of adolescent socially defeated rodents is lower with respect to adolescent non-stressed animals (Watt et al, 2009, 2014). Adult amphetamine injection elicits a larger increase in NAc core DA tissue content after mid-adolescent social defeat stress, without other changes taking place (Burke et al. 2010). Similarly, only adolescent stress, and not adult stress, increases DA content in the NAc of

amphetamine-injected mid-adolescent rats (Cruz et al. 2012) and increases locomotor response to psychostimulants in said animals (McCormick et al. 2005).

We have measured the expression of the transcription factors Nurr1 and Pitx3, both involved in the development and maintenance of the dopaminergic phenotype, in the VTA of socially defeated adolescent and adult mice. We have observed that the expression of these transcription factors was not altered by cocaine administration in socially defeated adult animals when compared with non-stressed mice. However, animals exposed to social stress during adolescence exhibited lower expression of Nurr1 and Pitx3 in the VTA after repeated injections of cocaine. The transcription factor Nurr1, together with its potentiator Pitx3, regulates crucial proteins for DA metabolism, such as DAT and D2DR, among others (Bissonette and Roesch 2015; Jacobs et al. 2009; Reddy et al. 2012). Our data showed no significant modifications of D2DR or DAT levels in the NAc of cocaine-treated vs. non-stressed mice following social defeat during adolescence. However, in cocaine-treated adult defeated animals we observed enhanced D2DR and DAT expression, although these increases were not statistically significant. We should point out that our study did not differentiate between presynaptic and postsynaptic D2DR, and that the projections of dopaminergic neurons of the VTA containing DAT and D2DR autoreceptors can be found in several nuclei, including NAc, mPFC and striatum, among others (Burke et al. 2011; Novick et al. 2011; García-Pérez et al. 2016). Therefore, regulation of DAT and D2DR expression by Pitx3 and Nurr1cannot be ruled out.

DAT activity - clearing synaptic DA - and expression in the mPFC in adulthood are influenced by exposure to stress during adolescence (Novick et al. 2011). It has been reported that adolescent social defeat evokes an increase in DA extracellular levels in the mPFC of rats, which has been postulated to provoke long-term over-activation of presynaptic D2 autoreceptors, which in turn would induce a decrease in DA through an enhancement of DAT expression and/or function in early adulthood (Watt et al. 2014). In conflict with the aforementioned, Burke et al. (2011) reported no alterations in D2DR expression in the mPFC, NAc core or shell, or striatum of adult rats exposed to social defeat during adolescence. However, they observed an enhancement of D2DR expression in the NAc core of amphetamine-conditioned socially defeated animals. Although we did not detect changes in D2DR levels in the NAc of adolescent socially defeated mice injected with cocaine, D2DR and DAT expression was found to be higher

in adult socially defeated animals. The increase of D2DR expression might have resulted from a loss in the capacity of D2 autoreceptors to modulate DA synthesis through maturation (Andersen et al. 1997). As previously suggested, the activation of D2 autoreceptors would lead to an increase of DAT expression and/or function in order to enhance DA clearance in the synaptic cleft. Our results also support an increase in postsynaptic D2DR as an adaptive response to cocaine-induced increases in synaptic DA.

In parallel with the alterations of dopaminergic pathways, our results also revealed an increased expression of pERK 1 and 2, and pCREB and BDNF in the DG of defeated mice exposed to cocaine, and an enhanced expression of BDNF in the BLA but no changes in that of pERK or pCREB. However, we detected a correlation between increased expression of ERK and pCREB in the DG only in adolescent defeated mice. In contrast, adult mice showed increased levels of pCREB without any changes in those of ERKs, indicating that other pathways, such as PKA or CaMK-IV, but not ERK, could be implicated in the activation of CREB. The activation of CREB triggers the transcription of target genes - including BDNF – in order to alter the behavior of animals and promote neurogenesis. Different results were observed in the BLA, in which socially defeated mice showed an increased expression of BDNF without any changes in pCREB or ERKs. It has been described that social defeat stress induces PKA activation in the BLA (Yang et al. 2016), which activates CREB for the transcription of BDNF (Markham et al. 2014). Altogether, these results confirm the hypothesis that social defeat stress induces different neuroadaptations to social stress depending on brain area and the stage of brain development.

An intracellular cascade of molecular events in the VTA-NAc-amygdala/hippocampus circuit includes several candidate mechanisms for persistent neuroplastic adaptations to social defeat and other types of stress; primarily BDNF. Social defeat stress leads to the heightened phasic firing of VTA dopaminergic neurons projecting to the NAc, resulting in the activity-dependent release of BDNF and the activation of BDNF signaling in the NAc (for revision see Krishnan 2014).

Specifically, BDNF in the BLA is implicated in fear conditioning (Rattiner et al. 2004) and regulates the consolidation of defeat-related memories (Dulka et al. 2016). According to previous reports (Fanous et al. 2010; Taylor et al. 2011), our data show

that defeated adult and adolescent animals exposed to cocaine present increased of BDNF in the BLA, suggesting that social defeat primarily activates fear and flight circuitry. In contrast with our results, it has been demonstrated that submissive animals show a progressive decrease of BDNF gene expression in the BLA, which may be influenced by fear conditioning (Smith et al. 2014). These discrepancies are very likely to be the result of differences in experimental design (animal strain, type of stress and environment). Moreover, it is important to bear in mind that our mice were exposed to cocaine, which can alter the brain changes induced by social defeat stress.

The effect of social stress on BDNF signaling in the hippocampus appears to be more complicated, with studies providing contrasting results. Using a more intense protocol than that of our study, Tsankova and co-workers (2006) observed a long-lasting reduction of BDNF mRNA in the hippocampus of socially defeated mice. In addition, it has been demonstrated that social conflict-induced learning leads to the largest increases in BDNF in the DG when the conflict is won (Taylor et al. 2011). In contrast, our results demonstrate that defeat social stress induces an enhancement of BDNF in the DG of defeated adult and adolescent mice. In agreement with our results, low-novelty-seeking rats have been reported to display increased levels of BDNF in the DG following social defeat (Duclot and Kabbaj 2013). Moreover, soluble epoxide hydrolase KO mice show increased BDNF in the prefrontal cortex and hippocampus, but not in the NAc (Ren et al. 2016). While higher BDNF levels in the NAc promote vulnerability to social defeat in mice (Krishnam et al. 2007), higher BDNF levels in the hippocampus promote resilience to a chronic mild stress (Begström et al. 2008; Taliaz et al. 2011).

The amygdala is the brain structure for the storage of fear memories and the hippocampus is especially important for the processing of contextual information, being necessary for both fear acquisition and extinction (Myers et al. 2006; Sierra-Mercado et al. 2011). In this regard, recent studies suggest that BDNF promotes extinction of fear memory (Andero and Ressler 2012; Rodríguez-Serrano et al. 2014), emphasizing that BDNF activity underlies memory extinction and supporting the idea that BDNF is a key regulator and mediator of long-term synaptic modifications (Rodriguez-Serrano et al. 2014). It has been shown that aversive experiences lead to modifications in the capacity to express subsequent synaptic plasticity like LTP, a persistent strengthening of synaptic efficacy that underlies learning and memory (Hirata et al. 2009; Rudy and Matus-Amat 2009). In our study, in parallel with the augmented BDNF expression in the DG and

BLA, we also demonstrate an enhancement of sensitivity to the conditioned rewarding effects of cocaine, which indicates that BDNF may extinguish aversive memory and improve reward memory processes in mice submitted to social defeat stress and exposed to cocaine. Altogether, these findings reinforce the idea of important brain reactions to stress in the form of neuronal plasticity, implicating BDNF as an important biomarker.

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Table 1. Experimental procedure.

			Social defeat							
			1st	2nd	3rd	4th	3 weeks	CPP Pre-C test	CPP Conditioning	CPP Post-C Test
1st set of mice	Adolescent	PND	27	30	33	36		58-60	61-64	65
	Adult	PND	47	50	53	56		78-80	81-84	85

			Social defeat						
			1st	2nd	3rd	4th	3 weeks	Cocaine administration	Brain samples
2nd set of mice	Adolescent	PND	27	30	33	36		61-64	65
	Adult	PND	47	50	53	56		81-84	85

Table 2. Behavior of mice during agonistic encounters. Mean cumulative times (±S.E.M.) devoted to different behavioral categories by adolescent and young adult experimental mice (avoidance/flee, defence/submission and latency to initiate these behaviors) and by aggressive opponents confronted with adolescent and young adult experimental mice (threat, attack and latency to initiate these behaviors), during the first (1) and the fourth (4) agonistic encounter. *** p <0.001, ** p< 0.01 significant difference with respect to adult mice. +++ p <0.001, significant difference with respect to the first encounter.

	Adolescent mice		Adult mice	
Social defeat	**1st**	**4th**	**1st**	**4th**
Intruder mice				
Avoidance	11 ± 2	12 ± 1	26 ± 5	17 ± 3
Latency Avoidance	17 ± 4	5 ± 2	38 ± 20	5 ± 1
Defence/Submissive	22 ± 4***	47 ± 5***+++	93 ± 10	87 ± 11
Latency Defence/Submissive	54 ± 7	11 ± 4	39 ± 20	9 ± 4
	Adolescent mice		**Adult mice**	
Social defeat	**1st**	**4th**	**1st**	**4th**
Resident mice				
Threat	12 ± 2	14 ± 2	23 ± 4	18 ± 2
LatencyThreat	21 ± 5,4**	5 ± 1**+++	2 ± 0	3 ± 1
Attack	12 ± 2***	17 ± 3***	23 ± 3	17 ± 2
LatencyAttack	23 ± 7***	1 ± 0***+++	8 ± 5	3 ± 1

Table 3. Effect of repeated social stress on corticosterone levels. Mean corticosterone levels (±S.E.M.) in blood (pg/ml) of adolescent and young adult mice after exploration (control) or repeated social defeat exposure (defeated), 0 or 30 min after the first (1st-0, 1st-30) and fourth (4th-0, 4th-30) social defeat or exploration and 3 weeks after. *** p <0.001, * p< 0.05 significant difference with respect to controls of the same age. ++ p <0.01, significant difference with respect to adult mice.

		1st social defeat		4th social defeat		3 weeks
		0 min	30 min	0 min	30 min	
Adolescent	Control	1329 ± 277	1499 ± 204	1447 ± 163	1829 ± 219	1249 ± 86
	RSD	1218 ± 206 +++	1113 ± 213 +++	4300 ± 529 ***+++	2709 ± 360 +++	1320 ± 171
Adult	Control	1671 ± 340	2016 ± 231	2882 ± 192	2561 ± 336	771 ± 115 ++
	RSD	4709 ± 752***	6429 ± 1247***	6317 ± 722***	3974 ± 577*	872 ± 155 ++

Fig 1. Effects of social defeat on acquisition of the CPP induced by 1 mg/kg of cocaine in adolescent and adult mice. The bars represent the time (in seconds) spent in the drug-paired compartment before conditioning sessions in the pre-conditioning test (white bars), after conditioning sessions in the post-conditioning test (black bars), in the last extinction session (light grey bars) and during the reinstatement test (dark grey bars). *** $p < 0.001$, significant difference in the time spent in the drug-paired compartment vs pre-conditioning test; + $p < 0.05$ significant difference in the time spent in the drug-paired compartment vs extinction test.

Fig 2. Chronic social defeat during adolescence decreases protein levels of Nurr1 and Pitx3 in the VTA of mice. Semi-quantitative analysis and representative immunoblots of Pitx3 (A) and Nurr1 (B) in the VTA and D2DR (C) and DAT (D) in the NAc of socially defeated mice receiving cocaine. Each bar corresponds with mean ± SEM (% of control). n = 5-12 animals/group. *$p < 0.05$ vs socially defeated adult mice; +$p < 0.05$ vs control adolescent mice.

Fig 3. pERK 1 (A) and 2 (B), pCREB (C) and BDNF (D) expression in the dentate gyrus (DG) in adult and adolescent mice exposed to cocaine after repeated social defeat (RSD). Each bar corresponds with mean ± SEM (n=6-8). *$p<0.05$, **$p<0.01$ versus socially defeat adult mice; +$p<0.05$, ++$p<0.01$ versus adolescent control mice; &$p<0.05$ versus adult control mice.

Fig 4. pERK 1 (A) and 2 (B), pCREB (C) and BDNF (D) expression in the basolateral amygdala (BLA) in adult and adolescent mice exposed to cocaine after repeated social defeat (RSD). Each bar corresponds to mean ± SEM (n=4-6). + $p<0.05$ versus adolescent control mice; & $p<0.05$ versus adult control mice.

Fig. 1

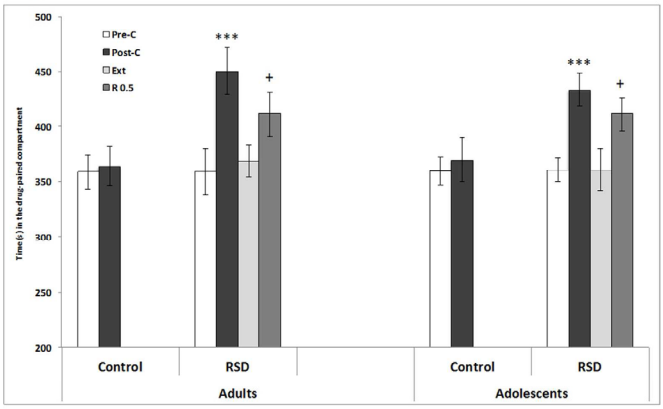

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Fig. 2

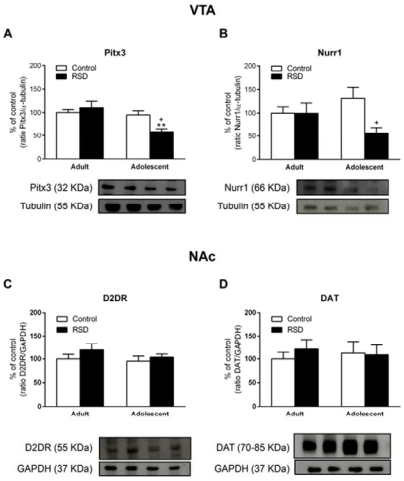

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Fig. 3

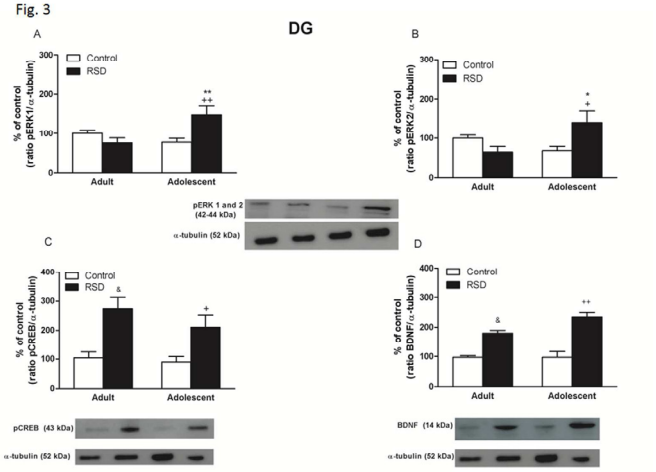

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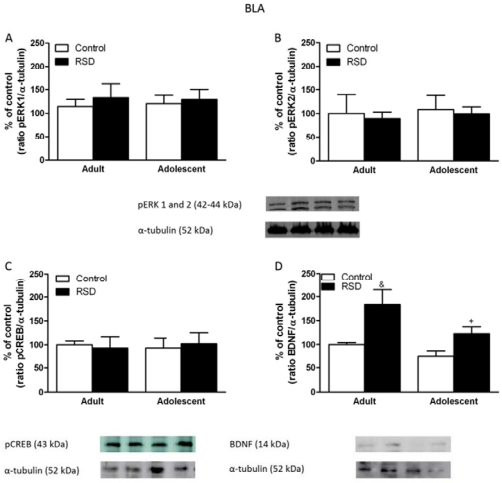
Fig. 4



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STUDY 6

How genetics and experience can influence in social dominance and patterns of agonistic behavior in mice.

S. Montagud-Romero, M.C. Blanco-Gandía, J. Miñarro, M.Rodríguez-Arias.

In preparation

How genetics and experience can influence in social dominance and patterns of agonistic behavior in mice.

Montagud-Romero S[1], Blanco-Gandía MC[1], Guerri C[2], Rodríguez de Fonseca F[3], Miñarro J[1] and Rodríguez-Arias M[1]

Department of Psychobiology, Facultad de Psicología, Universitat de Valencia, Avda. Blasco Ibáñez, 21, 46010 Valencia, Spain. [2]Department of Cellular Pathology, Centro de Investigación Príncipe Felipe, C/ Eduardo Primo Yúfera 3, 46012, Valencia, Spain. [3]Instituto IBIMA de Málaga, Unidad de Gestión Clínica de Salud Mental, Hospital Regional Universitario, Málaga, Spain.

1. Introduction

Aggression is a complex social behavior present in most animal species, such as insects, fish and most mammals, including humans. It has been defined as ‘‘behavior that inflicts harm and injury or threatens to do so’’ (Berkowitz, 1993) or ‘‘any form of behavior directed toward the goal of harming or injuring another living being who is motivated to avoid such treatment’’ (Baron and Richardson, 1994). It exhibits different dimensions in terms of origins, motivations, expressions, and functions and includes a variety of different behavioral patterns (Miczek et al., 2007). Using animal models, the investigation on aggression has evaluated the ethological implication of the behavior, such as its involvement in the survival and animal reproduction, and its phylogenetic and ontogenetic development (Miczek, 2014).

The prevalent practices of aggression in male mice occur in situations of social conflict, when a male defends against a territorial intruder (e.g. territorial aggression, intermale aggression). In such confrontations, indices of the individual aptitude such as social status or access to resources are improved (Miczek et al., 2001). Aggressive behavior in male mice (between conspecifics) is classified in two levels: the offensive and the defensive behavior. The first one is ritually organized, composed of chasing, tail rattling, threats, defensive upright postures, and attack bites (Miczek and O’Donnell, 1978) often lead to body areas like the back and flanks of the adversary (Blanchard and Blanchard 1977; Blanchard et al., 1979, 2001). However, the defensive aggression includes escape and freezing behaviors, defensive postures, and threats, with defensive attacks (Blanchard and Blanchard 2003).

In the animal models paradigms, social defeat stress or subordination is considered a stressor with a relevance ethology that faithfully mimics the real life situations (Tornatzky and Miczek, 1993). It is now commonly used to model the effects of social stress in humans (Brown and Harris, 1989; Björkqvist, 2001). To simulate that stress in the laboratory, the

resident-intruder paradigm (Covington and Miczek, 2001) has been used as a model of repeated social defeat (RSD), where a set of rodents are used as dominant subject and another set as subordinate. In each of the experimental sessions, animals are placed into a dominant territorial aggressor cage and experiencing repeated attacks from the home-cage animal. The experimental rodent, with the repeated experience of these meetings will present conduct of flight, defense or submission. This model is based on the development of dominance hierarchies based on such interactions (Huntingford and Turner, 1987). This paradigm has been used multiple times with laboratory rodents, in order to study the physiological, behavioral and neurobiological changes caused by social experiences of stress, both acute and chronic (Holly et al., 2015; Burke and Miczek, 2015; Garcia-Pardo et al, 2015; Montagud-Romero et al, 2015).

Currently, there is great evidence of behavioral differences among strains. Behavioral phenotypes for C57BL/6 and 129S6/SvEv strains showed differences in anxiety, locomotor activity and social interaction regardless of pre-experimental housing conditions (Abramov et al., 2008). Specifically, the C57BL/6 mice are more reactive and adventurous when compared with the strain 129S6/SvEv, which are inactive and anxious (Crabbe et al., 1999; Tarantino et al., 2000). The depressive test behavior showed that 129S6/SvEv were more vulnerable to develop this feature (Liu and Gershenfeld 2001, 2003). On the other hand, CBA/Lac and C57BL /6J strains also displayed behavioral differences; where C57BL /6J showed higher levels of movement and investigative activity than CBA/Lac, which is also observed after being exposed to an acute physical stress (Avgustinovich et al., 2007). In addition, BALB/c mice have been studied for their high level of aggression (Dow et al., 2011) and their low level of sociability compared with C57BL/6 mice (Fairless et al., 2008), which may contribute to their overall phenotype.

In laboratory mice, the genotype could markedly influence social dominance and the patterns of agonistic behavior in social partners (Kudryavtseva et al., 2006; Nevison et al., 1999). Significant differences in the levels of dominance were found between BALB, CBA and PT strains, using

the ethological model of social hierarchy (Osadchuk et al., 2009). To study behavioral responses, different strains interacted in the same environment, so it was observed that the inbred ICR (CD-1) strain and the outbred BALB/c strain were more aggressive than other strains, such as C57BL / 6, CBA /Ca and DBA/2, which exhibited lower levels of aggression, as is usually the case with most inbred strains (Kudryavtseva et al., 2006; Nevison et al., 1999). In addition, in that interaction, the strain DBA/2 was the ones that showed lower levels of anxiety (Kudryavtseva et al., 2002; Kudryavtseva, 2006; Vishnivetskaia et al., 2013). The exposure to chronic social defeat for 21 days in strains like CBA/Lac and C57BL/6J, increases anxiety (Kovalengo et al., 2015; Kudryavtseva et al., 2006; Kudryavtseva and Avgustinovich 1998), while the answer to depressive behavior is not so clear (Kudryavtseva and Avgustinovich 1998; Berton et al., 2006; Krishnan et al., 2007; Golden et al., 2011). Furthermore, BALB/c mice demonstrated reduced social interaction following a 10 day social stress paradigm when compared to the C57BL/6 (Savignac et al., 2011).

However, mice behavior could be regulated by the experience of different social-environmental situations. There are variables than can modulate aggression, as housing conditions, food restriction and social experiences. Although housing rodents in an enriched environment has been considered to have beneficial effects on the well-being and cognitive functioning of the animals, in some strains of mice, it has also been reported to elicit aggression and to promote stress-related outcomes (Marashi et al., 2003; Abou-Ismail, 2011; McQuaid et al., 2012). Moreover, individually or isolated housed rodents heighten aggression (Rodríguez-Arias et al., 1998; Montagud-Romero et al., 2015, 2016; García-pardo et al., 2015) and cohabitating with a female could also modulate their agonistic behavior (Han et al., 2015; and Holly et al., 2015). Furthermore, food restriction was used as the ecological stressor because a relationship exists between food deprivation and elevated aggression (Nakamura et al., 2008). Together with all these social-environmental situations, male inbred rodents exposed to a positive fighting experience in daily agonistic interactions increased aggressive

behaviors (Kudryavtseva et al., 2014); while, adolescent rats exposed to social defeat, did not show changes in aggressive behavior in adulthood (Coppens et al., 2014).

The aim of our study was to assess the social dominance and patterns of agonistic behavior in the different strains and evaluate how the experience to the agonistic encounters could change the behavioral patterns depending on the genetics. Most of the studies that employ the resident-intruder paradigm use rats of the Long-Evans strain (Miczek and Mutschler, 1996; Tidey and Miczek, 1997; Covington and Miczek, 2001, 2005; Covington et al., 2008; Quadros and Miczek, 2009; Cruz et al., 2011; Boyson et al., 2014). However, when performing social defeat in mice, there is a great variability in the strain employed (OF1, CD1, CFW etc) (Rodríguez-Arias et al., 2015; Montagud-Romero et al., 2016; García-pardo et al., 2015; Han et al., 2015). For this reason, our work is focused on analyzing and clarifying dominance and defeat in different strains of mice, in order to elucidate which of these strains would be more convenient to perform studies on social defeat stress and obtain reliable results.

2. Material and Methods

2.1. Animals

A total of 96 male OF1, CD1, B6.129X1 (Charles River, France) and C57BL/6 WT (Harlan Ibérica, Barcelona, Spain) arrived at our laboratory at 42 days of age. All mice (except those used as aggressive opponents n=12 in each strain) were housed in groups of four in plastic cages (25×25×14.5 cm) for 8 days before the experiments began. Aggressive opponents were individually housed in plastic cages (23×13.5×13 cm) for a month prior to experiments in order to heighten aggression (Rodríguez-Arias et al., 1998). Mice were housed in controlled laboratory conditions with the temperature maintained at 21±1 ºC and humidity at 55±10%. All test took place during the first hours of the dark phase of a reversed light/dark cycle (lights off at 08:00 and on at 20:00). Food and water were available ad libitum for the mice used. All procedures were conducted in compliance with the guidelines of

the European Council Directive 2010/63/UE regulating animal research and were approved by the local ethics committees (University of Valencia).

2.2. Procedure: Repeated Social Defeat encounters

Animals in the corresponding group were exposed to 4 episodes of social defeat lasting 25 min each on PND 47, 50, 53 and 56. Each episode consisted of three phases, which began by placing the intruder animal in the home cage of the aggressive opponent or resident for 10 min. During this initial phase, the intruder was protected from attacks by a wire mesh wall that permitted social interaction and species-typical threats from the male aggressive resident (Covington and Miczek, 2001). In the second phase, the wire mesh was removed from the cage and a 5-min period of confrontation began. The second phase of each social defeat protocol was video-recorded and ethologically analyzed. Threat and attack behaviors were scored in resident mice and avoidance/flee and defensive/submissive behaviors were evaluated in intruder mice. In the third phase, the wire mesh was replaced for a further 10 minutes to allow social threats from the resident. Each resident confronted with its respective intruder strain, without mixing them.

All agonistic encounters were videotaped and evaluated using a computerized system by an observer who was blind to the treatment (Brain et al., 1989). This custom-developed program allows estimation of the time engaged in different broad functional categories of behavior—threat, attack, avoidance/ flee and submission—each of which is characterized by a series of diverse postures and elements (Rodríguez-Arias et al., 1998).

2.3. Statistical analyses

For each of the behaviors studied a two-way ANOVA was performed, with a between subjects variable – Strain (OF1, CD1, B6.129X1 and C57BL/6 WT) – and a within-subjects variable – Days (fist and fourth social defeat). Post hoc comparisons were performed with Bonferroni tests.

A linear correlation analysis was employed to determine the association between the aggressive behavior levels and the submissive ones.

3. Results

3.1. Behavioral characterization of repeated social defeat in resident mice

In the resident mice, the ANOVA revealed a significant effect of the interaction Days X Strain for the time spent in attack and threat [$F(3,38)$ 2.573; $p = 0.05$] and [$F(3,38) = 11.946$; $p < 0.001$]. When confronted with the intruders, the B6.129X1 mice spent more time in threat and attack behaviors ($p<0.001$, $p<0.05$ respectively) than the other strains in the first social defeat (see Figure 1a). However, in the fourth social defeat the OF1 strain spent less time in threat behaviors when compared with B6.129X1 and C57BL/6 ($p<0.001$ in both cases)(see Figure 1b). Therefore, the experience of being exposed to the different agonistic encounters changed the patterns of the aggressive behavior. OF1 and B6.129X1 spent less time in threat behavior in the fourth than in the first agonistic encounter ($p<0.05$, in both cases) (Figure 2a and 2b); the opposite is observed in the C57BL/6 strain ($p<0.001$) for threat behavior (Figure 2c). Furthermore, B6.129X1 spent less time in attack behavior in the fourth social defeat than in the first agonistic encounter ($p=0.01$) (see Figure 2a).

The ANOVA also revealed a significant effect in the latency to perform the first attack behavior for the interaction Days X Strain [$F(3, 38) = 11.929$; $p < 0.001$]. In the first social defeat, C57BL/6 mice showed longer latencies to perform attack than the other strains ($p<0.01$); while OF1 mice displayed shorter latencies than C57BL/6 and CD1 strains ($p<0.001$ and $p<0.01$, respectively) (see Fig 1a). In addition, in the first social defeat, C57BL/6 and CD1 mice showed longer latencies to perform agonistic behaviors than in the fourth agonistic encounter ($p<0.001$ in both cases) (see Figure 2d).

3.2. Behavioral characterization of repeated social defeat in intruder mice

In the intruder mice, the ANOVA revealed a significant effect in the time spent in avoidance and defeated/submissive behavior for the interaction Days X Strain [$F(3,38) = 26.600$; $p < 0.001$] and [$F(3, 38) = 7.762$; $p < 0.001$],

respectively. In the first social defeat, B6.129X1 and OF1 strains spent more time in defense/submissive behavior than the C57BL/6 mice ($p<0.001$); and B6.129X1 spent more time in avoidance behavior than the other strains ($p=0.01$ in all cases) (see Figure 3a). However, in the fourth encounter, the OF1 strain spent less time in the avoidance pattern behavior when compared with B6.129X1 and C57BL/6 ($p=0.01$; $p=0.001$, respectively) and also less time in defense/submissive behavior than all the other groups ($p<0.001$) (Figure 3b). Furthermore, the experience changed some behavioral patterns, since in the fourth agonistic encounter, the OF1 and B6.129X1 mice spent less time showing defeated ($p<0.01$, $p<0.001$, respectively) an avoidance behaviors ($p<0.05$, $p<0.001$, respectively) than in the first social experience, while the opposite is observed in the C57BL/6 mice ($p<0.05$) (see Figure 4a, b, c).

The ANOVA revealed a significant effect in the latency to perform the submissive behavior for the interaction Days X Strain [$F(3,38) = 7.896$; $p = 0.001$]. In the first social defeat, C57BL/6 mice took longer latency to perform defense/submissive than the OF1 strain ($p=0.05$). Furthermore, in the fourth agonistic experience, the latency to perform the defense/submissive patterns was higher in OF1 strain when compared with the C57BL/6 and CD1 mice ($p<0.05$ in both cases) and when compared to its first social defeat ($p<0.001$) (See Figure 3 and 5c). In addition, C57BL/6 took shorter latencies to perform avoidance in the fourth social defeat ($p<0.05$) than in the first one (Figure 5a).

3.3. Correlations

Taking together the data of the four strains, the Pearson correlation showed that the agonistic behavior of the resident mice had a significant effect on the defensive behavior observed in the defeated animals in the first ($\chi^2= 0.700$, $p < 0.001$) and the fourth defeat ($\chi^2= 0.852$, $p < 0.001$). There was a positive lineal correlation between the aggressive and submissive behaviors, which means that the higher aggressive behavior in the residents induces higher levels of defensive behavior in the intruders.

The correlation analysis studied in each strain independently revealed that

the level of aggressive behavior showed by the resident had a significant effect on the development of submissive behavior in the intruders, only in the C57BL/6 strain (χ^2= 0.969, $p < 0.001$). There was no effect in the other strains ($p>0.05$) in all cases.

4. **Discussion**

Social stress is one of the most potent stressful stimuli in mammals of all species (Blanchard et al., 2001). In this study, we investigate the behavioral effects of four intermittent episodes of social defeat separated by 72h (on days 1, 4, 7 and 10). On day one of this experiment, all resident mice (in all strains) displayed aggressive behaviors when exposed to the agonistic encounter against the intruder, which in turn, displayed submissive and defensive behaviors. These responses determined the status of winners for the residents and losers for the intruders. We have observed that genetics established different behavioral patterns when different kind of mice are exposed to an agonistic situation. Although genetics determine the behavior, the experience could change it, depending on the animal strain. Genetically, the strains that showed higher levels of behavioral aggressive patterns were B6.129X1 and OF1. However, their aggression was reduced by the experience of being exposed to different encounters. The opposite effect was observed in the C57BL6/J, which decreased the latencies to perform agonistic behaviors (threat and attack faster) by the repeated defeat stress encounters. The intruder's behaviors were related with the resident's agonistic patterns, decreasing the defensive/submissive behavior when the threat and attack were reduced.

Our data showed that when confronted with the intruders, during the first social defeat, resident B6.129X1 mice spent more time in threat and attack than the other three strains, showing that it was the most genetically aggressive strain. Nevertheless, the fastest strain to perform an attack was the OF1 mice, while the lowest one was the C57BL/6. In line with these results, C57BL6/J males neither show higher aggressive score than other mice strain such as CD1, nor become dominant in the subordination stress

protocol (Bartolomucci et al., 2009). The CD1 strain showed intermediate levels of aggression. However, that kind of mice has demonstrated high levels of intermale aggression, as well as maternal aggression in female rodents (Parmigiani et al., 1999; Van Loo et al., 2003). These contradictory results could be explained by the different procedures used to analyze the aggressive behavior, as well as their periodicity of the encounters. For example, Van Loo and co-workers (2003) measured the aggressive behavior after cage cleaning in the week prior to and immediately following the introduction of two new enrichment items. As the studies used different social-environmental situations, the strain could respond in a behaviorally different way to them.

On the other hand, the intruder mice which spent more time in defense/submissive behavior were the B6.129X1 and OF1 strains, while avoidance behavior was more prominent in the B6.129X1. Social avoidance is a multifaceted behavior that allows the individual to withdraw from hostile situations (Blanchard et al., 2005). It reflects a depressive and anxiety state in the animal (Henriques-Alves and Queiroz et al., 2015), and might be originated from the disrupted motivational processes associated with social interaction or from the activation of the neuronal pathways related with fear and responses to social stimulus (Steimer, 2011; Toth et al., 2012; Toth and Neumann, 2013). As we have previously mentioned, resident mice of these two strains showed higher aggressive response, therefore the submissive behaviors are presented proportionally.

As we commented above, the experience of being exposed to different agonistic experiences modulates the patterns of the aggressive behavior differently depending on the genetics of each strain. While the OF1 and B6.129X1 strains reduced the time spent in aggressive behaviors, the C57BL/6 strain of mice decreased the latencies to perform agonistic behaviors (threat and attack faster). Several studies have reported the elevate aggressiveness of the outbred strain OF1 (Montagud-Romero et al., 2015; 2016; García-Pardo et al., 2015; Ribeiro do Couto et al., 2006; Rodríguez-Arias et al., 1998), but this is the first study showing that the agonistic patterns of the OF1 mice was modulated by the exposure to repeated-social encounters.

The intruder mice of the strains B6.129X1 and OF1 consequently decreased defensive/submissive behavior. Again, the opposite was observed in the C57BL/6 mice, which increased defensive patterns showing them faster. Correlation also confirm the relation between resident and intruder behaviors only for this strain. In line with these results, it has been shown that a prolonged exposure to chronic social defeat stress led to increased immobility behavior and pronounced anxiety in the C57BL6/J (Kudryavtseva, 2002; Kudryavtseva, 2006) with a long-lasting reduction in social interaction (Berton et al., 2006 and Tsankova et al., 2006).

Together, these findings suggest that the experience of suffering repeated episodes of social stress would modulate the social dominance and the defensive behaviors depending on the genetic of the subjects. Different mice strains would show distinct behavioral patterns genetically determined and the experience could change them. Therefore, our results found that although aggressive behavior can be learned, the consequences are not equal in all animals. While, for some strains, experience reduced aggression, in others, it induced the opposite effect. The impact of social stress in humans is a risk factor for both mental and physical diseases and most findings highlight the beneficial impact of social support (House, 2001; Lederbogen et al., 2013). Stress research, neuroscience and epidemiology have substantially contributed to elucidating the role of social stress as a risk factor for mental disorders. As we commented above, the resident-intruder paradigm is an important procedure used in animal studies because of its ethological validity in mice that closely mimics real life situations (Tornatzky and Miczek, 1993), and it allows to study the neurobiology of stress. Therefore, it is important for the researchers who work on stress using preclinical models to know the sensitivity of the different strains to this stress, and how the response can change throughout repeated episodes. This would clarify the findings of these studies and would reduce many discrepancies that currently can be observed.

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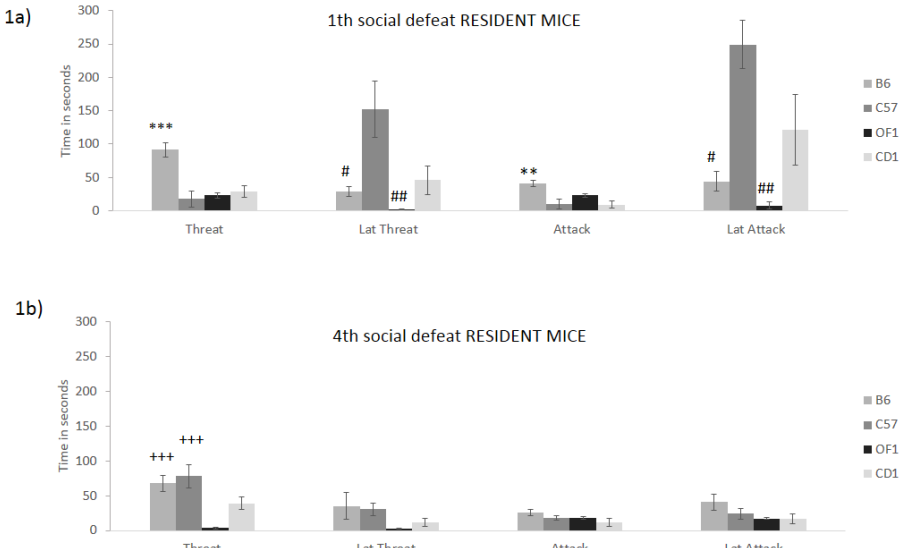

Fig 1. Behavior of resident mice during **a)** the 1st and **b)** the 4th agonistic encounters with their respective intruder mice. The bars represent the time (in seconds) that mice spent engaging in different behavioral categories (threat, attack, latency threat and latency to attack).

***p<0.001, **p<0.01, significant difference with respect the other strains.

##p<0.01, #p<0.05, significant difference with respect the C57BL6/J strain.

+++ p<0.001, significant difference with respect the OF1 strain.

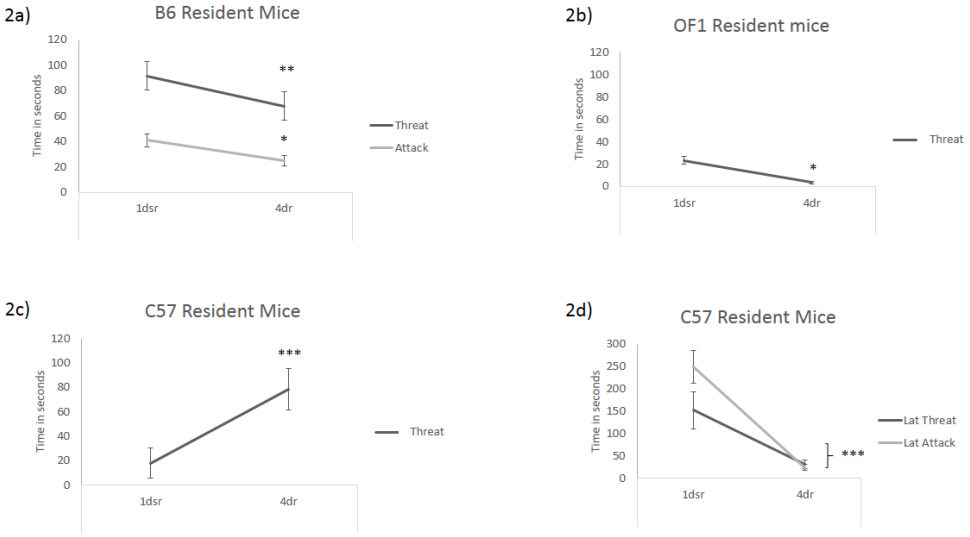

Fig 2. Behavioral changing patterns of resident mice between the 1st and the 4th agonistic encounters in adult mice. The bars represent the time (in seconds) that mice spent engaging in different behavioral categories (threat, attack, latency threat and latency to attack).

***p<0.001, **p<0.01, p< p<0.001 significant difference with respect the 4th social defeat encounter.

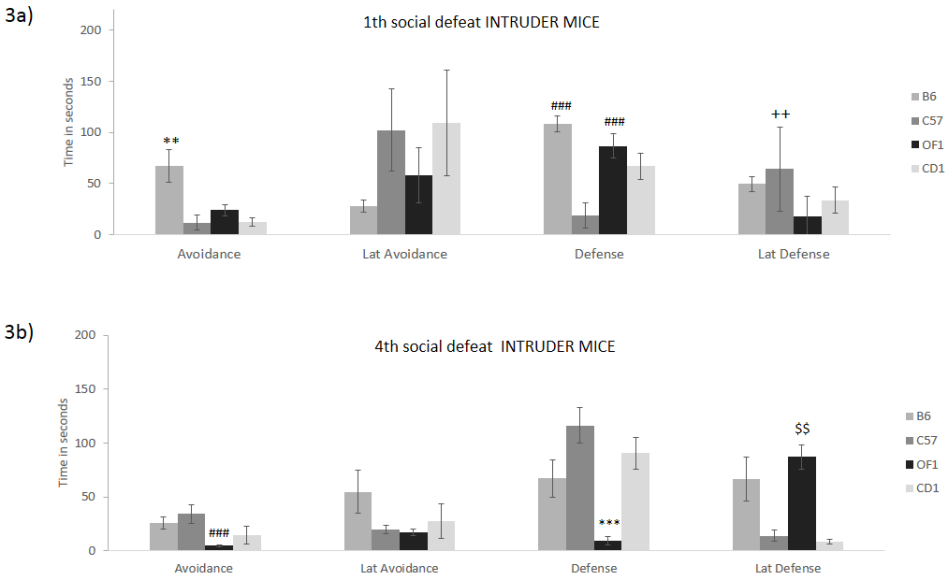

Fig 3. Behavior of intruder mice during **a)** the 1st and **b)** the 4th agonistic encounters with their respective resident/aggressive mice. The bars represent the time (in seconds) mice spent engaging in different behavioral categories (avoidance, defense, latency to avoidance and latency to defense).

***p<0.001, **p<0.01, significant difference with respect the other strains.

###p<0.001, significant difference with respect the C57BL6/J strain.

++ p<0.01, significant difference with respect the OF1 strain.

$$ p<0.01, significant difference with respect the CD1 and C57BL6/J strain.

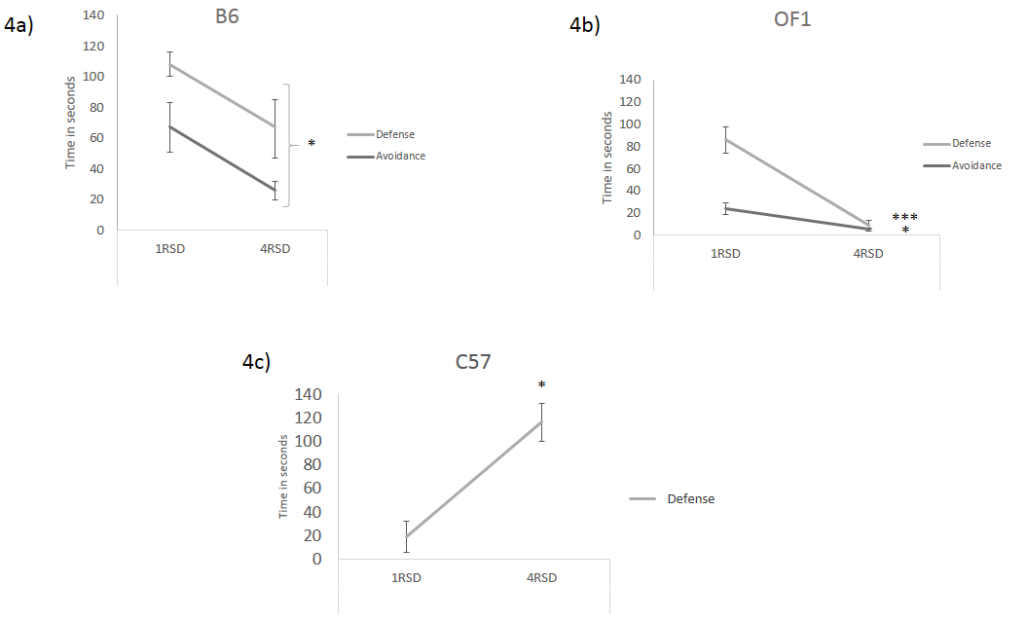

Fig 4. Behavioral changing patterns of intruder mice between the 1st and the 4th agonistic encounters in adult mice. The bars represent the time (in seconds) mice spent engaging in different behavioral categories (defense).

***p<0.001, *p< p<0.001, significant difference with respect the 4th social defeat encounter.

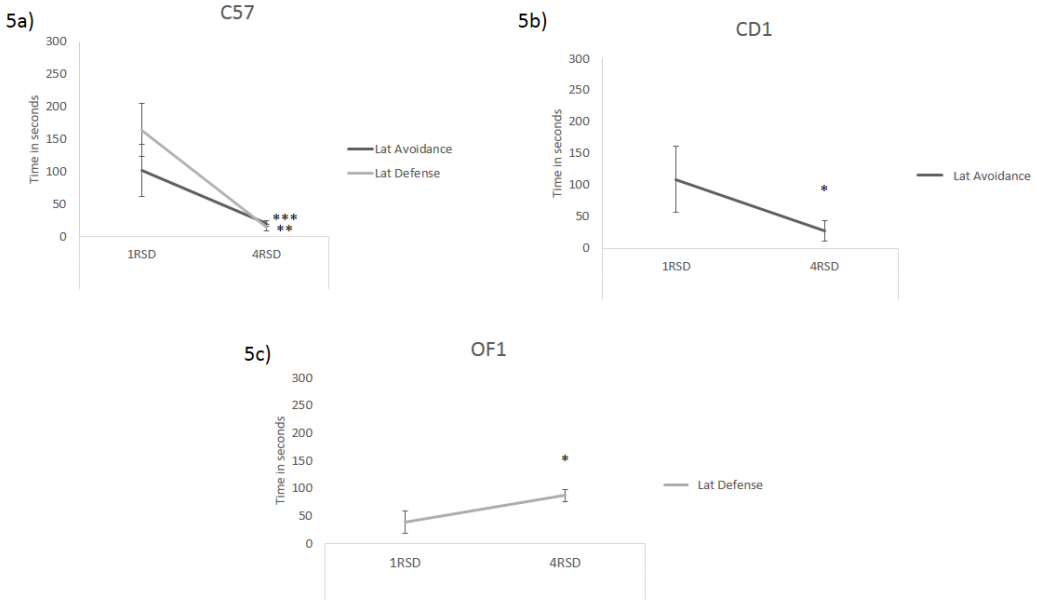

Fig 5. Behavioral changing patterns of intruder mice between the 1st and the 4th agonistic encounters in adult mice. The bars represent the time (in seconds) mice spent engaging in different behavioral categories (latency to avoidance and latency to defense).

***p<0.001, **p<0.01, *p<0.001, significant difference with respect the 4th social defeat encounter.

STUDY 7

Episodic Social Stress-Escalated Cocaine Self-Administration: Role of Phasic and Tonic Corticotropin Releasing Factor in the Anterior and Posterior Ventral Tegmental Area

E.N. Holly, C.O. Boyson, **S.Montagud-Romero**, D.J. Stein, K.L. Gobrogge, J.F. DeBold, K.A. Miczek

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The paper "Episodic Social Stress-Escalated Cocaine Self-Administration: Role of Phasic and Tonic Corticotropin Releasing Factor in the Anterior and Posterior Ventral Tegmental Area" explores how CRF is released and interacts with its receptors in specific regions of the VTA. This action is studied both during and after stress, to fuel later escalated cocaine taking and seeking behavior. Understanding these acute and persistent changes to the VTA CRF system may lead to better therapeutic interventions for addiction. This work has been divided in several experiments. First, it was explored the nature of phasic and tonic CRF increases in the VTA during acute and repeated stress (Experiment 1). Next, we investigated whether CRF actions on its receptors in the anterior VTA and posterior VTA during stress are necessary for subsequent escalated cocaine self-administration (Experiment 2). Finally, we investigated whether the effects of repeated stress on VTA CRF and its receptors persist long after social defeat and affect later cocaine seeking after forced abstinence, which has been proposed as a translational model of relapse (Reichel and Bevins, 2009)(Experiment 3). Within this last experiment, I was involved in the study of intra-VTA antagonism of CRF receptors during cocaine seeking.

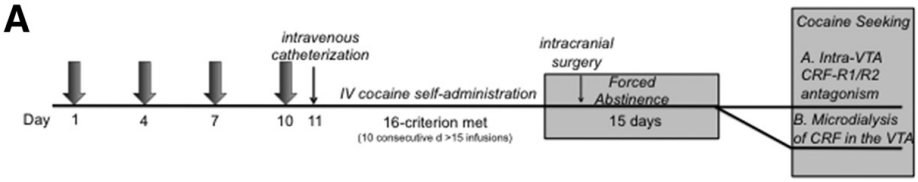
Male Long-Evans rats underwent intermittent social defeat stress or handling (control groups) on days 1, 4, 7 and 10, and then underwent cocaine self-administration followed by 15 d forced abstinence. Rats were returned to the cocaine self-administration chamber and responses were recorded on the lever previously paired with cocaine reinforcement. Upon reintroduction to the cocaine self-administration chamber, rats were microinjected in their temporary housing room with vehicle (aCSF), CRF-R1 antagonist (CP376395, 500 ng/side), or CRF-R2 antagonist (Astressin2B, 1000 ng/side) in the aVTA or pVTA 20 min before reintroduction to the cocaine self-administration chamber.

Our results showed that all rats acquired and reliably self-administered cocaine at an FR3 schedule of reinforcement and there was no effect of prior stress history on the number of infusions in daily cocaine self-administration sessions. There was also no significant difference between groups on cumulative cocaine earned before forced abstinence. In addition, previously stressed rats were microinjected with vehicle and demonstrated augmented cocaine seeking after forced abstinence, which was prevented in previously stressed rats treated with antagonists of CRF-R1 in the pVTA and CRF-R2 in the aVTA before the return to the cocaine self-administration chamber. Three-way ANOVA revealed a significant interaction of VTA subregion X drug ($F(2,38) = 6.479$, $p = 0.004$), as well as stress group X drug ($F(2,38) = 5.910$, $p = 0.006$), meaning that the effect each CRF receptor antagonist or vehicle in the VTA is dependent on both which VTA subregion it is delivered in and whether the animals were previously stressed.

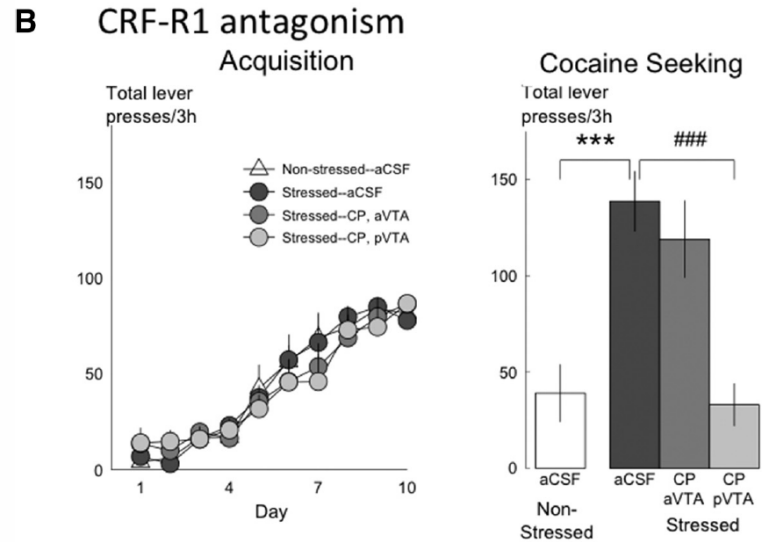
Previous intermittent social defeat stress resulted in significantly more lever pressing upon return to the cocaine self-administration chamber compared with non stressed controls. Previously stressed rats microinjected with aCSF pressed the lever previously associated with cocaine significantly more than their last day of cocaine self-administration (one-way repeated-measures ANOVA $F(1,17) = 10.549$, $p=0.012$), an effect not seen in aCSF-treated rats with no stress history. The stressed aCSF group also exhibited significantly more cocaine seeking compared with the non stressed aCSF group ($p =0.001$).

Although CRF actions on VTA CRF-Rs during repeated stress contribute to the development of later maladaptive cocaine self-administration, the current findings also demonstrate that VTA CRF continues to play a significant role in cocaine seeking after stress. Prior stress increased cocaine seeking >1 month after the last defeat. Because most humans undergo abstinence—either voluntary or forced—instead of extinction (Katz and Higgins, 2003), the current study attempted to translate the human condition to rats through reexposure to cocaine self-administration chambers after forced abstinence.

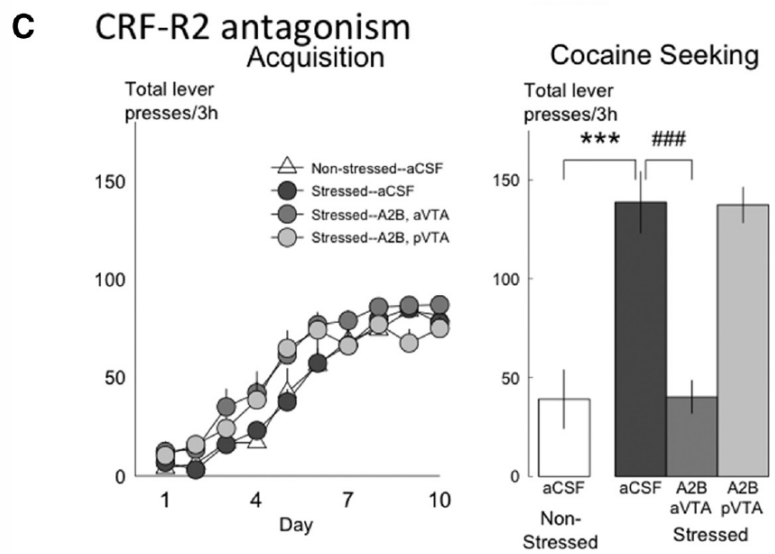
Antagonism of either pVTA CRF-R1 or aVTA CRF-R2 after stress-induced neuroadaptations had already occurred prevented augmented cocaine seeking after forced abstinence in previously stressed rats.



A. Experimetnal timeline. Rats underwent intermittent social defeat or handling, after which they were catheterized for cocaine self-administration, where they were allowed to self-administer cocaine (0.75 mg/kg/infusion) in 3 h sessions until they met a criterion of 10 consecutive days of > 15 infusions. Next, rats were placed in forced abstinence for 15 d, after which they were reintroduced to their cocaine self-administration chambers and assessed for cocaine seeking after intra-VTA CRF-R1 or CRF-R2 antagonism.



B. After intermittent social defeat or handling, there was no difference in acquisition (left) between the previously nonstressed rats treated with aCSF in the aVTA + pVTA (n=7) and previously stressed rats microinjected with aCSF in the aVTA + pVTA (n = 9) or CRF-R1 antagonist (CP) in the aVTA (n = 4) or pVTA (n = 4). Upon return to the cocaine self-administration chamber (right), stressed rats microinjected with aCSF pressed the previously cocaine-paired lever significantly more than nonstressed aCSF animals, which was prevented by intra-pVTA, but not intra-aVTA, CRF-R1 antagonism.



C. Similarly, there were no differences in acquisition (left) between rats before abstinence in rats treated with CRF-R2 antagonist (A2B) before the return to the cocaine selfadministration chamber (right). Intra-aVTA (*n*=5), but not intra-pVTA (*n*=4), CRF-R2 antagonism prevented the increased lever responding in previously stressed rats.

STUDY 8

Maladaptive choices by defeated rats: link between rapid approach to social threat and escalated cocaine self-administration

C.O. Boyson, E.N. Holly, A.R. Burke, **S. Montagud-Romero**, J.F. DeBold, K.A. Miczek

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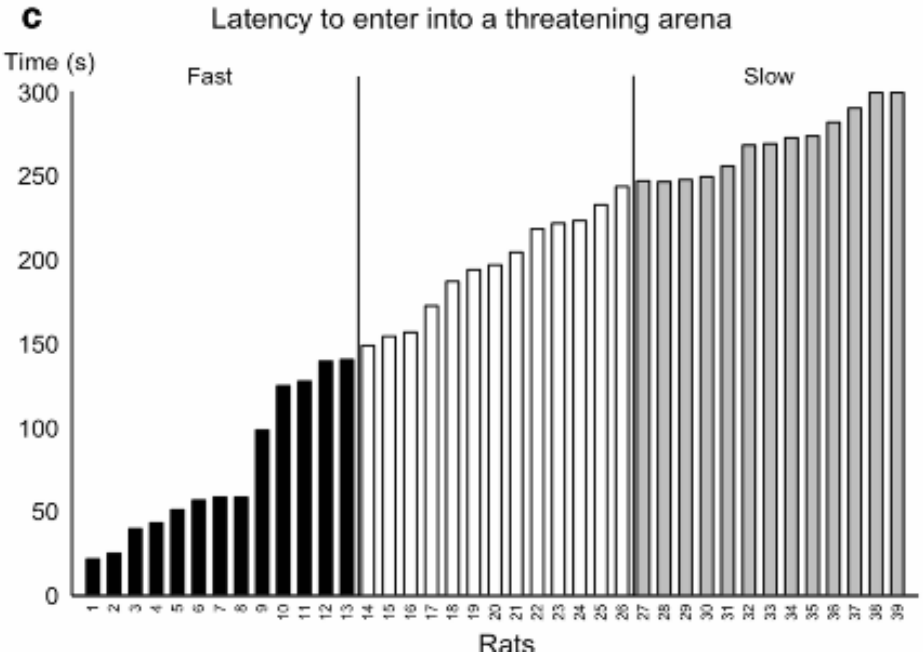
The paper titled "Maladaptive choices by defeated rats: link between rapid approach to social threat and escalated cocaine self-administration" was designed with the aim of dissected the discrete behavioral phases of social defeat and analyzed which behavioral characteristics may be predictive of subsequent cocaine self-administration. This work was carried out in Tuft University, in the Psychology Department. It developed different experiments to get the objective, but during my stay I was involved in the experiment number 5 "corticosterone measurement" which aim was to determine whether individual differences in latency to enter the threat zone could affect the physiological stress response.

Upon arrival, Male Long-Evans rats were randomly assigned to two groups: defeat stressed or non-defeated control. Stressed rats were subjected to nine intermittent social defeat episodes over 21 days in a modified version of our previously described procedure (Covington and Miczek 2001), while non-defeated controls were briefly handled. Defeats occurred in a modified resident home cage consisting of two compartments (the resident's home compartment or "fight zone" and a neutral compartment or "threat zone" with clean bedding) connected by a porthole, with a second porthole allowing access to the experimental rat's home cage ("safe zone"). During each defeat, the experimental intruder's home cage was moved adjacent to the resident cage, the porthole opened, and the rat allowed up to 5 min to voluntarily move to the threat zone. After 5 min in the threat zone, the intruder was placed into the fight zone with the resident with the escape porthole closed until it showed 8 s continuous supine posture, received ten attack bites, or 5 min had elapsed. A screen was then used to separate the aggressive resident from the intruder and the escape porthole opened.

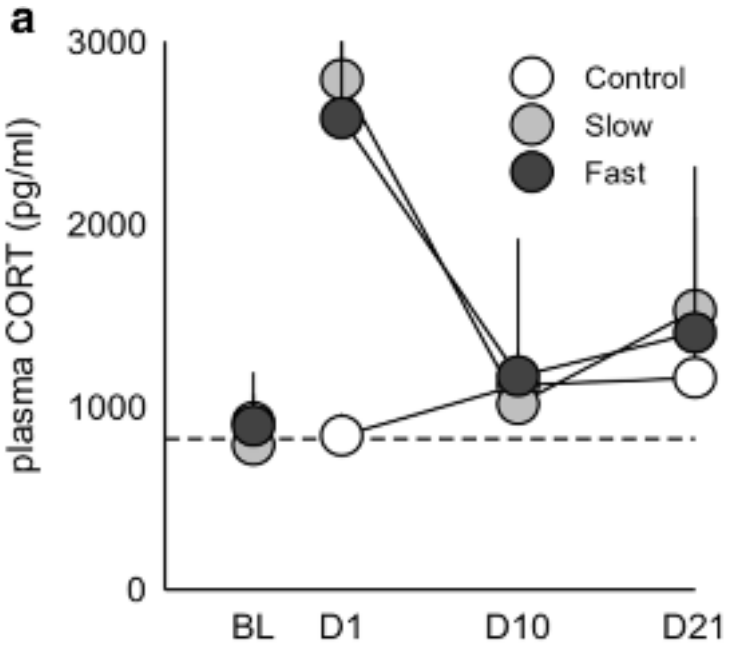
Plasma corticosterone was measured at baseline and 20 min following social defeat on days 1, 10, and 21. The results showed that corticosterone social stress resulted in a group x day interaction in plasma corticosterone 20 min after defeat ($F_{3, 94} = 5.93$, $p < 0.001$). This difference appears to be a

result of increased corticosterone in stressed rats limited to Day 1. Fast and slow rats did not differ in the corticosterone response to stress.

We have demonstrated that fast and slow rats do not differ in physiological measures of responsivity to social defeat stress, indicating that fast and slow rats may generally experience the same level of stress. This is significant because it precludes the possibility that fast rats self-administer more cocaine due to an increased physiological stress response and points to the important role of other factors, such as decision-making or altered cocaine responsivity.



Mean latency to enter the threat zone across nine social defeat encounters was a continuous distribution, and with the upper 1/3 (n = 13) of the distribution considered slow and the lower 1/3 (n = 13) of the distribution considered fast. Data shown are mean ± SEM.



Latency to enter the threat zone was not associated with altered physiological responses to stress. Plasma corticosterone (CORT) did not differ between groups at baseline (BL), and while there was a significant effect of stress on the first day of defeat (D1), there was no difference between slow (n = 6, gray) and fast (n = 6, black) groups (control n = 16, white). There was no effect of stress on plasma corticosterone on day 10 or 21.

4. GENERAL CONCLUSIONS

The main aim of this work was to study the influence of social stress on the rewarding effects of cocaine. As a model of social stress, we have chosen the social defeat paradigm as a naturalistic model of stress that involves an agonistic encounter between conspecifics. The social defeat paradigm closely mimics real life situations and represents a stressor of ecological and ethological validity in mice (Tornatzky and Miczek, 1993). This social stressor induces robust physiological, behavioral and endocrine responses, including circadian rhythm disturbances, avoidance behavior and elevated levels of corticosterone (Lumley et al., 2000; Meerlo et al., 2002). Under these circumstances, animals develop social dominance based on hierarchies constructed as a consequence of their agonistic interactions (Huntingford and Turner, 1987). Using this procedure we have produced two different kinds of social stress: ASD and RSD. This has allowed us to discriminate between immediate and long-lasting effects of stress. The rewarding effects of cocaine have been evaluated mainly using the CPP procedure, which evaluates the importance of conditioned cues associated with the drug (Tzschentke, 2007). In this model, the primary motivational stimulus (drug) properties are used as an unconditioned stimulus (US). Repeated exposure to a US with a set of environmental cues (initially neutral) makes these contextual cues acquire secondary motivational properties, which constitutes a conditioned stimulus (CS) (Aguilar et al., 2009). When the animal is exposed again to the context in which the substance was given, it spends more time in the associated compartment (if the stimulus is appetitive) or away from it (if the stimulus is aversive). In animal models, the association between stress and psychostimulant addiction has been evaluated mainly through the SA paradigm. The SA is an experimental model that evaluates the primary reinforcing effect of drugs according to the effort made by the animal to obtain the drug. In this context, the animal obtains the drug (reinforcement) by performing an operant response, such as pressing a lever. The effects of social defeat on cocaine self-administration have been extensively studied in adult rats (Tidey and Miczek, 1997; Covington and Miczek, 2005; Covington et al., 2005; Miczek et al., 2011; Boyson et al., 2014), but few studies have evaluated the effect of social defeat on adolescent animals. SA together

with the CPP paradigm represent the addictive process in animal models, measuring both the motivation role and influence of the environmental cues.

Due to the particular neurobiological and behavioral characteristics of adolescence, we have studied the effect of social defeat in both adult and adolescent mice, in which it is considered a model of bullying (Björkqvist, 2001; Koolhas et al., 2013). With this design we have tried to clarify how the rewarding effects of cocaine are affected by social defeat stress depending on the age at which exposure to stress takes place.

We have observed that the effects of social defeat stress on the CPP induced by cocaine vary depending on the social stress procedure used (ASD or RSD) and the age of the animals when they experience this stress (***studies 1, 2, 3 and 5***). Acute social defeat undermines the rewarding effects of cocaine when administered during adolescence. Defeated adolescent mice do not develop CPP with 1 mg/kg of cocaine, while control non-stressed mice do. One mg/kg of cocaine is considered a subthreshold dose, as it is ineffective in inducing CPP in adult mice (Vidal-Infer et al., 2012; Arenas et al., 2014; Montagud-Romero et al., 2014), although it is effective in adolescent subjects, which are more sensitive to drugs of abuse (Schramm-Sapyta et al., 2009). Comparable results were observed with 25 mg/kg of cocaine; although control and stressed adolescent mice that developed CPP showed a reinstated preference with a priming dose of 12.5 mg/kg of cocaine, this preference was extinguished faster in those exposed to ASD than in controls. Therefore, the ability of cocaine to establish conditioned memories is diminished in acutely defeated adolescent animals. On the contrary, RSD induced an increase in the rewarding effects of cocaine regardless of the age at which exposure to the stress occurs. After ASD or RSD, adult mice developed CPP with 1 mg/kg of cocaine, and required more sessions than non-stressed animals for the CPP induced by an effective dose of cocaine to be extinguished. RSD experience during adolescence or adulthood induced a lasting increase in the conditioned rewarding effect of cocaine. Although it is well known that RSD also increases cocaine SA in adult animals, in study 2 we found that RSD during adolescence delayed this learning. One possible

explanation is that RSD increased the sensitivity of the mice to the rewarding effects of cocaine. The subthreshold dose of cocaine administered in the CPP may have been experienced more intensely by defeated mice. Consequently, these mice would have acquired CPP with a non-effective dose. However, as an effective dose was used in the self-administration procedure (0.5mg per injection), defeated mice would have experienced a stronger subjective effect, which would have induced them to self-administer this cocaine dose at a lower rate. Further studies using subthreshold cocaine doses may be of interest to clarify this hypothesis.

We should take into consideration that the lack of effects we have observed in adolescent defeated mice could have been related with a less intense agonistic encounter. Adolescent mice showed lower increases in corticosterone levels after social defeat than defeated adults. It is possible that, when exposed to such encounters, adolescent mice experienced them as play-fighting rather than social defeat. Despite this less intense defeat experience, RSD induced the same increase in cocaine reward than that observed in adult defeated mice, highlighting adolescence as a period of high sensitivity to social stressful events.

However, despite substantial evidence linking stress and compulsive drug- taking and -seeking, not all individuals exposed to high levels of stress engage in drug use. A possible underlying basis for this variability in drug taking are individual differences in behavior during social stress. Social defeat encompasses a myriad of behaviors, such as approach to a threatening situation, reaction to a fight encounter, escape behavior, and return to a safe environment (Koolhaas et al. 2010). In ***study 8***, we observed that the latency to enter the threatening environment (the first phase of social confrontation) was highly predictive of later cocaine self-administration during the 24-h binge. However, this behavior was not associated with physiological measures of stress, including plasma corticosterone and CRF in the extended amygdala, which indicate that both fast and slow rats generally experience the same level of stress. Nevertheless, consistent with previous works (Bergstrom et al., 2008; Taliaz et al., 2011; Duclot and Kabbaj, 2013), latency to enter the

threatening environment altered BDNF in the hippocampus.

Behavior may also be regulated by genetics and by experience of different social-environmental situations. We have demonstrated (***study 6***) that different strains of mice showed different behavioral patterns when exposed to an agonistic situation. Although genetics determine behavior during the first social encounter, the experience of repeated defeat may differentially condition the behavior observed. During the first encounter, the B6.129X1 strain was the most aggressive, while OF1 animals were faster in showing aggression. However, experience of RSD reduced the time that both strains spent engaged in aggression. The opposite response was observed in the C57BL/6 strain of mice, which showed increased aggression after repeated agonistic encounters. Intruder mice responded accordingly to the resident's level of aggression, with the more aggressive strains showing higher levels of submissive and avoidance behaviors. Therefore, we conclude that genetics determine the basal level of aggression, but that thus behaviour is also modulated by experience.

To sum up the first part of our work, we have expanded the knowledge about the effects of social defeat on the subsequent effects of cocaine. The age of the animal when social defeat is suffered determines the effects observed after ASD stress. However, independently of whether stress is suffered during adolescence or adulthood, RSD increases the rewarding effects of cocaine. These effects can be modulated by genetics, which modify the response to defeat and/or influence personality traits such as impulsivity.

In the second part of this work, we attempted to elucidate some of the mechanisms involved in the aforementioned effects. Our first candidate was CRF and its role in mediating the response to social stress (***study 7***). We next performed a pharmacological and biochemical study of the role of DA neurotransmission (***study 3 and 5***). We also made a first approach (there are almost no previous studies in this field) to explore the role of

neuroinflammation in social defeat effects and its interaction with cocaine (***study 2)***. Finally, we evaluated the principal epigenetic changes induced by RSD stress (***study 4)***.

The key role played by CRF in the effects induced by social stress is well defined (Wang et al., 2005; Boyson et al., 2011). Recently, CRF within the VTA has emerged as a likely candidate molecule underlying the fundamental link between a history of stress and escalated drug self-administration (Boyson et al., 2014). Our work, besides corroborating the implication of the CRF-R1 and 2 in cocaine self-administration, suggested dynamic, shifting roles of CRF and its receptors within VTA subregions, during and after stress, which promoted later escalated cocaine taking and seeking. Acute stress promotes a phasic increase in CRF within the posterior VTA (pVTA), whereas repeated stress recruited a phasic CRF response in the anterior VTA (aVTA) and elevated CRF tone throughout the VTA. CRF acts on CRF-R1 in the pVTA and CRF-R2 in the aVTA during RSD stress to cause increased cocaine self-administration. CRF tone may remain elevated in the VTA, thus contributing to cocaine-seeking after forced abstinence in previously stressed animals through amplified actions on pVTA CRF-R1 and aVTA CRF-R2. CRF-R1 within the pVTA seems to be more relevant for the initial response to stress, whereas CRF-R2 within the aVTA may be recruited with repeated exposure to stress (Holly et al., 2015).

Numerous studies have found that social defeat stress not only alters CRF, but also DA neurotransmission (Tidey and Miczek, 1996; Cabib et al., 2000; Isovich et al., 2001; Razzoli et al., 2011; Shimamoto et al., 2015). Classically, social stressors have been related with increases in extracellular DA release in the shell of the NAcc (Tidey and Miczek, 1997; Piazza and LeMoal, 1998; Han et al., 2015; Holly et al., 2015). In relation to this, the results we obtained in ***study 3*** demonstrated that both DA receptors (DR1 and DR2) were involved in the long-lasting effect of social stress on the rewarding effects of cocaine. While the increased sensitivity to acquire cocaine-induced CPP was fully impeded by blocking D1R or D2R before each social defeat, increased vulnerability to the reinstatement of cocaine-seeking was only diminished after blockade of D1R. Brain DA receptor levels were also altered in response

to social defeat, which induced an immediate increase of D2R levels in the cortex that was sustained in the hippocampus 3 weeks after the last social defeat. However, D1R levels were only slightly modified, with a momentary decrease in the hippocampus only after the last social defeat.

These results confirm the critical role of DA neurotransmission in the effects of social defeat and encouraged us to continue investigating the alterations that social defeat produce in the DA system. To do this, we studied the principal transcription factors that modulate DA gene expression (***study 5***). The transcription factor Nurr1, together with its potentiator Pitx3, regulates proteins that are crucial for DA metabolism, such as DAT and D2DR, among others (Jacobs et al., 2009; Reddy et al., 2012; Bissonette and Roesch, 2015). Although RSD induced an increase in the rewarding effects of cocaine in both adult and adolescent mice, as we have previously reported, only defeated adolescent mice exhibited decreased levels of the transcription factors Pitx3 and Nurr1 in the VTA. Therefore, although the behavioral consequences of social defeat may be comparable in adolescent and adult mice (increased sensitivity to cocaine CPP), there was a more pronounced alteration in the genetic control of DA markers among defeated adolescent animals.

Social defeat stress leads to the heightened phasic firing of VTA dopaminergic neurons projecting to the NAcc, resulting in the activity-dependent release of BDNF and the activation of BDNF signaling in the NAcc (for revision see Krishnan, 2014). Specifically, BDNF in the BLA is implicated in fear conditioning (Rattiner et al., 2004) and regulates the consolidation of defeat-related memories (Dulka et al., 2016). In agreement with this, we found that defeated mice showed an increased expression of BDNF in the DG and BLA, regardless of their age. Among the pathways that affect BDNF gene expression when activated are ERK and CREB. ERK phosphorylate CREB and active (phosphorylated) CREB stimulate the expression of target genes, including BDNF (Kandel et al., 2001; Barco et al., 2002; Bramham and Messaoudi, 2005). Depending on the age of the mice when exposed to RSD, the increase in the BDNF was accompanied or not by alterations in pERK. pERK1 and pERK2 expression in the DG increased only in socially defeated

adolescent mice. However, increased expression of pCREB in the DG was observed in socially defeated adult and adolescent mice. Socially defeated mice showed an increased expression of BDNF in the BLA, but no changes in pCREB or ERKs.

To summarise, *studies 3* and *5* confirm and extend the role of DA neurotransmission in the effects of RSD on cocaine reward. Both DA receptors are involved, although D1R seems to play a more important role. Transcription factors regulating DA gene expression are differentially altered by social defeat depending on the age at which exposure takes place, thereby highlighting adolescence as a sensitive period. Modifications of DA neurotransmission may modulate the increased expression in BDNF via ERK/CREB or other pathways, which in turn would mediate neuroplastic changes in brain areas related to reward.

Numerous reports show that proinflammatory signaling in the brain affects mood, cognition, and behavior and is linked with the etiology of psychiatric disorders such as anxiety or depression. It is now known, that stress-induced bidirectional communication pathways between the CNS and peripheral immune system, converge to promote a heightened neuroinflammatory environment. Microglia-dependent neuroinflammatory events promote myeloid cell-trafficking to the brain that reinforces stress-related behavior, and is argued to play a role in stress-related psychiatric disorders. The blood-brain barrier is critical in propagating peripheral-to-central immune communication.

The current literature suggests that psychostimulant drugs of abuse alter the function of the BBB, which is likely to contribute to the neurotoxicity associated with these drugs. Psychostimulants produce BBB dysfunction through alterations in tight junction protein expression and conformation, increased glial activation, increased enzyme activation related to BBB cytoskeleton remodeling, and induction of neuroinflammatory pathways (Fiala et al., 2005, 2008). These detrimental changes lead to increased permeability of the BBB and subsequent vulnerability of the brain

to peripheral toxins (Kousik et al., 2012). Based on the results previously obtained, in ***study 2*** we focused on potential alterations of the BBB caused by social defeat suffered during adolescence, reporting for the first time that mice exposed to RSD undergo significant changes in BBB structure. RSD reduced the expression of the tight junction protein claudin-5 and produced an increase in basal laminina degradation in the NAcc. Consequently, there was an increase in IgG extravasation, indicating that social defeat increases BBB permeability, probably through alterations in structural proteins. This disruption of BBB was not brain region-specific, as similar results were obtained in subfields of the hippocampus, dentate gyrus, CA1 and CA3. Although the mechanisms underlying these effects have not been evaluated in our study, we believe that the effect of social defeat on BBB may be produced by an increase in the activation of MMPs (the matrix metalloproteinases). Release of proinflammatory cytokines, particularly IL-1β, may be implicated in augmented MMP activity (Gottschall and Deb, 1996; Vecil et al., 2000). Current studies in our laboratory continue this line of research; for instance, we are exploring the role of anti-inflammatory drugs in preventing the effects of social defeat.

Finally, in ***study 4*** we tackled a general mechanism that underlies RSD effects; i.e. epigenetic modification. Changes in chromatin remodeling form part of many physiological and pathological processes, including carcinogenesis, brain development, synaptic plasticity and addiction (Levenson and Sweatt, 2005). Evidence reported over the last decade has revealed that, when exposed to social defeat experiences, the brain undergoes remodeling and functional modifications, which leads in turn to behavioral changes (for review see Hammels et al., 2015). Using the RSD paradigm, we showed for the first time that the epigenetic changes induced by social stress are associated with an increase in the rewarding and reinstating effects of a threshold dose of cocaine in the CPP paradigm. We described how up-regulation of histone acetylation H4(K12) was accompanied by an increase in HAT activity in the hippocampus three weeks after the last social encounter. Moreover, the increase in the conditioned rewarding effects of cocaine was blocked by

administration of the HAT inhibitor (curcumin) prior to each social defeat. Accordingly, administration of the HDAC inhibitor (valproic acid) before each SD potentiated the long-term effects of social stress. These results suggest that blockade of acetylation by inhibition of HAT impedes the behavioral effects of social defeat.

To sum up, the results obtained in *study 2* and *4* throw new light on the mechanisms of response to stress and highlight novel therapeutic targets that work against the development of stress-related psychiatric disorders.

The present work offers a wide picture of the short and long-term effects of social defeat on the motivational properties of cocaine, making an important contribution to current knowledge about the effects of stress on drug addiction and behavior. Furthermore, we have described new pharmacological targets that could modify the effects of social stress on the rewarding effects of cocaine. In future years, we expect our basic research to be translated to develop better prevention and treatment strategies to reduce the negative influence of stress on addictive disorders.

5. REFERENCES

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6. ANNEXES

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Neurobiology of Disease

Episodic Social Stress-Escalated Cocaine Self-Administration: Role of Phasic and Tonic Corticotropin Releasing Factor in the Anterior and Posterior Ventral Tegmental Area

Elizabeth N. Holly,[1,2] Christopher O. Boyson,[1] Sandra Montagud-Romero,[3] Dirson J. Stein,[4] Kyle L. Gobrogge,[1] Joseph F. DeBold,[1] and Klaus A. Miczek[1,5]

[1]Department of Psychology, Tufts University, Medford, Massachusetts 02155, [2]McGovern Institute for Brain Research and Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, [3]Unidad de Investigación Psicobiología de las Drogodependencias, Departamento de Psicobiología, Facultad de Psicología, Universitat de València, València, Spain 46010, [4]Programa de Pós-Graduação em Ciências Médicas, Psiquiatria, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil 90050, and [5]Departments of Neuroscience and Pharmacology, Tufts University School of Medicine, Boston, Massachusetts 02111

Intermittent social defeat stress escalates later cocaine self-administration. Reward and stress both activate ventral tegmental area (VTA) dopamine neurons, increasing downstream extracellular dopamine concentration in the medial prefrontal cortex and nucleus accumbens. The stress neuropeptide corticotropin releasing factor (CRF) and its receptors (CRF-R1, CRF-R2) are located in the VTA and influence dopaminergic activity. These experiments explore how CRF release and the activation of its receptors within the VTA both during and after stress influence later cocaine self-administration in rats. *In vivo* microdialysis of CRF in the VTA demonstrated that CRF is phasically released in the posterior VTA (pVTA) during acute defeat, but, with repeated defeat, CRF is recruited into the anterior VTA (aVTA) and CRF tone is increased in both subregions. Intra-VTA antagonism of CRF-R1 in the pVTA and CRF-R2 in the aVTA during each social defeat prevented escalated cocaine self-administration in a 24 h "binge." VTA CRF continues to influence cocaine seeking in stressed animals long after social defeat exposure. Unlike nonstressed controls, previously stressed rats show significant cocaine seeking after 15 d of forced abstinence. Previously stressed rats continue to express elevated CRF tone within the VTA and antagonism of pVTA CRF-R1 or aVTA CRF-R2 reverses cocaine seeking. In conclusion, these experiments demonstrate neuroadaptive changes in tonic and phasic CRF with repeated stress, that CRF release during stress may contribute to later escalated cocaine taking, and that persistently elevated CRF tone in the VTA may drive later cocaine seeking through increased activation of pVTA CRF-R1 and aVTA CRF-R2.

Key words: cocaine seeking; cocaine self-administration; corticotropin releasing factor; social defeat; stress; ventral tegmental area

Significance Statement

Corticotropin releasing factor (CRF) within the ventral tegmental area (VTA) has emerged as a likely candidate molecule underlying the fundamental link between stress history and escalated drug self-administration. However, the nature of CRF release in the VTA during acute and repeated stress, as well as its role in enduring neuroadaptations driving later drug taking and seeking, are poorly understood. These experiments explore how CRF is released and interacts with its receptors in specific regions of the VTA both during and after stress to fuel later escalated cocaine taking and seeking behavior. Understanding these acute and persistent changes to the VTA CRF system may lead to better therapeutic interventions for addiction.

Introduction

A history of stress has long been associated with enhanced susceptibility to the initiation, escalation, and relapse of drug use in both preclinical and clinical populations (Erb et al., 1996; Sinha, 2008). In particular, intermittent social defeat stress in rats engenders long lasting neuroadaptations, resulting in behavioral and dopaminergic cross-sensitization to cocaine, accelerated ac-

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quisition of cocaine self-administration, increased breakpoints on a progressive ratio schedule of reinforcement, and escalated cocaine self-administration in a 24 h "binge" (Miczek and Mutschler, 1996; Tidey and Miczek, 1997; Covington and Miczek, 2001; Holly et al., 2012; Boyson et al., 2014). Understanding how stress initiates a cascade of neural processes in stress and cocaine sensitive systems, as well as the enduring neuroadaptations, may lead to improved targets for therapeutic intervention. It is particularly significant to investigate a neural point of intersection whereby stress and reward mechanisms may interact to potentiate later behavior.

The ventral tegmental area (VTA) notably stands out due to its prominent role in reward-related behavior. However, in addition to its integral role in reward processing, the VTA is also highly responsive to stress/aversion. Dopamine neurons within the VTA are heterogeneous in cytoarchitecture, electrophysiological characteristics, dopaminergic content, and afferent/efferent connectivity (Ikemoto, 2007; Lammel et al., 2014; Holly and Miczek, 2016) and morphological heterogeneity may extend to function as well. Of particular functional importance is heterogeneity between VTA subregions along the anterior/posterior axis. Drugs of abuse are self-administered into the posterior (pVTA), but not anterior (aVTA), portions of the VTA (for review, see Ikemoto, 2007). Rostrocaudal distinctions in VTA function also extend to aversion. A discrete population of dopamine neurons in the pVTA, but not the aVTA, is rapidly and potently excited by acute foot shock (Brischoux et al., 2009).

One potential mediator of stress-induced changes in VTA dopamine neuron activity is the stress neuropeptide corticotropin releasing factor (CRF). Due to their key function in the initiation of the physiological stress response, CRF and its receptors (CRF-R1 and CRF-R2) may play an important role in the behavioral and neural interactions of stress and reward. Although one primary role of CRF is the initiation of the hypothalamic–pituitary–adrenal axis stress response, CRF is also found in widespread extrahypothalamic regions, including the VTA (Swanson et al., 1983). CRF actions on CRF-Rs within the VTA directly and indirectly influence the mesolimbic dopamine system. CRF rapidly increases VTA dopamine neuron firing rate (Korotkova et al., 2006), which is at least partially dependent on postsynaptic CRF-R1 activation (Wanat et al., 2008). Postsynaptic CRF-R2 activation also enhances VTA dopaminergic neuronal excitability through transient potentiation of NMDA currents and metabotropic glutamate receptor current (Fiorillo and Williams, 1998; Ungless et al., 2003). Together, acute activation of VTA CRF-R1 and CRF-R2 leads to increased dopamine neuronal activity, which may in turn cause long-lasting synaptic neural and behavioral adaptations.

Activation of both CRF-R1 and CRF-R2 in the VTA during stress is necessary for the induction of later behavioral and neural cross-sensitization to cocaine and escalated cocaine self-administration (Boyson et al., 2014). However, the nature of CRF release in the VTA and long-lasting adaptations within the CRF system as a result of repeated stress remain to be determined. As yet, it is unclear whether CRF is phasically released into the VTA during repeated stress, which VTA subregion CRF is exerting its actions, and whether there are neuroadaptive changes in CRF release within the VTA with repeated stress exposure—all of which may be responsible for stress-escalated drug use. Using *in vivo* microdialysis, we first explored the nature of phasic and tonic CRF increases in the VTA during acute and repeated stress (Experiment 1). Next, we investigated whether CRF actions on its receptors in the aVTA and pVTA during stress are necessary for subsequent escalated cocaine self-administration (Experiment 2). Finally, we investigated whether persistent alterations in CRF/CRF-R interactions within the aVTA and pVTA play a role in later escalated cocaine-seeking behavior long after stress exposure (Experiment 3).

Table 1. Group sizes

Experiment	Group	Drug	Target	*n*
1. Microdialysis for CRF in the VTA during stress	Nonstressed	μD	aVTA	5, 5[a]
	Nonstressed	μD	pVTA	5, 5
	Stressed	μD	aVTA	5, 5
	Stressed	μD	pVTA	5, 6
2. Role of VTA CRF during stress on later cocaine taking	Nonstressed	aCSF	aVTA	6
	Nonstressed	aCSF	pVTA	7
	Nonstressed	CP	aVTA	4
	Nonstressed	CP	pVTA	4
	Nonstressed	A2B	aVTA	4
	Nonstressed	A2B	pVTA	9
	Stressed	aCSF	aVTA	4
	Stressed	aCSF	pVTA	7
	Stressed	CP	aVTA	5
	Stressed	CP	pVTA	5
	Stressed	A2B	aVTA	4
	Stressed	A2B	pVTA	5
3. Role of VTA CRF after stress on cocaine seeking after abstinence	Nonstressed	aCSF	aVTA	4
	Nonstressed	aCSF	pVTA	4
	Nonstressed	CP	aVTA	6
	Nonstressed	CP	pVTA	4
	Nonstressed	A2B	aVTA	4
	Nonstressed	A2B	pVTA	4
	Nonstressed	(μD)	aVTA	3
	Nonstressed	(μD)	pVTA	4
	Stressed	aCSF	aVTA	5
	Stressed	aCSF	pVTA	4
	Stressed	CP	aVTA	4
	Stressed	CP	pVTA	4
	Stressed	A2B	aVTA	5
	Stressed	A2B	pVTA	4
	Stressed	(μD)	aVTA	3
	Stressed	(μD)	pVTA	4

Total $N = 151$.

[a]First number represents number included in analysis of day 1; second number represents number included for day 10.

Materials and Methods

General methods and design

Subjects

Male Long–Evans rats ($N = 151$, Experiment 1, $n = 21$; Experiment 2, $n = 64$; Experiment 3, $n = 66$; see Table 1 for group sizes; rats were obtained from Charles River) weighed 225–250 g upon arrival and were individually housed in custom-built acrylic chambers (30 × 20.5 × 24.5 cm) and given food and water *ad libitum*. Rats were allowed to habituate to the vivarium for at least 1 week before surgery or experimental manipulations. Stimulus "resident" rats were housed in male–female pairs in large stainless steel cages (71 × 46 × 46 cm) in a separate room as described previously (Miczek, 1979). All procedures were approved by the Tufts University Institutional Animal Care and Use Committee following the guidelines set forth in the *Guide for Care and Use of Laboratory Animals* (National Research Council, 2011).

Correspondence should be addressed to Elizabeth Holly, PhD, McGovern Institute for Brain Research and Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, 46-2171A, 77 Massachusetts Ave., Cambridge, MA 02139. E-mail: eholly@mit.edu.

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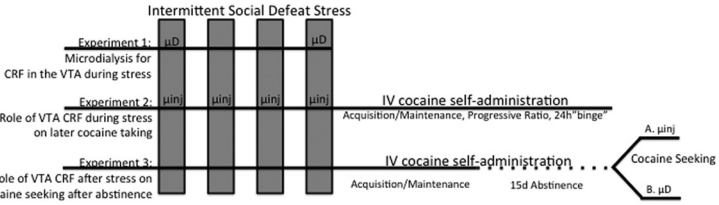

Figure 1. Experimental design. Social defeat (SD, gray bars) occurred on days 1, 4, 7, and 10. In Experiment 1, microdialysis (uD) for CRF within the VTA occurred concurrently with SD on days 1 and 10 to assess the CRF response to acute and repeated stress. In Experiment 2, the functional roles of CRF receptors during stress on later cocaine self-administration were assessed through intra-VTA microinjection (uinj) of aCSF, CRF-R1 antagonist, or CRF-R2 antagonist before each social defeat. In Experiment 3, the enduring role of CRF in the VTA on cocaine seeking after forced abstinence in previously stressed animals was assessed through intra-VTA microinjection of aCSF, CRF-R1, or CRF-R2 antagonist before reinstatement (***A***) or microdialysis for CRF in the VTA (***B***).

Experimental design

The experimental design is shown in Figure 1. Rats underwent intermittent social defeat stress (stressed groups) or handling (control groups) on days 1, 4, 7, and 10. In Experiment 1, rats concurrently underwent *in vivo* microdialysis for extracellular CRF in the VTA during social defeat on days 1 and 10 to examine the CRF response to acute and repeated social defeat stress or handling.

Experiments 2 and 3 examined the impact of these phasic and tonic VTA CRF responses to repeated social defeat stress on subsequent cocaine taking and seeking behavior. In Experiment 2, the role of CRF receptors in the aVTA vs pVTA during social defeat was investigated. Rats were microinjected with vehicle, a CRF-R1 antagonist, or a CRF-R2 antagonist into the aVTA or pVTA before each social defeat or control handling and subsequently catheterized for intravenous cocaine self-administration, culminating in a 24 h binge.

Finally, Experiment 3 evaluated the role of VTA CRF and its receptors long after stress in a translational model of cocaine seeking. Rats underwent intermittent social defeat stress or handling and then underwent cocaine self-administration followed by 15 d forced abstinence. Rats were returned to the cocaine self-administration chamber and responses were recorded on the lever previously paired with cocaine reinforcement. Upon reintroduction to the cocaine self-administration chamber, vehicle, CRF-R1 antagonist, or CRF-R2 antagonist was microinjected into the aVTA or pVTA (Experiment 3A) or extracellular CRF was measured by *in vivo* microdialysis (Experiment 3B).

Social defeat stress

A modified version of a previously described resident–intruder paradigm (Tornatzky and Miczek, 1993; Boyson et al., 2014; Holly et al., 2015) was used for all experiments. Rats in the stressed groups were exposed to 4 brief social defeats separated by ~72 h (days 1, 4, 7, and 10). The female co-resident rat was removed before each defeat, which consisted of three phases. The first phase was instigation, in which the experimental animal ("intruder") was placed in a wire mesh enclosure inside the resident's home cage for 10 min, allowing for visual and olfactory instigation, but preventing tactile contact. The second phase was defeat, in which the protective enclosure was removed and the experimental rat was placed in the resident cage until the experimental rat was held in supine for 10 s, was bitten 10 times, or 5 min had elapsed. For Experiment 1, all defeats were 5 min in duration to account for microdialysis sampling time. Attack latency and number of bites were recorded and no statistical difference in latency, bites, or defeat duration was observed between experiments or drug treatment groups. The third phase was threat, in which the experimental rat was then returned to the protective cage inside the resident's home cage for an additional 10 min, after which it was returned to its home cage.

Intracranial surgery

Rats underwent intracranial surgery under ketamine (100 mg/kg, i.p.) and xylazine (6 mg/kg, i.p.) anesthesia when indicated below in the detailed methods for each experiment. Rats were and implanted with either a unilateral microdialysis guide cannula (8 mm length; Synaptech) in Experiments 1 and 3B or bilateral microinjection cannluae (23 Ga, 11 mm length; PlasticsOne) in Experiments 2 and 3A at a 10° angle 5.0, 5.2, or 5.4 mm posterior from bregma and 1.8 mm lateral from the midline at a depth of 7.5 mm from the skull surface. Rats were allowed to recover for at least 1 week before further manipulation.

Histology

At the termination of experiments, rats were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were removed and placed in 4% paraformaldehyde for at least 24 h, after which they were sliced into 50 μm sections, mounted onto microscope slides, stained with cresyl violet, and coverslipped as described previously (Holly et al., 2012; Holly et al., 2015). Slides were examined under light microscopy for verification of microdialysis probe and microinjection cannula placement. It has been a challenge to define the boundaries of VTA subregions consistently because early characterizations of dopaminergic neurons in the rat VTA (Lindvall and Björklund, 1974; Fallon and Moore, 1978; Swanson, 1982). Although some have defined the aVTA/pVTA more caudally, the emergence of the paranigral nucleus of the VTA beginning around the interpeduncular fossa and nucleus (−5.20 mm from bregma) is a common division in pharmacological, anatomical, and behavioral studies (Ikemoto et al., 1998; Rodd-Henricks et al., 2000; Ikemoto and Wise, 2002; Ikemoto, 2007; Hauser et al., 2014; Sanchez-Catalan et al., 2014; Holly and Miczek, 2016). Therefore, the present studies set the emergence of the interpeduncular fossa and nucleus as the boundary between the aVTA and pVTA.

Experiment 1: Microdialysis during social defeat for CRF in the VTA

Experiment 1 measured CRF in the VTA during acute and repeated social defeat stress. Rats underwent intracranial surgery, after which they were exposed to intermittent social defeat stress on days 1, 4, 7, and 10, with microdialysis performed concurrently on days 1 and 10. Nonstressed controls underwent microdialysis on days 1 and 10, but were transferred to a clean, empty cage and briefly handled in lieu of social defeat. A timeline for Experiment 1 is shown in Figure 2*A* and histology is shown in Figure 3.

In vivo *microdialysis*

Rats underwent intracranial surgery 1 week before the first microdialysis day. *In vivo* microdialysis for CRF in the VTA was performed from the same site on both days 1 and 10 of the social defeat protocol as described above. On the night before sample collection, the microdialysis guide cannula was removed and replaced with a microdialysis probe (2 mm active polyacrylonitrile membrane, 0.36 mm OD, 20 kDa cutoff; Synaptech), which was perfused with artificial CSF (aCSF, 147 mmol/L NaCl, 2.7 mmol/L KCl, 1.2 mmol/L $CaCl_2$, 0.85 mmol/L $MgCl_2$) at a flow rate of 0.5 μl/min overnight. The following morning, the aCSF was replaced

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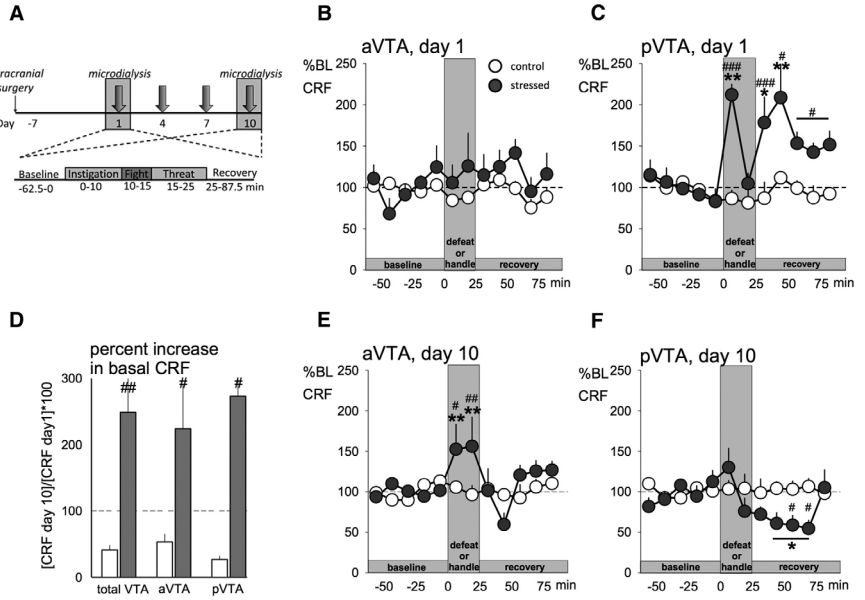

Figure 2. Experiment 1: Microdialysis for CRF in the VTA during acute and repeated social defeat stress. ***A***, Experimental timeline. Top, Microdialysis occurred during social defeat stress on days 1 and 10 of the intermittent social defeat protocol, consisting of defeats (arrows) on days 1, 4, 7, and 10. Bottom, During microdialysis, five baseline samples were collected, followed by social defeat stress (instigation, fight, and threat), and an additional five recovery samples were collected after the termination of defeat. ***B***, During acute defeat, CRF was not significantly changed from baseline in the aVTA during social defeat stress (gray circles, $n = 5$) or control handling (white circles, $n = 5$). ***C***, During acute defeat, CRF was phasically increased in the pVTA both during the initial part of social defeat and in response to stress termination (gray circles, $n = 5$), but was not changed from baseline during control handling (white circles, $n = 5$). ***D***, On day 10, compared with nonstressed controls (white bars, $n = 5$ in aVTA, $n = 5$ in pVTA), stressed rats (gray bars, $n = 5$ in aVTA, $n = 6$ in pVTA) exhibited significantly greater tonic baseline levels of CRF in the VTA, measured in the five baseline samples before defeat stress, expressed as a percentage of average baseline CRF concentration on day 1 compared with day 10. ***E***, On day 10, CRF was increased in the aVTA during the last social defeat episode (gray circles, $n = 5$), whereas it remained unchanged during control handling (white circles, $n = 5$). ***F***, Within the pVTA, CRF is suppressed during the final social defeat (black circles, $n = 6$), but unchanged during control handling (white circles, $n = 5$). $^{*}p < 0.05$, $^{**}p < 0.01$ versus baseline. $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ versus nonstressed controls.

with aCSF containing 0.2% bovine serum albumin (BSA) and the flow rate increased to 2.0 μl/min 2 h before sample collection. Samples were collected by hand into Eppendorf Protein LoBind tubes every 12.5 min and immediately stored at −80°C. Tonic levels of CRF were measured in five baseline samples before experimental manipulations. Experimental rats were then transferred to a resident rat's home cage for social defeat as described above, with samples collected ongoing throughout defeat. After the rat was removed from the threat phase, five additional samples were collected to evaluate the time course of CRF changes during "recovery." On days 4 and 7, social defeats occurred in an identical manner without microdialysis. Nonstressed controls underwent a similar procedure, but were placed in a clean, empty cage for the 10 min instigation and threat periods and briefly handled in lieu of social defeat.

Microdialysis probe recovery

Recovery of microdialysis probes was determined *in vitro* at the end of the experiment. After removal from the rat brain at the end of the experiment, probes were immersed in a vial containing 0.5255 nM CRF and aCSF + 0.2% BSA perfused at a flow rate of 2.0 μl/min. After at least 30 min, 12.5 min samples were collected into 0.5 ml Eppendorf Protein LoBind tubes and both the recovery sample and standard were stored in the −80°C freezer until CRF concentration was determined by enzyme immunoassay (EIA). Mean probe recovery was 12.6%.

CRF EIA

CRF from dialysate or recovery samples was measured by a commercially available EIA kit (S-1169.0001; Peninsula Laboratories). Some steps of the provided protocol were adjusted to optimize detection. Standards were diluted with aCSF + 0.2% BSA rather than the provided standard diluent buffer, such that standards and samples were treated equally. Either 25 μl (Experiment 1) or 20 μl (Experiment 3B) standards or samples were pipetted into each well, as opposed to the recommended 50 μl, but all other reagent volumes were identical to the recommended protocol. After the addition of TMB solution, the developing color was read approximately every 5 min at 630 nm, and the reaction terminated using 2 N HCl and absorbance reread at 450 nm. Initial pilot experiments determined that ~15–20 min (as opposed to the recommended 30–60 min) was the optimal time to terminate the TMB reaction. All other incubation times were identical to the recommended EIA protocol.

All samples were greater than the limit of detection (LoD) and within the linear range of the standard curve, near the half-maximal inhibitory concentration (IC_{50}). The LoD was 0.0046 nM, calculated according to the Clinical and Laboratory Standards Institute published guideline EP17, *Protocols for Determination of Limits of Detection and Limits of Quantitation* (Clinical and Laboratory Standards Institute, 2004). The interplate coefficient of variance (%CV) of the standard concentration

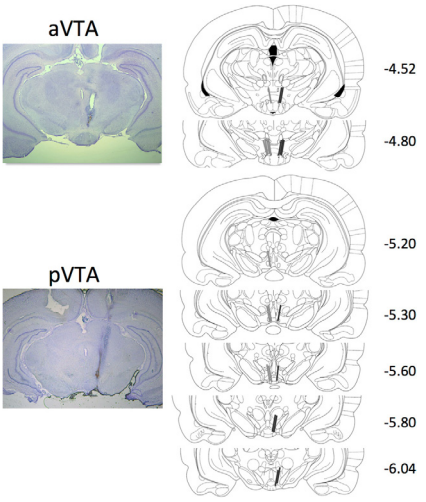

Figure 3. Histology for Experiment 1. Representative photopictomicrographs of probe placement in the aVTA and pVTA are shown on the left, with schematics on the right depicting all probe placements of control (shown on left side of brain, aVTA $n = 5$, pVTA $n = 5$) and stressed (aVTA $n = 5$, pVTA $n = 6$) rats.

closest to actual values obtained (0.525 nM) was 5.571% and the mean intraplate %CV was 1.386% (range: 0.367–3.479%).

Experiment 2: Role of VTA CRF during stress on later cocaine taking

Experiment 2 evaluated whether intra-VTA antagonism of CRF receptors during social stress prevents later escalated cocaine self-administration. To meet this objective, rats were microinjected with vehicle (aCSF), a CRF-R1 antagonist (CP376395, 500 ng/side), or CRF-R2 antagonist (Astressin2B, 1000 ng/side) into the aVTA or pVTA 10 min before the instigation phase of each defeat or handling for non-defeated controls. The doses of each drug were chosen based on previous *in vivo* work (Henry et al., 2006; Blacktop et al., 2011; Boyson et al., 2014). Rats were then catheterized for subsequent intravenous cocaine self-administration and ultimately exposed to a 24 h binge (for timeline, see Fig. 4*A*). Histology is shown in Figure 5.

Microinjections and social defeat stress

Rats underwent intracranial surgery 1 week before the first microinjection. Microinjections occurred before social defeat or brief handling for nondefeated controls on days 1, 4, 7, and 10. Drugs were microinjected into the VTA with an infusion pump (CMA 102; CMA Microdialysis) using 33 Ga microinjectors protruding 1 mm beyond the guide cannulae (PlasticsOne). All drugs and vehicle were administered in a volume of 0.25 μl/side for 1 min. Injectors were left in place for an additional 1 min to allow for diffusion from injection site and prevent backflow. Social defeat, as described above, began 10 min after the beginning of the microinjection. Nonstressed control rats were briefly handled in lieu of defeat stress.

Intravenous catheterization

Rats were catheterized for cocaine self-administration 11–12 d after the last social defeat episode. Rats were implanted with a catheter (SILASTIC silicon tubing, inner diameter 0.63 mm, outer diameter 1.17 mm; Dow-Corning) in the right jugular vein under ketamine (100 mg/kg, i.p.) and xylazine (6 mg/kg) anesthesia as described previously (Holly et al., 2012; Boyson et al., 2014). The catheter was passed subcutaneously over the shoulder and exited from a small incision between the scapulae, where it was affixed to a pedestal mounted inside a harness (SAI Infusion Technologies). Rats were allowed to recover for at least 5 d and were then moved from their home cage to permanent housing in cocaine self-administration chambers. Rats were housed in custom-built acrylic cages (30 × 20.5 × 24.5 cm) lined with Cellu-Dri pellet bedding (Shepherd Specialty Papers). The cage had a removable panel on one wall, which was replaced with a custom-built panel containing one cue light, one session light, and two retractable levers. The cages and panels were contained in larger sound-attenuating chambers. To ensure catheter patency, catheters were flushed daily with 0.2 ml of saline and 0.2 ml of heparinized saline (20 IU/ml); 0.17 ml pulses of saline were delivered every 30 min when self-administration sessions were not running. If patency was questioned, propofol (10 mg/ml) was infused to test the catheter.

Cocaine self-administration

Rats were allowed to self-administer cocaine (0.75 mg/kg/infusion) freely without priming or autoshaping during daily self-administration sessions that began ~2 h into the dark cycle. Sessions were signaled by a stimulus light and two retractable levers were extended from one wall of the home cage. Pressing the active lever resulted in a cocaine infusion paired with a cue light. Cocaine infusions were followed by a 30 s timeout period, during which stimulus and cue lights were off and lever presses were recorded, but had no effect. Throughout the session, pressing the inactive lever was recorded, but was neither reinforced nor punished.

Acquisition/maintenance. Rats were initially trained on a fixed ratio 1 (FR1) schedule of reinforcement, in which each lever press resulted in an intravenous infusion of cocaine. After 2 consecutive days of 15 infusions at FR1, the schedule was gradually increased to FR5. If rats did not meet acquisition criteria within the first 2 d of cocaine self-administration, they were behaviorally shaped with female urine or Fruit Loops on the active lever. Due to behavioral shaping, group differences in acquisition performance could not be assessed. Daily FR sessions were terminated after 15 infusions or 5 h. Rats were maintained on the FR5 schedule of reinforcement for 5 d to ensure stable and consistent responding across all stress and drug treatment groups.

Progressive ratio. Sessions then alternated between FR5 maintenance sessions and progressive ratio (PR) sessions for 6 d (3 d each). During PR sessions, rats were required to respond with an increasing number of lever presses to achieve an infusion of cocaine (0.3 mg/kg/infusion). The PR schedule of reinforcement, adapted from Richardson and Roberts (1996), was 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178. Sessions ended after 60 min without cocaine infusion.

24 h binge. After the last PR session, rats were given 1 more FR5 maintenance day and, the following day, were given unlimited access to cocaine (0.3 mg/kg/infusion) on an FR5 schedule for 24 h. After completion of the binge, catheter patency was tested by propofol injection. Data presented here are a reanalysis of previously published data (Boyson et al., 2014) because the prior publication did not assess or account for probe placement location within the VTA.

Experiment 3: Role of VTA CRF after stress on cocaine seeking after abstinence

Experiment 3 investigated whether the effects of repeated stress on VTA CRF and its receptors persist long after social defeat and affect later cocaine seeking after forced abstinence, which has been proposed as a translational model of relapse (Reichel and Bevins, 2009). Compared with nonstressed control rats, we demonstrate that previously stressed rats show greater lever pressing after forced abstinence.

Experiment 3A first evaluates whether the increased cocaine seeking in previously stressed rats can be blocked by CRF-R antagonism in the aVTA or pVTA. One possible explanation for intra-VTA CRF-R antagonism preventing cocaine seeking after forced abstinence in previously stressed rats is that the reintroduction to the context previously associated with cocaine stimulates a greater phasic CRF increase in the VTA of previously stressed rats compared with nonstressed controls. To test this hypothesis, a separate co-

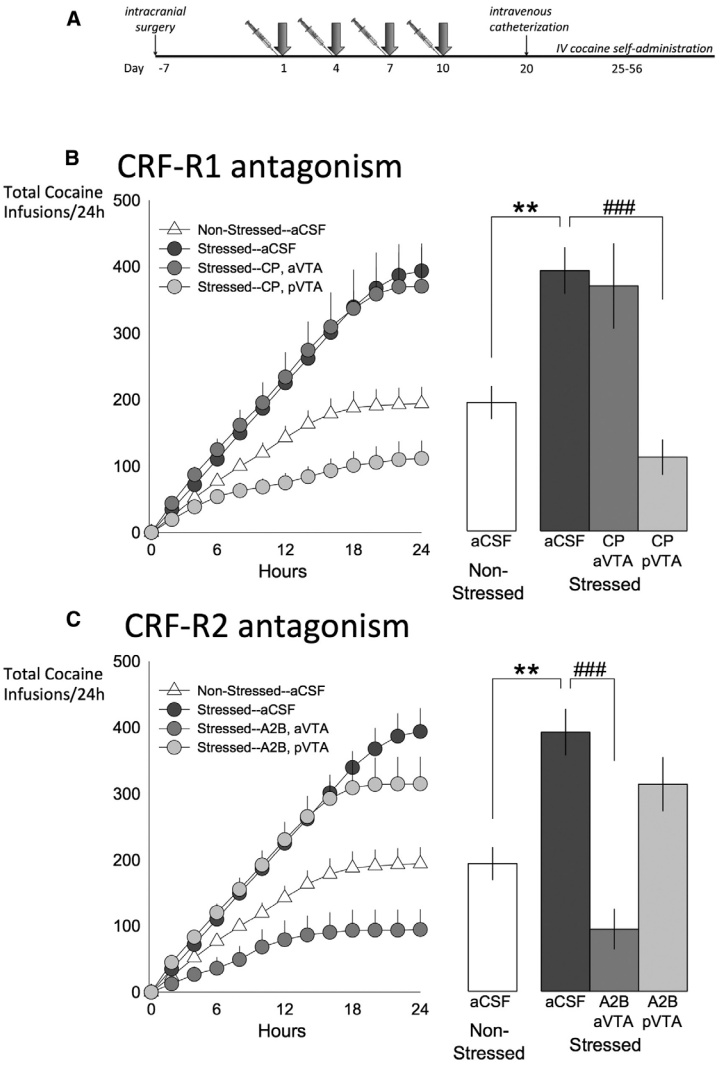

Figure 4. Experiment 2: Role of VTA CRF during stress on later cocaine self-administration. ***A***, Experimental timeline. Vehicle (aCSF), CRF-R1 antagonist (CP376395, CP), or CRF-R2 antagonist (Astressin2B, A2B), was microinjected into the VTA before each social defeat (arrows), after which animals were catheterized for intravenous cocaine self-administration, culminating in a 24 h binge. ***B***, Stressed rats pretreated with aCSF before each social defeat (dark gray, $n = 11$) self-administered significantly more cocaine during the 24 h binge compared with aCSF-pretreated nonstressed controls (white, $n = 13$). This was prevented with intra-pVTA antagonism of CRF-R1 (light gray, CP, $n = 4$), but not intra-aVTA CRF-R1 antagonism (medium gray, CP, $n = 4$). Cumulative infusions in 2 h bins are shown on the left, with total infusions shown on the right. ***C***, Conversely, intra-aVTA CRF-R2 antagonism (medium gray, A2B, $n = 4$), but not intra-pVTA CRF-R2 antagonism (light gray, A2B, $n = 5$), prevented stress-escalated cocaine self-administration during the 24 h binge. Complete data with all control groups are shown in Table 3. $^{**}p < 0.01$ versus aCSF-nonstressed, $^{###}p < 0.001$ versus aCSF-stressed.

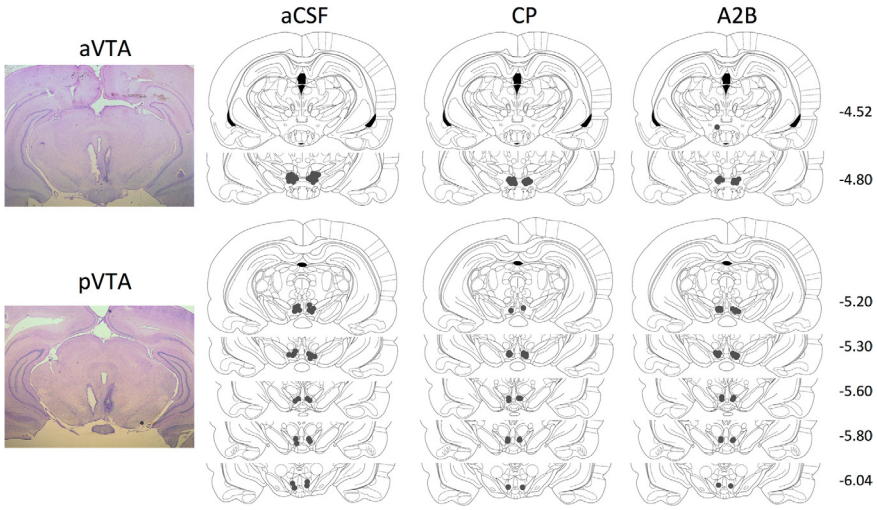

Figure 5. Histology for Experiment 2. Representative photopictomicrographs of microinjection cannula placement in the aVTA and pVTA are shown on the left, with schematics on the right depicting spread of all placements for animals pretreated with aCSF (aVTA $n = 10$, pVTA $n = 14$), CP376395 (CP, aVTA $n = 9$, pVTA $n = 9$), and Astressin2B (A2B, aVTA $n = 8$, pVTA $n = 14$).

hort of rats underwent *in vivo* microdialysis of CRF in the VTA during reinstatement testing in Experiment 3B. The experimental timeline is shown in Figure 6*A* and histology is shown in Figure 7.

Cocaine self-administration

Rats underwent intermittent social defeat stress or brief handling on days 1, 4, 7, and 10, as described above. One to 2 d after the last social defeat episode (or handling for nonstressed controls), rats underwent intravenous catheterization as described in Experiment 2. After 5 d of recovery, rats were transferred to sound-attenuating chambers, where they remained until abstinence and then began cocaine self-administration. Daily self-administration sessions were modified slightly from Experiment 2 to be more similar to other reports of cocaine seeking after forced abstinence (Fuchs et al., 2006; See et al., 2007; Hearing et al., 2008). Rats were initially trained on an FR1 schedule of reinforcement for cocaine (0.75 mg/kg/infusion) and sessions were terminated after 30 infusions or 3 h. After 2 consecutive days of >15 infusions, the schedule of reinforcement was gradually increased to FR3, where rats were maintained until they reached a criterion of 10 consecutive days of >15 infusions.

Forced abstinence. After the criterion was met, rats were moved from the self-administration room into a separate vivarium room for 15 ± 1 d of forced abstinence. The panel containing the session light, cue light, and retractable levers was removed and replaced with a wire mesh panel and the cages were moved from the sound-attenuating chambers in the self-administration room into a separate vivarium room. The sound-attenuating chambers were not cleaned or used during the abstinence period to retain any potential contextual odor cues, but each rat's bedding was changed weekly. Intracranial surgery was performed ~7 d into the abstinence period.

Cocaine seeking after abstinence. Rats were brought from their temporary housing room to their previous cocaine-self-administration sound-attenuating chamber. The wire mesh panel on their cage was replaced with their previous self-administration panel and a 3 h session began. During this session, the two levers were extended and the stimulus light was illuminated; however, now, pressing the active or inactive lever was recorded but did not result in any reinforcement, previously paired cue light, or timeout period.

Experiment 3A: Intra-VTA antagonism of CRF receptors during cocaine seeking

Rats were microinjected in their temporary housing room with vehicle (aCSF), CRF-R1 antagonist (CP376395, 500 ng/side), or CRF-R2 antagonist (Astressin2B, 1000 ng/side) in the aVTA or pVTA 20 min before reintroduction to the cocaine self-administration chamber.

Experiment 3B: In vivo *microdialysis for CRF in the VTA during cocaine seeking*

The night before reintroduction to the cocaine self-administration chamber, a separate group of rats was prepared for *in vivo* microdialysis in their temporary housing room. Microdialysis was performed and CRF measured by EIA as described in Experiment 1, except samples were collected every 10 min. After 5 baseline samples, rats were quickly brought to their self-administration chamber and samples continued to be collected throughout the 3 h cocaine-seeking session.

Results

Experiment 1: Microdialysis during social defeat for CRF in the VTA

Acute stress phasically increases extracellular CRF in the pVTA, but not aVTA

Regardless of probe placement within the VTA, baseline nanomolar concentrations of CRF did not vary between groups on day 1 (Table 2). Due to slight variability in nanomolar concentrations across multiple EIA runs and significantly different baseline values on day 10 (described below), data reported here were standardized as the percentage change from the average concentration of the five baseline samples (subsequently referred to as percentage change from baseline). Within both the aVTA and pVTA, there were no effects of stress group or sample or stress

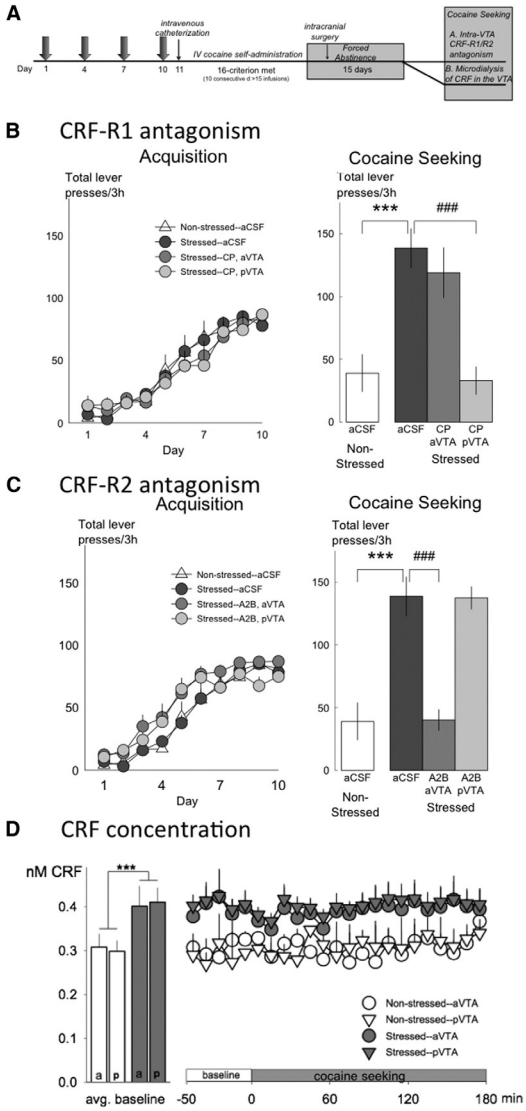

Figure 6. Experiment 3: Role of CRF in the VTA after stress on cocaine seeking after abstinence. ***A***, Experimental timeline. Rats underwent intermittent social defeat or handling, after which they were catheterized for cocaine self-administration, where they were allowed to self-administer cocaine (0.75 mg/kg/infusion) in 3 h sessions until they met a criterion of 10 consecutive days of >15 infusions. Next, rats were placed in forced abstinence for 15 d, after which they were reintroduced to their cocaine self-administration chambers and assessed for cocaine seeking after intra-VTA CRF-R1 or CRF-R2 antagonism (Experiment 3A) or with concurrent microdialysis for CRF in the VTA (Experiment 3B). ***B***, After intermittent social defeat or handling, there was no difference in acquisition (left) between the previously nonstressed rats treated with aCSF in the aVTA + pVTA ($n = 7$) and previously stressed rats microinjected with aCSF in the aVTA + pVTA ($n = 9$) or CRF-R1 antagonist (CP) in the aVTA ($n = 4$) or pVTA ($n = 4$). Upon return to the cocaine self-administration chamber (right), stressed rats microinjected with aCSF pressed the previously cocaine-paired lever significantly more than nonstressed aCSF animals, which was prevented by intra-pVTA, but not intra-aVTA, CRF-R1 antagonism. ***C***, Similarly, there were no differences in acquisition (left) between rats before abstinence in rats treated with CRF-R2 antagonist (A2B) before the return to the cocaine self-administration chamber (right). Intra-aVTA ($n = 5$), but not intra-pVTA ($n = 4$), CRF-R2 antagonism prevented the increased lever responding in previously stressed rats. ***D***, Microdialysis for CRF in the VTA revealed that, although previously stressed animals (aVTA, $n = 3$; pVTA, $n = 4$) showed a significantly greater average baseline concentration of CRF (left) compared with controls (aVTA, $n = 3$; pVTA, $n = 4$), there was no phasic increase of CRF throughout cocaine seeking (right). Complete data are shown in Table 4. $^{***}p < 0.01$ versus nonstressed control. $^{\#\#\#}p < 0.05$ versus stressed aCSF.

group × sample interaction within the first five baseline samples, so subsequent analyses excluded the first four baseline samples.

Probe placement within the VTA had a significant effect on the stress-induced efflux of CRF on day 1 (Fig. 2*B*,*C*). Within the aVTA, CRF did not change from baseline within the control or stressed groups (Fig. 2*B*) and two-way repeated-measures ANOVA revealed no main effects of stress group or sample nor a stress group × sample interaction.

Within the pVTA, however, extracellular CRF was increased in the stressed group during and after social defeat, whereas it remained unchanged in the control group (Fig. 2*C*). Two-way repeated-measures ANOVA revealed significant main effects of stress group ($F_{(1,8)} = 20.225$, $p = 0.002$), sample ($F_{(7,56)} = 4.984$, $p < 0.001$), as well as a stress group × sample interaction ($F_{(7,56)} = 3.027$, $p = 0.009$). *Post hoc* Holm–Sidak tests for multiple comparisons on the stress group × sample interaction revealed that, within the stressed group, CRF was significantly increased above baseline during the first half of social defeat stress (Holm–Sidak $t = 5.381$, $p < 0.001$), as well as during the first and second samples immediately after social defeat was terminated (first sample: Holm–Sidak $t = 3.880$, $p = 0.007$; second sample: Holm–Sidak $t = 5.228$, $p < 0.001$). CRF did not change significantly

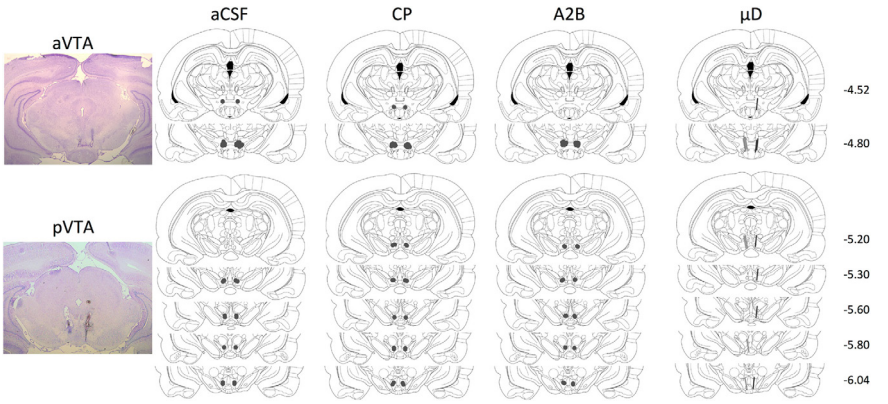

Figure 7. Histology for Experiment 3. Representative photopictomicrographs of microinjection cannula placement in the aVTA and pVTA are shown on the left, with schematics on the right depicting spread of all placements for animals pretreated with aCSF (aVTA $n = 8$, pVTA $n = 7$), CP376395 (CP, aVTA $n = 10$, pVTA $n = 8$), and Astressin2B (A2B, aVTA $n = 9$, pVTA $n = 8$). Schematics on the far right depict all microdialysis (uD) probe placements of control (shown on left side of brain, aVTA $n = 3$, pVTA $n = 4$) and stressed (aVTA $n = 3$, pVTA $n = 4$) rats in Experiment 3B.

Table 2. Baseline CRF concentration (nM)

Group	Region	Day 1	Day 10
Nonstressed	aVTA	0.325 ± 0.043	0.083 ± 0.008
	pVTA	0.293 ± 0.012	0.115 ± 0.025
Stressed	aVTA	0.294 ± 0.038	0.617 ± 0.192##
	pVTA	0.216 ± 0.063	0.502 ± 0.126

Data are represented as mean ± SEM.
##$p < 0.01$ versus day 1.

from baseline within the control group. Furthermore, also probing the interaction, extracellular CRF in the stressed group was significantly greater than in the control group during the first half of social defeat stress (Holm–Sidak $t = 4.947$, $p < 0.001$), as well as during all samples after social defeat ended (first sample: Holm–Sidak $t = 3.601$, $p < 0.001$, second sample: Holm–Sidak $t = 3.820$, $p < 0.001$, third sample: Holm–Sidak $t = 2.133$, $p = 0.038$, fourth sample: Holm–Sidak $t = 2.166$, $p = 0.035$, fifth sample: Holm–Sidak $t = 2.335$, $p = 0.024$).

Repeated stress increases tonic CRF in the VTA

On the last day of intermittent social defeat stress, tonic levels of CRF were increased in stressed rats regardless of probe placement within the VTA, whereas they were decreased within nonstressed controls (Table 2).

Two-way repeated-measures ANOVA of nanomolar baseline concentrations revealed a significant main effect of stress ($F_{(1,15)} = 4.906$, $p = 0.043$) and an interaction of stress condition and day ($F_{(1,15)} = 13.247$, $p = 0.002$), but no main effect of day. Within the stressed group, there was a significant increase in tonic CRF from day 1 to day 10 (Holm–Sidak $t = 3.182$, $p = 0.006$), but with the current sample size, the decrease in tonic CRF observed within nonstressed controls did not reach statistical significance (Holm–Sidak $t = 2.083$, $p = 0.055$). There was, however, a significant effect of stress group on day 10, with tonic CRF levels substantially greater within stressed animals than those of nonstressed controls (Holm–Sidak $t = 3.617$, $p < 0.001$).

Separate analysis of VTA subregions revealed the same statistical effects and trends. Within the aVTA, there was a significant interaction between stress group and day ($F_{(1,7)} = 6.825$, $p = 0.035$) and CRF tone was significantly greater in stressed compared with nonstressed rats on day 10 (Holm–Sidak $t = 3.357$, $p = 0.005$). There were no significant effects in the pVTA.

Baseline CRF concentration on day 10 was also analyzed as a percentage increase from the day 1 baseline concentration within each rat. In this manner, the change in CRF concentration from day 1 to day 10 was readily apparent, with stressed rats exhibiting an increase in CRF tone compared with day 1 and a decrease in nonstressed rats (Fig. 2*C*). Two-way ANOVA revealed a significant main effect of stress ($F_{(1,13)} = 9.451$, $p = 0.009$) with no effects of VTA subregion or stress × subregion interaction. There was a significant effect of stress group within the pVTA (Holm–Sidak $t = 2.424$, $p = 0.031$), but not aVTA (Holm–Sidak $t = 1.905$, $p = 0.079$).

Repeated stress phasically increases extracellular CRF in the aVTA, but not pVTA

After exposure to repeated stress, extracellular CRF is altered within both the aVTA and pVTA during the last defeat. During the final defeat, CRF is slightly elevated above baseline in both the aVTA and pVTA during the first half of social defeat stress, after which they diverge (Fig. 2*E*,*F*).

Within the aVTA, CRF remains elevated, after which it returns to baseline (Fig. 2*E*). Two-way repeated-measures ANOVA revealed a significant main effect of sample ($F_{(7,49)} = 3.090$, $p = 0.009$) and a stress group × sample interaction ($F_{(7,49)} = 3.175$, $p = 0.008$), but no main effect of stress. *Post hoc* Holm–Sidak tests for multiple comparisons on the significant interaction revealed that, within the stressed group, extracellular CRF was significantly increased from baseline during both samples during social defeat stress (first sample: Holm–Sidak $t = 3.535$, $p = 0.006$; second sample: Holm–Sidak $t = 3.356$, $p = 0.009$), but did not differ from baseline after the termination of social defeat stress. In contrast, extracellular CRF did not differ from baseline within the control group. In addition, extracellular CRF was significantly

Table 3. Effects of intra-VTA CRF-R antagonism during stress on cocaine self-administration FR performance rate, progressive ratio breakpoint (PRBP), and infusions during a 24 h "binge"

Group	Drug	Region	FR rate	PRBP	Binge
Nonstressed	aCSF	aVTA	0.59 ± 0.09	7.49 ± 1.13	179.99 ± 44.64
Nonstressed	aCSF	pVTA	0.64 ± 0.08	9.71 ± 0.56	216.00 ± 32.80
Nonstressed	CP	aVTA	0.85 ± 0.21	10.67 ± 2.03	183.33 ± 85.47
Nonstressed	CP	pVTA	0.99 ± 0.22	10.79 ± 1.36	252.00 ± 51.80
Nonstressed	A2B	aVTA	0.55 ± 0.23	9.71 ± 0.83	233.00 ± 67.42
Nonstressed	A2B	pVTA	0.90 ± 0.13	11.67 ± 1.07	228.50 ± 63.29
Stressed	aCSF	aVTA	0.76 ± 0.22	10.42 ± 1.32	342.50 ± 41.19**
Stressed	aCSF	pVTA	0.82 ± 0.05	10.87 ± 0.85	423.00 ± 48.69**
Stressed	CP	aVTA	1.19 ± 0.15	10.67 ± 0.68	370.40 ± 64.36
Stressed	CP	pVTA	0.79 ± 0.12	8.83 ± 1.06	111.40 ± 26.72###
Stressed	A2B	aVTA	0.73 ± 0.08	8.38 ± 1.21	94.50 ± 30.68####
Stressed	A2B	pVTA	0.88 ± 0.06	11.92 ± 0.88	314.67 ± 41.03

Data are represented as mean ± SEM.

**$p < 0.01$ versus nonstressed aCSF; ###$p < 0.001$ versus stressed aCSF within the same subregion.

greater in the stressed compared with control group in both samples during social defeat (first sample: Holm–Sidak $t = 2.159$, $p = 0.037$; second sample: Holm–Sidak $t = 2.749$, $p = 0.009$).

Conversely, within the pVTA, extracellular CRF was suppressed after the termination of social defeat (Fig. 2*F*). Two-way repeated-measures ANOVA revealed a significant stress group × sample interaction ($F_{(7,63)} = 2.281$, $p = 0.038$), but no main effects of stress group or sample. Testing the significant interaction with Holm–Sidak tests for multiple comparisons, a significant decrease from baseline CRF was observed within the stressed group during the second, third, and fourth samples after social defeat stress ended (second sample: Holm–Sidak $t = 2.755$, $p = 0.038$; third sample: Holm–Sidak $t = 2.864$, $p = 0.034$; Holm–Sidak $t = 3.097$, $p = 0.020$) and percentage baseline CRF was significantly lower in the stressed group compared with control group during the third and fourth samples after social defeat (third sample: Holm–Sidak $t = 2.021$, $p = 0.048$; fourth sample: Holm–Sidak $t = 2.382$, $p = 0.021$).

Experiment 2: Role of VTA CRF during stress on later cocaine taking

Intra-VTA antagonism of CRF-R1 in the pVTA or CRF-R2 in the aVTA during each defeat prevents later escalated cocaine self-administration

The response rate during fixed ratio sessions and the breakpoint in progressive ratio sessions were not affected by stress or pretreatment with CRF receptor antagonists into either VTA subregion before each stress or handling episode (Table 3).

However, stress significantly increased cocaine self-administration during the 24 h binge and this effect could be prevented with CRF-R1 antagonism in the pVTA or CRF-R2 antagonism in the aVTA (Fig. 5*B*,*C*, Table 3). Three-way ANOVA revealed significant interactions of stress group × drug pretreatment ($F_{(2,51)} = 6.366$, $p = 0.003$), VTA subregion × drug pretreatment ($F_{(2,51)} = 3.167$, $p = 0.05$), and stress group × VTA subregion × drug pretreatment ($F_{(2,51)} = 10.279$, $p < 0.001$).

Because there was a significant three-way interaction, main effects and two-way interactions cannot be interpreted. The effect of one or more factor is not consistent at all combinations of the other two factors such that an unambiguous interpretation of main effects and two-way interactions is not possible. To interpret the three-way interaction, further two-way *post hoc* analyses were performed evaluating two-way interactions across one level of the third factor. First, the effects of stress group and VTA subregion were tested within the vehicle-pretreated animals. Within the vehicle group, previously stressed rats self-administered significantly more cocaine during the 24 h binge compared with nonstressed controls regardless of VTA subregion (Holm–Sidak $t = 2.898$, $p = 0.006$).

Because there was a significant effect of stress within the vehicle-pretreated animals, we next analyzed the effects of drug pretreatment and VTA subregion within the stressed group. Two-way ANOVA revealed a significant effect of drug pretreatment ($F_{(2,28)} = 8.521$, $p = 0.001$) and drug pretreatment × VTA subregion interaction ($F_{(2,28)} = 18.581$, $p < 0.001$) within the previously stressed rats. Although the number of binge infusions was significantly less in stressed rats pretreated with the CRF-R1 antagonist (Holm–Sidak $t = 3.181$, $p = 0.007$) and the CRF-R2 antagonist (Holm–Sidak $t = 3.902$, $p = 0.002$) compared with vehicle-pretreated stressed animals, this was driven by a significant effect of where the antagonists were microinjected. Within stressed rats, CRF-R1 antagonism within the pVTA significantly prevented increased cocaine taking compared with vehicle pretreatment in the pVTA (Holm–Sidak $t = 5.316$, $p < 0.001$), as well as CRF-R1 antagonism within the aVTA (Holm–Sidak $t = 4.091$, $p < 0.001$). In addition, stressed rats pretreated with CRF-R1 antagonist in the pVTA self-administered significantly less than pVTA CRF-R1-pretreated nonstressed controls (Holm–Sidak $t = 4.840$, $p < 0.05$), which may be a result of antagonism of a phasic rather than tonic CRF response to social stress. Conversely, CRF-R2 antagonism within the aVTA of stressed rats significantly prevented increased cocaine taking compared with vehicle pretreatment in the aVTA (Holm–Sidak $t = 3.778$, $p = 0.002$) and CRF-R2 antagonism within the pVTA (Holm–Sidak $t = 4.502$, $p < 0.001$).

Experiment 3: Role of CRF in the VTA after stress on cocaine seeking after abstinence

Rats with a history of repeated stress exhibit greater cocaine seeking after forced abstinence compared with nonstressed controls

All rats acquired and reliably self-administered cocaine at an FR3 schedule of reinforcement and there was no effect of prior stress history on the number of infusions in daily cocaine self-administration sessions (Fig. 6*B*,*C*, left). Two-way repeated-measures ANOVA revealed a main effect of self-administration day ($F_{(9,459)} = 177.259$, $p < 0.001$), but no effect of stress group or stress group × self-administration day interaction. There was also no significant difference between groups on cumulative cocaine earned before forced abstinence (data not shown).

Previously stressed rats microinjected with vehicle demonstrated augmented cocaine seeking after forced abstinence, which was prevented in previously stressed rats treated with antagonists of CRF-R1 in the pVTA (Fig. 6*B*, right, Table 4) and CRF-R2 in the aVTA (Fig. 6*C*, right, Table 4) before the return to the cocaine self-administration chamber. Three-way ANOVA revealed a significant interaction of VTA subregion × drug ($F_{(2,38)} = 6.479$, $p = 0.004$), as well as stress group × drug ($F_{(2,38)} = 5.910$, $p = 0.006$), meaning that the effect each CRF receptor antagonist or vehicle in the VTA is dependent on both which VTA subregion it is delivered in and whether the animals were previously stressed. *Post hoc* Holm–Sidak tests for multiple comparisons were then run to interpret these significant interactions.

Previous intermittent social defeat stress resulted in significantly more lever pressing upon return to the cocaine self-administration chamber compared with nonstressed controls (Fig. 6*B*,*C*, right, Table 4). Previously stressed rats microinjected with aCSF pressed the lever previously associated with cocaine

Table 4. Effects of intra-VTA CRF-R antagonism during cocaine seeking after forced abstinence in previously stressed or nonstressed rats

Group	Drug	Region	Lever presses
Nonstressed	aCSF	aVTA	30.00 ± 17.01
Nonstressed	aCSF	pVTA	66.67 ± 17.80
Nonstressed	CP	aVTA	62.83 ± 20.30
Nonstressed	CP	pVTA	59.25 ± 26.88
Nonstressed	A2B	aVTA	66.86 ± 24.81
Nonstressed	A2B	pVTA	59.67 ± 11.17
Stressed	aCSF	aVTA	134.40 ± 13.79***
Stressed	aCSF	pVTA	144.00 ± 33.71***
Stressed	CP	aVTA	119.00 ± 20.10
Stressed	CP	pVTA	33.00 ± 11.14##
Stressed	A2B	aVTA	40.20 ± 8.42##
Stressed	A2B	pVTA	137.33 ± 9.12

Data are represented as mean ± SEM.

***$p < 0.001$ versus nonstressed aCSF; ##$p < 0.01$ versus stressed aCSF within the same subregion.

significantly more than their last day of cocaine self-administration (one-way repeated-measures ANOVA $F_{(1,17)} = 10.549$, $p = 0.012$), an effect not seen in aCSF-treated rats with no stress history. The stressed aCSF group also exhibited significantly more cocaine seeking compared with the nonstressed aCSF group (Holm–Sidak $t = 3.879$, $p < 0.001$).

Heightened cocaine seeking in previously stressed rats is associated with increased tonic CRF in the VTA acting on pVTA CRF-R1 and aVTA CRF-R2

The cocaine seeking observed in the stressed group was prevented by antagonism of CRF-R1 in the pVTA and CRF-R2 in the aVTA (Fig. 6*B*,*C*, right, Table 4). Within the pVTA, responses on the lever previously associated with cocaine reinforcement were significantly reduced in animals treated with CRF-R1 antagonist compared with those treated with aCSF (Holm–Sidak $t = 3.274$, $p = 0.011$), but no difference between CRF-R2 antagonist- and aCSF-treated rats was observed. Conversely, within the aVTA, CRF-R2 antagonism resulted in significantly fewer lever presses compared with aCSF-treated rats (Holm–Sidak $t = 3.092$, $p = 0.016$), with no difference between stressed rats administered aCSF and CRF-R1 antagonist.

Unexpectedly, there was no phasic increase from baseline in extracellular CRF in the VTA during cocaine seeking in either the previously stressed or the nonstressed group (Fig. 6*D*). However, nanomolar concentrations of CRF were significantly greater in previously stressed compared with nonstressed rats (one-way ANOVA $F_{(1,11)} = 6.529$, $p = 0.027$). There was no difference between VTA subregion in either group.

Discussion

The current experiments suggest dynamic, shifting roles of CRF and its receptors within VTA subregions during and after stress, promoting later escalated cocaine taking and seeking. Acute stress promotes a phasic increase in CRF within the pVTA, whereas repeated stress recruits a phasic CRF response in the aVTA and elevates CRF tone throughout the VTA. CRF acts on CRF-R1 in the pVTA and CRF-R2 in the aVTA during repeated social defeat stress to cause increased cocaine self-administration during a 24 h binge. Finally, changes in VTA CRF are persistent after stress exposure such that CRF tone remains elevated and may contribute to cocaine seeking after forced abstinence in previously stressed animals through amplified actions on pVTA CRF-R1 and aVTA CRF-R2.

Only one other study reports phasic extracellular CRF changes in the VTA during stress. Wang et al. (2005) demonstrated that acute foot shock stress increases CRF within the VTA. Consistent with this prior work, the current study found increased extracellular CRF in the pVTA during acute stress. Although Wang et al. (2005) did not differentiate between the aVTA and pVTA, our results suggest that CRF efflux differs between these VTA subregions during acute stress.

The time course of phasic CRF fluctuations within the pVTA during acute defeat resemble the pattern of dopaminergic increases in the medial prefrontal cortex (mPFC) and nucleus accumbens shell (NAcSh) (Holly et al., 2015). Here, we show that, after a burst in CRF at the initiation of social defeat, extracellular CRF returns to baseline for the second half of social defeat. Although this return to baseline is difficult to interpret, the variability of this data point is low. Moreover, the pVTA CRF changes during acute defeat parallel our report demonstrating that extracellular mPFC and NAcSh dopamine concentrations are highest in the first half of social defeat stress, but not significantly different from baseline during the final threat period (Holly et al., 2015). Unlike our observations of extracellular dopamine, however, the present data indicate that CRF is again elevated at the termination of defeat, possibly signaling negative reinforcement.

VTA dopamine neurons responsive to acute foot shock are primarily localized in the pVTA (Brischoux et al., 2009). The present observations of phasic CRF increase in the pVTA during acute social defeat stress may be one mechanism underlying stress-induced dopamine neuron activation because CRF bath application increases VTA dopamine neuron firing rate (Korotkova et al., 2006; Wanat et al., 2008) and blockade of VTA CRF-R2 can prevent acute social-stress-induced dopamine efflux in the NAcSh (Holly et al., 2015).

Future work should identify sources of CRF into the aVTA and pVTA and determine which neural circuit(s) is responsible for the observed increase in pVTA CRF during acute stress. However, neuropeptides are not solely released from axon terminals, but may be released from the entire surface of the neuron (Pow and Morris, 1989). Grieder et al. (2014) described CRF-containing dopaminergic cell bodies within the pVTA, and their activation may be a source of the efflux in extracellular CRF in the pVTA during acute stress. Therefore, although microdialysis captures increased extracellular neuropeptide concentration, the source of the increase cannot be determined conclusively even if distinct inputs are uncovered (Wotjak et al., 2008).

Repeated, as opposed to acute, social stress has been associated with increased psychostimulant self-administration, so the shifts in CRF activity within the VTA with repeated stress are of particular interest. With repeated social defeat, tonic extracellular CRF was significantly increased by ~200% within stressed animals (Fig. 2*D*). This increased tone could be due to the recruitment of previously silent CRF neurons (George et al., 2012); increased vesicular storage or increased firing rate of CRF neurons located in, projecting to, or passing through the VTA (van den Pol, 2012); or increased CRF production and somatodendritic release from CRF neurons within the VTA itself (Grieder et al., 2014). However, although CRF remains elevated >1 month after social defeat stress ends (Fig. 6*D*) and we recently demonstrated no change in mesocorticolimbic dopamine tone with repeated defeat (Holly et al., 2015), a conditioned response to anticipation of defeat may contribute to the elevated CRF tone.

Phasic CRF responses are also altered with repeated stress. CRF was not significantly altered in the aVTA during the first defeat, but CRF was significantly increased in the aVTA during the last defeat. This may also reflect the recruitment of previously silent neurons or neuroadaptations resulting in a phasic response

of already active CRF neurons that do not respond to acute stress. In addition, there was a shift in the phasic response of CRF within the pVTA; after the last defeat CRF is significantly depressed below baseline. Neuropeptides are slower to regenerate within vesicular stores than canonical neurotransmitters (van den Pol, 2012), so this suppression may represent complete depletion of vesicular stores at the beginning of social defeat. However, evoked CRF release during repeated stress may be altered by prior probe implantation.

Phasic CRF responses within the VTA during social defeat stress have important functional consequences on later cocaine self-administration behavior. Intra-VTA CRF-R1 and CRF-R2 antagonism during social defeat prevents the induction of cross-sensitization to cocaine and escalated cocaine self-administration during a 24 h binge (Boyson et al., 2014). No effects on PR breakpoints were observed, suggesting a possible dissociation between escalated intake and motivation. However, the present reanalysis clearly differentiates the effects of CRF-R1 and CRF-R2 antagonism between the aVTA and pVTA on binge self-administration (Fig. 4). Within the aVTA, CRF-R2, but not CRF-R1, antagonism prevented increased binge cocaine self-administration, whereas the converse was true of the pVTA.

The distribution of CRF-R1 and CRF-R2 along the rostrocaudal axis of the VTA has not yet been reported, but recent work suggests that CRF-R1 within the pVTA may be more relevant for the initial response to stress, whereas CRF-R2 within the aVTA may be recruited with repeated stress exposure (Holly et al., 2015). Within the locus ceruleus and dorsal raphe nucleus, stress induces internalization of CRF-R1 and externalization of CRF-R2 to the membrane (Reyes et al., 2008; Waselus et al., 2009) and a similar effect may be occurring within the aVTA in combination with recruitment of a phasic aVTA CRF response with repeated stress.

Although CRF actions on VTA CRF-Rs during repeated stress contribute to the development of later maladaptive cocaine self-administration, the current findings also demonstrate that VTA CRF continues to play a significant role in cocaine seeking after stress. Prior stress increased cocaine seeking >1 month after the last defeat (Fig. 6). Because most humans undergo abstinence—either voluntary or forced—instead of extinction (Katz and Higgins, 2003), the current study attempted to translate the human condition to rats through reexposure to cocaine self-administration chambers after forced abstinence. Antagonism of either pVTA CRF-R1 or aVTA CRF-R2 after stress-induced neuroadaptations had already occurred prevented augmented cocaine seeking after forced abstinence in previously stressed rats. This parallels the findings of Experiment 2, furthering the hypothesis that there may be rostrocaudal differences in CRF-R expression as a result of stress exposure.

VTA CRF-R1 and CRF-R2 have both been implicated in stress-induced reinstatement to cocaine seeking after extinction (Wang et al., 2007; Blacktop et al., 2011), so it was initially hypothesized that cocaine seeking after forced abstinence in previously stressed animals was serving as an additional stressor, phasically increasing extracellular CRF within the VTA. However, the present results indicate that, although previously stressed animals still had significantly greater CRF tone throughout the VTA compared with previously nonstressed rats, there were no phasic CRF changes in the VTA within either group. This suggests that intra-VTA CRF-R antagonism, rather than preventing any phasic CRF effect, may prevent the increased extracellular tonic CRF from exerting effects, contributing to cocaine seeking after forced abstinence in previously stressed animals. Future work should elucidate a causal, as opposed to a correlational, link between augmented CRF tone and selective changes in expression of CRF within the VTA. Nonetheless, CRF is not the only endogenous ligand for CRF-R1 and CRF-R2 and the urocortin system may contribute to increased cocaine seeking (Ryabinin et al., 2012).

Addiction is a chronic relapsing disorder intricately entwined with stress and characterized by cycles of abstinence/withdrawal followed by reinitiation of drug taking (Koob and Kreek, 2007). In later phases of addiction, negative, as opposed to positive, reinforcement may be a more critical process behind escalated drug taking and seeking behavior (Koob and Le Moal, 2001, 2008). As we demonstrate here, pVTA CRF is significantly different from baseline upon the termination of both acute and repeated social stress, indicating a possible role of pVTA CRF in negative reinforcement. The current series of experiments suggests a critical role of VTA CRF acting on aVTA CRF-R2 and pVTA CRF-R1, not only in stress, but also in escalated cocaine intake and relapse after abstinence. This work complements a growing literature implicating extrahypothalamic CRF, particularly in the extended amygdala, in animal models of escalated drug self-administration (Zhou et al., 1996; Funk et al., 2006; Funk and Koob, 2007; George et al., 2007; Specio et al., 2008; Greenwell et al., 2009).

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ORIGINAL INVESTIGATION

Maladaptive choices by defeated rats: link between rapid approach to social threat and escalated cocaine self-administration

Christopher O. Boyson[1] · Elizabeth N. Holly[1,2,3,4] · Andrew R. Burke[1,5] · Sandra Montagud-Romero[1,6] · Joseph F. DeBold[1] · Klaus A. Miczek[1,7,8,9]

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Abstract
Rationale Intermittent social defeat stress engenders persistent neuroadaptations and can result in later increased cocaine taking and seeking. However, there are individual differences in stress-escalated cocaine self-administration behavior, which may be a direct result of individual differences in the manner in which rats experience social defeat stress.

Objective The present study dissected the discrete behavioral phases of social defeat and analyzed which behavioral characteristics may be predictive of subsequent cocaine self-administration.
Methods Male Long-Evans rats underwent nine intermittent social defeat episodes over 21 days in a three-compartment apparatus permitting approach to and escape from a confrontation with an aggressive resident rat. Rats then self-administered intravenous cocaine, which culminated in a 24-h unlimited access "binge." Behaviors during social defeat and cocaine self-administration were evaluated by principal component analysis (PCA).
Results PCA revealed that the latency to enter the threatening environment was highly predictive of later cocaine self-administration during the 24-h binge. This behavior was not associated with other cocaine-predictive traits, such as reactivity to novelty in an open field, saccharin preference, and motor impulsivity. Additionally, there was no effect of latency to enter a threatening environment on physiological measures of stress, including plasma corticosterone and corticotropin releasing factor (CRF) in the extended amygdala. However, latency to enter the threatening environment was negatively correlated with brain-derived neurotropic factor (BDNF) and its receptor, tyrosine kinase B (TrkB) in the hippocampus.
Conclusion These data suggest that latency to enter a threatening environment is a novel behavioral characteristic predictive of later cocaine self-administration.

Keywords Social defeat stress · Cocaine self-administration · BDNF · Impulsivity · Behavior · Individual differences

Christopher O. Boyson and Elizabeth N. Holly are co-first authors.

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✉ Klaus A. Miczek
Klaus.Miczek@tufts.edu

[1] Department of Psychology, Tufts University, Bacon Hall, 530 Boston Ave, Medford, MA 02155, USA

[2] McGovern Institute for Brain Research, Massachusetts Institute of Technology, Cambridge, MA, USA

[3] Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA, USA

[4] Current affiliation: Department of Neuroscience, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

[5] Current affiliation: Department of Psychiatry, Indiana University School of Medicine, Indianapolis, IN, USA

[6] Unidad de Investigación Psicobiología de las Drogodependencias, Departmento de Psicobiología, Universitat de València, València, Spain

[7] Department of Psychiatry, Tufts University School of Medicine, Boston, MA, USA

[8] Department of Pharmacology, Tufts University School of Medicine, Boston, MA, USA

[9] Department of Neuroscience, Tufts University School of Medicine, Boston, MA, USA

Introduction

Stress is both a predisposing, triggering factor and consequence of drug taking. Individuals with high levels of previous stress and

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adverse life events are more likely to engage in drug use, initiate drug use at an earlier age, transition from occasional use to compulsive drug addiction more quickly, and relapse (Sinha 2001). Preclinical studies have largely paralleled these findings in both nonhuman primates and rodents. In particular, a model of intermittent social defeat stress in rats engenders long-lasting neuroadaptations, resulting in faster acquisition of cocaine self-administration, greater responding under progressive ratio schedules of reinforcement, increased cocaine self-administration during a 24-h unlimited access "binge," and heightened context-induced reinstatement after abstinence (Covington and Miczek 2001; Holly et al. 2016; Miczek and Mutschler 1996; Miczek et al. 2011; Tidey and Miczek 1997).

However, despite substantial evidence linking stress and compulsive drug taking and seeking, not all individuals exposed to high levels of stress engage in drug use. Similarly, there is considerable variability in cocaine self-administration patterns and levels of consumption in rats exposed to models of intermittent social defeat stress (Covington and Miczek 2005; Covington et al. 2008; Kabbaj et al. 2001; Shimamoto et al. 2015). Understanding the behavioral sources and underlying mechanisms of individual differences in augmented cocaine self-administration following stress may lead to earlier and more effective therapeutic interventions for vulnerable individuals.

A possible underlying basis for this variability in drug taking is individual differences in behavior during social stress. Wood et al. (2010) found that active vs. passive coping during social defeat (operationally defined by latency to submit to the resident) was predictive of subsequent resilience or vulnerability, respectively, to maladaptive behavioral and physiological effects of stress. However, social defeat encompasses a myriad of behaviors, such as approach to a threatening situation, reaction to a fight encounter, escape behavior, and return to a safe environment (Koolhaas et al. 2010). How each of these distinct behaviors relates to subsequent drug self-administration has yet to be explored. In the current experiment, we dissected these phases of social confrontation and examined resulting effects on stress-associated physiological and behavioral measures. We designed an apparatus which allowed us to assess rats for their latencies to engage in each component of social defeat stress: entering a threatening zone, engaging in a confrontation, submitting to social defeat, escape, and return to a safe zone. After nine encounters, rats were tested for behavioral cross-sensitization to cocaine and catheterized for cocaine self-administration, which culminated in a 24-h binge. As other factors, such as reactivity to novelty, saccharin preference, impulsivity, and resistance to punishment have also been correlated with individual differences in cocaine self-administration (Belin et al. 2008; Carroll et al. 2002; Deroche-Gamonet et al. 2004; Piazza et al. 1989), we also examined whether these measures were associated with defeat-related behaviors.

Additionally, we evaluated whether behaviors during defeat were associated with neurophysiological differences in brain regions associated with stress and drug reward. Chronic stress downregulates hippocampal brain-derived neurotrophic factor (BDNF) transcription (Duclot and Kabbaj 2013; Haenisch et al. 2009; Komatsu et al. 2011; Patki et al. 2013; Zhang et al. 2015), with individual differences in chronic stress-induced changes in hippocampal BDNF and its receptor, tyrosine receptor kinase B (TrkB) (Duclot and Kabbaj 2013). Individual differences in the physiological response to stress may also contribute to individual differences in stress-escalated (as opposed to drug history-escalated) cocaine self-administration. Clinical work has shown that cortisol release in response to stress is positively correlated with positive feelings in response to amphetamine (Hamidovic et al. 2010). Corticotropin releasing factor (CRF) is a neuropeptide involved in the initiation of the hypothalamic-pituitary-adrenal axis stress response, but also has widespread extrahypothalamic sites of action (Swanson et al. 1983). In particular, CRF in the extended amygdala and mesocorticolimbic dopamine system has been strongly implicated in stress-induced psychiatric disorders, including drug addiction (Zorrilla et al. 2014). Therefore, we also examined whether hippocampal BDNF and TrkB transcript, CRF expression, or corticosterone levels were correlated with defeat behaviors.

Methods

General methods

Subjects

Male Long-Evans rats (Charles River Laboratories, Wilmington, MA, $n = 213$) weighing 225–250 g upon arrival were individually housed in custom-built acrylic chambers (30.5 × 30.5 × 24.5 cm) lined with Cellu-Dri™ bedding (Shepherd Specialty Papers, Kalamazoo, MI) and provided food and water ad libitum. Rats were housed on a reverse light cycle (lights on 2000–0800 hours), with controlled temperature (21 ± 1 °C) and humidity (30–60 %). All behavioral testing was conducted during the dark phase, and all experimental procedures were approved by the Tufts Institutional Animal Care and Use Committee following the Guide for the Care and Use of Laboratory Animals (National Research Council 2011).

Experimental design

In experiment 1, rats were first tested for baseline saccharin preference and open field activity, after which they were exposed to social defeat stress or control handling, with weekly measurements of saccharin preference and locomotion in the open field. Saccharin preference and open field activity were tested again 1 week after the last social defeat. Rats were then assessed for

locomotor cross-sensitization to cocaine, after which rats were implanted with intravenous catheters and trained to self-administer cocaine. In experiment 2, rats underwent social defeat or handling, followed by Go/No-Go testing for impulsivity. In experiment 3, rats were assessed for BDNF and TrkB messenger RNA (mRNA) expression in the prefrontal cortex and hippocampus after the last social defeat, while experiment 4 measured CRF protein expression in the nucleus accumbens, amygdala, and ventral tegmental area. Finally, experiment 5 measured plasma corticosterone after the first, fifth, and ninth defeat. Experimental design is shown in Fig. 1a.

Social defeat stress

Upon arrival, rats were randomly assigned to two groups: defeat stressed or non-defeated control. Stressed rats were subjected to nine intermittent social defeat episodes over 21 days in a modified version of our previously described procedure (Covington and Miczek 2001), while non-defeated controls were briefly handled. Defeats occurred in a modified resident home cage (Fig. 1b) consisting of two compartments (the resident's home compartment or "fight zone" and a neutral compartment or "threat zone" with clean bedding) connected by a porthole, with a second porthole allowing access to the experimental rat's home cage ("safe zone"). See Electronic supplementary material for additional details.

During each defeat, the experimental intruder's home cage was moved adjacent to the resident cage (Fig. 1b), the porthole opened, and the rat allowed up to 5 min to voluntarily move to the threat zone. After 5 min in the threat zone, the intruder was placed into the fight zone with the resident with the escape porthole closed until it showed 8 s continuous supine posture, received ten attack bites, or 5 min had elapsed. A screen was then used to separate the aggressive resident from the intruder and the escape porthole opened. The rat was allowed up to 5 min to voluntarily move from the fight zone to

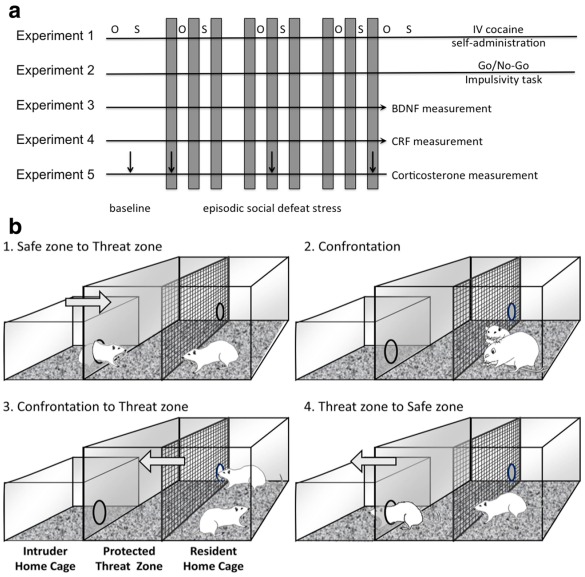

Fig. 1 Experimental and apparatus design. **a** In the first experiment, rats were tested for baseline open field activity (*O*) and saccharin drinking (*S*), which continued weekly throughout the course of intermittent social defeat stress (defeats represented by *gray bars*). After the last defeat, rats were catheterized and allowed to self-administer cocaine. In the second experiment, separate rats were tested for impulsivity in the Go/No-Go task after social defeat stress. Physiological measures were taken in the final three experiments, with one cohort assessed for BDNF and TrkB mRNA, another for extrahypothalamic CRF content, and a final cohort tested for corticosterone at baseline and after the first, fifth, and ninth defeat. **b** Three-chamber apparatus permitting entry and escape for the intruder. *1*, The intruder voluntarily leaves the safe zone (home cage) and enters the threat zone. *2*, After 5 min in the threat zone, the intruder is placed into the resident's compartment for social defat. *3*, After ten bites, 8 s supine, or 5 min, the porthole is opened and the intruder allowed to escape back to the threat zone. *4* After 5 min, the porthole to the safe zone is opened and the intruder allowed to return to its home cage

the threat zone. After 5 min in the threat zone, the safe zone porthole was opened and the intruder allowed up to 5 min to voluntarily return to its home cage. If an intruder did not move from one compartment to another after 5 min, the rat was placed into the adjacent compartment by the experimenter and the porthole closed.

Experiment 1: analysis of behaviors predictive of increased cocaine self-administration after social defeat

The aim of experiment 1 was to elucidate which behaviors during social stress were predictive of later augmented cocaine self-administration during the binge. Additionally, as individual differences in both saccharin drinking and open field locomotor activity have been associated with increased psychostimulant self-administration, we also investigated whether these factors were predictive of stress-escalated cocaine self-administration. Design for experiment 1 is shown in Fig. 1a, and a timeline shown in Fig. 2a.

Saccharin drinking and open field testing

Rats were randomly assigned to stressed ($n = 39$) or control ($n = 43$) groups and were tested for 0.02 % saccharin preference in a weekly 1-h two bottle choice procedure. Rats were also tested weekly for locomotor activity in an open field. Rats were removed from their homecage during the dark phase and placed in the center of a large open field (71 × 45 × 45 cm). After 30 min habituation, a novel object was placed into the arena and one 5-min video sample was recorded for novel object exploration. Total distance traveled throughout the habituation and novel object exploration was measured by Ethovision. Baseline measurements were performed 1 week prior to the social stress phase. Further testing occurred once per week throughout the stress period and 1 week after stress exposure (for additional details, see Electronic supplementary material).

Locomotor cross-sensitization to cocaine

Ten days after the final social defeat episode (day 31), rats were tested for cross-sensitization to acute cocaine challenge (Boyson et al. 2014; Covington and Miczek 2001).

Intravenous cocaine self-administration

Methods for cocaine self-administration have been previously described (Boyson et al. 2014; Holly et al. 2016). Timeline for self-administration methods is shown in Fig. 2a and additional details in Electronic supplementary material.

Rats were permanently implanted with an indwelling catheter (Silastic® silicon tubing, ID 0.63 mm, OD 1.17 mm) into the right jugular vein under ketamine (100 mg/kg) and xylazine (6 mg/kg) anesthesia. Rats were allowed to recover for at least 5 days, then were moved from their home cage to permanent housing inside cocaine self-administration chambers.

Rats were initially allowed to self-administer cocaine (0.75 mg kg^{-1} $infusion^{-1}$, administered 0.15 ml/kg at 0.018 ml/s) without priming or autoshaping on a fixed ratio 1 (FR1) schedule of reinforcement, followed by a 30-s timeout. Each daily session terminated after 15 infusions or 5 h. Once animals demonstrated reliable, stable responding at FR1, operationally defined as two consecutive days of maximal responding (15 infusions), the schedule was gradually advanced to FR5 to ensure rats were reliably performing for cocaine. Rats were maintained on a limited access FR5 schedule for at least five consecutive days. Rats were then subjected to three progressive ratio (PR) sessions (0.3 mg kg^{-1} $infusion^{-1}$), alternated with three FR5 sessions (0.75 mg kg^{-1} $infusion^{-1}$) across 6 days. The day after the final FR5 maintenance session, rats were given unlimited access to cocaine (0.3 mg kg^{-1} $infusion^{-1}$) for 24 h.

Experiment 2: Go/No-Go task

The aim of experiment 2 was to assess whether individual differences in impulsivity could explain the individual differences in behavior during social defeat stress. A separate cohort of rats underwent social defeat ($n = 21$) or control handling ($n = 11$) as described above and was subsequently tested using a modified version of the Go/No-Go task (Helms et al. 2008).

Each session of the Go/No-Go task ended after 60 trials. For each trial of the Go/No-Go task, there was a variable precue period (9–24 s), signaled by the house light, and responses were recorded but not reinforced. During the initial training phase, the precue period was followed by a 30-s "Go" cue (constant light above the Go hole). When the Go cue was displayed, the first nosepoke response resulted in the delivery of 0.07 ml 0.02 % saccharin. After rats successfully completed >30 trials within 1 h for four consecutive sessions, the cue duration was reduced from 30 to 10 s. After completion of >30 trials within 1 h for four consecutive sessions at 10 s, the cue light was reduced to 5 s.

After stable responding was again demonstrated, the "No-Go" cue (flashing cue light of a different color above the opposite nosepoke) was introduced on five consecutive sessions. When the No-Go cue was presented, rats must inhibit nosepoke responding during the cue. A nosepoke response (false alarm) terminated the trial and no saccharin was delivered. Go and No-Go trials were randomly ordered, with Go trials occurring 75 % of the time. After either trial type, a 10-s intertrial period occurred with all lights off and no reward available.

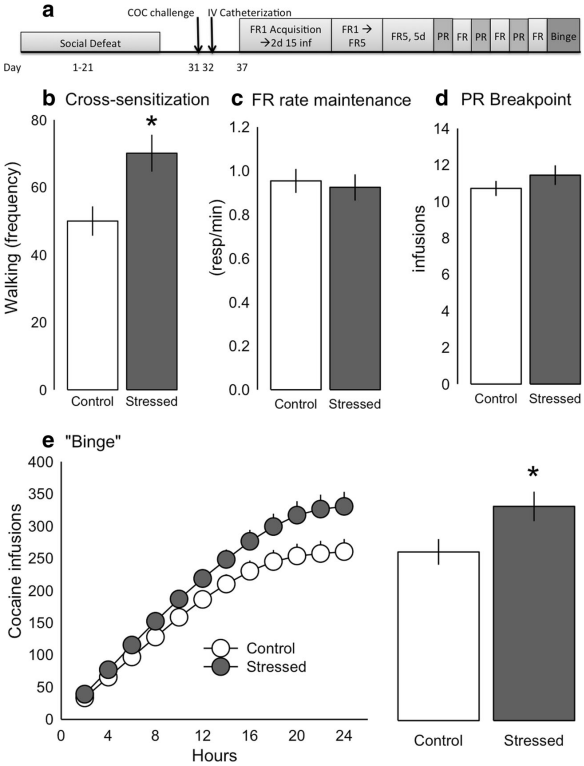

Fig. 2 Social defeat stress induces behavioral cross-sensitization to cocaine and escalated cocaine self-administration during a 24-h "binge." **a** Timeline for the cocaine self-administration methods. Animals were challenged with cocaine (10 mg/kg, ip) 10 days after the last social defeat to assess for behavioral cross-sensitization and were catheterized for intravenous cocaine self-administration the following day. After 5 days recovery, animals were initially allowed to self-administer cocaine (0.75 mg kg^{-1} $infusion^{-1}$) on a fixed ratio 1 (*FR1*) schedule until they obtained the maximal 15 infusions in two consecutive days. The FR schedule was then gradually increased to FR5, followed by 5 days FR5 maintenance. Three progressive ratio (*PR*) sessions were then alternated with three FR sessions across 6 days, and the experiment culminated in a 24-h binge. **b** Stressed rats ($n = 39$) had a significantly greater walking frequency in response to cocaine compared with non-stressed controls ($n = 43$), indicative of behavioral cross-sensitization. **c** There were no differences between stressed and control rats in mean response rate (responses/min) across the last three FR5 maintenance sessions **d** nor were there any differences in median breakpoint (defined as infusions self-administered) across the three PR sessions. **e** Stressed rats self-administered significantly more cocaine across the 24-h binge compared with controls. Data shown are mean ± SEM; $*p < 0.01$ vs. control

Experiment 3: BDNF and TrkB mRNA measurement

The aim of experiment 3 was to determine whether altered BDNF signaling in the hippocampus or prefrontal cortex (PFC) was an underlying mechanism behind individual differences in social defeat behavior. One hour after the last defeat, a separate group of rats ($n = 20$ control, $n = 20$ stressed) were anesthetized with isofluorane and rapidly decapitated for quantification of BDNF and TrkB mRNA through quantitative reverse transcription polymerase chain reaction (qRT-PCR, see Electronic supplementary material).

Experiment 4: CRF measurement

The aim of experiment 4 was to evaluate whether individual differences in social defeat behavior resulted in altered CRF expression in limbic regions associated with stress and reward. A separate cohort of rats ($n = 12$ control, $n = 12$ stressed)

was rapidly decapitated under isofluorane anesthesia 1 h after the final defeat to measure extrahypothalamic CRF content using a commercially available enzyme immune assay kit (EIA; Peninsula Laboratories, San Carlos, CA, see Electronic supplementary material).

Experiment 5: corticosterone measurement

Finally, experiment 5 aimed to determine whether individual differences in stress-escalated cocaine self-administration could be simply explained by differences in the physiological response to social defeat. A separate cohort of rats (control $n = 16$, stressed $n = 18$) was used to measure plasma corticosterone at baseline and 20 min following social defeat on days 1, 10, and 21. Blood was collected via tail vein puncture and centrifuged to obtain plasma. Corticosterone concentration (ng/ml) was determined using a commercially available EIA kit (Arbor Assays Detect X, Ann Arbor, MI). Standard curve range was 78.125–10000 pg/ml.

Statistical analysis

A principle component factor analysis was used to determine the relationship between specific behaviors during social defeat stress and subsequent cocaine self-administration (SAS, SAS Institute). To assess behaviors during aggressive encounters, the average latencies for the slow and fast groups were collapsed across the nine encounters and then were followed by a one-way analysis of variance (ANOVA). A two-way (group × time) repeated measures ANOVA was used to assess differences in body weight, saccharin drinking, and locomotor activity. Locomotor sensitization and IV cocaine self-administration were assessed by a one-way ANOVA. All post-hoc tests were analyzed by Holm-Sidak corrections for multiple comparisons. In experiment 2, a two-way (group × time) repeated measures ANOVA was used to assess precue, go hits, and false alarms (Sigma Plot version 11.0, Systat Software). All post-hoc tests were analyzed with Holm-Sidak corrections for multiple comparisons. In experiments 3–5, one-way ANOVAs were used to assess total BDNF and TrkB receptor mRNA and CRF content, while a two-way (group × time) repeated measures ANOVA was used to determine corticosterone secretion. A linear regression was used for correlational analyses of BDNF and average latency to enter into a threat zone as well as TrkB receptor mRNA and average latency to enter into a threat zone. All post-hoc tests were analyzed by Holm-Sidak corrections for multiple comparisons.

Results

Experiment 1: analysis of behaviors predictive of increased cocaine self-administration after social defeat

Rats with a history of social defeat stress showed behavioral cross-sensitization to cocaine, as measured by walking frequency (Student's $t = -2.826$, $df = 83$, $p = 0.006$, Fig. 2b) and self-administered significantly more cocaine under 24 h unlimited access binge condition (Student's $t = -2.328$, $df = 72$, $p = 0.023$, Fig. 2e). FR response rate (Fig. 2c) and PR breakpoint (Fig. 2d) were not affected by stress history.

Principal component analysis: latency to enter threat zone is predictive of stress-escalated cocaine self-administration

We next assessed whether any behaviors during social defeat (latency to enter the threat zone, fight duration, latency to leave fight, return to safe zone) were predictive of subsequent cocaine self-administration behavior (progressive ratio breakpoint, rate of fixed ratio maintenance, total binge infusions, binge duration). Principal component analysis (PCA) revealed two factors with an eigenvalue >1 (Fig. 3a). Five behavioral elements loaded onto factor 1; four of these behaviors were related to cocaine self-administration, and only one was related to the social defeat procedure ($r = -0.45$–0.90). The three other social defeat-related behaviors loaded onto separate factor with minimal cross-loading ($r = 0.41$–0.84). Based on the results from the PCA, latency to enter the threat zone was then used as a predictive factor to assess all subsequent behavioral and neurophysiological results. Differences in latency to enter the threatening zone emerged by the second defeat (Fig. 3b). To enhance contrast between groups, the upper and lower tails of the distribution for the latency measure were used to separate socially defeated rats, with the lower 33rd percentile termed "fast," and upper 33rd percentile termed "slow." The middle 33 % were removed from all further analyses (Fig. 3c).

Individual differences in latency to enter threat zone did not affect other defeat behavior

The fast rats had significantly lower latencies to enter the threat zone compared with the slow rats (Student's $t = 14.328$, $df = 24$, $p < 0.001$), however, these groups did not differ in fight duration, latency to escape the fight, or latency to return to the safe zone (Table 1). Furthermore, there were no significant differences between fast and slow rats on specific behaviors before, during, or after the fight period (see Electronic supplementary material).

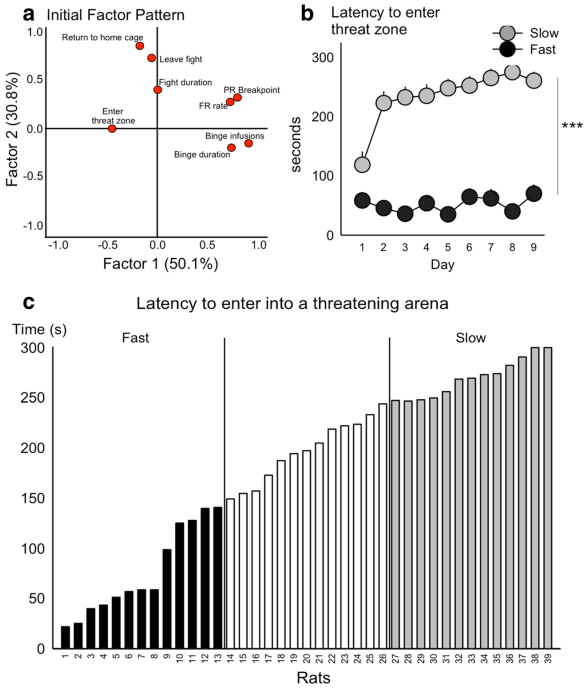

Fig. 3 Principle component analysis (*PCA*) reveals latency to enter threat zone is correlated with cocaine self-administration behavior. **a** Two factors were extracted from the PCA; factor 1, on the *x*-axis, accounts for 50.1 % of the total variance, while factor 2, on the *y*-axis, accounts for 30.8 % of the total variance. Latency to enter the threat zone was the only behavioral component of social defeat that loaded onto the factor with cocaine self-administration variables. **b** Latency to enter the threat zone significantly differed between "fast" (*black*, $n = 13$) and "slow" (*gray*, $n = 13$) rats across the nine social defeats, and group differences emerged by the second defeat. **c** Mean latency to enter the threat zone across nine social defeat encounters was a continuous distribution, and with the upper 1/3 ($n = 13$) of the distribution considered slow and the lower 1/3 ($n = 13$) of the distribution considered fast. Data shown are mean ± SEM. ***$p < 0.001$

Individual differences in latency to enter the threat zone do not affect body weight gain, saccharin preference, and open field activity

Body weight Overall, in regard to body weight (g, Fig. 4a), there was a significant main effect of week (two-way repeated measures ANOVA $F_{4,\ 264} = 516.630$, $p < 0.001$), with a significant main effect of group (control, fast, and slow, $F_{2,\ 66} = 3.874$, $p = 0.026$) and week × group interaction ($F_{8,\ 264} = 5.689$, $p < 0.001$). Post-hoc analyses revealed no significant group differences in body weight at baseline. When collapsed across all weeks tested (baseline, weeks 1–3 of stress, and 1 week post-stress), fast rats weighed significantly less than both controls (Holm-Sidak $t = 2.575$, $p = 0.036$) and slow stressed rats (Holm-Sidak $t = 2.425$, $p = 0.036$). However, when data were normalized to percent of baseline for each rat, there were no significant differences in weight gain across groups (Fig. 4b).

Saccharin drinking There were no significant differences between fast- and slow-defeated rats and non-stressed

Table 1 Latencies of behaviors in "slow" (bottom 33 %, $n = 13$) and "fast" (top 33 %, $n = 13$) rats

	Slow	Fast
Latency to leave home cage prior to fight (s)	269.6 ± 5.4	75.9 ± 12.2*
Fight duration (s)	131.8 ± 17.6	126.0 ± 20.7
Latency to escape fight (s)	32.8 ± 9.4	31.4 ± 4.0
Latency to return to home cage after fight (s)	74.0 ± 9.9	72.2 ± 18.0

All values are means ± SEM

*$p < 0.001$ slow vs. fast

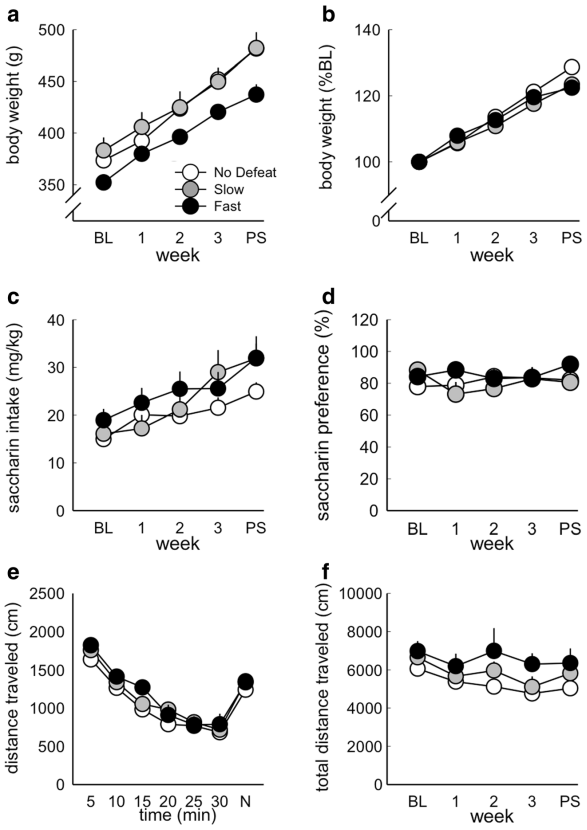

Fig. 4 Latency to enter the threat zone does not affect body weight gain, saccharin preference, or open field activity. **a** Body weight (g) increased in all groups from baseline (*BL*), through the 3 weeks of social defeat stress, to 1 week post-stress (*PS*), although overall, "fast" (*black circles*, $n = 13$) rats weighed significantly less than "slow" (*gray circles*, $n = 13$) and control (*white circles*, $n = 43$) rats (*$p < 0.05$). **b** Percent change in body weight from baseline (*%BL*) did not differ between groups. **c** Saccharin intake (mg/kg body weight) increased from BL to the 1 week PS in all groups, but no differences in saccharin consumption between groups were observed. **d** Saccharin preference (%, saccharin intake/(saccharin intake + water intake) also did not differ between groups. **e** On the first day of baseline open field testing, distance traveled (cm) in 5 min bins decreased across the 30-min habituation period but did not differ between groups. Insertion of a novel object (*N*) into the open field caused increased locomotor activity, but distance traveled did not differ between groups. **f** Average locomotor activity at BL, during each of the 3 weeks of stress, or PS did not differ across groups. Data shown are mean ± SEM

controls in saccharin preference (Fig. 4d). However, there was a significant main effect of day for total saccharin intake (two-way repeated measures ANOVA $F_{1, 62} = 24.118$, $p < 0.001$, Fig. 4c), suggesting all groups increased total consumption across time.

Open field There was a significant main effect of time on total locomotor activity in an open field during the first session of testing ("baseline", two-way repeated measures ANOVA $F_{5, 283} = 124.673$, $p < 0.001$), but no effect of group (Fig. 4e). There was a significant main effect of day on total distance traveled across the five weeks of testing (Two-way repeated measures ANOVA $F_{4, 231} = 3.350$, $p = 0.011$; Fig. 4f), suggesting no locomotor activity deficits in slow rats.

Individual differences in latency to enter the threat zone affect cocaine self-administration

Cross-sensitization to cocaine Escapable intermittent social defeat stress increased locomotor activity in response to an acute cocaine challenge. There was a significant main effect of group (control, fast, and slow, one-way ANOVA $F_{2, 64} = 3.846$, $p = 0.026$, Fig. 5a), with stressed groups exhibiting significantly increased walking frequency in response to cocaine compared with controls (Holm-Sidak $t = 2.715$, $p = 0.008$).

Acquisition/maintenance There were no significant differences in acquisition among the fast, slow, and control groups. However, once rats acquired self-administration,

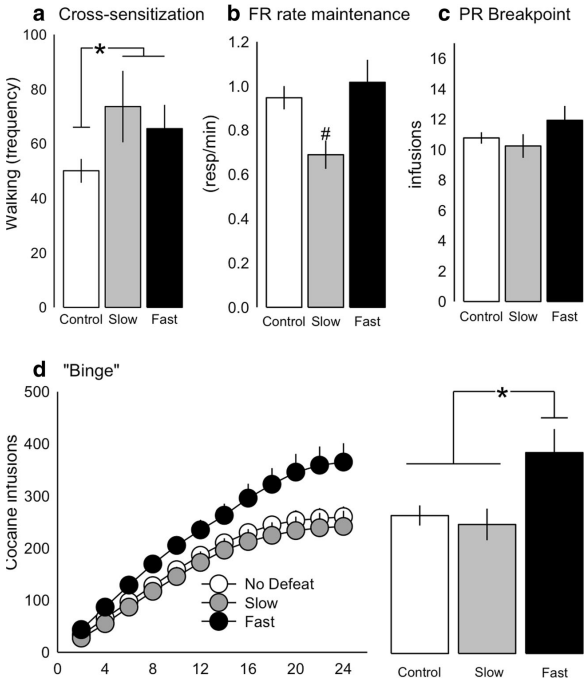

Fig. 5 Latency to enter the threat zone was predictive of cocaine self-administration. **a** Both "fast" ($n = 13$) and "slow" ($n = 13$) rats showed stress-induced cross-sensitization to cocaine compared with controls ($n = 43$) as assessed by walking frequency after cocaine challenge (10 mg/kg, ip). **b** Rate of cocaine self-administration (responses/min) during fixed ratio (*FR*) was lower in slow rats compared with fast rats. **c** There was no effect of group on breakpoint (number of infusions obtained) in a progressive ratio (*PR*) schedule of reinforcement. **d** Cumulative cocaine infusions self-administered across a 24-h period was significantly lower in slow rats compared with fast rats (*left*) with fast rats self-administering significantly more total cocaine infusions compared with both slow rats and non-defeated controls (*right*). Data shown are mean ± SEM. $^{*}p < 0.05$ vs. non-defeated controls; $^{\#}p < 0.05$ vs. "fast" rats

there was a significant main effect of group on the average rate of cocaine self-administration during the last 3 days of maintenance (one-way ANOVA $F_{2, 65} = 3.896$, $p = 0.025$). Post-hoc analyses revealed a significantly lower response rate in the slow-defeated rats compared with the fast-defeated rats (Holm-Sidak $t = 2.544$, $p = 0.039$; Fig. 5b) and non-defeated controls (Holm-Sidak $t = 2.475$, $p = 0.032$).

Progressive ratio There were no significant differences among the fast, slow, or non-defeated control rats on measures of motivation as assessed by total cocaine infusions (breakpoints) under a PR schedule of reinforcement (Fig. 5c).

Twenty-four-hour binge There was a significant main effect of group (one-way ANOVA $F_{2, 62} = 5.117$, $p = 0.009$) on the number of cocaine infusions self-administered during a 24-h continuous access binge. Post-hoc analyses fast rats self-administered significantly more infusions than both the slow-defeated rats and non-defeated controls (Holm-Sidak $t = 2.766$, $p = 0.015$; $t = 2.965$, $p = 0.013$, respectively; Fig. 5d).

Experiment 2: Go/No-Go task

Individual differences in latency to enter the threat zone do not affect impulsivity as measured by the Go/No-Go task

No significant differences between the fast, slow, and non-defeated control groups were found in any of the impulsive-like measures in the Go/No-Go task (precue responses, hits, and false alarms), suggesting that fast latency to enter the threat zone is not indicative of motor impulsivity (Fig. 6).

Experiment 3: BDNF and TrkB mRNA measurement

Individual differences in latency to enter the threat zone is associated with altered hippocampal BDNF and TrkB mRNA transcription

There was no significant effect of group on BDNF or TrkB mRNA in the prefrontal cortex, but there was a significant effect of group on BDNF and TrkB mRNA within the hippocampus. Hippocampal BDNF was significantly

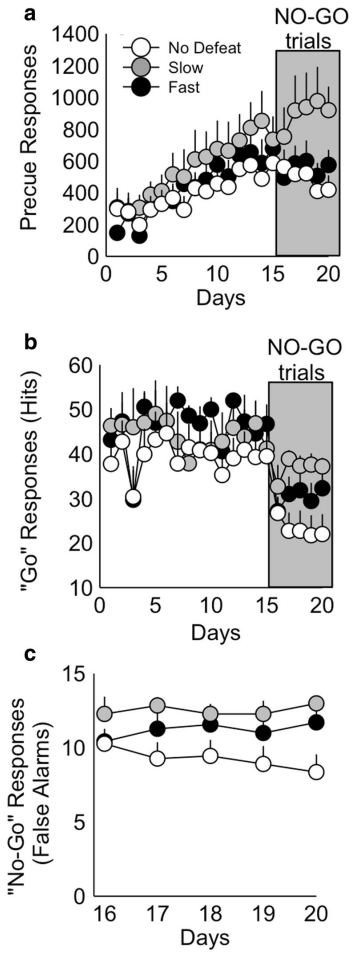

Fig. 6 Latency to enter the threat zone did not significantly affect impulsivity as measured by the Go/No-Go task. **a** Precue responses during training (days 1–15) as well as No-Go testing (*shaded box*, days 16–20) for fast (*black circles*, $n = 7$), slow (*gray circles*, $n = 7$), and non-defeated controls (*white circles*, $n = 11$) did not significantly differ. **b** Go "hits" during the training phase (days 1–15) and Go/No-Go phase (days 16–20, *gray box*), did not significantly differ between groups. **c** False alarms during the Go/No-Go phase (days 16–20) also did not differ between groups. Data shown are mean ± SEM

affected by group (one-way ANOVA $F_{2,\ 29} = 4.178$, $p = 0.025$), with fast rats expressing significantly more BDNF mRNA in the hippocampus relative to slow rats ($p = 0.023$). Similarly, hippocampal TrkB mRNA was affected by group ($F_{2,\ 29} = 3.534$, $p = 0.042$), with fast rats again expressing significantly more hippocampal TrkB mRNA relative to slow rats ($p = 0.037$). There was a significant negative correlation between average latency to enter the threat zone and hippocampal BDNF ($r = 0.621$, $p = 0.003$) and TrkB (0.660, $p = 0.002$) mRNA (Fig. 7).

Experiments 4 and 5: CRF and corticosterone measurement

Individual differences in latency to enter the threat zone do not affect the physiological stress response

Corticosterone Social stress resulted in a group x day interaction in plasma corticosterone 20 min after defeat ($F_{3,\ 94} = 5.93$, $p < 0.001$). This difference appears to be a result of increased corticosterone in stressed rats limited to Day 1. Fast and slow rats did not differ in the corticosterone response to stress (Fig. 8a).

Extrahypothalamic CRF There was no effect of group on CRF content in the nucleus accumbens (NAc), amygdala, or ventral tegmental area (VTA). However, there was a main effect of brain region regardless of group (two-way repeated measures ANOVA $F_{2,\ 33} = 22.262$, $p < 0.001$), with significantly more CRF in the amygdala and VTA compared with the NAc (Holm-Sidak $t = 6.488$, $p < 0.001$; $t = 4.580$, $p < 0.001$, respectively; Fig. 8b).

Discussion

Through dissecting the cluster of behaviors involved in social defeat stress, we have shown that latency to enter into a threatening zone is highly predictive of the subsequent development of stress-escalated cocaine self-administration. Rats with a short latency to enter a threatening environment ("fast" group) self-administer cocaine at a higher rate and accumulate more cocaine infusions during a 24-h binge compared with rats with a long latency to enter the threatening environment ("slow" group). Importantly, separating animals based upon this measure did not result in significant differences in saccharin preference, open field locomotor activity, or motor impulsivity, all of which have been linked with increased rates of psychostimulant self-administration (Belin et al. 2008; Carroll et al. 2002; Piazza et al. 1989). This indicates that latency to enter a threatening environment is a distinct individual difference in behavior related to escalated self-administration. Furthermore, differences in cocaine self-administration between fast and slow rats are not the result of altered behavioral (immobility, reaction to attack bite) or physiological (CRF, corticosterone) responses to social defeat

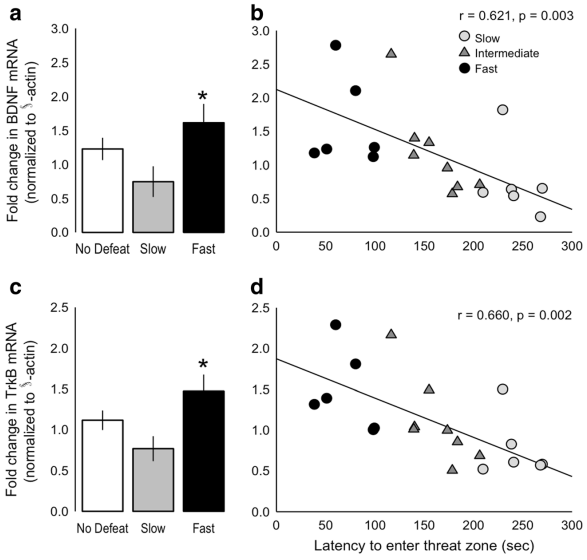

Fig. 7 Latency to enter the threat zone was associated with altered hippocampal BDNF and TrkB mRNA. **a** "Fast" ($n = 7$, *black*) rats showed significantly greater hippocampal BDNF mRNA expression compared with "slow" ($n = 7$, *gray*) rats and non-defeated controls ($n = 20$, *white*), **b** resulting in a significant negative correlation between latency to enter the threat zone and hippocampal BDNF mRNA (intermediate 1/3 ($n = 7$) of defeated rats shown as *gray triangles*). **c** Similarly, fast rats showed significantly greater hippocampal TrkB mRNA expression compared with slow rats, **d** which also resulted in a significant negative correlation between latency to enter threat zone and hippocampal TrkB mRNA. Data shown are mean ± SEM. $^{*}p < 0.05$ vs. slow

stress. Finally, we demonstrate that fast and slow rats show altered BDNF and TrkB transcription in the hippocampus, which may be an underlying mechanism for the persistent neuroadaptations engendered by social defeat stress which promote escalated cocaine self-administration.

Individual differences in factors that promote enhanced cocaine self-administration in rodents have received considerable attention. Of note, high preference for and locomotor reactivity to a novel environment, preference for sucrose, and impulsivity have all been associated with increased psychostimulant self-administration (Belin et al. 2008; Carroll et al. 2002; Piazza et al. 1989). The current series of experiments complement this growing literature, indicating that a consistent decision to rapidly and voluntarily enter into a threatening social environment, despite known consequences, is also highly predictive of intensified cocaine self-administration. Differences between rats fast and slow to approach social threat emerge by the second social defeat encounter and are sustained through the 21 days of social stress. Importantly, this maladaptive choice by some stressed rats is distinct from other previously studied innate traits associated with subsequent maladaptive behavior.

As a motor behavior, rapid approach to social threat could be function of high-responder (HR) and low-responder (LR) rats, which has been established as a highly predictive trait for faster acquisition of psychostimulant self-administration (Kabbaj et al. 2001; Piazza et al. 1989). Innate traits, such as HR or increased locomotor behavior in a novel open field environment, are highly associated with acquisition rates of cocaine self-administration, although there have been no reported effects on other measures of psychostimulant self-administration (Bardo et al. 2013; Blanchard et al. 2009; Piazza et al. 1989). Although the current study used different methods from previous HR/LR experiments, fast and slow rats showed no significant differences in reactivity to a novel open field and novel object exploration, as assessed by total distance traveled. This suggests that latency to enter a threatening environment may be a distinct trait from HR and LR phenotypes, although future work should assess this in a circular arena using methods initially promoted by Piazza et al. (1989). Importantly, however, differences in general locomotor behavior and locomotor activity in response to a novel object cannot explain differences in latency to enter the threat zone exhibited by fast and slow rats.

An alternative explanation for the behavioral differences observed between fast and slow rats is individual differences in motor impulsivity, also established as a predisposing factor for the transition from initial cocaine self-administration to escalated, compulsive self-administration (Belin et al. 2008; Murray et al. 2014). However, following social defeat stress, fast and slow rats did not differ in impulsivity from non-stressed controls in the Go/No-Go task. In contrast to motoric impulsivity, fast and slow rats may be exhibiting increased and decreased levels of social impulsivity, as exhibited by

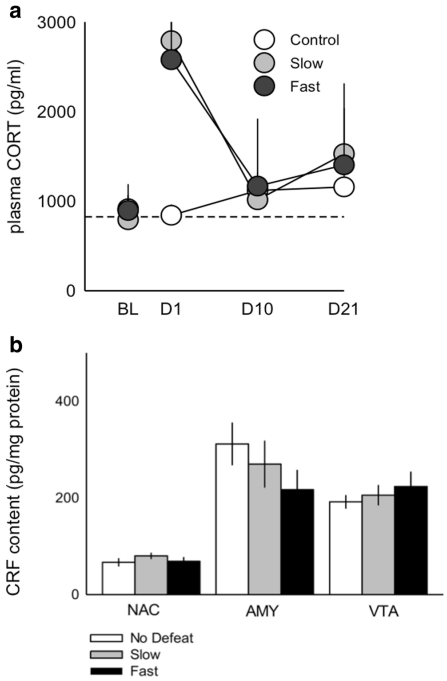

Fig. 8 Latency to enter the threat zone was not associated with altered physiological responses to stress. **a** Plasma corticosterone (*CORT*) did not differ between groups at baseline (*BL*), and while there was a significant effect of stress on the first day of defeat (*D1*), there was no difference between "slow" ($n = 6$, *gray*) and "fast" ($n = 6$, *black*) groups (control $n = 16$, *white*). There was no effect of stress on plasma corticosterone on day 10 or 21. **b** Total CRF content (pg/mg protein) in the nucleus accumbens (*NAc*), amygdala (*AMY*), and ventral tegmental area (*VTA*) also did not differ between groups (control $n = 12$, fast $n = 4$, slow $n = 4$). Data shown are mean ± SEM

consistent low or high latency to approach a novel stimulus animal despite possible negative consequences.

Furthermore, we have demonstrated that fast and slow rats do not differ in two physiological measures of responsivity to social defeat stress, namely corticosterone secretion and extrahypothalamic CRF concentration. Although these physiological measures are not necessarily direct correlates of the amount of stress the animals experience and the single time point measurement cannot rule out differences in HPA axis negative feedback, they indicate that fast and slow rats may generally experience the same level of stress. This is significant because it precludes the possibility that fast rats self-administer more cocaine due to an increased physiological stress response and points to the important role of other factors, such as decision-making, altered cocaine responsivity, or altered hippocampal BDNF/TrkB signaling.

Latency to enter a threatening zone was positively correlated with hippocampal BDNF and TrkB (Fig. 5). This is consistent with other work demonstrating individual differences are regulated by hippocampal BDNF signaling. Greater hippocampal BDNF is associated with the promotion of resilience to anhedonia-like symptoms induced by chronic stress (Bergstrom et al. 2008; Duclot and Kabbaj 2013; Taliaz et al. 2011). Duclot and Kabbaj (2013) report that altered BDNF signaling is a key mechanism underlying individual differences in vulnerability to chronic stress; enhanced hippocampal BDNF signaling promotes resilience, whereas disruption promotes vulnerability to chronic social defeat-induced anhedonia-like behavior.

In the present series of experiments, as opposed to investigating individual differences in anhedonic-like behavior after chronic stress, we focused on individual differences in hedonic-like behavior (i.e., cocaine self-administration) following intermittent social defeat, which does not induce an anhedonic-like phenotype (Miczek et al. 2011). We demonstrate that BDNF and TrkB transcription is altered within the slow group, resilient to the hedonic-promoting effects of intermittent social defeat, but remains equivalent to controls in the fast or vulnerable group. BDNF/TrkB signaling may be functioning similarly to promote resilience to the divergent behavioral effects of chronic and intermittent stress. BDNF facilitates synaptic plasticity and enhances postsynaptic responsivity (Cordeira et al. 2010; Fortin et al. 2012; Madara and Levine 2008; Park and Poo 2013), so enhanced hippocampal BDNF/TrkB signaling observed in "resilient" rats may promote synaptic and behavioral adaptations after stress. We initially investigated hippocampal BDNF due to the well-described role of hippocampal plasticity in response to stress and notable glucocorticoid sensitivity (McEwen 1999), but it will be important to evaluate BDNF and TrkB in other limbic brain regions associated with drug self-administration, particularly the VTA and NAc. Additionally, in the current experimental design, it is unclear whether BDNF signaling is causal or resultant of individual differences in defeat, as individual differences in latency to enter the threatening zone by definition cannot be ascertained until after defeat experience.

This is the first study to our knowledge to thoroughly explore the full repertoire of specific behaviors involved in social defeat stress and link them with later cocaine self-administration. Understanding behavioral indices of stress associated with later maladaptive behaviors, as well as neurobiological differences between vulnerable and resilient subjects, may lead to more targeted and effective therapeutic interventions for stress-related disorders, such as addiction or depression. Ultimately, we propose that individual differences in latency to enter into a threatening environment are highly predictive of subsequent stress-related maladaptive behavior. Future

work will continue to explore how this particular aspect of social defeat stress plays a role in escalated cocaine self-administration.

Compliance with ethical standards

Conflicts of interest All authors declare no conflicts of interest.

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