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Role of Intracellular Calcium on Ethanol-Induced Activation of Protein Kinase A: Molecular Model and Behavioral Consequences

Doctoral Thesis

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A mis padres y hermanos

A Kiran

El Doctor D. Carlos Manuel González Aragón, Catedrático de Psicobiología del Departamento de Psicología Básica, Clínica y Psicobiología de la Universitat Jaume I

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Abbreviation list

5-HT*5-Hydroxytryptamine*

A2a Adenosine A2a receptor

ACh *Acetylcholine*

AChR *Acetylcholine receptors*

aPKCs *Atypical protein kinase C*

Ca^{2+} *Calcium*

CaM, *Calmodulin*

CaMK *Ca^{2+} calmodulin kinase*

cAMP *Cyclic adenosine monohosphate*

Ca_Vs *Voltage-dependent Ca^{2+} channels*

CBP *CREB binding protein*

CICR *Ca^{2+}-induced Ca^{2+} release*

CNS *Central nervous system*

cPKCs *Conventional protein kinase C*

CRE *cAMP response elements*

CREB *cAMP response element binding protein*

DAG *Diacylglycerol*

DARPP-32 *Dopamine- and cAMP regulated-phosphoprotein*

DMPX *3,7-dimethyl-1-propargylaxanthine*

EGTA *Ethylene glycol tetraacetic acid*

ER *Endoplasmic reticulum*

GABAA *γ-Aminobutyric acid type A receptors*

GAD *Glutamic acid decarboxylase*

h *Hours*

i.p. *Intraperitoneal*

IC_{50} *Inhibitor concentration 50*

InsP3 *Inositol-1, 4, 5-trisphosphate*

$InsP_3R$ *Inositol-1, 4, 5-trisphosphate receptor*

Kd *Dissociation constant*

kDa *Kilodaltons*

K_vs *Voltage-dependent potassium channels*

LGICs *Ligand gated ion channels*

MiCa *Mitochondrial Ca^{2+} channel*

mM *Millimolar*

NAADP *Nicotinic acid adenine dinucleotide phosphate*

NAc *Nucleus accumbens*

nACh *Nicotinic acetylcholine*

Na_vs *Voltage-dependent sodium channels*

nm *Nanomolar*

NMDA *N-methyl-D-aspartate glutamate*

nPKCs *Novel protein kinase C*

min *Minutes*

PA *Phosphatidic acid*

PC *Phosphatidylcholine*

PC12 *Pheochromocytoma*

pCREB Phospho *CREB*

PDE *Phosphodiesterase*

PDK1 *Phosphoinositide-dependent kinase 1*

PEth *Phosphatidylethanol*

PIP_2 *Phosphatidylinositol 4,5-bisphosphate*

PKA *cAMP dependent protein kinase*

PKC *Protein kinase C*

PLD *Phospholipase D (PLD)*

PMCA *Plasma membrane Ca^{2+} ATPase*

PTKs *Protein Tyrosine Kinases*

RACK *Receptor for Activated C Kinase*

RyR *Ryanodine receptor*

SERCA *Sarco(endo)plasmic reticulum Ca^{2+}-ATPase*

SR *Sarcoplasmic reticulum*

VOCs *Voltage operated channels*

VTA. *Ventral Tegmental Area*

µM *Micromolar*

W7 *N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide*

TABLE OF CONTENTS

I- INTRODUCTION

CHAPTER 1 - Ethanol

I- INTRODUCTION

1
Ethanol

Background

Ethyl alcohol (ethanol) abuse, and problems related to excessive ethanol consumption vary widely around the world. The World Health Organization (WHO) "*Global Status Report on Alcohol* " (2004 and 2009) estimates that 76 million people worldwide have diagnosable ethanol-abuse disorders. Ethanol is a causal factor in 60 types of diseases and injuries and a component cause of 200 others. Almost 4% of all deaths worldwide are attributed to ethanol, which is greater than the number of deaths caused by HIV/AIDS, violence or tuberculosis. Ethanol abuse is also associated with many serious social issues, including violence, child neglect, and workplace absenteeism. While ethanol abuse has deep social and economic impacts, and exerts a wide range of psychopharmacological effects through its interaction with numerous elements on the CNS, to date research has failed to identify a unique cellular target of ethanol or molecular mechanism of action. Despite the scale of the problem, there are a few pharmacological treatments for alcohol abuse disorders. Thus, the understanding of the mechanisms by which ethanol alters the CNS function is critical for a rational approach to the development of new medications.

One reason for the difficulty in identifying a central mechanism of ethanol or a specific ethanol receptor is the low binding affinity of ethanol to proteins. Ethanol is distributed into total body water, so that alcohol concentrations in all organs and cellular compartments are similar to the concentration in the blood. Alcohol can dramatically affect both the structure and function of a wide variety of cellular proteins and, ultimately, complex biological activities such as neurotransmission and immune responses.

At high concentrations, ethanol can modify membranes and cause nonspecific changes in neural activity; however, more interestingly, it has

specific interactions with proteins that occur at physiologically relevant concentrations. At this level, current research suggests that the neurobiological response to ethanol involves different hormone- and neurotransmitter-dependent signaling transduction cascades that may explain some of ethanol's acute and chronic changes in neuronal function. For example, it has been described that at a very high concentrations (> 0.5 M) ethanol elicits changes in the protein structure ranging from promoting protein stability to protein denaturation (Dwyer and Bradley, 2000). Conversely, at low concentrations (1-80 mM), ethanol has been shown to modify the function of different ion channel proteins (Messing et al., 1986) neurotransmitter receptors (Bradley et al., 1980), enzymes (Chen and LaBella, 1997) and adhesion molecules (Charness et al., 1994).

1. Cytoplasmic ethanol targets

Drugs of abuse have very different acute mechanisms of action but all converge on the brain's reward pathways by producing a series of common functional effects after both acute and chronic administration. The behavioral abnormalities associated with the development of addictive behavior are a result of different stable and (in some cases) irreversible cellular and molecular structural changes in the brain. This implies a "molecular and structural switch" from controlled drug intake to compulsive drug abuse (Spanagel and Heilig, 2005; Lee and Messing, 2008). In this respect, recent genetic analysis has identified some common molecular pathways that contribute to changes in synaptic function. Some of these activated signaling cascades seem to be pivotal in the neurobiological effects of ethanol.

1.2 Dopamine- and cAMP regulated-phosphoprotein (DARPP-32)

The 32 kDa, dopamine- and cAMP regulated-phosphoprotein (DARPP-32) is described as an important downstream modulator of dopaminergic signaling, however, it has been proposed as an important contributor to ethanol mechanism of action through the modulation of NMDA receptors. Ethanol inhibition of NMDA receptors is thought to participate as a mediator of the intoxicating/sedative effects of ethanol (Lovinger et al., 1989). To the contrary it has been proposed that the activation of NMDA receptors participate prominently on ethanol reward and reinforcement (Biala and Kotlinska, 1999). This apparent contradiction is better explained by the fact that NMDA receptors present different sensitivity to the inhibition effects of ethanol depending on the brain area. DARPP-32 is abundant in striatal neurons with abundant dopaminergic inputs. When DARPP-32 becomes phosphorylated at Thr-34 it becomes a potent inhibitor of the serine/threonine phosphatase PP1. The action of phospho-DARPP-32 inhibiting the action of PP1 promotes the activation of NMDA that remain phosphorylated on NR1. This increased phosphorylation of NMDA counteracts the inhibition of the receptor function by ethanol. At the behavioral level, DARPP-32 knockout mice presented decreased ethanol self-administration did not conditioned for ethanol-induced place preference. To the contrary, presented enhanced locomotor stimulation and ethanol-induced conditioned taste aversion (Risinger et al, 2001).

1.3 Fyn protein

Fyn element is a member of the Src protein tyrosine kinase family. This family of PTKs is capable of communicating with a large number of different cellular receptors and many distinct cellular targets. Is expressed throughout the brain, with moderate expression in the amygdala, NAc,

VTA. Fyn is composed of six domains and constitutively inactive. This state is maintained by a Tyr-527 phosphorylation autoinhibitory mechanism that blocks the catalytic region of Fyn. Activation of Fyn involves dephosphorylation of Tyr-527 and other Tyr-416 autophosphorylation. Interestingly, Fyn activation is commonly measured as an increase in autophosphorylation at Tyr-416.

Fyn kinase modulates ethanol neurobehavioral effects through an NMDA-dependent mechanism. As we have commented above, ethanol has an inhibitory effect on NMDA receptor-mediated excitatory postsynaptic potentials. This effect is counteracted by the phosphorylation of the NR2B subunit that enhances NMDA receptor activity. Fyn kinase is forming a complex with the scaffolding protein RACK1and NMDA NR2B. Thus, RACK1 protein acts inhibiting the Fyn-induced phosphorylation of NR2B. The dissociation of this complex promoted by ethanol is the responsible of the NR2B subunit phosphorylation by Fyn and thereby increase NMDA activity. In animals lacking Fyn kinase do not show acute tolerance to ethanol. These animals show an increased response to the hypnotic effects of ethanol, as measure by the loss of righting reflex (Miyakawa et al., 1997). Interestingly, a human Fyn single nucleotide polymorphism (T-to-C change) exist that significant correlates with increased ethanol consumption when compared to control subjects (Schumann et al., 2003).

Despite the fact that the functional consequences of such polymorphism are still poorly understood, Fyn expression is thought to be affected.

1.4 Protein kinase C (PKC)

Protein kinase C (PKC) is a family of serine-threonine phospholipid-dependent protein kinases encoded by nine genes, all of which are expressed in the central nervous system with isozyme-specific patterns of

expression (Lee and Messing, 2008). This family can be classified into three subclasses based on differences in structure and activation by second messengers. Conventional PKCs (α, β, γ), novel PKCs (δ, ε, η, θ) and atypical PKCs (ζ, ι/λ). The first group, conventional PKCs (cPKCs) is activated by DAG and Ca^{2+}. The second group novel PKCs (nPKCs); is activated by DAG but not Ca^{2+}. The third, atypical PKCs (aPKCs) are insensitive to DAG or Ca^{2+}but are activated by other lipid such as arachidonic and phosphatidic acid. Different elements are involved in this pathway. The hydrolysis of phosphatidylinositol-4,5-bis-phosphate by phospholipase C generates IP3 and DAG. The intracellular stored Ca^{2+} is release by the action of IP3 that can synergize with DAG to activate conventional PKCs. This family of protein kinases presents regional brain distribution. PKC γ is highly expressed in the frontal cortex and hippocampus, PKC in thalamus, PKC βII and PKC ε are found widespread throughout the brain (Newton and Messing, 2006). Conversely, PKC θ has not yet been found in the brain. PKC activation is dependent on different factors: autophosphorylation, membrane association, and second messenger binding (Mochly-Rosenand and Gordon, 1998; Liu and and Heckman, 1998). While these factors may vary for the different PKC isozymes, there are certain key principles that are thought to be critical to all. PKCs must itself be phosphorylated at two or three sites within its kinase domain. Thus it requires phosphorylation by PDK1 and subsequent autophosphorylation in the catalytic domain to be fully active. Furthermore, the activation of PKC isozymes is associated with their translocation to a specific subcellular compartment where they interact with their substrates after associating with some specific anchoring proteins (Schechtmanand and Mochly-Rosen, 2001).

All the different isoforms of PKC have been pointed out as key mediators of the acute and chronic effects of ethanol.

1.4.1 cPKCs α, β, γ

Different experiments *in vitro* have suggested a brain region specific effect of ethanol on signaling mediated by these PKCs. It is known that PKCα, is the conventional PKC least studied in terms of its contribution to ethanol neurobehavioral effects either in vitro or in animal model. However, some authors have assessed the effects of ethanol administration in vivo on PKCα and γ in several brain regions. Animals exposed to a liquid diet of ethanol (5% v/v) for several days, demonstrated a time effect within these PKCs expression on different brain areas. Thus, the abundance of membrane-associated PKCα and γ was modified after ethanol exposure by increasing 10-20% in the hippocampus and reduced by 20-25% in the frontal cortex.

With regard to PKC β, ethanol exposure to cell cultures, resulted in a blockade of PKC βII translocation due to the mislocalization of the PKC βII scaffolding protein, RACK1 (Ron et al., 2000). Moreover, Kumar and cols, 2006 reported that systemic ethanol administration (2 g/kg. i.p.) to rat, caused an acute increase in the abundance of membrane associated

PKCβ in the cortex, that rose above 144% of control levels by 1 h. In contrast, the membrane bounded PKCβ, the cytosolic PKCβ was not modified within the same experimental conditions. This suggests that under these circumstances, ethanol treatment causes a very rapid increase in the cytosolic synthesis of PKCβ or attenuates the degradation of membrane-associated PKCβ within the areas of the study (Kumar et al., 2006).

At the behavioral level, the role of PKCα and PKCβ on ethanol neurobehavioral effects, has not been yet examined in detail. However, PKCγ knockout mice have been extensively studied due to their key role on mediating the ethanol behavioral effects. Thus, these mice show significantly altered behavioral responses to ethanol, such as decreased sensitivity to the hypnotic and anxiolytic effects of ethanol (Harris et al., 1995; Bowers et al., 2001), increased sensitivity to activating doses of ethanol (Hix et al., 2003), increased ethanol preference and impulsivity (Bowers and Wehner, 2001).

It has been proposed that these behavioral traits are mediated by an altered regulation of GABAA receptors. This has been demonstrated in PKCγ-Knockout mice tissue preparations that did not present the same GABAA response to ethanol when compared to its control (Harris et al., 1995; Proctor et al., 2003). Interestingly, this reduced sensitivity of GABAA receptors appears to be specific for ethanol since biochemical and behavioral measures of sensitivity to different positive allosteric modulators of GABAA such the benzodiazepines are unchanged in mice lacking PKCγ (Harris et al., 1995).

1.4.2 nPKCs (δ, ε)

In different cell preparations such as the neuroblastoma-glioma hybrid cell line NG108-15, chronic ethanol exposure induces translocation of PKCδ and PKCε to intracellular compartments in a PKA-dependent complex with εRACK (Gordon et al., 1997; Yao et al., 2008). Moreover, in PC12 primary cultures, chronic ethanol exposure ranging from 50 to 200 mM produced an increase in the density of δ and ε PKC isoforms (Messing et al., 1991). These two isoforms have also been involved in the ethanol-induced regulation of voltage-dependent Ca^{2+} channels (Ca_vs). In PC12 cell, chronic

ethanol treatment up-regulates the L-type Ca_vs density by a PKCδ dependent mechanism (Gerstin et al., 1998). Moreover, up-regulation of PKCε is required for ethanol-induced increase in the abundance of N-type Ca_vs and the nerve growth factor-induced neurite outgrowth in PC12 cells (McMahon et al., 2000; Hundle et al., 1997).

At the behavioral level, effects of ethanol in mice lacking PKCε have been extensively studied compared to those dependent of PKCδ. Different authors have found that the behavioral effects of ethanol are substantially altered in mice lacking PKCε. The loss of righting reflex is reduced in PKCε knockout mice (Hodge et al., 1999), acute functional tolerance to the motor-impairing effects of ethanol is reduced in PKCε null mice when compared with its control wild-type littermates (Wallace et al., 2007). These mutant mice, also consume approximately 75% less ethanol and show reduced ethanol preference compared with wild-type mice in a 2-bottle choice paradigm (Hodge et al., 1999). Furthermore, mice lacking this PKC isotype showed reduced anxiety-like behavior, in both elevated plus-maze and open-field paradigms. These animals also presented a reduction in corticosterone and adrenocorticotrophic hormone levels (Hodge et al., 2002). At this respect, the mechanism by which PKCε regulates responses to ethanol may, parallel to those observed in PKCγ, be related to effects in GABAA receptors (Newton and Messing, 2006; Proctor et al., 2003). Interestingly, similar to its effects on GABAA receptors, ethanol modifies glycine repceptors. It has been described that ethanol potentiates the effects of glycine at glycine receptors by a PKCε-dependent mechanism. At this respect, PKCε blockade prevents ethanol-induced potentiation of glycine receptors without altering the effect of glycine by itself. Thus, suggests that ethanol-induced potentiation of glycine receptors is a PKCε dependent mechanism (Jiang and Ye, 2003).

1.4.3 aPKCs (ζ, ι/λ)

Multiple lines of evidence have demonstrated that aPKCs inhibition resulted in a dopamine-dependent spiny neurons decreased of the firing ratio in the shell region of the NAc (Gonzales et al., 2004). Cell lines exposed chronically to ethanol (50-200mM), did not show an altered levels of aPKCs (McIntyre et al., 1999; Mahadevand and Vemuri, 1998). In contrast, genetic mRNA microarray studies have shown an ethanol-dependent PKC ζ upregulation in the frontal cortex in a human model (Lewohl et al., 2000). Is worth mentioning that the role of aPKC isoforms in cellular and behavioral responses to ethanol has not been studied in detail yet. However, these results point out a key role for atypical PKCs in mediating the neurobehavioral effects of ethanol.

1.5 Phospholipase D (PLD)

Despite the suggested ethanol mechanism of action is not thought based in a classical interaction enzyme/substrate, meaning enzymes are able to use ethanol as a substrate, there are a few enzymes that can directly interact with ethanol such as the PLD. However, behavioral consequences of such interaction have not been studied in detail yet. These enzymes, are ubiquitous in almost all living creatures, from bacteria to mammals (Liscovitch et al., 2000; Exton, 2002). These enzymes cleave phospholipids, commonly PC, releasing the freebase, choline, and PA. In the presence of ethanol, the PLDs form PEth using the PA as a substrate. There are two common subtypes PLD 1 and 2 found in the mammal brain and are abundant in the hippocampus (Colley et al., 1997). The PLD1 is a 120 kDa protein found in a perinuclear distribution. This protein is known to be synergistically activated by different other protein kinases and small G-proteins (Exton 2002). The PLD2 is lighter, approximately 100 kDa found

prominently in the plasma membrane. In contrast to PLD1, this enzyme is not activated by interaction with PKC or the small G-proteins (Colley et al. 1997). The activation of PLD is thought regulated by protein–protein interactions.

Experiments exposing cultured cells to ethanol have found cells can generate micromolar concentrations of PEth that result in biological activity (Aradottir et al., 2002; Varga and Alling, 2002). In this respect, in vitro studies have found that a PLDs suitable mechanism of action may be based on the physical properties that the PEth produces on cell membranes. Thus, PEth incorporation into membranes increases membrane fluidity, as well as a tolerance to the membrane-fluidizing effects of ethanol (Omodeo-Sale et al., 1991). The PEth is thought to have biological activity by itself (Asaoka, 1989; Chang et al., 2000; Nishida et al., 1997).

Despite the lack of pharmacological agents with which to manipulate PLD, different bodies of evidence demonstrate that alterations in PLD signaling and PEth accumulation underlie some of the behavioral responses to ethanol. For example, it has been proposed that the tolerance to the membrane-fluidizing effects of ethanol mediated by PEth plays a key role in the development of tolerance to the intoxicating effects of ethanol (Rottenberg et al., 1992; Gustavsson, 1995).

1.6 Adenylyl Cyclases (AC)

The cAMP synthesis is determined by the activity of the AC, which convert ATP to cAMP. Thus make the AC as the effector molecule of one of the most widely utilized signal transduction pathways. In mammals, nine distinct AC genes differing in their patterns of expression and regulatory properties have thus far been identified. All the isoforms share a primary

structure consisting of two trans-membrane regions, M1 and M2, and two cytoplasmic regions, C1 and C2. The trans-membrane M1 and M2 regions have an unknown function; conversely, C1 and C2 regions that are subdivided into C1a and C1b; and C2a and C2b are well conserved and contain all of the catalytic machinery (Hurley, 1999). ACs can be regulated in several different ways. Some ACs are activated by $Ca^{2+/}$CaM, others are inhibited by low concentrations of Ca^{2+} and others are inhibited by calcineurin, de Ca^{2+}-dependent protein phosphatase, or by phosphorylation mediated by Ca^{2+}/CaMK II.

Various in vitro experiments have demonstrated that ethanol is able to regulate AC activity through a variety of different stimulus, including other G proteins (Gαi and Gβγ), different protein kinases such as PKA, PKC, and CaM, calcineurin, Ca^{2+}, and Ca^{2+}/CaM (Hanoune and Defer, 2001, Nelson et al., 2003, Merchenthaler et al., 1993).

Interestingly, at the behavioral level, it has been described that the Ca^{2+}/CaM-dependent AC 1 and 8 are critical for the behavioral effects of ethanol (Cali et al., 1994; Wang et al., 2003, Maas et al., 2005). Mice lacking AC1 and 8 show altered responses to ethanol. Specifically, doublé AC 1 and 8 null mice, showed reduced voluntary ethanol consumption but increased sensitivity to the sedative hypnotic effects of etanol (Maas et al., 2005).

1.7 cAMP dependent protein kinase (PKA)

It has been demonstrated that the cAMP signaling pathway is a major target for ethanol (Diamond and Gordon, 1997; Wand et al., 2001; Thiele et al., 2000). The cAMP- dependent kinase A (PKA) is a heterodimer consisting of two regulatory (R) and two catalytic subunits (Cα). In mammals, the PKA family consists of four regulatory subunit, RIα, RIβ,

RIIα, and RIIβ, and three different catalytic subunits, Cα, Cβ and Cγ, each encoded by a unique gene (Brandon et al., 1997; Skalhegg and Tasken, 2000). Type I or type II PKA is classically defined by their specific regulatory subunits.

When cAMP binds to the PKA R subunits promotes Cα release, and then phosphorylates different intracellular substrates. Importantly, the main PKA action of Cα translocation to the nucleus is the phosphorylation of its specific transcription factor, CREB, initiating changes in expression of genes containing CRE (Brandon et al., 1997). Cytoplasmic targets vary from a wide range of different proteins; however, Cα can interact with many of the main receptors expressed in the brain such as acetylcholine, dopamine, norepinephrine, serotonin, histamine, somatostatin, opioids and cannabinoids (Brandon et al., 1997; Waldhoer et al., 2004, Howlett et al., 2004; Newton and Messing, 2006). Another important intracellular target of PKA involves the regulation of voltage dependent ion channels. In this respect, it has been described that PKA regulates the Ca_vs through an AC/ Ca^{2+}-CaM-dependent mechanism (Zühlke et al., 1999; Yan et al., 2011). Thus, the altered Ca^{2+} flux through Ca_vs is critical for the neurotransmitter release into the synaptic cleft and so, in mediating the neurobehavioral effects of ethanol.

The ethanol-dependent modulation of the cAMP signaling cascade and the PKA has been suggested by two mechanisms. One involves ethanol inhibition of the cell surface, type I equilibrative nucleoside transporter, which leads to the accumulation of extracellular adenosine. Thus, the stimulation of A2a receptors and G proteins mediate downstream cAMP cascade activation. Another mechanism for ethanol regulation of cAMP signaling is mediated by the stimulation by ethanol of some specific isoforms of AC (Newton and Messing, 2006). It has been shown that AC 1

and 8 among others play a critical role in mediating the cAMP-dependent signal transduction in response to ethanol (Wand et al., 2001; Maas et al., 2005; Conti et al., 2009).

In vitro, it has been demonstrated that ethanol treatment of NG108-15 cell cultures promotes the translocation of Cα and RIIβ into the nucleus in a time dependent fashion. In the nucleus, Cα phosphorylates CREB to stimulate gene transcription.

At the behavioral level, in knockout mice with decreased PKA activity promoted by the overexpression of R(AB), these mice are more sensitive to ethanol-induced sedation and show lower preference for ethanol compared to wild-type littermates (Wand et al., 2001). These R(AB), knockout mice showed a dominant inhibitor isoform of the regulatory subunit of PKA under the control of CaMKIIa. To the contrary, knockout mice (RIIβ (-/-)) lacking RIIβ, which are predicted to have increased brain PKA activity; showed increased voluntary ethanol consumption and reduced sensitivity to the hypnotic effects of ethanol (Thiele et al., 2000; Ferraro et al., 2006). Interestingly, pharmacological manipulations of PKA activity demonstrated that increasing PKA activity prior to ethanol administration produced an increase in the loss of righting reflex duration (Kumar et al., 2012), effect that appears to be mediated, in part, by PKA-dependent regulation of GABAA receptor subunit.

1.8 cAMP response element binding protein (CREB)

Probably, one of the best-described molecular mechanisms, which mediates the drug-induced neural plasticity, involves the widely known transcription factor CREB. In particular, CREB plays one of the most known and prominent roles in the neurobehavioral effects of ethanol.

CREB is part of a superfamily of transcription factors known as bZIP. It is composed of a C-terminal domain that is responsible for binding to DNA and a leucine zipper domain that mediates dimerization with itself or other members of the CREB family of transcription factors. CREB has a kinase-inducible domain at Ser-133 that can be phosphorylated in response to stimuli. In vitro experiments, have demonstrated that either acute or chronic ethanol administration to cell cultures increases pCREB levels in rodents (Asher et al., 2002; Constantinescu et al., 1999). This phosphorylation is regulated by PKA and by other protein kinases such as Ca2+/CaMK (Matthews et al., 1994), and PKC (Muthusamy and Leiden, 1998; Roberson et al., 1999). Thus, CREB is a target for several different signaling pathways (Matthews et al., 1994; Impey et al., 1998; Muthusamy and Leiden, 1998; Roberson et al., 1999). The Ser-133 phosphorylation promotes CREB binding to CBP, which can trigger the expression of numerous genes and therefore a functional response mediated by CRE. At this respect, many genes have CRE sites in their promoters, including neuropeptides, neurotransmitter receptors, signaling proteins, and many other transcription factors, providing a role for CREB in many biological processes (Lonze and Ginty, 2002; Mayr and Montminy, 2001).

At the behavioral level, it has been described that different CREB-regulated genes including enkephalin, dynorphin, corticotrophin-releasing factor, brain-derived neurotrophic factor, and neuropeptide Y may mediate the response to ethanol in animals (Pandey, 2004). Thus, it has been demonstrated that ethanol-induced up-regulation of pCREB levels, appear to modulate different ethanol elicited behaviors such as ethanol self administration, ethanol sedative effects and anxiety-like behavior (Newton and Messing, 2006; Pandey et al., 2003; Pandey, 2004).

2. Ethanol actions on ion channels

Ion channels are proteins which form pores across the plasmatic membrane. Their main function is to establish and control the ion-dependent voltage gradient that exists across the plasma membrane of all living cells by allowing the flow of ions down their electrochemical gradient. Moreover, ion channels can be observed in the membranes of intracellular organelles which are important for regulating different intracellular processes such as for example cytoplasmic Ca^{2+} concentration and acidification of specific subcellular compartments.

Ion channels also play a critical role in cell signaling, electrical excitability, neurotransmitter release, gene transcription, fluid transport, and can also act as drug targets.

Ion channels can be grouped into two main classes, voltage- and ligand-gated channels, and can exist in multiple states such as the closed, open, and inactivated states. Both classes present a transition between states depending on changes in the membrane potential or in response to the binding and unbinding respectively.

2.1 Ligand-gated ion channels

In the brain, ligand-gated ion channels (LGICs) are among the postsynaptic receptors that contain integral ion channels that open in response to neurotransmitter binding. Presynaptic cells release neurotransmitters that excite or inhibit postsynaptic cells, depending on which neurotransmitter is released and to what postsynaptic receptors it binds.

Among these receptors, NMDA, GABA-A, 5-HT3, and neuronal nACh in ethanol actions on the brain have drawn special attention (Harris et al., 2008).

2.1.1 NMDA

NMDARs are the glutamate receptor channels that mediate most of the fast excitatory synaptic transmission in the mammalian central nervous system. These receptors channels are highly Ca^{2+} permeable and blocked by Mg^{2+} in a voltage-dependent manner (Mayer et al., 1984; MacDermott et al., 1986; Ascher and Nowak, 1988). These properties indicate that NMDA channels play a key role on different important physiological functions such as synaptic plasticity, learning and memory, gene expression, development of neural networks during the developmental process (Mayer and Westbrook, 1987; Volianskis et al., 2013). Distinct NMDAR subtypes have been described differing in their sensitivity to endogenous and exogenous ligands, block by divalent ions, and interaction with intracellular proteins.

To date, a great variety of NMDAR subunits have been identified. This ion channels appear to function as a heterometic unit composed by the ubiquitously expressed NR1 subunit; a family of four distinct NR2 subunits (A, B, C and D); and two NR3 subunits (Yamakura and Shimoji, 1999). All the subunits present several isoforms determined by three sites of alternative splicing (Dingledine et al., 1999).

Ethanol effects on NMDARs have been described in different neuronal cell populations. Acute ethanol exposure inhibits NMDARs in a concentration-dependent manner within a range of 5–50 mmol/L (Alfonso-Loeches and Guerri, 2011). The molecular mechanism by which ethanol inhibits NMDARs remains to be fully understood. However, it has been proposed that a conformational site-dependent inhibition of such receptors exists. Thus, mutational and molecular exploration of the ethanol sites in NMDARs suggests that there exists an ethanol-binding site at the trasnsmembrane regions 3 and 4 at the NR1 and NR2A subunits, respectively (Yamakura and Shimoji, 1999; Loeches and Guerri, 2011). On the other hand, ethanol

when chronically administered, increases the number of NMDARs as a homeostatic compensation mechanism. Thus, at the behavioral level, several studies have demonstrated that NMDARs participate in ethanol dependence, tolerance, and withdrawal (Loeches and Guerri, 2011; Lovinger, 1995; Chandrasekar, 2013).

2.1.2 GABA

Conversely to other amino acids such as glutamate that participate in many cellular functions; the only function of GABA is to serve as a neurotransmitter. GABA is synthetized from glutamate by a single biochemical step, which is catalyzed by the enzyme GAD. There are three types of known GABA receptors located in the postsynaptic membrane: GABAA, GABAB and GABAC. GABAA is a ligand-gated chloride channel, and considered the most common inhibitory neurotransmitter receptor in the brain. GABAB is a G protein-coupled transmembrane receptor that activates second messenger system and K^+, Ca^{2+} channels. GABAC receptor, composed of GABAρ subunits, is a newly discovered GABAA-like receptor, and is also a kind of ligand-gated chloride channel (Lovinger and Crabbe, 2005; Chebib and Johnston, 1999).

GABAA receptors represent one of the several important pharmacological targets of ethanol (Kumar et al., 2009; Weiner and Valenzuela, 2006). Numerous in vitro studies have demonstrated that ethanol administration potentiates GABAA receptor function (Weiner & Valenzuela, 2006). This ethanol elicited increase in the GABAergic synaptic activity may be critical for the neurobehavioral effects of ethanol. Thus, different behaviors such ethanol intake (Nie et al., 2011; Rewal et al., 2011) and tolerance (Liang et al., 2007), sedative-hypnotic, anticonvulsant, cognitive-impairing and

anxiolytic actions (Kumar et al., 2009) have been described to be, in part, mediated by the ethanol-induced enhancement of GABAA activity in the central nervous system.

2.1.3 nAChR

Central AChR can be divided in two main families: muscarinic and nicotinic. Muscarinic AChR are G-coupled protein receptors, while nicotinic AChR are ligand-gated ion channels. When Ach is released from a presynaptic neuron into the synaptic cleft it binds to presynaptic receptors leading to an activation or inhibition that modulates presynaptic cholinergic neuron. ACh can also bind to postsynaptic receptors leading to a cholinergic response. Moreover, Ach released into the synaptic cleft can be also degraded by acetylcholinesterase and thus terminating its effect.

It has been described that ethanol-induced stimulation of the mesolimbic DA system involves the activity of central nAChR (Ericson et al.,1998; Söderpalm et al., 2000; Molander et al., 2005). Central nAChR are ligand-gated ion channels that consist of pentameric combinations of α and β subunits. These receptors are distributed throughout the brain and highly expressed in areas underlying the drug abuse circuitry. It is generally accepted that ethanol potentiates the nAChR activity and plays a key role in regulating the motivated behavior (Vangeliene et al., 2008; Ericson et al., 1998, 2003). It has been described that nAChR modulate ethanol self-administration in a rodent model. Cholinesterase inhibitors have been proved to reduce ethanol intake and preference in ethanol-preferring rats (Doetkotte et al., 2005). Furthermore, pharmacological manipulations affecting different subunits of the nAChR reduced voluntary ethanol

consumption in rats and mice (Larsson and Engel, 2004; Jerlhag et al., 2006b).

2.1.4 5-HT

A different criterion based on biochemical and pharmacological properties is used to classify the 5-HT receptors. These receptors are designated as 5-HT1, 5-HT2, 5-HT4, and 5-HT7, which, are G-protein-coupled, and the 5-HT3 subtype that represents a ligand-gated ion channel. 5-HT and its receptors are found both in the central and peripheral nervous system, as well as in a number of non neuronal tissues. In the brain 5-HT receptors participate in many specific physiological responses, ranging from modulation of neuronal activity and transmitter release to behavioral change (Hoyer et al., 2002).

It is known that ethanol targets the serotonergic system prominently through its interaction with 5-HT3, 5-HT1B, 5-HT1A receptors, and 5-HTT. For example, it has been shown that ethanol specifically acts on 5-HT3 receptor (Lovinger, 1997). Moreover, different studies have shown ethanol consumption affects the functionality and expression of 5-HTT in areas involved in the rewarding effects of ethanol (Mantere et al., 2002; Storvik et al., 2006). Thus, 5-HT removal is modified and remains active at the postsynaptic cleft in these areas.

At the behavioral level, the 5-HT3, the 5-HT1B, and the 5-HT1A receptors have been implicated in ethanol intake (Sari et al., 2011). These receptors are distributed throughout the nervous system and possess different molecular and pharmacological characteristics. Moreover, the selective ethanol elicited activation of the 5-HT3 receptor on GABA interneurons has been described to be critical in mediating the acute intoxicating effects produced by ethanol (Lovinger, 1997; Vengeliene et al., 2008).

2.2 Voltage-dependent ion channels (VDICs)

VDICs are transmembrane proteins that conduct ions across the membrane in a voltage-dependent manner. These channels are highly related in structure and in function suggesting a common evolution from and ancestral gene. This kind of ion channels, play a fundamental role in the generation and propagation of the nerve impulse and in cell homeostasis. Three fundamental properties of VDICs enable nerve cells to conduct the electric impulse: 1- opening in response to changes in the membrane potential (voltage gating); 2- subsequent channel closing and inactivation, 3- specificity for those ions that will permeate and those that will not.

All the VDICs are selectively permeable to each of the main physiological ions such as Na^+, K^+, Ca^{2+}, and Cl^-. The function of these channels is based in the movement of a voltage sensor initianting a conformational change in the channel gate and thus controlling the ions flow. This voltage-sensitive region is composed of charged amino acids that relocate upon changes in the membrane electric field. Although these VDIC channels have been the focus of much of the research, voltage-dependent cation channels (Na^+, K^+, Ca^{2+}) appear to be of especial relevance as an ethanol molecular target being affected by at relatively low ethanol concentrations.

2.2.1 Voltage-dependent potassium channels (K_vs)

A particular form of the K_vs called BK channel appear to be very responsive to ethanol (Dopico et al., 1999). Interestingly, BK channel is widely expressed in the brain, and plays a prominent role in many aspects of the neuronal physiology such as neurotransmitter release, generation of action potential and dendritic excitability-dependent synaptic plasticity (Dopico et

al., 1999; Treistman and Martin, 2009). Moreover, BK channels have been proved to be a key element in mediating the neurobehavioral effects of ethanol (Treistman and Martin, 2009; Nicoll and Madison, 1982). These channels are composed of two parts, a core and auxiliary subunits. The pore forming, α subunit is responsible for most of the fundamental channel properties (Dopico et al., 1999).

Ethanol interaction with BK channels has been widely discussed, it has been demonstrated that very high ethanol concentrations were able to trigger BK current response. Thus, in vitro experiments using mouse brain synaptosomes reported an intracellular Ca^{2+}-dependent modulation of BK flux after ethanol (400mM) exposure (Harris, 1984). Interestingly, other groups have shown that ethanol at biologically relevant concentrations (5-80 mM) was able to modulate BK flux in vitro (Carlen et al., 1985; Carlenm 1987; Niesen et al., 1988).

At the behavioral level, it has been proved that the BK subunit composition is critical in mediating ethanol acute tolerance and consumption. Thus, animals lacking BK β4 subunit showed enhanced ethanol consumption when compared to wild type mice. Moreover, BK β4 mutant mice appear to present acute behavioral tolerance to the depressing effects of ethanol, when measured in a locomotor activity paradigm (Treistman and Martin, 2009).

2.2.2 Voltage-dependent sodium channels (Na_vs)

Na_vs are large (≈ 250 kDa) membrane proteins, composed of a α subunit and one or more smaller auxiliary β subunit. At the functional level, nine α subunits have characterized (*$Na_v1.1$- $Na_v1.9$*) and three β (β1, β2 and/or β3) (Yu and Caterall, 2003).

Na_vs play a specific and particular role in the upstroke of the action potential. The initiation and propagation of the action potentials in neurons and other excitable cells is determined by the *Na_vs* activity. When the cell membrane is discretely depolarized, sodium flux through the *Na_v* activates the channel to further depolarize the membrane initiating the rising phase of the action potential. After opening, *Na_vs* rapidly undergo inactivation, a crucial process through which sodium conductance is negatively regulated (Petege et al., 2012; Yu and Caterall, 2003).

In vitro, it has been demonstrated that ethanol produces an inhibitory effect on *Na_vs* gating activity. Thus, different studies showed that ethanol administration to neuronal cell cultures decreased Na+ currents and channel open probability in a concentration dependent manner, and impaired the action potential firing (Xiao et al., 2008; Klein et al., 2007).

Despite *Na_vs* have been widely studied regarding its critical role on action potential dynamics and neuronal transmission, the behavioral consequences of *Na_vs* manipulations in mediating the neurobehavioral effects of ethanol, remain to be fully elucidated.

2.2.3 Voltage-dependent Ca^{2+} channels (Ca_vs)

Ca_vs have been widely studied among time due to its prominent/principal role on many physiological functions, especially those involving neurotransmission dynamics. Thus, a great body of evidence has identified Ca_vs as a contributor in mediating some of the neurobiological proprieties of different drugs of abuse. Particularly, over the past three decades *Ca_vs* have emerged as critical mediators to ethanol effects on the neural function and behavioral response.

Neuronal Ca_vs are multimeric membrane complexes conformed by at least three types of subunits: α1, α2, β, γ, and δ. Different studies have

proposed a model of a channel organization comprising a principal transmembrane α1 subunit of 190 kDa in association with a disulfide-linked α2δ dimer of 170 kDa, an intracellular phosphorylated β subunit of 55 kDa, and a transmembrane γ subunit of 33 kDa (Takahashi et al. 1987; McClesky, 1994, Walter and Messing, 1999). Different classes of Ca_vs have been described based on its α1 subunit-dependent pharmacological and physiological features. The α1 subunit contains the Ca^{2+} pore and binding sites for selective channel antagonists. This particular subunit is composed of four homologous repeats (I-IV) each comprising six transmembrane segments (S1-S6). There are six genes known to encode α1 subunits in brain (α1A, α1B, α1C, α1D, α1E, and α1G). Further Ca_vs classifications are based on three different molecular, electrophysiological or pharmacological criteria (Table 1).

Table 1
Neuronal Ca_vs classification

Channel type	Electrophysiological activation range	Gene product (α1 subunit)	Pharmacology
L	High voltage activated	α1C	Dihydropyridines, Cd^{2+} ($IC_{50} \approx 1\ \mu M$)
L	High voltage activated	α1D	Dihydropyridines, reversibly sensitive to ω-conotoxin GVIA and ω-agatoxin IVA, Cd^{2+} ($IC_{50} \approx 1\ \mu M$)
N	High voltage activated	α1B	ω-conotoxin GVIA (100-500 nM), ω-conotoxin MVIIC (>100 nM), ω-agatoxin IIIA ($IC_{50} \approx 1$ nM), Cd^{2+} ($IC_{50} \approx 1\ \mu M$)
P	High voltage activated	α1A	ω-agatoxin IVA (≤10 nM), Cd^{2+} ($IC_{50} \approx 1\ \mu M$)
Q	High voltage activated	α1A	ω-agatoxin IVA (>10 nM), ω-conotoxin MVIIC (>100 nM), ω-agatoxin IIIA (IC_{50} unknown), Cd^{2+} ($IC_{50} \approx 1\ \mu M$)
R	High voltage activated	α1E	Ni^{2+} ($IC_{50} \approx 50\ \mu M$), Cd^{2+} ($IC_{50} \approx 1\ \mu M$)
T	Low voltage activated	α1G	Ni^{2+} (≤ 100 μM), Ethosuximide (< 500 μM; incomplete), Amiloride (< 500 μM)

Briefly, L-type channels are activated by high voltage, inactivate slowly, and are blocked by dihydropyridine Ca^{2+} channel antagonists. N-, P-, and Q-type channels are activated by high voltage and are blocked by the peptide neurotoxins *ω*-conotoxin GVIA (N), *ω*-conotoxin MVIIA (N), *ω*-agatoxin IVA (P and Q) and *ω*-conotoxin MVIIC (N, P and Q). R-type channels are activated by high voltage and are also resistant to organic Ca^{2+} channel blockers (Randall and Tsien, 1997). T- type channels activate at low voltage, inactivate quickly, and are blocked by low concentrations of Ni^{2+} (Walter and Messing, 1999).

Despite the existence of the above-described wide range of Ca_vs (L-, P/Q, N-, R- or T-type), L-type Ca^{2+} channel are the most extensively localized in the mammalian central nervous system. These channels have also been found of interest due to its participation in the neurobiological response to different drugs of abuse (Baliño et al., 2010; Bhutada et al., 2012; Newton et al., 2008; Bisagno et al., 2010). Interestingly, different studies have demonstrated that L-type *Ca_vs* activity and thus neurotransmitter release can be shaped by different posttranscriptional modifications such as kinase-dependent phosphorylation (Trimmer and Rhodes, 2004; Yan et al., 2011; Zühlke et al., 1999).

A great body of evidence supports an activating effect of Ca_vs activity after ethanol exposure as a very early physiological response. The majority of the investigation has focused on a time- and concentration- range of ethanol exposure to cell cultures, which proves an ethanol-elicited inhibition of Ca_vs. All these studies however, failed to explain a mechanism of action by which ethanol through its interaction with L-type Ca_vs, can shape and modulate the ethanol-elicited behavior. In this respect, different studies

Shown that a single exposure to ethanol increases the function of Ca_vs. Thus, it has been demonstrated in vitro using different preparations such as brain hypothalamic cultures (Simasko et al., 1999), PC12 cells (Belia et al., 1995), and in brain synaptosome homogenates after in vivo injections of ethanol (Bergamaschi et al., 1998).

Furthermore, other studies in neural cell preparations have reported an increased number of binding sites for radiolabeled Ca_vs blockers after chronic exposure to ethanol (Katsura et al., 2002).

At the behavioral level, antagonists of L-type Ca_vs reduce voluntary ethanol consumption (DeBeun et al., 1997; Fadda et al., 1992), ethanol discrimination (Colombo et al., 1994), manifestations of ethanol withdrawal (Bone et al., 1989; Littleton et al., 1990) and locomotor activity (Baliño et al., 2010). Moreover, development and expression of behavioral sensitization to ethanol is also affected by L-type Ca_vs inhibition (Broadbent, 2013). Mutant mice lacking of N-type Ca_vs (Newton et al., 2004), showed reduced ethanol consumption and altered ethanol responses after an acute administration.

Chapter Summary

In summary, in this section we have described the capacity for ethanol to interact with different core elements of the neuronal substrate. Most of the proposed substrate conforms to the principal mechanisms by which cells can transmit and propagate information. While it has been proposed that many different cellular molecular targets interact with ethanol, to date research has failed to identify a unique mechanism of action of ethanol.

Acute behavioral effects of ethanol are observed within very short periods of time < 5 min, with brain ethanol concentrations ranging from 5 to 200mM. One possibility which explains the rapid effects of ethanol is based on first and immediate interactions of ethanol with some receptors, excitable membrane proteins, or second messenger molecules that allow for rapid transmission of information. In this respect, it has been described that ethanol interacts and modulates some excitable membrane proteins, promoting changes in the cellular flux dynamics of several ions. These disturbances in the transient *cellular electrical potential* may derive in the modulation of the cellular receptor potential, synaptic potential and/or action potential, therefore modulating the chemical signals that control different biological processes. Being so, we have described some ethanol interactions with the cellular substrate that conforms the excitable pool responsible for the electric transmission of information.

Most of these cellular elements are formed by integral membrane proteins that maintain equilibrium on ion concentrations across the plasmatic membrane (VOCs and LGICs). Thus, we have described how ethanol, through changes in different cellular ion homeostasis, can modulate

different elements of the cellular neurotransmission machinery. In fact, these rapid changes can explain some of the molecular and behavioral responses to ethanol. However, the particular mechanism by which ethanol interacts within these substrates and thus modulates behavior remains to be fully clarified.

Some of the particularly interesting actions of ethanol described previously relate to the Ca^{2+} ion. Apart from its determinant contribution to neuronal excitability via its role in action potential dynamics (Berridge et al., 2000; Poage and Meriney, 2002), Ca^{2+} is the most ubiquitous intracellular signal, responsible for controlling numerous neurobiological processes such as second messenger cascades, gene expression and neurotransmitter release (Berridge et al., 2000; Dunlap et al., 1995; Ghosh and Greenberg, 1995). Moreover, Ca^{2+} ion participates as a critical mediator in some of these described ethanol molecular targets.

2
Calcium

Background

Calcium (Ca^{2+}) remains the most ubiquitous intracellular signal and second messenger responsible for controlling a wide range of cellular processes (Berridge, 1997; Clapham, 1995). Despite its importance in numerous cellular processes, prolonged rises in [Ca^{2+}] are incredibly hazardous to the cell physiology and may result in an irreversible cell damage and death. Because of its cytotoxicity, cells held at rest narrow Ca^{2+} concentrations ranging from 20-100 nM, when intracellular concentrations rise to roughly 500-1000 nM, cells are activated to trigger a series of different processes that restore the cell resting state. Thus, cells have an extensive molecular repertoire of signaling components, which comprise a Ca^{2+} signaling elements that can be assembled in combinations to create signals with widely different spatial and temporal profiles. Another mechanism, by which Ca^{2+} can trigger changes in cell function, is due to its positive double charge. Similarly to phosphorylation that imparts negative charges, Ca^{2+} alters local electrostatic fields and protein conformations (Clapham, 2007).

1. Calcium homeostasis

Cells generate their Ca^{2+}-mobilizing signals, by using two sources. Firstly, it can enter from the outside through a variety of channels such as the Ca_vs (*see chapter I)*, or some types of LGICs (*see chapter I*). Secondly, Ca^{2+} can be released from internal stores such as ER or SR and mitochondria. Despite ER/SR and mitochondria are the most important and widely described, different studies have identified new emerging Ca^{2+} release stores. Thus, endosomes, Golgi vesicles, lysosomes, secretory granules, and melanosomes are single membrane-bound compartments that could

contribute to cell Ca^{2+} homeostasis (Rizzuto and Pozzan, 2006; Clapham, 2007).

1.1 Endoplasmic reticulum

Internal Ca^{2+} homeostasis from this compartment is controlled by $InsP_3R$, RyR and the SERCA. In this respect, $InsP_3R$ and RyR, extrude Ca^{2+} to the cytoplasm and SERCA pump ensures that cytoplasmic [Ca^{2+}] remains low by removing it against a gradient. Usually a local event that brief burst [Ca^{2+}] is responsible for cell activation and promotes a coordinated opening of either RYRs or $InsP_3R$ by a phenomenon called CICR. Thus this discrete rise on [Ca^{2+}] has a biphasic effect on these two receptors; at first, it exerts a positive feedback by enhancing the channel opening (CICR). Next, the feedback switches from positive to negative and Ca^{2+} then inhibits the channel avoiding the cytoplasm from being flooded with this cytotoxic agent.

There are three RyRs and at least four $InsP_3R$, it seems that both receptors evolved from a common ancestor because their structural and functional similarities.

In the brain, the $InsP_3R$ are distributed throughout the brain, the ER network is especially abundant in the synaptic terminal, and prominently within Purkinje cells and spiny neurons. From the three isoforms of RyR, the predominant RyR isoform in the brain is RYR2 that is know as the cardiac isoform.

Regulation of $InsP_3R$ is determined by the hydrolysis of PIP_2 to form the two-second messengers $InsP_3$ and DAG. Despite RYRs regulation can be affected by second messenger such as cyclic ADP-ribose, this receptor is very sensitive to a Ca^{2+} environment that enters either from outside or from

neighboring receptors. The basic idea of neuronal Ca^{2+} regulation by $InsP_3R$ or RyR, comes from the factor that Ca^{2+} entering through either the voltage-operated channels or receptor-operated channels provides the trigger Ca^{2+} to stimulate Ca^{2+} release from the internal stores (Lipscombe et al., 1988; Berridge, 1998). Increasing the resting cytosolic level of Ca^{2+} greatly increases the sensitivity of $InsP_3R$ or RyR. Thus, a most effective response of the receptor is produced when Ca^{2+} and some of its agonists such as $InsP_3$ or cyclic ADP-ribose are presented together.

At this point is worth mentioning the role of ATPase pumps as key contributors to the Ca^{2+} intracellular homeostasis. Thus, to maintain low cytoplasmic [Ca^{2+}], SERCA pumps Ca^{2+} into ER, in contrast to PMCA that pumps Ca^{2+} out of the cell. These ATPases exchange protons for two (SERCA) or one (PMCA) Ca^{2+} per ATP hydrolyzed to ensure cellular levels of Ca^{2+}.

1.2 Mitocondria

Similarly to ER, mitochondria can also store cellular Ca^{2+}. Mitocondrial stored Ca^{2+} readily diffuses through large pores in the outer membrane and passes through the inner mitochondrial space via ion channels and transporters. The most studied mechanism by which mitochondria regulates Ca^{2+} levels is based on a highly Ca^{2+}-selective ion conductance channel that pump Ca^{2+} across the inner mitochondrial membranes. This channel, named MiCa, binds Ca^{2+} with high affinity (Kd ≤ 2nM), enabling high Ca^{2+} selectivity despite relatively low cytoplasmic [Ca^{2+}]. MiCa is impermeable to the abundant cytoplasmic Mg^{2+} and K^+ ions, insuring that only Ca^{2+} dissipates mitochondrial potential.

Despite mitochondria can play distinct roles on shaping intracellular Ca^{2+} signals; the functional significance of mitochondria as important regulator of

intracellular Ca^{2+} homeostasis, is due to its ability for local Ca^{2+} buffering of Ca^{2+} released from the ER (Rizzuto and Pozzan, 2006; Hajnoczky et al., 2002). Thus, exists a functional association between ER (or other organelles) and mitochondria to further activate mitochondrial Ca^{2+} metabolism when local Ca^{2+} microdomains are produced.

1.3 Acidic stores

As we have commented, Ca^{2+} is derived from the extracellular space and/or intracellular Ca^{2+} stores, being the best studied the ER. Recently, different organelles rich in both H^+ and $Ca^{2+,}$ called "acidic Ca^{2+} stores" have been described to participate in the control of intracellular Ca^{2+} dynamics in organisms. These cellular compartments are diverse including, acidocalcisomes (best characterized in protists), vacuoles, (present in many organisms including plants and yeast), lysosomes and endosomes. Acidic stores Ca^{2+} mobilization, is driven by Ca^{2+} ATPases and Ca^{2+} /H^+ exchangers. In addition, the cofactor NAADP plays a key role in mediating these mobilizing signals from acidic Ca^{2+} stores through activation of two pore channels. Ca^{2+} release from these acidic compartments, are important for some physiological processes including muscle contraction and differentiation. Interestingly, inappropriate Ca^{2+} mobilization from these stores is thought to result cell damage or death induction pathways. (Patel and Muallem, 2011; Patel and Docampo, 2010).

2. Ethanol and calcium

Effects of ethanol on Ca^{2+} flux and distribution have been studied in different neuronal cell preparations. It has been demonstrated that ethanol administration to different cell preparations alters Ca^{2+} homeostasis,

however, how ethanol modulates and shapes the cellular Ca^{2+} dynamics remains to be fully understood.

These studies demonstrated that ethanol exposure at physiologically relevant ethanol concentrations (< 80 mM) increase intracellular Ca^{2+} levels (Daniell and Harris, 1989; Mironov and Hermann, 1996; Xiao et al., 2005). Thus, pheochromocytoma (PC12) cultured cells exposed to ethanol (20-120 mM), presented with an initial rise in the cellular Ca^{2+} levels that was abolished at greater concentrations (120-360 mM). In addition, similar results were found in cultures from the neuroblastoma (NCB-20) cell line (Chan and Greenberg, 1991) and in primary cultures from septohippocampal and hippocampal neurons (Webb et al., 1997a, b).

Together, all these studies mark a mechanism by which a prominent ethanol-induced rise in the cellular Ca^{2+} levels is achieved by L-type Ca_vs activity. These channels are widely distributed throughout the brain and participate in many biological functions with regard to cell physiology. Moreover, Ca_vs have been described as the main cell components in maintaining and modifying Ca^{2+} homeostasis from the extracellular side to the cytoplasm.

Thus, many authors have suggested the possibility of ethanol, through Ca_vs interaction as the main mechanism involved in the cellular Ca^{2+} changes elicited after ethanol exposure. In this respect, these previous findings appear to be controversial.

A great body of evidence has signaled an alternative cellular mechanism by which ethanol shapes intracellular Ca^{2+} homeostasis and dynamics.

To ascertain the role of intracellular Ca^{2+} sources on ethanol-induced intracellular Ca^{2+} increase, neuronal cell cultures were first challenged with ethanol in the absence of extracellular Ca^{2+} (no Ca^{2+} was added to the

media, which was supplemented with 0.5 mM EGTA). Secondly, cell cultures were incubated in the presence of the SERCA-inhibitor (1 mM) in a Ca^{2+} free medium. Thirdly, in order to discard an ethanol-unespecific effect on membrane physicochemical properties, cell cultures were challenged with methanol.

Under these circumstances, ethanol administration evoked an increase in the cytoplasmic Ca^{2+} levels indicating that the source for Ca^{2+} release in response to ethanol is the **ER** (Dallwig and Deitmer, 2002; Salazar et al., 2008 González el al., 2007; Kelm et al., 2007; Mironov and Hermann, 1996; Salazar et al., 2008, Daniell and Harris, 1989). Moreover, this effect appears to be selective for ethanol interaction within the intracellular Ca^{2+} compartments since no modulation of the cellular Ca^{2+} levels was observed after methanol administration.

Given this, ethanol modulation of cellular Ca^{2+} levels, appears to be mediated by a cooperative mechanism that has not yet been elucidated in detail. Firstly, ethanol would promote an initial and rapid Ca^{2+} release response from the ER; then followed by a higher Ca^{2+} flux current through the L-type Ca_vs.

Chapter summary

In this section, we have described the role of Ca^{2+} ion in many biological processes. In part due to its cellular toxicity, Ca^{2+} homeostasis remains strictly controlled under a series of pumps and cellular compartments. This ion not only contributes to the electrical cellular state involved in the action potential and neurotransmitter release; Ca^{2+} acts as an important second messenger transmitting information across the cell.

Recent findings have pointed out the role of different intracellular compartments as important contributors to cellular Ca^{2+}dynamics, nonetheless the ER remains the main cell structure that participates actively in the maintenance of intracellular Ca^{2+}homeostasis. The ER is an interconnected network of sacs, branching tubules, vesicles, and cisternae that extends throughout the cytoplasm. These properties confer to the ER a perfect candidate by which ethanol can have rapid and immediate interactions.

It has been described that ethanol, in a time and dose-dependent manner, modulates cytoplasmic Ca^{2+}levels through different mechanisms. One of these is with respect to ethanol interacting with extracellular-to-cytoplasm Ca^{2+} flux through VOCs and/or LGICs. However, in spite of the great number of investigations carried out to study the action of ethanol action on ion channels, less attention has been paid to the involvement of intracellular stored Ca^{2+} in ethanol-evoked effects.
In this respect, the other mechanism by which ethanol can shape intracellular Ca^{2+} homeostasis refers to a selective release of Ca^{2+} from intracellular stores, particularly the ER.

All this evidence points to a cooperative cell mechanism by which ethanol shapes cellular Ca^{2+} homeostasis. However, this mechanism appears to be controversial; it would start with an initial and rapid Ca^{2+} release response from the ER; followed by a higher Ca^{2+} flux current through the L-type Ca_vs.

INTRODUCTION EPILOGUE

When all the information in this introduction is taken together, it seems that ethanol modulates different neuronal substrates but a general central ethanol mechanism remains to be fully elucidated. This is in part due to the inherent physical-chemical properties of ethanol.

In this respect, ethanol can be considered a weak drug when compared to most other drugs of abuse. The molecule of ethanol has no asymmetric carbon. Being so, it does not interact with the biological substrates in a stereoselective way as most of the receptor ligand drugs do. Moreover, ethanol has a very simple chemical structure. Thus, the hydroxyl group provides a dipole that allows the formation of hydrogen bonds. It is the formation of hydrogen bonds that makes ethanol soluble in water. In contrast, ethanol presents an aliphatic moiety that provides a lipophilic group that can interact with non-polar cellular elements. However, contrary to what it is generally believed, ethanol has low lipid solubility. All these chemical properties of ethanol have led different authors to consider the first oxidative metabolite of ethanol as the biologically active compound. Nonetheless, ethanol produces quite complex effects on neuronal systems.

Behavioral changes observed after ethanol administration are produced within very short periods of time (< 5 min). In this respect mobilization and/or modulation of some of these described ethanol neuronal substrates, requires reorganization of protein structures and/or in some cases early gene expression. It is unlikely that ethanol actions after acute administered are explained by processes such as induction of gene expression or new protein synthesis. As such, primary ethanol interactions among the neuronal substrate must be mediated by cellular mechanisms that can be controlled and triggered almost immediately. Interestingly, one of these

posttranscriptional mechanisms that lead to protein modification is the *phosphorylation*, carried out by the activity of *protein kinases.* They represent one of the major classes of cell-regulatory molecules and are important mediators in signal-transduction pathways that affect cell physiology and behavior. In this respect, most of the previously described ethanol molecular targets require posttranscriptional phosphorylation to be fully active (DARPP-32, Fyn, etc).

One key protein kinase that has been described as an important mediator involved in the neurobehavioral responses to ethanol is the cAMP dependent protein kinase (PKA). This kinase is the end point of a cAMP-dependent intracellular pathway. Moreover, this cAMP-dependent cascade is conformed by different intermediates that are Ca^{2+} sensitive (Hanoune and Defer, 2001, Nelson et al., 2003; Cali et al., 1994; Wang et al., 2003, Maas et al., 2005). All together, this pathway is conformed by different elements that control and modulate the activity of the PKA. The activated PKA transmits information rapidly across the cell and promotes changes in the cellular machinery and also is involved in the modulation of several ethanol neurobehavioral effects. Manipulations of the cAMP-dependent cascade modulate the acute stimulant effects of ethanol, ethanol-induced behavioral sensitization (Freund and Palmer, 1997; Tolliver et al., 1999; Hayes et al., 2012), ethanol sensitivity (Thiele et al., 2000; Wand et al., 2001), and ethanol intake (Pandey et al., 2005).

Given this, in the present work we aimed to study the role of cellular Ca^{2+} distribution and flux on the modulation of a PKA/cAMP-dependent cascade as an ethanol central mechanism of action.

II- HYPOTHESIS AND OBJECTIVES

General hypothesis

Ethanol interacts with multiple molecular targets but a particular specific mechanism of action by which ethanol can exert its actions at a central level remains to be identified. The participation of the cAMP-PKA in the neurobehavioral response to ethanol has been described. Intracellular Ca^{2+} appears to be a second messenger mechanism critical to signal and information transduction across the cell. Moreover, a great body of evidence has showed Ca^{2+} to be a critical mediator in the cAMP-PKA cascade.

Based in all the data presented on the theoretical introduction, we hypothesized that *in vivo* cellular Ca^{2+} homeostasis disruption would be critical to further activating the cAMP-PKA cascade and therefore to determining the neurobehavioral response to ethanol. For this reason we propose that ethanol administration elicits modulation of the cellular Ca^{2+} fluxes to further activate CaM. This enhances the activity of AC leading to an increase in the cellular levels of cAMP which produces an activation of the PKA. As a result of this PKA activation, different substrates are modified, particularly the Ca_Vs, which play a key role in mediating the behavioral effects elicited by ethanol.

EtOH ➔ Ca^{2+} ➔ CaM ➔ AC ➔ cAMP ➔ PKA

The **general aim** of this work is:

First, to study the role of cellular Ca^{2+} in the activation of a cAMP-dependent signaling pathway by ethanol *in vivo.*

- To investigate the role of extracellular-to-cytoplasm Ca^{2+} flux.
- To investigate the role of ER inner Ca^{2+} currents.
- To investigate the role of different cAMP-PKA Ca^{2+}-dependent mediators.

Second, to study the functional consequences of Ca^{2+} and cAMP-PKA pathway manipulations on different ethanol elicited behaviors.

- To investigate the role of general cytoplasm free Ca^{2+}.
- To investigate the role of extracellular-to-cytoplasm Ca^{2+} flux.
- To investigate the role of ER inner Ca^{2+} currents.
- To investigate the role of different cAMP-PKA Ca^{2+}-dependent mediators.

The **specific aims** of this work are:

At the molecular level, to study,

- The role of Ca^{2+} flux through ER RyR in the ethanol-induced activation of PKA in different brain areas.
- The role of Ca^{2+} flux through Ca_vs in the ethanol-induced activation of PKA in different brain areas.

- The role of the A2a receptors, in the ethanol-induced activation of PKA in different brain areas.
- The role of Ca^{2+} CaM on ethanol ethanol-induced activated PKA in different brain areas.
- The role phosphodiesterase 4 on ethanol-induced activated PKA in different brain areas.

At the behavioral level, to study,

- The role of free cell Ca^{2+} pool on ethanol-induced behavioral stimulation, ethanol intake, ethanol incoordinating effects and loss of righting reflex.
- The role of extracellular-to-cytoplasm Ca^{2+} flux through Ca_vs on ethanol-induced behavioral stimulation and motor incoordination.
- The role of inner Ca^{2+} flux through ER RyR on ethanol-induced behavioral stimulation, ethanol intake and loss of righting reflex.
- The role of Ca^{2+} activated CaM on ethanol-induced behavioral stimulation and ethanol intake.
- The role of phosphodiesterase 4 on ethanol ethanol-induced behavioral stimulation and ethanol intake.

III- RESULTS

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Study 1

Study 2

Study 3

Study 4

Study 5

Study 6

IV- DISCUSSION

The present work was designed to study a new ethanol mechanism of action at a central level that involves Ca^{2+} mobilization from the endoplasmic reticulum to further activate a cAMP mediated signaling cascade.

We have evaluated the molecular and behavioral consequences of cellular Ca^{2+} manipulations (intracellular and extracellular) on ethanol-induced activation of protein kinase A. Moreover, we have extended our investigation to ascertain the role of different intracellular cAMP-PKA pathway mediators on the ethanol induced PKA-dependent molecular response as well as its participation on the ethanol elicited behavioral response.

At the molecular level, different approaches were used to evaluate the participation of the main cellular Ca^{2+} sources on the ethanol-mediate PKA molecular response. We have also extended our investigation to ascertain the role of different cAMP cascade mediators on this ethanol-dependent PKA response.

In this respect, it has been described that cells generate their intracellular mobilizing Ca^{2+} signals by using external and/or internal sources of Ca^{2+}. The external and never-ending source is regulated mainly by the Ca^{2+} flux through Ca_vs. Conversely, the inner source is limited and tidily controlled. Nowadays different Ca^{2+} sources such mitochondria and acidic stores have been proposed as key contributors to the cell Ca^{2+} dynamics, however the ER remains the major system that maintains the intracellular cell Ca^{2+} homeostasis.

Thus, in the first work, we studied in vivo the participation of cellular Ca^{2+} on the ethanol-elicited response of cAMP-PKA in different brain areas. Here, we have focused on Ca^{2+} flux through L-type Cavs, and intracellular Ca^{2+} release from ER RyR. We further extended our investigation to

ascertain the role of adenosine A2a receptors on this ethanol-induced activation of PKA. Results from this work demonstrated that ethanol administration increased the activity of PKA in a time-dependent fashion in cortex, hypothalamus, hippocampus and striatum. We further showed that intracellular Ca^{2+} from the ER plays a critical role in this ethanol-elicited PKA response when compared to extracellular-to-cytoplasm Ca^{2+} blockade. Manipulations of Ca^{2+} flux through L-type Ca_vs did not modify the ethanol-induced activation of PKA. At this point is worth mentioning that other authors have found that the activating effects of ethanol in this central cAMP-PKA route may be regulated by an adenosine A2a receptor-dependent mechanism (Mailliard and Diamond, 2004). To evaluate the participation of A2a receptors on the PKA response elicited by ethanol, we have designed an experiment where animals were challenged with the A2a receptor antagonist DMPX. Here, we have found that A2a antagonism failed to prevent the ethanol-induced activation of PKA in all the neuroanatomical areas of the study.

Together, this data suggests that the ability of ethanol to induce PKA activation and phosphorylation of PKA suitable substrates is explained, in part, by the intracellular Ca^{2+} released from the ER through the RyR; at least in the neuroanatomical areas of the study. Furthermore, extracellular-to-cytoplasm Ca^{2+} flux through L-type Cavs appears not to be determinant in the ethanol elicited PKA activation response. Given this, these results support the idea of an alternative mechanism of action for ethanol that elicits PKA response. In our hypothesis, ethanol-induced activation of PKA would imply a selective Ca^{2+} dependent release from the ER RyR as an initiator of the cellular response in vivo compared to extracellular Ca^{2+} currents trough Ca_vs.

Following this line of argumentation, several groups have provided evidence that disruptions of the cAMP signaling pathway system modulate the response to ethanol, *in vitro* and *in vivo*. The cAMP synthesis is determined by the activity of the AC. It has been demonstrated that ACI and ACVIII play a key role in mediating the cAMP-PKA dependent response to ethanol (Wand et al., 2001; Maas et al., 2005; Conti et al., 2009). Interestingly, these two isoforms are Ca^{2+} sensitive via CaM activation (Cali et al., 1994; Wang et al., 2003). This cAMP-PKA pathway mediator has been described as a cellular Ca^{2+} sensor and one of the key proteins responsible for transducing a signal in response to intracellular Ca^{2+} increase.

Given this, in the second work we have studied the participation of CaM in the ethanol-induced PKA signaling activation as an early cellular response. Results from the present study demonstrated a decreased intensity of the PKA footprint when the CaM inhibitor W7 was administered prior to ethanol. This effect appears to be the result of a decreased Ca^{2+}-CaM dependent PKA activation by ethanol. These results would indicate that CaM activity is critical to further activate this cAMP-PKA cascade in response to ethanol.

The cellular levels of the second messenger cAMP determine the activity of PKA. The cAMP synthesis is determined by the activity of the AC, which convert ATP to cAMP. These enzymes are widely distributed within the brain and among all the subtypes, the AC 1 and 8 have been shown to play a pivotal role in mediating the molecular response to ethanol (Wand et al., 2001; Maas et al., 2005; Conti et al., 2009). It is well established that Ca^{2+} and cAMP are interrelated second messengers for many processes. In this respect, it has been described that Ca^{2+} increases the cAMP levels by a CaM- and AC-dependent mechanism (Gnegy et al., 1984; Cheung, 1980).

Interestinlgy, Ca^{2+} directly stimulates AC1 and AC8 via CaM activation (Cali et al., 1994; Wang et al., 2003). CaM has been described as an ubiquitous intracellular Ca^{2+} receptor (Chin and Means, 2000) and one of the key proteins responsible for transducing signals in response to an intracellular Ca^{2+} level increase. This protein also mediates many aspects of neuronal function including synaptic plasticity, gene expression, and neurotransmitter synthesis and release. Furthermore, Ca^{2+}-CaM kinases have been described as important molecular mechanisms involved in the neurobiological response to drug-abuse (Robison et al., 2013; Jackson et al., 2012).

In summary, results from these experiments point out the prominent role of intracellular ER Ca^{2+} currents (when compared to those from the extracellular side) in mediating the molecular response to ethanol. Thus, our results support our proposed linear pattern of events initiated by Ca^{2+} release from the ER as an early cellular response to further activate the intracellular Ca^{2+} sensor CaM and so the cAMP-PKA signaling pathway.

Following the same approach used in the above molecular studies, at the behavioral level, different studies were designed to evaluate the participation of the main cellular Ca^{2+} sources on the ethanol-elicited behavioral response. Furthermore, we have extended our investigation to ascertain whether the different mediators of the proposed ethanol mechanism of action participate in the ethanol-dependent behavioral response.

In this respect, great body of evidence has pointed out Ca^{2+} as one of the most ubiquitous, universal and versatile intracellular signaling molecules responsible for controlling numerous cellular processes. In the previous experiments we have demonstrated that intracellular ER Ca^{2+} release in response to ethanol acts as a second messenger by initiating a cascade of events leading to PKA activation. The versatility of signaling mechanisms operated by Ca^{2+} results in an ample variety of Ca^{2+}-dependent cellular events, including gene expression processes, metabolic responses and the release of neuroactive substances. How all these changes translate into behavior remains to be fully solved.

Ethanol effects on Ca^{2+} flux and distribution have been widely studied *in vitro* using different cell preparations (Belia et al., 1995; Chan and Greenberg, 1991; Davidson et al., 1988). Thus, acute ethanol exposure *in vitro* modulates Ca^{2+} flux and it is generally accepted that physiologically relevant ethanol concentrations (< 80 mM) increase intracellular Ca^{2+} levels (Daniell and Harris, 1989; Mironov and Hermann, 1996; Xiao et al., 2005). As we have previously stated, cellular Ca^{2+} homeostasis is achieved either by its release from internal stores (González et al., 2007; Kelm et al., 2007; Mironov and Hermann, 1996; Salazar et al., 2008) or by Ca^{2+} flux from the extracellular side. In this respect, Ca^{2+} flux through Ca_vs is of special relevance (Belia et al., 1995; Chan and Greenberg, 1991; Leslie et al., 1990).

Given this, in the first work, we investigated the role of intracellular Ca^{2+} chelation on different ethanol elicited behaviors such as motor activity, ethanol intake and the sedative effects of ethanol. Here, we demonstrated that pretreatment with the membrane permeable chelating agent BAPTA-

AM, reduced ethanol-induced stimulation in a dose dependent manner. Moreover, this effect seems to be specific to the stimulating actions of ethanol since no interaction was observed with other drugs with motor-stimulating properties. Furthermore, doses of BAPTA-AM that reduced the ethanol-induced locomotor activity did not enhance the incoordinating effects of ethanol in an accelerating rotarod paradigm. This would suggest that the suppressant effect of BAPTA-AM on ethanol-induced locomotor activity may not be due to an enhancement of the motor ataxic effects of ethanol. In this work we also have studied the effects of intracellular Ca^{2+} pool chelation on a voluntary ethanol intake paradigm. Our results showed that pretreatment with the intracellular Ca^{2+} chelator BAPTA-AM reduced voluntary ethanol intake in a dose dependent manner. Moreover, BAPTA-AM pretreatment did not modify intake of plain tap water or a sweetened solution, indicating that BAPTA-AM effects are selective to ethanol intake.

The effect of cellular Ca^{2+} chelation was also addressed in a loss of righting reflex paradigm. In this respect it has been described that PKA inhibition at a central level decreased the ethanol-induced loss of righting reflex (Lai et al., 2007). Thus, if intracellular Ca^{2+} mediates the ethanol-induced mobilization of PKA, BAPTA-AM administration may result in reduced PKA activity and so a reduced sleeping time in mice. Results from this experiment showed that the duration of LORR was significantly reduced in animals pretreated with BAPTA-AM. Moreover, as we have previously demonstrated, the effects of intracellular Ca^{2+} chelation seems to be relatively selective for the hypnotic effects of ethanol since no differences were observed in the LORR of pentobarbital-treated animals. Together, these data suggest that the central actions of ethanol require normal distribution and flux of intracellular Ca^{2+}.

Following our proposed mechanism of action of ethanol, in accordance with the literature we have demonstrated that EtOH-induced increase in Ca^{2+} inward currents may be triggered by Ca^{2+} release from different cellular internal stores. The role of ER is of special interest in this regard (Cofan et al., 2000; Salazar et al., 2008; Kelm et al., 2007). If, as we are proposing here, ethanol requires the participation of ER RyR as part of its primary mechanism of action, we suggest that impairment of Ca^{2+} release from ER RyR would affect some neurophysiological responses observed after ethanol administration in mice.

Thus, in the second work, we have studied the effects of dantrolene, an ER RyR blocker, on ethanol-induced locomotor activity, ethanol intake and ethanol sedative effects. Dantrolene administration prevented ethanol-induced locomotor activity in a dose dependent manner. Moreover, basal locomotion or the suppression of motor activity produced by high doses of this alcohol was not affected by dantrolene pretreatment. Again, specific Ca^{2+} release blockade from the ER RyR seems to have a prominent role in the stimulating behavioral effects of ethanol when compared to other drugs with motor-stimulating properties.

The ingestive behavior was also studied in the present work, demonstrating that dantrolene pretreatment was effective in reducing the ethanol intake dose-dependently. Furthermore, ER RyR blockade appears to be selective to the consumption of ethanol because dantrolene administration within the dose-range that blocked ethanol intake did not modify consumption of plain tap or sweetened water.

Results obtained in this work showed that Ca^{2+} release from the ER RyR may be involved in the sedative effects of ethanol since animals pretreated with dantrolene presented a reduction in the loss of righting reflex time.

In summary, results from the present work, indicate that the central actions of ethanol require normal distribution and flux of intracellular Ca^{2+} since we have used dantrolene as a RyR blocker that does not directly affect the general cell Ca^{2+} pool or the extracellular-to-cytoplasm Ca^{2+} flux.

Accordingly to different published studies, Ca^{2+}-activated CaM appears to be a determinant factor in the molecular response elicited by administration. Interestingly, CaM has been described as the "Ca^{2+} sensor" and is one of the main molecules that regulate intracellular Ca2+ homeostasis. The Ca^{2+}-activated CaM interacts with many cellular target proteins such as the AC (Klee et al., 1980) among others, and modulates their activity (Sola et al., 1999; Chin and Means, 1998). In addition, it has been shown that this cellular response to ethanol is mediated through the direct stimulation of the AC, especially AC1 and AC8 (Conti et al. 2009; Maas et al. 2005) leading to an increase in the cellular cAMP levels and therefore PKA activity. The results of our molecular experiments are in agreement with regard to the role of CaM in mediating the molecular response to ethanol. Animals that were pretreated with the CaM inhibitor W7 did not present an activation of PKA after ethanol administration.

Given this, we hypothesize that CaM activity may be a key factor in mediating the neurobehavioral response to ethanol. Thus, in the third work we aimed to study the participation of the intracellular Ca^{2+} sensor CaM in different ethanol elicited behaviors. Results from the present work showed that CaM blockade reduced the locomotor stimulation induced by ethanol. This effect appears to be selective for the stimulating properties of ethanol since locomotor activity induced by other psychostimulant drugs was not affected by W7. We further extended our investigation to assess the effects

of W7 on ethanol voluntary intake. Here we have demonstrated that manipulations of CaM reduced ethanol intake in a dose dependent fashion. These manipulations were selective for the motivational properties of ethanol since the intake of other solutions such as water or saccharine were not affected.

It seems that the manipulations that were effective in reducing the ethanol-induced activation of PKA were also determinant in the neurobehavioral response to ethanol. If we assume that a reduction in the PKA activity induced by ethanol mediates different ethanol elicited behaviors, a potentiation of such PKA activity would also be critical in determining the neurobehavioral response to ethanol. To further clarify the role of the cAMP-PKA pathway in the molecular and behavioral response to ethanol administration *in vivo*, we have designed an experiment aimed at potentiating PKA activity by a cAMP dependent mechanism.

The rate of cAMP production is mediated by an equilibrium between AC and PDE activity. The PDEs comprise a group of enzymes that degrade the phosphodiester bond in the second messenger molecules such as cAMP. Moreover, AC activity acts as a cAMP production-limiting factor (De Arcangelis et al., 2010). In summary, cAMP homeostasis is depending on the cooperative activity of these two enzymes. Thus, in order to investigate the suggested ethanol mechanism of action at a central level, using this molecular and behavioral approach we aimed to study the role of cAMP production rate mediated by PDE4 on the ethanol-induced activation of PKA.

At the molecular level, results from this work demonstrated that ethanol administration to control animals produced an increase in the enzymatic PKA activity measured as the nmol of phosphate transferred to a substrate

by min. Moreover, a slight boost in PKA activity was observed when the PDE4 inhibitor Ro 20-1724 was injected to control animals. When Ro 20-1724 was administered prior to ethanol challenge, observed PKA activity was greater than in control animals (saline-ethanol and Ro 20-1724-saline groups). This would indicate that ethanol administration promotes an increase of the cAMP levels that is highly boosted by the inhibition of the PDE4 by the agent Ro 20-1724. At the behavioral level, greater PKA activity resulted in an increase of ethanol-induced locomotor stimulation. Thus, the potentiation effect appears to be selective for the stimulating effects of ethanol since other drugs with motor stimulation properties were not affected.

In summary, all these data suggest that the molecular and behavioral response to ethanol is determined, in part and at least in all the dependent variables studied here, by strictly controlled intracellular Ca^{2+} homeostasis. Manipulations affecting the general cytoplasm Ca^{2+} pool (BAPTA-AM) or its release from the ER RyR (dantrolene) appear to determine the molecular and behavioral responses to ethanol. Moreover, the modulation of different cAMP-PKA cascade mediators such CaM or cAMP levels appear to play a prominent role in this ethanol-elicited neurobehavioral response.

These results strongly support the proposed hypothesis of a Ca^{2+}-dependent cAMP-PKA-mediated molecular ethanol mechanism of action at a central level. Thus, ethanol administration provokes a linear cascade of events started by Ca^{2+} release from the ER RyR which further activates PKA activity. This increase in PKA activity is caused by an elevation of the cAMP levels due to Ca^{2+}-CaM dependent AC activation.

This ethanol-elicited PKA activation appears to be the end point in a line of events started by an initial Ca^{2+} release from the ER. Thus, manipulations that tended to reduce ER Ca^{2+} flux or cytoplasm Ca^{2+} availability compromised the ethanol-induced activation of PKA and therefore different ethanol elicited behaviors. Moreover, manipulations of the cAMP-PKA pathway mediators also appear to be critical to cellular and behavioral responses to ethanol. CaM blockade resulted in decreased ethanol-induced PKA activity and therefore a reduction in the neurobehavioral effects of ethanol. Furthermore, PDE4 antagonism boosted ethanol-induced PKA activity and therefore a potentiation of the behavioral response to ethanol in the studied paradigm.

As can be seen in molecular studies, ethanol, through the proposed Ca^{2+} dependent mechanism, is able to activate PKA. That being so, there may be an "upstream" mechanism by which the activated PKA modulates the behavioral response to ethanol. In this respect, previously published studies indicate the role of PKA as a key mediator of the behavioral responses to ethanol. Thus, it has been demonstrated that mutant mice with decreased PKA activity were more sensitive to ethanol-induced sedation. Moreover, these animals presented lower preference for ethanol (Wand et al., 2001). To the contrary, knockout mice with elevated brain PKA activity showed increased voluntary ethanol consumption and decreased sensitivity to the hypnotic effects of ethanol (Thiele et al., 2000; Ferraro et al., 2006).

The cAMP-PKA signaling has been implicated in the anxiety response. Animals with an increased level of PKA showed anxiogenic-like behavior. Moreover, it has been demonstrated that the PKA RIIβ subunit of PKA plays a protective role against the stimulant effects of ethanol and ethanol-

induced behavioral sensitization (Freund and Palmer, 1997; Hayes et al., 2012; Keil et al., 2012). Given this, seems that PKA signaling may be critical to the neuroplastic changes underlying the neurobehavioral response to ethanol.

At this point is worth mentioning that a great body of evidence suggests PKA as one of the key factors in the regulation of L-type Ca_vs activity. Different *in vitro* studies have demonstrated that Ca_vs are one of the first discovered ion channels regulated by protein phosphorylation. Among other systems, this channel regulation occurs through a CaM and PKA-dependent pathway that results in the phosphorylation of the channels (Kavali et al., 1997; Hosey et al., 1996). As a result of such PKA mediated phosphorylation, the L-type Ca_vs current is dynamically regulated producing an increase in its activity (Trautwein and Hescheler, 1990; Yatani et al.,1999; Reuter, 1983; Zhülke et al., 1999).

Following our line of evidence, we hypothesize that the PKA activated by ethanol may be critical to the Ca_vs activity. This PKA-mediated Ca_vs channel activation would promote an increase in the Ca^{2+} flux through the extracellular side to the cytoplasm that would be a key factor in the neurobehavioral response to ethanol but not a limiting factor of the intracellular activated PKA response.

Given this, in the fourth experiment we assessed the participation of Ca_vs in ethanol activated PKA and the role of Ca_vs in ethanol-induced behavioral stimulation and motor incoordination. Despite the existence of a wide range of VGCC; L-, P/Q, N-, R- or T-type (Catterall et al., 2005), we have focused in the L-type Ca^{2+} channel subtype due to its prominent role in different critical cellular dynamics. Furthermore molecular isoforms of Ca2+ channel α1 subunit polypeptides (which correspond with the L-type family) have

been extensively localized in the mammalian central nervous system (Trimmer and Rhodes, 2004).

At the molecular level, results from the present study showed that manipulations that blocked Ca^{2+} flux through the L-type Ca_vs did not prevent the ethanol-induced activation of PKA. In other words, extracellular-to-cytoplasm Ca^{2+} flux appears not to participate in the PKA activation response elicited by ethanol.

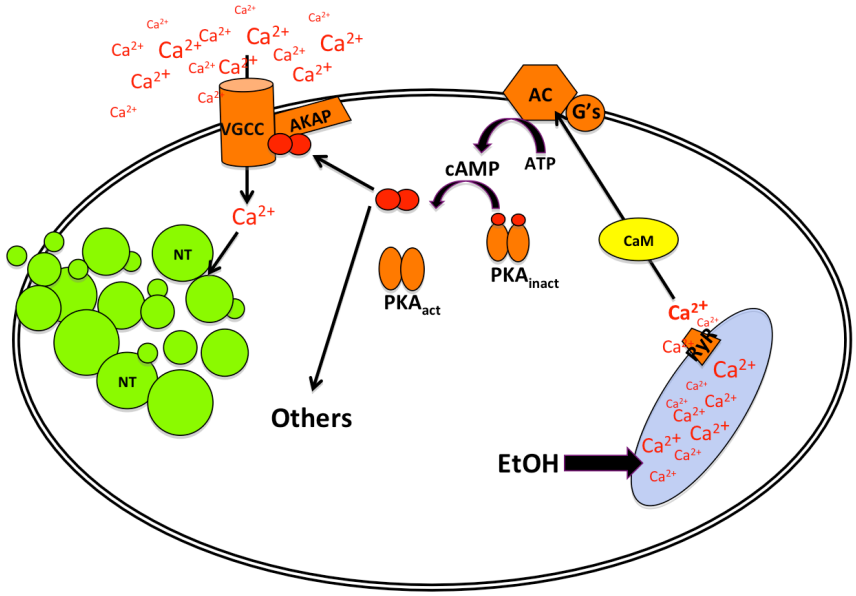
At the behavioral level, L-type Ca_vs blockade resulted in a selective reduction of ethanol-induced locomotor stimulation. This reduction appears to be selective for the stimulating effects of ethanol since locomotor stimulating properties of other drugs were not affected by L-type Ca_vs blockade at doses that prevented the ethanol-induced locomotor activity. Moreover, pretreatment with these two L-type Ca_vs did not increase the motor-incoordinating effects of ethanol in an accelerating rotarod paradigm.

In summary, when all this evidence is considered, our results fully support the proposed hypothesis. Manipulations designed to block or decrease the PKA were also efficient in blocking several ethanol-induced behaviors. In contrast, manipulations that enhanced PKA activity resulted in a potentiation of the behavioral response to ethanol. Moreover, L-type Ca_vs manipulations were able to modulate the behavioral response elicited by ethanol but did not prevent the ethanol-induced PKA activation.

Thus, we finally propose a new mechanism of action of ethanol at a central level based on a selective Ca^{2+} release from the ER RyR. This Ca^{2+}-dependent early cellular response to ethanol promotes the mobilization of different intracellular mediators that lead to an increase in the PKA activity.

This activated PKA is a key factor in mediating the behavioral response to ethanol through a L-type Ca_Vs dependent mechanism

The proposed ethanol-signaling cascade is depicted in the following figure:



V. CONCLUSIONS

At the molecular level:

- *In vivo* ethanol administration, promotes PKA activation in a time dependent manner in different brain areas. The result of such activation measured as a PKA footprint remained for longer periods of time.
- Intracellular ER RyR Ca^{2+} flux blockade with dantrolene prevented the ethanol-elicited activation of PKA. Moreover, this manipulation was also efficient in blocking the PKA footprint.
- Diltiazem blockade of extracellular-to-cytoplasm Ca^{2+} flux through L-type Cavs did not prevent the ethanol-induced activation of PKA. The PKA footprint was also unaffected by this manipulation.
- Selective blockade of the adenosine A2a receptors with DMPX, did not prevent the ethanol-induced activation of PKA and also the consequences of such activation measured as a PKA footprint.
- Blockade of the intracellular Ca^{2+} sensor CaM with W7 prevented the ethanol-induced activation of PKA and PKA promoted footprint.
- Manipulations of the cAMP cellular levels with the PDE4 inhibitor resulted in an increase in the PKA activity in different brain areas.

At the behavioral level:

- Intracellular cytoplasmic chelation with BAPTA-AM, selectively reduced the behavioral stimulation induced by ethanol in a dose dependent fashion. Rotarod motor performance of animals treated with ethanol was not affected after pretreatment with BAPTA-AM. Moreover, BAPTA-AM administration reduced ethanol intake in a dose dependent manner. The consumption of water or sucrose was

not affected by BAPTA-AM pretreatment. Yet again, BAPTA-AM pretreatment decreased selectively the duration of the ethanol-induced sleeping time in a LORR paradigm.

- The ER RyR blockade with dantrolene selectively reduced the ethanol-induced locomotor stimulation in a dose dependent manner. Moreover, ethanol intake was also decreased in a dose dependent manner. No effect was observed on the intake of other fluids such water or saccharine water. Dantrolene administration was also efficient in reducing the ethanol-induced loss of righting reflex.
- The blockade of the intracellular Ca^{2+} sensor CaM reduced the ethanol-induced locomotor stimulation in a dose-dependent manner. Ethanol intake was also decreased after pretreatment with the CaM blocker W7 selectively since no interaction was observed with the intake of water or sweetened water.
- Manipulations of the cAMP levels by the use of a PDE4 inhibitor produced a potentiation effect in ethanol-induced locomotor stimulation.
- Extracellular-to-cytoplasm Ca^{2+} flux blockade through L-type Cavs with diltiazem and verapamil was efficient in reducing dose-dependent ethanol-induced behavioral stimulation. These manipulations did not increase the motor incoordinating effects of ethanol measured using a rotarod paradigm.

General conclusions

- *In vivo*, ethanol administration, promotes an activation of the cAMP-PKA pathway, which is dependent on intracellular Ca^{2+} release from the ER RyR.
- The Ca^{2+}-dependent PKA activation appears to be a key element in mediating the neurobehavioral response to ethanol.
- Extracellular-to-cytoplasm Ca^{2+}flux through L-type Cavs is also a factor that mediates ethanol neurobehavioral effects. However, we suggest that this effect may be consequence of such PKA activation.

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APPENDIX I

Resumen ampliado en Español

UNIVERSITAT DE VALÈNCIA

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Papel del calcio intracelular en la activación de la proteina quinasa A: Modelo molecular y consecuencias conductuales.

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Índice

I- INTRODUCCIÓN

Capítulo 1 - Etanol

Capítulo 2 - Calcio

Antecedentes

1. Homeóstasis de caliio.

    1.1 Retículo endoplásmico

    1.2 Mitocondria

    1.3 Compartimentos acídicos

2. Etanol y calcio.

3. *Resumen del capítulo.*

Epílogo a la introducción

II- HIPÓTESIS Y OBJETIVOS

III- RESULTADOS

IV- DISCUSIÓN

V- CONCLUSIONES GENERALES

INTRODUCCIÓN

Antecedentes

El abuso del etanol y los problemas derivados del consumo excesivo de este, varían de forma diferente en todo el mundo. El informe de la Organización Mundial de la Salud sobre la situación mundial del Alcohol (2004 y 2009) estima que 76 millones de personas en el mundo tienen algún trastorno diagnosticable producido por el excesivo consumo de etanol. El etanol es la causa directa en al menos 60 tipos de enfermedades , y la causa indirecta en otras 200. Casi el 4% de todas las muertes en el mundo se atribuyen al etanol, porcentaje mayor que las muertes derivadas de la tuberculosis, violencia o VIH / SIDA.El abuso del etanol también está asociado con graves problemas sociales tales como la violencia, abandono, abuso infantil y absentismo laboral. A pesar de que el abuso del etanol produce un gran impacto económico y social; y que sus efectos psicofarmacológicos a través de su interacción con numerosos elementos en el sistema nervioso central están ampliamente descritos; hasta la fecha , no se ha podido identificar todavía una diana celular específica o un mecanismo de acción molecular del etanol. A pesar de la magnitud de este problema, existen diferentes tratamientos farmacológicos para el alcoholismo. Por tanto, la correcta comprensión de los mecanismos moleculares por los cuales el etanol altera la función del sistema nervioso central es un factor determinante a la hora de desarrollar futuros medicamentos.

Una de las principales razones que dificulta la identificación del mecanismo central de acción del etanol, radica en la baja afinidad de unión que este tiene hacia las proteínas. Debido a su coeficiente de reparto el etanol se

distribuye de forma equitativa en el componente hídrico corporal, de modo que las concentraciones de etanol los órganos y los diferentes compartimentos celulares son similares a la concentración en sangre . El etanol puede afectar de forma dramática tanto a la estructura como a la función de una amplia gama de proteínas celulares y , en última instancia , muchas actividades biológicas complejas tales como la neurotransmisión o la respuesta inmune.

A altas concentraciones, el etanol puede interactuar y modificar las membranas, provocando cambios inespecíficos en la actividad neural. Sin embargo, existen interacciones mucho mas específicas con proteínas celulares que se producen a concentraciones de etanol fisiológicamente relevantes. Actualmente, hay evidencias que señalan que la respuesta neurobiológica al etanol debería involucrar diferentes cascadas de señalización celular dependientes de hormonas y/o de neurotransmisores. De esta manera se podrían explicar algunos de los cambios agudos y crónicos observados tras la administración del etanol en la función neural. Por ejemplo, se ha descrito que a muy altas concentraciones (> 0,5 M), el etanol provoca cambios en la estructura proteica. Cambios que implican desde la estabilidad proteica hasta a la desnaturalización de proteínas (Dwyer y Bradley , 2000). Por el contrario, a concentraciones bajas (1-80 mM), el etanol modifica la función de diferentes canales iónicos (Messing et al., 1986), receptores de neurotransmisores (Bradley et al . , 1980), enzimas (Chen y LaBella , 1997) y moléculas de adhesión celular(Charness et al . , 1994).

1. Dianas citoplasmáticas del etanol.

Las drogas de abuso tienen diferentes mecanismos de acción pero coinciden en que muchas de sus acciones involucran rutas cerebrales relacionadas con el refuerzo tanto a nivel crónico como a nivel agudo. Importantes cambios tanto moleculares como de la fisiología celular, subyacen al desarrollo de la conducta adictiva. En muchos casos estos cambios resultan no solo ligados a esta conducta adictiva anormal si no que son irreversibles. Estudios genéticos recientes han identificado diferentes cascadas intracelulares que no solo son responsables de cambios en la función sináptica si no que intervienen como dianas moleculares del etanol.

1.1 Fosfoproteína regulada por Dopamina y AMPc de 32 KDa

Esta fosfoproteína es una diana moduladora en rutas dopaminérgicas. Se ha propuesto que su interacción con el etanol, se produce a través de la modulación de los receptores NMDA.

1.2 Proteina Fyn

El elemento Fyn es un miembro de la familia Src de tirosinas quinasas (Src TKs). Esta familia media muchos procesos de señalización intracelular. Se piensa que su interacción con el etanol como mecanismo de acción, esta mediado por los receptores NMDA.

1.3 Proteina Kinasa C (PKC)

Esta proteína pertenece a una familia de serina/treonina quinasas dependientes de fosfolípidos. En función de su estructura y de los segundos mensajeros que la activan, la PKC se clasifican en

covencionales PKCs (α, β, γ), nuevas PKCs (δ, ε, η, θ) y atípicas PKCs (ζ, ι/λ). El primer grupo se activa por diacilglicerol (DAG) y calcio (Ca^{2+}). El segundo grupo, las nuevas se active por DAG pero no por Ca^{2+}. El tercer grupo, las atípicas no se activan ni por DAG ni por Ca^{2} si no por otros lípidos tales como acido araquidónico of fosfatídico. Todas las isoformas se ha demostrado que actúan como dianas del etanol tanto in vivo y en vitro, tanto de forma aguda como crónica.

1.4 Fosfolipasa D (PLD)

A pesar de que las acciones del etanol con las dianas moleculares propuestas no están basadas en la clásica interacción enzima/substrato; la PLD es una de las pocas enzimas que actúa directamente a través de este mecanismo con el etanol.

1.5 Adenilil ciclasas (AC).

Estas enzimas se agrupan dentro de las conocidas como liasas, y se encargan de sintetizar cAMP a partir de ATP. Se ha demostrado que el etanol puede regular estas AC a través de estímulos tales como proteínas G, otras quinasas, Ca^{2+}, etc.

1.6 Protein quinasa dependiente de cAMP (PKA).

Esta quinasa, es el punto final de una cascada intracelular dependiente de AMPc. Se ha descrito que la PKA es una importante diana molecular del etanol. Esta quinasa esta formada por cuatro subunidades de las cuales dos son reguladoras y dos son catalíticas. Cuando el AMPc se une a las subunidades reguladoras, se libera la subunidad catalítica que fosforila diferentes substratos celulares (Brandon et al., 1997). Una de las acciones

principales de la PKA es fosforilar el elemento nuclear CREB (cAMP Response Element Binding) (Brandon et al., 1997). A nivel conductual, se ha descrito que PKA media algunos de los efectos psicofarmacológicos del etanol. De esta manera, se sabe que animales con una actividad PKA disminuida son mas sensibles a los efectos sedativos del etanol, o tienen menos preferencia por este cuando se comparan con animales control (Wand et al., 2001). De forma opuesta, animales con mayor actividad PKA presentan mayor preferencia e ingesta de etanol y son mas sensibles a los efectos sedativos del etanol (Thiele et al., 2000; Kumar et al., 2012).

1.7 cAMP response element binding protein (CREB)

Este elemento, es uno de los mecanismo celulares mejor descritos y conocidos de plasticidad neural inducida por drogas. Sabemos que la expresión de CREB juega un papel crítico en la respuesta neurobiológica al etanol. In vitro, tanto la administración aguda como crónica de etanol induce los niveles de CREB fosforilado (Asher et al., 2002; Constantinescu et al., 1999). Una vez fosforilado, CREB induce la expresión de genes relacionados con la síntesis de neurotrasnmisores, receptores, porteinas de señalización celular, etc (Lonze and Ginty, 2002; Mayr and Montminy, 2001). A nivel conductual, se sabe que CREB fosforilado modula diferentes conductas inducidas por etanol (Newton and Messing, 2006; Pandey et al., 2003; Pandey, 2004).

2. Canales iónicos

Los canales iónicos son proteínas que forman poros a través de la membrana plasmática . Su función principal , recae en establecer y controlar el flujo de voltaje que existe a ambos lados de la membrana de todas las células vivas permitiendo el flujo de iones en contra de su

gradiente electroquímico. Por otra parte, los canales iónicos se observan también en las membranas de orgánulos intracelulares, donde son importantes para la regulación de diferentes procesos intracelulares, tales como por ejemplo la concentración de Ca^{2+} citoplasmático y la acidificación de los compartimentos subcelulares específicos. Los canales iónicos también desempeñan un papel crítico en la señalización celular, la excitabilidad eléctrica, liberación de neurotransmisores, la transcripción de genes, el balance hídrico, y también pueden actuar como dianas de fármacos .

Los canales iónicos se pueden agrupar en dos clases principales , los canales operados por voltaje, o operados por ligando. Pueden existir en varios estados tales como cerrado, abierto o inactivado. Tanto los operados por voltaje como por ligando, presentan una transición entre estos estados en función de los cambios en el potencial de membrana o en respuesta a la unión y/o separación de un determinado ligando.

2.1 Canales iónicos operados por Ligando

En el cerebro, los canales iónicos activados por ligando (LGICs) se encuentran entre los receptores post-sinápticos formados por proteínas. Las células pre-sinápticas liberan neurotransmisores que excitan o inhiben las células post-sinápticas en función del neurotransmisor liberado y a que tipo de receptor post-sináptico se une. Entre estos receptores, los de glutamato N-metil-D-aspartato (NMDA), el de ácido γ-aminobutírico tipo A (GABA-A), 5-hidroxitriptamina-3 (5-HT3), y los de acetilcolina de tipo nicotínico (nACh) son especialmente interesantes en relación al etanol (Harris et al. , 2008).

2.1.1 NMDA

Los canales NMDA son los receptores de glutamato (NMDAR) que median la mayor parte de la transmisión sináptica excitadora en el sistema nervioso central de los mamíferos. Estos receptores son altamente permeables al Ca^{2+} y son bloqueados por Mg^{2+} de forma voltaje dependiente (Mayer et al . , 1984 , McDermott et al . , 1986 , Ascher y Nowak , 1988). Estas propiedades indican que los canales NMDA desempeñan un papel fundamental en importantes funciones fisiológicas, tales como la plasticidad sináptica, el aprendizaje y la memoria, la expresión génica o el desarrollo de las redes neuronales durante el proceso de desarrollo (Mayer y Westbrook, 1987; Volianskis et al, 2013).

Los efectos del etanol sobre los NMDAR se han descrito en diferentes poblaciones de células neuronales. De forma aguda, el etanol inhibe los NMDAR en función de la concentración en un rango de concentración de 5 a 50 mmol / L (Alfonso-Loeches y Guerri, 2011). La exposición crónica al etanol aumenta el número de NMDARs como mecanismo homeostático de compensación. A nivel conductual se ha descrito que estos canales median diferentes aspectos relacionados con la tolerancia o la retirada del etanol (Loeches y Guerri, 2011; Lovinger, 1995; Chandrasekar, 2013)

2.1.2 GABA

De forma opuesta a otros aminoácidos tales como el glutamato que participa en diversas funciones celulares, la única función del GABA es servir como neurotransmisor. GABA es sintetizado a partir del glutamato en un solo paso bioquímico catalizado por la enzima glutamato GAD.

Los receptores GABAA representan uno de los mas importantes objetivos farmacológicos del etanol (Kumar et al., 2009; Weiner y Valenzuela, 2006).

Numerosos estudios in vitro han demostrado que la administración de etanol potencia la función GABAAR (Weiner & Valenzuela , 2006). Así, a nivel conductual, se ha descrito que conductas tales como la ingesta de etanol (Nie et al., 2011; Rewal et al., 2011), tolerancia (Liang et al., 2007), efectos hipnóticos, anticonvulsivos, ansiolíticos (Kumar et al. , 2009) están mediadas por un aumento de la actividad GABAergica inducida por etanol.

2.1.3 nAChR

Los receptores de acetilcolina cerebrales (AChR) se dividen en dos principales familias: muscarínicos (mAChR) y nicotínicos (nAChR). Los mAChR son receptores acoplados a proteína G , mientras que los nAChR son canales iónicos activados por ligando.

Se ha descrito que la estimulación dopaminérgica del sistema mesolímbico inducida por etanol implica la actividad de los nAChR (Ericson et al., 1998; Söderpalm et al. , 2000; Molander et al., 2005). De forma general, el etanol potencia la actividad de nAChR desempeñando un papel clave en la regulación de la conducta motivada (Vangeliene et al., 2008; Ericson et al., 1998, 2003). Evidencias experimentales, demuestran que nAChR modulan la autoadministración de etanol. Manipulaciones farmacológicas que afectan a diferentes subunidades de nAChR reducen la ingesta voluntaria de etanol en un modelo de roedor (Larsson y Engel, 2004; Jerlhag et al., 2006b).

2.1.4 5 - HT

En el cerebro los receptores 5-HT participan en muchas respuestas fisiológicas tales como la modulación del comportamiento, la actividad neuronal o la liberación de neurotransmisor (Hoyer et al. , 2002).

A nivel conductual, se ha descrito que los receptores 5-HT3 están implicados en procesos de ingesta de etanol (Sari et al., 2011) y en diferentes procesos que median los efectos sedativos del mismo (Lovinger, 1997; Vengeliene et al., 2008).

2.2 Canales iónicos dependientes de voltaje

Los canales iónicos dependientes de voltaje (VOCs) son proteínas integrales de membrana que conducen iones de una forma voltaje dependiente. Este tipo de canales de iónicos juegan un papel fundamental en la generación y propagación del impulso nervioso y en la homeostasis celular. Tres propiedades fundamentales de los VOCs permiten a las células nerviosas llevar a cabo el impulso eléctrico: 1 - apertura en respuesta a los cambios en el potencial de membrana, 2 - posterior de cierre de canal e inactivación, 3 – Permeabilidad selectiva para un tipo de iones.

Todos los VOCs permean selectivamente los principales iones fisiológicos, Na^+, K^+, Ca^{2+} y Cl^-.

2.2.1 Los canales de potasio dependientes de voltaje (K_vs)

En relación a las interacciones del etanol con los Kvs, cabe destacar una forma particular de estos Ca^{2+} dependiente llamado canal BK (BK) (Dopico et al., 1999). Los canales BK se expresan en el cerebro y desempeñan un papel importante en muchos aspectos de la fisiología neuronal como la liberación de neurotransmisor, la generación de la excitabilidad dendrítica y la plasticidad sináptica dependiente de potencial (Dopico et al., 1999; Treistman y Martin 2009). Por otra parte, se ha demostrado que los canales BK son un elemento clave en la mediación de los efectos

neuroconductuales del etanol (Treistman y Martin, 2009; Nicoll y Madison, 1982).

A nivel conductual, se ha demostrado que los canales BK median procesos como consumo de etanol o tolerancia aguda al etanol (Treistman y Martin, 2009).

2.2.2 Los canales de sodio dependientes de voltaje (Na_vs)

Los canales de sodio dependientes de voltaje (Na_vs) son proteínas grandes (≈ 250 kDa) integrales de membrana. Estos canales juegan un papel especial en la generación de la *espiga* del potencial de acción. El inicio y la propagación de los potenciales de acción en las neuronas y otras células excitables se determina por la actividad de los Na_vs.

In vitro, se ha demostrado que el etanol produce un efecto inhibitorio sobre la actividad de los Na_vs. Diferentes estudios demuestran que la administración de etanol a cultivos de neuronas disminuye las corrientes de Na^+, la probabilidad de apertura y altera el disparo del potencial de acción (Xiao et al., 2008, Klein et al., 2007). A pesar de que los Na_vs han sido ampliamente estudiados en relación a la generación de potenciales de acción en las neuronas, las consecuencias conductuales derivadas de manipulaciones de los Na_vs están todavía por esclarecer.

2.2.3 Canales de Ca^{2+} dependientes de voltaje (Ca_vs)

Los canales de Ca^{2+} dependientes de voltaje (Ca_vs) han sido ampliamente estudiados debido a su destacado papel en muchas funciones fisiológicas, sobre todo aquellas involucradas en procesos de neurotransmisión. Existen evidencias que señalan el papel de los Ca_vs como intermediarios en la respuesta neurobiológica a diferentes drogas de abuso. En particular,

durante las últimas décadas, los Ca_vs se han convertido en elementos determinantes en la respuesta fisiológica y conductual del etanol.

A pesar de la existencia de diferentes tipos de Ca_vs (tipo L, P/Q, N, R o T), los de tipo L destacan por ser los mas abundantes en el SNC de mamífero. Se ha demostrado que estos canales participan la respuesta neurobiológica a diferentes drogas de abuso (Baliño et al., 2010; Bhutada et al., 2012; Newton et al., 2008; Bisagno et al., 2010).

A pesar de que los efectos del etanol sobre Ca_vs aún no ha sido aclarada, existen evidencias experimentales que indican que la administración de etanol tiene un efecto potenciador de la actividad Ca_vs. Este efecto se ha demostrado in vitro utilizando diferentes cultivos de células de hipotálamo (Simasko et al.,1999), células PC12 (Belia et al., 1995) y en sinaptosomas de homogenados cerebrales (Bergamaschi et al., 1998).

A nivel conductual, antagonistas de los Cavs de tipo L reducen el consumo voluntario de etanol (DeBeun et al., 1997; Fadda et al., 1992), modulan la discriminación al etanol (Colombo et al , 1994), reducen las manifestaciones del síndrome de abstinencia (Bone et al . , 1989 ; Littleton et al , 1990) y modulan la actividad locomotora inducida por etanol (Baliño et al , 2010).

Resumen del capítulo.

En resumen , en esta sección hemos descrito la capacidad del etanol para interactuar con diferentes elementos que conforman el sustrato neural. La mayor parte de este sustrato forma parte de los mecanismos por los que las células transmiten y propagan la información. A pesar de que las dianas moleculares descritas anteriormente se han propuesto como posibles mecanismos de interacción con el etanol, todavía no se ha l

logrado identificar un mecanismo único de acción que permita explicar los efectos psicofarmacológicos del etanol. Sabemos que los efectos conductuales agudos de etanol se observan en un rango de tiempo muy corto (< 5 min) y en un rango de concentraciones cerebrales de etanol que van desde 5 a 200 mM.

Una posibilidad para explicar estos efectos rápidos del etanol podría basarse en una interacción inmediata con receptores, proteínas excitables de membrana, o segundos mensajeros de tal manera que se produzca una rápida transmisión de la información. A este respecto , se ha descrito que el etanol interactúa y modula algunas proteínas excitables de membrana que promueven cambios en el flujo de diferentes iones que participan en la homeostasis eléctrica celular. Estas perturbaciones transitorias del potencial eléctrico de la célula podrían modular potenciales de receptor o el potencial de acción y por consiguiente ejercer una modulación de las señales químicas que median diferentes procesos biológicos.

Hemos descrito que el etanol a través de cambios en la homeostasis celular de diferentes iones promueve cambios en el estado eléctrico de la célula y por lo modula elementos relacionados con el proceso de neurotransmisión. De hecho, estas modificaciones que se producen de forma rápida nos permiten explicar algunas de las respuestas moleculares y conductuales del etanol. Sin embargo, todavía queda por descifrar el mecanismo particular por el cual el etanol interactúa con el sustrato neural propuesto y modula el comportamiento.

Algunos de estos hallazgos descritos anteriormente son especialmente interesantes en relación al ión Ca^{2+}. Aparte de la contribución que el Ca^{2+} tiene en procesos de excitabilidad (Berridge et al , 2000 ; Poage y Meriney, 2002), el Ca^{2+} es una de las señales intracelulares más ubicuas responsable de controlar numerosos procesos neurobiológicos tales como,

cascadas de segundo mensajero, expresión génica diferencial, liberación de neurotransmisor, etc (Berridge et al , 2000 ; Dunlap et al , 1995; Ghosh y Greenberg, 1995) . Por otra parte, el Ca^{2+} participa como un intermediario de especial relevancia en algunas de las dianas moleculares del etanol previamente descritas.

CAPÍTULO II- Calcio

1. Homeóstasis de calcio

El calcio (Ca^{2+}) es una de las moléculas biológicas mas ubicuas y segundo mensajero responsable de numerosos procesos celulares. (Berridge, 1997; Clapham, 1995). A pesar de su relevancia funcional, los niveles prolongados de concentraciones elevadas de Ca^{2+}, son altamente tóxicos para la fisiología celular llegando a causar daños irreversibles e incluso muerte. Esto hace que la célula haya desarrollado diferentes mecanismos homeostáticos para el control de los niveles intracelulares de Ca^{2+} (Clapham, 2007).

Las células movilizan el Ca^{2+}, mediante el uso de dos fuentes. En primer lugar, el Ca^{2+} se puede introducir desde el exterior a través de una gran variedad de canales tales como los canales operados por voltaje (véase el capítulo I) , o canales operados por receptores (véase CAPITULO I) . En segundo lugar, el Ca^{2+} puede ser liberado de los almacenes internos tales como retículo endoplásmico / sarcoplásmico (ER , SR) y las mitocondrias. A pesar de que ER y mitocondria son los compartimentos celulares de mayor importancia en la regulación de los niveles de Ca^{2+}, se han identificado otros orgánulos celulares que participan en este proceso.

Endosomas , vesículas de Golgi , lisosomas , gránulos secretores y melanosomas son compartimentos individuales que pueden contribuir a la homeostasis celular del Ca^{2+} (Rizzuto y Pozzan , 2006, Clapham, 2007).

1.1 Retículo endoplasmático

La homeostasis interna de Ca^{2+} mediada por este compartimiento , se controla principalemnte por inositol - 1 , 4 , receptor de 5 -trifosfato (InsP3R), receptor de rianodina (RyR) y el sarco (endo) retículo endoplásmico Ca^{2+}-ATPasa (SERCA).

1.2 Mitocondria

Las mitocondrias también pueden almacenar Ca^{2+} que se libera de forma pasiva difundiendo fácilmente a través de grandes poros en la membrana mitocondrial externa. Posteriormente pasa a través del espacio mitocondrial por medio de canales iónicos y transportadores.

1.3 Compartimentos acídicos

Recientemente, diferentes orgánulos ricos en H^+ y Ca^{2+} se han descrito como elementos que participan en el control de las dinámicas de Ca^{2+} intracelular en los organismos. Estos compartimentos celulares son diversos, e incluyen: acidocalcisomes, vacuolas, lisosomas y endosomas (Patel y Muallem , 2011 ; Patel y Docampo , 2010).

2. Etanol y calcio

Los efectos del etanol sobre el flujo y distribución de Ca^{2+} han sido ampliamente estudiados en diferentes preparaciones celulares. Se ha demostrado que la administración de etanol a cultivos celulares altera la

homeóstasis de Ca^{2+} aunque los mecanismos concretos de este proceso son todavía desconocidos. En general se asume que la exposición de cultivos celulares a concentraciones de etanol biológicamente relevantes (< 80mM), aumenta los niveles intracelulares Ca^{2+} (Daniell and Harris, 1989; Mironov and Hermann, 1996; Xiao et al., 2005). La gran mayoría de estudios apuntan a que estos cambios en la homeostasis celular están mediados por el flujo de Ca^{2+} extracelular a través de los canales de calcio operados por voltaje. Sin embargo, existen evidencias de un mecanismo celular alternativo que media la homeostasis intracelular de Ca^{2+}. Así, se ha demostrado que la administración de etanol en cultivos de células modifica los niveles intracelulares de Ca^{2+} gracias a un mecanismo de liberación dependiente de las reservas de Ca^{2+} intracelulares (González et al , 2007 ; . Kelm et al , 2007 ; . Mironov y Hermann , 1996 ; . Salazar et al , 2008 , Daniell y Harris , 1989).

Resumen del capítulo

En esta sección , hemos descrito el papel del iones Ca^{2+} en muchos procesos biológicos. Debido a su toxicidad celular, la homeostasis de Ca^{2+} ha de ser estrictamente controlada por una serie de bombas y compartimentos celulares. El Ca^{2+} no sólo contribuye al estado eléctrico de la célula y tiene un papel determinante en el potencial de acción y liberación de neurotransmisores, si no que el Ca^{2+} actúa como un segundo mensajero crítico que media la transmisión de información a través de la célula. Estas propiedades convierten a este elemento celular en candidato perfecto que permitiría explicar los efectos cuasi inmediatos del etanol. Además, el Ca^{2+} resulta un elemento determinante en muchos de los procesos anteriormente descritos.

EPÍLOGO

Tomada en conjunto toda la información expuesta en esta introducción teórica , parece que el etanol modula diferentes sustratos neurales aunque seguimos sin proponer un único mecanismo de acción general del etanol a nivel central.

Parte de esta dificultad está relacionada con las propiedades físico-quimicas del etanol. A este respecto, el etanol puede ser considerado una droga débil en comparación con la mayoría de las drogas de abuso . La molécula de etanol no interactúa con los sustratos biológicos de forma estereoselectiva como la mayoría de los fármacos basados en un mecanismo ligando/receptor. El etanol tiene una estructura molecular muy simple que le confiere poca reactividad química. Esta misma estructura le confiere un carácter anfipático cosa que le dota de un coeficiente de distribución del 50%. Esto hace que actúe de forma inespecífica en el SNC. No obstante, estas características resultan paradójicas cuando se le compara con el resultado conductual tan organizado y complejo. Una proteína quinasa clave que ha sido descrita como un intermediario implicado en las respuestas neuroconductuales del etanol, es la PKA. Esta quinasa es el punto final de una vía intracelular dependiente de AMPc. La PKA activada transmite información de forma rápida a través de la célula y promueve cambios en la maquinaria celular que están involucrados en la modulación de varios efectos conductuales del etanol. Teniendo en cuenta esto , en el presente trabajo nos proponemos estudiar el papel de la distribución y flujo de Ca^{2+} celular en la modulación de la vía intracelular AMPc/PKA como mecanismo central de la acción de etanol.

HIPÓTESIS Y OBJETIVOS

Hipótesis general

El etanol interactúa con múltiples dianas moleculares, pero un mecanismo de acción especifico que permita explicar las acciones del etanol a nivel central, sigue siendo desconocido. Se ha descrito la participación de la cascada AMPc-PKA como elemento mediador de la respuesta neuroconductual al etanol. El Ca^{2+} intracelular es un segundo mensajero implicado en la transducción de señales e información celular a través de la célula . Sabemos que el Ca^{2+} actúa como elemento clave en la cascada de señalización AMPc-PKA.

Basándonos en la información presentada en la introducción teórica, hipotetizamos que la modulación de la homeostasis celular de Ca^{2+} *in vivo*, es un factor crítico que determina la respuesta de la cascada AMPc-PKA y por tanto la conducta inducida por el etanol. Por esta razón se propone que la administración de etanol in vivo, modificará los flujos de Ca^{2+} celular para posteriormente activar la CaM que aumentara la actividad AC y así los niveles de AMPc que activarán a la PKA .

Como resultado de esta activación PKA, diferentes sustratos serán modificados, en particular los Cavs , que desempeñan un papel clave en las conductas inducidas por etanol.

El **objetivo general** de este trabajo es:

Primero, estudiar el papel del calcio celular en la activación de la vía AMPc-PKA inducida por etanol in vivo.

- Estudiar el papel del flujo de calcio extracelular hacia el citoplasma.
- Estudiar el papel del flujo de calcio del retículo endoplásmico.
- Estudiar el papel de intermediarios calcio dependientes de la via AMPc-PKA.

Segundo, estudiar las consecuencias conductuales de manipulaciones del calcio y de la via AMPc-PKA en diferentes conductas inducidas por etanol.

- Estudiar el papel del calcio citoplasmático general.
- Estudiar el papel del flujo de calcio extracelular hacia el citoplasma.
- Estudiar el papel del flujo de calcio del retículo endoplásmico.
- Estudiar el papel de intermediarios calcio dependientes de la via AMPc-PKA.

Los **objetivos específicos** de este trabajo son:

A nivel molecular:

- Estudiar el papel de flujo de Ca^{2+} a través de ER RyR en la activación de la PKA inducida por el etanol en diferentes áreas del cerebro.
- Estudiar el papel del flujo de Ca^{2+} a través de Cavs en la activación de la PKA inducida por el etanol en diferentes áreas del cerebro.
- Estudiar el papel de los receptores A2a, en la activación de la PKA inducida por el etanol en diferentes áreas del cerebro.
- Estudiar el papel de la CaM en la activación de la PKA inducida por el etanol en diferentes áreas del cerebro.

- Estudiar el papel de la fosfodiesterasa 4 en la activación de la PKA inducida por etanol en diferentes áreas del cerebro.

A nivel conductual:

- Estudiar el papel del calcio celular en la estimulación locomotora inducida por etanol, en los efectos incoordinantes del etanol, la perdida del reflejo de enderezamiento y en la ingesta de etanol.
- Estudiar el papel del flujo de calcio extracelular hacia el citoplasma a través de los Cavs en la estimulación locomotora inducida por etanol e incoordinación motora.
- El papel del flujo de Ca2 + intracelular a través de RyR en la estimulación locomotora inducida por etanol, ingesta de etanol y perdida del reflejo de enderezamiento.
- El papel de la CaM activada por Ca2+ en la estimulación locomotora inducida por etano y en la ingesta de etanol.
- El papel del AMPc a través de la modulación dependiente de fosfodiesterasa 4 en la estimulación locomotora inducida por etanol.

DISCUSION

El trabajo presentado ha sido diseñado para estudiar un nuevo mecanismo de acción del etanol a nivel central que implica la movilización de Ca^{2+} desde el retículo endoplásmico para activar posteriormente una vía señalización mediada por cAMP. Además, en este trabajo se ha evaluado las consecuencias moleculares y conductuales de manipulaciones del Ca^{2+} celular (intracelular y extracelular) en la activación de la proteína quinasa A inducida por etanol. En este trabajo, se ha estudiado también el

papel de diferentes intermediarios de la vía cAMP-PKA la respuesta de la PKA inducida por etanol, así como la participación de estos intermediarios a nivel conductual.

A nivel molecular.

En el primer trabajo, se aborda in vivo, la participación del Ca^{2+} celular en la respuesta AMPc-PKA dependiente de etanol en diferentes áreas del cerebro. En este sentido, este trabajo se ha centrado en el papel del flujo de Ca2 + a través los Cavs de tipo L y en el flujo de Ca2 + intracelular de los RyR del RE.

Resultados obtenidos en el presente trabajo, demostraron que la administración de etanol aumentó la actividad PKA de forma tiempo dependiente en la corteza , hipotálamo , hipocampo y estriado. Además, manipulaciones de flujo de Ca^{2+} de los RyR del RE bloquearon la inducción de la PKA por el etanol. De forma opuesta, manipulaciones del Ca^{2+} a través de los Cavs de tipo L no modificaron la activación de la PKA inducida por el etanol.

El segundo trabajo, se ha estudiado la participación de la CaM en la activación de la vía PKA inducida por etanol. Resultados de este estudio demostraron que el tratamiento con un inhibidor (W7) de la actividad CaM previo a la exposición al etanol, es capaz de prevenir la activación PKA inducida por etanol.

En resumen, los resultados de estos experimentos apoyan nuestro modelo propuesto de acción lineal del etanol. De tal manera que la liberación de Ca^{2+} a través de los RyR del RE sería una respuesta inicial temprana inducida por el etanol para activar la vía de señalización cAMP-PKA.

A nivel conductual.

En el primer trabajo, se investigó el papel de la quelación intracelular de Ca^{2+} en diferentes conductas inducidas por etanol. Hemos demostrado que el pretratamiento con un agente permeable de membrana quelante de calcio (BAPTA-AM) redujo de forma selectiva la estimulación locomotora inducida por etanol y la ingesta voluntaria. Además, dosis de BAPTA-AM que redujeron la actividad locomotora inducida por etanol no incrementaron los efectos incoordinantes de este, sugiriendo que esta reducción en la actividad locomotora no es debida a un incremento en los efectos atáxicos del etanol. También se evaluó en este trabajo el efecto de la quelación de Ca^{2+} intracelular en la perdida del relejo de enderezamiento inducida por etanol. A este respecto, animales pretratados con BAPTA-AM fueron menos sensibles a los efectos hipnóticos del etanol ya que la pérdida del reflejo de enderezamiento fue menor que en animales control.

En el segundo trabajo se estudió el papel del dantrolene, un inhibidor de la liberación de calcio a través de los RyR del RE, en diferentes conductas inducidas por etanol. A este respecto, la administración de dantrolene produjo una reducción selectiva en la actividad locomotora inducida por etanol de forma dosis dependiente. En este trabajo se estudió también el rol de este compuesto en la ingesta de etanol. De forma similar a los experimentos de actividad locomotora, la administración de dantrolene redujo de forma selectiva la ingesta de etanol en un modelo de acceso restringido. También la perdida del reflejo de enderezamiento inducida por etanol fue revertida tras el tratamiento con este inhibidor de los RyR del RE.

En el tercer trabajo, se estudió el papel del sensor intracelular de $Ca^{2+,}$ CaM, en diferentes conductas inducidas por etanol. Así, manipulaciones donde se bloqueo la actividad CaM bloquearon la respuesta motora

estimulante del etanol. Además, estas manipulaciones fueron efectivas en paradigmas de ingesta de etanol. La administración del inhibidor de la CaM (W7), redujo los niveles de ingesta de etanol en un paradigma de acceso restringido.

Teniendo en cuenta estos resultados, parece que las manipulaciones que a nivel molecular tienden a disminuir la actividad PKA, resultan en un bloqueo de diferentes conductas inducidas por el etanol. Con esto en mente, proponemos que la potenciación de esta actividad quinasa debería producir una potenciación de los efectos estimulantes del etanol y una mayor ingesta. Para ello, en el tercer experimento usamos una manipulación bioquímica diseñada a elevar los niveles intracelulares de AMPc y por tanto potenciar la actividad PKA. Nuestra estrategia se basa no en aumentar la sintesis de AMPc si no en impedir su degradación. Manipulaciones orientadas a bloquear la fosfodiesteras 4 demostraron a nivel bioquímico niveles mas altos de actividad PKA. A nivel conductual, estas manipulaciones de la PDE4 resultaron en una potenciación selectiva de los efectos estimulantes del etanol. Estos resultados apoyan nuestra hipótesis propuesta ya que manipulaciones destinadas a bloquear la actividad PKA resultaron un bloqueo de las conductas inducidas por el etanol. De forma opuesta, manipulaciones orientadas a aumentar la actividad PKA resultaron en una mayor potenciación de los efectos estimulantes del etanol.

Teniendo en cuenta los resultados obtenidos, proponemos un nuevo mecanismo de acción para el etanol basado en la liberación selectiva de Ca^{2+} de los RyR del RE que posteriormente actúa sobre una serie de proteínas intracelulares que conforman la vía de señalización del AMPc activando finalmente la PKA. Una de las acciones celulares de esta PKA

sería aumentar la actividad de los canales de Ca^{2+} dependientes de voltaje. Esto permitiría explicar porque manipulaciones de estos canales son suficientes para bloquear conductas inducidas por el etanol pero no resultan efectivas a la hora de impedir la activación de la PKA inducida por etanol. Esto solo sería posible si la activación de la PKA es un paso previo a activación de los canales de calcio dependientes de voltaje.

CONCLUSIONES GENERALES

- La administración de etanol *in vivo* promueve la activación de la vía AMPc-PKA de forma dependiente de la liberación de Ca^{2+} intracelular de los RyR del RE.
- La activación Ca^{2+}-dependiente de la PKA parece ser un elemento clave en las efectos neuroconductuales inducidos por el etanol.
- Los efectos conductuales derivados de la modulación de los Cavs de tipo L son consecuencia de la activación previa de la PKA.

APPENDIX II

ESTUDIO 1

Baliño P, Ledesma JC, Aragon CM. *In Vivo Study of Ethanol-Activated Brain Protein Kinase A: Manipulations of Ca2+ Distribution and Flux.* Alcohol Clin Exp Res. 2013 Oct 11. doi: 10.1111/acer.12289.

Introducción

Se ha demostrado que la vía de señalización de proteína quinasa dependiente de AMPc (PKA) desempeña un papel importante en la modulación de varias conductas inducidas por etanol. In vivo , la exposición aguda etanol regula la vía de señalización del AMPc. Curiosamente, diferentes intermediarios calcio dependientes de esta vía desempeñan un papel crítico en la respuesta neuroconductual al etanol. De especial relevancia es el caso de las adenilil ciclasas calcio dependientes 1 y 8. Postulamos pues que la activación calcio dependiente de la PKA inducida por etanol. De esta manera, en el siguiente trabajo se trata de estudiar el papel del calcio celular (interno y externo) en la respuesta de la vía AMPc/PKA.

Procedimiento

Ratones Swiss macho de 6 semanas recibieron una inyección intraperitoneal de etanol (0 o 4 g/kg). Siguiendo un patrón temporal (7 , 15 , 30 , 45 , 90 , o 120 minutos) se diseccionaron los cerebros.

Una vez obtenidas las muestras, se analizó la actividad enzimática de la PKA o la huella que esta deja en un conjunto de proteínas celulares. Los ratones fueron pretratados con diltiazem (0 o 20 mg/kg) , dantrolene (0 o 5

mg/kg), o 3,7-dimetil-1-(2-propinil) xantina (CMPX) (0 o 1 mg/kg) 30 minutos antes de la administración de etanol (4 g/kg). Después de 45 minutos de la administración de etanol, se diseccionaron los cerebros en diferentes áreas para medir la actividad de la PKA o su huella en un conjunto de sustratos.

Resultados

Resultados de estos experimentos demostraron que existe una activación dependiente de etanol de la PKA en diferentes áreas de cerebro. Manipulaciones farmacológicas que bloquearon la liberación de calcio del retículo endoplásmico a través de los receptores de rianodina, produjeron un bloqueo en la activación de la PKA inducida por etanol. De forma opuesta, manupulaciones farmacológicas que bloquearon el flujo de calcio a través de los canales operados por voltaje de tipo L , no evitaron la respuesta de activación de la PKA promovida por la administración de etanol.

Conclusiones

En conjunto, estos resultados demuestran el papel fundamental del calcio intracelular como un intermediario clave en las acciones neurobiológicas

del etanol. Proporcionando además una prueba más del posible rol del calcio como diana del etanol en el sistema nervioso central.

ESTUDIO 2

Baliño P, Monferrer L, Pastor R, Aragon CM*. Intracellular calcium chelation with BAPTA-AM modulates ethanol-induced behavioral effects in mice.* Exp Neurol. 2012 234, 446-453.

Introducción

El calcio se ha caracterizado como una de las moléculas de señalización intracelulares más ubicuas y universales. Los efectos del etanol sobre la distribución y el flujo de calcio han sido ampliamente estudiado in vitro. Estos trabajos muestran que la administración aguda de etanol modula de manera dependiente de la concentración. In vivo, la relación entre las manipulaciones de los niveles de calcio celulares y los efectos conductuales del etnaol se han centrado en el flujo de calcio a través de los canales de calcio acctivados por voltaje. En el presente estudio se trató de elucidar el papel de los flujos intracelulares de calcio en los efectos psicomotores inducidas por etanol (estimulación y sedación). En una segunda serie de experimentos, se investigaron los efectos de la quelación de calcio intracelular en la ingesta de etanol.

Procedimiento

La herramienta farmacológica usada en el siguiente estudio es una molécula quelante de calcio (BAPTA-AM) constitutivamente inactiva.

Esta molécula lleva asociado un residuo acetometil-ester de manera que le confiere la propiedad de ser permeable a la membrana celular. Una vez ha penetrado en la célula, las esterasas citoplasmáticas cortan este residuo

acetometil-ester haciendo la molécula activa. De esta manera nos aseguramos que la acción de este compuesto es una y exlcusivamente intracelular.

Ratones *swiss* macho de 6 semanas fueron pretratados con BAPTA-AM (0-10 mg/kg) 30 minutos antes de la administración de etanol (0-4 g/kg). Inmediatamente, se situó a los animales en un campo abierto para monitorizar la distancia horizontal recorrida. Para el estudio de los efectos sedativos del etanol, los animales se situaron en lechos de narcosis hasta la recuperación del reflejo de enderezamiento.

En los experimentos de ingesta de etanol se usaron ratones macho de la estirpe C57. Estos animales se pretrataron itraperitonealmente con BAPTA-AM (0-5 mg/kg) y 30 min después se expusieron a etanol (20%, v/v). Durante 2 horas se monitorizó la ingesta en ml. Posteriormente los datos fueron convertidos a g/kg.

Resultados

Los resultados de este estudio muestran que el pretratamiento con BAPTA-AM bloqueo la estimulación locomotora inducida por etanol sin alterar la locomoción basal. Por el contrario, el pretratamiento con BAPTA-AM revirtion los efectos hipnóticos inducidos por etanol ya que los

animales pretratados con BAPTA-AM recobraron el reflejo de enderezamiento antes que los animales control.

Resultados de los experimentos de ingesta de etanol, revelaron que el pretratamiento con BAPTA-AM (0-5 mg/kg) redujo el consumo de etanol de manera dosis dependiente sin afectar el consumo de agua o una sustancia azucarada.

Conclusiones

Nuestros resultados apoyan el papel activo de las corrientes intracelulares de calcio en la mediación de diferentes respuestas conductuales inducidas por etanol. Nuestros resultados muestran también que el etanol parece ser mas sensible a modulación de los niveles intracelulares de calcio que otras drogas psicoactivas.

ESTUDIO 3

Tarragon E, **Baliño P**, Aragon CM. *Dantrolene blockade of ryanodine receptor impairs ethanol-induced behavioral stimulation, ethanol intake and loss of righting reflex.* Behav Brain Res. 2012 233, 554-562.

Introducción

El calcio se ha caracterizado como una de las señales intracelulares más ubicuas, universales y versátiles. Entre diferentes sustancias que tienen la capacidad de alterar los niveles de calcio intracelular, el etanol se ha descrito como de especial relevancia debido a su impacto social y económico. Los efectos del etanol sobre la distribución y flujo de calcio in vitro han sido ampliamente estudiados, demostrando que la administración aguda de etanol

modula los niveles de calcio intracelular de manera dosis dependiente. El calcio intracelular liberado por retículo endoplásmico juega un papel determinante en varios procesos celulares. En el siguiente trabajo, estudiaremos el papel del *dantrolene,* un antagonista específico del receptor de rianodina, en tres conductas suscitadas por el etanol: actividad locomotora, pérdida del reflejo de enderezamiento e ingesta de etanol.

Procedimiento

Ratones Swiss macho de 6 semanas, fueron inyectados intraperitonealmente con dantrolene (0-5 mg/kg) 30 min antes de la administración de etanol (0-4 g/kg).

Inmediatamente, los animales se colocaron en un campo abierto para medir la distancia horizontal recorrida. En los experimentos de perdida del reflejo de enderezamiento los animales se colocaron en lechos de narcosis para medir el tiempo en recuperar el reflejo de enderezamiento.

La ingesta de etanol se estudió mediante un paradigma de acceso restringido en oscuridad. Ratones macho de la stirpe C57 se pretreataron intraperitonealmente con dantrolene (0-5 mg/kg) 30 minutos antes de la exposición al etanol (20%, v/v). Durante 2 horas se monitorizó la ingesta en ml. Posteriormente los datos fueron convertidos a g/kg.

Resultados

Nuestros resultados demuestran que el pretratamiento con dantrolene reduce dosis dependiente y de forma selectiva la estimulación locomotora inducida por etanol y la ingesta de etanol. Además disminuye la sensibilidad a los efectos sedativos de altas dosis de etanol ya que el pretratamiento con dantrolene redujo selectivamente el tiempo de perdida del reflejo de enderezamiento..

Conclusiones

En conjunto, estos datos sugieren que el calcio intracelular liberado por retículo endoplásmico juega un papel crítico en los efectos conductuales

causadas por el etanol y apuntan al calcio como un posible mecanismo de acción molecular del etanol .

ESTUDIO 4

Baliño P, Ledesma JC, Aragon CM. *Role of Ca2+/Calmoduline on ethanol neurobehavioral effects*. Psychopharmacology (Berl). 2013 *[In press]*

Introducción

La vía de señalización de AMPc y en concreto, la PKA sean descrito como importantes moduladores en diferentes conductas inducidas por el etanol. Se ha demostrado que el Ca^{2+} intracelular es un intermediario importante en la activación de la PKA en respuesta al etanol. La proteína Ca^{2+} dependiente calmodulina (C.M), no solo participa como elemento crítico en la vía del AMPc si no que se ha demostrado que esta implicada en los efectos centrales del etanol. Por tanto, en este estudio pretendemos evaluar el papel de la CaM en la activación de la PKA inducida por etanol. Además se estudiará el papel de la CaM en diferentes conductas inducidas por el etanol tales como la estimulación locomotora inducida por etanol y la ingesta de etanol.

Procedimiento

Ratones *Swiss* macho de 6 semanas se pretrataron intraperitonealmente con un inhibidor selectivo de la CaM, el compuesto W7 (0-10 mg/kg), 30 min. antes de la administración de etanol (0-3.5 g/kg). Inmediatamente los animales fueron colocados durante 20 minutos en un paradigma de campo abierto donde se registró la actividad locomotora durante 20 min.

La ingesta de etanol se evaluó mediante un paradigma de acceso restringido en oscuridad. En este estudio, se utilizaron ratones macho de la

estirpe C57 de 6 semanas. Los animales se pretrataron con w7 (0-10 mg/kg) 30 min. antes de exponerlos al etanol (20% v/v). La ingesta en ml se monitorizo cada 30 min. durante 2 h. Los ml consumidos en 2 h. se transformaron a g/kg.

Para los experimentos de *Western blot* , el W7 (0 o 5 mg/kg) se administró 30 min. antes de la inyección de etanol (0 o 2.5 g/kg). La extracción de los cerebros se realizo 45 min. después de la administración de etanol.

Resultados

Los resultados de este estudio mostraron que el tratamiento previo con W7, redujo de forma selectiva la estimulación y dependiente de dosis, la actividad locomotora inducida por el etanol y la ingesta de etanol. El pretratamiento con W7 resulto efectivo a la hora de bloquear la activación de la PKA inducida por el etanol.

Conclusiones

Estos resultados demuestran la CaM, es un intermediario crítico en las acciones conductuales del etanol. La inhibición selectiva de la CaM resultó en un bloqueo de los efectos estimulantes del etanol y redujo los niveles de ingesta en un paradigma de acceso restringido. La activación de la PKA inducida por el etanol también fue inhibida en animales tratados con W7.

Estos resultados apoyan la literatura previa y aportan una prueba mas del papel de la vía intracelular del AMPc como posible diana de acción del etanol in vivo.

ESTUDIO 5

Baliño P, Ledesma JC, Aragon CM. *Increased cAMP cellular levels by inhibition of phosphodiesterase-4 potentiate ethanol's effects on locomotor activity in mice. 2013 [Under revission]*

Introducción

La ruta de señalización mediada por aMPc, se ha propuesto un importante sistema que media los efectos conductuales del etanol. Además, sabemos que uno de los elementos que conforman esta vía, la PKA, es responsable de la modulación de diferentes conductas inducidas por el etanol. La sísntesis del AMPc viene determinada por la actividad enzimática de las adenilil ciclasas que sintetizan AMPc a partir de ATP. De forma opuesta, la degradación del AMPc viene determinada por la actividad de otras enzimas denominadas fosfodiesterasas (PDE). De toda la familia de PDEs, resulta de especial interés la PDE4 ya que se ha descrito que es especifica para la degradación de AMPc en el cerebro. En el presente trabajo, se pretende abordar el rol de la PDE4 en los efectos estimulantes del etanol.

Procedimiento

Ratones *Swiss* macho de 6 semanas fueron inyectados con el Ro 20-1724 (0-5 mg/kg; i.p) a diferentes intervalos de tiempo antes de la administración del etanol (0, 1.25, 2.5, 3.75 g/kg; i.p). El compuesto Ro 20-1724 es un inhibidor selectivo de la PDE4. De esta manera se consiguen aumentar los niveles de AMPc de una manera mas duradera y estable que activando las enzimas encargadas de su síntesis. Inmediatamente después de la administración de etanol, los animales se situaron durante 20 min. en un

paradigma de campo. Durante este tiempo se monitorizo la actividad locomotora del animal.

Resultados

La administración de Ro 20-1724, potenció la respueta locomotora inducida por etanol de manera dependiente de dosis. La actividad basal del animal se modificó por la administración previa de este compuesto. Los efectos sedativos producidos por dosis altas de este compuesto, no fueron afectados por el pretratamiento con Ro 20-1724.

La acción de este compuesto parece ser selectiva para los efectos estimulantes, en este caso, del etanol ya que la actividad locomotora de otras drogas estimulantes (anfetamina y cocaína) no se vió afectada tras la administración de Ro 20-1724.

Conclusiones

Resultados de este estudio, confirmaron que la inhibición selectiva de la PDE4 produjo una potenciación de los efectos estimulantes del etanol en un modelo de roedor. Estos resultados proporcionan nuevas evidencias al rol de la vía de señalización de AMPc en los efectos centrales del etanol.

ESTUDIO 6

Baliño P, Pastor R, Aragon CM *Participation of L-type calcium channels in ethanol-induced behavioral stimulation and motor incoordination: effects of diltiazem and verapamil*. Behav Brain Res. 2010 209, 196-204.

Introducción

El flujo de calcio a través de los canales de calcio activados por voltaje (CCAV) está involucrado en muchos procesos neuronales tales como la despolarización de membrana, expresión génica, secreción de hormonas y liberación de neurotransmisores entre otros. Varios estudios han demostrado que tanto la exposición aguda como crónica a etanol modifica la entrada de calcio a través de los CCAV. De especial relevancia son los CCAV de tipo L. Se ha demostrado que manipulaciones farmacológicas de los CCAV de tipo L afectan a la ingesta de etanol, la discriminación etanol y manifestaciones de síndrome de abstinencia. El presente estudio investiga el papel diferentes inhibidores de los CCAV de tipo L en los efectos psicomotores (estimulación y sedación/ataxia) del etanol.

Procedimiento

Ratones Swiss macho de 6 semanas, fueron pretratados intraperitonealmente con diltiazem (0-40 mg/kg) o verapamil (0-30 mg/kg) 30 minutos antes de la administración de etanol (0-3,5 g/kg). Inmediatamente después de la inyección de etanol los animales se pusieron en un campo abierto durante 20 min. Donde se midió la actividad locomotora horizontal.

Resultados

Los dos inhibidores testados, diltiazem y verapamil, modularon dosis dependiente la estimulación locomotora inducida por etanol pero no la supresión locomotora producida por dosis sedativas de este. Dosis de diltiazem y verapamil que redujeron la estimulación motora inducida por etanol, no alteraron la locomoción espontánea del animal. Los efectos atáxicos del etanol (1,25 g/kg) medidos en un paradigma coordinación/incoordinación motora, no se vieron afectados tras la administración de diltiazem (20 mg/kg) o verapamil (15 mg/kg). Además, nuestros resultados indican que el etanol es más sensible al antagonismo de los CCAV de tipo L que otros fármacos con propiedades estimulantes. Dosis de diltiazem y verapamil que bloquearon la estimulación locomotora inducida por etanol no afectaron los efectos psicomotores de la anfetamina, cafeína y cocaína .

Conclusiones

En conclusión, estos datos proporcionan nueva información y amplían la literatura sobre los efectos del bloqueo de los CCAV de tipo L en las conductas inducidas por etanol.