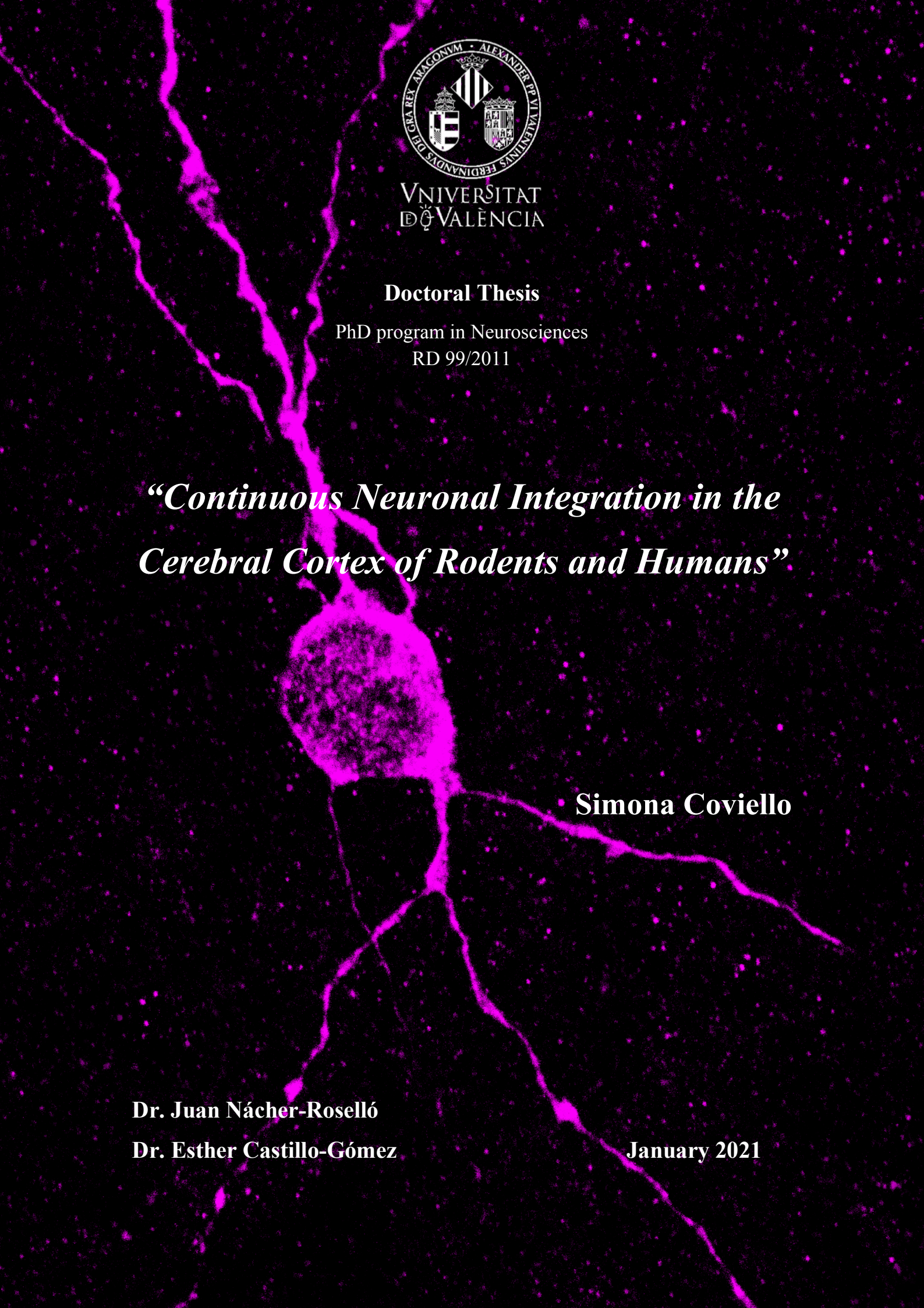

VNIVERSITAT DE VALÈNCIA

Doctoral Thesis

PhD program in Neurosciences
RD 99/2011

"Continuous Neuronal Integration in the Cerebral Cortex of Rodents and Humans"

Simona Coviello

Dr. Juan Nácher-Roselló
Dr. Esther Castillo-Gómez

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Juan Nàcher Roselló, Catedrático del Instituto Universitario de Biotecnología y Biomedicina (BIOTECMED) de la Universitat de València y Esther Castillo Gómez, Profesora Ayudante Doctora del Departamento de Medicina de la Universitat Jaume I de Castelló,

Certifican

Que Simona Coviello, ha realizado bajo su dirección el presente trabajo de investigación, correspondiente a la Tesis Doctoral titulada: “Continuous Neuronal Integration in the Cerebral Cortex of Rodents and Humans”.

Una vez revisado el trabajo, consideran que puede ser presentado al Tribunal que ha de juzgarlo para optar al grado de Doctora.

Y para que así conste, en cumplimento de la legislación vigente, firman el presente certificado a 11 de Enero de 2021.

Juan Nàcher Roselló, PhD

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A mia madre,
per avermi trasmesso
la passione e l'amore
per la scienza.

"Rare sono le persone che usano la mente,
poche coloro che usano il cuore e uniche coloro che usano entrambi."

Rita Levi-Montalcini

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ABBREVIATIONS

5-HT	5-hydroxytrypatamine
5-HTT	5-hydroxytrypatamine transporter
6-OHDA	6-hydroxydopamine
aa	Amino acid
ABC	Avidin-biotin-peroxidase complex
AIS	Axonal initial segment
Ank-G	Ankyrin-G
AON	Anterior olfactory nucleus
Bcl-2	B-cell lymphoma 2 protein
BrdU	Bromodeoxyuridine
CAMK-II	Ca^{2+}/calmodulin-dependent protein kinase II
CB	Calbindin
CCK	Cholecystokinin
CNGA-3	Cyclic nucleotide-gate ion channel 3
CNS	Central nervous system
CoA	Cortical amygdala
CR	Calretinin
CTIP2	COUP TF1-interacting protein 2
CUX1	Cut like homeobox 1
D2r	Dopamine type 2 receptor
DA	Dopamine
DAT	Dopamine transporter
DCX	Doublecortin
DG	Dentate gyrus
DGCs	Dentate gyrus granule cells
DLL	Pan distalles
DLPNs	Deep pyramidal neurons
DNA	Deoxyribonucleic acid
E	Embryonic day
EGFP	Enhanced green fluorescent protein
Endo-N	Endoneuraminidase-N

FCD	Focal cortical dysplasia
GABA	γ-Amynobutiric acid
GAD67	Glutamatergic acid decarboxylase 67
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
i.p.	Intraperitoneal
IBA1	Ionized calcium binding adaptor molecule 1
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IN	Interneurons
IP	Immunoprecipitation
kDa	KiloDalton
KO	knockout mice
LEnt	Lateral entorhinal cortex
LHX6	LIM/homeobox protein 6
LOT	Lateral olfactory tract
LV	Lateral ventricle
MAP2	Microtubule associated protein 2
MCA	Middle cerebral artery
MGCs	Mature granule cells
mPFC	Medial prefrontal cortex
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NCAM	Neural cell adhesion molecule
NE	Norepinephrine
Neu-N	Neuronal nuclear protein
NG2	Neural/glial antigen-2
NMDA	N-metil-D-aspartato
NMDAR	N-metil-D-aspartato receptor
NPY	Neuropeptide Y
NSCs	Neural stem cells
OB	Olfactory bulb
p-CREB	Phosphorylated cAMP response element binding protein
PB	Phosphate buffer
PBS	Phosphate buffer saline

PCs	Pyramidal cells
PCX	Piriform cortex
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PPHT	2-(N-phenethyl-N-propyl) amino-5-hydroxytetralin hydrochloride
PSA	Polysialic acid
PV	Parvalbumin
RMS	Rostral migratory stream
s.c.	Subcutaneous
SBB	Sudan B black
SGZ	Subgranular zone
SLNs	Semilunar neurons
SLPTNs	Semilunar pyramidal transitional neurons
SPNs	Superficial pyramidal neurons
ST	Somatostatin
ST8SiaII	Polysialytransferase II
ST8SiaIV	Polysialytransferase IV
SVZ	Subventricular zone
SYN	Synaptophysin
TBR1	T-box brain transcription factor 1
TH	Tyrosine hydroxylase
TLE	Temporal lobe epilepsy
TuJ1	Neurospecific class III beta-tubulin
VGAT	Vesicular γ-aminobutyric acid (GABA) transporter
VGLUT1	Vesicular glutamate transporter 1
VIP	Vasoactive intestinal polypeptide
VPA	Valproic acid
VTA	Ventral tegmental area
WB	Western blotting
β3-AR	β3-adrenergic receptor

RESUMEN EN CASTELLANO

INTRODUCCIÓN

La capacidad básica del sistema nervioso para realizar cambios funcionales adaptativos durante el desarrollo y la edad adulta se denomina "*plasticidad neuronal* " (Zilles, 1992). El término plasticidad estructural neuronal adulta se refiere específicamente a todo tipo de cambios que modifican la estructura de las neuronas y células gliales en el sistema nervioso central adulto (SNC). Estos cambios morfológicos pueden afectar a la célula completa o algunas de sus partes y, en consecuencia, se pueden clasificar en diferentes niveles: (a) *plasticidad sináptica* (sinaptogénesis y eliminación sináptica); (b) *remodelación de neuritas* (crecimiento/retracción axonal y dendrítica) y espinas (aparición/desaparición, crecimiento o estabilización); (c) *plasticidad neurona-glía* (plasticidad en la comunicación neuro-glial); (d) *neurogénesis* (proliferación de precursores neuronales, migración neuronal e incorporación a circuitos neuronales funcionales) (Xerri, 2008).

En esa tesis me centraré en el último nivel de plasticidad, la neurogénesis, para revisar la información actual disponible sobre los procesos neurogénicos que ocurren en el cerebro de los mamíferos adultos.

NEUROGENESIS ADULTA EN MAMÍFEROS

La neurogénesis es un tipo de plasticidad estructural que se produce en diversas regiones del cerebro de los mamíferos adultos y consiste en la capacidad de las células madre neurales (NSC) de autorrenovarse, proliferar y diferenciarse en todos los tipos de células del SNC (Doetsch et al., 1999). Tradicionalmente se creía que las NSC se reducían poco después del nacimiento y, por tanto, la neurogénesis cesaba perinatalmente. Desde principios de la década de 1960, cuando se describieron por primera vez neuronas recién generadas en el hipocampo de ratas adultas (Altman and Das, 1965), se ha acumulado abundante conocimiento sobre la capacidad de los progenitores, precursores y células madre neurales para generar nuevas neuronas, astrocitos y oligodendrocitos en ciertas áreas del cerebro durante la edad adulta (Emsley et al., 2005). Dos tipos principales de neurogénesis tienen lugar en el SNC de mamíferos adultos: (a) una neurogénesis espontánea (constitutiva) "completa", sustancialmente limitada a la zona subventricular

de los ventrículos laterales (SVZ) (Lim and Alvarez-Buylla, 2016) y la zona subgranular de la circunvolución dentada en el hipocampo (SGZ) (nichos neurogénicos "canónicos") (Gonçalves et al., 2016), y (b) una neurogénesis "incompleta" (no constitutiva), que generalmente ocurre en el parénquima (no-regiones neurogénicas o nichos neurogénicos "no canónicos"). En ambos tipos de neurogénesis se han observado muchas diferencias debido a la notable diversidad en la anatomía y función del cerebro a lo largo de la escala evolutiva (Bonfanti and Peretto, 2011). Se ha demostrado ampliamente que la neurogénesis puede verse influida por modificaciones genéticas y ambientales (Kempermann and Gage, 2002). Se sabe, de hecho, que si bien el enriquecimiento ambiental, la dieta, el ejercicio y el embarazo pueden aumentar significativamente el número de neuronas recién nacidas en muchas áreas del cerebro (Van Praag et al., 1999; Shingo et al., 2003; Kobilo et al., 2011; Boitard et al., 2012; Tharmaratnam et al., 2017), por otro lado, el aislamiento, el estrés, la depresión o el envejecimiento pueden actuar como moduladores negativos de los procesos neurogénicos (Gould and Tanapat, 1999; Fowler et al., 2002; Kempermann and Kronenberg, 2003; Warner-Schmidt and Duman, 2006; Klempin and Kempermann, 2007; Lieberwirth et al., 2012). Además, alteraciones en la neurogénesis adulta también se han asociado con algunas enfermedades neurológicas y neuropsiquiátricas; en consecuencia, la modulación de los procesos implicados en estas alteraciones puede ser considerada como una estrategia válida para su tratamiento (Apple et al., 2016; Rusznák et al., 2016).

LOS NICHOS NEUROGÉNICOS "CANÓNICOS" y "NO CANÓNICOS"

En los mamíferos adultos, la neurogénesis espontánea ocurre en dos regiones cerebrales específicas, conocidas como nichos neurogénicos "canónicos": la SVZ y la SGZ, que son sitios derivados de la capa germinal (Emsley et al., 2005). En ambos nichos, las NSC, que en su mayoría son células parecidas a astrocitos en reposo (Apple et al., 2016), dan origen a precursores neuronales altamente proliferativos (progenitores intermedios) que producen una gran cantidad de neuroblastos. Desde la SVZ, estos neuroblastos migran a través del flujo migratorio rostral (RMS) para integrarse en circuitos preexistentes del bulbo olfatorio (OB), como neuronas inhibidoras: células granulares o periglomerulares. Por el contrario, las células recién nacidas en la SGZ migran a la capa granular del giro dentado, donde se convierten en células granulares

excitadoras (Kempermann et al., 2015; Lim and Alvarez-Buylla, 2016). Los procesos neurogénicos que ocurren en la SVZ y SGZ se han relacionado con el aprendizaje y la memoria (Kempermann et al., 2004; Lledo et al., 2006) y, aunque existen diferencias de especificidad de especie, se han descrito en todas las especies animales estudiadas hasta el momento, incluidos los humanos (Eriksson et al., 1998; Lindsey and Tropepe, 2006; Bonfanti and Peretto, 2011; Kempermann, 2012; Ernst et al., 2014).

Además de la SVZ y SGZ, la neurogénesis adulta también se ha descrito en muchas regiones del parénquima del SNC, los denominados nichos neurogénicos "no canónicos" (Emsley et al., 2005; Feliciano et al., 2015). La neurogénesis en el parénquima no está directamente relacionada con las capas germinales y las células madre/progenitoras, a diferencia de las que se encuentran en la SVZ y SGZ. Además, este tipo de neurogénesis es más heterogénea, considerando su naturaleza y potencialidades (Bonfanti and Peretto, 2011). De hecho, en el parénquima del SNC adulto pueden ocurrir diferentes "tipos" de neurogénesis dentro de la escala evolutiva (Bonfanti and Peretto, 2011; Feliciano et al., 2015) y no entran en una clasificación simple por su alta heterogeneidad que involucra muchas variables (Feliciano et al., 2015). La neurogénesis espontánea en el parénquima se ha considerado un fenómeno poco común en mamíferos, aunque se han hallado varios ejemplos en la neocorteza y en el cuerpo estriado de roedores (Dayer et al., 2005) y conejos (Luzzati et al., 2006), en el núcleo caudado y en el cerebelo de conejos (Luzzati et al., 2006; Ponti et al., 2008) y en la neocorteza, corteza piriforme y amígdala de primates (Gould et al., 1999; Bernier et al., 2002). La mayor parte de la neurogénesis en el parénquima parece ocurrir en condiciones fisiológicas o patológicas específicas. La activación de progenitores endógenos en respuesta a formas específicas de lesión y en ciertas condiciones/modelos experimentales se ha descrito en la neocorteza, así como en el cuerpo estriado, séptum, tálamo e hipotálamo tanto en ratones como en ratas (Magavi et al., 2000; Pencea et al., 2001; Arvidsson et al., 2002; Chen et al., 2004; Kokoeva et al., 2005; Ohira et al., 2010; Pierce and Xu, 2010; Pérez-Martín et al., 2010; Matsuzaki et al., 2017). De manera similar, también se han descrito bajos niveles de neurogénesis que implican la proliferación de progenitores y la incorporación de nuevas neuronas a los circuitos en el tubérculo olfatorio de ratones (Shapiro et al., 2007b) y monos (Bédard et al., 2002), en el complejo vagal dorsal del tronco cerebral de rata (Bauer et al., 2005), en la sustancia negra y en el cuerno de Amón del hipocampo del ratón (Rietze et al., 2000; Zhao et al., 2003; Zhao and Lang, 2009). Se ha descrito proliferación celular y producción de nuevas neuronas en el hipotálamo de

mamíferos con cerebros más grandes y complejos como las ovejas (Migaud et al., 2010), así como en otras estructuras, incluida la eminencia medial, y la pars tuberalis de la glandula pituitaria y del tálamo (Migaud et al., 2010) Además, también se han descrito progenitores locales similares a células gliales, que retienen cierta capacidad proliferativa, en los nichos neurogénicos "no canónicos" del SNC maduro (Horner et al., 2000; Butt et al., 2005; Ponti et al., 2008; Trotter et al., 2010).

Recientemente, la atención se ha centrado en una forma de neurogénesis retardada lenta ("neurogénesis sin división") (La Rosa et al., 2020), que difiere de las descritas hasta ahora. Este tipo de neurogénesis involucra neuronas inmaduras en el cerebro adulto, que no están proliferando. Estas neuronas pueden representar un potencial "*reservorio*" de células inmaduras que completarían su programa de diferenciación en diferentes circunstancias (Bonfanti and Nacher, 2012; Piumatti et al., 2018; La Rosa et al., 2019) para finalmente integrarse en los circuitos neuronales (Rotheneichner et al., 2018; Benedetti et al., 2020).

LA CORTEZA PIRIFORME DE ROEDORES ADULTOS

La corteza piriforme de roedores adultos (PCX) es una estructura paleocortical trilaminar involucrada en funciones olfativas, que contiene una población de neuronas inmaduras distribuidas principalmente dentro de la capa II (Gómez-Climent et al., 2008; Rubio et al., 2015). Algunos estudios sugirieron que un subconjunto menor de estas neuronas inmaduras podría ser de generación reciente, probablemente en la SVZ (Pekcec et al., 2006; Shapiro et al., 2007b), pero hay pruebas sólidas de que la gran mayoría de ellas han sido generadas durante el desarrollo embrionario y permanecen en un estado indiferenciado hasta la edad adulta (Gómez-Climent et al., 2008; Rubio et al., 2015). En consecuencia, la capa II de la PCX de roedores adultos puede considerarse un nicho neurogénico "no canónico".

La PCX es el área más grande de la corteza olfativa de los mamíferos, ubicada en la superficie ventrolateral del cerebro, cerca del tracto olfatorio lateral (LOT). Está compuesta por tres capas: la capa más superficial o capa I que esta formada por la capa Ia, que contiene las dendritas distales de las células piramidales PCX (PC) y recibe información de los axones de las células mitral y copetuda en el OB (Shipley and Ennis, 1996; Gire et al., 2012) y la capa más profunda Ib, que incluye axones asociativos locales y axones comisurales de las neuronas corticales olfativas contralaterales. La capa II está

organizada en dos capas sublaminares: IIa y IIb, que contienen el mayor número de neuronas liberadoras de glutamato. Estas neuronas excitadoras se clasifican principalmente morfológicamente en neuronas semilunares (SLN) y piramidales superficiales (SPN) (Suzuki and Bekkers, 2011). Las SLN están ubicadas en la parte superficial de la capa IIa, proyectan 2 dendritas principales a la capa I y carecen de un árbol dendrítico basal. Muestran una alta densidad de espinas dendríticas en la región distal de sus dendritas apicales. Estas neuronas reciben fuertes aferencias excitadoras de los axones del tracto lateral olfativo (LOT) y su actividad se correlaciona con la estimulación del OB (Suzuki and Bekkers, 2011). Las SPN, ubicadas en la capa IIb, extienden sus dendritas basales a la capa III y las dendritas apicales a la capa I, reciben solo escasos inputs del OB a través del LOT y están involucradas en la integración de múltiples olores (Klingler, 2017). También se pueden observar otros tipos de neuronas excitadoras en la capa II que se clasifican como neuronas de transición entre piramidales semilunares (SLPTN) (Haberly and Behan, 1983)(Haberly y Behan, 1983). Finalmente, la capa III contiene los cuerpos celulares de las neuronas piramidales profundas (DLPN), dos poblaciones de células multipolares y una gran cantidad de fibras asociativas de la PCX y otras estructuras cerebrales. Las DLPN reciben aferencias débiles de los axones del LOT (Hagiwara et al., 2012) y considerables impulsos excitadores intracorticales (Klingler, 2017). Además, se describen diferentes tipos de interneuronas liberadoras de GABA uniformemente dispersas en todas las capas de la PCX, las cuales proporcionan inhibición sináptica por retroalimentación (feedback and feedforward) a las células principales (Suzuki and Bekkers, 2007). La PCX codifica, procesa y finalmente transmite la información proveniente principalmente del OB, pero también envía proyecciones dentro de la corteza olfatoria, incluyendo al núcleo olfatorio anterior (AON), el tubérculo olfatorio, la amígdala cortical (CoA) y la corteza entorrinal lateral (LEnt). Además, la PCX envía proyecciones al OB, al núcleo mediodorsal del tálamo y a varias subdivisiones de la corteza prefrontal (Klingler, 2017).

LAS NEURONAS INMADURAS EN LA CORTEZA CEREBRAL DE ROEDORES

En roedores adultos las neuronas inmaduras se distribuyen mayoritariamente en la capa II de las cortezas piriforme y entorrinal lateral y también aparecen, con una densidad mucho menor, en la capa II de la perirrinal y la región más ventral de las cortezas

agranular insular y ectorinal (Seki and Arai, 1991a; Gómez-Climent et al., 2008; Bonfanti and Nacher, 2012).

En la PCX, las neuronas inmaduras están esencialmente restringidas a la capa II, particularmente a la capa IIb, donde sus somas están densamente organizados (Gómez-Climent et al., 2008; Luzzati et al., 2009; Bonfanti and Nacher, 2012). Dependiendo de sus características morfológicas, se pueden clasificar en 2 poblaciones principales de células: a) células enmarañadas (tipo I), que son muy pequeñas (con un diámetro del soma alrededor de 9 μm), carecen de axones y solo presentan algunas neuritas cortas con trayectorias irregulares, generalmente restringidas a la capa II y b) células complejas (tipo II), con somas más grandes (diámetro alrededor de 15 μm), que generalmente muestran segmentos iniciales de axón (AIS) y dendritas apicales típicas que se expanden hacia la capa I. Las células complejas más desarrolladas muestran ramificaciones dendríticas típicas y espinas dendríticas con escasas sinapsis excitadoras. También existen neuronas con características intermedias entre las células enmarañadas y las complejas, lo que indica una maduración progresiva entre estos 2 tipos de células (Gomez-Climent et al., 2010). Además, las células complejas también tienen una mayor densidad de puncta que expresan marcadores presinápticos asociados a su región perisomática, lo que sugiere un aumento en su conectividad con la red circundante (Rotheneichner et al., 2018). Aunque más escasas que en la capa II, también se observaron neuronas inmaduras en la capa III de la PCX, en el núcleo endopiriforme y en las capas profundas de la corteza entorrinal lateral. Estas células son monopolares o bipolares y frecuentemente presentan procesos cortos orientados verticalmente; también se pueden observar formando agregados de 2-5 células, especialmente en el extremo ventral de la cápsula externa del cuerpo calloso (Nacher et al., 2001a, 2002a, 2010). Curiosamente, el análisis detallado con microscopía electrónica también ha revelado que las neuronas inmaduras en la PCX, especialmente las células enmarañadas, tienen sus somas y neuritas cubiertas por procesos astrogliales (Gómez-Climent et al., 2008).

LAS NEURONAS INMADURAS EN OTROS MAMÍFEROS

En los mamíferos con cortezas cerebrales más grandes, las neuronas inmaduras muestran una distribución más amplia que en los roedores. En conejos, gatos y cobayas, se ha observado la expresión concurrente de marcadores neuronales inmaduros típicos como PSA-NCAM y DCX, en las capas II y III del PCX, así como en las cortezas

perirrinal y entorrinal y en la región de transición piriforme-amigdalar. Además, también se encontraron neuronas inmaduras en la neocorteza de conejos, gatos y monos, particularmente en la corteza somatosensorial y en diferentes regiones de la ínsula (Bonfanti, 2006; Cai et al., 2009; Luzzati et al., 2009; Varea et al., 2011). Se describieron neuronas inmaduras en gatos adultos, que pueblan muchas áreas corticales, no sólo en la PCX, siendo particularmente abundantes en la corteza entorrinal y en las porciones ventrales de los lóbulos frontal y temporoparietal. Por el contrario, en las secciones del lóbulo occipital, son menos abundantes en las regiones dorsales, como la corteza visual primaria, mostrando un gradiente decreciente desde el área 20 (V4), el área 19 (V3), el área 18 (V2) y al área 17 (V1) (Cai et al., 2009; Varea et al., 2011). También se han observado neuronas inmaduras en primates no humanos adultos, específicamente en la capa II y la capa superior III de la mayoría de las áreas corticales analizadas (Cai et al., 2009). También se han descrito recientemente neuronas inmaduras tanto en la neocorteza de especies lisencefálicas de cerebro pequeño como el sengi (un tipo de musaraña) y los murciélagos, así como en en mamíferos de cerebro grande como chimpancés, zorros, ovejas y gatos, en los que su densidad tiende a asociarse generalmente con el tamaño del cerebro (Gatome et al., 2010; La Rosa et al., 2020).

En el cerebro humano, la presencia de neuronas inmaduras se ha observado exclusivamente en la corteza entorrinal de niños (Dhúill et al., 1999) y en las cortezas temporal y frontal en adulto, donde se han descrito predominantemente en el borde superior de la capa II de la neocorteza (Cai et al., 2009). Además, células inmaduras que expresaban DCX se han descrito también entre las capas I y II del lóbulo temporal y frontal en niños con epilepsia (Srikandarajah et al., 2009).

NEURONAS INMDURAS: MARCADORES, ORIGEN, DESTINO y FUNCIÓN

Entre los numerosos marcadores de neuronas inmaduras, uno de los más utilizados es la forma polisializada de la molécula de adhesión de células neurales (PSA-NCAM). PSA-NCAM es una molécula relacionada con la plasticidad y generalmente considerada un promotor de la migración y diferenciación celular y un facilitador del crecimiento axonal, de la remodelación estructural neuronas-glia y de la sinaptogénesis (Kiss and Rougon, 1997; Maarouf and Rutishauser, 2003; Bonfanti, 2006; Gascon et al., 2007; Rutishauser, 2008; Hildebrandt and Dityatev, 2015). En el cerebro de los mamíferos adultos, PSA-NCAM está abundantemente presente en los nichos

neurogénicos “canónicos”, la SVZ y SGZ, donde la expresan neuroblastos durante su migración y primera fase de diferenciación (Seki and Arai, 1991a; Bonfanti and Theodosis, 1994; Rousselot et al., 1995). Sin embargo, la expresión de PSA-NCAM no solo está restringida a esta población de células, si no que también se expresa abundantemente en células que pueblan nichos neurogénicos “no canónicos”, como las de la capa II de la PCX de roedores (Seki and Arai, 1991a; Nacher et al., 2002a; Gómez-Climent et al., 2008; Rubio et al., 2015). Otro marcador que se usa con frecuencia para identificar neuronas inmaduras es la doblecortina (DCX), una proteína asociada a microtúbulos que se expresa en el sistema nervioso central y periférico durante el desarrollo embrionario y posnatal. Desempeña un papel clave en la estabilización de microtúbulos (Gleeson et al., 1999), en la translocación nuclear y en el mantenimiento de la morfología bipolar durante la migración neuronal (Koizumi et al., 2006), así como en la dinámica de crecimiento de los conos axonicos (Burgess and Reiner, 2000). La DCX se expresa transitoriamente en células progenitoras en proliferación y neuroblastos recién generados dentro de los dos nichos canónicos en el cerebro adulto, la SVZ y SGZ (Nacher et al., 2001a) y en la PCX de roedores adultos (Nacher et al., 2001a; Rubio et al., 2015).

Muchos estudios han demostrado claramente que la mayoría de las neuronas inmaduras en la capa II de la PCX son células residentes inmaduras posmitóticas que no expresan marcadores de actividad proliferativa. Los estudios realizados en roedores (Gómez-Climent et al., 2008; Rubio et al., 2015) no han logrado encontrar evidencia de células en división o recién generadas en la capa II de la corteza cerebral adulta. Curiosamente, la mayoría, si no todas, de las neuronas inmaduras en la capa II de la PCX se generan durante el desarrollo embrionario, principalmente en E15.5 en ratas (Gómez-Climent et al., 2008) y en E13.5 y E14.5 en ratones (Rubio et al., 2015). Se propusieron dos hipótesis principales sobre el destino de estas neuronas inmaduras. Una hipótesis es que tengan una existencia transitoria y mueran progresivamente durante el curso del envejecimiento, la otra es que se integren progresivamente como neuronas maduras funcionales. Varios estudios realizados en roedores excluyen la muerte como un posible destino: de hecho, no se encontraron evidencias de un número sustancial de células moribundas en la capa II de la PCX (Friedman and Price, 1986; Xiong et al., 2008; Gómez-Climent et al., 2011a). En consecuencia, la segunda hipótesis parece más probable.

Aunque se ha llevado a cabo una extensa investigación sobre la distribución, morfología, fenotipo y origen de las neuronas inmaduras en la capa II de la corteza

cerebral, su destino putativo solo se ha aclarado recientemente. Por esta razón, ha sido difícil descifrar su función en el SNC adulto, que aún permanece en duda. Se ha propuesto que esta población de neuronas inmaduras puede constituir un "*reservorio*" de células postmitóticas de origen embrionario que pueden completar su programa de diferenciación durante la edad adulta bajo diferentes condiciones fisiológicas o patológicas (Bonfanti and Nacher, 2012). En un estudio reciente, el uso del ratón transgénico DCX-CreER^{T2}/ Flox-EGFP, en el que las células que expresan DCX se marcan permanentemente después de la administración de tamoxifeno y se controlan tras la expresión de la proteína fluorescente verde intensificada (EGFP) (Zhang et al., 2010), ha permitido estudiar el destino de la población de las células inmaduras en la capa II de la PCX (Rotheneichner et al., 2018). Curiosamente, la administración de tamoxifeno en diferentes momentos ha confirmado que las células EGFP+ de la capa II de la PCX no mueren en el curso del envejecimiento, sino que continúan su maduración y adquieren una morfología más compleja. De hecho, a los 6 meses de maduración, las dendritas apicales de las células EGFP+ mostraron un aumento en su grosor y en la densidad de sus espinas, lo que sugiere una mayor conectividad sináptica con la red circundante en la que se integran como neuronas principales glutamatérgicas maduras (Rotheneichner et al., 2018). Estos hallazgos estaban de acuerdo con un estudio previo que informaba que las células DCX+ en la capa II de la PCX de ratones adultos exhibían un perfil electrofisiológico típico de neuronas en proceso de maduración (Klempin et al., 2011). Un estudio reciente ha demostrado claramente que las neuronas inmaduras con una morfología compleja maduran en neuronas funcionales y que la expresión de marcadores para espinas y botones sinápticos glutamatérgicos indican su integración en la conectividad excitadora de redes neuronales adultas (Benedetti et al., 2020).

Hasta ahora, la función de esta población de células inmaduras descrita tanto en roedores adultos como en mamíferos girencefálicos más grandes sigue siendo poco conocida. Dado que la distribución de estas neuronas inmaduras en roedores se describió principalmente en la paleocorteza, una región del cerebro especializada en el olfato (Gómez-Climent et al., 2008), la primera hipótesis plausible fue que estas células estaban involucradas en funciones olfativas. Posteriormente, considerando la distribución más amplia de estas células en la neocorteza de especies de mamíferos con cortezas girencefálicas más grandes, incluidos los humanos, es posible que participen activamente en procesos cerebrales integrativos de orden-superior, lo que sugiere un plausible papel

estratégico en funciones corticales cerebrales avanzadas (Bonfanti and Nacher, 2012; König et al., 2016; La Rosa et al., 2020).

Diferentes líneas de evidencia han sugerido la participación de las neuronas inmaduras de la capa II de la corteza cerebral en enfermedades neurológicas y psiquiátricas. El sistema monoaminérgico está profundamente involucrado en la etiopatología y el tratamiento de enfermedades neurológicas y psiquiátricas y parece ser un potente modulador de la población neuronal inmadura en la capa II de la corteza cerebral. Un estudio reciente ha demostrado que la estimulación crónica de los receptores α2-adrenérgicos induce un aumento en el número de neuronas inmaduras que expresan tanto PSA-NCAM como DCX en la capa II de la PCX de roedores (Vadodaria et al., 2017). Además, algunos otros tratamientos farmacológicos que incluyen antagonistas del receptor *N*-metil-D-aspartato (NMDAR) (Nacher et al., 2002a) antiepilépticos como el ácido valproico (VPA) (Murphy et al., 2001) o los antidepresivos imipramina (Sairanen et al., 2007) y la fluoxetina (Varea et al., 2007a) afectan a las neuronas inmaduras en la capa II de la corteza cerebral. Por lo tanto, estos datos sugieren que las neuronas inmaduras en la capa II de la corteza cerebral pueden estar involucradas en diferentes funciones, no solo relacionadas con el procesamiento olfativo, y pueden representar un objetivo para hormonas, neurotransmisores y tratamientos farmacológicos. Sin embargo, aún se necesitan más estudios para proporcionar evidencia relevante para aclarar su función en el cerebro de los mamíferos adultos, tanto en condiciones fisiológicas como patológicas.

OBJETIVOS

El objetivo principal de esta tesis doctoral es estudiar la población de neuronas inmaduras en la capa II de la corteza cerebral adulta tanto en roedores como en humanos. En concreto, me centraré en los mecanismos que subyacen a las etapas finales de desarrollo de estas células inmaduras y a los factores que favorecen su diferenciación e integración en los circuitos cerebrales, así como la implicación de la molécula de plasticidad PSA-NCAM en estos procesos.

Para lograr este objetivo principal, seguiré diferentes objetivos específicos, que se detallan a continuación:

1. Evaluar los efectos de la modulación farmacológica de la neurotransmisión dopaminérgica sobre la diferenciación de las neuronas inmaduras en la capa II de la PCX, estudiando la distribución del D2r en esta región y la relación entre fibras dopaminérgicas y neuronas inmaduras.

2. Estudiar el impacto de la depleción enzimática de PSA de NCAM por la enzima EndoN en la población de neuronas inmaduras en la capa II de la PCX, utilizando una línea inducible de ratón DCX-Cre (DCX-CreER^{T2}/Flox-EGFP) como herramienta para mapear el destino de estas células.

3. Investigar la presencia de neuronas inmaduras en la capa II de la corteza cerebral humana, utilizando muestras quirúrgicas de pacientes epilépticos y tejido *post-mortem*, empleando marcadores neuroquímicos específicos para estudiar su distribución, morfología y fenotipo, así como su destino y función.

METODOLOGÍA Y RESULTADOS

ESTUDIO 1. EFFECTO DE LA DOPAMINA SOBRE LAS NEURONAS INMADURAS DE LA CORTEZA PIRIFORME DE LA RATA ADULTA

Con el fin de evaluar los efectos hipotéticos de la modulación farmacológica de la neurotransmisión dopaminérgica sobre la diferenciación de las neuronas inmaduras de la PCX adulta, primero he estudiado la expresión del D2r en estas células y su relación con los axones dopaminérgicos. A continuación, he explorado si la manipulación experimental de la señalización dopaminérgica a través de antagonistas y agonistas del D2r podía alterar la tasa de diferenciación de las neuronas inmaduras en la PCX cuantificando los cambios en la densidad de células que expresaban PSA-NCAM en la capa II. Los resultados obtenidos demuestran la presencia de muchas fibras dopaminérgicas en estrecha aposición a las neuronas que expresan PSA-NCAM, que también coexpresan el D2r. Además, he visto que el tratamiento crónico con haloperidol aumenta significativamente el número de células inmunoreactivas para PSA-NCAM, mientras que el tratamiento con PPHT lo reduce.

ESTUDIO 2. LA DEPLECIÓN DE PSA INDUCE LA DIFFERENCIACIÓN DE LAS NEURONAS INMADURAS EN LA CORTEZA PIRIFORME EN RATONES ADULTOS

Para estudiar la implicación de PSA-NCAM en las etapas finales del desarrollo de las neuronas inmaduras en la capa II de la PCX he eliminado PSA de NCAM mediante una inyección intracraneal de EndoN. Para rastrear la maduración de las células complejas en la PCX adulta, he utilizado una línea de ratones transgénicos inducibles por tamoxifeno con Cre recombinasa bajo el control de un promotor DCX (DCX-CreER^{T2}/ Flox-EGFP). En estos ratones, las células que expresan DCX se marcan permanentemente con EGFP después de la administración de tamoxifeno. Para desarrollar el objetivo principal de ese estudio, primero he clasificado las células EGFP+ en diferentes grupos, según el diámetro del soma y su estadio de maduración. A continuación, he cuantificado su porcentaje y densidad comparando ambos hemisferios después de la inyección de EndoN. Para investigar si la depleción de PSA podría acelerar la maduración de las células EGFP+ en el PCX, he cuantificado la presencia del AIS en estas células. Luego, he estudiado el efecto de la eliminación de PSA sobre la complejidad dendrítica y la densidad de espinas, así como sobre la expresión de marcadores presinápticos en la superficie de las espinas. También he analizado la densidad de los puntos que expresan VGLUT1, VGAT y PV en la región perisomática de las células complexas EGFP+. Finalmente, he estudiado la expresión de marcadores de neuronas inmaduras (DCX) o maduras (NeuN, CAMK-II) en las células EGFP+, así como la densidad total de núcleos inmunoreactivos para NeuN y células que expresan CAMK-II.

Este estudio demuestra que la depleción enzimática de PSA en el ratón transgénico DCX-CreER^{T2}/Flox-EGFP influye en las etapas finales de desarrollo de la población de neuronas inmaduras en la capa II de la PCX adulta. He descubierto que la eliminación de PSA de NCAM promueve la maduración de las células EGFP+, como queda demostrado en el aumento de la densidad de las células complejas EGFP+ que muestran un AIS. Además, he encontrado una mayor densidad de dendritas EGFP+ y espinas dendríticas en la capa I de la PCX. Finalmente, la eliminación de PSA aumenta también el porcentaje y la densidad de las células EGFP+ que expresan el marcador neuronal maduro NeuN, así como la densidad de núcleos que expresan NeuN en la capa II de la PCX adulta.

ESTUDIO 3. LA CAPA II DE LA CORTEZA CEREBRAL HUMANA: UN "*RESERVORIO*" DE NEURONAS INMADURAS EN EL CEREBRO ADULTO

Para estudiar la población de neuronas inmaduras en la capa II de la corteza cerebral humana he usado muestras quirúrgicas de pacientes epilépticos y tejido *post-mortem.* He utilizado marcadores neuroquímicos para estudiar su distribución, morfología y fenotipo, así como su destino y función putativa. Para investigar si la epilepsia podría afectar a la población de células inmaduras en la capa II de la corteza cerebral humana, he utilizado un modelo de litio-pilocarpina en rata para analizar cambios en la distribución y morfología de las neuronas inmaduras en la capa II de la PCX. He estudiado si estas células expresan ciertas proteínas expresadas exclusivamente por neuronas maduras, neuronas principales e interneuronas. También he utilizado marcadores gliales para excluir la posibilidad de que estas células sean astrocitos, oligodendrocitos, polidendrocitos o microglía. Adicionalmente, dado que en el primer estudio de esta tesis he descrito la influencia de la inervación dopaminérgica sobre las neuronas inmaduras en la capa II de la PCX de las ratas adultas, también he investigado la expresión del D2r en estas células y su relación espacial con las fibras TH positivas.

Este estudio proporciona evidencia de la presencia de neuronas inmaduras en el cerebro humano, específicamente en la capa II de la corteza cerebral. Utilizando muestras quirúrgicas de pacientes epilépticos y tejido *post-mortem*, he encontrado células en diferentes etapas de desarrollo que expresan DCX y PSA-NCAM y que carecen de expresión del marcador neuronal maduro NeuN. En el modelo de la rata he encontrado una ligera reducción de las células inmaduras en la capa II de la PCX, mientras que la mayoría de las células inmaduras que expresan PSA-NCAM aún persistían después de la epilepsia. Las células inmaduras descritas en las muestras de humanos pertenecen al linaje excitador, como lo demuestra tanto la expresión de los factores de transcripción CUX1, CTIP2 y TBR1, así como la ausencia del marcador inhibidor GAD67. Además, las células en el estado de maduración más avanzado muestran puncta que expresan marcadores sinápticos inhibitorios y excitadores estrechamente asociados a sus regiones perisomáticas y peridendríticas. Curiosamente, también he demostrado que estas neuronas inmaduras no son células gliales, aunque he encontrado procesos astrogliales y microgliales en estrecha aposición a sus somas y dendritas. Por último, he descubierto

que estas neuronas inmaduras en la capa II de la corteza cerebral expresan D2r y muestran algunas fibras TH+ en las proximidades de sus somas.

CONCLUSIONES

1. En las tres capas de la PCX de rata adulta hay fibras dopaminérgicas y muchas de ellas están en estrecha aposición con las neuronas que expresan PSA-NCAM en la capa II.

2. Las neuronas inmunoreactivas para PSA-NCAM en la capa II de la PCX de rata adulta expresan el D2r.

3. El tratamiento crónico con haloperidol aumenta significativamente la densidad de células que expresan PSA-NCAM en la capa II de la PCX de la rata adulta.

4. El tratamiento crónico con PPHT disminuye significativamente la densidad de células que expresan PSA-NCAM en la capa II de la PCX de la rata adulta.

5. El uso del ratón transgénico inducible DCX-CreER^{T2}/Flox-EGFP permite seguir las diferentes etapas de desarrollo de la población de neuronas inmaduras en la capa II de la PCX adulta.

6. La enzima EndoN depleta de manera eficiente PSA de NCAM en la capa II de la PCX del ratón transgénico inducible DCX-CreER^{T2}/Flox-EGFP.

7. La eliminación enzimática de PSA de NCAM no induce cambios en la morfología, en el porcentaje de células en diferentes etapas de desarrollo o en la densidad de células EGFP + en la capa II de la PCX de ratones adultos.

8. La eliminación enzimática de PSA de NCAM aumenta significativamente la densidad de células complejas EGFP + que muestran un AIS en la capa II de las PCX de ratones adultos.

9. La eliminación enzimática de PSA de NCAM aumenta significativamente la densidad de las dendritas EGFP+ y de las espinas dendríticas en la capa I de la PCX de ratones adultos, pero no induce cambios específicos en la densidad de diferentes tipos de espinas: rechonchas, en forma de hongo y delgadas.

10. La eliminación enzimática de PSA de NCAM no induce cambios en el porcentaje de espinas dendríticas EGFP+ asociadas a puncta que expresan los marcadores presinápticos SYN+ y VGLUT1+ en la capa I de la PCX de ratones adultos.

11. La eliminación enzimática de PSA de NCAM no induce cambios en la densidad y en la intensidad de fluorescencia de los punctum que expresan VGLUT+ en la región perisomática de las células complexas EGFP+ ubicadas en la capa II de la PCX de ratones adultos.

12. La eliminación enzimática de PSA de NCAM no induce cambios en la densidad y en la intensidad de fluorescencia de los punctum que expresan PV y / o VGAT en la región perisomática de las células complexas EGFP+ ubicadas en la capa II de la PCX de ratones adultos.

13. La eliminación enzimática de PSA de NCAM aumenta significativamente la densidad de núcleos que expresan NeuN en la capa II de la PCX de ratones adultos.

14. La eliminación enzimática de PSA de NCAM no induce cambios en la densidad de células que expresan CAMK-II en la capa II de la PCX de ratones adultos.

15. La eliminación enzimática de PSA de NCAM aumenta significativamente el porcentaje y densidad de las células EGFP+ que expresan NeuN en la capa II de la PCX de ratones adultos.

16. La eliminación enzimática de PSA de NCAM no induce cambios en el porcentaje y densidad de las células EGFP + que expresan DCX en la capa II de la PCX de ratones adultos.

17. La eliminación enzimática de PSA de NCAM no induce cambios en el porcentaje y densidad de células EGFP + que expresan CAMK-II en la capa II de la PCX de ratones adultos.

18. En la capa II de la corteza cerebral humana, específicamente en los lóbulos frontal, temporal, parietal y occipital, existe una población ampliamente distribuida de células que expresan DCX y PSA-NCAM.

19. La distribución, morfología y fenotipo de las células que expresan DCX y PSA-NCAM en la capa II de la corteza cerebral humana es similar en pacientes controles, epilépticos y no epilépticos.

20. El estado epiléptico inducido por la pilocarpina no afecta a la densidad de las células que expresan PSA-NCAM en la capa II de la PCX de rata.

21. En la capa II de la corteza cerebral humana, hay células que expresan DCX con diferentes morfologías, las cuales sugieren diferentes etapas de desarrollo. Estas células se pueden clasificar principalmente como de tipo I o de tipo II, pero también existen células con morfología intermedia.

22. Las células que expresan DCX de tipo I son células pequeñas y bipolares con un diámetro de soma entre 3-9 μm y con un único proceso corto restringido a la capa II.

23. Las células que expresan DCX con una morfología intermedia son células con un diámetro de soma entre 9-11 μm, un proceso basal delgado y 1 o 2 dendritas cortas sin espinas ni excrecencias.

24. Las células que expresan DCX de tipo II son células grandes y multipolares con un diámetro de soma entre 11-17 μm y arborizaciones dendríticas más largas, orientadas principalmente en paralelo a la capa II. Estas células de tipo II muestran un proceso delgado que se asemeja a un axón, aunque en ninguna de ellas el AIS es detectable. Las dendritas de las células de tipo II muestran algunas

protuberancias (longitud ≤ 1 μm) que se asemejan a espinas dendríticas rechonchas.

25. La mayoría de las células DCX de la capa II de la corteza cerebral humana coexpresan PSA-NCAM y no expresan el marcador neuronal maduro NeuN; por lo tanto, pueden considerarse neuronas inmaduras.

26. Las neuronas inmaduras de la capa II de la corteza cerebral humana pertenecen al linaje excitador: expresan los factores de transcripción CUX1, CTIP2 y TBR1 y carecen del marcador inhibidor GAD67.

27. Las células que expresan DCX de tipo II de la capa II de la corteza cerebral humana muestran punctum que expresan marcadores sinápticos inhibitorios y excitatorios en estrecha aposición a sus somas y dendritas.

28. Las neuronas inmaduras de la capa II de la corteza cerebral humana no expresan marcadores típicos de células gliales como GFAP, NG2 e IBA1.

29. En la capa II de la corteza cerebral humana, las neuronas inmaduras muestran procesos astrogliales y microgliales estrechamente asociados a sus somas y dendritas.

30. En la capa II de la corteza cerebral humana, hay algunas fibras que expresan TH, muchas de las cuales están en estrecha aposición a las neuronas inmaduras. Estas neuronas inmaduras expresan D2r.

Chapter 1

INTRODUCTION

1. STRUCTURAL PLASTICITY IN THE ADULT MAMMALIAN CENTRAL NERVOUS SYSTEM

The basic ability of the nervous system to make adaptive functional changes during development and adulthood is called "*neuronal plasticity"* (Zilles, 1992). The term adult neuronal structural plasticity refers specifically to all types of changes, which modify the shape and structure of neurons and glial cells in the adult central nervous system (CNS). These morphological changes can affect the whole cell or some of its parts and, consequently, they can be classified into different levels: *(a) synaptic plasticity* (synaptogenesis and synaptic removal); *(b) neurite* (axonal and dendritic outgrowth/retraction) and *spine remodeling* (appearance/disappearance, enlargement or stabilization); *(c) neuron-glia plasticity* (plasticity in neuron-glial communication); *(d) neurogenesis* (proliferation of neuronal precursors, neuronal migration and incorporation to functional neuronal circuits) (Xerri, 2008, for review).

In the next paragraphs I will focus on the last level of plasticity, neurogenesis, to review the current information available on the neurogenic processes occurring in the adult mammalian brain.

1.1 Adult Neurogenesis in mammals

Neurogenesis is a type of structural plasticity that occurs in various regions of the adult mammalian brain and consists in the ability of neural stem cells (NSCs) to self-renew, proliferate, and differentiate into all cell types of the CNS (Doetsch et al., 1999). It was traditionally believed that NSCs are depleted shortly after birth and thus that neurogenesis ceases perinatally. From the early 1960s, when newly generated neurons were described for the first time in the adult rat hippocampus (Altman and Das, 1965), abundant knowledge has been accumulated on the capacity of neural progenitors, precursors, and stem cells to generate new neurons, astrocytes, and oligodendrocytes in certain areas of the brain during adulthood (Emsley et al., 2005). Two main types of neurogenesis take place in the adult mammalian CNS: (a) a "complete" spontaneous (constitutive) neurogenesis, substantially limited to the subventricular zone of the lateral ventricles (SVZ) (Lim and Alvarez-Buylla, 2016) and the subgranular zone of the dentate gyrus in the hippocampus (SGZ)("canonical" neurogenic niches)(Gonçalves et al., 2016), and (b) a rather "incomplete" (no constitutive) neurogenesis, generally occurring in the

parenchyma (non-neurogenic regions or "non-canonical" neurogenic niches). In both types of neurogenesis, many differences were observed due to the notable diversity in brain anatomy and function throughout the evolutive scale (Bonfanti and Peretto, 2011). It has been widely demonstrated that neurogenesis can be influenced by genetic and environmental modifications (Kempermann and Gage, 2002). It is known, in fact, that while environmental enrichment, diet, exercise and pregnancy can significantly increase the number of new-born neurons in many areas of the brain (Van Praag et al., 1999; Shingo et al., 2003; Kobilo et al., 2011; Boitard et al., 2012; Tharmaratnam et al., 2017), on the other side, isolation, stress, depression or aging can act as negative modulators of the neurogenic processes (Gould and Tanapat, 1999; Fowler et al., 2002; Kempermann and Kronenberg, 2003; Warner-Schmidt and Duman, 2006; Klempin and Kempermann, 2007; Lieberwirth et al., 2012). Moreover, altered adult neurogenesis has been also associated with some neurological and neuropsychiatric diseases; consequently, the modulation of the processes involved in these alterations may be considered as a valid strategy for their treatment (Apple et al., 2016; Rusznák et al., 2016).

In the next paragraphs I will describe in detail adult neurogenesis in brain regions classified as "canonical" or "non-canonical" neurogenic niches.

1.1.1 The "canonical" neurogenic niches

In adult mammals, spontaneous neurogenesis occurs in 2 specific brain regions, known as "canonical" neurogenic niches: the SVZ and SGZ, which are germinal layer-derived sites (Emsley et al., 2005). In both niches, NSCs, which are mostly quiescent astrocyte-like cells (Apple et al., 2016), give origin to highly proliferative neuronal precursors (intermediate progenitors) that produce a large number of neuroblasts. From the SVZ, these neuroblasts migrate through the rostral migratory stream (RMS) to be integrated into pre-existing circuits of the olfactory bulb (OB), as inhibitory neurons: granule or periglomerular cells. By contrast, newly-born cells in the SGZ migrate to the granular layer of the dentate gyrus, where they become excitatory granule cells (Kempermann et al., 2015; Lim and Alvarez-Buylla, 2016) **(Figure 1)**.

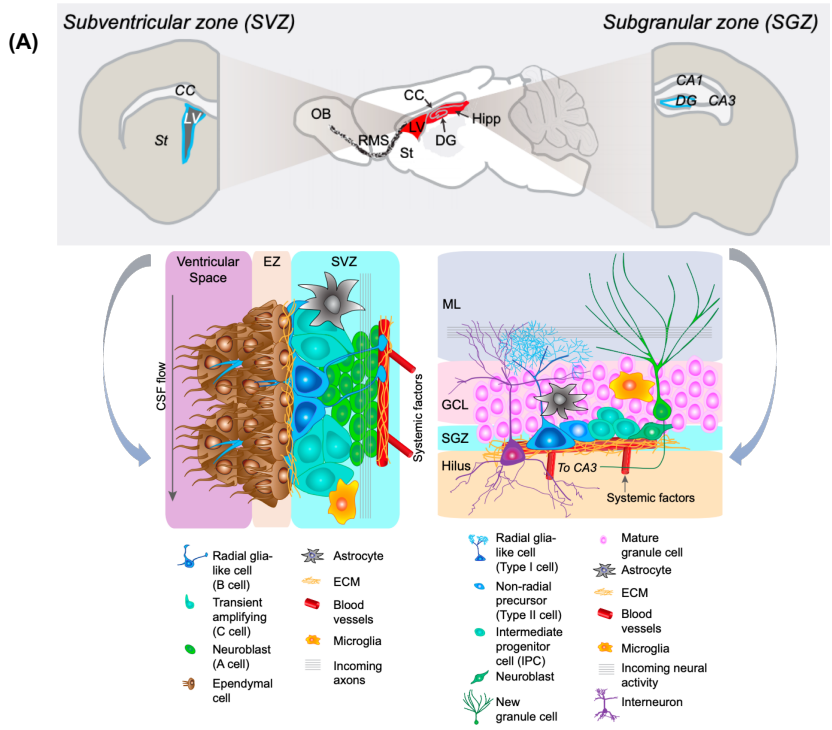

Figure 1. Adult neurogenesis in the 2 "canonical" neurogenic niches the SVZ and SGZ. (A) A sagittal view of the adult rodent brain, focusing on SVZ and SGZ where adult NSCs reside. The SVZ is located along the lateral ventricle in the forebrain, while the SGZ is located in the hippocampus along the dentate granule cell layer where it abuts the hilus. **(A,** panel left**)** A schematic representation showing cellular and molecular components of the SVZ niche. Radial glia-like neural stem cells (NSCs) generate neuroblasts (C cells). Neuroblasts migrate down the RMS to the OB where they differentiate into OB neurons. **(A,** panel right**)** A schematic diagram depicting cellular and molecular components of the SGZ niche. Radial glia-like NSCs generate intermediate progenitor cells, which generate neuroblasts. These neuroblasts differentiate into dentate granule cells (DGCs), which migrate into the granule cell layer of the dentate gyrus. (CC: corpus callosum; DG: dentate gyrus; ECM: extracellular matrix; EZ: ependymal zone; GCL: granule cell layer; Hipp: hippocampus; LV: lateral ventricle; ML: molecular layer; NSC: neural stem cell; OB: olfactory bulb; RMS: rostral migratory stream; SC: stem cell; St: striatum; SGZ: subgranular zone; SVZ: subventricular zone) (Modified from Bond et al., 2005)

The neurogenic processes occurring in the SVZ and SGZ have been related to learning and memory (Kempermann et al., 2004; Lledo et al., 2006) and, although species-specificity differences exist, they have been described in all animal species studied so far, including humans (Eriksson et al., 1998; Lindsey and Tropepe, 2006; Bonfanti and Peretto, 2011; Kempermann, 2012; Ernst et al., 2014).

1.1.2 The "non-canonical" neurogenic niches

In addition to the SVZ and SGZ, adult neurogenesis has been also described in many CNS parenchymal regions, the so-called "non-canonical" neurogenic niches (Emsley et al., 2005; Feliciano et al., 2015). Parenchymal neurogenesis is not directly related to germinal layers and the stem/progenitor cells, in contrast with those found in the SVZ and SGZ. Moreover, this type of neurogenesis is more heterogeneous, considering its nature and potentialities (Bonfanti and Peretto, 2011). In fact, in the adult

CNS parenchyma, different "types" of neurogenesis may occur among the evolutive scale (Bonfanti and Peretto, 2011; Feliciano et al., 2015) and they do not fall into a simple classification due to their high heterogeneity involving many variables (Feliciano et al., 2015). Spontaneous parenchymal neurogenesis has been considered a rare phenomenon in mammals, although several examples have been reported in the neocortex and striatum of rodents (Dayer et al., 2005) and rabbits (Luzzati et al., 2006), in the caudate nucleus and cerebellum of rabbits (Luzzati et al., 2006; Ponti et al., 2008) and in the neocortex, piriform cortex and amygdala of primates (Gould et al., 1999; Bernier et al., 2002). Most parenchymal neurogenesis seems to occur in specific physiological or pathological conditions. Therefore, the activation of endogenous progenitors in response to specific forms of injury and in certain experimental conditions/models has been described in the neocortex as well as in the striatum, septum, thalamus and hypothalamus in both mice and rats (Magavi et al., 2000; Pencea et al., 2001; Arvidsson et al., 2002; Chen et al., 2004; Kokoeva et al., 2005; Ohira et al., 2010; Pierce and Xu, 2010; Pérez-Martín et al., 2010; Matsuzaki et al., 2017). Similarly, low levels of neurogenesis involving proliferation of progenitors and incorporation of new neurons to the circuitry have been also described in the olfactory tubercle of mice (Shapiro et al., 2007b) and monkeys (Bédard et al., 2002), in the dorsal vagal complex of the rat brainstem (Bauer et al., 2005), in the substantia nigra and in the Ammon's horn of the mouse hippocampus (Rietze et al., 2000; Zhao et al., 2003; Zhao and Lang, 2009). Cell proliferation and production of new neurons have been reported in the hypothalamus of mammals with larger and more complex brains such as sheep (Migaud et al., 2010), as well as in other structures including the median eminence, the pars tuberalis of the pituitary gland, and the thalamus (Migaud et al., 2011). In addition, local glia-like progenitors, retaining some proliferative capacity, have been also described in the "non-canonical" neurogenic niches of the mature CNS (Horner et al., 2000; Butt et al., 2005; Ponti et al., 2008; Trotter et al., 2010).

Recently, attention has been focused on a form of slow delayed neurogenesis ("neurogenesis without division") (La Rosa et al., 2020), which differs from those described so far. This type of neurogenesis involves immature neurons in the adult brain, which are not proliferating. These neurons may represent a potential "*reservoir* "of immature cells that complete their differentiation program under different circumstances (Bonfanti and Nacher, 2012; Piumatti et al., 2018; La Rosa et al., 2019) to be ultimately integrated into the circuitry (Rotheneichner et al., 2018; Benedetti et al., 2020).

1.1.3 The adult rodent piriform cortex layer II: a "non-canonical" neurogenic niche

The adult rodent piriform cortex (PCX) is a trilaminar paleocortical structure involved in olfactory functions, which contains a population of immature neurons mostly distributed within the layer II (Gómez-Climent et al., 2008; Rubio et al., 2015). Some studies suggested that a minor subset of these immature neurons might be recently generated, probably in the SVZ (Pekcec et al., 2006; Shapiro et al., 2007b), but there is solid proof that the vast majority of them have been generated during embryonic development and remain in an undifferentiated state until adulthood (Gómez-Climent et al., 2008; Rubio et al., 2015). Consequently, the adult rodent PCX layer II can be considered a "non-canonical" neurogenic niche.

The PCX is the largest area of the mammalian olfactory cortex, located on the ventrolateral surface of the brain, close to the lateral olfactory tract (LOT) **(Figure 2)**. It is composed by 3 layers: the most superficial layer I consisting in the layer Ia, which contains the distal dendrites of PCX pyramidal cells (PCs) and receives input from the mitral and tufted cell axons in OB (Shipley and Ennis, 1996; Gire et al., 2012), and the deeper layer Ib, which includes local associative axons and commissural axons from the contralateral olfactory cortical neurons. The layer II is organized in 2 sublaminar layers: IIa and IIb, which contain the highest number of glutamate-releasing neurons. These excitatory neurons are mainly classified morphologically into semilunar (SLN) and superficial pyramidal (SPN) neurons (Suzuki and Bekkers, 2011). The SLN are located in the superficial part of layer IIa, project 2 principal dendrites to layer I and lack a basal dendritic tree. They display a high density of dendritic spines in the distal region of their apical dendrites. These neurons receive strong excitatory afferents from LOT axons and their activity is correlated with OB stimulation (Suzuki and Bekkers, 2011). The SPNs, located in layer IIb, extend their basal dendrites into layer III and apical dendrites into layer I, receive only sparse LOT input from the OB and are involved in the integration of multiple odors (Klingler, 2017, for review). Other excitatory neuronal types can be also observed in layer II and are classified as semilunar pyramidal transitional neurons (SLPTNs) (Haberly and Behan, 1983). Finally, the layer III contains the cell bodies of deep pyramidal neurons (DLPNs), 2 populations of multipolar cells and a large number of associational fibers from the PCX and other brain structures. The DLPNs receive weak afferents from LOT axons (Hagiwara et al., 2012) and substantial intracortical excitatory

inputs (Klingler, 2017). In addition, different types of GABA-releasing interneurons are uniformly scattered across all layers of the PCX, in which they provide feedforward or feedback synaptic inhibition to the principal cells (Suzuki and Bekkers, 2007). The PCX encodes, processes and finally transmits the information coming mainly from the OB, but it also sends projections within the olfactory cortex, including the anterior olfactory nucleus (AON), the olfactory tubercle, the cortical amygdala (CoA) and the lateral entorhinal cortex (LEnt). In addition, the PCX also sends projections to the OB, to the mediodorsal nucleus of the thalamus and to several subdivisions of the prefrontal cortex (PFC)(Klingler, 2017) **(Figure 2)**.

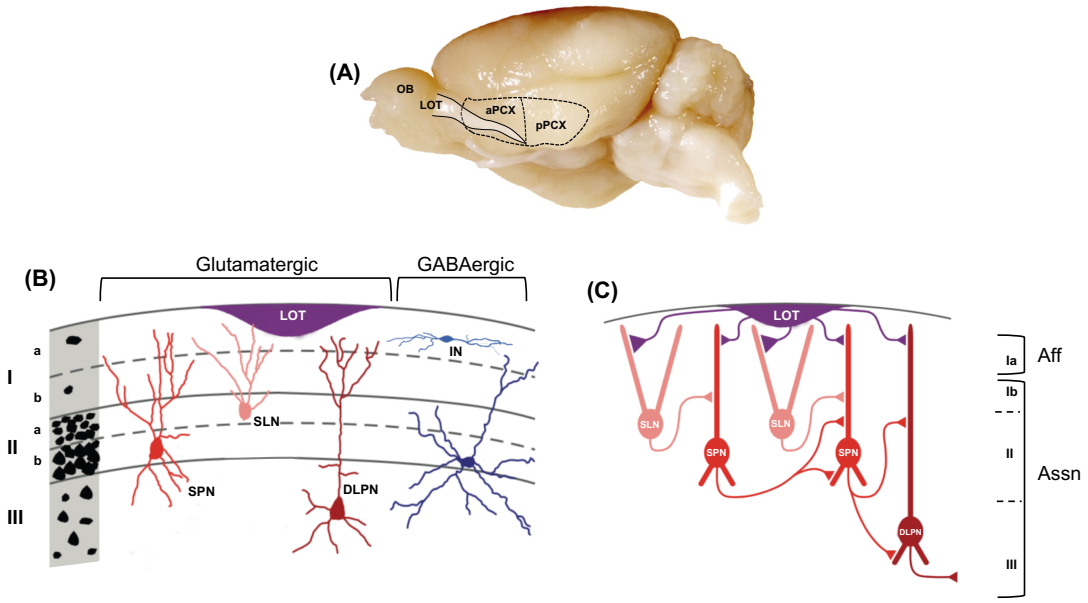

Figure 2. Location, cytoarchitecture, and circuitry of the PCX. (A) Ventrolateral aspect of the rat brain, showing the olfactory OB, the LOT and the aPCX and pPCX, which are approximately marked by the dashed line. **(B)** Schematic illustration of the three-layered PCX. At left is a schematic representation of the density of neuronal somata in each layer, showing the high density of mainly principal cells in layer II and a lower density of neurons in layers I and III. SLNs and SPNs have their somata concentrated in layers IIa and IIb, respectively. DLPNs are found at lower density in layer III. GABA-releasing INs are distributed more sparsely and uniformly across all layers. **(C)** Schematic connectivity of glutamatergic neurons in the PCX. SLNs and SPNs receive afferent (Aff) input from the LOT in layer Ia, but SLNs cells receive a stronger Aff input (larger triangle, representing a bouton). SPNs cells receive intracortical associational (Assn) inputs in layers Ib, II, and III from SLNs and SPNs, whereas Assn inputs to SLNs are weak. DLPNs have been less studied but their connectivity likely resembles that of SPNs. (aPCX: anterior piriform cortex; DLPNs: deep pyramidal neurons; INs: interneurons; LOT: lateral olfactory tract; OB: olfactory bulb; pPCX: posterior piriform cortex; SPNs: superficial pyramidal neurons; SLNs: semilunar neurons) (Modified from Bekkers et al., 2013)

2. IMMATURE NEURONS IN THE ADULT RODENT CEREBRAL CORTEX

2.1 Distribution and morphology

In adult rodents the immature neurons are mostly distributed in the layer II of the piriform and lateral entorhinal cortices and they also appear, with a much lower density, in the layer II of the perirhinal and the most ventral region of the agranular insular and ectorhinal cortices (Seki and Arai, 1991a; Gómez-Climent et al., 2008; Bonfanti and Nacher, 2012).

In the PCX, immature neurons are essentially restricted to layer II, particularly to layer IIb, where their somata are densely organized (Gómez-Climent et al., 2008; Luzzati et al., 2009; Bonfanti and Nacher, 2012). Depending on their morphological characteristics, they can be classified in 2 main population of cells: a) tangled (type I) cells, which are very small (with a soma diameter around 9 µm), lack axons and only display some short neurites with irregular trajectories, usually restricted to layer II and b) complex (type II) cells, with larger somata (diameter around 15 µm), which usually display axon initial segments (AIS) and typical apical dendrites expanding into layer I: the most developed complex cells show typical dendritic branching and dendritic spines with sparsely excitatory synapses. Neurons with intermediate characteristics between tangled and complex cells have also been found, indicating a progressive transition between these 2 cells types (Gomez-Climent et al., 2010) (**Figure 3)**.

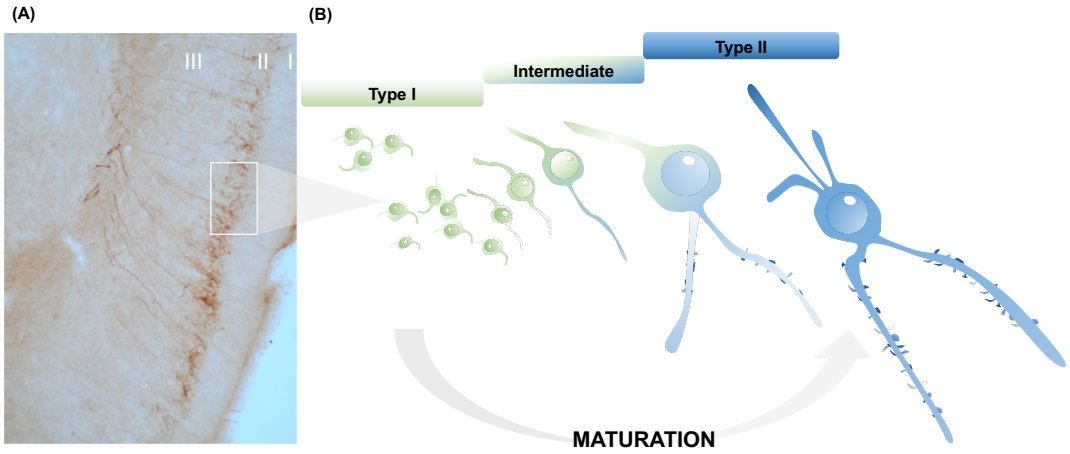

Figure 3. Immature neurons in the adult rat PCX. (A) Optical microphotograph showing the three-layered architecture of the PCX. Note the high density of neuronal somata in layer II. **(B)** Schematic drawing representing different stages of cell maturation in the PCX layer II. Bipolar, small type I cells (green), which are frequently associated in clusters (2-5 cells). Larger type II cells (blue) displaying 1 or 2 long dendrites with protrusions resembling thin dendritic spines or typical spines and some basal processes. Cells with intermediate morphology can also be distinguished indicating a progressive transition between type I and type II cells. (Created in BioRender.com)

Moreover, complex cells have also a higher density of puncta expressing pre-synaptic markers in their perisomatic region, which suggests an increase in their connectivity with the surrounding network (Rotheneichner et al., 2018). Although scarcer than in layer II, immature neurons were also observed in the PCX layer III, the endopiriform nucleus and in the deep layers of the lateral entorhinal cortex. These cells are monopolar or bipolar and frequently display short processes oriented vertically; they can also be observed forming aggregates of 2-5 cells, especially at the ventral end of the external capsule of the corpus callosum (Nacher et al., 2001a, 2002a, 2010). Interestingly, detailed analysis with electron microscopy has also revealed that the immature neurons in the PCX, specially the tangled cells, have a substantial surface of their somata and neurites covered by astroglial processes (Gómez-Climent et al., 2008).

2.2 Phenotypic characterization

In the adult CNS, immature neurons transiently express a complex set of genes that encode molecules involved in neurodevelopmental events. The expression of these genes is not exclusively found in immature neurons, in fact, they can also be detected in mature neurons or glial cells. Therefore, the co-expression of different neurodevelopmental related-molecules, as well as the lack of expression of mature neuronal and glial markers, is a useful instrument to demonstrate their immature phenotype.

2.2.1 Immature neuronal markers

Among the numerous markers of immature neurons, one of the most widely used is the polysialylated form of the neural cell adhesion molecule (PSA-NCAM). PSA-NCAM is a plasticity-related molecule generally considered a promoter of cell migration, cell differentiation and a facilitator of axonal outgrowth, neuronal-glial structural remodeling and synaptogenesis (Kiss and Rougon, 1997; Maarouf and Rutishauser, 2003; Bonfanti, 2006; Gascon et al., 2007; Rutishauser, 2008; Hildebrandt and Dityatev, 2015). In the adult mammalian brain, PSA-NCAM is abundantly present in the “canonical” neurogenic niches, the SVZ and SGZ, in which it is expressed by neuroblasts during their migration and first phase of differentiation (Seki and Arai, 1991a; Bonfanti and Theodosis, 1994; Rousselot et al., 1995). However, PSA-NCAM expression is not only restricted to these population of cells, in fact, it is also abundantly expressed by cells

populating “non-canonical” neurogenic niches, like that of the rodent PCX layer II (Seki and Arai, 1991a; Nacher et al., 2002a; Gómez-Climent et al., 2008; Rubio et al., 2015). Another marker frequently used to identify immature neurons is doublecortin (DCX), a microtubule associated protein heavily expressed in the central and peripheral nervous systems during embryonic and postnatal development. It plays a key role in microtubule stabilization (Gleeson et al., 1999), nuclear translocation and maintenance of bipolar morphology during neuronal migration (Koizumi et al., 2006), as well as in growth cone dynamics (Burgess and Reiner, 2000). DCX is transiently expressed in proliferating progenitor cells and newly generated neuroblasts within the 2 canonical niches in the adult brain, the SVZ and SGZ (Nacher et al., 2001a). Cells expressing DCX are found in the adult rodent PCX (Nacher et al., 2001a; Rubio et al., 2015). Other molecules that are transiently expressed in newly generated neurons during development can also be used as markers of immature neurons during adulthood. For example, TUC4, a protein of the TOAD/ Ulip/CRMP family of axonal guidance proteins (Minturn et al., 1995), the cyclic nucleotide-gated ion channel 3 (CNGA-3) (Gutièrrez-Mecinas et al., 2007), the phosphorylated cAMP response element-binding protein (p-CREB) (Nakagawa et al., 2002), the class III beta-tubulin (TuJ1) (Menezes and Luskin, 1994) or the antiapoptotic B-cell lymphoma 2 protein (Bcl-2) (Bernier and Parent, 1997). However, in the adult CNS, some of these molecules specifically used to identify immature neurons can also be expressed by mature neurons and/or glial cells: PSA-NCAM expression is also found in mature interneurons of the hippocampus and the neocortex (Nacher et al., 2002a; Varea et al., 2005; Gómez-Climent et al., 2011a), CNGA3 expression can be detected in mature principal and inhibitory neurons in the OB (Gutièrrez-Mecinas et al., 2007) and p-CREB expression was found in mature neurons of the cerebral cortex (Thome et al., 2000). TUC4 expression was revealed in a subset of cortical oligodendrocytes (Nacher et al., 2000).

2.2.2 Markers for mature neurons and glial cells

Immature neurons in the adult CNS can also be identified by the lack expression of mature neuronal and glial markers. Several molecules are intensely expressed by mature neurons in the adult CNS, among them the neuronal nuclear protein (NeuN) (Mullen et al., 1992) and the microtubule associated protein 2 (MAP2) (Murphy et al., 1977; Weisshaar and Matus, 1993). Most immature neurons in the PCX layer II lack both

NeuN and MAP2 expression (Nacher et al., 2002a; Gómez-Climent et al., 2008; Xiong et al., 2008; Cai et al., 2009; Zhang et al., 2009). However, the expression of NeuN was detectable, albeit at low levels, in some of the nuclei of the immature neurons belonging to the complex cell population (Nacher et al., 2002a; Gómez-Climent et al., 2008; Xiong et al., 2008). Several markers of astrocytes, oligodendrocytes, and microglial cells are also commonly used to discriminate between neurons and glial cells. Studies conducted in rats, guinea pigs and cats have demonstrated that these glial markers are absent from the population of immature neurons in the PCX layer II (Gómez-Climent et al., 2008; Xiong et al., 2008; Cai et al., 2009). However, a small proportion of PSA-NCAM expressing tangled cells is positive for the Neural/glial antigen-2 (NG2), a marker of polydendrocytes and oligodendrocyte progenitors (Gómez-Climent et al., 2008; Rubio et al., 2015).

2.3 Origin and fate

Several hypotheses have been proposed on the origin of immature neurons in the cerebral cortex layer II, which have been matter of debate until recently. One of these hypotheses is based on the possibility that the immature neurons may be generated *de novo* during adulthood. Experiments using bromodeoxyuridine (BrdU), a thymidine analogue, which is incorporated into the DNA of mitotic cells, have revealed a very discrete number of new neurons in the adult PCX of rodents (Pekcec et al., 2006; Shapiro et al., 2007a, 2007b). Particularly, some scarce NeuN+/BrdU+ labeled cells have been described in the PCX of adult mice when animals were sacrificed 4 days after BrdU administration, but not after 12 weeks (Pekcec et al., 2006). BrdU+ cells expressing NeuN, MAP2 and TuJ1 have been found in the PCX of *Old/New world* primate 28 days after BrdU injection (Bernier et al., 2002). Moreover, DCX+/BrdU+ and NeuN+/BrdU+ labeled cells have been described in the adult rodent PCX, 21 days (Shapiro et al., 2007b) and 7 days (Shapiro et al., 2007a) after BrdU injection, but without a precise cortical layer location. Importantly, other studies using similar experimental approaches have failed to find recently generated neurons in the adult PCX (Gómez-Climent et al., 2008; Rubio et al., 2015). Consequently, based on these findings, it appears that the number of newly-generated neurons in the adult PCX is extremely low and that the majority of these cells have an uncertain location and disappear soon after their generation, probably because they do not become incorporated to the circuitry and degenerate. It is possible

that the site of origin of these cells would be the SVZ and that they migrate from this niche to the PCX. In fact, in early postnatal mice neurons originated in the SVZ still migrate to the PCX (De Marchis et al., 2004). Another hypothesis for the intrinsic origin of the immature neurons in the PCX is related to the possibility that some of these cells may originate from local progenitor cells. In the adult cerebral cortex, actively dividing Ng2+ cells can generate both oligodendrocytes and cells with a neuronal phenotype (Belachew et al., 2003). Some studies have suggested that Ng2+ precursors might be capable to differentiate into pyramidal neurons or interneurons in the cerebral cortex of adult mice (Guo et al., 2010) and rats (Dayer et al., 2005), respectively. In addition, some populations of DCX+/Ng2+ and PSA-NCAM+/Ng2+ cells have been found in the PCX and the entorhinal cortex of adult rats and mice (Tamura et al., 2007; Gómez-Climent et al., 2008; Rubio et al., 2015). In a similar way, a study proposed that some of the immature neurons in the PCX layer II of young adult guinea pigs might be generated by progenitors residing in layer I (Xiong et al., 2010). In contrast with these hypotheses suggesting *de novo* generation of neurons in the adult PCX, other studies analyzing BrdU labeling and the expression of proteins that are commonly found in neuronal precursor cells, such as nestin (Doetsch et al., 1997; Nacher et al., 2001b) or Pax6 (Maekawa et al., 2005; Nacher et al., 2005) have not confirmed generation or incorporation of newly-generated neurons from precursor cells. Moreover, BrdU analysis has also suggested that NG2+ precursors, which might be able to generate pyramidal neurons, cease to proliferate since the first postnatal month (Guo et al., 2010). All these previous "*de novo* generation" hypotheses have been challenged by studies that have clearly demonstrated that the majority of immature neurons in the PCX layer II are post-mitotic immature resident cells that do not express markers of proliferative activity. Studies conducted in rodents (Gómez-Climent et al., 2008; Rubio et al., 2015) have failed to find evidence of dividing or newly-generated cells in the adult cerebral cortex layer II. Interestingly, most, if not all, immature neurons in the PCX layer II are generated during embryonic development, mainly at E15.5 in rats (Gómez-Climent et al., 2008) and at E13.5 and E14.5 in mice (Rubio et al., 2015). In the work published by Rubio et al., (2015), only very few cells in layer II were either cycling or newly-generated, but none of them expressed markers of immature neurons, in line with was previously described in rats (Gómez-Climent et al., 2008). The number of immature neurons in the piriform cortex layer II drastically declines during aging. Several studies have described an age dependent decrease in the number of PSA-NCAM+/DCX+ expressing cells in rats (Abrous et al., 1997; Murphy et al., 2001;

Varea et al., 2009) and guinea pigs (Xiong et al., 2008). Consequently, 2 main hypotheses have been proposed on the putative fate of these immature neurons. They may either have only a transient existence and progressively die during the course of aging or they progressively become functionally integrated as mature neurons. Several studies conducted in rodents exclude death as a possible fate: no evidences of a substantial number of dying cells was found in the PCX layer II (Friedman and Price, 1986; Xiong et al., 2008; Gómez-Climent et al., 2011b). Consequently, the second hypothesis appears more likely. These immature neurons might become principal or inhibitory neurons. Most of these cells in the PCX layer II expressed the T-box brain transcription factor 1 (TBR1), specific for pallium-derived principal neurons (Gómez-Climent et al., 2008; Luzzati et al., 2009; Rubio et al., 2015), while almost none of them expressed markers of subpallium-derived interneurons, such as the LIM/homeobox protein 6 (LHX6) or pan distalless (DLL) (Luzzati et al., 2009). The study of the expression of molecules typical of either principal or inhibitory neurons has clarified the neuronal phenotype of these immature neurons. PSA-NCAM+/DCX+ cells in the PCX layer II of adult rats and mice, lacked the expression of inhibitory markers such as γ-amynobutiric acid (GABA), glutamatergic acid decarboxylase 67 kDa (GAD67), calbindin (CB), parvalbumin (PV), calretinin (CR), somatostatin (ST), neuropeptide Y (NPY), cholecystokinin (CCK) or the vasoactive intestinal polypeptide (VIP) (Gómez-Climent et al., 2008; Rubio et al., 2015). Nevertheless, a study suggested a major interneuronal fate for immature neurons in the PCX layer II of adult guinea pig (Xiong et al., 2008). However, this affirmation was based on the study of large-sized mature neurons exhibiting weak DCX reactivity and not exactly located in the PCX layer II. Interestingly, PSA-NCAM+/DCX+ cells in the PCX layer II of adult rats do not show colocalization with Ca (2+)/CaM-dependent protein kinase II (CAMK-II), a marker of mature excitatory neurons (Gómez-Climent et al., 2008). This protein is only found in fully mature excitatory neurons and this is why it is absent from the PSA-NCAM+/DCX+ cells. However, the vast majority of type II immature neurons in the PCX layer II do not show morphological characteristic of interneurons (Suzuki and Bekkers, 2010), but are similar to SLPTN, SLN or SPN, which are excitatory neurons commonly found in the PCX layer II. All these findings, also supported by a recent study demonstrating continuous maturation into excitatory neurons (Rotheneichner et al., 2018), as well as 2 studies describing the electrophysiological properties of DCX+ cells in the PCX layer II (Klempin et al., 2011; Benedetti et al., 2020), have provide solid evidences that the vast majority of immature neurons in the

PCX layer II are integrated in the local circuitry as excitatory neurons and only a small subpopulation may differentiate into interneurons.

3. IMMATURE NEURONS IN OTHER MAMMALS

In mammals with larger cerebral cortices, immature neurons show a wider distribution than in rodents. In rabbits, cats and guinea pigs, the concurrent expression of typical immature neuronal markers such as PSA-NCAM and DCX, has been observed in layers II and upper III of the PCX, as well as in the perirhinal and entorhinal cortices and in the amygdaloid-piriform transitional region. In addition, immature cells were also found in the neocortex of rabbits, cats, and monkeys, particularly in somatosensory cortex and in different regions of the insula (Bonfanti, 2006; Cai et al., 2009; Luzzati et al., 2009; Varea et al., 2011). Immature neurons were described in adult cats, populating many cortical areas, in which they appear more dispersed than in the PCX, being particularly abundant in the entorhinal cortex and in ventral portions of the frontal and temporoparietal lobes. By contrast, in the occipital lobe sections, they were less abundant in dorsal regions, such as the primary visual cortex, showing a lower gradient from area 20 (V4), to area 19 (V3), to area 18 (V2), and to area 17 (V1) (Cai et al., 2009; Varea et al., 2011). Immature neurons have also been observed in adult non-human primates, specifically in layer II and upper layer III of most cortical areas analyzed (Cai et al., 2009). Immature neurons have been also recently described both in the neocortex of small-brained lissencephalic species such as sengi and bats and in large-brain mammals including chimpanzees, foxes, sheep and cats, in which their density tends to be generally associated with brain size (Gatome et al., 2010; La Rosa et al., 2020).

In human brain, the presence of immature neurons has been exclusively observed in the entorhinal cortex of infants (Dhúill et al., 1999) and in the adult temporal and frontal cortices, where they were prevalently located in the upper border of neocortical layer II (Cai et al., 2009). Interestingly, in cortical malformative lesions associated with epilepsy, DCX+ cells were found in 2 years-old patients at the border between layers I and II in the temporal and frontal cortices (Srikandarajah et al., 2009).

4. PSA-NCAM: A MOLECULE INVOLVED IN BRAIN PLASTICITY

The PSA-NCAM, is involved in different forms of neural plasticity, spanning from embryonic to adult nervous system, including axonal growth and fasciculation, cell migration, synaptic plasticity, activity-induced plasticity, neuronal-glial plasticity and embryonic and adult neurogenesis (Bonfanti, 2006; Rutishauser, 2008; Hildebrandt and Dityatev, 2015).

4.1 The Polysialylated form of the NCAM molecule

The cell adhesion molecules (CAMs) play a crucial role in the architecture of the developing CNS, modulating cellular interactions and stabilizing neural circuitry (Gumbiner, 1996). Among CAMs, neural CAM (NCAM) is the most widely present in the CNS and it is expressed on the surface of most neurons (Hoffman et al., 1982; Edelman, 1986). NCAM was identified as a cell surface glycoprotein more than 30 years ago (Rutishauser et al., 1988). It is traditionally viewed as a key mediator of cell adhesion in the CNS. In fact, it is involved in stabilizing and modulating cellular interactions with other cells or with the extracellular matrix through a homophylic Ca2+-independent binding mechanism (Edelman, 1986). NCAM belongs to the immunoglobulin superfamily of adhesion molecules, encoded by a 26-exon-containing gene found at a single locus in the genome (Brümmendorf and Rathjen, 1995; Rougon and Hobert, 2003; Walmod et al., 2004). From the single copy gene, alternative splicing and post-translational modifications give rise at least 20-30 distinct forms of NCAM, differing in the size of their cytoplasmic domains, their mode of membrane attachment or the structure of the extracellular domains (Goridis and Brunet, 1992). Among these, the 3 splice isoforms NCAM 180, NCAM 140 and NCAM 120, named according to their apparent molecular weight, share identical extracellular structures, consisting of 5 immunoglobulin (Ig)-like domains (Ig I–V) followed by 2 fibronectin type III domains (denoted F3I and F3II). However, while both NCAM 140 and NCAM 180 isoforms contain a transmembrane and an intracellular domain, the NCAM 120 isoform lacks those domains and is attached to the cell surface through a glycosylphosphatidylinositol (GPI) anchor (He et al., 1986) (**Figure 4**). The functional properties of NCAM are strongly influenced

by the addition of long chains of a complex sugar, the polysialic acid (PSA). The polysialic acids are polymers of derivates of 9 carbon sugar neuraminic acids which can be found in a wide range of biological components (Rougon, 1993; Mühlenhoff et al., 1998). Due to the negative charge of the nine-carbon monosaccharide, PSA forms a hydration shell, which increases the hydrodynamic radius of the PSA carrier and enlarges the space between adjacent cells (Hildebrandt and Dityatev, 2015). In mammals, PSA is a linear homopolymer of α-2,8-linked N-acetylmeuraminic acid (NeuNAc), which is typically found as terminal residues on cell surface glycoconjugates, thus playing important roles in cellular recognition and adhesive properties (Bonfanti, 2006). Although NCAM has 6 N-glycosylation sites, the attachment of sialic acid residues is restricted to sites 5 and 6 located in the fifth Ig-like domain (Ig5) (**Figure 4**).

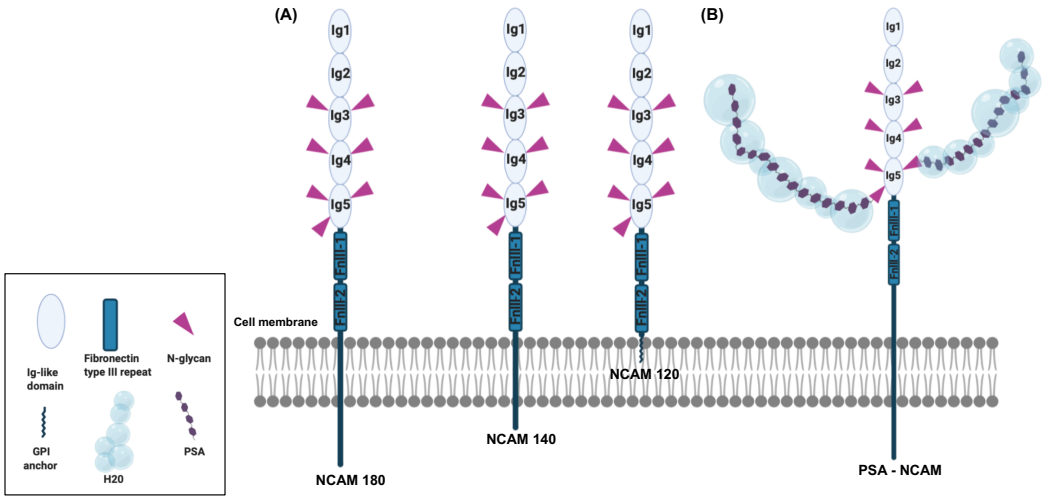

Figure 4. NCAM and PSA-NCAM structure. (A) Scheme of the 3 major NCAM isoforms with identical extracellular domain, composed by 5 immunoglobulin (Ig)-like domains (light-blue) and 2 fibronectin type III repeats (FnIII, blue). Arrows in violet represent the N-glycosylation sites. Differences can be observed in their intracellular domains (NCAM 180 and 140) or in the mode of binding to the cell membrane (NCAM 120), which is attached through a glycosylphosphatidylinositol (GPI) anchor. **(B)** Polysialylated form of NCAM. Only the fifth and sixth N-glycosylation sites located in Ig5 are substrates for PSA attachment. (Modified from Hildebrandt et al., 2010) (Created in BioRender.com)

The addition of PSA to NCAM is catalyzed by 2 independent Golgi-resident polysialyltransferases, the polysialyltransferase II (ST8Sia II, also known as STX) and the polysialyltransferase IV (ST8Sia IV, also known as PST) (Hildebrandt et al., 2007). These enzymes show a high sequence homology and are typical members of the mammalian sialyltransferase family (Angata and Fukuda, 2003; Hildebrandt et al., 2010; Hildebrandt and Dityatev, 2015). In vitro, each enzyme independently is capable of

synthesizing PSA on NCAM. During brain development, both enzymes are expressed with partially overlapping distribution but distinct time course (Hildebrandt et al., 2007). ST8SiaII expression is high in embryonic and perinatal stages but subsequently it decreases and remains low in young and adult animals. By contrast, the expression of ST8SiaIV, although reduced as well, remains high and more persistent in the adult brain (Hildebrandt et al., 1998; Ong et al., 1998; Oltmann-Norden et al., 2008). As reported by Nacher et al., (2010), ST8SiaIV is exclusively responsible for the addition of PSA to NCAM in mature interneurons and in most regions of cortical neuropil, while ST8SiaII plays this role predominantly in immature neurons of the paleocortex layer II and in the SVZ and SGZ of adult mice. However, analysis of PSA-NCAM and DCX expression in mice deficient for either of the 2 polysialyltransferases, ST8SiaIV and ST8SiaII, has demonstrated that both of them seem to play a role on the development and maintenance of the immature neuronal population in the adult PCX layer II (Nacher et al., 2010).

Although NCAM is the preferred substrate for these polysialyltransferases, PSA has been also found on other proteins, including the α-subunit of the rat brain voltage-sensitive sodium channel (Zuber et al., 1992), the scavenger receptor CD36 in human milk (Yabe et al., 2003), the neuropilin-2 on human dendritic cells (Rey-Gallardo et al., 2011) and the synaptic cell adhesion molecule (SynCAM 1) (Mühlenhoff et al., 2013). Remarkably, ST8Sia II and IV are able to polysialylate their own *N*-glycans and this autopolysialylation seems to be essential for proper activity of these enzymes on their target proteins (Close and Colley, 1998; Close et al., 2001; Mühlenhoff et al., 2001).

4.2 Distribution and role of PSA-NCAM in the central nervous system

In the mammalian CNS, the highest levels of PSA-NCAM have been described during neuronal development. The most prominent expression of PSA-NCAM has been found in neural migrating precursors and seems to be crucial for their correct final position and differentiation (Bonfanti and Theodosis, 1994; Bonfanti, 2006). Moreover, PSA-NCAM plays a permissive role in axon guidance reducing the fasciculative interaction between axons and allowing them to respond more effectively to a variety of extrinsic signals and to reach their target, both in the peripheral and in the CNS (Rutishauser, 2008). PSA-NCAM expression has been also detected in radial glia of the developing cortex and mesencephalon (Li et al., 2004; Schiff et al., 2009) and during synapse formation of hippocampal neurons (Dityatev et al., 2000, 2004).

Although commonly considered as a molecule abundant in the developing nervous system, consistent levels of PSA-NCAM have been also described in brain regions that display a high degree of neuronal plasticity during adulthood (Bonfanti, 2006; Gascon et al., 2007; Rutishauser, 2008). As extensively described in the previous paragraphs, PSA-NCAM is intensely expressed in both the SGZ and the SVZ (Seki and Arai, 1991b; Bonfanti and Theodosis, 1994; Rousselot et al., 1995), as well as in the layer II of the cerebral cortex of several mammalian species (Gómez-Climent et al., 2008; Xiong et al., 2008; Bloch et al., 2011; Varea et al., 2011; Rubio et al., 2015; La Rosa et al., 2020).

In the adult CNS, besides immature neurons, a subpopulation of mature cortical interneurons express PSA-NCAM (Gómez-Climent et al., 2011b). Interneurons expressing PSA-NCAM has been described in different cortical areas, including the PFC (Varea et al., 2005, 2007b), the septum (Foley et al., 2003), the hippocampus (Seki and Arai, 1993; Nacher et al., 2002b) and the amygdala (Nacher et al., 2002c; Cordero et al., 2005). Intense PSA-NCAM expression can also be observed in glial cells and neurons of the hypothalamus (Theodosis et al., 1991), as well as in the suprachiasmatic nuclei (Glass et al., 1994) and in the adult brainstem (Bouzioukh et al., 2001). PSA-NCAM expression has been described in a subpopulation of primary sensory neurons (Quartu et al., 2008) and in the adult spinal cord grey matter (Seki and Arai, 1993). PSA-NCAM has been also expressed in certain regions of sub-ependymal tissue and at the surface of CNS ventricular cavities, on both sides of the ependymal wall (Aaron and Chesselet, 1989; Bonfanti et al., 1992; Szele et al., 1994). Expression of PSA-NCAM has been also described in adult optic nerve and retina by astrocytes and Muller cells, as well as in the neuropil of specific retinal layers (Bartsch et al., 1990; Sawaguchi et al., 1999). Interestingly, PSA-NCAM expression has not only been described on the surface of neuronal cell somata and processes, but certain populations of excitatory neurons may express it in their axons or their terminal boutons (Seki and Arai, 1999). Furthermore, wide areas of the adult brain may retain a diffuse pattern of PSA-NCAM expression (Bonfanti, 2006), corresponding mainly to inhibitory elements (Nacher et al., 2013).

In the adult brain, PSA-NCAM plays an important role in neuronal structural plasticity, which includes the remodeling of the structure and connectivity of neurons (Nacher et al., 2002a, 2013; Rutishauser, 2008). PSA-NCAM is involved in synaptic plasticity, especially in synapse formation, but also in the synaptic remodeling associated to changes in synaptic strength and in long term potentiation (LTP) (Gascon et al., 2007; Hildebrandt and Dityatev, 2015). Moreover, PSA-NCAM participates in the remodeling

of dendritic and axonal arbors, including branch formation/elimination, as well as collateral branching (Gascon et al., 2007). It is also important for neurogenesis and recruitment of new neurons to functional networks. In postnatal neurogenic sites, PSA-NCAM regulates the biological properties of new neurons as well as their proper migration, growth and survival (Gascon et al., 2007). Interestingly, PSA-NCAM through its anti-adhesive properties, may have a possible insulating role as proposed by studying the population of immature neurons in the adult rat PCX layer II (Gómez-Climent et al., 2008) and subpopulations of mature cortical interneurons (Gómez-Climent et al., 2011b; Nacher et al., 2013). Moreover, PSA-NCAM seems to have also a key role in brain tissue repair (Nguyen et al., 2003; Maarouf and Rutishauser, 2010) and neuroprotection (Duveau et al., 2007).

4.3 Effects of PSA manipulation on neuronal structural plasticity

The crucial role of PSA-NCAM in several aspects of neural plasticity has been widely demonstrated in *vitro* and *in vivo* studies by the use of the enzyme Endoneuraminidase-N (EndoN). The EndoN is a bacteriophage derived enzyme that cleaves PSA from live cells or tissues without detectable toxicity or gross morphological disruption (Vimr et al., 1984; Rutishauser et al., 1985). Several studies have reported that the specific removal of PSA from NCAM by EndoN may disrupt the induction of LTP in the hippocampus (Muller et al., 1996; Dityatev et al., 2004; Gascon et al., 2007; Hildebrandt and Dityatev, 2015). After removing PSA with EndoN, mossy fiber interaction and synapses formations are perturbed (Seki and Rutishauser, 1998). The formation of cortico-spinal tract axon collaterals appear strongly reduced (Saegusa et al., 2004). In the hippocampus, enzymatic removal of PSA by EndoN induces a remarkable expansion of dendritic arbors in pyramidal neurons (McCall et al., 2013) and increases dendritic spine density in interneurons (Guirado et al., 2014). Conversely, in the medial prefrontal cortex (mPFC), EndoN treatment decreases spine density in apical dendrites of pyramidal neurons (Castillo-Gómez et al., 2016b) and interneurons (Castillo-Gómez et al., 2016a) and increases perisomatic inhibition in both cell types (Castillo-Gómez et al., 2011b, 2016b). Moreover, the enzymatic removal of PSA plays a role in the onset of critical periods during development (Karl et al., 2006) and, interestingly, in the 2 "canonical" neurogenic niches, promotes the differentiation of subventricular (Petridis et al., 2004) and hippocampal (Burgess et al., 2008) neuronal progenitors cells.

5. THE ROLE OF MONOAMINES IN NEURONAL DEVELOPMENT

In the adult rodent brain, the proliferation of NSCs in the SVZ and the SGZ, is highly influenced by monoaminergic neurotransmitters such as 5-hydroxytryptamine (5-HT), dopamine (DA) and norepinephrine (NE). The 5-HT originates predominantly from serotonergic neurons located in the raphe nuclei of the brainstem, which project extensively to multiple brain regions. The NSCs, in both the SVZ and the SGZ, are highly dependent upon serotonergic projections (Mongeau et al., 1997; Brezun and Daszuta, 1999). Lesion studies in which these projections from the raphe were ablated in adult animals have revealed a subsequent decrease in cell proliferation and neurogenesis (Brezun and Daszuta, 1999; Banasr et al., 2004). The proliferation of progenitor cells is altered in mice that lack the 5-HT transporter (5-HTT), which is required for 5-HT re-uptake into the presynaptic cell, as well as after acute or chronic pharmacological manipulation of 5-HT receptors (Radley and Jacobs, 2002; Santarelli et al., 2003; Banasr et al., 2004; Soumier et al., 2010).

DA, similar to 5-HT, is a monoaminergic neurotransmitter that plays a key role in the regulation of adult neurogenesis, as indicated by its experimental manipulation (Höglinger et al., 2004). Dopaminergic projections from the substantia nigra pars compacta and ventral tegmental area (VTA) contact NSCs in the "canonical" neurogenic niches (Höglinger et al., 2014). Ablation of midbrain dopamine neurons by injection of selective neurotoxins, such as 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), as well as treatments with dopamine receptor antagonists, result in reduced proliferation of progenitor cells and neurogenesis in the SVZ (Baker et al., 2004; Höglinger et al., 2004; O'Keeffe et al., 2009; Winner et al., 2009). Several studies have demonstrated that this reduced proliferation is restored by increasing DA receptor signaling in the lesioned brain and by the administration of DA receptor agonists (Höglinger et al., 2004; Kampen et al., 2004; O'Keeffe et al., 2009; Kim et al., 2010). A similar dopaminergic regulation of precursor cell proliferation has been also described in the SGZ (Höglinger et al., 2004; Mu et al., 2011).

NE is also a monoaminergic neurotransmitter implicated in regulating the activation and proliferation of NSCs in the adult brain. Several studies have indicated different responses of progenitor cells to NE signaling. A single intrahippocampal injection of a β3-adrenergic receptor (β3-AR) agonist or the systemic administration over 7 days of isoproterenol, a nonselective β-AR agonist, increased proliferation of hippocampal

progenitor cells (Jhaveri et al., 2010). Intracerebral infusion of exogenous NE and the ablation of noradrenergic neurons reduced the proliferation of progenitor cells in the SVZ (Weselek et al., 2020) and in the SGZ (Kulkarni et al., 2002).

In humans, neurogenic regions receive a similar innervation by monoaminergic projections as that observed in rodents. However, there are not many reports describing how NSCs in the SGZ and SVZ respond to neurotransmitter signaling in our species (Santarelli et al., 2003). The stimulating effect of DA on precursor cell proliferation in the adult SVZ seems to be functional and phylogenetically conserved from rodents to primates (Freundlieb et al., 2006) and it is well established that SGZ neurogenesis increases in response to antidepressant treatment in animal models (Santarelli et al., 2003). Given the highly conserved nature of neurotransmitter signaling in higher mammals, it is reasonable to presume that similar effects on NSC proliferation and migration may eventually be observed in human neurogenic regions.

The monoaminergic neurotransmitters are involved in the regulation of several aspects of adult neurogenesis including proliferation, migration and differentiation of neuronal progenitor cells, as well as in dendritic and axonal growth, spinogenesis and synaptogenesis (Herlenius and Lagercrantz, 2001; Ronald E. van Kesteren and Gaynor E. Spencer, 2003; Money and Stanwood, 2013). Accumulating evidence has demonstrated that monoamines may play a key role in the response to stress and the etiopathology and treatment of major depression (Mongeau et al., 1997; Santarelli et al., 2003; Belujon and Grace, 2017). Some reports have also described the role of these neurotransmitters in the modulation of plasticity-related molecules expressed by immature neurons in the cerebral cortex layer II (Bonfanti and Nacher, 2012). Interestingly, a recent study has provided a novel evidence for a role for NE in the regulation of plasticity markers expressed by immature neurons within the PCX layer II, in both mice and rats (Vadodaria et al., 2017). The adult rodent PCX, in fact, receives a dense noradrenergic innervation, as well as abundant serotonergic and dopaminergic projections (Pazos et al., 1985; Boyson et al., 1986). However, the mechanism through which serotonergic and dopaminergic inputs may modulate the development of immature cells in the PCX layer II is largely unknown. Therefore, future work will be required to clarify whether 5-HT and DA may be involved in the regulation of the final stages of development of this population of immature neurons in the PCX layer II and, consequently, which may be the influence of the pharmacological modulation of monoaminergic neurotransmission on this population of immature neurons.

6. NON-NEWLY GENERATED IMMATURE NEURONS IN THE ADULT BRAIN: PUTATIVE FUNCTION AND IMPLICATION IN NEUROLOGICAL AND PSYCHIATRIC DISEASES

Although extensive research has been carried out on the distribution, morphology, phenotype and origin of immature neurons in the cerebral cortex layer II, their putative fate has been only recently clarified. For this reason, it has been difficult to decipher their function in the adult CNS, which still remains in question. It has been proposed that this population of immature neurons may constitute a "*reservoir*" of post-mitotic cells of embryonic origin that can complete their differentiation program during adulthood under different physiological or pathological conditions (Bonfanti and Nacher, 2012). In a recent study, the use of the transgenic DCX-CreER^{T2}/Flox-EGFP mouse, in which DCX expressing cells are permanently labeled after tamoxifen administration and monitored with the enhanced green fluorescent protein (EGFP) reporter (Zhang et al., 2010), has allowed to clarify the fate of the population of immature cells in the PCX layer II (Rotheneichner et al., 2018). Interestingly, the administration of tamoxifen at different time points has confirmed that EGFP+ cells in the PCX layer II do not die in the course of aging, instead they continue their maturation and acquire a more complex morphology. In fact, upon 6 months of maturation, the apical dendrites of EGFP+ cells showed an increase in their thickness and in the linear density of spines, suggesting an increased synaptic connectivity with the surrounding network in which they are integrated as mature glutamatergic principal neurons (Rotheneichner et al., 2018). These findings were in accordance with a previous study reporting that DCX+ cell in the PCX layer II of adult mice exhibited a typical electrophysiological profile of neurons undergoing maturation (Klempin et al., 2011). A recent study has clearly showed that the immature neurons with a complex morphology mature into functional neurons and that the expression of markers for spines and glutamatergic synaptic boutons indicate their integration into the excitatory connectivity of adult neuronal networks (Benedetti et al., 2020).

Some studies have shown that the immature neurons in the cerebral cortex layer II may be affected by intrinsic or extrinsic factors. Several behavioral paradigms seem to have an impact on these cells. Avoidance conditioning and spatial learning paradigms increase the number of PSA-NCAM expressing cells in the rat PCX layer II (Fox et al., 2000). The distribution of immature neurons in regions that receive a major input from the OB, suggests that the function of these cells may be directly related to olfactory information processing. However, it is worth noting that while in rodents the distribution

of these cells is restricted to cerebral areas involved in olfactory functions, in species with larger and gyrencephalic brains such as cats and primates, these cells are widely dispersed in higher-order integrative cortical areas where no direct olfactory inputs are found, suggesting their implication in non-olfactory brain functions (Bonfanti and Nacher, 2012).

Different lines of evidence have suggested the involvement of the immature neurons of the cerebral cortex layer II in neurological and psychiatric diseases. Rats submitted to transient middle cerebral artery (MCA) occlusion (Hayashi et al., 2001) and humans with temporal lobe epilepsy (TLE) (Mikkonen et al., 1998) had an increased number of immature cells expressing PSA-NCAM in the cerebral cortex layer II. On the contrary, transgenic murine models of Huntington disease showed a decreased number of immature neurons expressing both DCX and PSA-NCAM in the PCX (Phillips et al., 2006; Lazic et al., 2007). Animal models of major depression showed an altered number of immature neurons. While chronic stress increased the number of PSA-NCAM expressing cells in the layer II of the adult PCX, chronic corticosterone treatment decreased it (Nacher et al., 2004). The effects of corticosterone should be indirect because immature neurons do not express glucocorticoid receptors and it is possible that the effects of stress were mediated by *N*-metil-D-aspartate receptors (NMDAR), since they have been found in complex cells (Gómez-Climent et al., 2008). The monoaminergic system is deeply involved in the etiopathology and treatment of neurological and psychiatric diseases and appears to be a potent modulator of the immature neuronal population in the cerebral cortex layer II. A recent study have demonstrated that the chronic stimulation of α2-adrenergic receptors induced an increase in the number of immature neurons expressing both PSA-NCAM and DCX in the rodent PCX layer II (Vadodaria et al., 2017). Additionally, some other pharmacological treatments including NMDAR antagonists (Nacher et al., 2002a), anti-epileptics such as the valproic acid (VPA) (Murphy et al., 2001) or the antidepressants imipramine (Sairanen et al., 2007) and fluoxetine (Varea et al., 2007a) affected immature neurons in the cerebral cortex layer II. Therefore, these data strongly suggest that immature neurons in the cerebral cortex layer II may be involved in different functions, not only related to olfactory processing, and they may represent a target for hormones, neurotransmitters and pharmacological treatments. However, further studies are still necessary to provide relevant evidence to clarify their function in the adult mammalian brain in both physiological and pathological conditions.

Chapter 2

OBJETIVES

The main objective of this doctoral thesis is to study the population of immature neurons in the adult cerebral cortex layer II in both rodents and humans. Specifically, I will focus in the mechanisms that underlie the final stages of development of these immature cells and the factors that promote their differentiation and integration in brain circuits, as well as the involvement of the plasticity-related molecule PSA-NCAM in these processes.

In order to achieve this main goal, I will follow different specific objectives, which are detailed below:

1. To evaluate the effects of the pharmacological modulation of dopaminergic neurotransmission on the differentiation of immature neurons of the adult rat PCX layer II, studying the distribution of the dopamine type 2 receptor (D2r) in this region and the relationship between dopaminergic fibers and immature neurons.

2. To study the impact of the enzymatic depletion of PSA from NCAM by the enzyme EndoN on the population of immature neurons in the PCX layer II, using an inducible DCX-Cre mouse line (DCX- $CreER^{T2}$/Flox-EGFP) as a fate mapping tool for these cells.

3. To investigate the presence of immature neurons in the human cerebral cortex layer II, using surgical samples of epileptic patients and *post-mortem* tissue, employing specific neurochemical markers to study their distribution, morphology and phenotype, as well as their putative fate and function.

Chapter 3

MATERIAL & METHODS

STUDY 1. EFFECTS OF DOPAMINE ON THE IMMATURE NEURONS OF THE ADULT RAT PIRIFORM CORTEX

1.1 Animals

Twenty-four male Sprague–Dawley rats (3 months old, 300 ± 15 g, Harlan Iberica) were used in this study. Two rats were used to study the expression of D2r in PSA-NCAM immunoreactive cells and the spatial relationship between these cells and fibers immunopositive for tyrosine hydroxylase (TH) and the dopamine transporter (DAT). Twenty-two rats were used for experimental manipulations of the dopamine D2 receptors: 10 rats were used for the D2r antagonist (haloperidol) experiment and 12 rats were used for the D2r agonist (2-(N-Phenethyl-N-propyl) amino-5-hydroxytetralin hydrochloride, PPHT) experiment. Animals were housed in groups in a temperature and humidity-controlled environment and were maintained on a 12 h light/dark cycle with free access to food and water. Rats were allowed to habituate to our facilities at least 1 week prior to the start of the experiments.

All animal experimentation was performed in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and was approved by the Committee on Bioethics of the University of Valencia. Every effort was made to minimize the number of animals used and their suffering.

1.2 Chronic treatment with haloperidol

Ten rats were divided randomly in 2 groups: control (vehicle-treated; n = 5) and haloperidol treated (0.5 mg/kg/day, n = 5). The injections were administered intraperitoneally (i.p.) once daily for 26 consecutive day (MacDonald et al., 2005; Castillo-Gómez et al., 2008). Haloperidol (Sigma-Aldrich) was dissolved in 0.1 N acetic acid, pH 5–6. Animals were perfused transcardially 24 h after the final injection and their brains were processed as described below for PSA-NCAM immunohistochemistry.

1.3 Chronic treatment with PPHT

Rats were randomly separated in 2 groups and administered i.p. either the selective D2r agonist PPHT (n = 6, 1.5 mg/ kg/day in 0.9% NaCl; Sigma-Aldrich) or saline (n = 6) once daily for 7 consecutive days (Sugahara and Shiraishi, 1998; Castillo-Gómez et al., 2008, 2011). Animals were sacrificed 24 h after the final injection. Their brains were processed, and sections were analyzed using immunohistochemistry for PSA-NCAM as described below.

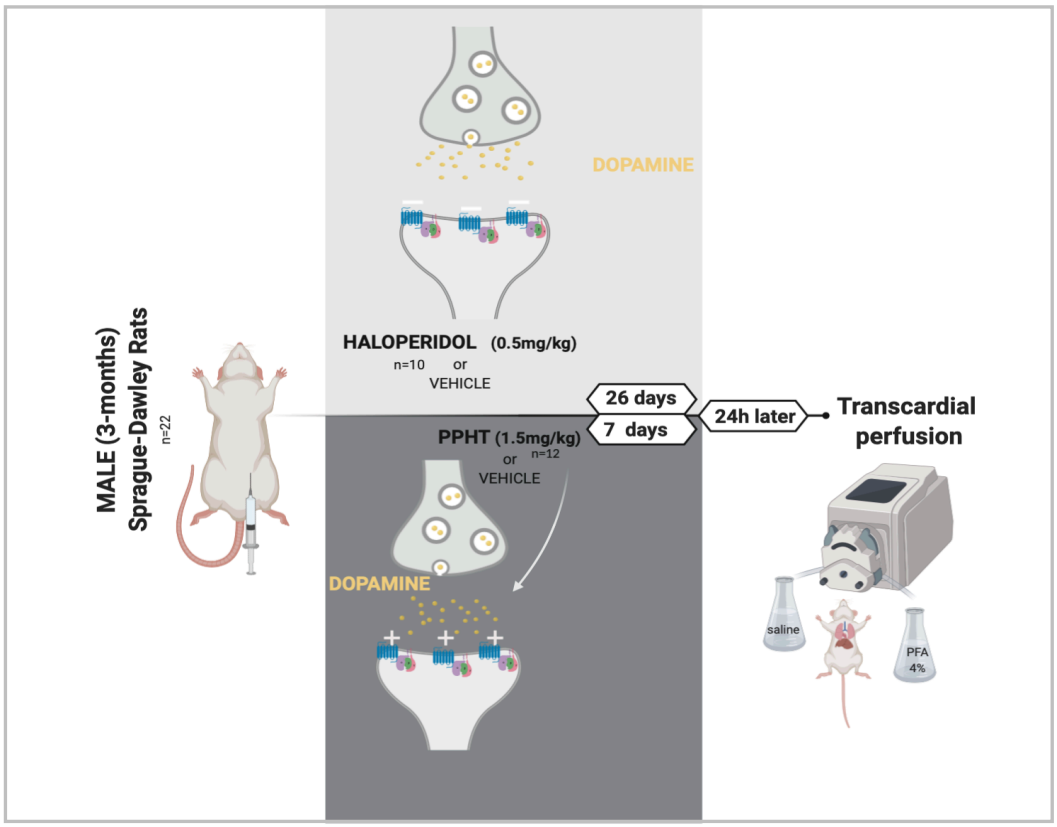

Figure 5. Experimental protocol used in the study 1. (Created in BioRender.com)

1.4 Histology

All rats (n=24) were perfused transcardially under deep chloral hydrate anesthesia, first with saline and then with 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS) 0.1 M, pH 7.4. Thirty minutes after perfusion, the brains were extracted and cryoprotected for 48h with 30% sucrose in phosphate buffer (PB) 0.1 M. Coronal sections (50 µm) were obtained from frozen brains (-30°C) with a freezing-sliding

microtome (Leica SM2000R), collected in 10 subseries and stored at -20 °C in 30% glycerol, 30% ethylene glycol in PB until used.

1.5 Immunohistochemistry for conventional light microscopy

Tissue was processed "free-floating" for immunohistochemistry as follows. Briefly, sections were incubated for 1 min in an antigen unmasking solution (0.01 M citrate buffer, pH 6) at 100 °C. After cooling down the sections to room temperature, they were incubated with 10% methanol, 3% H_2O_2 in PBS for 10 min to block endogenous peroxidase activity. After this, sections were treated for 1 h with 10% normal donkey serum (NDS) (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS with 0.2% Triton-X100 (Sigma-Aldrich) and were incubated overnight at room temperature in monoclonal mouse IgM anti-PSA-NCAM antibody (**Table 1**). After washing, sections were incubated for 2 h with donkey anti-mouse IgM biotinylated antibody (**Table 1**), followed by an avidin-biotin-peroxidase complex (ABC; Vector Laboratories, Peterborough, UK) for 1 h in PBS. Color development was achieved by incubating with 3,3′ diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) and H_2O_2 for 4 min. PBS containing 0.2% Triton-X100 and 5% NDS was used for primary and secondary antibodies dilution. All sections passed through all procedures simultaneously in order to minimize any difference from immunohistochemical staining itself.

1.6 Immunohistochemistry for confocal microscopy

In general, sections were processed as described above but omitting the endogenous peroxidase block. The first day, all sections were incubated overnight at room temperature with the following primary antibody cocktails: a) monoclonal mouse IgM anti-PSA-NCAM and polyclonal rabbit anti-Human D2r or b) monoclonal mouse IgM anti-PSA-NCAM, monoclonal rat IgG anti-DAT and monoclonal mouse IgG1 anti-TH. (**Table 1**) Forty-eight hours later, sections were washed and incubated for 2 h with the appropriate fluorescent secondary antibodies (**Table 1**). All sections were mounted on slides and coverslipped using Dako Cytomation fluorescent mounting medium (Dako).

Primary antibodies	*Anti*	*Host*	*Isotype*	*Dilution*	*Cat. number*	*Company*
	D2r	Rabbit	IgG	1:200	AB5084P	Sigma-Aldrich
	DAT	Rat	IgG	1:200	MAB369	Sigma-Aldrich
	PSA-NCAM	Mouse	IgM	1:1400	MAB5324	Sigma-Aldrich
	TH	Mouse	IgG_1	1:200	MAB318	Sigma-Aldrich

Secondary antibodies	*Anti*	*Host*	*Conjugate*	*Dilution*	*Cat. number*	*Company*
	Mouse IgM	Donkey	Biotin	1:250	715066020	Jackson Immunoresearch
	Mouse IgM	Goat	AF555	1:200	A21246	Molecular Probes
	Rabbit IgG	Donkey	AF488	1:200	A32790	Invitrogen
	Rat IgG	Goat	AF488	1:200	A11006	Molecular Probes
	Mouse IgG_1	Goat	AF647	1:200	A21240	Life Technologies

Table 1. Primary and secondary antibodies used in the study 1.

1.7 Antibody characterization

All antibodies used in this thesis were previously validated by the manufacturers and extensively used by other researchers on rodent (manufacturer's information). The omission of the primary antibodies during the immunohistochemical protocols resulted in total absence of immunostaining. Below there is a detailed description of the antibody characterization.

The D2r antibody was raised against a 28-amino acid (aa) fragment (284-311) within the cytoplasmic third loop (aa 284-311) of human D2r (Hersch et al., 1995). The immunogen peptide has no significant homology with other subtypes of dopamine receptors (D1, D3-D5), and the antiserum recognizes both short and long forms of D2r (Boundy et al., 1993). The specificity of the antiserum for the immunogen peptide was shown in rats by the absence of immunoreactivity in controls of pre-immune antiserum and immunogen peptide adsorption (Boundy et al., 1993).

The DAT antibody used in this study corresponds to the DAT/Nt antibody generated against human DAT N-terminus aa 1-66. The specificity of this antibody has been

demonstrated by western blotting (WB), immunoprecipitation (IP), pre-adsorption followed by WB and immunohistochemistry (IHC), and several IHC assays, and it has been validated for the detection of DAT in rats (Wersinger et al., 2004; Bianco et al., 2008; García-Cabezas et al., 2009; Pandolfo et al., 2013).

The mouse monoclonal anti-TH antibody was raised against purified TH from the PC 12 pheochromocytoma cell line; this antibody recognizes recombinant TH by WB (Wolf and Kapatos, 1989). The anti-TH antibody labels a single protein band in the molecular weight range of 56-60 kDa on WB prepared from VTA protein homogenates (Shepard et al., 2006). In addition, this anti-TH antibody was used to demonstrate that midbrain unilateral lesion with 6-hydroxydopamine (6-OHDA) results in lack of TH immunolabeling in the lesioned site, whereas it is preserved in the contralateral unlesioned midrain (Sarabi et al., 2001). The clone LNC1 has been validated for use in IHC with more than 700 product citations (manufacturer's information).

1.8 Confocal analysis and quantification

The sections were observed under a confocal microscope (Leica TCS-SPE). Z-series of optical sections (0.5 μm apart) of the whole thickness of the histological sections were obtained using sequential scanning mode. These stacks were processed with FIJI/ImageJ software (Schindelin et al., 2012). Fifty PSA-NCAM immunoreactive cells were randomly selected from all the sections containing PCX (10 sections between Bregma -1.40 mm to -3.80 mm (Herman and Watson, 1987) to analyze their spatial relationship with TH and DAT immunoreactive fibers. To obtain the percentages describing their colocalization with D2r, the quantification was performed blindly by first evaluating PSA-NCAM neurons in individual detection channels to assess the distribution of D2r population, followed by merging the single images to identify and count doubly-labeled neurons. In addition, 25 TH fibers were also selected from the same sections to analyze their colocalization with DAT.

1.9 Quantification of the density of PSA-NCAM expressing cells

In order to study the density of PSA-NCAM expressing cells in the PCX layer II (haloperidol and PPHT experiments), 2 sections per animal were selected from the following coordinates: Bregma -1.40 mm and Bregma -3.60 mm (Herman and Watson,

1987). Cell counting was performed manually, using an Olympus CX41 microscope and results were expressed as density of immunopositive cells per mm^2.

1.10 Statistical analysis

After checking the normality and homoscedasticity of the data, unpaired Student's t-tests were used to compare control and experimental groups. Analysis were performed using the statistical package SPSS v22.0 (IBM). In every test, α was set to 0.05 and the animal was considered as the "n".

STUDY 2. PSA DEPLETION INDUCES THE DIFFERENTIATION OF IMMATURE NEURONS IN THE PIRIFORM CORTEX OF ADULT MICE

2.1 Transgenic animals

Fourteen young-adult transgenic DCX-CreER^{T2}/Flox-EGFP mice (Zhang et al., 2010) were used for the study of neuronal maturation in the PCX after PSA depletion by EndoN. In this transgenic strain the expression of the EGFP reporter gene was induced in DCX-expressing cells by tamoxifen administration (100mg/Kg dissolved in corn oil, Sigma-Aldrich). Tamoxifen was administered orally by gavage for 5 consecutive days at the age of 2 months (**Figure 6**).

Experiments were performed in agreement with the "Directive 2016/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes" and were approved by Austrian animal care authorities: protocol number: 66.019/0025-V/3b/2019.

2.2 Polysialic acid depletion

Two days after the last injection of tamoxifen, mice were first anesthetized with ketamine (75mg/Kg, i.p.) and medetomidine (1.5mg/Kg, i.p.) and received afterward carprofen (5mg/kg, subcutaneous, s.c.). They were then placed in a stereotaxic instrument (David Kopf Instruments) and received an intracranial injection of 1µl of EndoN (0.7 U/µL in glycerol; AbCys, France), using a Hamilton syringe, in the right hemisphere and of vehicle solution (saline and glycerol 1:1) in the left hemisphere. EndoN is a phage enzyme that specifically cleaves α-2,8-linked N-acetylneuraminic acid polymers with a minimum chain length of 8. It diffuses rapidly throughout the brain and removes all detectable PSA within 1 day for 3-4 weeks (Troy et al., 1987). Since Endo-N can diffuse long distances in the brain parenchyma, the enzyme was injected in the ectorhinal cortex (Bregma coordinates: 1.58mm; lateral ± 4mm; deep -3.4mm) (Franklin and Paxinos, 2008) in order to avoid lesion or edema caused by the intracerebral injection in the PCX. One µl of the enzyme EndoN was injected in the right hemisphere during 4 min. After the injection was completed, the needle was left in place for 1min to reduce reflux of the solution into the track of the injection needle and then withdrawn. Next, the vehicle

solution was injected following the same procedure in the right hemisphere. After surgery, the animals received atipamazol (5mg/Kg, i.p.) and were kept in individual cages. To prevent the occurrence of infection, enrofloxacin (10mg/Kg, s.c.) was also administered on the day of surgery until the fifth day post-operative. Recovery and survival rates of mice after surgery were improved by careful management of postoperative care (**Figure 6**).

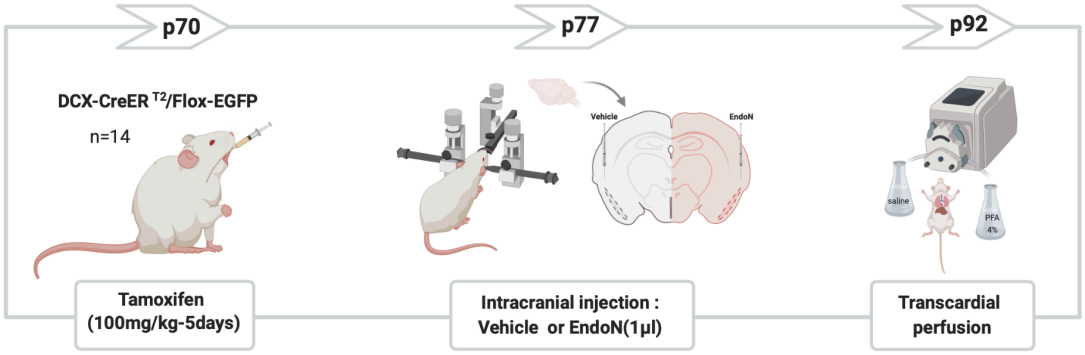

Figure 6. Experimental protocol used in the study 2. (Created in BioRender.com)

2.3 Histology

Two weeks after surgery, mice were deeply anesthetized by i.p. injection of ketamine (205mg/kg), xylazine (53.6mg/kg) and acepromazine (2.7 mg/Kg). They were transcardially perfused, first for 1 min with NaCl 0.9% and then for 30 min with 4% PFA in PBS 0.1M, pH 7.4 (**Figure 6**). Thirty min after perfusion brains were extracted from the skull, post-fixed in PFA solution for 24 h, followed by washout and their hemispheres were separated. Coronal sections were sliced with a Leica VT1200s microtome at a thickness of 50 µm. They were collected in 6 subseries and stored in PB 0.1 M with sodium azide 0.05% as preservative at 4°C until used.

2.4 Immunohistochemistry for confocal microscopy

Tissue sections were processed "free-floating" as follows: briefly, sections were incubated for 1 min in an antigen unmasking solution (0.01 M citrate buffer, pH 6) at 100 ˚C. After cooling down the sections to room temperature they were incubated with 10% NDS (Sigma-Aldrich) in PBS with 0.2% Triton-X 100 (Sigma-Aldrich) for 1h, then they were incubated 48 h at 4˚C with a cocktail of primary antibodies (**Table 2**). After washing, sections were incubated for 2 h at room temperature with the appropriate

fluorescent secondary antibodies (**Table 2**). Nuclei were counterstained with DAPI (0.5µg/mL; Sigma-Aldrich). Finally, sections were mounted on slides and coverslipped using Dako (Agilent Technologies) mounting medium

Primary antibodies	***Anti***	***Host***	***Isotype***	***Dilution***	***Cat. number***	***Company***
	Ank-G	Mouse	IgG_1	1:200	SC12719	Santa Cruz
	CAMK-II	Mouse	IgG_1	1:1000	ab22609	Abcam
	DCX	Rabbit	IgG	1:2000	ab207175	Abcam
	GFP	Chicken	IgY	1:1000	A10262	ThermoFisher
	NeuN	Mouse	IgG_1	1:1000	MAB377	Sigma-Aldrich
	PSA-NCAM	Mouse	IgM	1:1400	MAB5324	Sigma-Aldrich
	PV	Guinea pig	IgG	1:2000	195004	SySy
	SYN	Mouse	IgG_1	1:1000	S5768	Sigma-Aldrich
	VGAT	Rabbit	IgG	1:1000	131002	SySy
	VGLUT1	Guinea pig	IgG	1:1000	AB5905	Sigma-Aldrich

Secondary antibodies	***Anti***	***Host***	***Conjugate***	***Dilution***	***Cat. number***	***Company***
	Chicken IgY	Donkey	CF488	1:400	20020	Biotium
	Guinea pig IgG	Goat	AF647	1:400	106605003	Jackson Immunoresearch
	Mouse IgG_1	Goat	CF647	1:400	20252	Biotium
	Mouse IgG_1	Goat	AF555	1:400	A21127	ThermoFisher
	Mouse IgM	Goat	CF555	1:400	20485	Biotium
	Rabbit IgG	Donkey	CF555	1:400	20038	Biotium

Table 2. Primary and secondary antibodies used in the study 2.

2.5 Confocal analysis and quantification

Fluorescence images were acquired using an LSM 710 confocal microscope with ZEN 2011 Black software (Carl Zeiss) and a TCS-SPE confocal microscope (Leica). All the images were analyzed using the FIJI/ImageJ software (Schindelin et al., 2012).

2.5.1 Classification and phenotypic characterization of EGFP+ neurons

To analyze the percentage and density of EGFP expressing neurons and their double immunolabeling with anti-NeuN, -DCX or -CAMK-II antibodies, cells were divided in 5 subgroups according to their soma diameter and maturational stage: a: small tangled cells (ø ≤ 5µm) b: tangled cells with short dendritic arborizations (ø =6-7µm); c: tangled cells with longer dendritic arborizations (ø =8-10µm); d: larger complex cells with thin basal processes and dendritic spines (ø =11-13µm) e: mature complex cells with larger dendrites and mature dendritic spines (ø ≥ 14µm). To calculate the percentage of each subtype, 50 EGFP+ cells in the PCX layer II were randomly selected in different brain sections (Bregma interval: -1.58 mm; -2.54mm). The density of EGFP+ cells and their double labeling with the markers of interest was measured in 3 randomly located squares per section (single confocal planes, 100 x 100 µm) and results were expressed as the number of cells per μm^2.

To study whether the injection of EndoN could accelerate the maturation of EGFP+ cells in the PCX layer II, I investigated the effect of EndoN treatment on the density of complex neurons displaying an AIS. For this purpose, I used an antibody against ankyrin-G (Ank-G) and the analysis was performed in a single square (single confocal planes, 200 x 200 µm) of a single section randomly selected from the Bregma interval: -1.58 mm; -2.54mm.

2.5.2 Analysis of EGFP+ dendrites and dendritic spines

In order to analyze the density of EGFP+ dendrites in the PCX layer I, a single confocal plane was obtained using a 63x oil immersion objective. The density was assessed in a single section per animal (Bregma interval: -1.58 mm; -2.54mm) using 3 randomly located squares (50 x 50 µm) and results were expressed as the number of dendrites per μm^2. I also studied the density of spine on the dendrites of EGFP+ neurons in the PCX layer I. Six dendrites were randomly selected from each animal. To be suitable for dendritic spine analysis, the EGFP+ neurons bearing the selected dendrites had to fulfill the following features: (1) the soma should be located in the PCX layer II; (2) the dendrites should have a thickness > of 1µm; (3) no other dendrites should be found crossing their trajectory. A 63x oil immersion objective with a 3.5x additional digital zoom was used for the analyses using confocal stacks (Z-step size = 0.38µm). The spines were quantified in a longitudinal dendritic 3D segment of 50 µm length, selected at a

constant distance of 50-60 µm from the tissue's surface. The same analysis was also performed considering the different types of dendritic spines, classified manually according to the length of the protrusion and the diameters of their head and neck. Three different categories were established as described before (Guirado et al., 2014): (1) stubby, when the length of the protrusion was < 1.5 µm; (2) mushroom, when a clear head could be observed (maximum diameter of the head should be at least 1.5 times the average length of the neck) and the total length of protrusion was < 3 µm; and (3) thin, when the length of the protrusion was > 3 µm or when this length was between 1.5 and 3 µm and a clear head could not be distinguished. Overall spine density was expressed as the number of spine/µm length.

2.5.3 Analysis of presynaptic inputs on EGFP+ spines

To analyze pre-synaptic inputs on EGFP+ spines, the presence of closely apposed puncta (distance: ≤ 3µm) expressing synaptophysin (SYN) and vesicular glutamate transporter (VGLUT1) was manually quantified. Fifty EGFP+ spines per animal were evaluated. Results were expressed as the percentage of EGFP+ spines lacking apposed puncta, the percentage of EGFP+ spines apposed to SYN or VGLUT1 expressing puncta and the percentage of EGFP+ spines apposed to puncta co-expressing SYN and VGLUT1.

2.5.4 Analysis of perisomatic puncta expressing excitatory/inhibitory synaptic markers

I also studied the density of perisomatic puncta expressing VGLUT1, the vesicular γ-aminobutyric acid (GABA) transporter (VGAT) and PV on EGFP+ complex cells (ø ≥10.5µm). Between 10-15 neurons were counted per animal and the analyses were performed in 2 randomly selected sections corresponding to Bregma interval: -1.58 mm; -2.54 mm. Confocal z-stacks covering the whole depth of the section were taken with 0.38 µm step-size, from which a single plane with the optimal penetration levels for each antibody was selected. Images were processed using a customized macro for FIJI/ImageJ software (Guirado et al., 2018). The soma profile of the complex neurons was drawn manually and then the selection was enlarged 0.5 µm in order to include the puncta closely surrounding the somata. The puncta were defined as having an area not smaller than 0.15

μm^2 for VGLUT1 and VGAT and 0.30 μm^2 for PV (circularity between 0.30 -1.00). The data were expressed as the number of immunoreactive puncta per micron of soma perimeter.

2.5.5 Nuclear and cellular density of EGFP+ neurons

To perform analyses of nuclear and cellular density, 4-7 sections per animal were selected from the following coordinates: Bregma -1.58 mm to -2.54 mm. Three single microphotographs with a single confocal plane, covering all the extension of the PCX layer II, were acquired using a 20x dry objective. The density of NeuN expressing nuclei and CAMK-II expressing cells was measured using 15 randomly located squares per section (50 x 50 μm) and results were expressed as the number of structures per μm^2.

2.6 Statistical analysis

All analyses were performed comparing the right (EndoN) with the left (vehicle) hemisphere. To avoid any bias in the analyses, the slides were coded and the code was not broken until the analyses were completed. Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software Inc.). After assessing the normality of the data via Shapiro-Wilk test, 2-tailed paired Student's *t*-test was applied.

STUDY 3. THE HUMAN CEREBRAL CORTEX LAYER II: A "*RESERVOIR*" OF IMMATURE NEURONS IN THE ADULT BRAIN

3.1 Human tissue collection

Post-mortem and post-operative neurosurgical specimens were collected for this study as described below.

Frozen, 14 µm-thick, coronal sections containing the ventral region of the temporal lobe were obtained from the Stanley Foundation Neuropathology Consortium. This collection consists of 60 individuals divided into 4 groups: a) normal control subjects (n = 15) and patients with b) bipolar disorder (n = 15), c) major depression without psychotic features (n = 15) and d) schizophrenia (n = 15) (Group demographic summaries can be found in **Table 3**). All brains underwent clinical neuropathological examination by 2 neuropathologists and none of them demonstrated evidence of neurodegenerative changes or other pathological lesions. Sections were unfrozen and fixed by immersion in 2.5% PFA solution in lysine-phosphate buffer pH 7.4 for 20 min at room temperature. The lysine-phosphate buffer was prepared 1:1 from a solution of PBS 0.1 M, and a solution 0.2 M lysine adjusted to pH 7.4 using a solution of Na_2HPO_4 0.1 M. The buffer was mixed with a concentrated solution of PFA 3:1 and 0.214 g of sodium periodate was added for each 100 ml just before use. After fixation, sections were washed in PB pH 7.4 and processed for immunohistochemistry. All the sections studied passed through the procedures simultaneously to minimise any difference arising from histochemical and immunohistochemical protocols.

Seventeen post-operative neurosurgical samples including tissue from the frontal, temporal, parietal and occipital lobes were collected from patients with epilepsy in accordance with the Ethical Committee for Biomedical Investigation, Hospital la Fe (2015/0447) and the University of Valencia Ethical Commission for Human Investigation (for clinical and demographical data see **Table 4**). After surgical resections samples were immediately immersed in 10% formalin and post-fixed at 4°C in fresh 0.1 M PB, pH 7.4. Twenty-four hours later, tissue was washed in 0.1 M PB under gentle shaking for 24h at 4°C. Coronal sections were sliced with a Leica VT1200s microtome at a thickness of 50 µm and kept until use in PB 0.1M with sodium azide 0.05% as preservative.

Table 3. Clinical and demographical data of the Stanley Neuropathology Consortium

	MD	BD	SCHZ	Controls
N	15	15	15	15
Age years, mean (SEM), (range)	46.5 (2.4), (30-65)	42.3 (3.0), (25-61)	42.3 (3.0), (25-61)	48.1 (2.8), (29-68)
Sex male/female	8/7	9/6	9/6	9/6
PMI, hours, mean (SEM), (range)	27.5 (2.8), (7-47)	32.5 (4.2), (13-62)	33.7 (3.8), (12-61)	23.7 (2.6), (8-42)
Tissue pH, mean (SEM), (range)	6.2 (0.06), (5.8-6.5)	6.2 (0.06), (5.8-6.5)	6.2 (0.07), (5.8-6.5)	6.3 (0.06), (5.8-6.5)
Suicide	7	9	4	0
Alcohol Use	5	7	5	2
Psychosis	0	11	15	0
Brain Weight, gr, mean (SEM), (range)	1462 (37), (1240-1740)	1441 (44), (1130-1690)	1471 (28), (1270-1640)	1501 (42),(13051840)
Duration, years, mean (SEM), (range)	12.7 (2.9), (1-42)	20.1 (2.5), (6-43)	21.3 (3.0), (5-45)	

(BD, bipolar disorder; MD, major depression; PMI, post mortem interval; SCHZ, schizophrenia).

Table 4. Clinical and demographical data of neurological samples.

N	Age	Gender	Clinical History	Brain Region
1	5	M	Epilepsy intraoperative resection	TL
2	5	F	Epilepsy intraoperative resection	FL
3	43	F	Epilepsy intraoperative resection	FL
4	48	M	Epilepsy intraoperative resection	TL
5	34	F	Epilepsy intraoperative resection	FL
6	25	F	Epilepsy intraoperative resection	FL
7	55	M	Epilepsy intraoperative resection	TL
8	62	F	Epilepsy intraoperative resection	TL
9	44	M	Epilepsy intraoperative resection	TL
10	42	F	Epilepsy intraoperative resection	TL
11	47	M	Epilepsy intraoperative resection	TL
12	28	M	Epilepsy intraoperative resection	TL
13	45	M	Epilepsy intraoperative resection	TL
14	37	M	Epilepsy intraoperative resection	TL
15	20	M	Epilepsy intraoperative resection	FL
16	46	M	Epilepsy intraoperative resection	PL
17	13	F	Epilepsy intraoperative resection	OL

(FL, frontal lobe; OL, occipital lobe; PL, parietal lobe; TL, temporal lobe).

3.2 Immunohistochemistry for conventional light microscopy

For conventional PSA-NCAM immunohistochemistry, *post-mortem* tissue samples were processed on slides, while post-operative tissue samples were processed "free-floating", as follows. All tissue sections (*post-mortem* and post-operative) were incubated 15 min in an antigen unmasking solution (0.01 M citrate buffer, pH 6) at 100 °C. After cooling down sections to room temperature, they were incubated with 3% H_2O_2 in PBS for 10 min to block endogenous peroxidase activity. After this, sections were treated for 1 h with 5% NDS (Jackson Laboratories) in PBS with 0.2% Triton-X100 (Sigma-Aldrich) and were incubated overnight at room temperature in anti-PSA-NCAM antibody (1:1400; Abcys, Paris, France) **(table 5)**. After washing, sections were incubated for 1h with donkey anti-mouse IgM biotinylated antibody (1:250; Jackson ImmunoResearch Laboratories) **(table 5)**, followed by an avidin-biotin-peroxidase complex (ABC; Vector Laboratories, Peterborough, UK) for 30 min in PBS. Colour development was achieved by incubating with 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and 0.033% hydrogen peroxide in PB for 4 min. PBS containing 0.2% Triton-X-100 and 3% NDS was used for primary and secondary antibodies dilution.

3.3 Immunohistochemistry for confocal microscopy

Fluorescence immunohistochemistry was performed as described above but omitting the endogenous peroxidase block. Samples were incubated for 48h at 4°C with a mixture of primary antibodies (**Table 5**). After washing, sections were incubated for 2h (room temperature) with a specific secondary antibody cocktail (**Table 5**). Thereafter, sections were washed in PB 0.1 M and mounted on slides in the case of the "free-floating" sections. To remove autofluorescence caused by aldehyde fixation samples were treated with Sudan Black B (SBB; Sigma-Aldrich) (Schnell et al., 1999; Oliveira et al., 2010). To this end, slides were incubated with a solution of 0.1% SBB in 70% ethanol for 20 minutes followed by 3 washes for 5 minutes in PBS with 0.02% Tween 20 (Sigma-Aldrich). Finally, all sections were washed in PB 0.1 M and coverslipped using DakoCytomamation fluorescent mounting medium (Dako).

Primary antibodies	*Anti*	*Host*	*Isotype*	*Dilution*	*Catalog number*	*Company*
	Ank-G	Mouse	IgG_1	1:200	SC12719	Santa Cruz
	CTIP2	Rat	IgG_{2A}	1:1000	Ab18465	Abcam
	CUX1	Mouse	IgG_1	1:100	Ab115854	Abcam
	D2r	Rabbit	IgG	1:200	AB5084P	Sigma-Aldrich
	DCX	Rabbit	IgG	1:500	4604S	Cell signaling
	DCX	Mouse	IgG_1	1:500	SC27139	Santa Cruz
	GAD67	Mouse	IgG_{2A}	1:1000	MAB5406	Sigma-Aldrich
	GFAP	Chicken	IgG	1:4000	Ab7260	Abcam
	IBA1	Rabbit	IgG	1:4000	Ab178846	Abcam
	NeuN	Mouse	IgG_1	1:1000	MAB377	Sigma-Aldrich
	PSA-NCAM	Mouse	IgM	1:1400	AbC0019	Abcys
	PSA-NCAM	Mouse	IgM	1:1400	MAB5324	Sigma-Aldrich
	TBR1	Rabbit	IgG	1:1000	Ab31940	Abcam
	TH	Mouse	IgG_1	1:200	MAB318	Sigma-Aldrich
	VGLUT1	Guinea pig	IgG	1:1000	AB5905	Sigma-Aldrich

Secondary antibodies	*Anti*	*Host*	*Conjugate*	*Dilution*	*Catalog number*	*Company*
	Chicken-IgY	Goat	AF555	1:400	A32932	ThermoFisher
	Chicken-IgY	Goat	CF488	1:400	20166	Biotium
	Mouse-IgG_1	Goat	CF633	1:400	20250	Biotium
	Mouse-IgG_1	Goat	AF555	1:400	A21127	ThermoFisher
	Mouse-IgG_{2A}	Goat	AF555	1:400	A21137	ThermoFisher
	Mouse-IgM	Goat	AF488	1:400	A21042	ThermoFisher
	Mouse-IgM	Goat	Biotin	1:400	A2338587	Jackson ImmunoResearch
	Rabbit-IgG	Goat	AF555	1:400	A32732	ThermoFisher
	Rabbit-IgG	Donkey	CF555	1:400	20038	Biotium
	Rat-IgG_{2A}	Goat	CF647	1:400	20843	Biotium

Table 5. Primary and secondary antibodies used in the study 3.

3.4 Antibody characterization

All antibodies used in this study were previously validated by the manufacturers and extensively used by other researchers on rodent and human brain tissue (manufacturer's information). Additionally, I have tested each antibody in 4% PFA fixed rodent brain slices before conducting the experiments. They give a regional and cellular immunolabeling distribution comparable to that of their respective antigens with the same or other equally selective antisera. Additionally, in order to confirm that some of the immunostaining was not produced by the secondary antibodies or by the

immunocytochemical protocol, I have omitted primary antibodies or have substituted them by NDS. These controls resulted in a complete absence of immunostaining in every case. Below there is a detailed description of the antibody characterization. In the case of the anti-DCX and anti-PSA-NCAM antibodies I have been able to run additional controls for their specificity.

The anti-PSA-NCAM antibody is generated against rat embryonic spinal cord membranes. The specificity of immunostaining in my tissues was tested in 4 different ways, as described before (Gómez-Climent et al., 2008): (a) three different anti-PSA antibodies were used, and they rendered exactly the same pattern of immunostaining; (b) pretreatment of the antibody with a-2,8-linked sialic polymer (colominic acid, Sigma) overnight, or the primary antibody omission during the IHC prevented all the labelling in the rat piriform cortex layer II and in the human cortex; (c) PSA immunohistochemistry of sections from animals injected intracerebrally with EndoN resulted in a complete absence of PSA immunostaining in the area affected by the injection and (d) immunostaining was absent from adult CNS tissue of St8SiaII/St8SiaIV double knockout mice (KO).

The DCX antibodies detect endogenous levels of the protein DCX. It is generated against a synthetic peptide corresponding to human DCX. Antibodies are purified by protein A and peptide affinity chromatography. The staining pattern seen in this study using the DCX-rabbit polyclonal antibody (4604S; Cell signaling) is consistent with other studies in human tissue (Liu et al., 2008, 2018; Sorrells et al., 2019). Antibody specific signal was proved using either DCX-KO and the DCX-mouse monoclonal antibody (SC27139; Santa Cruz). In both cases antibodies showed immunopositive labeled and overlap of expression. The polyclonal antibody showed the most extensive labelling of cells and processes, for this reason it was used across all samples for the descriptive and quantitative analysis.

The CTIP2 protein is a reported synonym for the human gene BCL11B, encoding BAF chromatin remodeling complex subunit BCL11B. The full-length protein is 95.519 Daltons in mass with 2 identified isoforms. When used in WB, this monoclonal antibody recognizes 2 bands representing CTIP2 at about 120kDa (manufacturer's datasheet). The CTIP2 antibody has been validated for its use in human with more than 50 citations (manufacturer's information).

The CUX1 antibody was raised against recombinant fragment corresponding to aa 521-621 of Human CUTL1. This monoclonal antibody reacts with human

(manufacturer's information) and recognizes a single band of predicted molecular weight of 164 kDa on WB (manufacturer's datasheet).

The D2r antibody is raised against a 28-aa fragment (284-311) within the cytoplasmic third loop (aa 284-311) of human D2r (Hersch et al., 1995). The immunogen peptide has no significant homology with other subtypes of dopamine receptors (D1, D3-D5), and the antiserum recognizes both short and long forms of D2r (Boundy et al., 1993). The specificity of this antibody has been demonstrated by WB on human brain lysate that revealed 3 bands between 70 and ~100 kDa, as well as by IHC and IP (Stojanovic et al., 2017).

The Ank-G monoclonal antibody is raised against a synthetic peptide derived from the spectrin-binding domain of Ank-G human origin. The antibody recognized 2 bands, at 480 kDa and 270 kDa, which are thought to correspond to the 2 forms of ankyrin on WB of Caco 2 (human colonic carcinoma cell line) whole-cell lysates (manufacturer's data sheet). It is recommended for detection of Ank-G of mouse, rat and human origin by WB, IHC, IP (manufacturer's information).The staining pattern in the current study is consistent with other studies in human (Tian et al., 2014; Szegedi et al., 2020).

The GAD67 antibody clone 1G10.2, reacts with the 67kDa isoform of glutamate decarboxylase of rat, mouse and human origins. According to the manufacturers, no detectable cross reactivity with GAD65 have been found by WB on rat brain lysate when compared to blots probed with the antibody AB1511 (Merck-Millipore) that reacts with both GAD65 & GAD67 isoforms. It has been validated for its use in IHC, IP, and WB for the detection of GAD67 and has more than 75 product citations, including some in human (manufacturer's information). The staining pattern in the current study is consistent with other studies in human (Fish et al., 2008; Neman et al., 2014).

The GFAP antibody was generated against a recombinant full-length protein corresponding to human GFAP. Isotype 1 expressed in and purified from E. coli. It recognizes a band of approximately 55.48 kDa on WB (manufacturer's datasheet). The GFAP was validated in human with more than 50 citations (manufacturer's information).

The IBA1 antibody has been generated against a synthetic peptide corresponding to aa 100 to the C-terminus from mouse. It recognizes a band of approximately 10-15 kDa (predicted molecular weight: 17 KDa) (manufacturer's datasheet). The use of this antibody in human brain tissue was previously described by (Mendes et al., 2018).

The NeuN monoclonal antibody is routinely evaluated by manufacturers by means of IHC on brain tissue (avian, chicken, mouse, rat and human) and its use have been

validated by published studies for ICC, IHC, IP, including some in human and WB (manufacturer's information). By WB analysis this antibody recognized 2-3 bands in the 46-48 kDa range and possibly another band at approximately 66 kDa. The staining pattern in the current study is consistent with other studies in human tissue (Talbot et al., 2004; Martí-Mengual et al., 2013).

The TH antibody is raised against purified TH from the PC 12 pheochromocytoma cell line; this antibody recognizes a protein of approximately 59-61 kDa by WB. The clone LNC1 has been validated by other researchers (more than 700 product citations) for its use in IHC and show reactivity in a wide range of species, including rat, mice and human (Heaton et al., 2012; Henry et al., 2012; Chaudhry and Ahmed, 2013; Ray et al., 2014).

The TBR1antibody has been generated against a synthetic peptide corresponding to aa 50-150 conjugated to keyhole limpet hemocyanin from mouse. When used in WB, it recognizes a single band of approximately 74 KDa (predicted molecular weight: 74 KDa). This antibody has been validated by IHC for its use in human tissue (manufacturer's information).

The VGLUT1 antibody is generated against a synthetic peptide from rat VGLUT1 protein with no overlap to VGLUT2. WB reveal a single band at approximately 60 kDa (Melone et al., 2005). Moreover, pre-absorption of VGLUT1 antibody with immunogen peptide eliminates all immunostaining (manufacturer's product information). VGLUT1 immunopositive puncta were also abolished in sections from VGLUT1-KO animals (Siembab et al., 2016). This antibody has been used in previous studies in human brain tissue (Talbot et al., 2004; Alonso-Nanclares and Defelipe, 2005).

3.5 Observation and quantification of labelled cells

A Nissl stain with toluidine blue in alternate series of sections was used to determine the boundaries and layers of the cerebral cortex. I used conventional light microscopy to evaluate the presence of PSA-NCAM expressing cells in layer II. Five sections per subject were randomly selected and observed with an Olympus CX41 microscope under bright-field illumination at 40x magnification.

For the characterization of the neurochemical phenotype of cells in layer II, coronal sections were double or triple labelled for fluorescence immunohistochemistry and observed under a confocal microscope (Leica TCS-SPE). Z-series of optical sections

(1µm apart) covering the whole thickness of the histological sections were obtained using sequential scanning mode. Photographs were taken at 20x, 40x or 60x magnification and in some cases were digitally zoomed at 0.5x to 2x. All images were processed with FIJI/ImageJ software (Schindelin et al., 2012). Twenty-five cells, in a minimum of 5 sections per subject, were randomly selected and manually counted to determine the percentage of colocalization between the different markers. GraphPad Prism 9 software (GraphPad Software Inc.) was used to create graphs and to determine mean ± SEM.

3.6 Lithium-pilocarpine epilepsy model in rat

3.6.1 Animals

For the lithium-pilocarpine model, 8 adult male Sprague-Dawley rats (3 months old, 250-300 g; Charles River Laboratories, MA, USA) were used (**Figure 7**). The rats were housed per groups in a standard environment (12h light/dark cycle) with ad *libitium* access to food and water.

All animal experimentation was conducted in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and was approved by the Committee on Bioethics of the University of Valencia. Every effort was made to minimize the number of animals used and their suffering.

Rats received pilocarpine (30 mg/Kg, s.c; Sigma-Aldrich)(treated, n=4) or saline (control, n=4) 6 h later the administration of the lithium chloride (3 mEq/Kg, s.c; Sigma-Aldrich). The onset of fulminant status epilepticus occurred 15-30 min after pilocarpine administration. Animals were then administered diazepam (10 mg/kg s.c.; Henry Schein, NY, USA) approximately 90 min after seizures onset. After 72 hours, all animals were perfused transcardially under deep chloral hydrate anesthesia, first with saline and then with 4% PFA in PBS 0.1 M, pH 7.4. Thirty minutes after perfusion, the brains were extracted from the skull and cut in coronal 50 µm-tick sections using a vibratome (Leica VT 1000E). Slices were collected in 10 subseries and stored in 0.1 MPB with sodium azide 0.05% as preservative at 4°C. They were used for conventional PSA-NCAM immunohistochemistry. Sections were processed “free-floating” using the ABC method as described above for the human samples (**Figure 7**).

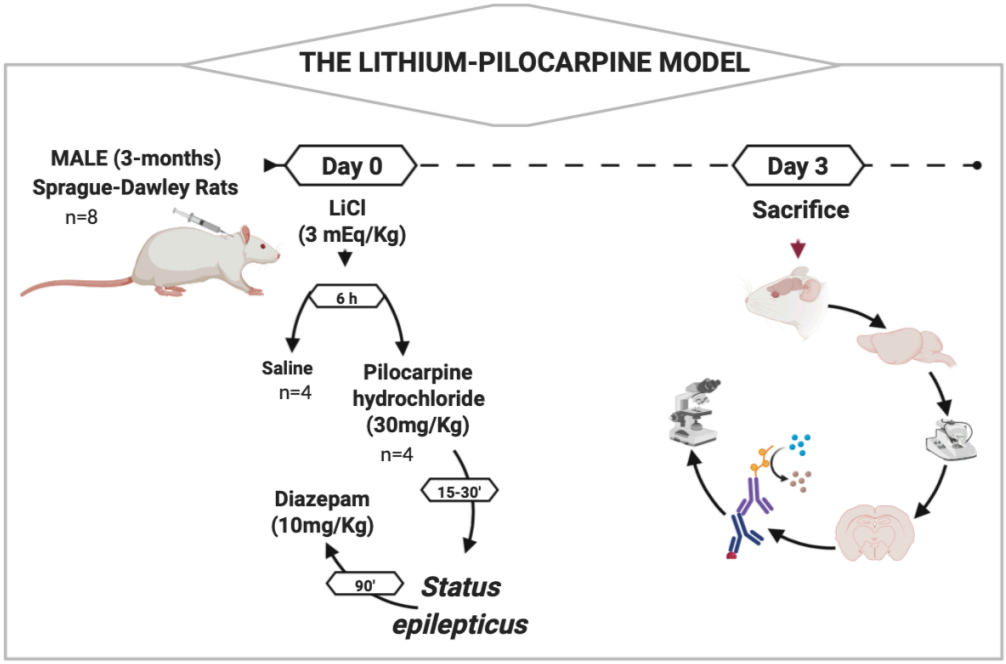

Figure 7. Experimental protocol used to induce epilepsy in rat in the study 3. (Created in BioRender.com)

3.6.2 Quantification

In order to study the density of PSA-NCAM expressing cells and processes in the rat PCX, 2 sections per animal were randomly selected into the Bregma interval: -1.40 mm; -3.60 mm (Herman and Watson, 1987). Cell counting was performed manually, using an Olympus CX41 microscope and results were expressed as density of immunopositive cells per mm^2. All slides were coded prior to analysis and the codes were not broken until the experiment was finished.

3.6.3 Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software Inc.). Mean ± SEM was determined and the resulting values were analyzed by unpaired Student's *t*- tests after checking the normality and homoscedasticity of the data.

Chapter 4

RESULTS

STUDY 1. EFFECTS OF DOPAMINE ON THE IMMATURE NEURONS OF THE ADULT RAT PIRIFORM CORTEX

1.1 Relationship between PSA-NCAM immature neurons and dopaminergic fibers in the rat piriform cortex layer II

I have analyzed whether TH immunoreactive fibers were closely apposed to PSA-NCAM immunoreactive neurons in the rat PCX layer II, using double immunostaining with antibodies against these 2 markers. Fibers containing TH were sparse in the three-layered PCX, but many of them were observed in close apposition to PSA-NCAM expressing neurons in layer II **(Figures 8A & A1-3)**. Since TH can also be found in noradrenergic axons, I have analyzed the co-expression of TH and DAT in fibers closely apposed to PSA-NCAM immunoreactive neurons, confirming their dopaminergic phenotype **(Figures 8B & B1-3)**.

1.2 Dopamine D2 receptors expression in PSA-NCAM immunoreactive neurons

Using double immunohistochemistry for PSA-NCAM and D2r, I have also analyzed whether PSA-NCAM immunoreactive neurons in the rat PCX layer II co-expressed this receptor. Interestingly, I found that 55% of these cells co-expressed D2r. The detailed analysis of its expression showed that D2r immunoreactivity was predominantly localized in the periphery of the somata, in the same compartment where PSA-NCAM immunoreactivity can be found **(Figures 8 C1-3)**.

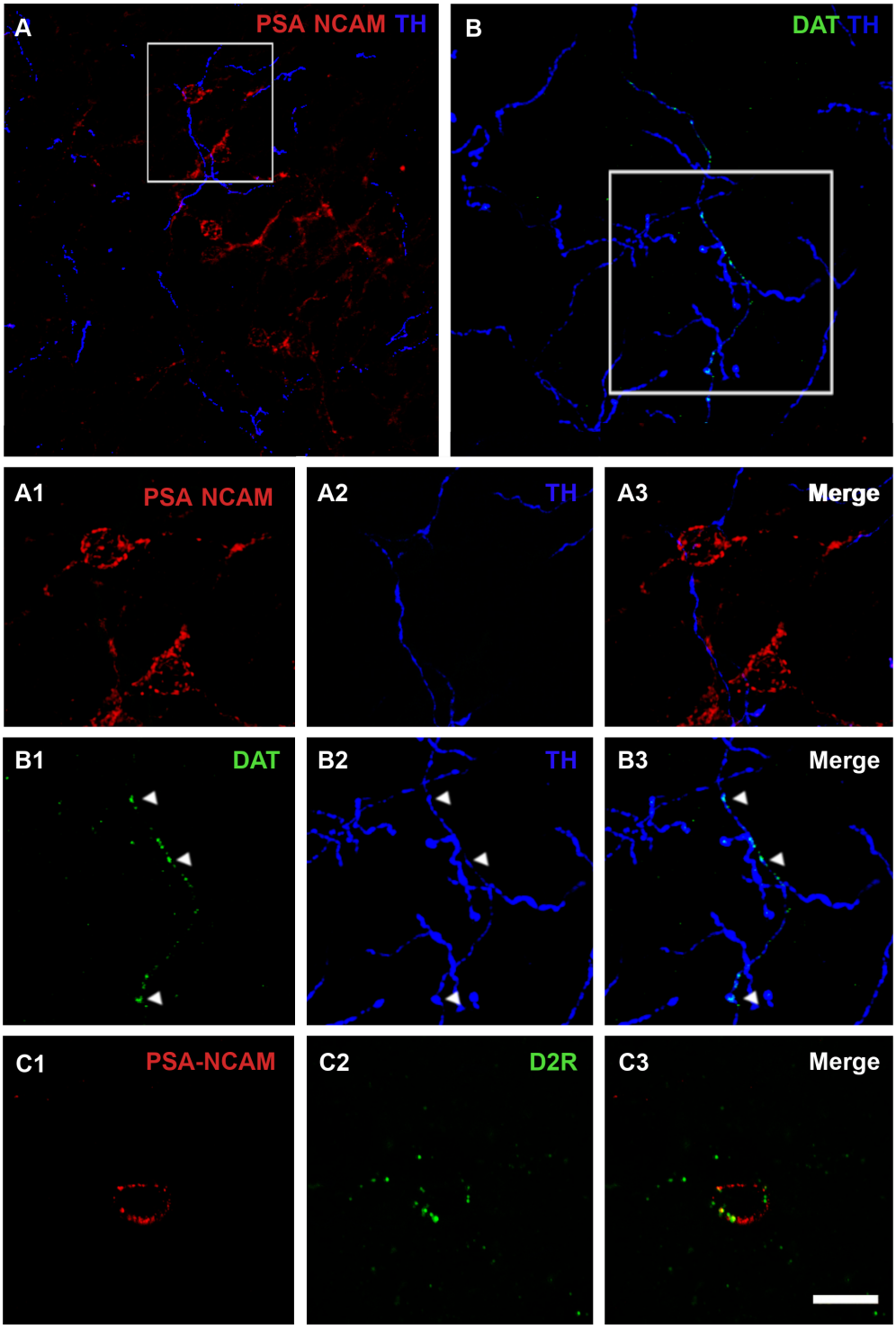

Figure 8. Confocal microscopic analysis of PSA-NCAM immunoreactive neurons and dopaminergic fibers in the piriform cortex layer II. (A) Double PSA-NCAM/TH immunohistochemistry. Note the TH immunoreactive fibers surrounding and contacting PSA-NCAM-expressing cells. In **(A1-A3)**, a detailed view of the magnification of the squared area in A can be observed. **(B)** Double DAT/TH immunohistochemistry. Observe the expression of DAT on TH fibers, squared area in B, and arrowheads in **(B1-B3)** images. **(C1)** Neuron expressing PSA-NCAM in the piriform cortex layer II. In **(C1-C3)** note the presence of D2r expression on PSA-NCAM-expressing cells. All the images in this figure are 2D projections of 25 consecutive confocal planes located 0.5 µm apart. Scale bars: 50 µm for **(A)**; 30 µm for **(B)**; 10 µm for **(C)**, and 15 µm for **(A1-A3, B1-B3)**.

1.3 Effects of chronic treatments with D2 receptors antagonists and agonists

I have next examined whether the pharmacological modulation of dopaminergic neurotransmission, using D2r antagonists and agonists, influenced the density of immature neurons expressing PSA-NCAM within the rat PCX layer II.

1.3.1 Density of PSA-NCAM immunoreactive cells after chronic haloperidol treatment

Twenty-six days of chronic haloperidol treatment induced an increase in the density of PSA-NCAM expressing cells in the rat PCX layer II **(Figures 9A & B)**. This increase was statistically significant (p=0.028) **(Figure 9E)**.

1.3.2 Density of PSA-NCAM immunoreactive cells after chronic PPHT treatment

Seven days of chronic PPHT treatment produced a decrease in the density of PSA-NCAM expressing cells in the rat PCX layer II **(Figures 9C & D)**. This reduction was statistically significant (p=0.015) **(Figure 9F)**.

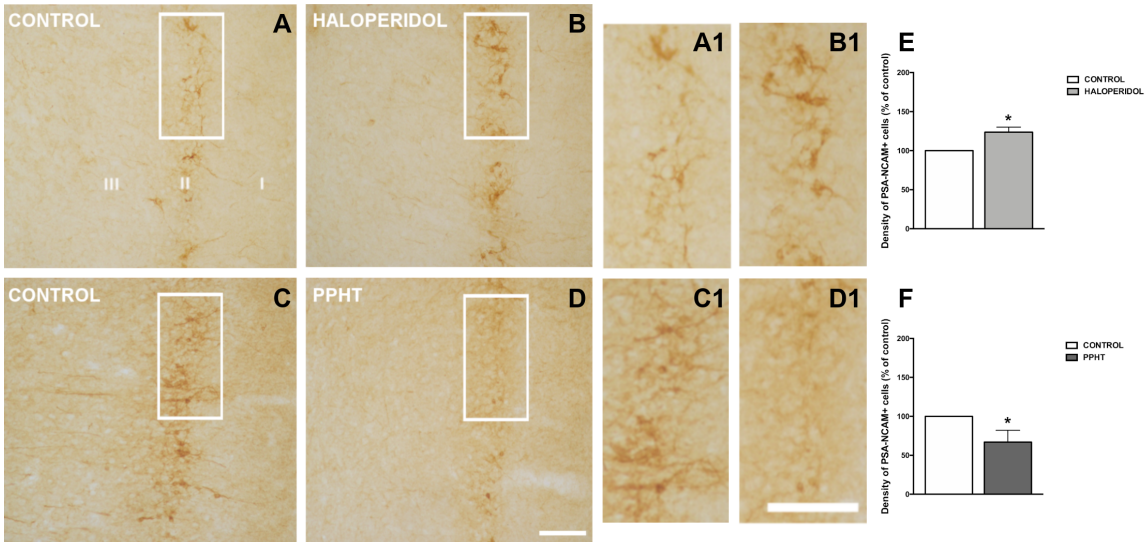

Figure 9. PSA-NCAM-expressing cells in the rat piriform cortex. Microphotographs showing PSA-NCAM immunostaining in control **(A & C)** and after chronic treatment with haloperidol **(B)** and PPHT **(D)**. **(A1-D1)** are higher magnifications of the squared areas in **(A-D)**. **(E & F)** Graphs showing changes in the density of PSA-NCAM-expressing cells in the piriform cortex layer II. Asterisks indicate statistically significant differences from the control group after unpaired Student's *t*-test (**p* < 0.05). Scale bars: 200 µm for **(A-D)**; 100 µm for **(A1-D1)**.

STUDY 2. PSA DEPLETION INDUCES THE DIFFERENTIATION OF IMMATURE NEURONS IN THE PIRIFORM CORTEX OF ADULT MICE

2.1 EndoN efficiently depletes PSA from the piriform cortex

Intracerebral injections of the enzyme Endo-N produced a clear depletion of PSA-NCAM. Two weeks after the injection of the enzyme in the ectorhinal cortex of the right hemisphere, the depletion covered all the extension of the PCX from -1.58 mm to 2.54 mm Bregma coordinates (Franklin and Paxinos, 2008) **(Figure 10)**.

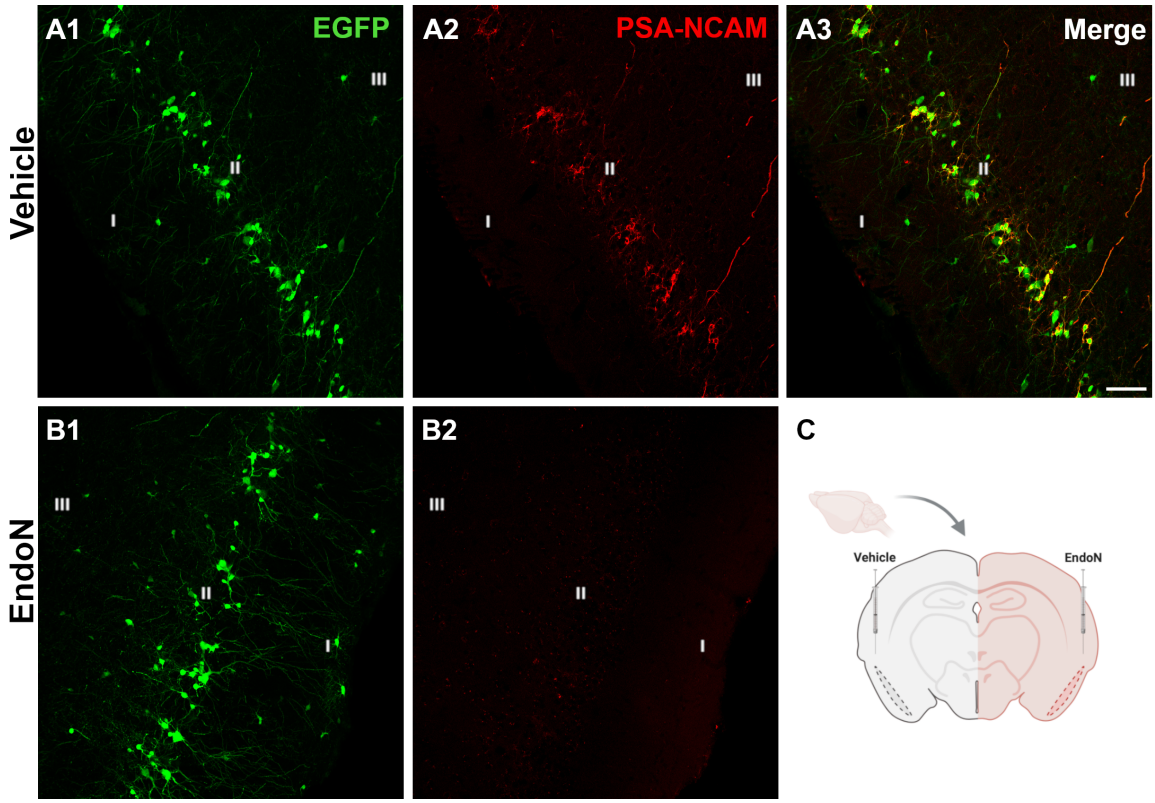

Figure 10. **Removal of PSA from the PCX by Endo-N. (A & B)** Confocal microscopic images of EGFP (green) and PSA-NCAM (red) immunoreactive neurons in the vehicle **(A1-3)** and EndoN **(B1-2)** injected hemispheres. Note the complete depletion of PSA-NCAM expression in the hemisphere injected with EndoN. **(C)** Drawing showing the hemispheres injected with vehicle (left) or EndoN (right). Images are single confocal planes. Scale bar: 70 µm.

2.2 Classification of EGFP+ neurons and impact of PSA depletion in the piriform cortex layer II

In the PCX layer II EGFP+ neurons were classified into tangled and complex cells as described in previous studies (Rubio et al., 2016; Rotheneichner et al., 2018). In addition, they were divided in 5 subgroups according to their soma diameter and maturational stage: a: small tangled cells (ø ≤ 5µm) b: tangled cells with short dendritic arborizations

(ø = 6-7µm); c: tangled cells with longer dendritic arborizations (ø = 8-10µm); d: larger complex cells with thin basal processes and dendritic spines (ø = 11-13µm) e: mature complex cells with larger dendrites and mature dendritic spines (ø ≥ 14µm) **(Figures 11 A & B)**.

In order to study the impact of PSA depletion on these cell subpopulations I analyzed their percentages in the vehicle and EndoN hemispheres. As expected, tangled cells were more abundant than complex cells in both hemispheres and no significant differences were observed in their percentages between hemispheres **(Figure 11C)**. Next, I analyzed the percentage **(Figure 11D)** and density **(Figure 11E)** of tangled and complex cells considering their diameter and maturational stage, comparing both hemispheres. Statistically significant decreases were observed in the percentage (p=0.02, **Figure 11D3**) and density (p=0.03, **Figure 11E3**) of tangled cells with 8-10 µm diameter and in the density of complex cells with diameters larger than 14 µm (p=0.009, **Figure 11E5**) in the EndoN hemisphere.

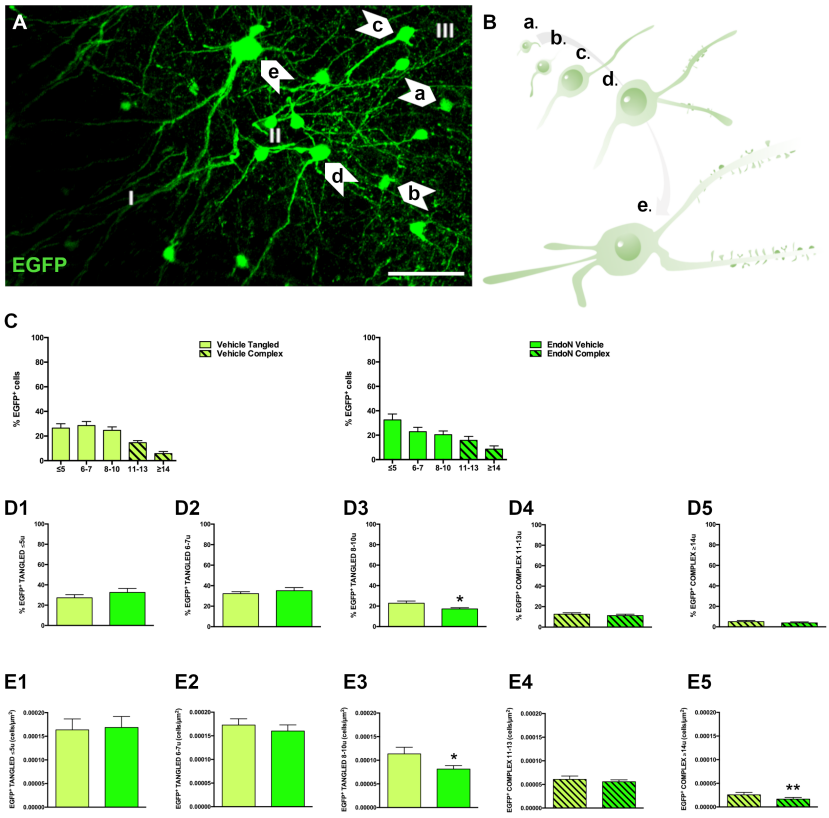

Figure 11. Classification of EGFP+ neurons and impact of PSA depletion. (A) Single confocal section showing the distribution of EGFP+ cells in the PCX layer II. Note the different subtypes of cells indicated by arrows. **(B)** Schematic representation showing the different maturational stages of EGFP+ cells: a: ≤5µm; b: 6-7µm; c: 8-10µm; d:11-13µm; e: ≥ 14µm **(C)** Graphs showing the percentage of EGFP+ neurons classified by their diameter and their maturational stage in the vehicle and EndoN hemispheres. **(D1-5 & E1-5)** Graphs representing the percentage and density of the different types and maturational stages of EGFP+ neurons in the PCX layer II. Error bars represent the mean ± SEM and asterisks in bars indicate statistically significant differences between hemispheres (Vehicle x EndoN) after paired Student's *t*-test (*p< 0.05, ** p<0.01). Scale bar: 100 µm.

Then, to understand whether PSA depletion could promote the differentiation of EGFP+ cells, through an increase in the density of cells displaying an AIS, I investigated the expression of Ank-G on EGFP+ complex cells. **(Figure 12A-B)**. Interestingly, I found a significantly higher density of complex cells expressing Ank-G in the EndoN hemisphere (p=0.04, **Figure 12C**).

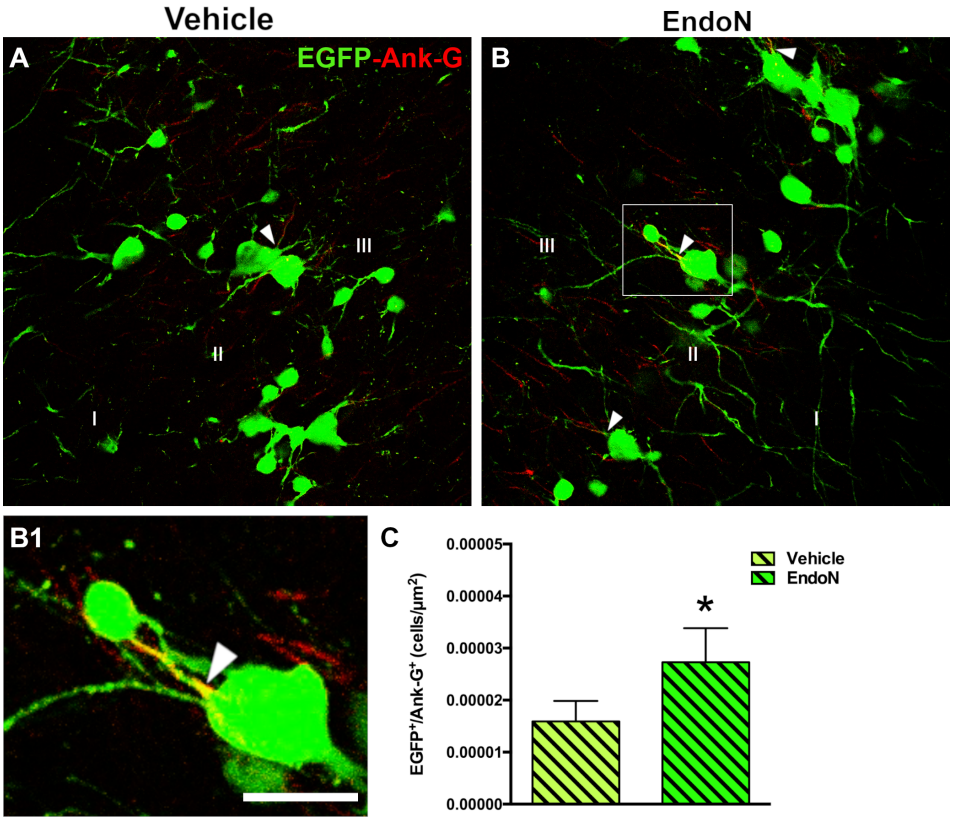

Figure 12. **Effects of Endo-N injection on the density of EGFP+ complex cells displaying an AIS. (A & B)** Panoramic views of the PCX layer II with EGFP+ cells (green) showing axon initial segments (AIS) (red) identified by the expression of the protein AnK-G. Note the presence of a larger number of EGFP+ complex cells displaying an AIS in the EndoN hemisphere. **(B1)** Higher magnification of the squared area in B. The AIS is indicated by an arrowhead. **(C)** Graph showing a higher density of EGFP+ complex cells expressing AnK-G in the EndoN hemisphere. Error bars represent the mean ± SEM and asterisks in bars indicate statistically significant differences between hemispheres (Vehicle x EndoN) after paired Student's *t*-test (*p< 0.05). Images are single confocal planes. Scale bar 20 µm for **(A & B)**; 55 µm for **(B1)**.

2.3 Effect of PSA depletion on the density of EGFP+ dendrites and dendritic spines in the piriform cortex layer II

In order to know whether the development of dendrites and dendritic spines of EGFP+ complex cells in the layer I of the PCX was affected by PSA depletion, I analyzed their density, comparing both hemispheres. PSA depletion resulted in a significantly higher density of EGFP+ dendrites in the PCX layer I in the right (EndoN) hemisphere (p=0.001, **Figure 13)**.

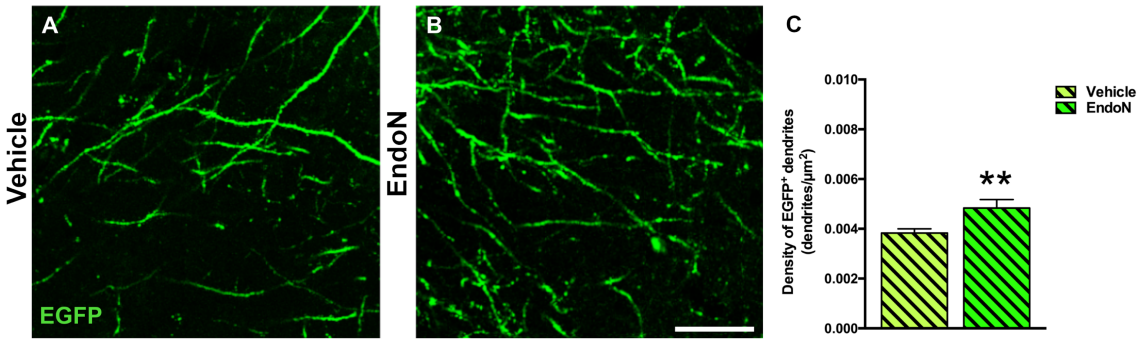

Figure 13. **Confocal microscopic analysis of the density of EGFP+ dendrites in the PCX layer I. (A & B)** Single confocal plane showing dendritic processes in the vehicle **(A)** and EndoN **(B)** hemispheres. **(C)** Graph showing a higher density of dendrites in the EndoN versus the vehicle hemisphere after Endo-N injection. Error bars represent the mean ± SEM and asterisks in bars indicate statistically significant differences between hemispheres (Vehicle x EndoN) after paired Student's *t*-test (** $p<0.01$). Scale bar: 10 µm.

Next, I analyzed the number of spines in 50 µm dendritic segments and I observed a significant increase in the EndoN hemisphere (p=0.03, **Figures 14A-C**). However, when considering specifically the different type of spines (mushroom, stubby and thin), no statistically significant changes were detected (data not shown).

To explore the connectivity and degree of maturation of the dendritic spines of EGFP+ neurons, I also analyzed the presence of closely apposed puncta expressing the presynaptic markers SYN and VGLUT1 **(Figures 14D-F)**. No statistically significant differences were found when considering the percentage of EGFP+ spines lacking SYN/VGLUT1 expressing puncta in their vicinity, although a tendency towards a decrease was detected in the EndoN hemisphere. By contrast, when considering the percentage of dendritic spines associated to puncta expressing only SYN or expressing both SYN and VGLUT1, I found tendencies towards a higher percentage in the EndoN hemisphere. No differences between hemispheres were found in the percentage of spines apposed to puncta expressing VGLUT1 exclusively.

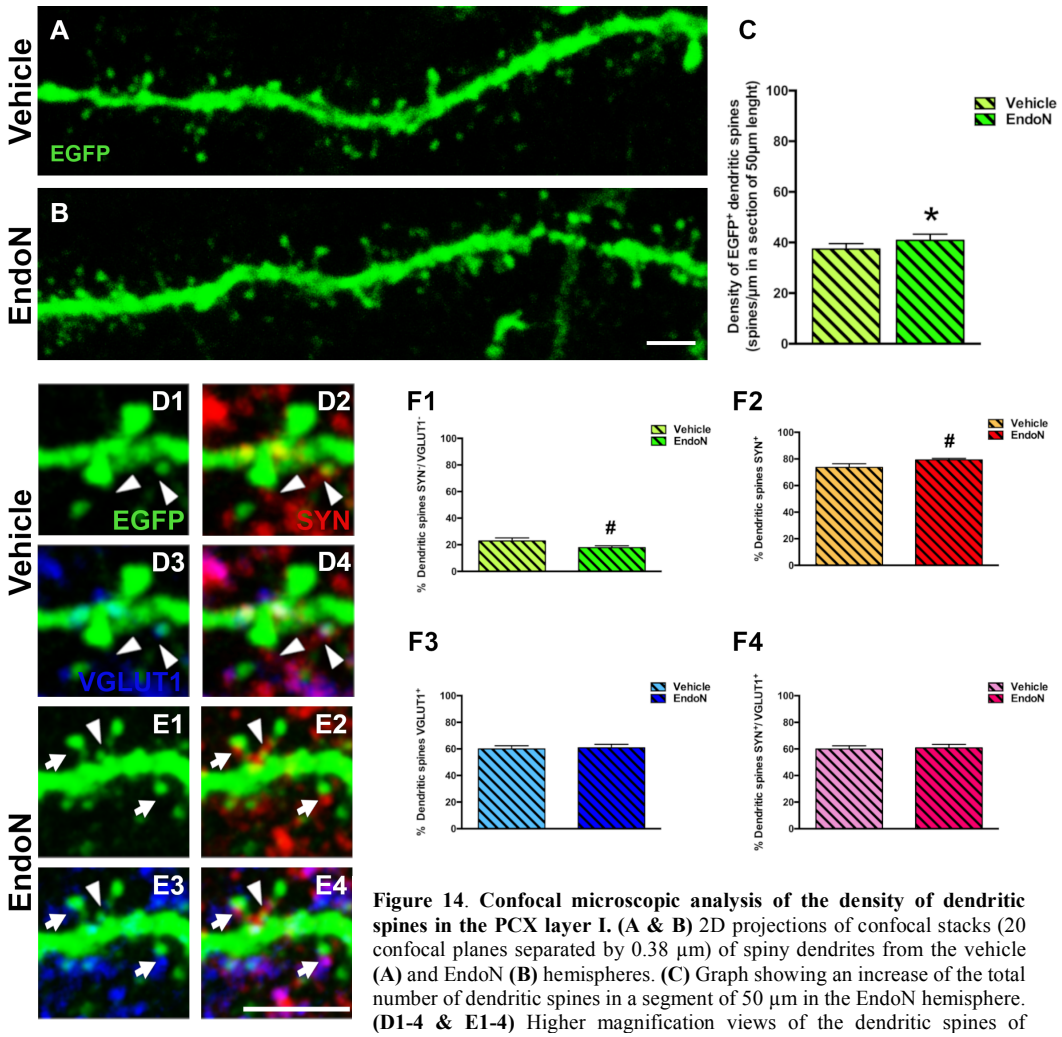

Figure 14. **Confocal microscopic analysis of the density of dendritic spines in the PCX layer I. (A & B)** 2D projections of confocal stacks (20 confocal planes separated by 0.38 µm) of spiny dendrites from the vehicle **(A)** and EndoN **(B)** hemispheres. **(C)** Graph showing an increase of the total number of dendritic spines in a segment of 50 µm in the EndoN hemisphere. **(D1-4 & E1-4)** Higher magnification views of the dendritic spines of cells in close apposition to puncta expressing the presynaptic markers SYN (red) and VGLUT1 (blue) on their surface. Note the presence of a larger number of puncta expressing SYN (arrowheads) or SYN-VGLUT1(arrows) in the EndoN hemisphere **(E1-4)** when compared to the vehicle hemisphere **(D1-4)**. **(F1-4)** Graphs showing the percentage puncta expressing synaptic markers apposed to EGFP+ spines. Error bars represent the mean ± SEM and asterisks in bars indicate statistically significant differences between hemispheres (Vehicle x EndoN) after paired Student's *t*-test (*p< 0.05; 0.05< #p <0.1). Scale bar: 5 µm for **(B)**; 15 µm for **(E4)**.

2.4 The enzymatic depletion of PSA does not change the density of excitatory and inhibitory puncta in the perisomatic region of EGFP+ cells

In order to investigate whether EGFP+ complex cells receive synaptic inputs in their perisomatic region and to investigate the nature of these afferences, I studied the presence and density of VGLUT1+ and VGAT+ puncta, which are markers of glutamatergic and GABAergic terminals, respectively and of PV+ puncta belonging to basket cells **(Figures 15A-D)**. In both hemispheres I found VGLUT1+, VGAT+ and PV+ puncta in the perisomatic region of EGFP+ complex cells. However, when I studied their density, I did not find statistically significant differences between hemispheres **(Figures 15E1-G1)**. Similarly, statistically significant differences were not found in the density of

puncta co-expressing VGAT and PV **(Figure 15H)**. In addition, I have analyzed the fluorescence intensity of perisomatic puncta immunoreactive for VGLUT1, VGAT and PV **(Figures 15E2-G2)** and only found a tendency toward an increase in VGAT+ puncta in the EndoN hemisphere (p=0.10).

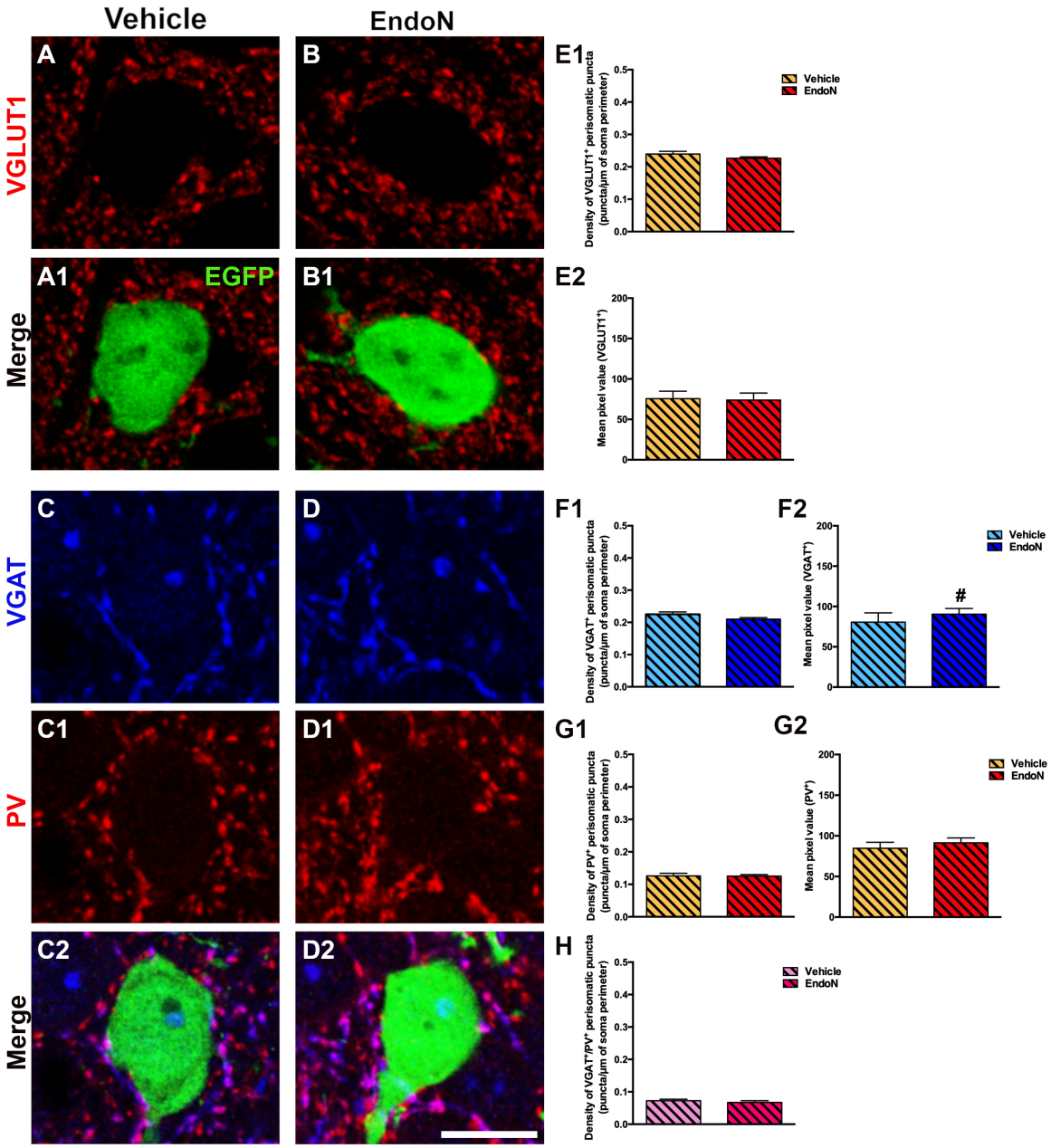

Figure 15. **Effects of Endo-N injection on the density and on the fluorescence intensity of excitatory and inhibitory puncta in the perisomatic region of EGFP+ complex cells in the PCX layer II. (A-D)** 2D projection of a single confocal stack showing VGLUT1+ (red), VGAT+ (blue) and PV+ (red) puncta in the perisomatic region of EGFP+ complex cells (green) in the vehicle **(A & C)** and EndoN **(B & D)** injected hemispheres. **(E1-2;F1-2;G1-2)** Graphs representing the density and the fluorescence intensity of VGLUT1+ **(E)**, VGAT+ **(F)** and PV+ **(G)** puncta in the perisomatic region of EGFP+ complex cells. **(H)** Graph representing the density of puncta coexpressing VGAT and PV in the perisomatic region of EGFP+ complex cells. Error bars represent the mean ± SEM. Symbol in bars indicate tendency toward an increase between hemispheres (Vehicle x EndoN) after paired Student's *t*-test ($0.05 < {}^{\#}p < 0.1$). Scale bar: 10 µm.

2.5 Effects of PSA depletion on phenotypic markers in EGFP+ cells in the piriform cortex layer II

2.5.1 PSA depletion increases the density of NeuN immunoreactive nuclei in the piriform cortex layer II

In order to know whether the depletion of PSA had an impact on the differentiation of EGFP+ neurons in the PCX layer II, I performed an immunohistochemical staining against NeuN, a marker of mature neurons (Mullen et al., 1992) and against CAMK-II a protein exclusively found in fully differentiated excitatory neurons (Wayman et al., 2008). I found a significantly higher density (p= 0.007) of NeuN immunoreactive nuclei in the EndoN versus the vehicle hemisphere **(Figure 16)**. In contrast, no statistically significant changes were observed in the density of CAMK-II expressing cells (data not shown).

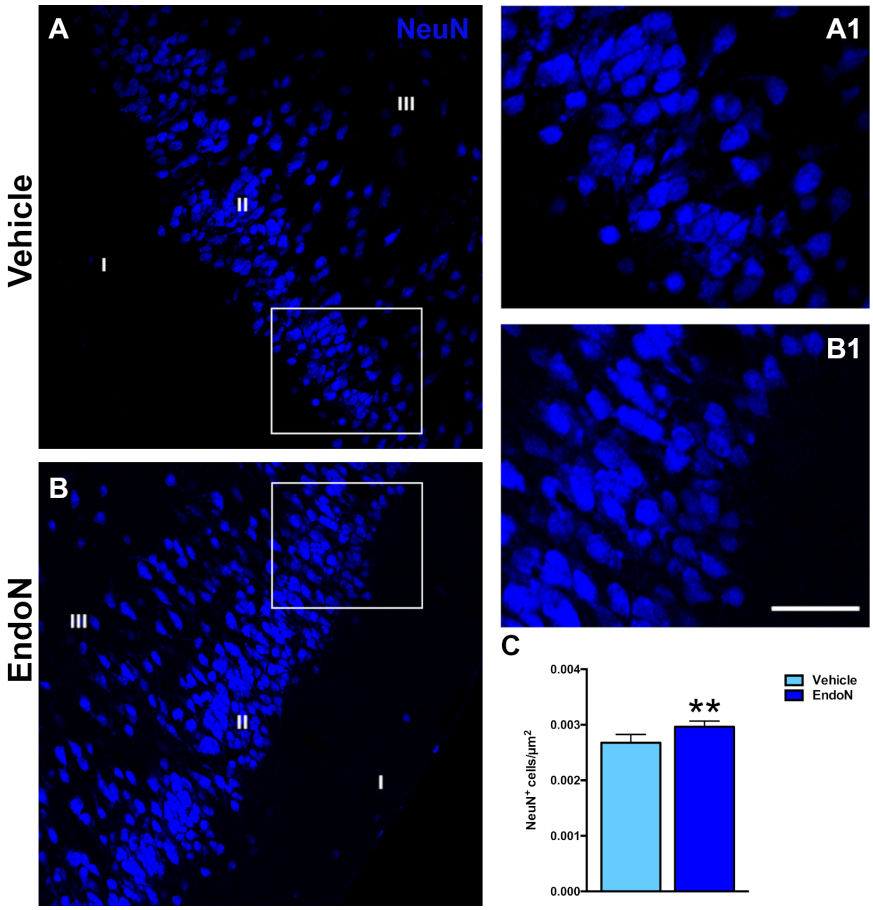

Figure 16. **Effects of Endo-N injection on the density of NeuN expressing nuclei. (A & B)** Single confocal sections showing the expression of NeuN in the PCX. **(A1& B1)** Higher magnifications of the squared areas in A and B, which show the different density of NeuN immunoreactive nuclei in the PCX layer II. **(C)** Graph showing the effect of EndoN injection on the density of NeuN immunoreactive nuclei in the PCX layer II. Error bars represent the mean ± SEM and asterisks in bars indicate statistically significant differences between hemispheres (Vehicle x EndoN) after paired Student's *t*-test (** p<0.01). Scale bar: 70 µm for **(A & B)**, 120 µm for **(A1 & B1)**.

2.5.2 *EndoN injection increases the percentage and density of EGFP+ cells displaying NeuN immunoreactive nuclei*

Immunohistochemical staining with NeuN was performed to study whether the EndoN injection could accelerate the final stages of development of EGFP+ cells. Many EGFP+ complex cells displaying a NeuN+ were found in both hemispheres **(Figures 17A & B)**. Comparing the vehicle and EndoN hemispheres, I found statistically significant differences in the percentage of EGFP/NeuN double labelled complex cells with 11-13µm diameter **(Figure 17C3**; p=0.05) and in the density **(Figure 17D1**; p=0.01) of tangled cells with 6-7µm soma diameter. The EGFP+ cells with ≤5µm diameter did not display NeuN immunoreactive nuclei, therefore corresponding graphs are not shown in the figure.

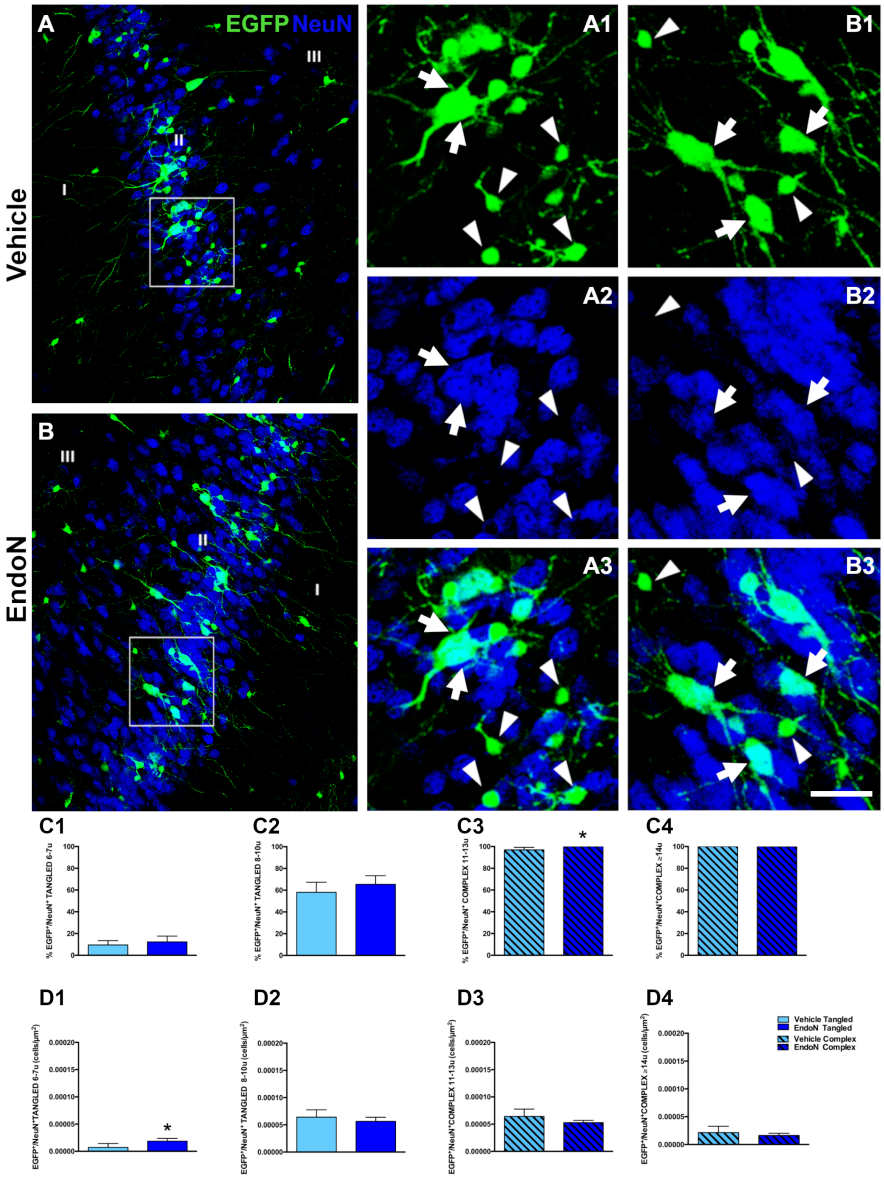

Figure 17. Effects of Endo-N injection on the percentage and density of EGFP+ cells displaying NeuN+ nuclei. (A & B) Single confocal sections showing EGFP+ cells (green) and NeuN expressing nuclei (blue). **(A1-3 & B1-3)** Higher magnifications of the squared areas in **(A & B)**. In these insets tangled and complex EGFP+ cells have been indicated by arrowheads and arrows respectively. Note the presence of a larger number of EGFP+ complex cells labelled with NeuN in the EndoN hemisphere. **(C1-4 & D1-4)** Graphs representing the percentage and density, respectively, of EGFP+/NeuN+ neurons classified as tangled or complex cells by their diameter. Error bars represent the mean ± SEM and asterisks in bars indicate statistically significant differences between hemispheres (Vehicle x EndoN) after paired Student's *t*-test (*p< 0.05). Scale bar: 70 µm.

2.5.3 Effects of EndoN injection on the percentage and density of EGFP+ cells immunoreactive for DCX

Comparing hemispheres, no statistically significant differences were found in the percentage **(Figure 18A)** of EGFP/DCX double labeled cells. In contrast, a tendency toward a higher density of these cells with diameter ≤5µm was found in the EndoN hemisphere **(Figure 18B1)**, as well as a significantly lower density of those with an 8-10 µm soma diameter (p=0.003, **Figure 18B3**).

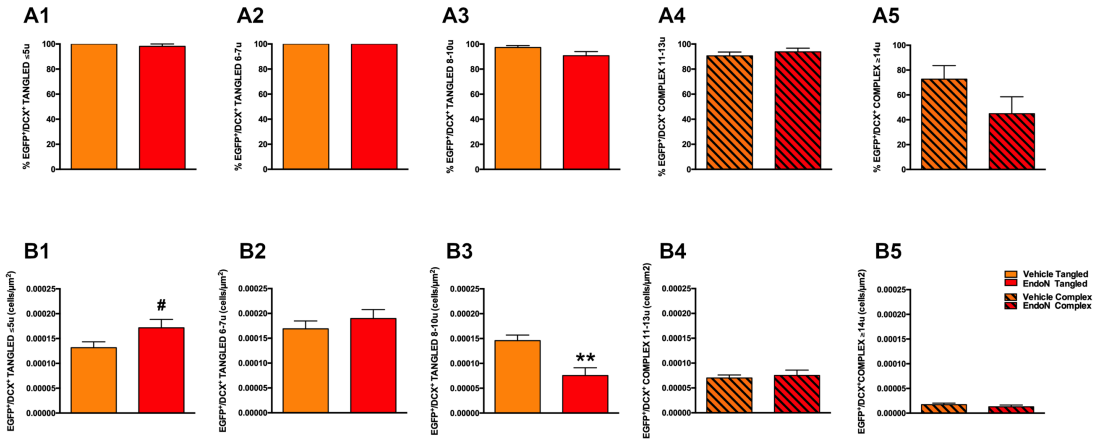

Figure 18. **Effects of Endo-N injection on the percentage and density of EGFP+ cells expressing DCX. (A1-5 & B1-5)** Graphs representing the percentage and density of EGFP/DCX double labeled neurons classified as tangled or complex cells by their diameter. Error bars represent the mean with ± SEM and asterisks in bars indicate statistically significant differences between hemispheres (Vehicle x EndoN) after paired Student's *t*-test (**p< 0.01).

2.5.4 Effects of EndoN injection on the percentage and density of EGFP+ cells displaying CAMK-II

In order to study the impact of EndoN on the final stages of development of EGFP+ cells, I performed immunohistochemical staining with CAMK-II. No statistically significant differences were found when comparing vehicle and EndoN hemispheres. Nevertheless, in the EndoN hemisphere I found a tendency toward a higher percentage of EGFP+/CAMK-II+ cells with 8-10 µm diameter and lower percentage and density of cells with ≥ 14µm diameter. As expected, when analysing the percentage and density of EGFP+ cells with ≤5µm and 6-7µm diameters I did not find colocalization with CAMK-II.

STUDY 3. THE HUMAN CEREBRAL CORTEX LAYER II: A" *RESERVOIR*" OF IMMATURE NEURONS IN THE ADULT BRAIN

3.1 Morphology and distribution of DCX immunoreactive cells in the human cerebral cortex layer II

Immunohistochemical analyses have revealed a population of DCX expressing cells in the layer II of the cerebral cortex of humans. I have analyzed samples from 2 sources: a) individuals included in the Stanley Neuropathology Consortium, which comprises controls, schizophrenia, bipolar disorder and major depression patients (n=15 per group) and b) seventeen post-operative neurosurgical samples from epileptic patients. From the Stanley Neuropathology Consortium, I only considered 20 individuals, in which positive cells could be detected in the layer II; the rest of samples were excluded due to low histological quality in this layer. The samples of the Consortium included the temporal cortical lobe (containing the entorhinal and perirhinal cortices and the fusiform gyrus). No qualitative differences were found in the distribution or morphology of these cells between the different groups of the Consortium (*see material and methods study 3*). The neurosurgical samples included tissue from the frontal, temporal, parietal and occipital lobes. In these samples, an anti-glial fibrillary acidic protein (GFAP) antibody was used to identify areas with reactive astrogliosis **(Figures 19A & B)** and all the analyses were performed outside these areas.

DCX immunoreactive cells could be found dispersed within the layer II of all cerebral lobes: frontal, temporal, parietal and occipital. These cells appeared frequently isolated, although occasionally could be detected in pairs **(Figure 19A)**. The quality of the samples from the Stanley Neuropathology Consortium only allowed the detection of scarce isolated cells due to the poor histological preservation of the tissue in the outer layers of the cerebral cortex; consequently, these samples were used only for double labeling experiments directed to analyze the phenotype of these cells. The histological preservation in the neurosurgical samples was much better and allowed an estimation of the linear density of these cells. Therefore, I assessed the number of DCX+ cells (3.75 ± 0.62) per mm of layer II perimeter.

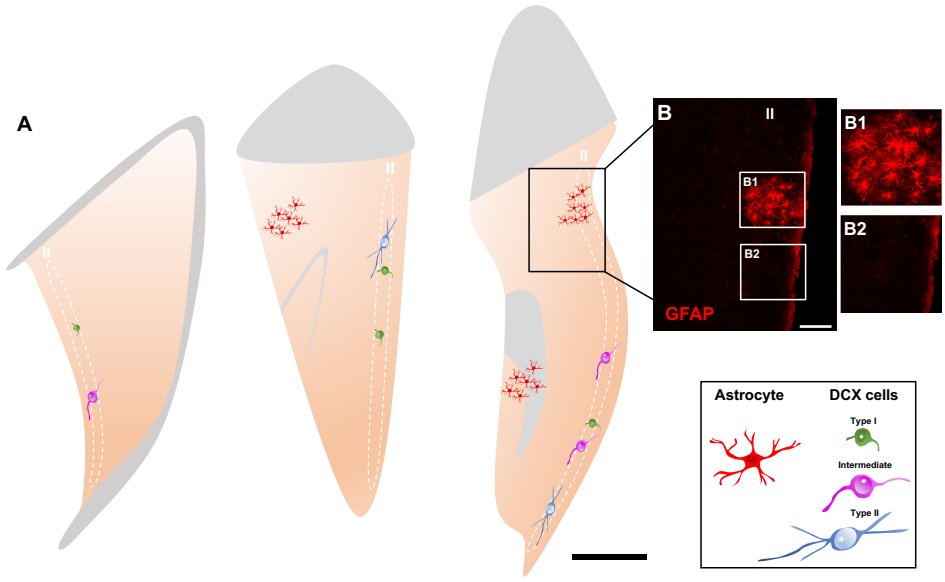

Figure 19. Distribution of DCX expressing cells and astrogliosis areas in human biopsies. (A) Schemes of 3 representative 50 µm coronal sections of temporal cortex, showing areas with reactive astrogliosis (red) and DCX immunoreactive cells in the layer II displaying different morphology (type I, green; intermediate, magenta; type II, blue). **(B)** Confocal image of the squared area in A, showing GFAP immunofluorescence. **(B1 & B2)** Higher magnifications of the squared areas in **(B)**, showing regions with **(B1)** or without **(B2)** reactive astrocytes. (White and gray matter correspond to gray and light-orange colour respectively). Scale bars: 30 mm for **(A)**; 70 µm for **(B)**; 35 µM for **(B1-2)**; 25 µm **(C & D)**.

Additionally, I used the rat-lithium pilocarpine model to analyze the effect of the epileptic status on the population of PSA-NCAM expressing neurons in the PCX layer II. Interestingly, my results demonstrated that the epileptic status induced by pilocarpine did not produce changes in the morphology and distribution of PSA-NCAM expressing cells **(Figures 20A & B)**. I only found a slight, but statistically significant (p=0.017) decrease in their density when comparing treated and control rats **(Figure 20C)**.

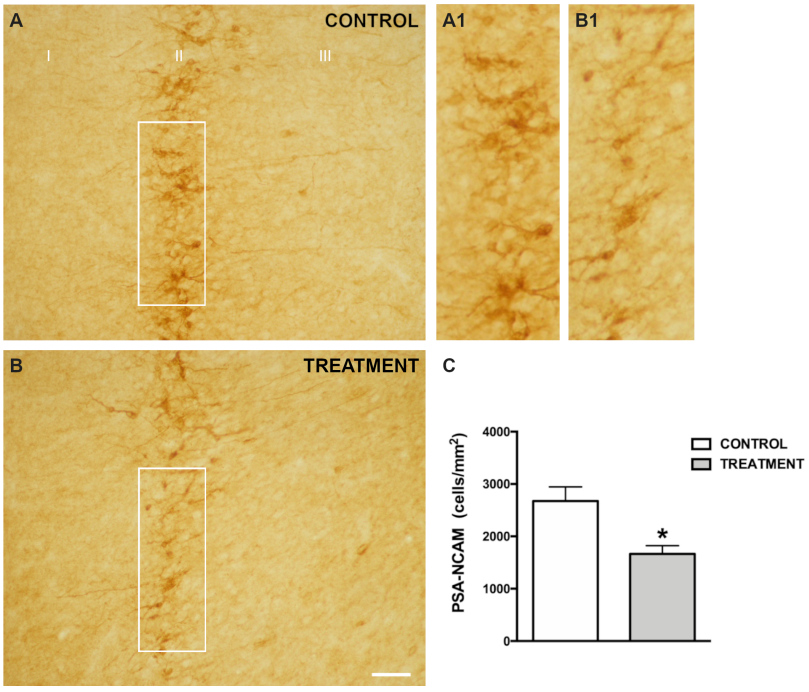

Figure 20. PSA-NCAM expressing cells in the three-layered rat PCX. (A & B) Microphotographs showing PSA-NCAM immunoreactive cells in control **(A)** and after treatment with pilocarpine **(B)**. **(A1-B1)** Higher magnification of the squared area in **(A & B)**. **(C)** Graph representing changes in the density of PSA-NCAM expressing cells in the PCX layer II. Asterisks in treatment bars indicate statistically significant differences from control groups after unpaired Student's *t*-test (*p< 0.05). Scale bars: 200 µm for **(A & B)**; 100 µm for **(A1 & B1)**.

The DCX expressing cells in the human cerebral cortex layer II had the typical morphology of the DCX positive cells recently described in the neocortex of several gyrencephalic mammalian species (La Rosa et al., 2019). This classification includes type I: small (soma diameter between 3-9 µm) and bipolar cells showing a single short process restricted to layer II **(Figure 21B1)** and type II: large (soma diameter between 9-17 µm) and multipolar cells with longer dendritic arborizations mainly oriented parallel to the layer II (**Figure 21B2**). These type II cells displayed a thin process resembling an axon, although the expression of Ank-G, which is usually used to identify the AIS, was not detected. The type II DCX expressing cells had both apical and basal dendrites, which frequently displayed some protrusions (length ≤ 1 µm) resembling stubby spines. Both cell types were present in the layer II (type I: 65.3 ± 10.32% and type II: 12.94 ± 5.0%) together with cells displaying intermediate morphologies (21.6 ± 6.27%), which usually showed a cell soma diameter between 9-11 µm, a thin basal process and only 1 or 2 short dendrites without spines or excrescences **(Figure 21B3)**.

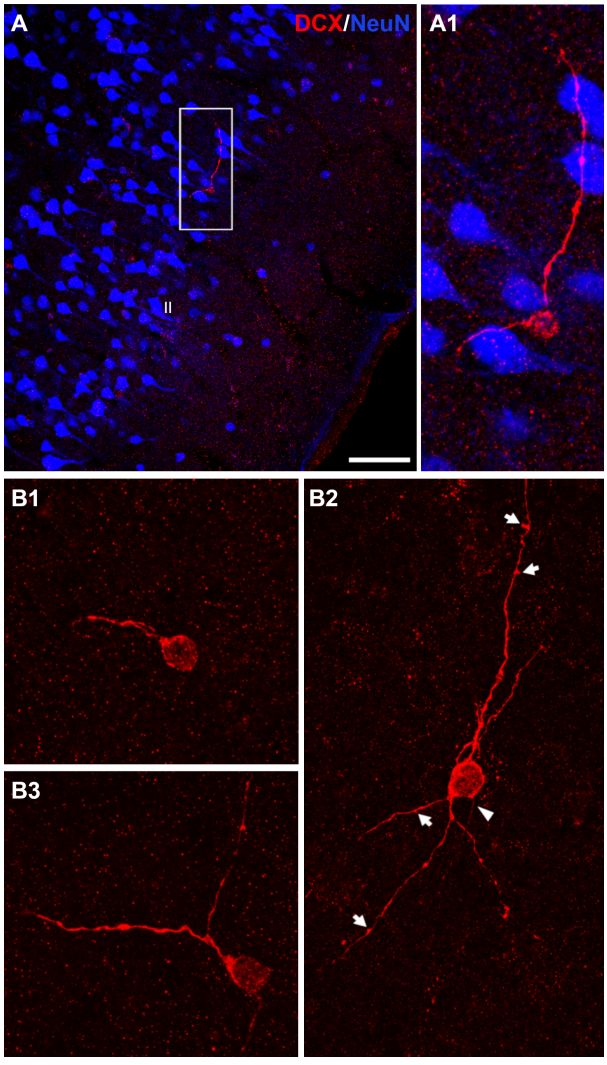

Figure 21. DCX immunoreactive cells in the human cerebral cortex layer II. (A) Double-immunofluorescence for DCX (red) and NeuN (blue) in the temporal cortex. Note a DCX cell with its soma located in the layer II and lacking a NeuN immunoreactive nucleus. The marker of mature neurons NeuN was used to define the six-layered cerebral cortex. **(A1)** Higher magnification of the squared area in A. **(B)** 2D projections of confocal stacks (5 confocal planes separated by 1 µm) showing DCX expressing cells (red) in different stages of development: type I **(B1)**, type II **(B2)** and intermediate **(B3)**. In the type II cell shown in figure **(B2)** it can be observed the presence of a thin process resembling an axon (arrowheads) and sparse protrusions similar to dendritic spines (arrows). Scale bars: 70 µm for (**A**); 20 µm for (**A1**); 30 µm for (**B1-3**).

3.2 The DCX expressing cells in the human cerebral cortex layer II are immature neurons

In order to reveal the phenotype of DCX expressing cells in the human cerebral cortex layer II, double immunofluorescence for DCX and NeuN, a marker of mature neurons (Mullen et al., 1992), was performed. DCX immunoreactive cells lack or have a very low expression of NeuN in their nuclei and thus could not be considered mature neurons **(Figures 21A & 22A)**.

Several studies, in both gyrencephalic mammals and rodents, have demonstrated that the co-expression of DCX and PSA-NCAM may be considered a solid evidence of an immature neuronal phenotype (Bonfanti and Nacher, 2012; Varea et al., 2012; König et al., 2016). In *post-mortem* and surgical samples, my results with conventional light microscopy have revealed a similar distribution of PSA-NCAM and DCX expressing cells in the cerebral cortex layer II. I have characterized the phenotype of DCX+ cells using immunofluorescence and antibodies against PSA-NCAM and NeuN. I found a population of cells that expressed only DCX and lacked PSA-NCAM and NeuN expression (41.6 ± 12.4 %) and another subset of cells that co-expressed DCX and PSA-NCAM but not NeuN in their nuclei (49.5 ± 16.3%). Only a very low percentage of DCX+/PSA-NCAM+ cells coexpressed NeuN (3.5 ± 2.3%) and the percentage of DCX^{+}/$PSA\text{-}NCAM^{-}$ cells coexpressing NeuN was also very low (5.3 ± 4.4%) **(Figure 22C)**.

When analyzing PSA-NCAM immunoreactive cells, I found a population that expressed only PSA-NCAM and lacked DCX and NeuN expression (19.8 ± 7.2 %) and another subset of cells that co-expressed PSA-NCAM and DCX but not NeuN in their nuclei (32.1 ± 15.7 %). I also found a high percentage of $PSA\text{-}NCAM^{+}$/ DCX^{-} cells that co-expressed NeuN (45.1 ± 14.4 %), although NeuN immunoreactivity was weak compared to pyramidal neurons located in the same layer **(Figure 22B)**. On the contrary, almost none of the $PSA\text{-}NCAM^{+}$/DCX^{+} cells coexpressed NeuN (1.4 ± 1.4 %) **(Figure 22D)**.

These results confirmed the immature phenotype of DCX expressing cells in the human cerebral cortex layer II.

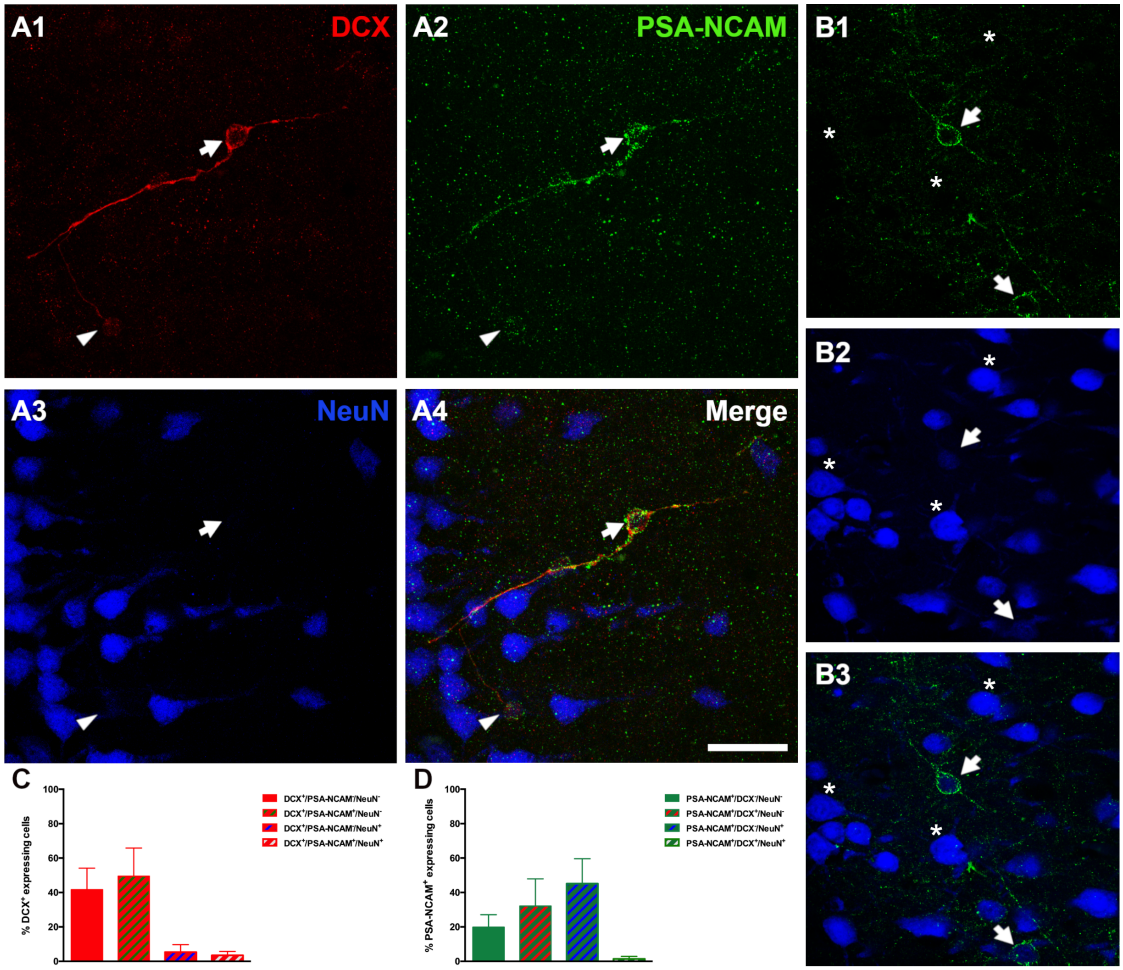

Figure 22. DCX and PSA-NCAM immunoreactive cells in the cerebral cortex layer II. (A1-4) Triple-immunofluorescence for DCX (red), PSA-NCAM (green) and NeuN (blue). Cells in the layer II of the occipital lobe colocalizing DCX and PSA-NCAM and lacking NeuN immunoreactivity in their nuclei. Note the presence of a large, more mature, DCX^{+}/$PSA\text{-}NCAM^{+}$ cell (arrow) and a smaller one (arrowhead). **(B)** PSA-NCAM/NeuN double labeled neurons (arrowheads) in the occipital lobe layer II. Note the lower intensity of NeuN immunoreactivity in their nuclei compared to that of neighboring pyramidal cells (asterisks). **(C-D)** Graphs showing the percentage of DCX **(C)** and PSA-NCAM **(D)** double labeled cells and their colocalization with NeuN immunoreactive nuclei. Error bars represent the mean ± SEM. All images in the figure are 2D projections of confocal stacks (5 confocal planes separated by 1 µm). Scale bar: 20 µm.

3.3 The immature neurons of the human cerebral cortex layer II belong to the excitatory lineage

The analysis of DCX expressing neurons in the cerebral cortex layer II revealed that none of them co-expressed GAD67, a marker of interneurons **(Figure 23)**. I found abundant presence of GAD-67 expressing somata in deep cortical layers, but they were very scarce in layer II **(Figure 23)**.

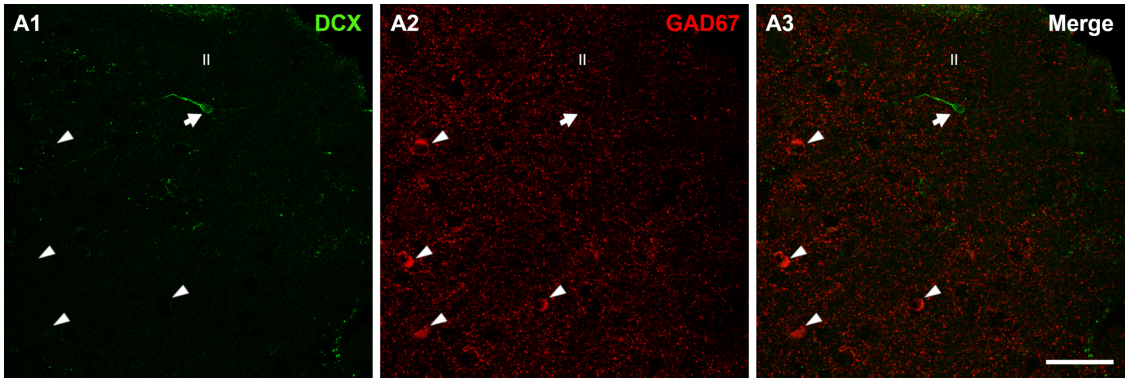

Figure 23. DCX immunoreactive cells are not interneurons. (A) Panoramic view of 2D projections (10 consecutive confocal stacks, 1 µm apart) showing the lack of co-localization between DCX (green) and GAD-67 positive cells (red). Note a single DCX immunopositive cell (arrow) in the temporal cortex layer II, lacking GAD67 expression in its soma. GAD-67 immunoreactive cells (arrowheads) can be detected in the deeper layers. Scale bar: 50 µm.

Interestingly, GAD-67 immunoreactive puncta were found apposed to the dendrites and the somata **(Figure 24A)** of DCX immunopositive cells. Moreover, I have analyzed the expression of the excitatory synaptic marker VGLUT1 and I found that DCX type II expressing cells showed VGLUT1 puncta apposed to their somata and dendrites **(Figure 24B)**.

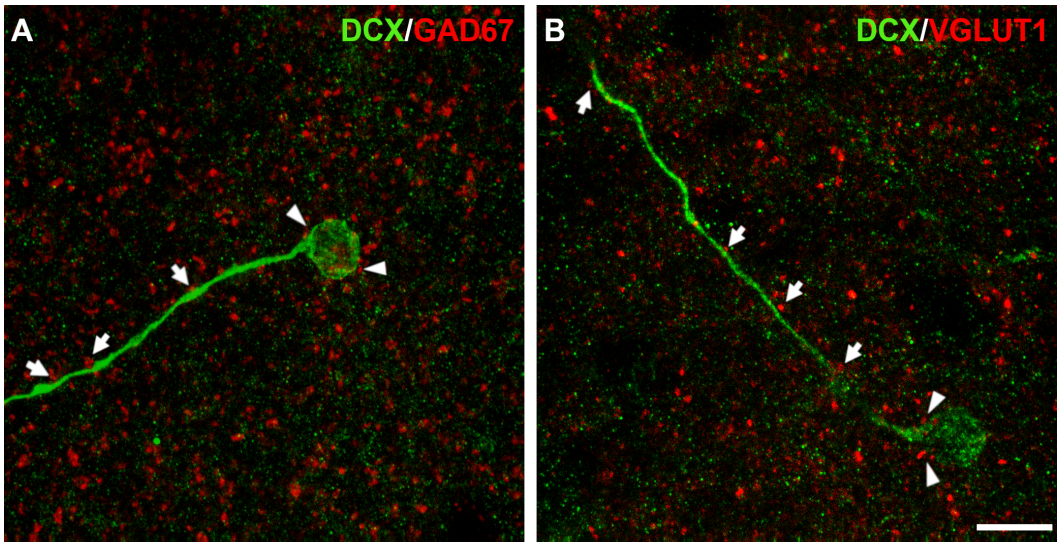

Figure 24. Inhibitory and excitatory puncta on DCX expressing cells. (A) DCX immunoreactive cell (green) in the temporal cortex layer II. Note the presence of GAD67 puncta (red) apposed to its soma (arrowheads) and dendrites (arrows). **(B)** DCX immunoreactive cell (green) in the occipital cortex layer II showing puncta expressing the excitatory marker VGLUT1(red) apposed to its soma (arrowheads) and apical dendrite (arrows). The images are 2D projection of 16 **(A)** and 18 **(B)** consecutive confocal stacks (1 µm apart). Scale bar: 10µm.

In the mammalian CNS, the transcription factors CUX1 and CTIP2 (also known as Bcl11b) are almost exclusively expressed by pyramidal neurons of the upper cortical layers (II-III-IV) (Cubelos et al., 2010; Li et al., 2010; Hulea and Nepveu, 2012; Lennon et al., 2017). Therefore, a triple immunohistochemistry for these 2 transcription factors and DCX was performed to confirm the excitatory lineage of the immature neurons

(Figure 25A). My data revealed that many DCX expressing cells expressed CUX1 but not CTIP2 (54.8 ± 9.9 %) and only a very low percentage expressed CTIP2 but not CUX1 (4.5 ± 3.0 %). Only a minor proportion of DCX positive cells expressed both transcription factors (21.4 ± 8.3%) **(Figure 25B)**.

Furthermore, I analyzed the expression of the neuron specific transcription factor T-box brain 1 (TBR1), exclusively expressed by pallial derived excitatory neurons (Hevner et al., 2001). Interestingly, I found that all DCX expressing cells in the cerebral cortex layer II expressed TBR1 **(Figure 25C)**.

Altogether, these results confirmed the excitatory lineage of the immature neurons in the cerebral cortex layer II.

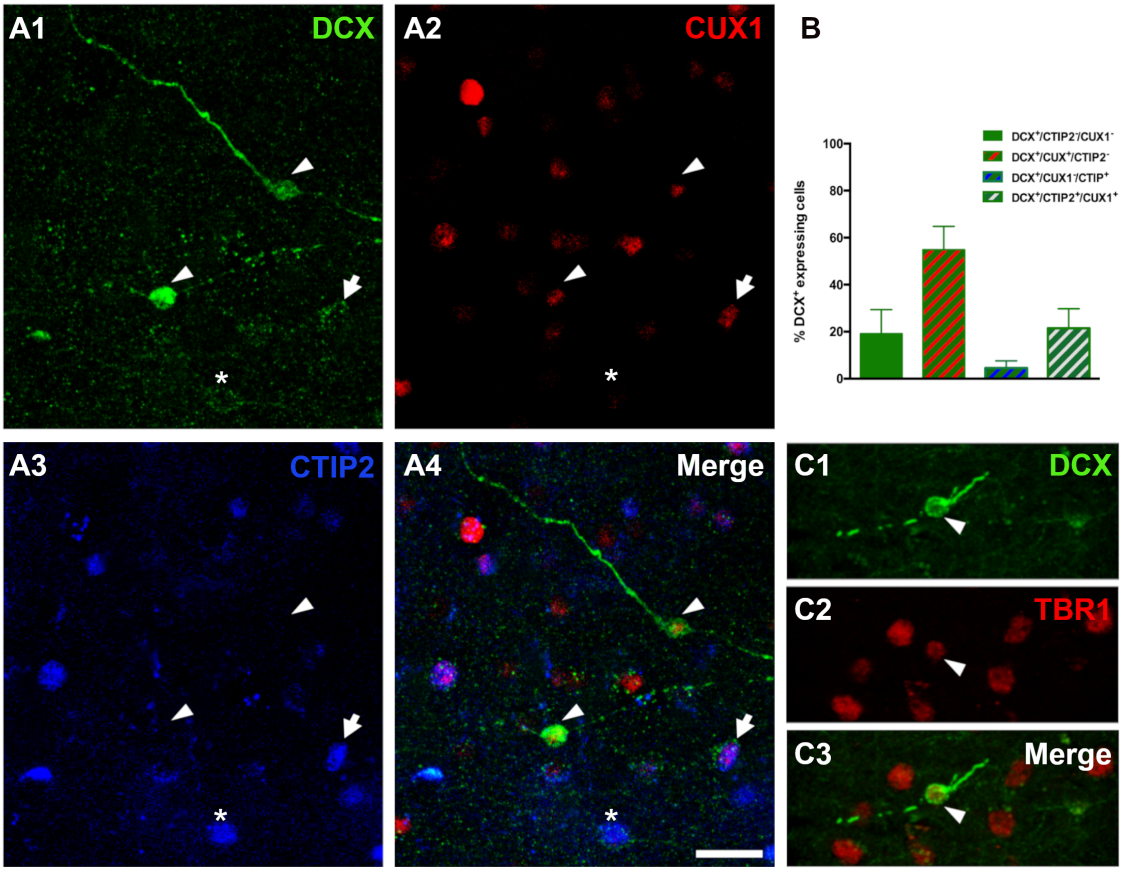

Figure 25. Expression of transcription factors specific for excitatory lineage in DCX+ neurons. (A) DCX immunoreactive cells (green) expressing the transcription factors CUX1 (red) and CTIP2 (blue). Observe DCX+ cells co-expressing CUX1 (arrowheads), CTIP2 (asterisk) or both (arrow). **(B)** Graph showing the percentage of DCX cells expressing each transcription factor. Error bars represent the mean with ± SEM. **(C)** Immunofluorescence staining for DCX expressing cells (green) and transcription factor TBR1 (red); arrowheads point to cells co-expressing both markers. Images are 2D projections of 10 **(A)** and 15 **(C)** consecutive confocal stacks (1 µm apart). Scale bar: 25 µm for **(A1-4)**; 10 µm for **(C1-3)**.

3.4 The immature neurons of the human cerebral cortex layer II are not glial cells

Different reports have described the presence of DCX immunopositive cells coexpressing GFAP in the cerebral cortex of human and nonhuman primates (Verwer et al., 2007; Bloch et al., 2011). In order to explore the possibility that some DCX expressing cells in the human cerebral cortex layer II correspond to astroglial cells, a combined fluorescence immunohistochemistry using anti-DCX and anti-GFAP antibodies was used. Since previous reports have indicated that the immature neurons in the PCX layer II of adult rodents appear covered by astroglial processes (Bonfanti and Nacher, 2012), I have also studied the relationship between GFAP processes and the immature neurons in the human cerebral cortex layer II. My results showed that the vast majority (97.2 ± 1.0 %) of DCX expressing cells in layer II did not express GFAP; only a very low percentage (2.7± 1.0%) of these cells co-expressed this astroglial-specific intermediate filament **(Figures 26A**, data not shown). Interestingly, GFAP immunohistochemistry showed astroglial processes apposed to the somata and the dendrites of DCX expressing cells in layer II **(Figure 26B)**. Interestingly, these processes were abundantly found in type I cells, whereas type II cells were far less covered.

Since a small proportion of immature neurons in the adult rodent PCX layer II was found positive for NG2, a marker of polydendrocytes and oligodendrocyte progenitors (Gómez-Climent et al., 2008; Rubio et al., 2015), I also performed a combined fluorescence immunohistochemistry using anti-DCX and anti-NG2 antibodies. My results excluded this eventuality, none of the DCX expressing cells in layer II were positive for NG2 **(Figure 26C)**.

In order to know whether some of the immature neurons in cerebral cortex layer II could correspond to microglial cells and to investigate whether there was a spatial relationship between them, I performed an immunohistochemistry with anti-PSA-NCAM and anti-IBA1 antibodies. For this immunohistochemistry I used the PSA-NCAM antibody due to species incompatibility between anti-DCX and anti-IBA1 antibodies. Since previous studies from our laboratory have described the expression of PSA-NCAM in a subpopulation of mature interneurons (Gilabert-Juan et al., 2011; Gómez-Climent et al., 2011b), I also used antibodies against the transcription factor CTIP2 to exclude interneurons from our analyses. I found that none of the PSA-NCAM^{+}/CTIP2^{+} cells in layer II expressed IBA1 **(Figure 26D)**. Interestingly, I have observed processes labelled

with IBA1 closely apposed to the surface of the somata of PSA-NCAM^{+}/CTIP2^{+} cells **(Figure 26E)**.

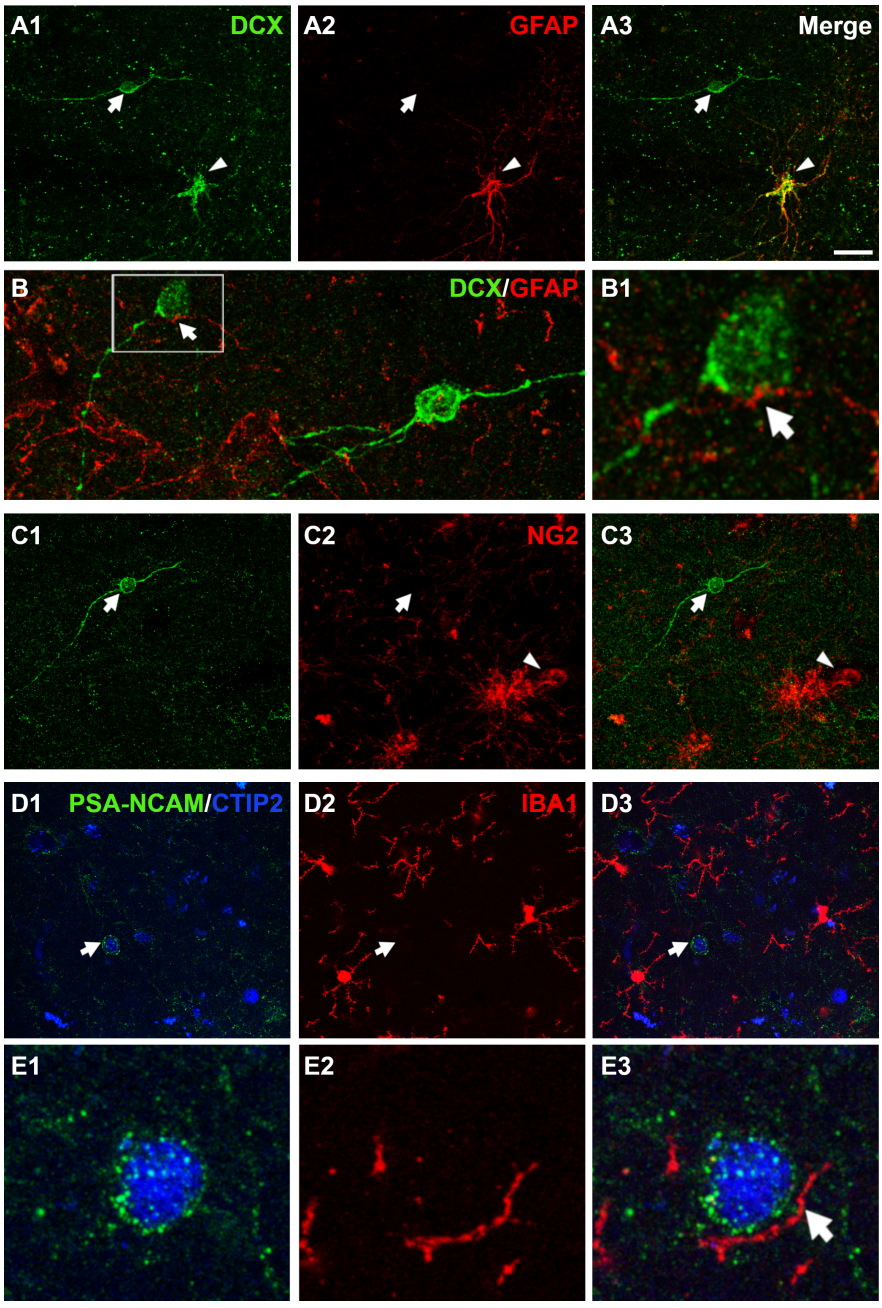

Figure 26. Expression of glial cell markers in the immature neurons of the human cerebral cortex layer II. (A1-3) Double DCX (green) and GFAP (red) immunohistochemistry. Arrow points to a DCX immunopositive cell devoid of GFAP expression. Arrowhead indicates a DCX/GFAP double labeled cell. **(B)** Confocal reconstruction of a DCX immunoreactive cell (green) contacted by different astroglial processes (red). **(B1)** Depicts a higher magnification view of the squared area in B, in which the arrow indicates a GFAP immunopositive process closely apposed to the PSA-NCAM+ soma. **(C1-3)** DCX immunopositive cell (green, arrow) devoid of NG2 expression (red, arrowhead). Note the presence of NG2 immunoreactive cells lacking DCX expression in the lower portion of the figure. **(D)** No co-localization was observed between PSA-NCAM/CTIP-2 double labeled neurons (arrow) and IBA1 immunopositive cells (red). The transcription factor CTIP2 was used to confirm the excitatory phenotype of PSA-NCAM immunoreactive neurons in the layer II. **(E)** High magnification view showing an IBA1+ process (arrow) closely apposed to the soma of a PSA-NCAM expressing cell. All mages are 2D projections of 15 confocal stacks (1µm apart). Scale bar: 30 µm for **(A**, **C**, **D)**; 10 µm for **(B)**; 5 µm for **(B1)**; 6 µm for **(E)**.

3.5 Dopamine innervation of immature neurons in the human cerebral cortex layer II

I used fluorescence immunostaining to investigate the dopaminergic innervation of immature neurons in the human cerebral cortex layer II. Due to the cross reactivity of antibody species, I studied the expression of the D2r and the presence of TH positive fibers identifying the immature neurons with anti-PSA-NCAM antibodies. Interestingly, I found that a 45.8 ± 5.2 % of PSA-NCAM positive cells expressed D2r on their surface **(Figure 27)**. TH immunoreactive fibers were found scattered in the cerebral cortex layer II, some of them in the close vicinity of the somata of PSA-NCAM expressing cells **(Figure 27)**.

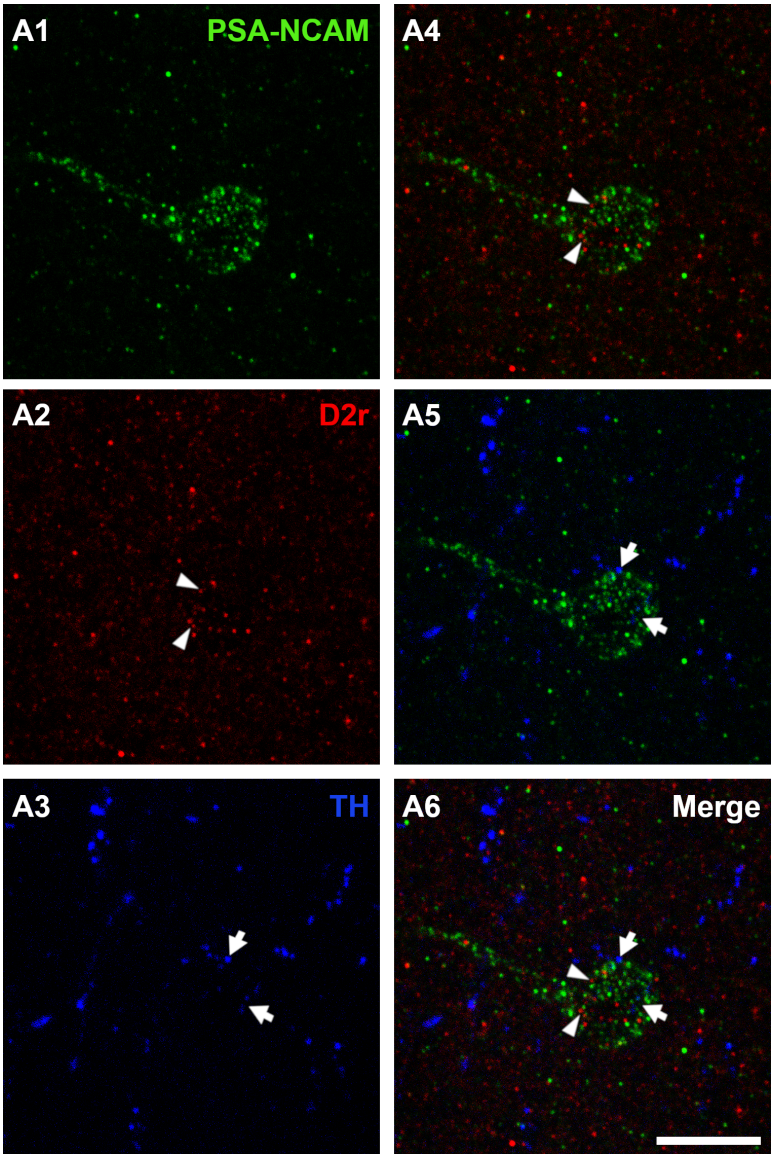

Figure 27. PSA-NCAM immunoreactive neurons, dopaminergic fibers and D2r in the layer II of the occipital lobe. **(A)** 2D projections showing a PSA-NCAM immunoreactive neuron (green) expressing D2r (arrowheads) on the surface of its soma (red) and closely apposed TH immunoreactive fibers (blue, arrows). Images are 2D projections of 10 confocal planes separated by 1 µm. Scale bar: 10 µm.

Chapter 5

DISCUSSION

STUDY 1. EFFECTS OF DOPAMINE ON THE IMMATURE NEURONS OF THE ADULT RAT PIRIFORM CORTEX

The present study describes the relationship of dopaminergic neurotransmission with the population of immature neurons in the PCX layer II of adult rats. In this study I have identified this population of cells by the expression of PSA-NCAM, a plasticity related molecule, which is strongly expressed in their somata and dendrites (Seki and Arai, 1991a; Bonfanti, 2006). The distribution and morphology of these cells are identical to those described previously in rats (Bonfanti et al., 1992; Nacher et al., 2002a). Most of the immature neurons in the adult PCX layer II have been generated during embryonic development and progressively mature into functional excitatory neurons in this layer (Rotheneichner et al., 2018; Benedetti et al., 2019), although the factors triggering or modulating these final steps of differentiation are still far from being understood.

I have decided to study the influence of DA on this immature neuronal population following 3 lines of evidence: 1) this monoamine regulates the expression of PSA-NCAM in the adult cerebral cortex (Castillo-Gómez et al., 2008), 2) another monoamine, NE, affects this immature neuronal population (Vadodaria et al., 2017) and 3) DA regulates neuronal differentiation during development (Money and Stanwood, 2013).

In this thesis, I have described the presence of fibbers expressing TH/DAT in the close vicinity of PSA-NCAM expressing somata. In accordance with previous studies in the PCX (Plantjé et al., 1987; Datiche and Cattarelli, 1996), I have observed that several immunoreactive fibers coexpressing TH and DAT, and consequently dopaminergic, could be found apposed to PSA-NCAM expressing somata. It is unlikely, however, that these fibers establish synapses with these immature neurons, because the presence of PSA-NCAM on their membranes prevents the establishment of synaptic contacts. There is evidence that only the cells in the most advanced stages of maturation show dendritic spines, in which some scarce synaptic contacts can be found (Gómez-Climent et al., 2008). However, monoamines, including DA, act in great part by volume transmission (Vizi et al., 2010), a type of paracrine signalling and thus, could exert their effect if receptors were present in the target neurons. In any case, further analysis using electron microscopy combined with immunohistochemistry would be necessary to unequivocally describe the relationship between dopaminergic fibers and the immature neurons. Since previous experiments in our laboratory have demonstrated that the expression of PSA-NCAM in the PFC can be modulated by DA through D2r, I have studied their expression

in the immature neurons in the PCX and confirmed their presence. This is consistent with previous data describing the expression of D2r in this cortical region (Meador-Woodruff et al., 1989). Although the cellular pattern of D2r expression in the PCX has not been explored yet, there is evidence of their presence in both inhibitory and excitatory neurons in other cortical regions (Santana et al., 2009). Consequently, some of the D2r-expressing cells in the PCX may be the immature neurons in layer II, which display an excitatory phenotype and differentiate into typical excitatory neurons of this region (Gómez-Climent et al., 2008; Rubio et al., 2015; Rotheneichner et al., 2018). It is important to note that I have only detected D2r in half of the PSA-NCAM positive cells, which raises questions on whether the expression of these receptors is restricted to certain phases of the development of these neurons. Since the presence of D2r has also been described in the PCX and in cortical excitatory cells, future works should also study their putative expression in the immature neurons of the PCX and the influence of their specific manipulation on these cells.

This study also shows that the pharmacological manipulation of D2r with specific antagonists and agonists produces opposite effects on the population of immature neurons in the PCX. I think that the decrease in the number of cells expressing PSA-NCAM induced by the D2r agonist PPHT may represent an increase in the rate of differentiation of these cells, which progressively lose PSA-NCAM expression as they reach their maturity (Rotheneichner et al., 2018). In fact, PSA-NCAM appears to have an insulating role in this immature neuronal population, limiting the formation of synaptic contacts (Gomez-Climent et al., 2011). On the contrary, the increase in the number of PSA-NCAM positive cells observed after treatment with haloperidol (a D2r antagonist), may be the consequence of a decrease in the rate of differentiation of these neurons. The number of immature neurons in the PCX decreases continuously as life progresses (Varea et al., 2009; Rotheneichner et al., 2018). Consequently, if the differentiation is slowed by haloperidol, at the end of the treatment, I will detect more PSA-NCAM expressing cells in the animals receiving this drug than in the controls, in which a larger cell population will have reached the PSA-NCAM-negative mature stage. However, further analyses with other markers of mature neurons such as NeuN or CAMK-II, and the use of transgenic animals similar to those used in the (Rotheneichner et al., 2018) study should be performed to demonstrate clearly this hypothesis. The effects of the pharmacological manipulation of D2r on PSA-NCAM expression has been already studied in the adult mPFC (Castillo-Gómez et al., 2008, 2011a). However, the effects of these manipulations

produce opposite effects to those detected in the PCX layer II. It has to be noted, however, that in the mPFC, PSA-NCAM is exclusively expressed by mature inhibitory neurons (Varea et al., 2005) and that the role of this molecule in these interneurons may be different than in the PCX immature neurons. In fact, the regulation of PSA-NCAM expression should also be different because the polysialyltransferase St8SiaII is responsible for the addition of PSA to NCAM in the immature neurons of the PCX, while St8SiaIV plays this role in mature interneurons (Nacher et al., 2010). Ongoing studies in our laboratory are exploring the effects of PSA depletion on the maturation of PCX layer II cells.

These results on the impact of the pharmacological modulation of D2r are particularly interesting, because there are different lines of evidence suggesting the involvement of the immature neurons in adult cortical layer II in psychiatric disorders and their treatment, especially in major depression. Although in rodents this cell population is restricted mainly to the PCX, they have a wider distribution in gyrencephalic animals (Varea et al., 2011; Piumatti et al., 2018), including humans (Ní Dhúill et al., 1999; Cai et al., 2009), covering most neocortical regions. Interestingly, similar cells have also been found in the human amygdala, a region critically involved in mood disorders (Martí-Mengual et al., 2013; Sorrells et al., 2019). Previous reports from our laboratory have shown that 2 animal models of major depression, chronic stress and chronic corticosterone treatment have a dramatic impact on the number of these immature neurons (Nacher et al., 2004). Olfactory bulbectomy, another accepted model of major depression, induces the differentiation of these cells (Gómez-Climent et al., 2011b). Moreover, chronic treatment with the antidepressant imipramine increases PSA-NCAM expression in the PCX layer II of adult rats (Sairanen et al., 2007). These results are also interesting because imipramine, besides its action as 5HT and NE reuptake inhibitor, also blocks D2 receptors (Smiałowski, 1991). This is consistent with the effects that I observe with haloperidol in this thesis. In addition, chronic treatment with the antidepressant fluoxetine, a 5HT reuptake blocker, also induces an increase in PSA-NCAM expression in the rat entorhinal cortex layer II, a region where some PSA-NCAM+ immature neurons can also be detected (Varea et al., 2007a). Finally, the modulation of α2-Adrenergic receptors, which are also involved in the pathogenesis of major depression and its treatment, also impacts the population of immature neurons in the PCX layer II (Vadodaria et al., 2017). Taking into account all these results, there is enough evidence

to promote the study of the immature neurons in the layer II of the cerebral cortex of humans, specially comparing major depression patients with healthy controls.

In conclusion, these results strongly indicate a prominent role of D2r and PSA-NCAM, on the differentiation of immature neurons in the adult PCX, although further analyses closely following the final development of these cells should be necessary to understand this mechanism. Interestingly, together with previous evidence, these data also suggest that this neuronal cell population is the target of drugs acting the monoaminergic systems and its involvement in the etiopathology of psychiatric disorders in which these systems are altered.

STUDY 2. PSA DEPLETION INDUCES THE DIFFERENTIATION OF IMMATURE NEURONS IN THE PIRIFORM CORTEX OF ADULT MICE

In the present thesis, I have explored the impact of PSA depletion in DCX-CreER^{T2}/Flox-EGFP transgenic mice to understand the role of this molecule in the differentiation of immature neurons in the adult PCX layer II. I have provided evidence that this enzymatic depletion influences the final stages of development of this neuronal population. Interestingly, I have found that the elimination of PSA from NCAM promoted the maturation of EGFP+ cells, as demonstrated by an increase in the density of EGFP+ complex cells displaying an AIS. Moreover, I have found a higher density of EGFP+ dendrites and dendritic spines in the PCX layer I. Finally, PSA removal was demonstrated to increase the percentage and density of EGFP+ cells expressing the mature neuronal marker NeuN, as well as the density of NeuN expressing nuclei in the adult PCX layer II.

This study has also demonstrated, for the first time, that the injection of the enzyme EndoN in the mouse ectorhinal cortex produced a clear removal of PSA from the PCX layer II 2 weeks after the injection. It is interesting to note that I have not observed evidence of neuronal death after EndoN treatment (absence of necrotic cells and pyknotic nuclei). These observations are in agreement with previous reports that have described an efficient disruption of the PSA side chain of NCAM in the adult rodent brain by EndoN (Rutishauser et al., 1985; Castillo-Gómez et al., 2008, 2016; Guirado et al., 2014, 2016), without induction of neuronal cell death (Nacher, unpublished observations).

Two previous studies have already used the DCX-CreER^{T2}/Flox-EGFP transgenic mouse to label and follow the fate of immature neurons in the adult PCX layer II (Rotheneichner et al., 2018; Benedetti et al., 2020). In this transgenic mouse, DCX expressing cells are permanently labelled after tamoxifen administration and monitored with the enhanced green fluorescent protein EGFP reporter (Zhang et al., 2010). The population of EGFP+ cells in the PCX layer II, showed morphological and functional features similar to those previously described in rodents using immunohistochemical markers and particularly in this transgenic strain (Gómez-Climent et al., 2008; Rubio et al., 2015; Rotheneichner et al., 2018; Benedetti et al., 2020). In this study, I classified EGFP+ cells in 5 different subcategories reflecting their stages of maturation. As it happens with the development and functional integration of new born DGCs during adult neurogenesis (Toni and Schinder, 2016), the process of maturation of EGFP+ cells occurs

through several intermediate steps, in which these cells progressively undergo a series of cellular events that are essential for their subsequent functional maturation. As demonstrated by the first study of EGFP+ cells in the PCX layer II, during maturation they progressive enlarge their somata (Rotheneichner et al., 2018). In the present thesis, after detailed morphological analyses of EGFP+ cells, I decided to add to the previous classification, which only included tangled and complex cells, different subcategories. Cells were included in each subcategory considering their soma diameter. This helped me to highlight the fact that these cells are subjected to a progressive process of maturation and to better monitor changes in their morphology. Unlike my expectation, the PSA removal did not alter the distribution of EGFP+ cells in the different subcategories, increasing the percentage of cells belonging to the categories with larger somata. In connection with this, I have found that in both hemispheres EGFP+ tangled cells were more abundant than complex cells. Longer times of survival after tamoxifen induction would be necessary to get a larger population of complex cells, by allowing the differentiation of more tangled cells. I believe that if the time between tamoxifen injection and sacrifice had been longer I probably would be able to observe stronger effects of EndoN in the subcategories. In fact, although there are no differences in the percentage or density of complex cells, there is a significant decrease of the larger tangled cells in the hemisphere injected with EndoN.

The role of EndoN as a promoter of the development of EGFP+ cells is also demonstrated by an increase in the density of EGFP+ complex cells showing structures related to neuronal maturity and functionality. In this study I have found an increase in the density of complex cells displaying an AIS, which is an electrogenic axonal domain essential for action potential initiation (Jamann et al., 2018). Moreover, it also has a unique molecular identity that makes it one of the master regulators of neural plasticity and excitability (Bolós et al., 2019). Therefore, the presence of the AIS can be considered a clear sign of structural remodeling associated to the final steps of neuronal maturation and its structural features have an enormous impact on the functionality of developing neurons (Evans et al., 2015; Yamada and Kuba, 2016; Bolós et al., 2019; Benedetti et al., 2020).

Other morphological changes of EGFP+ cells, which are correlated to their maturation and functionality, include the axon outgrowth, the development of dendritic trees and the occurrence of dendritic spines. As previously described by Rotheneichner at al., (2018), upon 6 months of maturation after tamoxifen injection, EGFP+ cells

increased their morphological complexity. In the present thesis we should expect also an increase in complexity, but to a lesser degree, since the time after tamoxifen is considerably shorter. The depletion of PSA from the PCX should accelerate this morphological maturation. In fact, in the EndoN hemisphere, besides the presence of an AIS, I observed increases in the density of dendritic branches and in the linear density of dendritic spines in the PCX layer I, suggesting an increase in the synaptic input on EGFP+ immature neurons. A similar effect induced by EndoN was previously described in the hippocampus, where the removal of PSA induced the extension of dendritic trees in CA3 pyramidal neurons (McCall et al., 2013). These findings are also similar to those observed in previous studies describing that new born DGCs undergo profound morphological transformations including dendritic and axonal outgrowth (Zhao et al., 2006; Rahimi and Claiborne, 2007; Bolós et al., 2019). Moreover, because mature DGCs receive most of their synaptic inputs through dendritic spines, the number and shape of dendritic spines are also indicative of the maturation and connectivity of these cells (Zhao et al., 2006; Rahimi and Claiborne, 2007; Bolós et al., 2019). All these morphological changes, that occur normally during neuronal development, may be accelerated in response to a variety of external stimuli (Alvarez et al., 2016; Bolós et al., 2019). Therefore, in this study PSA depletion positively modulates the dendritogenesis and spinogenesis of EGFP+ cells, accelerating their consequent maturation and integration in the local circuitry.

I have also analyzed the effects of PSA removal on the development of EGFP+ dendritic spines, focusing on their shape. In the adult CNS, spines are extremely diversified (Peters and Kaiserman-Abramof, 1970) and depending of their morphology are usually classified as: mushroom, thin and stubby (Sala, 2002; Von Bohlen und Halbach, 2009; Guirado et al., 2014), although a further category, the so-called filopodium also exists (Skoff and Hamburger, 1974). Mushroom spines are normally associated to mature neurons, whereas filopodia/thin and stubby spines are more abundant in immature neurons (Nimchinsky et al., 2002). However, in this thesis it was not possible to reveal any change in the morphology of EGFP+ dendritic spines. It is possible that this may be due to the short time available for maturation after tamoxifen administration, which probably did not produce a substantial number of fully developed complex cells bearing a considerable density of dendritic spines.

As the development of immature neurons progresses, besides the morphological changes, there is a parallel increase in connectivity, which is normally determined by both afferent input through dendrites and efferent output through axons. As described in

previous studies, only EGFP+ complex cells, which had undergone 6 months of maturation, were surrounded by SYN and VGAT immunoreactive puncta and showed expression of markers for spines and glutamatergic synaptic boutons, indicating their integration into the excitatory connectivity of adult neuronal networks (Rotheneichner et al., 2018; Benedetti et al., 2020). In the present thesis, using considerable shorter times after tamoxifen administration, I have also observed similar connectivity changes, although to a lesser extent. The dendritic spines on EGFP+ complex cells were closely apposed to puncta expressing pre-synaptic markers, such as SYN and VGLUT1, on their surface, indicating that they were functional and received a glutamatergic input.

Another important finding of this thesis is the description, for the first time of the development of perisomatic inhibitory input on complex cells in the PCX layer II. I have found that EGFP+ complex cells receive both excitatory and inhibitory input in their perisomatic region and that the latter belonged mainly to PV+ basket cells. Unfortunately, no significant changes in the density of VGAT+ or PV+ perisomatic puncta have been detected after EndoN administration. However, I observed a tendency for a higher intensity of fluorescence in VGAT+ puncta. It is possible that experiments with longer survival times, which will result in a larger population of fully developed complex cells, could render more solid results. In fact, it was previously described that the depletion of PSA induced an increase in the perisomatic input on pyramidal cells of the adult cerebral cortex (Castillo-Gómez et al., 2011b).

EGFP+ cells follow a pattern of morphological and functional maturation that resembles that occurring in DGCs. Newly born cells in the DG of the adult hippocampus express early neuronal markers. Later, during their maturation, they progressively lack this expression and start to express mature neuronal markers (Van Praag et al., 2002; Brown et al., 2003; Spampanato et al., 2012). The hypothesis that PSA removal may induce the differentiation of EGFP+ cells is supported by the increase in the density of NeuN expressing nuclei in the adult PCX layer II. This increase in the expression of this nuclear protein, which is widely used to confirm neuronal maturation (Mullen et al., 1992; Bonfanti and Nacher, 2012), may be considered as a sign that the EndoN treatment accelerates the maturation and consequent incorporation of EGFP+ cells to the circuitry. In addition, the percentage of EGFP+ cells double labeled with NeuN was significantly higher in the EndoN hemisphere, confirming that the new cells incorporated were EGFP+ and that consequently have matured after tamoxifen injection.

I have also found that the removal of PSA significantly decreased the density of EGFP+ cells double labeled with DCX. DCX is an immature neuronal marker, which is normally turned off when neurons reach maturity (Nacher et al., 2001b; Brown et al., 2003). In fact, the expression levels in complex cells are very reduced and almost undetectable in the most mature category (see **figure 11B**); the densities of DCX+ cells in the 2 categories of complex cells are extremely low. It is possible, as I have argued when discussing the results on the perisomatic input, that longer survival times after tamoxifen treatment, which will result in a larger population of EGFP+ complex cells, may detect differences in DCX expression between hemispheres. Interestingly, this reduction was observed only in tangled cells belonging to the group with larger soma diameter. It is possible that, given the short period after tamoxifen induction, this subcategory of cells might be the only one in which the reduction in DCX expression could be observed.

The current data and several previous studies (Gómez-Climent et al., 2008; Rubio et al., 2015; Rotheneichner et al., 2018; Benedetti et al., 2020), suggest that immature neurons in the PCX layer II and particularly the EGFP+ cells in the DCX- CreER^{T2}/Flox-EGFP transgenic mouse, have an excitatory fate and are integrated in the circuitry as pyramidal neurons. Therefore, I decided to study the effect of PSA removal on the expression of CAMK-II, an excitatory mature neuronal marker (Wayman et al., 2008). However, the EndoN treatment did not induce changes in the percentage and density of EGFP+ cells expressing CAMK-II or in the density of CAMK-II expressing cells in the adult PCX layer II. I think that this lack of effect is due to the fact that CAMK-II expression is highly restricted to fully mature excitatory neurons. As already discussed, given the short time after tamoxifen induction used in this thesis, it is possible that most EGFP+ cells did not have enough time to achieve a complete maturation and express this marker.

Different hypothesis can be postulated on the mechanisms by which PSA depletion may accelerate the differentiation of the immature neurons in the PCX. These hypotheses are connected to the roles that the presence of PSA-NCAM may have in these cells (Bonfanti and Nacher, 2012). First, PSA-NCAM may have an insulating role, preventing the formation of synaptic contacts on these immature neurons. This insulating role, could also affect NCAM signaling. In fact, different reports have demonstrated that the polysialylated form of NCAM, through its large hydration volume, decreased its homophilic bindings and this may influence the heterophilic interactions with other

molecules and receptors (Bonfanti, 2006; Gascon et al., 2007; Kochlamazashvili et al., 2010). This observation may shed light on the possibility that the removal of PSA from NCAM may facilitate a specific signaling transduction pathway that may promote the final development of immature neurons.

On the other hand, PSA-NCAM may act as an anti-adhesive molecule, which facilitates neurodevelopmental events, such as neurite outgrowth or spinogenesis. During the first stages of neuronal development, in fact, PSA-NCAM plays an important role promoting cell migration, cell differentiation and also acting as facilitator of axonal outgrowth, neuronal-glial structural remodeling and synaptogenesis (Kiss and Rougon, 1997; Maarouf and Rutishauser, 2003; Bonfanti, 2006; Gascon et al., 2007; Rutishauser, 2008; Hildebrandt and Dityatev, 2015).

Considering these non-exclusive hypotheses, it is possible that the disruption of the PSA chain promotes the final maturation of complex cells (or even the most developed tangled cells), creating spaces in the plasma membrane that allow the formation of new synapses and their integration in the local circuitry. The depletion of PSA may also favors signaling pathways that could start or promote neuritogenesis or synaptogenesis. On the other side, it has also to be considered that the lack of PSA may affect negatively the population of EGFP+ tangled cells, especially those in the earliest stages of development, which due to the absence of this molecule may have difficulties to extend their dendritic tree, their axon or construct their dendritic spines.

STUDY 3. THE HUMAN CEREBRAL CORTEX LAYER II: A" *RESERVOIR*" OF IMMATURE NEURONS IN THE ADULT BRAIN

This thesis provides evidence of the presence of immature neurons in the human brain, specifically in the layer II of the cerebral cortex. Using surgical samples from epileptic patients and *post-mortem* tissue, I have found cells in different stages of development expressing DCX and PSA-NCAM and lacking expression of the mature neuronal marker NeuN. These immature cells belonged to the excitatory lineage, as demonstrated both by the expression of CUX1, CTIP2 and TBR1 transcription factors and by the lack of the inhibitory marker GAD67. Moreover, cells in the most advanced state of maturation showed puncta expressing inhibitory and excitatory synaptic markers apposed to their perisomatic and peridendritic regions. Interestingly, I have also demonstrated that these immature neurons were not glial cells, although astroglial and microglial processes were found in close apposition to their somata and dendrites. Finally, I have found that these immature neurons in the cortical layer II expressed D2r and displayed sparse TH+ fibers in the close vicinity of their somata.

In this thesis, I have provided evidence of a numerous population of immature neurons in the human cerebral cortex layer II and I have also described in detail the distribution of these cells. In *post-mortem* tissue I found cells double labelled for DCX and PSA-NCAM in the layer II of the temporal cortical lobe, containing the entorhinal and perirhinal cortices and the fusiform gyrus, whereas in neurosurgical samples the distribution of these cells was described in the layer II of all cerebral lobes: frontal, temporal, parietal and occipital. The observation of immature neurons in these human samples considerably expands the restricted and poorly detailed description of these cells provided by previous studies. The presence of a single band of immature neurons expressing PSA-NCAM was primarily described in the entorhinal cortex in *post-mortem* samples of early infants (Dhúill et al., 1999). DCX expressing cells have been also found in human cortical samples of adult patients of a wide age range with intracranial tumors or recurrent temporal epilepsy. These DCX+ cells, which showed a morphology resembling those described in this study, were prevalently located in the upper border of neocortical layer II in the temporal and frontal cortices (Cai et al., 2009). Moreover, intensely labeled DCX+ cells were described in epilepsy surgical samples, including focal cortical dysplasia (FCD), of 2 years-old patients. These cells were described at the border

between cortical layers I and II, particularly in temporal and frontal cortices (Srikandarajah et al., 2009).

By analogy with what occurs in large-brained mammals (La Rosa et al., 2020), in which the number of immature neurons significantly increases in association with brain size, it may be presumed that the total number of immature neurons in the human brain must be impressive if considering the linear density of these cells distributed along the total extension of the layer II. The distribution of immature neurons in the human brain is clearly more expanded than in rodents, in which it is restricted to paleocortical regions (Bonfanti and Nacher, 2012) and this distribution is more similar to those found in species with larger gyrencephalic brains (Bonfanti, 2006; Cai et al., 2009; Luzzati et al., 2009; Varea et al., 2011; La Rosa et al., 2020). For instance, in rabbits, cats and monkeys these cells were described in the somatosensory cortex and in different regions of the insula, as well as in the associative frontal, occipital and tempoparietal lobes (Cai et al., 2009; Zhang et al., 2009; Varea et al., 2011).

Unlike *post-mortem* tissue, which belong only to adult individuals, the neurosurgical samples used in the present thesis also include brain tissue from pediatric and young adult patients. However, interestingly, in all samples analyzed, I did not observe differences in the distribution of immature cells in different age-groups. By contrast, in a previous work by Srikandarajah et al., (2009), a different DCX expression pattern was described in the human cerebral cortex layer II of patients of different ages (Srikandarajah et al., 2009). In the mentioned study, DCX+ cells were identified mainly in biopsies from pediatric patients with epilepsy and focal cortical dysplasia (FCD) malformations. By contrast, they were not present in significant numbers in other epilepsy pathologies or in the cortex of *post-mortem* tissue of patients of similar age. Furthermore, DCX+ cells were virtually absent from adult surgical samples and from *post-mortem* adult control tissue.

In the neurosurgical samples of epileptic patients used for this thesis, the analysis of the distribution of immature neurons, was conducted outside the epileptic foci, which were specifically identified by the use of an anti-GFAP antibody. The results obtained by Srikandarajah et al., (2009) in epileptic tissue, were partially similar to those described in this thesis. In fact, although I have found expression of DCX+ cells in the cerebral cortex layer II both in pediatric surgical samples and in surgical and *post-mortem* adult samples, Srikandarajah et al., (2009) failed to find DCX+ cells in their adult samples. One possible explanation for these discrepant results could be the use of paraffin embedding in their

study but not mine, which could have adversely affected the immunogenicity of their samples.

Since a detailed analysis revealed an absence of immature neurons inside these epileptic foci and considering that Srikandarajah et al.,(2009) have described only occasionally expression of DCX+ cells in adult epileptic patients, I have used a rat lithium-pilocarpine epileptic model, to exclude the possibility that epilepsy could be responsible of the disappearance of this population of immature cells in my area of study. Interestingly, I have only found a slight reduction of immature cells in the rat PCX layer II, whereas the majority of immature cells expressing PSA-NCAM still persisted after epilepsy. Therefore, the biopsies from epileptic patients constitute a valid approach to study, outside the epileptic foci, the distribution of immature cells within the layer II.

In general terms, the neurosurgical samples showed better histological quality than the samples collected from the Stanley Neuropathology Consortium. However, some intraoperative samples had to be excluded from the study, especially due to their restricted size, absence of superficial layers or the presence of widespread white matter. In general, the use of surgical biopsies provides many advantages, especially because it avoids sample fixation in formalin for prolonged periods, which negatively influences the performance of different antibodies, altering consequently the protein detection by immunohistochemistry procedures (Flor-García et al., 2020). P*ost-mortem* tissue is highly vulnerable to several factors that interfere with the molecular preservation, the processing of these samples and their histological examination. Specifically, some factors are related with *pre-mortem* events such as prolonged agonal state, hypoxia, acidosis, fever and seizures. Others are related with long *post-mortem* delay between death and sample processing for storage and fixation, temperature of the corpse, characteristics of the fixative solutions and processing of frozen material (Boekhoorn et al., 2006; Ferrer et al., 2008).

The immature phenotype of cells described in the human cerebral cortex layer II was defined by the expression of DCX and PSA-NCAM, which are both commonly used to identify immature neurons in the adult CNS of several mammalian species (Gómez-Climent et al., 2008; Cai et al., 2009; Luzzati et al., 2009; Varea et al., 2011; Rubio et al., 2015; König et al., 2016; La Rosa et al., 2020). Given the poor functioning of numerous antibodies on human tissue, the detection of DCX and PSA-NCAM has to be validated through different immunohistochemical controls. Ideally, the antibody signal should be validated using synthetic blocking peptides that eliminate the binding sites of the antibody

and permit comparison between the specific and unspecific signal of these molecules. Due to the lack availability of blocking peptides, I have had to use alternate methods to demonstrate the specificity, specially in the case of DCX antibodies. I used 2 commercially-available DCX antibodies, which were previously applied in other human studies in which DCX expression was found in the neocortex, hippocampus and amygdala (Liu et al., 2018; Sorrells et al., 2019; Flor-García et al., 2020), although no appropriate controls were performed in these studies. In order to have a better control of the specificity of the antibodies I have tested them successfully in DCX-KO mice. On the other side, the anti PSA-NCAM antibody was also previously used in human studies (Varea et al., 2007b, 2012) and its specificity was widely tested in different ways, including its blockade with the antigen (colominic acid) (*see material and methods, study 3)*.

The morphology of the DCX+ cells described in this study (type I: small cell somata and bipolar morphology; type II: large cell somata with multipolar morphology) resembled that previously described in gyrencephalic mammals (Cai et al., 2009; Srikandarajah et al., 2009; Zhang et al., 2009; Varea et al., 2011a; Bonfanti and Nacher, 2012; König et al., 2016; Piumatti et al., 2018; La Rosa et al., 2020). Moreover, DCX+ cells with intermediate morphological characteristics between type I and type II were also described, suggesting that these cells undergo a progressive process of maturation, similar to what has been found recently in rodents (Rotheneichner et al., 2018).

Interestingly, for the first time in humans, I have observed the presence of synaptic spine-like protrusions and thin axon-like basal processes on DCX+ type II cells. However, I failed to find expression of the cytoskeletal scaffold protein Ank-G, which is normally expressed by the AIS and is essential for its assembly and long-term maintenance (Hedstrom et al., 2008). Therefore, it is likely that these cells are not yet fully differentiated. The presence on type II immature cells of an extension resembling an axon was previously observed in rats (Gómez-Climent et al., 2008) and cats (Cai et al., 2009), whereas the existence of dendritic protrusions resembling spines on these cells was only reported in rats (Gómez-Climent et al., 2008). The presence of these structures is very important because they could indicate the appearance of nascent synaptic contacts. In any case, further studies will be needed to clarify the presence of these synapses. In fact, I have detected puncta expressing excitatory and inhibitory synaptic markers in close apposition to the dendritic shafts and somata of type II cells, suggesting that these cells are progressively being integrated in pre-existing neural circuits.

Most DCX expressing cells in layer II co-expressed PSA-NCAM and lacked NeuN immunostaining in their nuclei, confirming their immature phenotype. The neuronal marker NeuN is a nuclear antigen that is expressed by mature differentiated neurons (Mullen et al., 1992). The expression of the immature neuronal markers DCX and PSA-NCAM overlapped partially with NeuN only in type II cells. The expression of NeuN was only detectable in a small number of DCX+/PSA-NCAM+ cells with larger size and lighter reactivity, whereas it was completely absent in cells with type I morphology. This is a clear indication of a maturational process from type I cells to the most complex type II cells, which can be considered virtually mature neurons.

As it occurred in rodents (Gómez-Climent et al., 2008; Luzzati et al., 2009; Rubio et al., 2015), rabbits (Luzzati et al., 2009) and cats (Varea et al., 2011), the analysis of immature neurons in the human cerebral cortex layer II has revealed that none of these cells co-expressed GAD67, a marker of interneurons. In addition, the expression of specific transcriptional factors also confirmed their excitatory lineage. In fact, these immature cells expressed the transcription factors CUX1 and CTIP2, which are both exclusively expressed by pyramidal neurons of the upper cortical layers (II-III-IV) (Cubelos et al., 2010; Li et al., 2010; Hulea and Nepveu, 2012; Lennon et al., 2017) and TBR1, which is a transcription factor specific for pallium-derived principal neurons (Hevner et al., 2001). Therefore, the expression of these transcription factors, together with the presence of inhibitory and excitatory synaptic markers apposed to the somata and dendrites of DCX+ type II cells, suggest that these cells are progressively being integrated into the circuitry as excitatory neurons. These data are also in line with findings recently provided by the study of the population of immature neurons in the PCX layer II using the inducible transgenic mouse model DCX- CreER^{T2}/Flox-EGFP (Rotheneichner et al., 2018; Benedetti et al., 2020) and also with results previously discussed in this thesis regarding the EndoN experiment performed in this same transgenic mouse model.

The immature neurons of the human cerebral cortex layer II did not express GFAP, NG2 or IBA1 and, consequently, they cannot be classified as astrocytes, polydendrocytes or microglial cells, respectively. Nevertheless, it is very interesting to note that most of the immature neurons were covered by astroglial and microglial processes, which were found in close apposition to their surface, both in their somata and in their dendritic processes. The presence of these glial lamellae was abundantly found in type I cells, whereas type II cells were far less covered.

I have described the presence of microglial lamellae for the first time in this thesis, but astroglial processes were described in close apposition to immature neurons in the PCX of adult rodents in previous reports (Gómez-Climent et al., 2008). In fact, a putative insulating role was previously proposed for this glial coverage (Gómez-Climent et al., 2008). Consequently, it is possible that the type II cells in the most advanced stage of maturation lack this coverage, allowing them to start synaptogenic processes.

In the first experiment of this thesis, I have demonstrated the presence of D2r on the population of immature neurons expressing PSA-NCAM in the rat PCX layer II, as well as the presence of TH immunoreactive fibers in the close vicinity of the somata of these cells. In connection with this, studies in non-human primates have shown that DCX+ cells and processes around the border of layers I/II were surrounded by or in close proximity to axon terminals immunolabeled for TH (Zhang et al., 2009). Therefore, I considered interesting to explore the presence of D2r and TH fibers on the population of immature cells described in the layer II of the human cerebral cortex. Similar to what I have found in rats, in human samples the expression of D2r was detected in almost half of the PSA-NCAM-positive cells, confirming the previous hypothesis that the expression of these receptors may be restricted to certain phases of the development of these neurons. In addition, TH immunoreactive fibers were found in the close vicinity of these cells and this may also suggest that this population of immature neurons dispersed in the human cerebral cortex layer II might be influenced by monoaminergic neurotransmission. These findings may have clinical relevance, especially considering that the development and final maturation of these cells might be modulated by antidepressant or anti-psychotic drugs, most of which act on monoaminergic neurotransmission. It is known, in fact, that certain antidepressants that modulate monoaminergic neurotransmission influence the number of immature neurons expressing DCX or PSA-NCAM in the hippocampus and PFC of rodents (Sairanen et al., 2007; Varea et al., 2007b; Perera et al., 2008). Moreover, a recent study has also demonstrated the influence of noradrenergic neurotransmission on the immature neurons of the adult rodent PCX layer II (Vadodaria et al., 2017). Additional support for this hypothesis of a monoaminergic control of the development of the immature neurons in the adult brain, comes from results obtained in the first experiment described in this thesis, in which I have shown that the number of immature neurons expressing PSA-NCAM in the rat PCX layer II can be modulated by the pharmacological manipulation of D2r.

Until now, the function of this population of immature cells described both in adult rodents and in larger gyrencephalic mammals remains still poorly known. Since the distribution of these immature neurons in rodents was primarily described in the paleocortex, a brain region specialized for olfaction (Gómez-Climent et al., 2008), the first plausible hypothesis was that these cells were involved in olfactory functions. Subsequently, considering the wider distribution of these cells in the neocortex of mammalian species with larger, gyrencephalic cortices, including humans, is tempting to speculate on their putative involvement in the regulation of higher-order brain integrative abilities, suggesting a plausible strategic role in advanced cortical brain functions (Bonfanti and Nacher, 2012; König et al., 2016; La Rosa et al., 2020). In connection to this, there are still many questions to be answered, specially regarding the involvement of these immature cells in intracortical circuits, the possibility that they may receive thalamic inputs or inputs from monoaminergic sources other than dopaminergic. Since the connectivity of these cells remains still unclear, further studies combining immunohistochemical and tracing studies are needed to highlight these questions.

Unfortunately, to date the putative fate of these population of immature cells is still a matter of debate. Consequently, it is very difficult to establish their specific function within the neural circuits. At present the favorite hypothesis is that these cells, which retain in the adult CNS a molecular profile of immaturity, represent a *"reservoir"* of young plastic neurons that under physiological or pathological circumstances complete their differentiation program to be consequently recruited into the preexisting neural circuits (Gomez-Climent et al., 2010; Bonfanti and Nacher, 2012; König et al., 2016; Piumatti et al., 2018; La Rosa et al., 2019, 2020).

Chapter 6

CONCLUSIONS

1. In the three-layered rat PCX there are sparse dopaminergic fibers and many of them are in close apposition to PSA-NCAM expressing neurons in the layer II.

2. PSA-NCAM immunoreactive neurons in the rat PCX layer II express the D2r.

3. A chronic treatment with haloperidol significantly increases the density of PSA-NCAM immunoreactive cells in the rat PCX layer II.

4. A chronic treatment with PPHT significantly decreases the density of PSA-NCAM immunoreactive cells in the rat PCX layer II.

5. The use of the inducible DCX- CreER^{T2}/Flox-EGFP transgenic mouse allows to follow the different stages of development of the population of immature neurons in the adult PCX layer II.

6. The enzyme EndoN efficiently depletes PSA from NCAM in the PCX layer II of the inducible DCX- CreER^{T2}/Flox-EGFP transgenic mouse.

7. The enzymatic removal of PSA from NCAM does not induce changes in the morphology, the percentage of cells in the different stages of development or the density of EGFP+ cells in the PCX layer II of adult mice.

8. The enzymatic removal of PSA from NCAM increases significantly the density of EGFP+ complex cells displaying an AIS in the PCX layer II of adult mice.

9. The enzymatic removal of PSA from NCAM increases significantly the density of EGFP+ dendrites and dendritic spines in the layer I of the PCX of adult mice, but does not induces specific changes in the density of the different types of spines: stubby, mushroom and thin.

10. The enzymatic removal of PSA from NCAM does not induce changes in the percentage of EGFP+ dendritic spines apposed to puncta expressing the presynaptic markers SYN and VGLUT1 in the PCX layer I of adult mice.

11. The enzymatic removal of PSA from NCAM does not induce changes in the density and the fluorescence intensity of VGLUT1+ excitatory puncta in the perisomatic region of EGFP+ complex cells located in the PCX layer II of adult mice.

12. The enzymatic removal of PSA from NCAM does not induce changes in the density and in the fluorescence intensity of PV+ and/or VGAT+ inhibitory puncta in the perisomatic region of EGFP+ complex cells located in the PCX layer II of adult mice.

13. The enzymatic removal of PSA from NCAM increases significantly the density of NeuN expressing nuclei in the PCX layer II of adult mice.

14. The enzymatic removal of PSA from NCAM does not induce changes in the density of CAMK-II expressing cells in the PCX layer II of adult mice.

15. The enzymatic removal of PSA from NCAM increases significantly the percentage and density of EGFP+ cells expressing NeuN in the PCX layer II of adult mice.

16. The enzymatic removal of PSA from NCAM does not induce changes in the percentage and density of EGFP+ cells expressing DCX in the PCX layer II of adult mice.

17. The enzymatic removal of PSA from NCAM does not induce changes in the percentage and density of EGFP+ cells expressing CAMK-II in the PCX layer II of adult mice.

18. In the human cerebral cortex layer II, specifically in the frontal, temporal, parietal and occipital lobes, there is a widely distributed population of cells expressing DCX and PSA-NCAM.

19. The distribution, morphology and phenotype of cells expressing DCX and PSA-NCAM in the human cerebral cortex layer II is similar in control, epileptic and non-epileptic patients.

20. The epileptic status induced by pilocarpine does not affect the density of PSA-NCAM expressing cells in the rat PCX layer II.

21. In the human cerebral cortex layer II, there are DCX expressing cells with different morphologies suggestive of different stages of development. These cells can be mainly classified as type I or type II, but cells with intermediate morphology also exist.

22. The type I DCX expressing cells are small and bipolar cells with a soma diameter between 3-9 μm and with a single short process restricted to layer II.

23. The DCX expressing cells with an intermediate morphology are cells with a soma diameter between 9-11 μm, a thin basal process and 1 or 2 short dendrites without spines or excrescences.

24. The type II DCX expressing cells are large and multipolar cells with a soma diameter between 11-17 μm and longer dendritic arborizations, mainly oriented parallel to layer II. These type II cells display a thin process resembling an axon, even if in none the AIS is detectable. The dendrites of type II cells show some protrusions (length $\leq$ 1 μm) resembling stubby dendritic spines.

25. Most of the DCX+ cells of the human cerebral cortex layer II coexpress PSA-NCAM and do not express the mature neuronal marker NeuN; consequently, they can be considered immature neurons.

26. The immature neurons of the human cerebral cortex layer II belong to the excitatory lineage. They express the transcription factors CUX1, CTIP2 and TBR1 and lack the inhibitory marker GAD67.

27. The type II DCX expressing cells of the human cerebral cortex layer II show puncta expressing inhibitory and excitatory synaptic markers apposed to their somata and dendrites.

28. The immature neurons of the human cerebral cortex layer II do not express typical markers of glial cells, such as GFAP, NG2 and IBA1.

29. In the human cerebral cortex layer II, the immature neurons show astroglial and microglial processes apposed to their somata and dendrites.

30. In the human cerebral cortex layer II, there are sparse fibers expressing TH, many of which are in close apposition to immature neurons. These cells express D2r.

Chapter 7

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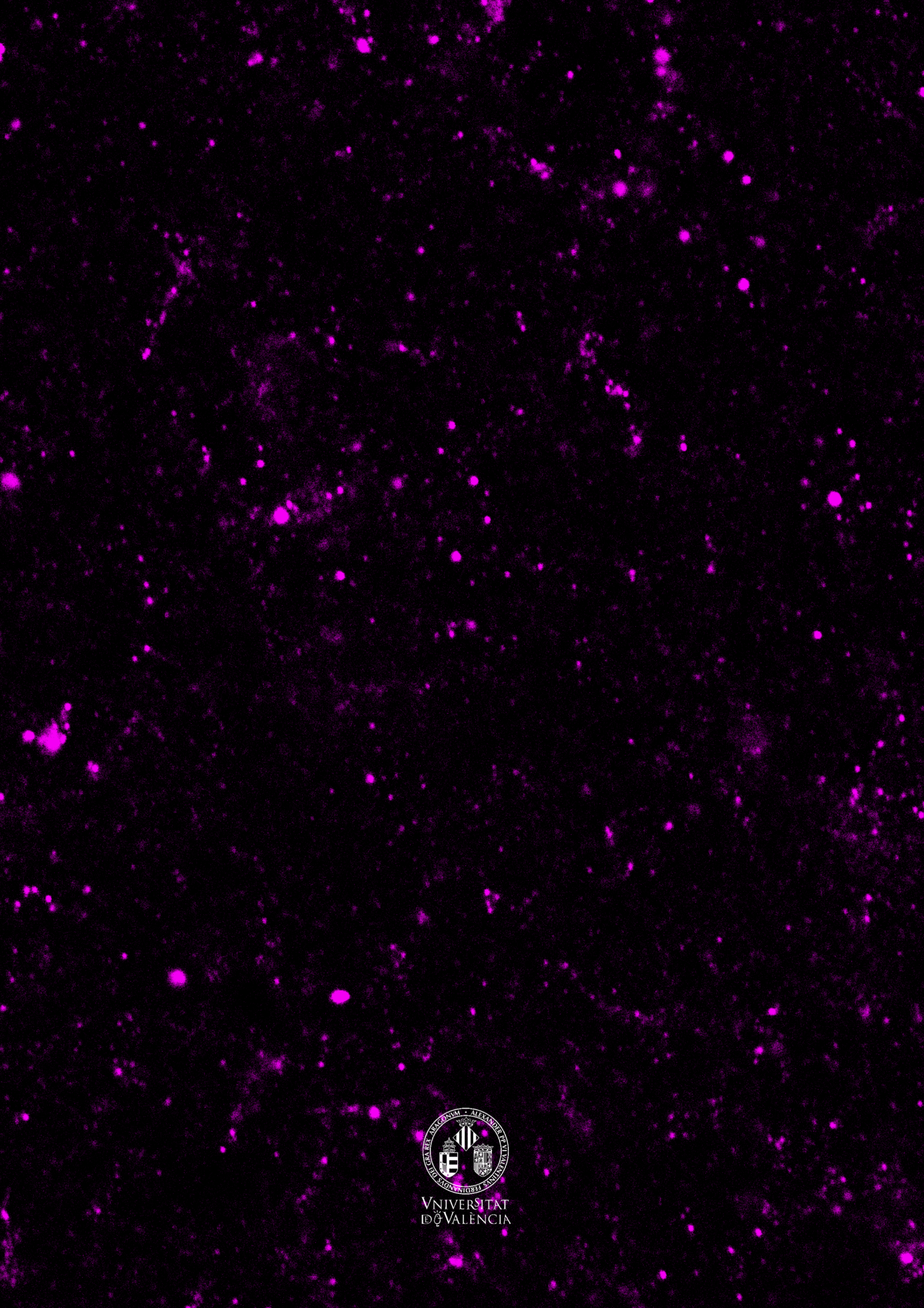
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