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The Choroid Plexus as a source and target of Prolactin in the brain

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Dedicatory

To my family, friends, and all that supported me throughout this journey. Thank you.

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Resumo Alargado

Os plexos coróides (CPs), localizados em cada um dos ventrículos cerebrais, são constituídos por uma camada de células epiteliais cubóides situadas sobre tecido conjuntivo altamente irrigado. Para além de serem os principais produtores de líquido cefalorraquidiano (CSF) no sistema nervoso central, os CPs são também responsáveis pela produção e secreção de péptidos com a capacidade de modular a função do cérebro. Os péptidos com origem no CP foram associados a diversas funções fisiológicas, incluindo inflamação e resposta imunológica, sinalização, proliferação e crescimento celular, morte celular, metabolismo e angiogénese, entre outras. As proteínas que constituem o secretoma do CP têm sido implicadas na modulação da neurogénese durante as fases de desenvolvimento bem como na idade adulta. A identificação de fatores com potencial neurogénico é extremamente relevante considerando o crescente envelhecimento populacional verificado a nível mundial e a alta incidência de doenças neurodegenerativas.

Dados preliminares de um estudo de *microarrays* com amostras de CP de rato, previamente realizado pelo nosso grupo de trabalho, sugerem a ocorrência da expressão de diversos fatores com potencial para promover a neurogénese, que não tinham sido anteriormente associados a esta estrutura do cérebro. Um dos transcritos mais relevantes, identificados neste estudo foi a prolactina. A prolactina é uma hormona polipeptídica sintetizada e secretada maioritariamente por células especializadas da glândula pituitária (ou hipófise). Para além das relacionadas com a reprodução nos mamíferos, esta hormona pleiotrópica está associada a centenas de funções biológicas distintas. As evidências científicas sugerem que a prolactina desempenha um papel como indutora da neurogénese em fases específicas da gestação e da amamentação em roedores. Para além disso, no nosso estudo prévio de *microarrays* foi também observado que a presença de transcritos de prolactina era mais elevada nos CPs recolhidos de fêmeas que nos CPs de machos, sugerindo que as hormonas sexuais podem desempenhar um papel relevante na modulação da expressão de prolactina no CP.

Com base na informação descrita anteriormente, um dos objetivos desta tese foi investigar se o CP é de facto uma fonte alternativa de prolactina no cérebro de rato. No Capítulo 4 apresentamos evidências de que os transcritos de prolactina estão presentes em CPs recolhidos de fêmeas gestantes, em células epiteliais de CP (CPEC) de rato e em

células da linha celular Z310 (linha celular de células epiteliais de CP de rato). Adicionalmente, foi ainda detetada, por *Western blot,* a presença de uma proteína imunorreativa, a prolactina com 63 kDa, em extratos proteicos de CP de rato, de extratos proteicos de CPEC e de células Z310, assim como em sobrenadantes de meio de cultura previamente incubado com pituitárias de rato e amostras de soro e de CSF de ratos. A utilização de um modelo *in vitro* da barreira sangue-CSF, com culturas primárias de CPEC de rato em insertos de placas de cultura, permitiu observar a presença da mesma proteína imunorreativa à prolactina tanto no compartimento superior (CSF) como no compartimento inferior (sangue) do referido modelo de cultura. Estes dados parecem indicar que as CPEC são capazes de secretar prolactina não só pelo lado apical das células, que se encontra em contacto com o CSF, mas também pelo lado basal das células em contacto com o sangue. Paralelamente, e utilizando o mesmo modelo *in vitro*, foi ainda possível observar por imunocitoquímica, que os recetores de prolactina (PRLR) se encontram distribuídos tanto no citoplasma como nas membranas basal e apical das CPEC.

Avaliámos também a possível influência das hormonas sexuais na produção de prolactina no CP de rato num modelo experimental de CP *ex vivo*. Para isso, explantes de CPs foram incubados durante 24 horas com diferentes concentrações de estradiol, progesterona e dihidrotestosterona, como descrito no Capítulo 5. Como esperado, a incubação com estradiol promoveu o aumento da secreção de prolactina nos explantes de pituitária, que serviram como controlos positivos da experiência. No entanto, nem a progesterona nem a dihidrotestosterona influenciaram a produção e a secreção da prolactina no tecido pituitário. Em relação ao CP, e contrariamente ao que tinha sido observado no estudo de *microarrays*, nenhuma das hormonas teve influência nos níveis de prolactina no CP, pelo menos nas concentrações testadas neste trabalho.

Por outro lado, além de ser uma fonte alternativa de prolactina, o CP é a estrutura do cérebro que expressa PRLR em maior quantidade. Inicialmente, a elevada expressão de PRLR no CP foi associada à existência de um mecanismo de transporte de prolactina para o cérebro mediado pelos PRLR presentes no CP. Contudo, os dados reportados recentemente, obtidos com um modelo de ablação de PRLR no CP de murganhos, mostraram que o transporte de prolactina para o cérebro não é mediado pelos PRLR. O mecanismo exato de transporte de prolactina para o sistema nervoso central é ainda desconhecido, embora o transporte a nível da microvasculatura seja apontado como a provável via de entrada para o cérebro. Por estes motivos, a informação relativa ao papel da prolactina e dos seus recetores no CP é atualmente escassa, especialmente em fases pós-natais. Consequentemente, outro dos objetivos principais desta tese consistiu

na avaliação do efeito da exposição de prolactina no transcriptoma do CP de ratos pós-natais. Como descrito ao longo do Capítulo 6, a incubação de explantes de CPs recolhidos de ratos recém-nascidos foi associada a uma diminuição da expressão de osteopontina (*Spp1*), uma proteína previamente implicada na indução de neurogénese mediada pelo CP. Adicionalmente, a exposição a prolactina reduziu a expressão de genes associados a proteínas de barreira, nomeadamente da claudina 5 (*Cldn5*), bem como de proteínas de proliferação celular, como a ciclina D1 (*Ccdn1*). Para além disso, a exposição à prolactina levou ao aumento da expressão de citocina pró-inflamatória interleucina 1 beta. Em suma estes dados parecem sugerir que a exposição à prolactina durante a fase pós-natal parece aumentar a permeabilidade do CP, reduzir a proliferação celular e ter um efeito pro-inflamatório no CP de rato.

Por último, este trabalho teve como objetivo identificar a existência de outros fatores com potencial neurogénico com origem no CP de rato. No Capítulo 7, são apresentadas evidências de que o CP de rato pode ser uma fonte alternativa de proteína secretada relacionada ao recetor *frizzled* 2 (SFRP2). Como consequência da semelhança com o domínio extracelular dos recetores *frizzled*, a SFRP2 compete com estes recetores pela ligação a fatores solúveis *Wnt*, interferindo com esta via de sinalização. A nível do funcionamento do cérebro, esta proteína foi precedentemente associada à modulação da proliferação, diferenciação e migração de células estaminais e progenitoras, assim como à regulação da densidade de espículas dendríticas, sendo também implicada na patologia de algumas doenças neurodegenerativas. Para além disso, os resultados descritos no Capítulo 7 parecem indicar que o estradiol é um dos reguladores dos níveis de SFRP2 no CP. Apesar da exposição ao estradiol aumentar os níveis de SFRP2 no CP, este aumento não é acompanhado por um aumento da secreção desta proteína.

Em resumo, as evidências reportadas ao longo deste trabalho reforçam a relevância do CP como produtor e secretor de péptidos com capacidade de modular a função do cérebro. Neste caso em particular, são apresentados resultados que suportam que o CP é uma fonte alternativa tanto de prolactina como de SFRP2 no cérebro. São necessários estudos adicionais para investigar as funções biológicas da prolactina e do SFRP2 produzidos pelo CP e avaliar se os mesmos têm alguma influência na modulação de neurogénese nos nichos neurogénicos adjacentes. Adicionalmente, será ainda necessário realizar estudos com o objetivo de identificar e investigar possíveis fatores com capacidade de regular a síntese, produção e secreção de prolactina e SFRP2 no CP de rato. Face ao crescente envelhecimento da população e ao aumento da incidência e da prevalência de doenças neurodegenerativas, a modulação da produção e secreção de fatores com potencial de indução de neurogénese com origem no CP pode ser

considerada como uma possível abordagem terapêutica. Nesse sentido, os resultados apresentados nesta tese podem servir como base de futuras investigações conducentes à identificação de novos alvos terapêuticos no tratamento de doenças neurodegenerativas.

Palavras-chave

Cérebro, Plexo coróide, Prolactina, Recetores da prolactina, Proteína secretada relacionada ao recetor *frizzled* 2, Hormonas sexuais.

Abstract

The choroid plexuses (CPs) are composed of a single layer of cuboid epithelial cells laying on highly irrigated connective tissue. In addition to being the main producer of cerebrospinal fluid (CSF) in the central nervous system, the CPs are also responsible for the production and secretion of peptides that modulate brain function. CP-derived peptides have been associated with several physiological functions, including inflammation and immune response, signaling, cell growth and cell proliferation, cell death, metabolism, and angiogenesis, among others. Proteins present in the CP secretome have been implicated in the modulation of neurogenesis during developmental stages and adult life. The identification of new neurogenic factors is highly relevant considering the increasing ageing of the population and the incidence of neurodegenerative disorders.

Preliminary data from a rat CP microarrays study from our research group suggested local expression of several factors with the potential to promote neurogenesis that had never been associated with the CP before. One of the most relevant transcripts identified was prolactin. Amongst the numerous distinct biological functions attributed to prolactin, this hormone has been described as a neurogenic factor at specific stages of pregnancy and lactation in rodents. In addition, the presence of prolactin transcripts was higher in CPs collected from female rats than male rats, suggesting that sex hormones could modulate prolactin expression in the CP.

The first aim of this thesis was to investigate if the rat CP could indeed be an alternative source of prolactin to the brain. In Chapter 4 we provide evidence that prolactin transcripts were present in pregnant rat CP, CP epithelial cells (CPEC) and in the rat immortalized CP cell line, Z310. Furthermore, a 63 kDa immunoreactive PRL was detected by Western blot in CP protein extracts as well as in culture medium supernatants after the incubation with rat pituitary and samples of rat cerebrospinal fluid and serum. Moreover, prolactin immunoreactive protein was present in both compartments of the blood-CSF barrier model which may indicate that CPEC can secrete prolactin not only through the apical membrane facing the CSF but also through the basal membrane facing the blood.

To ascertain the possible influence of sex hormones in the production of prolactin in the rat CP as initially hypothesized, we performed 24-hour incubations of CP explants with different concentrations of estradiol, progesterone, or dihydrotestosterone.

Nonetheless, neither of the hormones seem to modulate the levels of 63 kDa prolactin in the CP tissues, at least at the concentrations tested in this work, as described in Chapter 5.

Besides being a source of prolactin, the CP is the brain structure with the higher expression of prolactin receptors (PRLR). The high expression of PRLR at the CP was initially associated with the existence of a receptor-mediated mechanism responsible for prolactin transport to the brain present. Nevertheless, considering recent evidence, it is now accepted that prolactin uptake is independent of its receptors. Information about the exact function of prolactin in the CP is still very scarce, especially in the postnatal stages. As so, another main goal of this thesis was to evaluate the effects of prolactin in the transcriptome of postnatal rat CP. In Chapter 6 we observed that prolactin exposure was associated with a reduction in neurogenesis-factor osteopontin, barrier protein claudin 5, and proliferation-related cyclin D1. On the other hand, prolactin incubation also led to an increase in pro-inflammatory interleukin 1 beta expression, suggesting that at a postnatal stage, prolactin exposure may increase the CP permeability, reduce the cellular proliferation and have a proinflammatory effect on the rat CP.

Another goal of this thesis was to identify additional neurogenic factors secreted by the CP. In Chapter 7 we provide evidence that the rat CP may be a source of secreted frizzled-related protein 2 (SFRP2), previously described as a regulator of neural stem cells proliferation, differentiation and homeostasis. Notably, estradiol seems to modulate the levels of SFRP2 in the rat CP.

In summary, the evidence reported throughout this work supports the relevance of the CP as a source of new peptides with the potential to modulate brain function, like prolactin and SFRP2. Further studies are necessary to understand the relevance of CP-derived prolactin and SFRP2 in brain function, especially in neurogenesis.

Keywords

Brain, Choroid plexus, Prolactin, Prolactin receptor, Secreted frizzled-related protein, Sex hormones.

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Abbreviations

AKT	Protein kinase B
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenic protein
BCSFB	Blood-cerebrospinal fluid barrier
Ccnd1	Cyclin D1
cDNA	Complementary deoxyribonucleic acid
Cldn5	Claudin 5
CNS	Central nervous system
CP	Choroid Plexus
CPEC	Choroid plexus epithelial cells
CRD	Cysteine-rich domain
CSF	Cerebrospinal fluid
CypA	Cyclophilin A
DAB	Diaminobenzidine
DAVID	Annotation, Visualization and Integrated Discovery program
DG	Dentate gyrus
DHT	4,5α-dihydrotestosterone
DIV	Day *in vitro*
DMEM	Dulbecco's Modified Eagle medium
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
ERK	Extracellular signal regulated kinase
E2	17β-estradiol
FBS	Fetal bovine serum
FGF2	Fibroblast growth factor 2
FOXO3	Forkhead transcription factor 3
GAS	γ-interferon activated sequence
Grb2	Growth factor receptor-bound protein 2
IGF1	Insulin-like growth factor I
IGF2	Insulin-like growth factor II
IL1B	Interleukin-1 beta
Il6	Interleukin-6
JAK2	Janus protein kinase 2
KIR	Kinase-inhibitory region
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NRT	Netrin-related motif
NSC	Neural stem cells
Ocln	Occludin
OTX2	Homeobox protein OTX2
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol 3-kinase

PMSF	Phenylmethylsulfonyl fluoride
PRL	Prolactin
PRLR	Prolactin receptor
pSTAT5	Phosphorylated STAT5
P4	Progesterone
Raf	Rapidly accelerated fibrosarcoma
Ras	Rat sarcoma
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription Realtime quantitative PCR
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SFRP2	Secreted frizzled-related protein 2
SGZ	Subgranular zone
SHC	SHC-transforming protein 1
SH2	Src homology 2
SOCS	Suppressor of cytokine signaling
SOS	Son of sevenless
SPP1	Osteopontin
SRC	Proto-oncogene tyrosine-protein kinase Src
STAT	Signal transducer and activator of transcription
SVZ	Subventricular zone
T	Testosterone
TBS	Tris buffered saline
TEER	Transepithelial electrical resistance
TGFB2	Transforming growth factor beta-2
TIDA	Tuberoinfundibular
TTR	Transthyretin
TWSG1	Twisted gastrulation protein homolog 1
Wnt	Wingless
V-SVZ	Ventricular-subventricular zone

Chapter 1

Introduction – Part A

The choroid plexus as a source of neuropeptides

This chapter is part of the original review manuscript that will be submitted for publication as:

Costa-Brito AR, Quintela T, Gonçalves I, Santos CRA. The Choroid plexus secretome: relevance to brain function and homeostasis.

Some alterations to the original text were introduced to follow the aim of the thesis.

1.1. The choroid plexus structure and functions

The choroid plexuses (CPs), located in each of the four brain ventricles, are composed of a single layer epithelium of cuboid cells laying on highly irrigated connective tissue. They establish the blood-cerebrospinal fluid (CSF) barrier (BCSFB). The CP epithelial cells are bound together by tight junctions, which form a sealed surface on the apical side of the epithelia, adherens junctions and desmosomes [1–3]. The apical membrane of CP epithelial cells faces the CSF that fills the brain ventricles, and the basal membrane lays on a basal lamina at the interface with the stroma that is constituted by highly fenestrated capillaries, fibroblasts, connective tissue and other specialized cells, including immune cells, macrophages, microglia and even myeloid progenitor cells [4–9]. The BCSFB is a physical and biochemical barrier between the two compartments that allows the maintenance of the brain microenvironment homeostasis and provides neuroprotection (Figure 1.1) [10–13].

Over the past decades, the complexity of the CP functions gained new insights and has been extensively revised [7, 14–17]. Besides the core role in the production and clearance of CSF [11, 15, 18], several other functions have been assigned to the CP. It has a relevant function in immune surveillance as it is an immunological niche for T cells proliferation and stimulation within the brain [2, 6, 19, 20]. The CP contains a wide range of chemosensing receptors, transporters and detoxifying enzymes essential for chemical surveillance [21–24] and regulates metal homeostasis [7, 25, 26]. Moreover, all components of circadian clocks were identified in the CP, and some relevant peptides expressed in CP cells show well-defined circadian rhythms [27, 28], possibly regulating CSF production and clearance [29]. Of notice, many of the CP functions are sensitive to sex hormones and differ between male and female rats [30], including the circadian clock machinery, which is regulated by estrogens [27]. In addition to the tight control of the transport of molecules into and out of the brain, the CP is a highly secretory epithelium, a characteristic reflected by the high content of mitochondria and protein synthesis/secretion components associated with the exocytic pathway in the CP epithelial cells (CPEC) [11, 31]. The CP secretes approximately 500-600 mL per day of CSF into the brain ventricles in healthy adult humans, contributing to two-thirds of the total volume of CSF, making this brain structure the main producer of CSF [32, 33]. Besides the production of CSF, the CP contributes to the composition of this “nourishing liquor” with the release of numerous factors that reach the central nervous system [8, 9, 18, 31, 34, 35], including neurogenic peptides that regulate neurogenesis at the subventricular zone [8, 16–18]. Recently, the development of a cutting-edge model using two-photon imaging in awake mice, allowed for the first time the registration of some of the secretory mechanisms of CPEC and surveillance strategies of the CP cells *in vivo*. In response to external stimuli, CPEC calcium activity increases leading to the apocrine secretion of the cytoplasmic content through the apical side of these cells, directly modulating the CSF composition [36].

Alterations in the CP morphology, function and secretion have been described in several neurological pathologies like Alzheimer’s disease [37], schizophrenia [38], psychiatric disorders

[39], brain inflammation [40, 41] or even migraine [42]. However, the impact of neurodegenerative diseases on CP secretion and CSF composition has not been thoroughly studied, as depicted from the evident lack of studies dedicated to the study of the CP secretome.

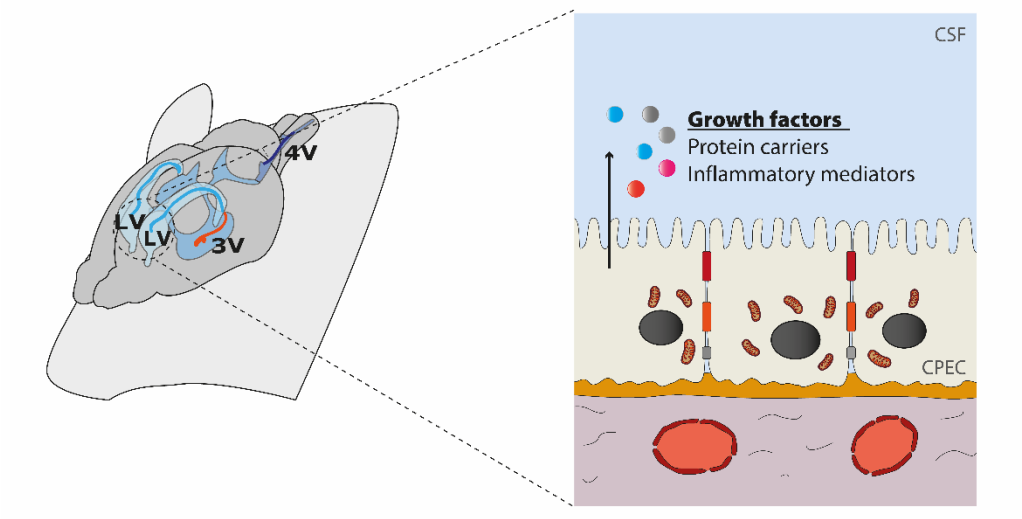

Figure 1.1. Location and structure of the choroid plexuses in the brain. The choroid plexuses (CPs) are structures located in each of the four brain ventricles. The CPs are composed of a single layer epithelium of cuboid cells laying on highly irrigated connective tissue, establishing a physical and biochemical barrier between the blood and the cerebrospinal fluid (CSF). The CP epithelial cells (CPEC) are bound together by tight junctions, which form a sealed surface on the apical side of the epithelia, adherens junctions and desmosomes. The CP contributes to the composition of the CSF with the release of numerous factors that reach the central nervous system, including neurogenic peptides and growth factors. LV: lateral ventricle; 3V: third ventricle; 4V: fourth ventricle.

1.2. The choroid plexus secretome

Although the amount of available information regarding the CP secretome is very limited, the number of published CP epithelium transcriptomic analyses, substantially more extensive, provide a relevant complementary tool for the study of protein expression and secretion by the CP. The analysis of CP samples from different species, using both proteomic and transcriptomic research approaches, unveiled how significant is the range of proteins originated from this brain structure which are released to the CSF.

Choroid plexus-derived proteins have been implicated in several physiological functions, including extracellular matrix or adhesion, inflammation and immune response, protein synthesis/degradation, signaling, transport, cell growth and cell proliferation, cell death, energy and metabolism, and angiogenesis [8, 9, 35, 43–49] (Table 1.1). Additionally, the CP is also a source of hormones to the brain, like growth hormone [8], insulin-like growth factor II (IGF2) [8, 46], melatonin [50], and the most recently discovered hormone augurin, also known as esophageal cancer-related gene 4 protein [51, 52]. It is important to mention that within the CP structure, other CP cells like fibroblasts and macrophages also express genes that encode growth factors and signaling molecules [4]. Overall, a total of 1400 proteins were identified in normal

Table 1.1. Most relevant molecules identified in the CP secretome.

Protein	Species	Stage	Sample	Technique	Reference
Extracellular matrix or adhesion					
Collagen alpha-1(XVIII) chain	Mouse	Adult	CP supernatant	Antibody arrays	[8]
Secreted phosphoprotein 1	Mouse	Adult	CP supernatant	Antibody arrays	[8]
Matrix metalloproteinase-3	Mouse	Adult	CP/CPEC cultures supernatant	MALDI-TOF MS, Antibody arrays	[8, 9]
Inflammation and immune response					
Complement C3	Mouse	Adult	CPEC cultures supernatant	MALDI-TOF MS	[9]
Complement factor H	Mouse	Adult	CPEC cultures supernatant	MALDI-TOF MS	[9]
C-X-C motif chemokine 16	Mouse	Adult	CP supernatant	Antibody arrays	[8]
Fractalkine	Mouse	Adult	CP supernatant	Antibody arrays	[8]
Interleukin-1 beta	Mouse	Adult	CP supernatant	Antibody arrays	[8]
Interleukin-6	Human	Adult	CP tissue	Immunoassay	[53]
Interleukin-8	Human	Adult	CP tissue	Immunoassay	[53]
Klotho	Mouse	Adult	CP tissue	IHC, WB	[54]
Moesin	Human	Adult	CP tissue	2D-DIGE	[55]
Pentraxin-related protein PTX3	Mouse	Adult	CPEC cultures supernatant	MALDI-TOF MS	[9]
Prostaglandin E2	Mouse	Juvenile	CP supernatant	ELISA	[56]
Proteasome activator complex subunit 1	Human	Adult	CP tissue	2D-DIGE	[55]
60 kDa heat shock protein	Human	Adult	CP tissue	IHC	[57]
90 kDa heat shock protein	Human	Adult	CP tissue	IHC	[57]
Protein synthesis/degradation					
Cathepsin B	Mouse	Development	CP supernatant	MS	[58]
Cathepsin D	Mouse	Development, adult	CPEC cultures/CP supernatants	MALDI-TOF MS, MS	[9, 58]
Carboxypeptidase E	Mouse	Adult	CPEC cultures supernatant	MALDI-TOF MS	[9]
Cystatin-C	Mouse	Development, adult	CPEC cultures/CP supernatants	MALDI-TOF MS, MS	[9, 58]
Serine protease inhibitor A3N	Mouse	Adult	CPEC cultures supernatant	MALDI-TOF MS	[9]
Plasminogen activator inhibitor 1	Mouse	Adult	CP/CPEC cultures supernatant	MALDI-TOF MS, Antibody arrays	[8, 9]
Signaling and transport					
Apolipoprotein E	Mouse	Adult	CPEC cultures supernatant	MALDI-TOF MS	[9]

Augurin	Human, rat	Adult	CPEC cultures supernatant	ELISA	[52]
Ceruloplasmin	Mouse	Adult	CPEC cultures supernatant	MALDI-TOF MS	[9]
Hepcidin	Mouse	Adult	CP tissue and CSF	qPCR	[26]
Neutrophil gelatinase-associated lipocalin	Mouse	Adult	CP supernatant	Antibody arrays	[8]
Neutrophil gelatinase-associated lipocalin	Mouse	Adult	CPEC cultures supernatant	MALDI-TOF MS	[9]
Proenkephalin-A	Mouse	Development	CP supernatant	MS	[58]
Retinol-binding protein 4	Mouse	Adult	CP supernatant	Antibody arrays	[8]
Serotransferrin	Mouse	Adult	CPEC cultures supernatant	MALDI-TOF MS	[9]
Transthyretin	Mouse	Adult	CPEC cultures supernatant	MALDI-TOF MS	[9]
Cellular growth and proliferation					
Amphiregulin	Mouse	Adult	CP tissue	IHC	[59]
Bone morphogenetic protein 5	Mouse	Adult	CP supernatant	Antibody arrays	[8]
Brain-derived neurotrophic factor	Rat	Neonatal	CPEC cultures supernatant	ELISA	[48]
C-X-C motif chemokine 12	Mouse	Adult	CP supernatant	Antibody arrays	[8]
Insulin-like growth factor I	Mouse	Adult	CP supernatant	Antibody arrays	[8]
Insulin-like growth factor II	Mouse	Adult	CP supernatant	Antibody arrays	[8]
Fibroblast growth factor 2	Human, mouse	Development, adult	CP tissues, CP supernatant	IHC, Antibody arrays	[8, 60]
Glial cell line-derived neurotrophic factor	Rat	Neonatal	CPEC cultures supernatant	ELISA	[48]
Growth differentiation factor 15	Rat	Adult	CP tissue	*In situ* hybridization	[61]
Insulin-like growth factor-binding protein 2	Mouse	Adult	CPEC cultures supernatant	MALDI-TOF MS	[9]
Insulin-like growth factor-binding protein 7	Mouse	Adult	CPEC cultures supernatant	MALDI-TOF MS	[9]
Metalloproteinase inhibitor 1	Mouse	Adult	CPEC cultures supernatant	MALDI-TOF MS	[9]
miR-204	Mouse	Adult	CP tissue, CSF supernatant	qPCR	[62]
Homeobox protein OTX2	Mouse	Adult	CP tissue	Immunoprecipitation, WB	[63]
Pigment epithelium-derived factor	Mouse	Adult	CPEC cultures supernatant	MALDI-TOF MS	[9]
Slit homolog 2 protein	Mouse	Development, Postnatal	CP	*Slit2* deletion	[64]
Sonic hedgehog protein	Mouse	Development	CP	*Shh* deletion	[65]

Transforming growth factor alpha	Rat, chicken	Neonatal, Adult	CP tissues	IHC	[43]
Transforming growth factor beta-2	Mouse	Adult	CP supernatant	Antibody arrays	[8]
Wnt-5a	Mouse	Development	CP supernatant	WB	[66]
Wnt-7b	Mouse	Adult	CPEC cultures supernatant	MALDI-TOF MS	[9]
Wnt-10A	Mouse	Adult	CPEC cultures supernatant	MALDI-TOF MS	[9]
Cellular death and apoptosis					
Amyloid-beta precursor protein	Human	Adult	CPEC cultures supernatant	ELISA	[67]
Annexin V	Human	Adult	CP tissue	2D-DIGE	[55]
Netrin-1	Mouse	Adult	CPEC cultures supernatant	MALDI-TOF MS	[9]
Energy metabolism					
Cytosolic 10-formyltetrahydrofolate dehydrogenase	Mouse	Development	CP supernatant	MS	[58]
Extracellular superoxide dismutase	Mouse	Development	CP supernatant	MS	[58]
Retinal dehydrogenase 2	Mouse	Development	CP supernatant	MS	[58]
Angiogenesis					
Matrix metalloproteinase-2	Mouse	Adult	CPEC cultures supernatant	MALDI-TOF MS	[9]
Vascular endothelial growth factor	Pig, Mouse	Neonatal, Adult	CPEC cultures/CP supernatant	ELISA, Antibody arrays	[8, 44]

CP: Choroid Plexus, CPEC: Choroid Plexus epithelial cells, IHC: Immunohistochemistry, MALDI-TOF MS: matrix assisted laser desorption ionization-time of flight mass spectrometry, MS: Mass Spectrometry, qPCR: Real-time quantitative Polymerase Chain Reaction, WB: Western Blot, 2D-DIGE: Two-Dimensional Difference Gel Electrophoresis.

adult rodent CP using multiple protein fractionation approaches [47], emphasizing the relevant contribution of the CP as a source of proteins and its importance in brain function.

After depletion of the most abundant proteins, approximately 56% of the total proteins identified in human CSF samples are considered CSF-specific (i.e., not derived from blood plasma), and are produced by the CP and other brain structures like astrocytes and neurons [68, 69]. Transthyretin (TTR), a protein involved in the transport of thyroid hormones and retinol (as a carrier of retinol-binding protein) in both plasma and CSF, is synthesized by CPEC, representing roughly up to 20% and 50% of the new synthesized and secreted proteins by the CP, respectively [9]. In this regard, the study of CSF composition is an additional valuable resource to investigate the composition of CP secretome. However, the identification of low abundance proteins present in the CSF is masked by the presence of high amounts of serum albumin isoforms, transferrin and immunoglobulins, accountable for the majority of the total protein mass present in this biological fluid [70–72] and by the existence of a large dynamic range of proteins concentrations [73, 74]. Nonetheless, the improvement of the sensitivity of proteomic techniques [75–77], together with the study of the proteome of extracellular vesicles present in the CSF [78–80], expanded our knowledge about the complexity of the CSF composition over the past two decades.

Since the first analysis of the human CSF proteome in the early 2000s [70, 81–83], a total of 3379 distinct proteins have been identified [75]. Interestingly, over-represented proteins identified in the CSF proteome belong to the apolipoprotein and insulin-like growth factor families or are involved in axon guidance regulation, complement and coagulation cascades, highlighting the considerable role of CSF proteins in brain function [75]. It is now accepted that CSF is composed of a combination of central nervous system-, immune- and growth-related proteins [68, 69, 71, 81, 82, 84–86].

Despite some knowledge of the CP secretome composition in rodents [9], not much information about the CP secretome is available in humans. However, it is possible to unveil some aspects of the human CP secretion profile by crossing the information of different public proteomic and transcriptomic databases. When we compared the proteins found in human CSF [75] that could be traced to known genes identified in transcriptomic databases of both human CP (accession number GSE110226) and human CPEC (accession number GSE61196) available at the NCBIs Gene Expression Omnibus repository (https://www.ncbi.nlm.nih.gov/geo/), it was possible to observe that the majority of proteins identified in the human CSF could be traced to CP and CPEC transcriptomes (2738/3357), possibly constituting the human CP secretome (Figure 1.2.A). Functional annotation analysis of the genes included in the intersection group of the three databases using the web-accessible program Annotation, Visualization and Integrated Discovery (DAVID) revealed that top Gene Ontology enriched terms with higher gene count include signal transduction and cell adhesion in biological processes, extracellular exosome and plasma membrane in cellular component, and protein binding and calcium ion binding in

molecular function categories (Figure 1.2.B). Additionally, top significantly enriched KEGG pathways include phosphatidylinositol 3-kinase (PI3K)/ Protein kinase B (Akt) signaling pathway, pathways in cancer, focal adhesion, axon guidance and endocytosis (Figure 1.2.C). On the other hand, functional annotation clustering analysis of the putative human CP secretome unveiled that top enriched clusters include genes associated with peptide signaling, epidermal growth factor-like domain and cell-cell adhesion (Figure 1.2.D).

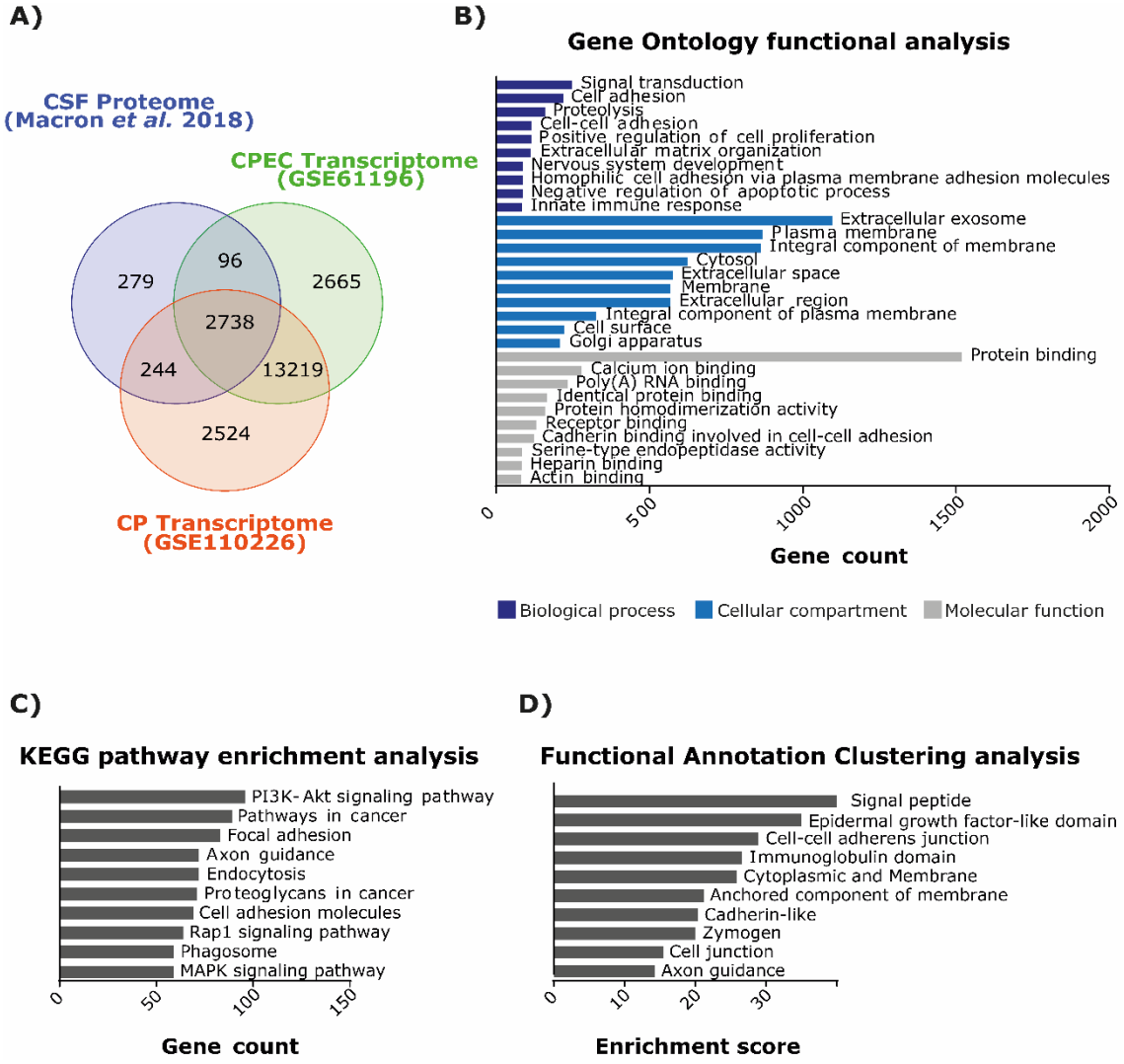

Figure 1.2. Summary of the main characteristics of the putative human choroid plexus secretome. (A) Venn diagram comparing the overlap of the human cerebrospinal fluid proteome [75], the human choroid plexus (CP) transcriptome (GSE110226) and the human CP epithelial cells (CPEC) transcriptome (GSE61196). The genes included in the obtained Venn diagram intersection group, constituting the putative human CP secretome, were analyzed using the web-accessible program Annotation, Visualization and Integrated Discovery (DAVID). The graphs show the top 10 Gene Ontology enriched terms with higher gene count included in biological process, cellular component and molecular function categories (B), the top 10 significantly enriched KEGG pathways (C) and the functional annotation clustering analysis of the putative human CP secretome (D).

1.3. Choroid Plexus secretome and neurogenesis

The presence of several growth factors in the CSF, with origin in the CP, during embryonic stages, including fibroblast growth factor 2 (FGF2), insulin-like growth factor I (IGF1), IGF2,

growth differentiation factor 4, sonic hedgehog, retinoic acid, bone morphogenic protein (BMP) and Wnt signaling family members highlighted the relevance of the CP-CSF system in prenatal brain development (reviewed by [17, 60, 65, 87–90]). However, some of these neurogenic factors, like FGF2, are also fundamental players in the regulation of adult neurogenesis, promoting the generation of oligodendrocytes and the differentiation of hippocampal new neurons [91–93].

During adulthood, the CP is near the subventricular zone (SVZ), which together with the subgranular zone (SGZ) of the dentate gyrus of the hippocampus, are the adult neurogenesis niches [94, 95]. In fact, due to the location of SVZ along the walls of the lateral ventricles and the privileged location of CPs within the same brain ventricles, the CP is considered a constituent of this particular neurogenic niche [88, 96]. Despite the lack of direct physical contact with CP or CSF, evidence support that CP and CSF-derived factors may also reach the SGZ (Figure 1.3) [18, 97]. Secreted factors present in conditioned medium from lateral ventricles' CP, more specifically BMP5 and IGF1, and to a less extent transforming growth factor beta-2 (TGFB2), endostatin, C-X-C motif chemokine 16, fractalkine, and osteopontin (SPP1), were able to increase the formation and proliferation of multipotent neurospheres in cell cultures from the ventricular-subventricular zone (V-SVZ), in an age-dependent manner [8]. Interestingly, while IGF1 promotes progenitor cell proliferation during adult neurogenesis, IGF2, highly expressed in the mice CP, is responsible for the maintenance of neuronal stem cells in the SVZ [98].

Similar results have been reported in a transcriptomic study conducted to profile the expression of ligands that regulate neurogenesis in distinct cell populations, with the most enriched CP specific ligands linked to secretome pathway being *Igf2*, *TGFb2*, twisted gastrulation protein homolog 1 (*Twsg1*), *Bmp6* and angiopoietin-related protein 2 [99]. In addition to the important role in neurogenesis during embryogenesis, the expression of BMP signaling and its regulator TWSG1 seem to remain high in the CP of adult mice [100]. Nonetheless, while BMP promotes the proliferation of neuronal precursors during development, it seems to encourage the maintenance of hippocampal stem cells' quiescence in adulthood (reviewed by [101]). Secreted factors from rat CP epithelial cells culture also seem to support neurite outgrowth and survival of hippocampus neurons [102]. Furthermore, upregulation of brain-derived neurotrophic factor (BDNF), IGF1, neurotrophin-3 and interleukin-10 in the CP, induced by copolymer-1, a peptide used in the treatment of multiple sclerosis with potential therapeutic effect in stroke, has been correlated with the increase in SVZ and SGZ neurogenesis after transient middle cerebral artery occlusion in rats [103].

In addition, IL1B expressed by the CP and secreted into the CSF, upregulates vascular cell adhesion molecule 1, involved in the regulation of SVZ lineage progression, promoting the maintenance of neural stem cells and consequently the reduction of proliferation and lineage progression [104]. Likewise, homeobox protein OTX2 (OTX2) secreted by the CP has also been associated with V-SVZ neurogenesis, regulating extracellular matrix and signaling factors after

being transferred into non-neurogenic astrocytes of the V-SVZ and rostral migration stream [63]. Moreover, OTX2 knockdown in the CP reduced the number of newborn neurons in the olfactory bulb, as a result of possible effects on neuroblast migration [63]. *In vitro*, in both mice and human models, SVZ neural progenitor cells migration is also regulated by slit homolog 2 protein, a protein with chemorepulsive activity secreted by the CP [64, 105]. Evidence suggests that CP is also a major source of prostaglandin E2, a potent regulator of glutamate release in astrocytes from the SVZ, involved in the control of neuroblasts proliferation and survival [56], and amphiregulin, a mitogen from the epidermal growth factor (EGF) family, involved in adult neural stem cells proliferation and neurogenesis [59]. Recently, the CP has also been associated with post-transcriptional control of the number of neural stem cells (NSC) in a quiescent state, promoting the maintenance of NSC during adulthood, mediated by CP release of microRNA 204 into the CSF [62].

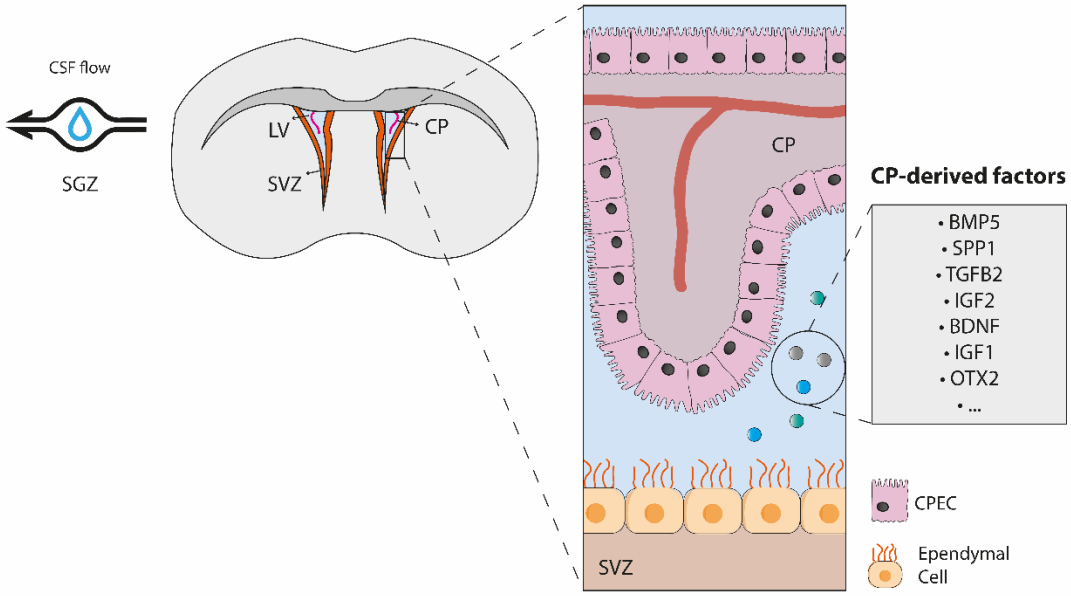

Figure 1.3. Schematic representation of the choroid plexus as a source of neuropeptides. Choroid plexus (CP)-derived peptides are fundamental players in the regulation of development and adult neurogenesis. The CP is near the subventricular zone (SVZ), which together with the subgranular zone (SGZ) of the dentate gyrus of the hippocampus, are the adult neurogenesis niches. Despite the lack of direct physical contact with CP or cerebrospinal fluid (CSF), evidence support that CP and CSF-derived factors may also reach the SGZ. CP-derived factors were previously associated with the increased formation and proliferation of neurospheres in cell cultures from the ventricular-subventricular zone, the promotion of the generation of oligodendrocytes and the promotion of the differentiation of hippocampal new neurons. BDNF: brain-derived neurotrophic factor; BMP5: bone morphogenic protein 5; IGF1: insulin-like growth factor I; IGF2: insulin-like growth factor II; OTX2: homeobox protein OTX2; SPP1: osteopontin; TGFB2: transforming growth factor beta-2.

Furthermore, in a study conducted to uncover the secretory molecule expression profile of mice SVZ niche cells, including the CP, the treatment of neurosphere cultures with exogenous TTR reduced the number of neurosphere formation and cell proliferation. This reduction may be a consequence of TTR binding/sequestering of triiodothyronine present in the culture medium, as suggested by the authors [96]. The same study provided evidence that other secreted molecules

are expressed in the CP (e.g., folate receptor alpha, insulin-like growth factor-binding protein 2, prostaglandin-H2 D-isomerase and prolactin receptor (*Prlr*)), but these factors were not validated for their potential function as neural stem cells niche signals in *in vitro* experiments [96]. Notwithstanding, *Ttr* knockout mice presented gender-independent increased oligodendrogenesis to neurogenesis ratio in the lateroventral SVZ (favoring glia/neuron balance), while this increase was only observed in the dorsal SVZ of male mice, suggesting that neuronal differentiation is possibly hindered in the absence of TTR [106]. Although not directly linked with neurogenesis, the CP is also the source of growth differentiation factor 15, a potent neurotrophic factor associated with the protection of neurons against iron cytotoxicity, promoting dopaminergic neurons proliferation both *in vitro* and *in vivo* in rats [61].

Besides the fundamental role in adult neurogenesis regulation, increasing evidence suggests that the CP itself may also harbor a variety of different multipotent progenitor cells [5, 107–111]. Overall, the CP is responsible for the secretion of growth factors that directly impact and regulate embryonic and adult neurogenesis. Moreover, the CP is also a source of neuronal, hematopoietic and myeloid progenitor cells, representing an additional source of pluripotent cells that possibly may have an important role under pathological conditions.

1.4. Conclusions

The CP is much more than just a mere BCSFB or the main producer of CSF. It is responsible for diverse functions, including the production and secretion of hundreds of factors that directly or indirectly impact brain homeostasis. The improvement of transcriptomic and proteomic techniques played a valuable contribution to broadening the knowledge of CP-derived peptides. The CP is not only fundamental to the proper function of the brain in healthy conditions but also plays a role in brain disease either by undergoing structural and physiological changes that contribute to disease onset or progression or by secreting factors that help mitigate some of the adverse effects observed in several pathologies, protecting the brain against disease. One of the most relevant roles of the CP is the production and secretion of neurogenic factors that are responsible not only for the induction of progenitor stem cell proliferation and differentiation but also for the maintenance of senescent states of NSC, contributing to the preservation of neuronal progenitor pools throughout life. Overall, the CP is responsible for the release of numerous peptides that influence normal and pathological brain function. However, further studies are necessary to better understand the complexity of the CP secretome and the impact of its alterations on brain function.

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Chapter 2

Introduction – Part B

The brain as a source and a target of prolactin in mammals

This chapter corresponds to the original review article:

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2.1. Abstract

Prolactin (PRL) is a polypeptide hormone associated with an extensive variety of biological functions. Among the roles of PRL in vertebrates, some were preserved throughout evolution. This is the case of its function in the brain, where PRL receptors (PRLR), are expressed in different structures of the central nervous system (CNS). In the brain, PRL actions are principally associated with reproduction and parental behavior, and involves the modulation of adult neurogenesis, neuroprotection, and neuroplasticity, especially during pregnancy, thereby preparing the brain to parenthood. PRL is mainly produced by specialized cells in the anterior pituitary gland. However, during vertebrate evolution many other extrapituitary tissues do also produce PRL, like the immune system, endothelial cells, reproductive structures and in several regions of the brain. This review summarizes the relevance of PRL for brain function, the sources of PRL in the CNS, as well as its local production and secretion. A highlight on the impact of PRL in human neurological diseases is also provided.

Keywords: Brain, Brain diseases, Choroid plexus, Neurogenesis, Neuroplasticity, Neuroprotection, Prolactin, Prolactin receptor

2.2. Introduction

Prolactin (PRL) is a pleiotropic hormone responsible for many biological functions that go far beyond its roles in reproduction and lactogenesis, like the modulation of the immune system, growth and metabolism, osmoregulation and regulation of brain function. These are well-documented roles of PRL in mammals, as well as in other vertebrates [1, 2]. The effects of PRL on the mammalian brain depend on factors such as age, gender, and reproductive status [3–5]. The functions of PRL in the brain are particularly relevant during pregnancy and lactation which are characterized by high levels of PRL [6]. PRL induces neurogenesis during pregnancy in mice [7–9] and parental behaviors, which have been conserved along evolution, from fish to mammals [2], and modulates other brain functions [10, 11].

The main goal of this review is to highlight the most recent findings on the effects of PRL in the function of the mammalian brain. A brief overview of the main signaling cascades elicited by PRL in the brain and the distribution of extrapituitary sources of PRL in the central nervous system (CNS) is initially provided followed by a thorough review on the role of PRL in the induction of neurogenesis, neuroprotection and neuroplasticity. The therapeutic potential of this neuropeptide in brain disorders is also discussed.

2.3. Search Strategy and Selection Criteria

Research articles published between January 2016 and May 2021 were retrieved from the PubMed database using the following terms: prolactin, brain, parental behavior, prolactin receptor signaling, neurogenesis, neuroprotection, neuroplasticity, and neurological disorders. Although bibliographic revision focused on the last five years earlier mandatory references in the field, were also considered.

2.4. PRL structure and prolactin variants

PRL is a polypeptide hormone mainly produced in the lactotrophs of the anterior pituitary gland [1]. The mature PRL protein of pituitary origin is composed of 197-199 amino acids in mammals [12]. PRL appeared early in evolution and remain relatively conserved [2], presenting high homology levels in mammals (Table 2.1). In vertebrates, PRL is encoded by a single gene composed of 5 exons and 4 introns [13]. However, despite sharing some common structural and regulatory features, the PRL gene is distinct between species. For instance, the human PRL gene contains an additional noncoding exon. This superdistal promoter region, firstly identified in the human decidua, is responsible for the regulation of its transcription at extrapituitary sites [14].

PRL is part of a family of polypeptide hormones that have similar structural and biological characteristics, which includes the growth hormone and placental lactogen. Human PRL is a 23 kDa protein, structurally composed by four antiparallel α-helices [15]. Moreover, several other PRL isoforms, resulting from proteolytic cleavage, alternative splicing and post-translational

modifications like glycosylation, dimerization and association with other circulating proteins, have also been described [13]. It is hypothesized that different PRL isoforms have distinct functions and biological activity, contributing in part to the plethora of PRL functions. For instance, in humans, macroprolactin (> 100 kDa) has reduced biological activity [16]. On the other hand, vasohinibin (a 16 kDa PRL isoform) is a potent anti-angiogenic factor, while 23 kDa PRL seems to have angiogenic properties [17].

Table 2.1. Comparison of prolactin gene/protein in different species.

Species	Exon Number	Size (amino acids)	Homology*	Accession number†
Human (*Homo sapiens*)	6	227	-	NP_001157030.1
Chimpanzee (*Pan troglodytes*)	6	227	98.68 %	XP_016810474.1
Rhesus monkey (*Macaca mulatta*)	6	227	97.80 %	NP_001040593.1
Rabbit (*Oryctolagus cuniculus*)	5	227	78.85 %	NP_001076144.1
Sheep (*Ovis aries*)	5	240	72.69 %	NP_001009306.1
Cattle (*Bos taurus*)	5	229	73.13 %	NP_776378.2
Chicken (*Gallus gallus*)	5	229	66.52 %	NP_990797.2
Rat (*Rattus norvegicus*)	5	226	62.22 %	NP_036761.1
Frog (*Xenopus laevis*)	5	230	61.23 %	NP_001093699.1
Mouse (*Mus musculus*)	5	228	60.89 %	NP_035294.2
Zebrafish (*Danio rerio*)	5	210	31.88 %	NP_852102.2

*Homology (percent identity) between human prolactin precursor protein and prolactin precursor protein from different species was analyzed using the multiple sequence alignment Clustal Omega program (runed online in EBI web server, https://www.ebi.ac.uk/Tools/msa/clustalo/).

†Input protein sequences were downloaded from NCBI protein database (https://www.ncbi.nlm.nih.gov/protein/).

2.5. PRL action is mediated by its receptors

PRL receptors (PRLR) belong to the type I cytokine receptor family and are composed by three domains: extracellular, transmembrane, and intracellular [18]. Similarly to other members of this family, the action of PRL is initiated when it binds to two PRLR units, forming a biological activated heterotrimeric complex [19]. It is important to notice that other hormones, like growth hormone and placental lactogen, also bind PRLR [13], and that in pregnancy, part of PRL signaling may be induced by placental lactogen [6]. Alternative splicing of the PRLR gene results in several PRLR isoforms with distinct intracellular domain lengths that vary between species. While rats have three distinct PRLR isoforms, long, short and intermediate [1, 15], several other additional PRLR isoforms, including two PRLR-short forms and a secreted soluble form, are present in humans [20]. However, the differences between the intracellular domain of the PRLR

do not have an impact in the elementary functions of the PRL, as observed in 84 mammalian species (Paré et al., 2021).

PRL binding to the long form of PRLR can elicit distinct signaling cascades mediated by tyrosine kinase activation (Figure 2.1), including Janus protein kinase 2 (JAK2) autophosphorylation and JAK2-mediated tyrosine phosphorylation of the receptors [13]. The JAK2 activation is the best characterized and one of the fastest mechanisms of action mediated by the PRLR-long form [21]. Binding of PRL to the PRLR-long form induces JAK2-mediated phosphorylation of signal transducer and activator of transcription (STAT) proteins that translocate to the nucleus. In the nucleus, STAT proteins bind to gene promoters that contain the γ-interferon activated sequence (GAS) DNA-binding motif and regulate the transcription of PRL target genes [22], constituting the canonical JAK/STAT pathway. In turn, JAK/STAT signaling is inhibited by proteins of the suppressor of cytokine signaling (SOCS) family (particularly SOCS1 and SOCS3), by a negative-feedback loop promoted by the activation of JAK/STAT pathway. SOCS proteins contain a Src homology 2 (SH2) domain and a kinase-inhibitory region (KIR) that inhibits the kinase activity of JAKs interfering with JAK/STAT signaling [23].

The mitogen-activated protein kinase (MAPK)/ extracellular signal regulated kinase (ERK) is another signaling pathway activated by PRL (Figure 2.1). Evidences from studies in human mammary epithelial cell lines, revealed that the activation of this pathway involves upstream intermediaries like SHC, growth factor receptor-bound protein 2 (Grb2), SOS, Ras and Raf [24], that lead to the phosphorylation of MAPK and ultimately regulate cell proliferation [25, 26]. Another signaling cascade associated with the PRLR-long form signaling described in human breast cancer cell lines, is phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (AKT) pathway (Figure 1). Briefly, PRL binding to PRLR activates Src, mediating the activation of PI3K and AKT that in turn modulates cell proliferation [26]. The exact mechanism of action of PRLR-short forms is unknown since the activation of JAK/STAT pathway is prevented. However, the PRLR-short isoform is able to activate the MAPK and PI3K/AKT pathways [15, 19, 27]. PRLR-short signaling was also associated with the inhibition of FOXO3 expression in the mice ovary, resulting in ovarian developmental defects [28]. On the other hand, the blockage of PRLR-short form with an antagonist in human uterine cancer cells lines was associated with a reduction in cell proliferation, possibly mediated by the reduction of PI3K/AKT activity and augmented FOXO3a nuclear translocation [27]. Conversely, the activation of the PRLR-short form in pancreatic ductal adenocarcinoma mice and in human models reduced cell proliferation. In this case the reduced cell proliferation was associated with decreased expression of genes involved in the pentose phosphatase pathway, through the activation of the Hippo signaling pathway [29]. Even though the available information regarding PRLR-short form signaling is conflicting, and that PRLR-short, like the PRLR-long signaling cascade, may be tissue and physiological state dependent, all data seem to reinforce that the PRLR-short form is involved in the modulation of relevant biological actions.

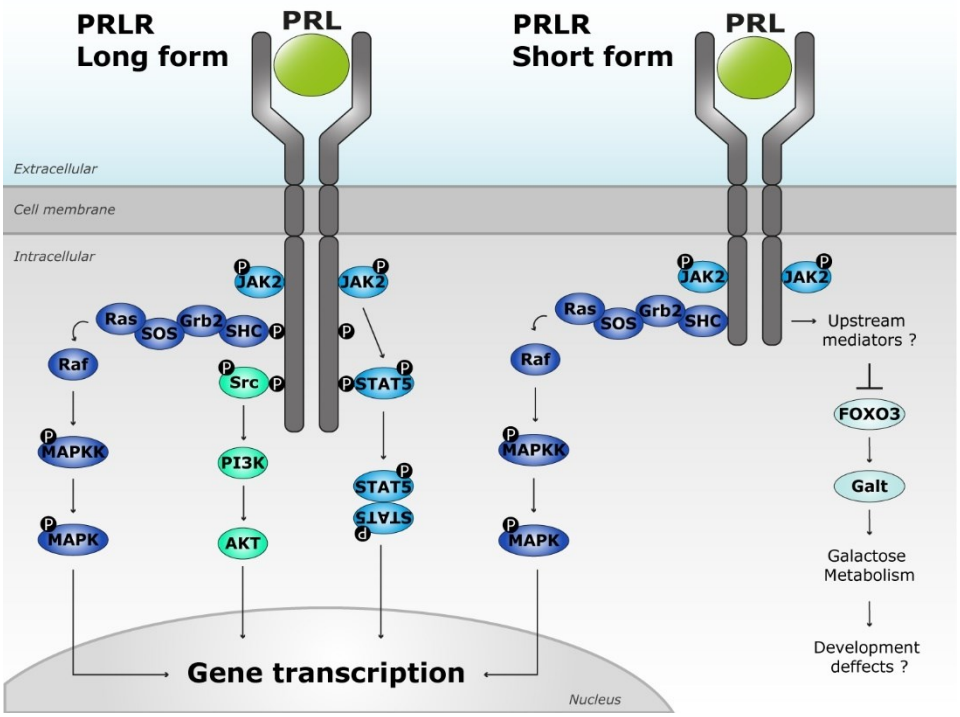

Figure 2.1. Main signaling pathways elicited by the activation of the long and short prolactin receptor isoforms. The action of prolactin (PRL) is initiated by its binding to a prolactin receptor (PRLR) homodimer, forming a heterotrimeric complex. PRL binding to the long form of PRLR triggers distinct signaling cascades that ultimately culminate in the regulation of gene transcription. The main activation pathway of PRL is the JAK/STAT pathway, but prolactin is also able to induce the PI3K/AKT pathway. The MAPK/ERK is another signaling pathway triggered by prolactin binding to both PRLR isoforms. The activation of this pathway involves the SHC, Grb2, SOS, Ras and Raf cascades. It is also believed that the activation of the PRLR-short form inhibits the expression of FOXO3 and Galt, involved in the metabolism of galactose to glucose, possibly leading to developmental defects. However, the exact mechanism of action of this pathway remains unidentified. AKT: Protein kinase B; ERK: Extracellular signal regulated kinase; FOXO3: Forkhead transcription factor 3; Galt: Galactose-1-phosphate uridyltransferase; Grb2: Growth factor receptor-bound protein 2; JAK2: Janus kinase 2; MAPK: Mitogen-activated protein kinase; MAPKK: MAPK kinase; PI3K: Phosphatidylinositol 3-kinase; Raf: Rapidly accelerated fibrosarcoma; Ras: Rat sarcoma; SHC: SHC-transforming protein 1; Src: Proto-oncogene tyrosine-protein kinase Src; SOS: Son of sevenless; STAT5: Signal transducer and activator of transcription 5.

Despite sharing the same signaling cascade, the target genes regulated by PRLR activation are cell type dependent. For instance, while in many cells PRL was associated with antiapoptotic and proliferative effects, in rat lactotrophs, PRL elicited proapoptotic and antiproliferative effects instead [30]. In this case, the PRL-induced apoptotic and antiproliferative effects were mediated by the JAK2/STAT5 pathway and inhibition of both ERK and Akt phosphorylation [30]. It is important to recognize that most studies dedicated to the investigation of type I cytokine receptor signaling cascades were performed in cancer cells, and that information regarding the biological actions mediated by this receptor family in healthy cells is still lacking.

2.6. PRL signaling in the brain

In mammals, PRLR are expressed in brain regions (Figure 2.2), like the amygdala, the preoptic area, the thalamus, the hypothalamus, the epithalamus and the brainstem [31–33]. It is

important to notice that most studies that investigated the expression of PRLR in the brain used probes that do not discriminate between the short and the long forms of PRLR. PRLR are also present in the subventricular zone (SVZ) and in the hippocampus in mice [34, 35], as well as in the circumventricular organs, like the median eminence, the subfornical organ and in the choroid plexus (CP) in rodents [32, 33, 36]. In fact, the CP is the region of the brain with the highest expression of PRLR-long form [33]. This PRLR form is located in the cytoplasm and both the apical and the basal membrane of rat CP epithelial cells [37].

Phosphorylation of STAT5, mediated by activated PRLR-long form, is considered to be the main PRLR signaling pathway in the mice brain [38], especially during pregnancy [39, 40]. However, neuronal ablation of STAT5 in mice revealed that STAT5 phosphorylation is not mandatory for the expression of nursing behaviors. In fact, the induction of faster responses observed in the medial preoptic area neurons, indicate that PRL-mediated effects in this brain region do not always involve the activation of transcription factors but rather other unidentified mechanisms of action [41].

Increasing evidences support the hypothesis that PRL elicits fast and transient calcium channels/transporters responses in specific subsets of hippocampal and hypothalamic neurons in mice [10, 42]. Recently, PRL-mediated action was associated with the modulation of membrane excitability in the neurons of the subfornical organ [36] and the arcuate nucleus [43] in rodents, with a particular relevant role of the transient receptor potential cation channel Trpc5 in the later. Other PRL-induced responses in the brain include the activation of ERK1/2 MAPK pathway in the hypothalamus and in hippocampal progenitor cells in rats [44, 45], or AKT phosphorylation in the hippocampus of male mice [46].

The expression of the PRLR-short form has been reported in hypothalamic areas, the amygdala, the thalamus and the CP of female rats [47, 48], and in the SVZ and the hippocampus of male mice [35]. Overall, PRLR-long and PRLR-short expression seem to overlap in several brain regions, but the expression of the PRLR-long form is usually higher than that of the PRLR-short [47]. Furthermore, the increase of both PRLR forms in the rat brain during lactation [49] and the observation of PRL actions mediated by PRLR-short form in the mice nervous system [50] have also been reported. Together, these evidences suggest that this PRLR-short isoform may be involved in PRL-mediated brain functions in rodents. In addition, PRLR isoforms can interact and regulate the action of each other in both mice and humans [50, 51]. For instance, in mice, the transient action of PRL mediated by the PRLR-short form in sensory neurons of the trigeminal ganglia is negatively regulated by the presence of PRLR-long form in these neurons [50]. However, most studies involving the investigation of PRLR activation in the brain do not discriminate between isoforms of these receptors, PRL signaling in the brain may not always be mediated by the activation of the canonical PRLR-long form pathways.

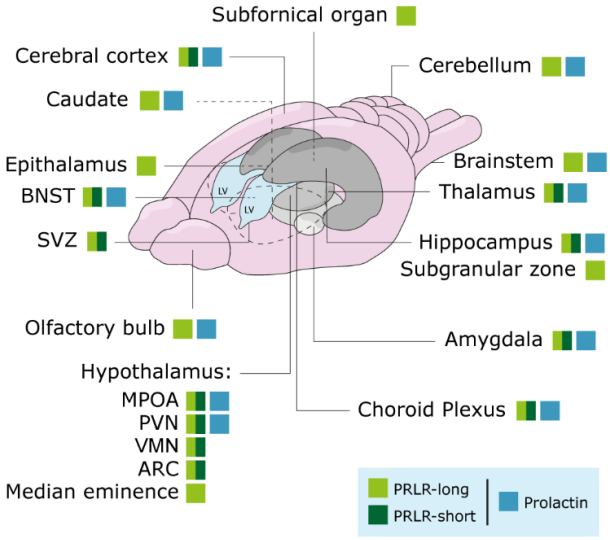

Figure 2.2. Schematic representation of the expression of prolactin and prolactin receptors in the brain. Reported sites of prolactin expression are signed by a blue square, while the expression of the long and the short form of the prolactin receptor (PRLR) are indicated in light green and dark green, respectively. Prolactin and PRLR expression are represented throughout several regions of the rat brain, since most of the available information is retrieved from studies conducted in rodents. ARC: Arcuate nucleus; BNST: Bed nucleus of the stria terminalis; LV: Lateral ventricle; MPOA: Medial preoptic area; SVZ: Subventricular zone; PVN: Paraventricular nuclei; VMN: Ventromedial hypothalamic nucleus.

2.7. Sources of PRL in the brain

Based on its size, PRL should not cross the blood-brain barrier (BBB). However, PRL is present in the cerebrospinal fluid (CSF), mimicking the fluctuations found in the peripheral circulation, reinforcing that PRL transport into the brain must occur [52]. For instance, in mice, transport of PRL into the brain is increased during lactation [53], a physiological state characterized by high levels of serum PRL. Based on the high expression of PRLR in the CP, and on the evidence depicted from studies in primates, a saturable receptor-mediated mechanism of transport of PRL across the CP has been considered the likely route of entrance of PRL into the CNS [54]. The CP, located within the brain ventricles, is responsible for the production of CSF and several peptides, as well as for controlling the passage of molecules into and out of the brain [55]. The CP is composed by a single layer of epithelial cells bound together by tight junctions, forming the blood-CSF barrier [55, 56].

Recently, the observation that PRL transport into the CSF occurred at normal rates in CP PRLR knockout mice, contradicted the theory that PRL transport into the brain was mediated by PRLR. Intriguingly, the rapid activation of PRL signaling in the brain observed after the peripheral administration of exogenous PRL was not accompanied by a rapid increase in the levels of this hormone in the CSF, leading the authors to propose that PRL transport into the brain could be mediated at the cerebral vasculature level [53]. The expression of PRLR and the observation of PRL-mediated action in circumventricular regions in mice, like the median eminence and subfornical organ [36, 57], that lack the conventional BBB [58], seem to

strengthen the hypothesis that PRL transport into the brain may also occur at brain regions were the BBB is more leaky [52]. However, although the BBB becomes more permeable with age [59], transport of PRL to the brain seems to be reduced by age in male mice [60]. This suggests that PRL transport into the brain is not entirely explained by leakier BBB regions. Overall, the lack of evidence to support that PRL is transported via PRLR in the CP, does not exclude the CP as a relevant gateway for the entrance of this hormone into the brain. In fact, increased PRL transport, mediated by CP epithelial cells, was recently reported under specific physiological conditions [61]. Notwithstanding, the saturable mechanism that is responsible for PRL entrance into the brain remains unidentified.

The first evidences of non-pituitary sources of PRL in the brain derived from studies demonstrating that PRL was still detectable in the CSF of rats that were subjected to hypophysectomy, contrasting with the observed reduction of plasma PRL levels [62]. Since then, immunoreactive-PRL were reported in distinct rodent brain regions, like several hypothalamic regions, the amygdala, brainstem, hippocampus, cerebellum and cerebral cortex [63–66] (Figure 2.2). Recently, our research group presented evidence that rather than being just a gateway for PRL, the CP itself constitutes an alternative source of PRL to the rat brain. Despite the expression of full-length PRL transcripts in the CP, identical to those expressed in the pituitary, the PRL detected in the CPs has a much higher molecular weight than pituitary PRL or PRL secreted to the conditioned media of pituitary cultures. This high molecular weight immunoreactive-PRL was observed in CP from pregnant female rats, in primary cultures of rat CP epithelial cells, and in CP-conditioned media and in the rat CSF [37]. The existence of high molecular weight PRL isoforms similar to those found in the CP was previously described in other cells like human monocytes [67]. However, the biological activity and relevance of this higher molecular weight PRL remains controversial. The recognition of non-pituitary sources of PRL in the brain is not consensual either, with some investigators defending that the reported expression of PRL mRNA based in high sensitivity techniques like RT-PCR may be the result of sample contamination with other cells that express PRL [19]. Overall, considering that the expression of PRL in brain regions other than the pituitary is very low, it is unlikely that PRL produced in extrapituitary tissues may have some impact in the circulating levels of PRL. Nevertheless, it is possible that brain PRL from extrapituitary sources could be produced in specific physiological circumstances, which require PRL autocrine and paracrine modulation of brain functions and account for the levels of PRL in the CSF.

2.8. PRL actions in the brain

The best documented actions of PRL in the mammalian brain, are associated with mechanisms that prepare the brain to motherhood/fatherhood [9, 35, 68, 69]. In female mice, the action of PRL in specific areas of the brain, including the medial preoptic area region of the hypothalamus and SVZ, is essential for the development of maternal behaviors like retrieving and crouching over the pups [9, 68]. In turn, male mice, a biparental species, and rat males, a uniparental species, display different paternal behaviors, which may be explained by distinct

release profiles of PRL between species [69]. Yet, PRL administration is able to elicit fatherhood behaviors even in males of non-paternal species like rats [69]. Furthermore, PRLR expression is relatively similar in the brain of male and female mice [33], reinforcing the theory that PRL may also play a relevant role in male brain functions of biparental species. On the other hand, in sheep, the increased expression of PRLR in the arcuate nucleus, contrast with the reduction of PRLR levels in the median eminence and in the adenohypophysis, during gestation. This may suggest that, during sheep gestation, PRL may have a predominant role in the regulation of appetite [31].

In rodents, the fine-tuned balance of PRL levels during pregnancy and postpartum is fundamental to the development of healthy maternal behavior of the progenitors and of the offspring in their adult life. Abnormal levels of PRL during prenatal and early-life in mice, have a negative impact in the nursing behaviors of the pups [70, 71]. More recently, the genes modulated by PRL in the hippocampus of female rats were examined by RNAseq. A total of 162 genes were differently expressed in the hippocampus of ovariectomized females exposed to PRL throughout 24 hours. Most differentially expressed genes were upregulated by PRL exposure and were correlated with cell cycle regulation and biological processes, like response to hypoxia, estradiol, or nutrients. Notably, PRL-modulated genes were associated with brain functions like learning, memory, plasticity, neuroprotection, neurogenesis and remodeling [72].

Interestingly, in mice, the development of mild maternal behavior is not always associated with higher peripheral PRL levels. Nulliparous female mice presented enhanced PRL responsiveness in the medial preoptic nucleus after prolonged exposure to pups, despite the lack of serum PRL rise as observed in puerperia's. This observation may be a consequence of alterations in PRL signaling or could be explained by the existence of brain extrapituitary PRL, as suggested by the authors [73]. However, further studies are necessary to understand if extrapituitary sources of PRL in the brain, play a relevant and direct role in the development of maternal behaviors.

2.8.1. Effects of PRL in neurogenesis

In mice, PRL modulates neurogenesis in the SVZ, where progenitor cells differentiate into interneurons that migrate to the olfactory bulb [7], and in the subgranular zone of the hippocampal dentate gyrus (Figure 2.3). These are the two brain neurogenic niches where progenitor cells continue to differentiate in new neurons and glial cells in adult life [35, 74].

In the SVZ, PRL plays an important role in the formation of the olfactory bulb by increasing the proliferation of neuronal stem cells during the early stage of pregnancy and early postpartum period in mice that further differentiate in olfactory neurons [7]. PRL also raises SVZ neurogenesis in male mice that interact with their pups [35]. In mice, PRL-induced adult SVZ neurogenesis is mediated by the ERK5 signaling pathway [8] and is fundamental for the development of healthy maternal behaviors [9], offspring recognition [7, 35] and choice of mates by females [75]. Additionally, exposure of females to male pheromones seems to be one of the factors that regulate PRL-induced neurogenesis. Besides increasing PRL levels in female

mice, pheromone exposure mediates the generation of new neurons in the SVZ in a PRLR dependent manner [75, 76]. Although olfactory bulb neurogenesis seemed to be fundamental for the development of maternal behavior and lamb recognition in sheep [77], no direct observation between PRL and olfactory bulb neurogenesis has been reported in this specie so far.

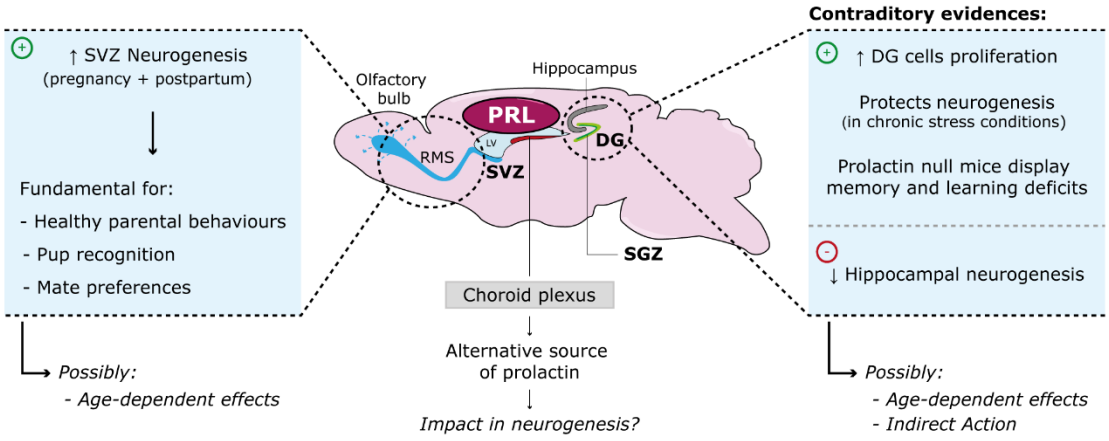

Figure 2.3. Summary of the main prolactin-mediated effects in the modulation of adult neurogenesis. Prolactin (PRL) has been associated with modulatory actions in the two major neurogenic niches in adult brain, the subventricular zone (SVZ) and subgranular zone (SGZ) of the dentate gyrus (DG). In the SVZ, progenitor cells differentiate into interneurons that migrate throughout the rostral migratory stream (RMS) to the olfactory bulb. During the early stage of gestation and lactation, PRL increases the number of proliferating cells in the SVZ and raises the amount of new interneurons in the olfactory bulb. The role of PRL in hippocampal progenitor cells neurogenesis is not as consensual. Some evidences support that PRL favors DG cells proliferation, while others suggest that PRL as either no effect or that negatively impacts hippocampal neurogenesis. In addition, recent evidence suggested that the choroid plexus may also be an alternative source of PRL in the rat brain. However, further studies are necessary to understand if choroid plexus derived PRL as any effects in SVZ neurogenesis. LV: Lateral ventricle.

Considering that the CP is near the SVZ, the possible contribution of the CP PRL induction of neurogenesis should also be considered. The CP epithelia is a remarkable source of peptides that modulate brain function and neurogenesis [56]. In that regard, in a recent RNAseq study conducted to examine transcriptomic differences induced by PRL, insulin-like growth factor 2 (Igf2) has emerged as a gene highly responsive to PRL in the adult mice CP. The upregulation of the *Igf2* gene during lactation, suggest that this particular growth factor is possibly involved in the postpartum increase of SVZ neurogenesis [4]. In fact, in the pioneer work of Shingo and colleagues, the authors suggested that due to the high expression of PRLR in the CP, that this structure could indirectly mediate PRL action in SVZ neurogenesis [7]. Local production of PRL in the rat CP [37], may explain the relevant role of the CP in PRL-induced neurogenesis in the SVZ.

The role of PRL in the neurogenesis in the dentate gyrus hippocampal progenitor cells is not consensual. Some studies describe that PRL enhances the proliferation of the dentate gyrus cells in both female and male mice [35, 74] and prevents the reduction of adult hippocampal

neurogenesis promoted by chronic stress in male mice [78]. Furthermore, PRL null mice display impaired learning and memory processes, and PRL infusion was able to restore the learning deficits observed in these mice. However, despite PRL-deficient mice presented lower generation of hippocampal precursor cells *in vitro*, the same was not observed *in vivo*, suggesting that in the absence of PRL, hippocampal neurogenesis may be sustained by other factors [74]. Other studies report that PRL has either no effect on rodents and human neurogenesis [7, 44, 79], or that in fact it has a negative impact on hippocampal neurogenesis in rats [3].

More recently, a study conducted in an *in vitro* model of human hippocampal stem cells revealed that PRL treatment was able to increase neuronal differentiation. However, this increase was only transient and continued PRL exposure actually decreased the number of proliferating cells, suggesting that fluctuations of PRL levels in different reproductive stages may induce short-term alterations or not contribute at all to adult hippocampus neurogenesis [79]. Interestingly, in an earlier study conducted with a distinct human neural stem cell *in vitro* model, distinct PRL levels were associated with different neuroblast and glial progenitor proliferation and migration rates [80], suggesting that the effects of PRL in neurogenesis may be dose dependent.

The lack of *in vitro* evidences that support the *in vivo* observations describing the valuable influence of PRL in hippocampal neurogenesis, may indicate that *in vivo*, the impact of PRL in this particular neurogenic niche may be mediated by indirect mechanisms [44]. As a matter of fact, the promoting of neurogenesis by PRL may be age- and species-dependent, since high levels of PRL during early-age were associated with a reduction in hippocampal neurogenesis in postnatal rats [3], differing from the proliferative effect observed in adulthood mouse [74]. Furthermore, the negative impact of PRL administration in both hippocampal and olfactory bulb neurogenesis in early life possibly contributes to the development of anxiety behaviors in males, but not females, during adulthood. However, the exact mechanism involved in such observations remained unknown [3]. A detailed review regarding the relation between motherhood, maternal experience and neurogenesis, not restricted to PRL-mediated effects, can be consulted elsewhere [81].

2.8.2. Effects of PRL in neuroplasticity

Recently, analysis of the proteome of postpartum maternal preoptic area revealed that the common regulators and targets of the significantly altered proteins found between mother and pup-deprived female rats, like AKT, MAPK1 and STAT3, could be traced to PRL. Thus, PRL may be in part responsible for motherhood-associated neuroplasticity in this region [82]. In fact, PRL, together with estrogen and progesterone, were considered to be possible intermediaries of the medial preoptic area plasticity observed during pregnancy in rats [83]. In addition, PRL may also be associated with brain plasticity modulation during fatherhood. In mandarin voles, experienced and first-time fathers showed increased spine density and greater dendrite length

in the medial prefrontal cortex than non-fathers. This alteration may be explained by the observed increase in PRL levels in both experienced and new fathers [84].

Exercise ameliorates the PRL response in chronically stressed mice, improving memory consolidation, through stimulation of the hippocampus [10]. At the synaptic level, it is believed that PRL modulates long- and short-term synaptic plasticity in the hippocampus of female mice at reproductive age. The enhancement of synaptic strength was not observed in immature females or male mice, suggesting that sex hormones are necessary to preserve long- and short-term plasticity response to PRL [85]. High serum PRL levels correlate with enhanced performance in learning and memory tasks possibly associated with the plasticity of the hippocampus in female mice [86].

2.9. PRL in neuroprotection and neurological and psychiatric disorders

2.9.1. Neuroprotection

In rodents, PRL protects the hippocampus against glutamate excitotoxicity in the kainic acid model *in vivo* and *in vitro* [34, 87, 88]. Recently, the detailed mechanisms of PRL-mediated neuroprotection have been extensively reviewed [15]. Briefly, in rodents, parenthood seems to protect the hippocampus against neurodegenerative insults [34, 89]. However, PRL neuroprotective properties were also observed in mice treated with PRL prior to kainic acid insult [46]. These protective effects were associated with increased AKT phosphorylation [46] and ERK1/2 activation [90].

The pretreatment of rat hippocampal neuronal cultures with PRL prior to glutamate incubation prevented cell death and mitochondrial disfunction, and inhibited the increase of intracellular calcium levels, triggered by the excitotoxic insult [87]. On the other hand, vasohinibin, generated by enzymatic cleavage of PRL in the hippocampus, prompts neuronal cell dead in cultures of mouse hippocampal primary cells [91]. These effects were reversed by the addition of PRL which was able to block the negative effects of vasohinibin, suggesting that PRL action in the hippocampus is complex [91].

Neuroprotective effects of PRL have also been reported in retinal cells. PRL reduces gliosis and favors the expression of survival factors in a rat model of light-induced retinal degeneration [92]. Furthermore, increased retinal dysfunction observed with age was correlated with the age-associated decrease of PRLR, and reduction of PRL signaling in the retina [93].

Based on its functions as a neuropeptide, PRL may have an important impact in other neurological disorders. Aside from the potential role as a therapeutic agent in excitotoxicity-mediated diseases like neurodegenerative disorders, as discussed above, the number of studies on the relevance of PRL in neurological disorders is still rather limited. Anew, the relevance of

PRL in brain health and disease seems to be complex, possibly tissue-specific, and once more, apparently dependent on a combination of factors such as age or reproductive stage.

2.9.2. Brain injury

PRL treatment reduced the cerebral infarct area and edema in a rat cerebral ischemia model, and restored brain glutamate levels and intracellular calcium homeostasis [11]. Although PRL administration failed to rescue loss of cortical neurons in hypoxic ischemic juvenile rats, increased PRL immunoreactivity was observed in the injured parietal cortex, and this hormone was associated with glial responses that led to the formation of the glial scare [94]. In the human brain, hypoxia- and ischemia-related death cases were associated with increased levels of PRL in the CSF, suggesting that the selective passage of PRL to CSF, possibly mediated by the CP, is augmented in these conditions, possibly to adjust osmotic pressure in the brain [61] or by disruption of the BBB associated with stroke [95]. Notwithstanding, as there is production of PRL in the CP that can be released to the CSF, the increase of this hormone levels in the CSF found in hypoxia can not be ruled out. On the other hand, prolactin was also associated with decrease of the BBB permeability by inducing the expression of tight junction proteins claudin-5 and occludin in primary cultures of bovine brain microvessel endothelial cells [96].

In a prospective cohort study, human patients with higher PRL levels during the first year after traumatic brain injury and aneurysmal subarachnoid hemorrhage, tended to perform worst in follow-up cognitive and behavioral tests. In this particular case, the higher PRL levels were considered a consequence of a major pituitary disfunction reported in these patients [97] rather than a neuroprotective response mechanism. Many of the putative mechanisms involved in the beneficial actions of PRL in brain injury are linked with neuroprotective properties of PRL, as described above. Although some studies report an increase of PRL levels after brain injury, further research is necessary to investigate if this increase has any protective role, especially in humans.

2.9.3. Multiple sclerosis

Multiple sclerosis is a CNS autoimmune disorder associated with axonal degeneration and demyelination processes [98]. In this regard, PRL is able to induce oligodendrocyte precursor cells proliferation and remyelination in adult female mice brain during pregnancy [99]. Although PRL can be associated with modulation and proinflammatory profiles in immune cells, the combined administration of PRL and interferon-ß seems to be beneficial as a short-term treatment of experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis [100]. On the other hand, PRL produced by immune cells in the late phases of experimental autoimmune encephalomyelitis mice model is responsible for the development of persistent neuroinflammation in this pathology [101], which may indicate that despite not being responsible for the onset of multiple sclerosis [102], PRL contributes to the pathological outcome of this disease, as previously reviewed [98].

2.9.4. Psychiatric disorders and stress

Higher levels of PRL are often observed in patients with depression, stress, and psychopathologies. However, this so-called hyperprolactinemia state is probably a consequence of pharmacological treatment with D2 dopamine receptors blockers and not a cause of psychiatric disorders [103]. Nonetheless, it has been suggested that higher PRL levels, associated with higher stress rates, in some risk-prone and first-episode psychosis groups, that had never been treated with antipsychotics, could be associated with emerging psychosis [104, 105]. This hypothesis lacks the support of robust evidence that directly link higher circulating PRL levels with the onset of mental disorders.

It has been considered that PRL has anxiolytic properties, particularly relevant in the perinatal and postpartum periods [9, 106, 107]. For instance, female mice exposed to trauma during gestation (exposure to contextual fear conditioning), presented lower levels of PRL during postpartum and decreased maternal behavior. Furthermore, pups of traumatized mothers, were born smaller and remained smaller throughout life when compared to non-traumatized animals and present anxiety-like behaviors [106]. Similar results have been reported in humans, where the reduction of PRL levels was also observed in the plasma of mothers with depressive and anxiety symptoms during the perinatal phase, which was associated with reduced social interactive behavior and irritability in newborns [108, 109]. Additionally, augmented brain PRL levels in rats exposed to stress and sleep-deprivation suggest that PRL is also associated with extended REM sleep periods and thus with stress coping mechanisms [110].

On the contrary, evidences that PRLR knockdown could be beneficial to the treatment of depression in mice subjected to chronic mild stress have also been reported [111]. In addition, the administration of exogenous PRL to rats during early-life stages, elicits depressive-like behaviors [3]. Also in rats, the administration of bromocriptine, a dopamine receptor agonist widely used to inhibit PRL secretion, during early lactation, was correlated with lower peripheral PRL levels and the development of increased anxiety-like behaviors in adult life, whereas the administration of the same compound in the late phase of lactation had the opposite effect [112]. Based on these findings, the effect of PRL in anxiety-like behaviors seems to be age and species dependent.

2.9.5. Glioblastoma

Some evidences suggest that PRL may also play an important role in the pathology of primary brain tumors. In the past, many studies focused on the pro-tumorigenic role of PRL signaling in breast and pancreatic cancers [113]. Recently, PRL and PRLR expression have been reported in human and rodents glioblastoma cells and induced PRLR overexpression was associated with increased proliferation, migration and chemoresistance of the tumors [114]. PRLR was found in all samples of human grade II and III gliomas and in glioblastoma, while only 12% of grade II and III gliomas and approximately 30% and glioblastoma samples, expressed PRL mRNA. Furthermore, the long-term patient survival rate was reduced in grade II and III and

glioblastoma that expressed PRL [114]. In addition, within the group of glioblastoma patients with tumors that express PRL, higher levels of PRLR expression were correlated with decreased survival in men. Interestingly, the opposite was observed in women, in which higher levels of PRLR correlate with extended survival. Remarkably, low levels of PRLR expression were associated with reduced long-term survival rates in grade II and III glioma in men, while no differences were observed in women, which may suggest that PRL and PRLR roles may be dependent of gender and tumor grade [114]. PRL treatment and PRLR expression in distinct glioblastoma cell lines, were positively correlated with increased invasion capacity of the cells [115]. Overall, the potential of PRL and PRLR as therapeutic targets and/or biomarkers is of particular interest in glioblastoma.

2.10. Concluding remarks

PRL is a pleiotropic hormone that mediates a considerable diversity of endocrine, autocrine and paracrine actions. Apart from its fundamental in reproduction and lactation, PRL has remarkable actions in the brain, particularly in the development of parental behavior, adapting brain circuits to the necessities imposed by normal parental care that ultimately promote survival of the offspring. PRL brain actions are wide and mediated through the activation of its receptors. Considering the role of PRL in the regulation of neurogenesis, neuroplasticity as well as in neuroprotection, it is tempting to suggest that its potential as a therapeutic agent should be investigated for the treatment of neurological disorders, including neurodegenerative diseases. Nonetheless, this approach should be carefully studied since PRL functions are wide and not restricted to a single target.

Despite some developments over the past decade, there is still a considerable lack of scientific evidence regarding the functions of PRL in the brain, especially in humans. It is important to notice that most studies devoted to the investigation of PRL-mediated action in the brain were performed in rodents and may not be alike in humans. Although *in vivo* studies using rodents have boosted our knowledge about brain function, the organization and complexity of the brain itself is very distinct between human and rodents. In fact, PRL systems are distinct in rodents and humans. These differences include distinct PRLR isoforms, which could be associated with the activation of different signaling cascades, and the existence of an additional superdistal promoter that modulates the expression of extrapituitary PRL in humans. An effort in the development of increasingly complex *in vitro* models that mimic human brain structures, like organoids, could be a practical tool for the study of PRL brain functions. Overall, several controversial issues like the biological relevance of brain extrapituitary PRL remain unanswered. Further studies are necessary to fully understand the dimension of PRL actions in the brain.

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Chapter 3

Global aims

3. Global aims

The choroid plexus (CP) is a relevant player in brain homeostasis. Besides constituting the blood-cerebrospinal fluid barrier, acting as a gatekeeper of the central nervous system (CNS), several other functions have been attributed to this brain structure over the past decades. As a highly secretory epithelium, CP is the main producer of cerebrospinal fluid in the CNS but it also contributes with the release of numerous factors that modulate brain function. Some of these factors have been implicated in the regulation of both development-related and adult neurogenesis. One of the neuropeptides previously associated with neurogenesis-induction is prolactin, a hormone mainly produced by the lactotroph cells, of the anterior pituitary. However, some evidence supports the existence of extrapituitary sources of prolactin in the brain.

Based on transcriptomic microarray data obtained in a research study previously performed by our group, we believe that the rat CP may be an alternative source of prolactin in the brain. In that study, designed to understand the relevance of sex hormone background in the rat CP transcriptome, prolactin transcripts were found in the rat CP. Moreover, the RNA expression of this hormone was found to be higher in intact females than in ovariectomized females or intact males, suggesting that prolactin gene expression in the CP is possibly modulated by sex hormones.

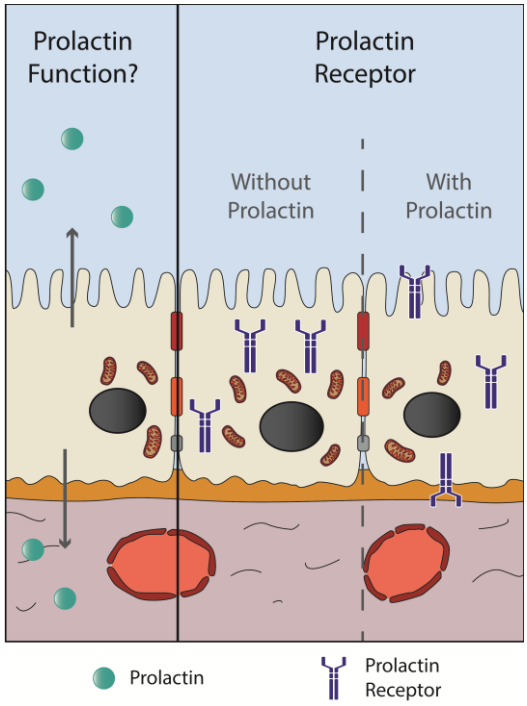
Besides being a putative source of prolactin, the CP is the brain structure with the higher expression of prolactin receptors. Though it was initially believed that the high expression of prolactin receptors was related to a receptor-mediated mechanism responsible for prolactin transport to the brain present at the CP, it is now accepted that prolactin uptake is independent of its receptors. In fact, information about the exact function of prolactin in the CP is still very scarce, especially in the postnatal stages. As so, the main goals of this doctoral thesis were to investigate the CP both as a source and a target of prolactin action.

Therefore, the exact goals of this thesis were:

- To analyze the expression, synthesis, and secretion of prolactin by the rat CP;
- To evaluate the sex hormones regulation of prolactin synthesis and secretion in rat CP;
- Assess the effects of prolactin exposure in the transcriptome of postnatal rat CP;
- Identify additional neurogenic factors secreted by the CP.

Chapter 4

The choroid plexus is an alternative source of prolactin to the rat brain



This chapter corresponds to the original research article:

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4.1. Abstract

Among the more than 300 functions attributed to prolactin (PRL), this hormone has been associated with the induction of neurogenesis and differentiation of olfactory neurons especially during pregnancy, which are essential for maternal behavior. Despite the original hypothesis that PRL enters the central nervous system through a process mediated by PRL receptors (PRLR) at the Choroid Plexus (CP), recent data suggested that PRL transport into the brain is independent of its receptors. Based on transcriptomic data suggesting that PRL could be expressed in the CP, this work aimed to confirm PRL synthesis and secretion by CP epithelial cells (CPEC). The secretion of PRL and the distribution of PRLR in CPEC was further characterized using an *in vitro* model of the rat blood-cerebrospinal fluid barrier. RT-PCR analysis of PRL transcripts showed its presence in pregnant rat CP, in CPEC and in the rat immortalized CP cell line, Z310. These observations were reinforced by immunocytochemistry staining of PRL in CPEC and Z310 cells cytoplasm. A 63 kDa immunoreactive PRL protein was detected by Western blot in CP protein extracts as well as in culture medium incubated with rat pituitary and samples of rat cerebrospinal fluid and serum. Positive immunocytochemistry staining of PRLR was present throughout CPEC cytoplasm and in the apical and basal membrane of these cells. Altogether, our evidences suggest that CP is an alternative source of PRL to the brain, that might impact neurogenesis of olfactory neurons at the subventricular zone, given its proximity to the CP.

Keywords: Choroid plexus, Prolactin, Prolactin receptor, Brain, Blood-cerebrospinal fluid barrier

4.2. Introduction

Prolactin (PRL) is a 23 kDa pleiotropic hormone mainly produced by specialized cells from the anterior pituitary. Since its discovery, PRL has been associated with more than 300 distinct physiological functions, ranging from reproduction to immunoregulation or metabolism control [1–3]. Additionally to the PRL from pituitary origin, extrapituitary PRL synthesis and secretion has been previously reported in several tissues including brain structures like the paraventricular and the supraoptic nuclei of the hypothalamus [4, 5].

Based on its molecular size, PRL should not be able to enter the brain across the blood brain barrier. Nevertheless, the presence of immunoreactive PRL in the cerebrospinal fluid (CSF) and the expression of PRL receptors (PRLR) in diverse brain tissues, including brainstem regions, have been previously reported [6–8]. One of the brain structures that strongly expresses PRLR is the choroid plexus (CP) [7]. The CP constitutes the blood-CSF barrier and is composed by a monolayer of cuboidal epithelial cells bounded by tight junctions, adherence junctions and desmosomes [9–11], lying on a highly irrigated connective tissue by fenestrated capillaries. Besides its barrier function between the periphery and the CSF, the CP has an important role in CSF production and in the synthesis and secretion of proteins and other signaling molecules that impact the development and functions of the brain, including neurogenesis [10, 12, 13].

One of the best demonstrated functions of PRL in the brain is its role in the development of maternal behavior (reviewed by [14]). PRL has been associated with the increase of subventricular zone (SVZ) neurogenesis [15] and raise of precursor cells in the hippocampus [16]. In fact, SVZ neurogenesis induced by PRL during the early stage pregnancy seems to be responsible for the incorporation of new interneurons in the olfactory bulb, influencing maternal recognition of offspring [15, 17] and normal levels of anxiety after delivery in rodents [18]. Other described functions of PRL in the brain comprise the development of healthy maternal responsiveness to pups in rats [19, 20], *in vitro* neuroprotection against glutamate-induced excitotoxicity in rat hippocampal neurons [21] and therapeutic potential in white matter diseases like multiple sclerosis [22].

For many years, it was believed that pituitary PRL gains access to the central nervous system through a transport-mediated process involving PRL binding sites in the CP [23]. However, the increase of PRL in the CSF in a PRLR knockout mice model abolished this theory and a cerebral vascular-mediated route was suggested as a possible alternative for the systemic PRL passage to the CSF [24]. Nevertheless, the possibility of local production of PRL at the CP and concomitant release into the CSF has never been suggested. Preliminary data from rat CP microarrays (GEO database, accession number GSE87045) suggested local expression of PRL [25]. Thus, this study aimed to investigate if the CP is indeed able to synthesize PRL, and if it is released through the apical membrane of CP epithelial cells (CPEC) to the medium, what would be compatible with its release to the CSF *in vivo*. Furthermore, the localization of PRLR in CPEC was evaluated using an *in vitro* model of the rat blood-CSF barrier.

4.3. Material and Methods

4.3.1. Animals

This study was conducted with the approval of the Animal Welfare and Ethics Committee of the Health Science Research Centre of the University of Beira Interior. In compliance with National and European Union rules for the care and handling of laboratory animals, no further licensing was required since the study did only involve collection of animal tissues without animal experimentation. The animals used for tissue collection were Wistar Han rats that were housed in appropriate cages at constant room temperature in a 12 h light/12 h dark photoperiod and given standard laboratory chow and water *ad libitum*. All efforts were made to minimize the number of animals used as well as their suffering.

All CPs included in this work were dissected exclusively from the brain lateral ventricles. CPs were collected from 3-month-old males, 3-month-old virgin females, 14—15 days gestation pregnant females and 3—6 days old postnatal rats.

4.3.2. Cell cultures and *ex vivo* studies

CPs were dissected from the lateral ventricles after the decapitation of 3—6 days old postnatal rats, previously anesthetized on ice, and used to perform free floating experiments (CP explants) or to establish CPEC primary cultures. All cultures were maintained in a humidified incubator in 95% air—5% CO_2 at 37 °C. In *ex vivo* experiments, postnatal CP explants (sixteen per well) and pituitaries (three per well), as well as CP explants collected from 14—15 days gestation pregnant females (two to six per well) were kept for 24—48 h with High Glucose Dulbecco's Modified Eagle medium (DMEM; Gibco, ThermoFisher Scientific, USA, Cat# 12800017) supplemented with 1% charcoal stripped fetal bovine serum (FBS; Capricorn Scientific, Germany, Cat# FBS-12B) and 100 U/mL of penicillin/streptomycin (Sigma-Aldrich, Germany, Cat# P4333) in 24 wells culture plates. CPs collected from the lateral ventricles of 14—15 days gestation pregnant female rats were also included in this study taking into account the reported role of PRL in SVZ neurogenesis during pregnancy in rodents [15].

Rat primary cultures of CP epithelial cells were established using 3—6 days old postnatal rats only, as previously described [26]. Dissociated cells were seeded into 12 (four CPs per well) or 24 (two CPs per well) culture plates and cultured in High Glucose DMEM supplemented with 100 U/mL antibiotics, 10% FBS, 10 ηg/mL epidermal growth factor (Sigma-Aldrich, Germany), 5 μg/mL insulin (Sigma-Aldrich, Germany, Cat# I9278) and 30 μM cytosine arabinoside (Sigma-Aldrich, Germany, Cat# C1768). Culture medium was replaced at day *in vitro* (DIV) 1, and every 2 days thereafter. All studies were performed using cultures established for at least 4—5 days.

The Z310 cell line, an immortalized murine choroidal epithelial cell line obtained by the transfection of rat primary choroidal epithelial cells with a viral plasmid containing SV40 large-T antigen [27], was kindly made available by Dr. Wei Zheng from the School of Health Sciences,

Purdue University (West Lafayette, IN, USA). The Z310 cell line was cultured as previously described [27, 28].

4.3.3. Establishment of CPEC cultures in inserts

To establish the rat *in vitro* blood-CSF model, freshly isolated CPEC were seeded in 35 mm culture Petri dishes (six animals per dish). Cell culture medium was replaced at DIV1. At DIV3, CPEC were incubated with trypsin-EDTA for 10 min at 37 °C and seeded into the upper compartment of collagen-precoated cell culture inserts at a density of approximately 600,000 cells per insert. Before seeding, transparent polyester cell culture inserts (12 mm diameter, 0.4 µm pore size, 1.12 cm^2 growth area; VWR, Germany, Cat# 734-2734) were incubated with 5.8 µg/cm^2 collagen from calf skin (Sigma-Aldrich, Germany, Cat# C8919) for 2 h at 37 °C, washed with sterile water and left to air-dry in the culture hood. Collagen-coated inserts were sterilized under UV light and stored at 4 °C until use. After seeding, cell culture medium was replaced every 2 days.

Transepithelial electrical resistance (TEER) was monitored from DIV6 onwards using an epithelial volt-ohmmeter EVOM2 (World Precision Instruments, USA). For each filter, independent measurements using STX2 electrode on three different cell culture areas were recorded and averaged. TEER values from collagen-coated filters without cells (blank) were subtracted from measured TEER values and multiplied by the filter surface area to obtain final unit area resistance (Ωcm^2). The formation of confluent monolayers was considered once measured TEER values across the insert barrier were higher than 75 Ωcm^2 [28], usually achieved at DIV6. After reaching confluence, cell culture medium was replaced and collected from both upper and lower compartments of the filters after 48 h. In all of these experiments, the formation of confluent monolayers was further confirmed by immunofluorescent staining of the basolateral or apical sides of CPEC using Abcc1 (1:200) or occludin (1:200; Thermo Fisher Scientific Cat# 331594, RRID:AB_2532186) primary antibodies, respectively. CPEC seeded in cell culture inserts were stained using the same immunofluorescence protocol applied to evaluate the subcellular location of PRLR, described later in this chapter.

4.3.4. Amplification of CP full-length PRL transcripts by reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from a pool of CPs dissected from five adult 14—15 days gestation pregnant rats, pituitary tissue of a single pregnant rat, CPEC and Z310 cells using TRI Reagent® (Sigma-Aldrich, Germany, Cat# T9424). RNA was quantified by spectrophotometry (NanoPhotometer™, Implen, Germany) and RNA integrity was assessed by agarose (1.5%) gel electrophoresis containing *GreenSafe* staining (NZYTech Ltd., Portugal, Cat# MB13201). Complementary DNA from CPs (1 µg), CPEC (500 ηg), Z310 (1 µg) and pituitary (1 µg) was synthesized using NZY First-Strand cDNA Synthesis Kit (NZYTech Ltd., Portugal, Cat# MB125, 20 µL reaction), following manufacturer's instructions. PCR reactions were carried out in a final volume of 10 µL using NZYTaq II 2x Green Master Mix (NZYTech Ltd., Portugal, Cat#

MB35801) and 0.4 μM of each primer. The PCR protocols consisted of an initial 3 min denaturing reaction at 95 °C followed by 30 s at 94 °C, 30 s at 62 °C, and 2 min at 72°C for 40 cycles with a final 10 min extension at 72°C using a MultiGene™ Optimax Thermal Cycler (Labnet International, USA). The following primers, designed using software tool Primer-BLAST (NCBI-NIH), were used: rat full-length PRL forward 5'-TTC TTG GGG AAG TGT GGT CC-3' and reverse 5'-AGC ATG TGC TGA AAG TTG TAA TGC-3', and β-2-microglobulin forward 5'-CCG TGA TCT TTC TGG TGC TTG TC -3' and reverse 5'-CTA TCT GAG GTG GGT GGA ACT GAG-3', resulting in amplicons of 804 and 150 bp, respectively. The PCR products were analyzed in a 1% agarose gel electrophoresis, visualized under UV with *GreenSafe* staining (NZYTech Ltd., Portugal, Cat# MB13201) and Sanger sequenced (STAB VIDA, Portugal) to confirm the gene identity (in case of CPs). A negative control consisting of a PCR reaction without template was included in all the assays.

4.3.5. Detection of PRL and PRLR by immunohistochemistry

Considering the small size of postnatal rat tissues, immunohistochemistry experiments were performed with lateral ventricle CPs and pituitary gland dissected from a 14—15 days pregnant female rat, in order to obtain more representative images of CP structure, as well as slices with higher tissue integrity. The collected tissues were fixed with 4 % paraformaldehyde immediately after collection. Paraffin-embedded slides were pre-treated with Trilogy™ (Cell Marque™, Millipore, USA, Cat# CMC920050030), according to the manufacturer's instructions. Then, slides were washed twice with 0.1 % Tween 20 in PBS (PBS-T 0.1 %) for 5 min and incubated with 3 % H_2O_2 for 10 min to block endogenous peroxidase activity. Slides were washed twice with PBS-T 0.1 % for 5 min and incubated for 1 h with anti-rat PRL antiserum IC-5 primary antibody (1:2,000; generously supplied by The National Hormone and Pituitary Program, NIDDK) or polyclonal rabbit anti-PRLR 122 (1:300; antibody kindly produced by Dr. Patricia Ingleton, University of Sheffield, UK), an antiserum specific to the residues 309 to 325 of the epitope of the intracellular domain of the long form of the PRLR [29]. Slides incubated in the absence of PRL and PRLR primary antibodies were used as negative controls. Afterwards, slides were washed with PBS-T 0.1% and treated with ready-to-use visualization HiDef Detection™ HRP Polymer System (Cell Marque™, Millipore, Cat# CMC954080040). Briefly, slides were incubated for 10 min HiDef Detection™ Amplifier, washed with PBS-T 0.1 % and incubated for another 10 min with HiDef Detection™ HRP Polymer Detector. After additional washes with PBS-T 0.1 %, chromogen staining was obtained using diaminobenzidine (DAB) for 10 min. Slides were then counterstained with hematoxylin for 3 min, dehydrated and mounted with Q Path® Coverquick 2000 (VWR, Germany, Cat# 05547530). All the described incubations were performed at room temperature. Images were acquired in an Axio Imager A1 microscope with an AxioVision camera and software (Carl Zeiss, Germany) using a magnification of 40x (A-Plan 40x/0.65) and 100x (A-Plan 100x/1.25 Oil).

4.3.6. Detection of PRL and PRLR subcellular distribution by immunofluorescence

CPEC and Z310 cells were cultured and grown to 70-80 % confluence on glass coverslips. Cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min, washed with PBS and treated with 1% Triton X-100 in PBS for 5 min at room temperature. Afterwards, cells were washed with PBS and incubated with blocking solution (10 % FBS in PBS-T 0.1 %) for 1 h at room temperature. Cells were then incubated overnight at 4 °C with polyclonal rabbit anti-rat PRL (1:2,500) diluted in PBS-T 0.1 % containing 1 % FBS and incubated for 1 h at room temperature with Alexa Fluor 488® goat anti-rabbit IgG conjugate (1:1,000; Molecular Probes Cat# A-11008, RRID:AB_143165A-11008). To confirm the specificity of anti-rat PRL primary antibody, the diluted antibody was pre-incubated with PRL from rat peptide (1 µg, Sigma-Aldrich, Germany, Cat# SRP4689) overnight at 4 °C before use.

To evaluate the subcellular location of PRLR, CPEC grown on cell culture filters were left unstimulated or stimulated over 8 h with 5 ηg/mL of rat PRL (Sigma-Aldrich, Germany, Cat# SRP4689) applied to the basal side of the inserts (lower compartment) prior to immunofluorescence experiments. Inserts were immediately incubated with blocking solution (3 % Bovine serum albumin, BSA, in PBS with 0.2 % Triton X-100) after fixation and incubated overnight at 4 °C with polyclonal rabbit anti-PRLR 122 (1:100) antibody diluted in PBS-T 0.01 % containing 1% BSA. Inserts were then incubated for 1 h at room temperature with Alexa Fluor 488® goat anti-rabbit IgG conjugate (1:1,000; Molecular Probes Cat# A-11008, RRID:AB_143165A-11008) and with monoclonal mouse anti-occludin Alexa Fluor 594® (1:200; Thermo Fisher Scientific Cat# 331594, RRID:AB_2532186), used to stain the apical side of the CPEC.

Cells nuclei were stained for 10 min with Hoechst 33342 (1:1,000; Thermo Fisher Scientific, USA, Cat# I34406). Finally, coverslips and cell culture inserts were washed several times with PBS-T 0.1 % or PBS-T 0.01 %, respectively, and mounted onto microscope slides with Dako Fluorescence Mounting Medium (Dako, USA, Cat# S3023). Coverslips and inserts incubated in the absence of primary antibody were used as negative controls. Slides were visualized under a confocal microscope LSM 710 (Carl Zeiss, Germany) using a magnification of 63x (Plan-Apochromat 63x/1.4 Oil DIC M27).

4.3.7. Detection of PRL by Western Blot in CP and cell culture media

Tissues or cultured cells were homogenized, on ice, using radioimmunoprecipitation assay buffer (RIPA) (150 mM sodium chloride, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 50 mM Tris pH 8.0) completed with 1 mM phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate and 10 µL/mL cOmplete™ EDTA Free protease inhibitor cocktail (Roche, Sigma-Aldrich, Germany, Cat# 11873580001). Lysates were centrifuged at 10,000 g for 10 min at 4 °C and supernatants were collected. Culture medium supernatants collected from the upper and the lower compartment of experiments performed using cell culture inserts were concentrated to a final volume of approximately 50 µL using Vivaspin® 500 µL spin columns (Sartorius, Germany, Cat# VS0112). Albumin was depleted

from concentrated culture medium supernatants (10 µL each) and rat serum sample (50 µL) using Pierce™ Albumin Depletion Kit (Thermo Fisher Scientific, USA, Cat# 85160), following the manufacturer's instructions. CSF samples collected from 3-month-old female and male rats and from 14—15 days gestation pregnant rat females, as well as a serum sample collected from a 14—15 gestation days pregnant rat, were used as positive controls to investigate the presence of the same higher molecular weight PRL isoform observed in CP. Total protein quantification was performed using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA, Cat# 23225). Protein extracts (20-25 µg), culture medium incubated with CP or pituitary explants (15 µL) and culture supernatants collected from experiments performed with inserts (30 µL) were diluted in 4X sample buffer containing freshly added 10 % β-mercaptoethanol, denatured at 100 °C for 5 min and separated by 10—12.5 % SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham™ Hybond™ 0.45µm, GE Healthcare, UK, Cat# 10600023). In Western blot experiments performed under non-reducing conditions, β-mercaptoethanol was not added to the sample buffer. Membranes were blocked for 1 h with 5 % skimmed milk in Tris buffered saline with 0.1 % Tween 20 (TBS-T 0.1 %), before being incubated overnight at 4 °C with anti-rat PRL (1:4,000 in TBS-T 0.1 %). After being washed with TBS-T 0.1%, membranes were incubated 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:20,000; Thermo Fisher Scientific Cat# 31466, RRID:AB_10960844). Protein bands were visualized using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, USA, Cat# 34577) and detected by Image Lab 5.1 software (Bio-Rad, USA, RRID:SCR_014210) with ChemiDoc™ MP (Bio-Rad, USA). The specificity of anti-rat PRL antibody was assessed with a pre-incubation of the diluted antibody with PRL from rat peptide (2.5 µg, Sigma-Aldrich, Germany, Cat# SRP4689) for 20 h at 4 °C. In quantitative experiments, 25 µg of protein extracts were separated, except for pituitary protein extracts where only a tenth of the total protein was loaded (2.5 µg), due to the high concentration of PRL in these tissues. Image Lab software was used to quantify band intensity and the ratio of the intensity of the bands of PRL and β-actin levels (1:20,000; Sigma-Aldrich Cat# A1978, RRID:AB_476692) was calculated and compared between groups. Only the 63 kDa molecular weight PRL isoform was quantified in CPs and cell culture samples and compared to the 23 kDa molecular weight PRL observed in pituitary. Three independent samples within each group were quantified.

4.3.8. Statistical analysis

Values are presented as mean ± standard error of mean (SEM). Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, CA, RRID:SCR_002798) and IBM SPSS Statistics 25 (SPSS Inc., IL, USA, RRID:SCR_002865). Data were tested for normality using Shapiro-Wilk test and for homogeneity of variance using Levene's test. Normally distributed data were analyzed with Student's unpaired *t* test. Differences between groups were determined using non-parametric Kruskal–Wallis test followed by Dunn's multiple comparison test in case of data that did not show homogeneity of variance. Significant differences between groups was set at $p < 0.05$.

4.4. Results

4.4.1. Prolactin is expressed and secreted by the rat CP

To examine if CP could be an alternative source of PRL in rat brain, and considering the role of PRL in SVZ neurogenesis during pregnancy [15, 17], we first performed RT-PCR with specific primers to amplify the full-length cDNA PRL using total RNA from a pool of lateral ventricle CPs from 14—15 days pregnant rat females. The amplicon obtained corresponded to the expected size of the full-length transcript (804 bp). Additionally, a PCR product of the same size was also obtained when total RNA from CPEC and Z310 cells were used, despite the lower band intensity of the Z310 transcript (Figure 4.1). The identity of the amplicon, which was identical to rat PRL (accession number NM_012629), was confirmed by Sanger sequencing (STAB VIDA, Portugal).

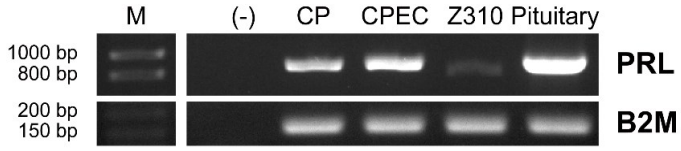

Figure 4.1. Full-length PRL transcripts are present in the CP. Gel electrophoresis of RT-PCR products show that PRL is expressed in pregnant rat CP, CPEC and Z310 cells. M: Molecular weight marker NZYDNA Ladder VIII (NZYTech Ltd., Portugal) in case of PRL and GRS Ladder 50 bp (Grisp, Portugal) in β-2-microglobulin (B2M). (-) Negative control.

The localization of PRL protein in the CP tissue was evaluated by immunohistochemistry using CPs collected from the lateral ventricles of a 14—15 days gestation pregnant rat female. Positive immunostaining was observed in the epithelial cells of the collected CP tissues (Fig. 4.2) and seems to be more evident in the apical membrane of the immunopositive cells. The absence of immunostaining in epithelial cells in negative control CP tissues (Figure 4.2 (-)), without PRL antibody, and the immunostaining in pituitary tissues (Figure 4.2 (+)) strengthened the reliability of these results.

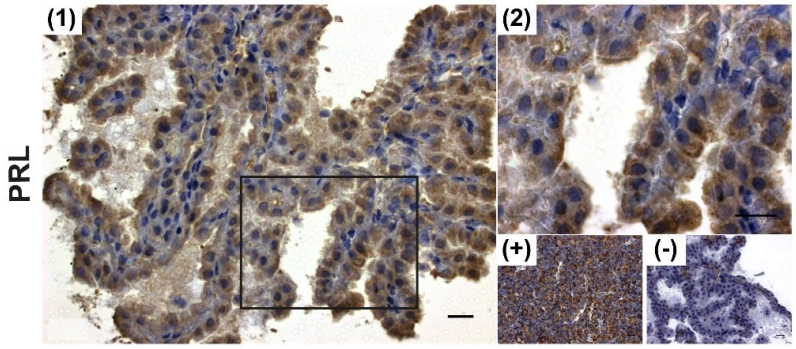

Figure 4.2. Immunostaining of PRL in the CP. CPs from a 14—15 days pregnant Wistar Han female were incubated with rabbit anti-rat PRL serum (1:2,000), followed by treatment with HiDef Detection™ HRP Polymer System and incubation with DAB. Nuclei are stained with Mayer´s Hematoxylin. Pituitary tissue was used as a positive control (+) and tissue sections incubated without anti-rat PRL antiserum were used as negative control (-). Images were acquired under a magnification of 40x, and (2) is a high-powered image (magnification 100x) of the boxed area in (1). Scale bar, 20 μm.

The localization of PRL protein was also evaluated by immunofluorescence. Positive immunostaining was observed in the cytoplasm of CPEC and in the Z310 cell line (Figure 4.3). The decrease of mean intensity fluorescence observed when anti-rat PRL antiserum was pre-incubated with rat PRL peptide confirmed the specificity of the primary antibody (data not shown). PRL protein was also detected in lysates from CPs collected from the lateral brain ventricles, CPEC, Z310 cells and culture medium supernatants as assessed by Western blot analysis (Figure 4.4.a). An ~63 kDa immunoreactive PRL protein, with higher molecular weight than pituitary PRL, was detected by Western blot in protein extracts from CP dissected from the lateral ventricles of 14—15 days pregnant female rats, postnatal rats (3—6 days old), 3-month-old male rats and 3-month-old virgin female rats, as well as in CPEC and Z310 cells (Figure 4.4.a). In some experiments, a ~45 kDa protein was also present in these protein extracts (Figure 4.4.a). The same 63 kDa immunoreactive PRL protein was also present in cell culture medium incubated for 24 h with CP explants dissected from the lateral ventricles of postnatal rats (3—6 days old), suggesting that PRL could be secreted by CP. An additional band with a higher molecular weight was also present in cell culture medium incubated with CP explants and protein extracts from CPs (Figure 4.4.a).

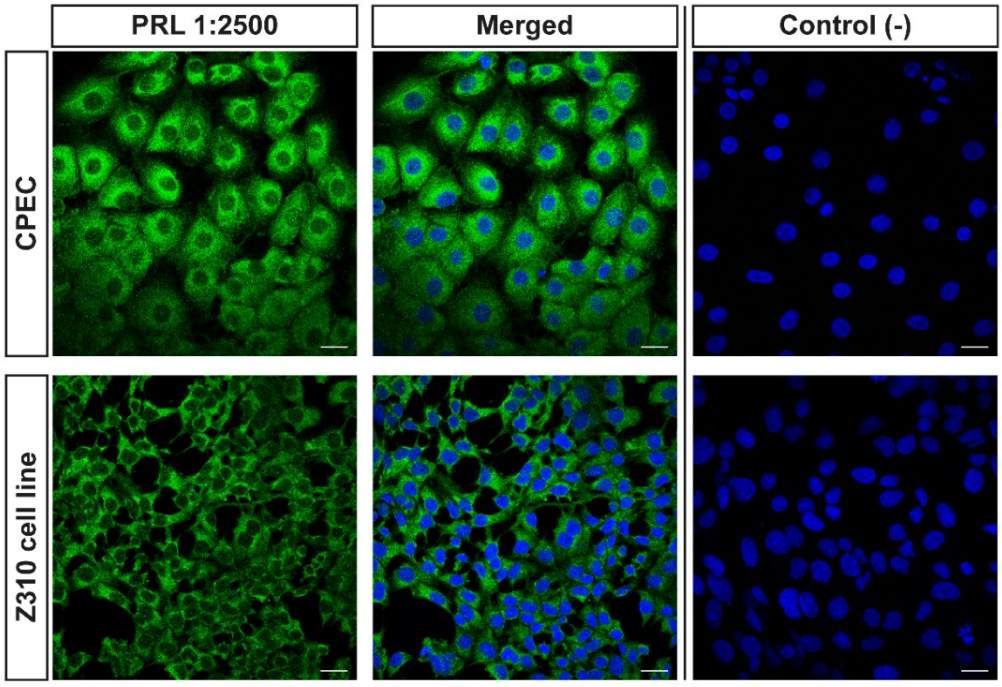

Figure 4.3. Confocal fluorescence images of PRL in CPEC and Z310 cells (63x). CPEC and Z310 cells incubated with rabbit anti-rat PRL primary antibody (dilution 1:2,500) and secondary antibody Alexa Fluor® 488 goat anti-rabbit (dilution 1:1,000). Nuclei were stained with Hoechst 33342 (dilution 1:1,000). Merged images of coverslips incubated without anti-rat PRL antiserum are shown (Control (-)). Images were obtained on a confocal fluorescence microscope. Immunocytochemistry staining of PRL in CPEC and Z310 cells cytoplasm was observed. Scale bar, 20 µm.

To assess if PRL was secreted to the membrane apical side, or to the basal side of the CP epithelium, the culture supernatants collected from the *in vitro* model used to mimic the blood-

CSF barrier were also analyzed by Western blot. Similarly to what was observed in the culture medium incubated with CP explants, the PRL protein with higher molecular weight (> 63 kDa) was also obtained in the supernatants collected from the upper and lower compartment of the inserts (Figure 4.4.a), suggesting that PRL is secreted both through the apical and the basal membrane of CPEC. These results are reinforced by the presence of immunoreactive PRL protein of the same size in culture medium incubated with pituitary explants, female and male CSF samples, and pregnant female serum samples. All tested CP tissue samples presented lower levels of PRL than the pituitary, as expected, but this difference only reached statistical significance in CP samples collected from 14—15 days pregnant female rats, where PRL levels were the lowest (Figure 4.4.b, 1.652 ± 0.419 versus 0.121 ± 0.043, $p < 0.05$).

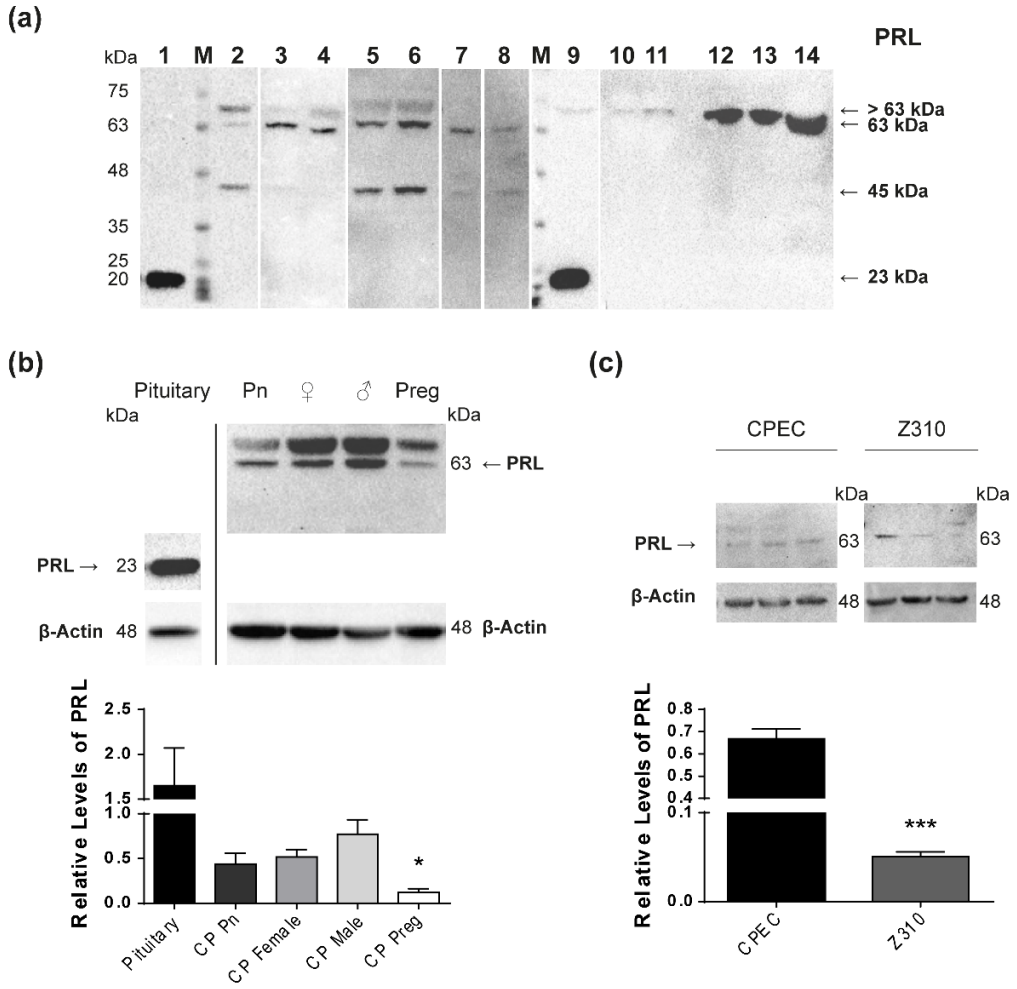

Figure 4.4. Western blot analysis and relative levels of PRL in rat CP. (a) L1: Pituitary collected from three postnatal rats (3—6 days old), 2.5 µg. L2: CPs collected from two pregnant rats, 25 µg. L3: CPs collected from eight postnatal rats (3—6 days old), 20 µg. L4: DMEM incubated for 24 h with CPs collected from eight postnatal rats (3—6 days old), 15 µL. L5: CPs collected from a 3-month-old virgin female rat, 25 µg. L6: CPs collected from a 3-month-old male rat, 25 µg. L7: Z310 cell line, 25 µg. L8: CPEC, 25 µg. L9: DMEM incubated with pituitaries collected from three postnatal rats (3—6 days old), 15 µL. L10: DMEM incubated with CPEC for 48 h, apical side, 30 µL. L11: DMEM incubated with CPEC for 48 h, basal side, 30 µL. L12: CSF collected from a 3-month-old female rat, 15 µL. L13: CSF collected from a 3-month-old male rat, 15 µL. L14: Serum collected from a 14-15 days gestation pregnant rat, 2.5 µL. M: GRS Protein Marker Multicolor (Grisp, Portugal). A protein band with the expected size (~23 kDa) is present in rat pituitary

(legend continued on next page)

(Positive Control). An ~63 kDa immunoreactive PRL protein, with higher molecular weight, was detected in protein extracts from CPs of pregnant, postnatal, 3-month-old virgin female and 3-month-old male rats, as well as in CPEC and Z310 cells. In CP samples and culture medium incubated for 24 h with CP explants, besides the 63 kDa immunoreactive PRL protein, a higher molecular band was also observed (> 63 kDa). (b) Quantitative results of PRL protein relative levels in postnatal pituitaries (2.5 µg), CPs collected from postnatal (CP Pn), 3-month-old female (CP Female), 3-month-old male (CP Male) and 14—15 days pregnant female (CP Preg) rats (25 µg) from three independent experiments (mean ± SEM). (c) Quantitative results of PRL protein relative levels in CPEC and Z310 cells (25 µg). Only 63 kDa bands were quantified in case of CPs and cells. Kruskal–Wallis test followed by Dunn's multiple comparison test against pituitary (* $p < 0.05$) in (b) and Student's unpaired t test (*** $p < 0.001$) in (c). ♀, Female; ♂ Male.

The levels of PRL protein were also compared between the two CP cell culture models used in this study (Figure 4.4.c), with CPEC cells presenting higher levels of PRL (0.669 ± 0.043) compared to the immortalized CP cell line Z310 (0.051 ± 0.005, $p < 0.001$). The absence of PRL bands in negative controls of culture medium and in membranes without primary antibody excluded the presence of PRL in the culture medium as well as the existence non-specific crosslinks caused by secondary antibody (data not shown). The specificity of the PRL antibody was tested and confirmed in CP and pituitary lysates by blocking the anti-rat PRL antibody with PRL peptide (Figure 4.5.a). The 63 kDa immunoreactive PRL was present in protein extracts from CPs collected from the lateral ventricles of postnatal rats and medium incubated with CP explants under reducing and non-reducing conditions (Figure 4.5.b).

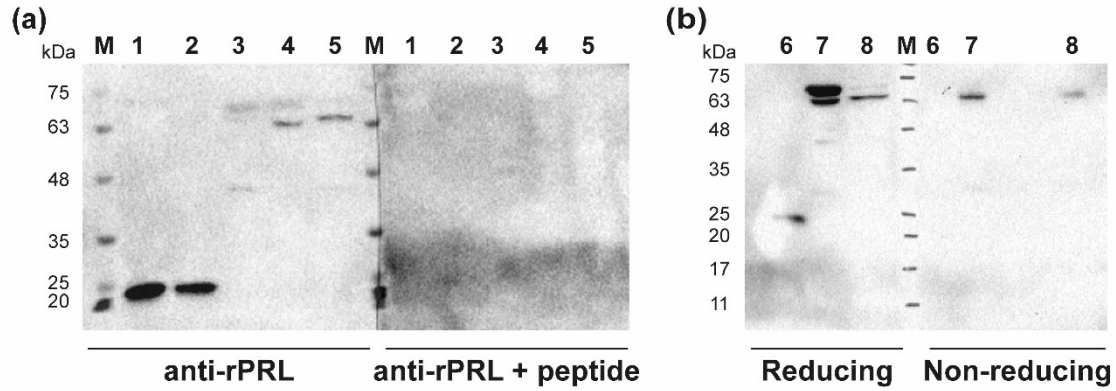

Figure 4.5. Western blot results using antibody pre-incubated with rat PRL peptide and reducing or non-reducing conditions. L1: DMEM incubated with pituitaries collected from three postnatal rats (3—6 days old), 15 µL. L2: Pituitary collected from three postnatal rats (3—6 days old) 2 µg. L3: CPs collected from pregnant rat, 20 µg. L4: DMEM incubated for 24 h with CPs collected from eight postnatal rats (3—6 days old), 15 µL. L5: CPs collected from eight postnatal rats (3—6 days old), 20 µg. L6: Pituitary collected from three postnatal rats (3—6 days old) 2.5 µg. L7: DMEM incubated for 24 h with CPs collected from eight postnatal rats (3—6 days old), 15 µL. L8: CPs collected from eight postnatal rats (3—6 days old), 20 µg. M: GRS Protein Marker Multicolor (Grisp, Portugal). (a) The specificity of the PRL antibody was confirmed by blocking the anti-rPRL antibody with PRL peptide over 20 h at 4 °C. No PRL immunoreactive bands were visible when the antibody was preincubated with the PRL peptide. (b) The ~63 kDa immunoreactive PRL protein was detected in protein extract from CPs and culture medium incubated with CPs in both reducing (with β-mercaptoethanol) and non-reducing conditions (without β-mercaptoethanol addition to sample buffer).

4.4.2. Prolactin receptor is located mainly in CPEC cytoplasm

The expression of PRLR in the CP is well known [6, 7, 24] and was confirmed in this study by the observation of cytosol positive staining in epithelial cells of the 14—15 days pregnant rat

female lateral ventricle CP tissues by immunohistochemistry (Figure 4.6.a). To investigate the subcellular localization of PRLR in the CP cells, the established *in vitro* model of the blood-CSF barrier was used. Positive immunocytochemistry staining of PRLR in CPEC isolated from postnatal rats was present in the cell cytoplasm, suggesting that at this stage of development PRLR are mainly present in the cytosol and probably in the endoplasmic reticulum (Figure 4.6.b). Nonetheless, even after the addition of 5 ηg/mL of rat PRL peptide stimulus to the basal side (lower compartment) of the *in vitro* blood-CSF barrier model over 8 hours, immunopositive staining of PRLR remained distributed throughout the cytoplasm of CPEC and was not restricted to the apical or to the basal side of these cells, although some positive staining was also observed in the cell membrane (Figure 4.6.b).

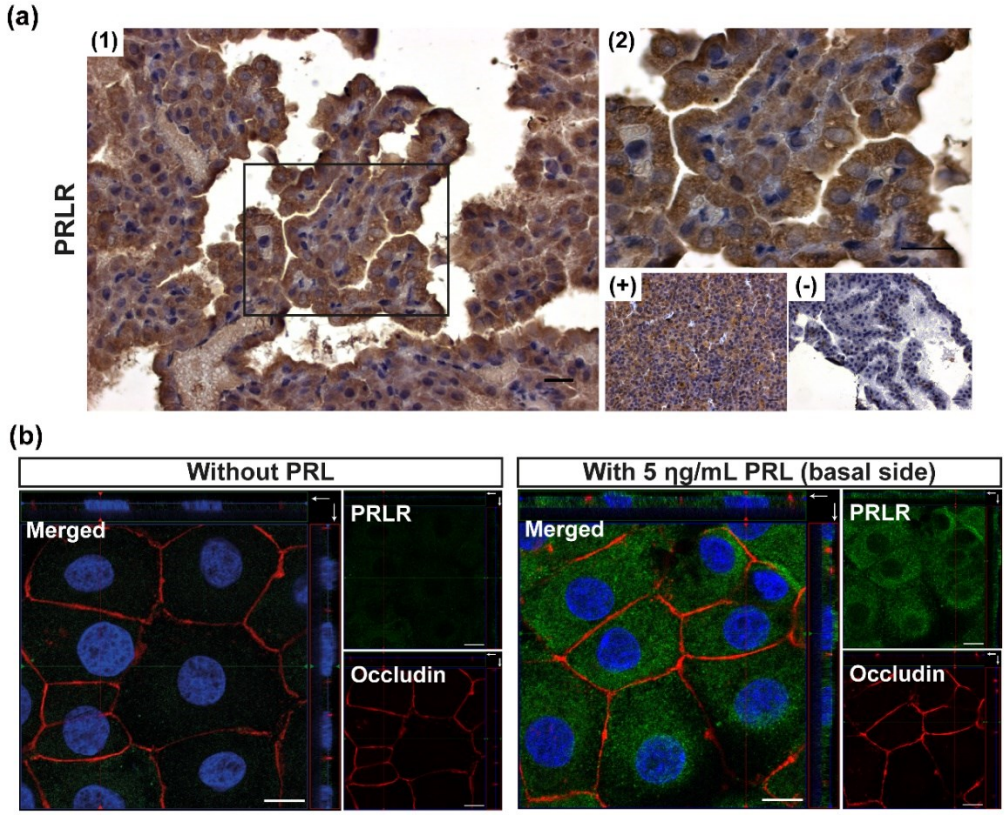

Figure 4.6. Immunostaining of PRLR in pregnant rat CP tissue and CPEC using an in vitro model of the blood-CSF barrier. (a) Representative images of PRLR immunostaining in CPs collected from a 14—15 days pregnant Wistar Han female incubated with rabbit anti-rat PRLR serum (1:300). Slides were visualized under a magnification of 40x and (2) is a high-powered image (magnification 100x) of boxed area in (1). Nuclei were stained with Mayer´s Hematoxylin. Pituitary tissue was used as a positive control (+) and tissue sections without PRLR antiserum were used as negative control (-). (b) CPEC cells in inserts were either left unstimulated or stimulated with 5 ηg/mL of rat PRL peptide applied in the basal side (lower compartment) for 8 hours. Afterwards, cells were incubated with rabbit PRLR antiserum (1:100), followed by incubation with Alexa Fluor® 488 goat anti-rabbit IgG conjugate (1:1000) and mouse anti-occludin Alexa Fluor® 594 (1:200). Nuclei were stained with Hoechst 33342 (1:1000). Membrane apical side is indicated by an arrow. Scale bar: (A) 20 μm, (B) 10 μm.

4.5. Discussion

In the past two decades, PRL has been associated with the development of maternal behavior and described as a neurogenesis-stimulating factor, especially during pregnancy [14, 15]. Yet, the origin of PRL in the central nervous system, in the CP and in the CSF that baths the SVZ remains unclear. It was previously thought that PRL was up taken by CP cells from the peripheral circulation via PRLR, and then delivered into the CSF. However, the finding that PRLR knockout mice still have similar levels of PRL in the CSF as their wild type littermates [24], compromised this view on the origin of PRL in the CSF.

The present study provides evidences that transcription of full-length PRL occurs in rat CPs dissected from the lateral ventricles of 14—15 days gestation pregnant rats, probably accounting for the presence of PRL protein in CP cells and in the CSF. These results confirm our previous detection of PRL transcripts in the rat CPs by cDNA microarrays [25], and add another site of PRL production to the brain in addition to the hypothalamus [30, 31], thalamus, caudate, amygdala and brainstem [32]. The presence of full-length PRL mRNA expression has also been described in bovine brain capillary endothelial cells [33] and could explain the presence of PRL transcripts in CP tissues collected from pregnant rat females. However, endothelial cells are absent in both primary and immortalized cultures of rat CP epithelial cells (highly pure cultures) used in this study, where the amplicon corresponding to the full PRL transcript was also present.

The existence of relevant extrapituitary sources of PRL in the brain of rodents is supported by several studies conducted in hypophysectomized animals. For instance, PRL levels remained detectable in CSF samples of hypophysectomized male rats two weeks after surgery, contrasting with the absence of PRL found in the plasma of these animals [34]. More importantly, nulliparous female rats subjected to hypophysectomy still displayed maternal behavior after prolonged exposure to pups [35, 36]. Since PRL action is fundamental for the development of maternal behavior [20], the presence of delayed maternal behavior in hypophysectomized rat females is supportive of the highly relevant physiological role played by PRL of non-pituitary origin in the brain. In addition, like the PRL mRNA in the CP, the PRL mRNA sequence found in other non-pituitary tissues from hypophysectomized rats, like the hypothalamus, is identical to the PRL mRNA of pituitary origin [37].

The synthesis and localization of PRL protein in CP, was also confirmed by immunohistochemistry, immunofluorescence and Western blot. As expected, immunohistochemistry analysis confirmed the presence of PRL positive staining in the cytoplasm of epithelial cells of CP tissues dissected from the lateral brain ventricles of a 14—15 days pregnant rat. Interestingly, PRL immunopositive staining was more evident in the apical membrane of CP epithelial cells, which is not an entirely surprising observation given the secretory nature of the CP. The presence of immunoreactive PRL protein in the brain [38, 39], including CP [40, 41], was previously described. However, to our knowledge, this is the first

work reporting the presence of immunofluorescence positive staining in CPEC and the Z310 cell line which excludes the possibility of uptake of PRL from the peripheral circulation, or from the cell culture media that was not immunoreactive to anti-PRL in Western blot experiments.

Western blot analysis performed in this report consistently revealed the presence of a higher molecular weight PRL immunoreactive protein, (~63 kDa), in extracts from CPs collected from the lateral ventricles of all tested animal groups, CPEC and Z310 cells. In some experiments, an additional 45 kDa PRL immunoreactive protein was also present in these extracts. The expected 23 kDa PRL band was only observed in pituitary extracts. The 63 kDa PRL protein and an additional band with slightly higher molecular weight were also found in medium incubated with CP explants or pituitaries, protein lysates of freshly collected pituitary tissues (data not shown) and in serum and CSF samples. This suggests that the CP is able to synthesize and secrete PRL and supports the results of this study. Although the presence of PRL protein was observed by immunofluorescence in Z310 cells, the low levels of PRL protein found in Western blot experiments, together with the low intensity band present in RT-PCR, suggest that this immortalized rat CP cell line may not be the most suitable model regarding the study of PRL expression and secretion by the rat CP.

The heterogeneity of PRL isoforms, including big PRL with a molecular weight of 40—60 kDa, is widely accepted among researchers and was reviewed in several seminal articles [42, 43]. Previous studies using Western blot reported the presence of this PRL isoform in cervical cancer cells [44], immune cells [45, 46] and plasma [47] from human origin. Big PRL represents approximately 15 to 30% of total PRL [43] and has been described as a dimer of covalently-linked subunits of monomeric PRL [48]. However, the presence of the 63 kDa PRL protein in Western blot experiments performed under reducing and non-reducing conditions may suggest that this isoform is not a dimer.

Other hypotheses concerning the origin of the 63 kDa PRL include the association of monomeric PRL with some type of PRL-binding protein and the possibility of other post-translational modifications. A variety of PRL-binding proteins have been reported in the literature [49–51] and were described as possible inhibitors/enhancers of PRL action or even as reservoirs of this polypeptide hormone in the circulation [51]. Additionally, PRL post-translational modifications were proposed as a possible explanation for the presence of the big PRL in peripheral blood mononuclear cells isolated from Systemic Lupus Erythematosus patients [52].

The biological activity of higher molecular weight PRL isoforms is a far more controversial issue. While some reports provide evidence of *in vitro* big PRL biological activity [46, 52], others support that this isoform presents reduced levels of bioactivity in bioassays conducted using Nb2 cells [53]. PRL bioactivity may be tissue or species dependent, as formerly suggested [53]. A thorough search for reports on the quantification of PRL in the serum or in the CSF did not show any data obtained by Western blot. Instead, comparisons of PRL levels are frequently carried out by radioimmunoassay or ELISA [54–57]. Thus, based on our findings, the possibility

that PRL measurements in these body fluids may be the result of the detection of the 63 kDa form rather than just the 23 kDa monomeric isoform of PRL cannot be ruled out. Whether the 63 kDa PRL protein here reported is the result of PRL binding to any carrier protein or if this isoform has any biological role are questions that deserve further investigation.

Evidences suggest that estradiol may play an important role in the upregulation of brain and pituitary PRL [2, 5]. Nonetheless, the presence of PRL RT-PCR transcripts in CPs collected from the lateral ventricles of mid-pregnancy female rats, characterized by low levels of estradiol [58], along with the lack of statistically significant differences in the levels of PRL protein in CPs collected from postnatal, virgin females and male rats and the presence of PRL protein in both male and female rat CSF samples, may indicate the existence of other regulators/inducers of PRL synthesis in CP, or of a constitutive expression of the hormone. Furthermore, the observation of full-length PRL in CPEC (isolated from postnatal rats) and Z310 cells, without estradiol exposure, seem to reinforce this hypothesis. Remarkably, the observation of similar PRL protein levels in the protein lysates of CPs collected from the lateral ventricles conflicts with the results of a previous transcriptomic analysis microarray study performed by our research group, reporting the presence of higher expression levels of PRL in CPs collected from the lateral ventricles of sham operated 2-month-old rat females when compared to 2-month-old ovariectomized females and same age sham operated rat males [25]. The differences between CP transcriptome and proteome suggest that PRL expression may suffer some type of post-transcriptional, translational or secretory regulation.

Another surprising finding of the Western blot analysis performed in this study was the presence of PRL in both apical and basal compartments of the established blood-CSF barrier model. The CP is a secretory tissue by nature contributing with the release of several peptides into the CSF [9, 59] leading us to initially hypothesize that PRL would be secreted through the apical membrane. However, the presence of PRL immunoreactive protein in both compartments of the blood-CSF model appears to indicate that CPEC are able to secrete PRL not only through the apical membrane facing the CSF, but also through the basal membrane facing the blood. The CP is not only a gateway for molecules into the brain but a structure responsible for brain detoxification and transport of molecules in the opposite direction [9, 11]. These observations put forward the possibility that CP-borne PRL may act as a paracrine in the connective tissue that lies beneath the basal membrane of CPEC and perhaps enter the fenestrated capillaries therein adding to the peripheral pool of circulating PRL. If so, the CP could also be considered a non-classical endocrine organ.

As stated above, the CP is the structure within the brain that expresses the highest amount of PRLR [42]. However, the exact localization of PRLR in CPEC cultured in inserts to mimic blood-CSF barrier has never been assessed before. Immunopositive staining was present in the cytoplasm of CPEC cultured in inserts which is in agreement with PRLR staining in experiments using rat ovary cells [60]. In an earlier work conducted to evaluate the developmental

expression of PRLR in rat CP, specific binding of PRL was only observed at postnatal day 14 [61], suggesting that PRLR may be present in vesicular compartments that later migrate and insert in the cell membrane [61]. Since CPEC cultures were isolated from 3—6 days old rats and based on former evidences reporting that PRL administration is able to increase the expression of PRLR mRNA in murine CP [62], we ascertained if PRL was able to induce membrane expression of PRLR *in vitro*. Although the majority of the immunopositive PRLR staining was still found in CPEC cytoplasm, some membrane staining was also observed after PRL stimulus. However, PRLR positive staining was not confined to either the apical or basal membrane of CPEC cells as initially hypothesized.

In conclusion, our results provide evidences that the lateral ventricles of CP synthesize and secrete PRL suggesting that this tissue might be an alternative source of PRL to the brain rather than just a gateway for its transport to the CSF. CP epithelial cells are not only able to secrete PRL through the apical membrane facing the CSF, but also trough the basal membrane side facing the blood. Furthermore, PRLR expression appears to be predominantly located in CPEC cytoplasm and appears to partially translocate to the cell membrane after the addition of PRL stimulus. Based on these data, we propose that the CP detects PRL changes in the periphery and based on those clues induces PRL secretion towards the CSF and perhaps back to the periphery to balance these alterations. Considering the proximity of CP to the SVZ, CP-borne PRL may play an important role in this neurogenic niche. Further studies are necessary in order to investigate this possibility and to better understand the biological function of CP-borne PRL as well as the possible regulation of PRL expression in the CP.

4.6. References

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Chapter 5

Sex hormones do not regulate the synthesis of prolactin in the postnatal rat choroid plexus

5.1. Introduction

The choroid plexus (CP) is a known target of sex hormones action (reviewed by [1]). In rats, sex hormone background modulates the expression of genes involved in a variety of functions, ranging from the barrier function to the immune and chemical surveillance [2, 3]. In a previous transcriptomic microarray study performed by our research group, prolactin expression in the rat CP was higher in intact females than in ovariectomized females or intact males, suggesting that sex hormones may modulate the expression of prolactin in this brain structure [2].

The hypothalamic dopaminergic system, the main inhibitory regulator of pituitary prolactin secretion, is composed of three populations of distinct dopaminergic neurons in rats: the periventricular hypophyseal, tuberoinfundibular (TIDA), tuberohypophyseal and periventricular hypophyseal neurons. Under normal physiological conditions, prolactin levels are monitored by TIDA neurons that release dopamine into the portal blood system in response to increased prolactin levels through a "short-loop" negative feedback that reduces prolactin secretion (reviewed by [4, 5]). However, the observation of increased prolactin levels during the late phase of pregnancy and lactation, indicates that the prolactin regulation mechanism by TIDA neurons suffers some type of alteration during these periods. Recently, Yip and colleagues suggested that prolactin is able to increase its own secretion during lactation by switching the phenotype profile of TIDA neurons from dopamine to met-enkephalin release and that this change is a consequence of placental lactogen-mediated modification of PRLR downstream signaling in TIDA neurons during pregnancy [6]. Many regulators of hypophyseal prolactin secretion, that either have a direct action in lactotrophs or mediate dopamine release and TIDA neurons activity, have been considered putative modulators of PRL expression, including triiodothyronine [7], thyrotropin-releasing hormone [8], oxytocin [9], vasoactive intestinal polypeptide [9, 10], kisspeptin [11], among many other antagonists of dopamine receptors.

Apart from physiological stimuli like suckling, mating, exercise and stress, that are associated with *in vivo* modulation of prolactin secretion [5, 12, 13], gonadal steroid hormones (Table 5.1), and particularly estradiol (E2), are also considered main regulators of pituitary prolactin secretion (reviewed by [4]). Depending on factors such as exposure time and concentration, estradiol is acknowledged for its stimulatory effect in pituitary PRL synthesis and secretion [14–16] and the modulation of TIDA neurons activity [17]. Both rat and human prolactin gene promoters have estrogen-responsive elements [18, 19]. In rodents, increased prolactin levels during the afternoon of proestrus and in the late stage of pregnancy are associated with increased circulating estradiol levels (reviewed by [4, 5]). On the other hand, the influence of progesterone (P4) is not as consensual as that of estradiol. Some data point to a potential inhibitory influence in prolactin release by pituitary cells [20], although a P4-induced increase of prolactin release in E2-treated ovariectomized female rats had also been reported [21]. Since prolactin is mainly associated with female reproduction, the possible role of androgens in the regulation of prolactin expression is understudied. However, there is evidence that testosterone can induce the rapid nongenomic release of prolactin in specific subsets of lactotrophs in male rats [22]. Testosterone (T) seems to be

fundamental for prolactin-mediated signaling in male mice brain and this action is presumably unrelated to testosterone conversion to estradiol by aromatase [23]. Conversely, 4,5α-dihydrotestosterone (DHT) seems to exert no influence on the levels of pituitary prolactin expression and secretion [24, 25].

While in rodents extrapituitary and pituitary prolactin gene expression is driven by the same gene promoter, in humans, the prolactin gene has an additional superdistal promoter region responsible for the regulation of its transcription at extrapituitary sites [26, 27]. This superdistal promoter region responsible for extrapituitary prolactin expression is not affected by estrogens [28]. Therefore, in humans, the expression of non-pituitary prolactin may be regulated by tissue-specific mechanisms, distinct from those observed in rodents. Several factors, including signaling molecules and transcription factors, have been implicated in the regulation of prolactin production in non-pituitary tissues like the immune system, the uterus, or the mammary tissue (reviewed by [29–31]). Like in the pituitary, sex hormones seem to influence the expression and secretion of prolactin in some extrapituitary tissues, and this action seems to be tissue-specific. For instance, E2 treatment increases the expression of prolactin in the brain of female rats [32, 33], while the incubation with E2 appears to not affect the level of prolactin secreted by human mammary tissue explants [34]. However, not much information regarding the modulation of prolactin expression in extrapituitary sources is available, especially in the brain, where besides the possible regulatory role of estradiol, only stress and suckling stimulus were identified as possible prolactin regulators [35].

Since evidence support that the CP is a source of prolactin in the rat brain, as earlier described in chapter 4, the main goal of this work was to evaluate if sex hormones, E4, P4 and DHT, modulate the levels of prolactin in the postnatal rat CP and pituitary as well.

Table 5.1. Summary of the effects of sex hormones in pituitary and extrapituitary PRL synthesis and secretion.

Hormone	Model	Hormone stimulus	Effect	Ref.
E2	Male rats	Single 80 μg/100 g of body weight SC injection	↑ Pituitary PRL mRNA levels (24—48 h after exposure)	[15]
	OVX rats	Single 1 μM SC	Day 1: ↓ Pituitary PRL mRNA and PRL serum levels	[14]
			Day 3: ↑ Pituitary PRL mRNA and PRL serum levels	
			Day 7: No effect in pituitary PRL and mRNA PRL serum levels	
	OVX rats	15 mg SC implantation	Day 8: ↑ Pituitary PRL levels	[16]
			Day 14: ↑ Serum PRL levels	
	OVX rats	0—5 μg/100 g body weight SC injection for 12 days	↑ Serum PRL levels (14—24 days after last exposure)	[36]
	Newborn female rats	Single 1—100 μg SC injection	↑ Serum PRL and pituitary PRL levels (8 weeks after treatment)	[37]
	GH3 cells	10 ρM – 1 μM for 7 days	↑ PRL secretion	[38]
	OVX rat pituitary explants	55—1500 ηg/mL for 4 hours	↑ Pituitary PRL levels	[39]
	Human breast tissue explants	1—10 ηM for 10 days	No effect on PRL release	[34]
	HPX female rats	1 μg/100 g of body weight for 4 days	↑ Hypothalamus and pons-medulla PRL mRNA content	[33]
	HPX female rats	SC implants for 10 days, concentration not available	↑ Hypothalamus PRL mRNA and PRL protein levels	[32]
P4	Rat pituitary explants GH3 cells	Explants: 100 nmol/L for 1 h GH3 cells: 20 nM for 30 min	↓ PRL secretion levels and ↑ PRL protein levels in pituitary explants	[20]
	GH3 cells	10 ρM – 1 μM for 7 days	↓ PRL secretion	[38]
	Rat pituitary primary cell cultures	10 nM for 4 days	No effect on the expression and secretion of PRL	[40]
	OVX E2-treated rats	Single 0—0.8 mg/100 g of body weight SC injection	↑ Serum PRL levels (4 h after treatment)	[21]
	Hypogonadal women	50 mg IM injection	↓ Serum PRL levels in 8 of the 12 women	[41]
	Human breast tissue explants	10—100 ηM for 1—10 days	↓ PRL release from glandular explants	[34]
	Human mifepristone-treated decidual explants	0—500 ηg/mL for 1—5 days	No effect on PRL secretion	[42]
	Human decidual explants	50 ηg/mL for 14—41 days	↑ PRL secretion	[43]

T	OOX rats	0—5 μg/100 g body weight SC injection for 12 days	↑ PRL serum levels (14—24 days after last exposure)	[36]
	Newborn female rats	Single 10—1000 μg SC injection	↑ Serum PRL and pituitary PRL levels (8 weeks after treatment)	[37]
	OVX pony mares	150 μg/kg of body weight SC injection for 21 days	No effect on pituitary PRL levels	[44]
	Klinefelter's syndrome patients	100 mg IV injection for 4 days	↑ Plasma PRL levels	[45]
	OOX rats	4 mg/kg of body weight SC injection for 3—10 days	Day 3: ↑ Serum PRL levels	[46]
			Day 10: No effect on serum PRL levels	
	Late-onset hypogonadism with macroprolactinemia* patients	100 mg/week IM injected for 4 months	No influence on PRL and macroprolactin serum levels	[47]
	Newborn and adult female rats	Single 250 μg SC injection at the day of birth and day 60	Neonatal treatment: ↓Pituitary PRL mRNA positive cells and PRL mRNA content/cell	[48]
			Adult treatment: No effect on pituitary PRL mRNA positive cells and PRL mRNA content/cell	
			Neonatal + Adult treatment: ↑ Pituitary PRL mRNA positive cells and PRL mRNA content/cell	
	Male rat pituitary explants	1—10 ηM for 5—20 minutes	↑ PRL secretion	[22]
	GH3 cells	1 μM for 7 days	↑ PRL secretion	[38]
DHT	OVX pony mares	150 μg/kg of body weight SC injection for 21 days	No effect on pituitary PRL levels	[44]
	Pregnant rats	4 mg pellet for 24 hours	↓ PRL nocturnal surge	[49]
	OVX ewes	Single 10—100 μg intracarotid injection	No effect of PRL serum levels (4 hours after treatment)	[25]
	Rat primary pituitary cell cultures	100 ηM for 3 days	No effect on pituitary PRL mRNA levels	[24]
	Klinefelter's syndrome patients	100 mg IV injection for 4 days	No effect on plasma PRL levels	[45]
	GH3 cells	1 μM for 7 days	No effect on PRL secretion	[38]

* Increased levels of macroprolactin (150—170 kDa PRL complexes).
DHT: 4,5α-dihydrotestosterone, E2: 17β-estradiol, GH3: Rat pituitary tumor cells, HPX: Hypophysectomized OVX: Ovariectomized, OOX: Orchidectomized, P4: Progesterone, Ref.: Reference, SC: subcutaneous injection, T: Testosterone.

5.2. Material and Methods

5.2.1. Animals

The pituitary and CP tissues were collected from Wistar Han rats aged between 3—6 postnatal days. Animals were housed in proper cages, at constant room temperature in a 12 h light/12 h dark photoperiod and given standard laboratory chow and water *ad libitum*. Animal tissue collection was approved by the Animal Welfare and Ethics Committee of the Health Science Research Centre of the University of Beira Interior, in compliance with National (Decree-law 113/2013, corrected by the Decree-law nº 1/2019) and European Union rules (Directive 2010/63/EU) for the care and handling of laboratory animals. No further licensing was required since the study did not involve animal experimentation. Before decapitation, Wistar Han rats up to 6 days old were anesthetized on ice, while older animals were anesthetized with isoflurane. All efforts were made to minimize the number of animals used as well as their suffering.

5.2.2. *Ex vivo* studies

For the *ex vivo* experiments, pituitary tissues (pituitary explants) and CP tissues (CP explants), the latter dissected from the brain lateral ventricles, were collected from 3—6 days old postnatal rats. In each experiment, pools of three pituitary and sixteen CPs were placed directly in 500 µL of high-glucose DMEM without phenol red (Gibco, ThermoFisher Scientific, USA, Cat# 12800017), supplemented with 10% charcoal-stripped fetal bovine serum (FBS; Capricorn Scientific, Germany, Cat# FBS-12B) and 100 U/mL of penicillin/streptomycin (Sigma-Aldrich, Germany, Cat# P4333) in 24-wells culture plates. To assess the influence of sex hormones in the regulation of prolactin levels, two hours after the incubation in culture medium, CP and pituitary explants were stimulated with different concentrations of E2 (17β-estradiol; Sigma-Aldrich, Germany, Cat# E8875: 0.1 and 1 ηM), P4 (Calbiochem; 10 and 100 ηM), DHT (Sigma-Aldrich, Germany, Cat# A8380: 1 and 10 ηM) or vehicle (0.0001—0.01% ethanol), and incubated for additional 24 hours. DHT was chosen in turn of testosterone to avoid any possible conversion of testosterone to E2 by aromatase present at the CP [50, 51]. All sex hormone concentrations included in this study were chosen according to previously reported *ex vivo* experiments [22, 34, 52, 53]. We opted to choose sex hormones concentrations closer to the female rat physiological levels [54, 55], and in the case of E2 and DHT, a higher concentration was also used. All cultures were maintained in a humidified incubator in 95% air—5% CO_2 at 37 °C.

5.2.3. RNA extraction and reverse transcription (RT)-PCR

Total RNA was extracted from the CP tissues collected from animals with 3—6, 14 and 21 days old. Except for rats with 4 days old, where total RNA was extracted from pools with 4 animals from the same litter, total RNA was extracted from the CPs of a single animal. Pituitary tissue collected from an adult pregnant rat was used as positive control. Total RNA was extracted using TRIzol (TripleXtractor, Grisp, Portugal, Cat# GB23.0100), according to the manufacturer's

instructions. RNA was quantified by spectrophotometry (NanoPhotometer™, Implen, Germany), and RNA integrity was assessed by agarose (1.5 %) gel electrophoresis stained with *GreenSafe* (NZYTech Ltd., Portugal, Cat# MB13201). To eliminate possible contaminations with genomic DNA, samples were treated with DNAse I (Sigma-Aldrich, Germany, Cat# AMPD1) before complementary DNA synthesis. One µg of total RNA of each sample was transcribed into complementary DNA using M-MuLV Reverse Transcriptase (NZYTech Ltd., Portugal, Cat# MB08301, 20 µL reaction), according to the manufacturer's instructions. PCR reactions were carried out in a final volume of 10 µL using NZYTaq II 2x Green Master Mix (NZYTech Ltd., Portugal, Cat# MB35801) and 0.4 µM of each primer, on a MultiGene™ Optimax Thermal Cycler (Labnet International, USA). The rat prolactin specific oligonucleotide primers were designed using the software tool Primer-BLAST (NCBI- NIH): forward 5'—TTG ACC GTG TGG TCA TGC TT—3' and reverse 5'—GGA GGG ACT TTC TGG GCT TG—3'. The PCR cycling protocol encompassed an initial 3-minute denaturing reaction at 95 °C, followed by 30 seconds at 94 °C, 30 seconds at 62 °C, and 30 seconds at 72°C for 40 cycles with a final 10 minutes extension at 72°C. Prolactin PCR products were analyzed on a 2 % agarose gel electrophoresis, visualized under UV with *GreenSafe* staining (NZYTech Ltd., Portugal, Cat# MB13201), and Sanger sequenced (STAB VIDA, Portugal) to confirm the gene identity. A negative control consisting of a PCR reaction without template was included in all the assays.

5.2.4. Total protein extraction and Western Blot

To evaluate the levels of prolactin in CP and pituitary explants, tissues collected at the end of *ex vivo* experiments were homogenized, on ice, using radioimmunoprecipitation assay buffer (RIPA) (150 mM sodium chloride, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 50 mM Tris pH 8.0) completed with 1 mM phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate and 40 µL/mL cOmplete™ EDTA Free protease inhibitor cocktail (Roche, Sigma-Aldrich, Germany, 25x stock, Cat# 11873580001). Lysates were centrifuged at 10,000 *g* for 10 minutes at 4 °C and supernatants were collected and stored at -20 °C until use. Pituitary conditioned media collected at the end of the *ex vivo* experiments were used to evaluate alterations in prolactin secretion. Total protein quantification was performed using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA, Cat# 23225). Pituitary (2 µg) and CP protein lysates (20 µg), as well as pituitary culture supernatants (15 µL) were diluted in 4X sample buffer containing freshly added 10 % β-mercaptoethanol, denatured at 100 °C for 5 minutes and separated by 10 % SDS-PAGE. Afterward, proteins were blotted to polyvinylidene difluoride membranes (Amersham™ Hybond™ 0.45 µm, GE Healthcare, UK, Cat# 10600023) and membranes were blocked for 1 hour with 5 % skimmed milk in Tris-buffered saline with 0.1 % Tween 20 (TBS-T 0.1%), before being incubated overnight at 4 °C with rabbit anti-rat PRL antiserum IC-5 primary antibody (1:4,000; generously supplied by The National Hormone and Pituitary Program, NIDDK). After being washed with TBS-T 0.1 %, membranes were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:20,000; Thermo Fisher Scientific Cat# 31466, RRID:AB_10960844). Protein bands were visualized by chemiluminescence using SuperSignal™ West Pico PLUS Chemiluminescent

Substrate (Thermo Fisher Scientific, USA, Cat# 34577) and detected using a ChemiDoc™ MP Imaging System (Bio-Rad, USA) and Image Lab 5.1 software (Bio-Rad, USA, RRID:SCR_014210). Densitometry of each protein band was measured using Image Lab software, and the relative intensity of each prolactin band was normalized against α-tubulin levels (1:10,000; Sigma-Aldrich Cat# T9026, RRID:AB_477593) and compared between groups. At least four independent samples within each group were quantified.

5.2.5. Statistical analysis

Values are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, CA, RRID:SCR_002798). The statistical significance between two groups was analyzed with Student's two-tailed unpaired *t*-test. Significant differences between groups was set at $p < 0.05$.

5.3. Results

5.3.1. Prolactin is expressed in the postnatal rat CP

Previously our research group presented evidence that prolactin mRNA is present in the CP of adult pregnant rat females as well as in CPEC [56]. To assess if prolactin was also expressed in the postnatal CP, RT-PCR was performed using CPs RNA samples from animals with distinct neonatal ages. As observed in RT-PCR experiments, in Figure 5.1, prolactin mRNA transcripts were present at all analyzed ages, suggesting that prolactin is already expressed in the postnatal stage.

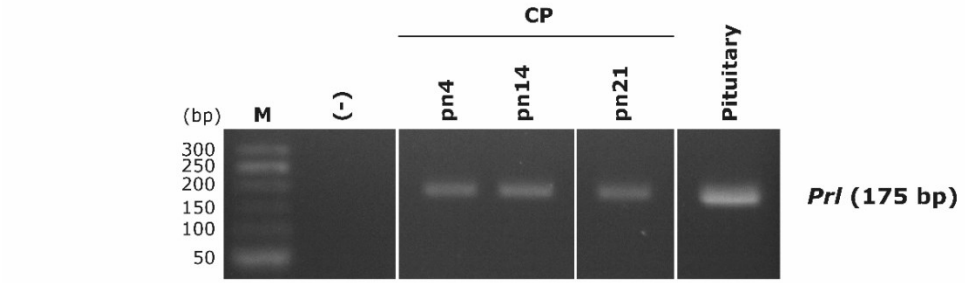

Figure 5.1. Prolactin is expressed in the postnatal rat choroid plexus (CP). Gel electrophoresis of RT-PCR products shows the presence of prolactin transcripts in the CP of a postnatal rat (pn) with 4, 14 and 21 days old. Total RNA extracted from pituitary tissue from an adult pregnant rat was used as a positive control. M: Molecular weight GRS Ladder 50bp (Grisp, Portugal, Cat# GL031.0050); (-) Negative control.

5.3.2. CP prolactin protein levels are not modulated by sex hormones

To evaluate the possible effect of sex hormones on the levels of prolactin in postnatal life, pituitary and CP explants were collected from 3—6 days old rats and incubated for 24 hours with different concentrations of E2, P4, or DHT. Despite none of the investigated sex hormones modulated the prolactin content in pituitary extracts (Figure 5.2), exposure to 1 ηM of E2 for 24 hours resulted in a statistically significant increase of prolactin secretion to the culture medium

supernatants (Vehicle versus E2 1 ηM: 125.2 ± 9.7 %, p = 0.0407). No further differences were observed regarding prolactin secretion levels by the pituitary.

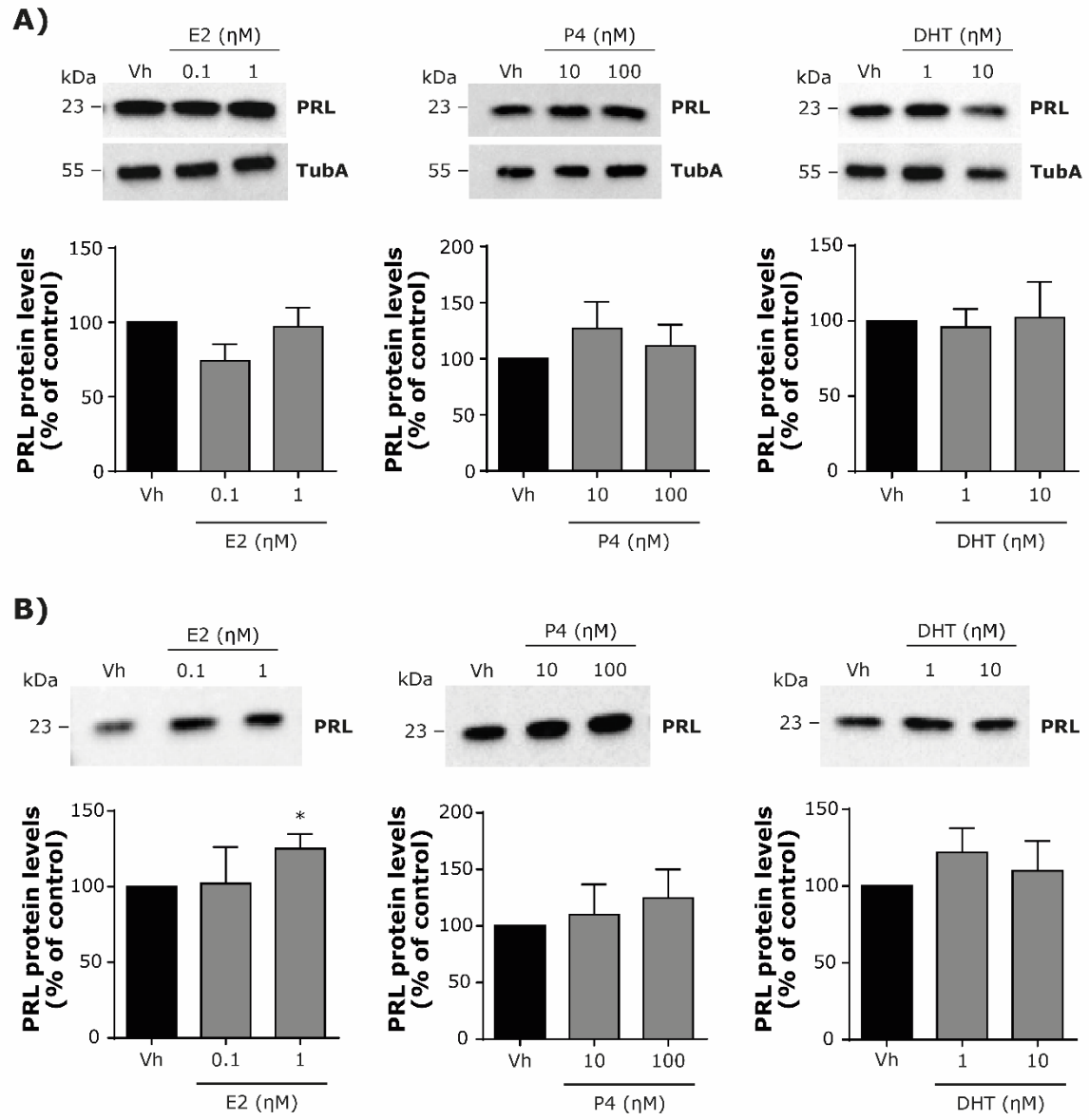

Figure 5.2. Effects of 17β-estradiol (E2), progesterone (P4), and 4,5α-dihydrotestosterone (DHT) on prolactin protein in the postnatal pituitary tissues and culture medium supernatants. In *ex vivo* experiments, pituitary explants were incubated for 24 hours with either vehicle (Vh), or 0.1 or 1 ηM of E2 (A), 10 or 100 ηM of P4 (B), or 1 or 10 ηM of DHT (C). After incubation, prolactin protein levels were analyzed by Western blot in (A) pituitary explants lysates (2 µg) and (B) culture medium (15 µL). Prolactin levels in tissue lysates were normalized by α-tubulin. Bar graphs represent means ± SEM from four independent experiments. Statistically significant differences to control were determined using Student's two-tailed unpaired *t*-test (* $p < 0.05$).

On the other hand, 24 hours of incubation with E2, P4, or DHT seem to have no effect on the levels of 63 kDa prolactin in the CP tissues, at least at the concentrations tested in this work.

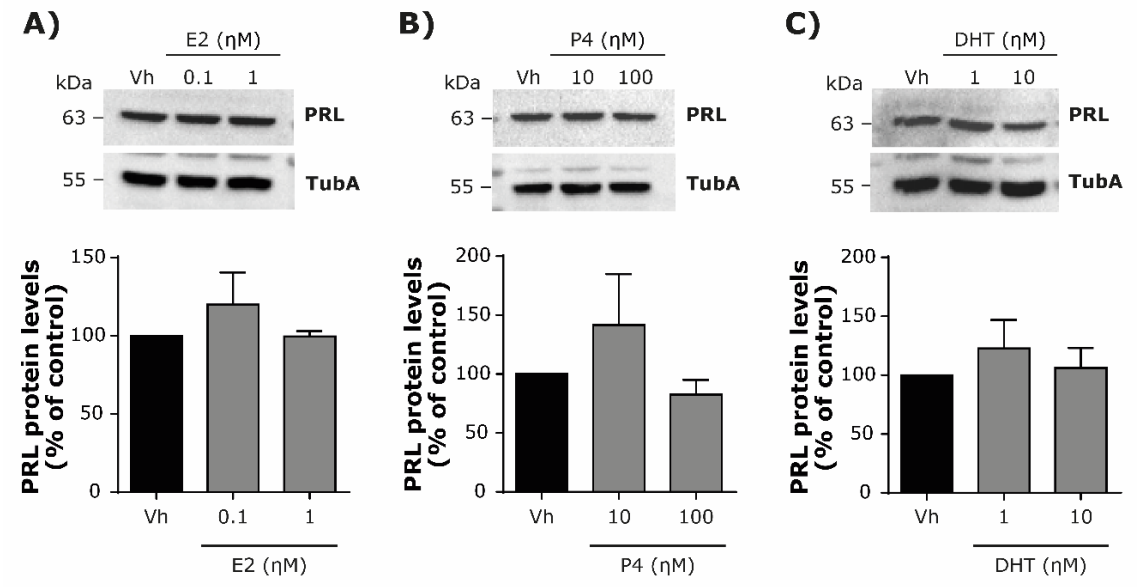

Figure 5.3. Effects of 17β-estradiol (E2), progesterone (P4), and 4,5α-dihydrotestosterone (DHT) on prolactin levels in the choroid plexus. In *ex vivo* experiments, CP explants were incubated for 24 hours with either vehicle (Vh), or 0.1 or 1 ηM of E2 (A), 10 or 100 ηM of P4 (B), or 1 or 10 ηM of DHT (C). After incubation, prolactin protein levels were analyzed by Western blot in both CP lysates (20 μg) and normalized by α-tubulin. Bar graphs represent means ± SEM from four independent experiments. Statistically significant differences to control were determined using Student's two-tailed unpaired *t*-test (* $p < 0.05$).

5.4. Discussion

There are conflicting results on the effects of exposure to E2 in pituitary prolactin. Either no effects, augmented or decreased levels of prolactin have been reported [14, 34], suggesting that factors such as the experimental model, time of exposure and concentration may influence the outcome of the experiments. In this work, we opted for an *ex vivo* experimental model, to investigate the possible influence of sex hormones exposure in both pituitary and CPs collected from postnatal rats. Besides keeping the *in vivo* tissue characteristics, preserving cell-cell communication, the use of pituitary and CP explants allowed the analysis of the direct action of sex hormones in these structures, avoiding the influence of a possible hypothalamic regulation [20].

As expected, the incubation of pituitary explants with the higher E2 concentration (1 ηM) was able to increase the secretion of prolactin. No statistically significant differences were observed in the prolactin levels in the pituitary tissue, suggesting that E2 exposure increases the release of prolactin in the postnatal pituitary possibly by increasing prolactin levels in this tissue. Although it had been reported that in culture, prolactin synthesis and secretion enhance throughout time in lactotrophs due to the removal from the *in vivo* dopamine tonic inhibition exerted by TIDA neurons [34], in this work, prolactin levels were only measured at a single time point. However, this possible increase would be observed in all experimental conditions, indicating the rise of prolactin levels present in the culture medium supernatants of pituitary explants incubated with E2 are a consequence of E2 exposure and not from the lack of dopamine inhibition.

Neither the exposure to P4 nor DHT seem to affect postnatal prolactin pituitary levels of secretion. Conflicting results regarding P4 influence in pituitary prolactin expression levels have also been reported. In line with our results, P4 addition to rat primary anterior pituitary cell cultures had no effect on the prolactin mRNA and prolactin secretion [40]. On the other hand, in another study performed using adult rat pituitary explants, incubation with P4 inhibited the secretion of prolactin [20]. These discrepancies may be explained by the different incubation times and P4 concentrations, as well as the *in vitro*/*ex vivo* model differences used in each study. In agreement with the previous work of Tong and colleagues, which reported that the addition of DHT to pituitary primary cells cultures isolated from adult male rats did not influence the amount of prolactin mRNA [24], no statistically significant differences were observed when postnatal pituitaries were incubated with DHT for 24 hours in our study. Notwithstanding, the use of an *ex vivo* model does not allow the exclusion of the hypothesis that sex hormones may have a distinctive effect *in vivo*, since E2, P4 and DHT may modulate prolactin expression and secretion at the hypothalamic level. Moreover, despite no influence on prolactin amount, sex hormones exposure during postnatal life may modulate the number of somatotrophs (growth hormone-producing cells) and lactotrophs cells of the anterior pituitary, regulating the cellular composition of this gland, as previously suggested [48]. The influence of sex hormones on the number and ratio of somatotrophs and lactotrophs cells was not evaluated in the present work, limiting the results obtained.

The initial goal of this work was to investigate if prolactin produced by the rat CP was regulated by sex hormones. As previously described, the rat CP may be an additional source of prolactin in the brain [56]. However, prolactin mRNA expression had only been investigated in CPs collected from adult pregnant females and CP epithelial cells primary cultures. In this study, we provide evidence that prolactin mRNA was present in the CPs of animals as early as 4 days old. In addition, the CP is a recognized target of sex hormone action, with implications for the function of this brain structure (reviewed by [1]). Furthermore, in an earlier transcriptomic study, the expression of prolactin in the adult rat CP seems to be modulated by sex hormones, with higher expression of this pleiotropic hormone being higher in intact females when compared to ovariectomized females and males [2]. Despite that observation, no statistically significant differences were observed in the levels of 63 kDa prolactin protein in the postnatal CP explants exposed to sex hormones in this work. These differences may suggest that prolactin protein levels may be regulated at the post-transcriptional level.

Another hypothesis is that prolactin may be differently regulated in postnatal CP than in adult rat CP. Age-associated differences in the transcriptome and protein secretion have been reported in the rodents' CP before [57, 58], with some of these differences influencing brain regions adjacent to the CP [58]. Nevertheless, the fact that the model used to perform the sex hormone influence studies may have some influence on the results obtained can not be excluded. Since in this work, we opted for an *ex vivo* model to directly expose the rat CPs to the action of sex hormones, and the previous cDNA microarrays study was performed using CPs

collected from ovariectomized, orchidectomized and sham rats [2], where the TIDA neuron system is present and functional, it is possible that, like in the pituitary, the regulation of prolactin synthesis and secretion in the CP may also be regulated by a combination of physiological factors. It was not possible to detect the 63 kDa prolactin protein in the culture medium supernatants collected at the end of the CP *ex vivo* experiments and the influence of sex hormones in the secretion of prolactin by the CP could not be evaluated (data not shown). We speculate that the higher concentration of albumin (66 kDa molecular weight) present in the CP—conditioned culture medium masked the signal of prolactin protein, a much less abundant protein than albumin in the culture medium supernatants.

Although prolactin is expressed in the rat CP at an early age, the findings reported in this work suggest that unlike in adult rat CP, the synthesis of prolactin in the postnatal CP may not be regulated by sex hormones, at least at the incubation period and sex hormones concentration used. Despite some studies supporting that sex hormones influence the expression of prolactin in extrapituitary tissues, this influence seems to be tissue-specific since opposing effects of the same sex hormone have been described in distinct non-pituitary tissues [34, 43]. Complementary studies are necessary to further investigate how the production and secretion of prolactin by the rat CP is modulated at this stage. Factors such as hypoxia [59] or exposure to other hormones and growth factors like thyrotropin-releasing hormone [8], epidermal growth factor [60], or kisspeptin [11], described as modulators of prolactin expression in lactotrophs and prolactin serum levels, should be considered for future analyses on the CP-derived prolactin.

5.5. References

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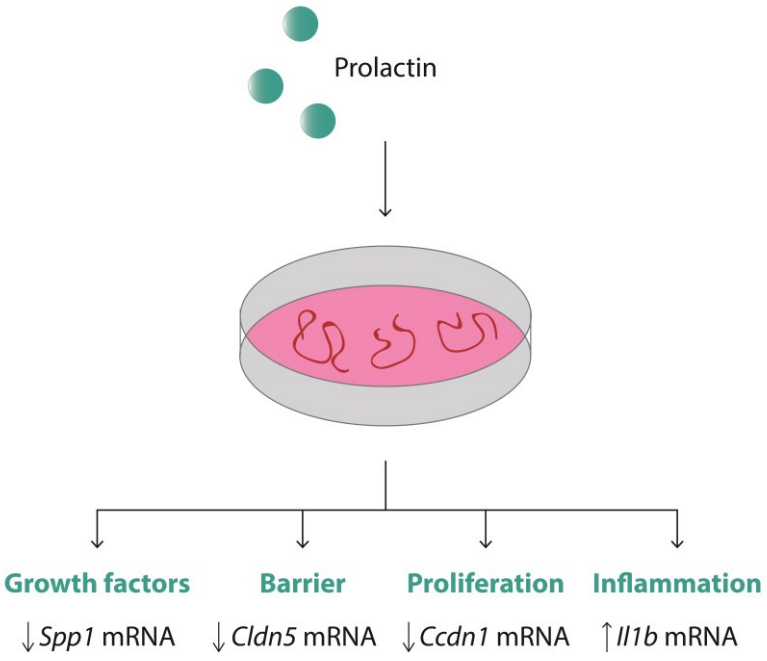
Chapter 6

The postnatal rat choroid plexus is a target of prolactin action



6.1. Introduction

Prolactin is a pituitary hormone with a primordial role in mammary cells proliferation and lactation in mammals and the development of parental behaviors in mammalian and nonmammalian species like birds and fishes (reviewed by [1–3]). Beyond the relevant role in reproduction, prolactin has been associated with hundreds of distinct biological actions in widespread target tissues. Under physiological and pathologic conditions, prolactin has been implicated in functions such as neurogenesis [4–7], neuroprotection [8–10], metabolism and energy homeostasis [2, 11, 12], cancer invasion and metastasis [3, 13, 14], bone growth [2, 15], among many other biological actions (reviewed by [2, 16–20]). Many of these biological actions are related to pregnancy and lactation, when the circulating levels of prolactin are higher [21, 22], and it is believed that prolactin is enrolled in the physiological adaptation to motherhood demands [11, 23–26]. Nonetheless, there is also evidence that prolactin is relevant in non-parental stages. For example, normal prolactin levels were associated with the maintenance of insulin and glucose balance in nonpregnant women [27], liver development in postanal mice [28], or osmoregulation in fishes [29].

The action of prolactin is mediated by the activation of prolactin receptors (PRLR), whose isoforms vary between species. For instance, while rats have a single short, an intermediate, and a long PRLR isoform [30], in mice one long and three short isoforms have been described [31, 32], and in humans, at least five distinct isoforms have been reported [33]. In addition to the canonical Janus kinases-signal transducer and activator of transcription (JAK/STAT) pathway, PRLR activation may also induce other downstream signaling cascades as the mitogen-activated protein kinase-extracellular signal regulated kinase (MAP/ERK) and the phosphatidylinositol 3-kinase-protein kinase B (PI3K/AKT) signaling pathways (reviewed by [18, 20, 34]). Ultimately, after PRLR activation of the JAK/STAT signaling cascade, STAT factors, particularly STAT5, translocate into the nucleus and modulate the transcription of target genes involved in functions such as cell growth and proliferation [35–39]. The target genes regulated by the PRLR signaling cascade activation seem to be cell-type dependent. In Nb2 T cells, a rat-derived cell line dependent on prolactin to proliferate, prolactin induces the expression of interferon regulatory factor 1 [36, 40], whilst in mammary epithelial cells, the same hormone induces the expression of β-casein, a gene involved in milk production [1, 41, 42]. In turn, prolactin elicits an apoptotic response in rat lactotrophs [43].

In mice, PRLR are present in a variety of tissues, such as nasal tissues, tongue, the vomeronasal organ, esophagus, small intestine, colon and reproductive system organs [44, 45], which may explain the wide range of prolactin functions. However, it is important to notice that PRLR also modulates the action of other hormones like placental lactogen in rodents and growth hormone in humans and that PRLR activation is not singly attributed to prolactin action (reviewed by [18, 21]). In the mice brain, the choroid plexus (CP) is the structure with the higher expression levels of PRLR [45–48]. Based on that, it was originally believed that PRLR were involved in receptor-mediated prolactin transportation mechanism, responsible for prolactin entrance to the

cerebrospinal fluid [49]. Nonetheless, the observation of no differences in the levels of prolactin transported to the cerebrospinal fluid in a mice PRLR knockout model contradicted that hypothesis [50]. Although the authors of this work have suggested that prolactin transport into the brain may occur at the microvasculature level [50], the exact mechanism of prolactin transport to the cerebrospinal fluid remains unknown.

The discovery that PRLR may not be involved in prolactin uptake into the brain, gave a new perspective on the role of both prolactin and its receptor in the CP. Recently, the transcriptional effects of prolactin in the CP were analyzed throughout different reproductive stages in adult female mice. During diestrus, exogenous prolactin induced a response that involved the downregulation of a higher number of protein-coding genes, contrasting with lactation, where prolactin was associated with gene upregulation. PRLR expression was increased, and insulin-like growth factor II (*Igf2*) was identified as a downstream target gene of prolactin in the mice CP during lactation [51]. Since prolactin was earlier associated with subventricular zone (SVZ) neurogenesis-induction [4, 52], and this neurogenic niche also includes the CP [53], the authors of this transcriptomic study suggested that *Igf2* produced by the CP may be a possible mediator of prolactin action in SVZ neurogenesis [51]. Despite some advances, available information supporting the precise function of prolactin in the CP is still scarce. Considering the many reported functions attributed to the CP and its relevance to brain function (reviewed by [54–56]), the main goal of this work was to investigate the effects of prolactin in the expression of genes associated with neurogenesis, barrier function, and immune surveillance in the postnatal rat CP.

6.2. Material and Methods

6.2.1. Animals

The CP tissues were collected from the lateral brain ventricles of Wistar Han rats aged between 3—21 postnatal days. Animals were housed in proper cages, at constant room temperature in a 12 h light/12 h dark photoperiod and given standard laboratory chow and water *ad libitum*. Animal tissue collection was approved by the Animal Welfare and Ethics Committee of the Health Science Research Centre of the University of Beira Interior, in compliance with National (Decree-law 113/2013, corrected by the Decree-law nº 1/2019) and European Union rules (Directive 2010/63/EU) for the care and handling of laboratory animals. No further licensing was required since the study did not involve animal experimentation. Before decapitation, Wistar Han rats up to 6 days old were anesthetized on ice, while older animals were anesthetized with isoflurane. All efforts were made to minimize the number of animals used as well as their suffering.

6.2.2. *Ex vivo* studies

For each *ex vivo* experiment, CP tissues (CP explants) were collected from 3—6 days old postnatal rats and placed directly in high-glucose DMEM without phenol red (Gibco,

ThermoFisher Scientific, USA, Cat# 12800017) supplemented with 10% charcoal-stripped fetal bovine serum (FBS; Capricorn Scientific, Germany, Cat# FBS-12B) and 100 U/mL of penicillin/streptomycin (Sigma-Aldrich, Germany, Cat# P4333) in 24-wells (ten CPs per well, in duplicate) or 96-wells culture plates (two CPs per well, in duplicate). Two hours after the tissues collection, the culture growth medium was replaced for free-serum high-glucose DMEM and CPs were incubated for sixteen hours. Then, CP explants were treated with rat prolactin (Sigma-Aldrich, Germany, Cat# SRP4689: 0, 5, 20, or 100 ηg/mL) for eight hours. Prolactin concentrations used in this work were within the physiological prolactin levels observed during different reproductive stages of female rats [57, 58]. All cultures were maintained in a humidified incubator in 95% air—5% CO_2 at 37 °C.

6.2.3. MTT assay

To assess the effect of prolactin treatment influenced in tissue viability, CP explants were rinsed once with phosphate-buffered saline (PBS) incubated with 50 µL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT; VWR, Germany, Cat# 0793: 1 mg/mL in PBS), for 3 hours at 37 °C, 95% air—5% CO2 [59]. CP explants incubated without prolactin or treated for 10 minutes with 4 % paraformaldehyde were used as negative and positive controls, respectively. After incubation, residual MTT medium was removed, and CP explants were washed once with PBS. After that, CP explants were incubated with isopropanol for 30 minutes, on an orbital shaker, to extract formazan crystals from the tissues. The optical density of formazan extracts was measured at 570 nm on a microplate spectrophotometer xMark™ (Bio-Rad, USA), and isopropanol was used as a blank.

6.2.4. RNA extraction and PCR reverse transcription (RT-PCR)

Total RNA was extracted from freshly collected CP tissues and CP explants at the end of the *ex vivo* experiments. In the last,duplicates of each condition were pooled (twenty CPs). Total RNA was extracted from all samples using TRIzol (TripleXtractor, Grisp, Portugal, Cat# GB23.0100), according to the manufacturer's instructions. RNA was quantified by spectrophotometry (NanoPhotometer™, Implen, Germany) and RNA integrity was assessed by agarose (1.5 %) gel electrophoresis containing *GreenSafe* staining (NZYTech Ltd., Portugal, Cat# MB13201). To prevent possible contaminations with genomic DNA, RNA samples were treated with DNAse I (Sigma-Aldrich, Germany, Cat# AMPD1) prior to complementary DNA synthesis. Five hundred ηg of CPEC and one µg of CPs and kidney total RNA was transcribed into complementary DNA using M-MuLV Reverse Transcriptase (NZYTech Ltd., Portugal, Cat# MB08301, 20 µL reaction), following the manufacturer's instructions. PCR reactions were carried out in a final volume of 10 µL using NZYTaq II 2x Green Master Mix (NZYTech Ltd., Portugal, Cat# MB35801) and 0.4 µM of each primer (Table 6.1), on a MultiGene™ Optimax Thermal Cycler (Labnet International, USA). The following PCR cycling protocol was used: an initial 3-minute denaturing reaction at 95 °C, followed by 30 seconds at 94 °C, 30 seconds at the corresponding annealing temperature, and 30 seconds at 72°C for 40 cycles with a final 10 minutes extension at 72°C. PCR products were analyzed in a 2 % agarose gel electrophoresis, visualized under UV

with *GreenSafe* staining (NZYTech Ltd., Portugal, Cat# MB13201) and Sanger sequenced (STAB VIDA, Portugal) to confirm the gene identity. A negative control consisting of a PCR reaction without template was included in all the assays.

6.2.5. Protein extraction and Western Blot

Phosphorylation of STAT5 is the main signaling molecule induced by PRLR-Long isoform activation in the rodents' brain [60] and is widely used to investigate prolactin action in the central nervous system [24, 50]. To evaluate if phosphorylated STAT5 (pSTAT5) levels were increased, thirty minutes after prolactin stimulus CP tissues were homogenized, on ice, using radioimmunoprecipitation assay buffer (RIPA) (150 mM sodium chloride, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 50 mM Tris pH 8.0) completed with 1 mM phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate and 40 µL/mL cOmplete™ EDTA Free protease inhibitor cocktail (Roche, Sigma-Aldrich, Germany, 25x stock, Cat# 11873580001). Lysates were centrifuged at 10,000 *g* for 10 minutes at 4 °C and supernatants were collected and stored at -20 °C until use. Total protein was quantified using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA, Cat# 23225). Protein extracts (25 µg) were diluted in 4X sample buffer containing freshly added 10 % β-mercaptoethanol, denatured at 100 °C for 5 minutes, and separated by 10 % SDS-PAGE. Then, proteins were transferred to polyvinylidene difluoride membranes (Amersham™ Hybond™ 0.45 µm, GE Healthcare, UK, Cat# 10600023) and membranes were blocked for 1 h with 5 % bovine serum albumin in Tris-buffered saline with 0.1 % Tween 20 (TBS-T 0.1%), before being incubated overnight at 4 °C with rabbit anti-phospho-STAT5 alpha (Tyr694) polyclonal primary antibody (1:1,000; Thermo Fisher Scientific, USA, Cat# 71-6900, RRID: AB_2533991). After being washed with TBS-T 0.1 %, membranes were incubated 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:20,000; Thermo Fisher Scientific, USA, Cat# 31466, RRID:AB_10960844). Immunoreactive proteins were visualized by chemiluminescence using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, USA, Cat# 34577) and detected using a ChemiDoc™ MP Imaging System (Bio-Rad, USA) and Image Lab 5.1 software (Bio-Rad, USA, RRID: SCR_014210). In quantitative experiments, densitometry of each protein band was measured using Image Lab software and the relative intensity of each pSTAT5 band was normalized against β-actin levels (1:20,000; Sigma-Aldrich Cat# A1978, RRID: AB_476692). Only two independent samples within each group were quantified.

6.2.6. Real-time quantitative PCR

To evaluate the effect of prolactin in the mRNA expression levels of PRLR long isoform (*PRLR-Long*), PRLR-short (*PRLR-Short*), *Igf2*, homeobox protein OTX2 (*Otx2*), osteopontin (*Spp1*), occludin (*Ocln*), cyclin D1 (*Ccnd1*), claudin 5 (*Cldn5*), interleukin-1 beta (*Il1b*) and interleukin-6 (*Il6*) real-time quantitative PCR (RT-qPCR) was performed using Xpert Fast SYBR 2X Mastermix (Grisp, Portugal, Cat# GE20.5100) on CFX Connect Real-Time PCR Detection System (Bio-Rad, USA). For each RT-qPCR, 1 µL of the synthesized cDNA, in a total volume of

10 µL reactions, was used. The following cycling program was used for RT-qPCR: Initial denaturation at 95 °C for 3 minutes, followed by 40 cycles of denaturation at 95 °C, annealing temperature for 30 seconds and extension at 72 °C for 10 seconds. Specific oligonucleotide primers sequences used for RT-qPCR are described in Table 6.1. Fluorescence was measured at the end of each cycle and the threshold amplification number (Ct) for each gene was determined using the CFX Manager™ Software (Bio-Rad, USA). The ΔCt was obtained by calculating the difference between the Ct of the gene of interest and the Ct of the reference gene Cyclophilin A (CypA). The relative expression of each gene was calculated using the $2^{-(\Delta\Delta Ct)}$ method [61, 62]. Four to six samples were used per group and all samples were assayed in duplicate. To ensure the specificity of the amplification, a melting curve analysis was performed at the end of the final cycle.

Table 6.1. Sequences of the oligonucleotide primers used in RT-PCR and RT-qPCR.

Gene	RefSeq	Primer sequence (5' – 3')	Amplicon size (bp)	AT (°C)
Ccnd1	NM_171992.5	Fw: CGCAAACATGCACAGACCTTT Rv: AGGCAGTCCGGGTCACA	197	58
Cldn5	NM_031701.2	Fw: GAACTACGTCTAAGGGCGGG Rv: ACCCAACCTAACTTGCCTCG	144	60
Igf2	NM_001190162.1	Fw: GAGAACCTTCCAGCCTTTTCC Rv: GGCCAAAGAGATGAGAAGCAC	125	62
Il1b	NM_031512.2	Fw: AAATGCCTCGTGCTGTCTGA Rv: AGGCCACAGGGATTTTGTCG	133	58
Ocln	NM_031329.3	Fw: TGAACAGCCCCCTAATGTGG Rv: TGCCATTCACTTTGCCGTTG	137	60
Otx2	NM_001100566.1	Fw: GCTTTAAGGAGTGCGGCTC Rv: GGTGGGTGGATTTGGAGTGAC	196	62
PRLR-Long	NM_001034111.1	Fw: GGATTTGATACCCATCTGCTGGA Rv: CATTAGCCGCTCGTCCTCAT	147	60
PRLR-Short	NM_012630.2	Fw: GGATTTGATACCCATCTGCTGGA Rv: TTGCGATGGTGGTAGAACCC	169	62
Spp1	NM_012881.2	Fw: GCCAGCCAAGGACCAACTAC Rv: GCTTCTGAGATGGGTCAGGC	178	60
CypA	NM_017101.1	Fw: GTCGCTAGTCCACGATGCT Rv: TGAGACTGTAGCTCTCCCGA	163	58-62

AT: Annealing temperature; Fw: Forward, Rv: Reverse.

6.2.8. Statistical analysis

Values are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, CA, RRID:SCR_002798). Before analysis, outliers were identified using Grubbs' test and removed from further calculations, and data were tested for normality using the Shapiro-Wilk test. Statistical significance of differences between two groups was analyzed with Student's two-tailed unpaired *t*-test, while one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to compare more than two groups. Significant differences between groups were set at $p < 0.05$.

6.3. Results

6.3.1. PRLR expression increase with age in the postnatal CP

Prolactin receptors are highly expressed in the brain of adult rodents [46, 47]. To analize the presence of PRLR in earlier life stages, the expression of *PRLR-Long* and *PRLR-Short* isoforms was evaluated by RT-PCR in the CPs of postnatal rats. As expected, the amplicons corresponding to *PRLR-Long* (147 bp) and *PRLR-Short* (169 bp) isoforms mRNA were observed in postnatal CP samples (Figure 6.1.A). Furthermore, the expression of both PRLR isoforms seems to increase throughout postnatal life, with higher expression levels of *PRLR-Long* (postnatal day 21: 10.23 ± 0.76 versus postnatal day 14: 3.68 ± 0.21 and postnatal day 4: 1.01 ± 0.08, $p < 0.001$) and *PRLR-Short* (postnatal day 21: 14.11 ± 2.59 versus postnatal day 4: 1.01 ± 0.09, $p < 0.01$) being observed by day 21 after birth.

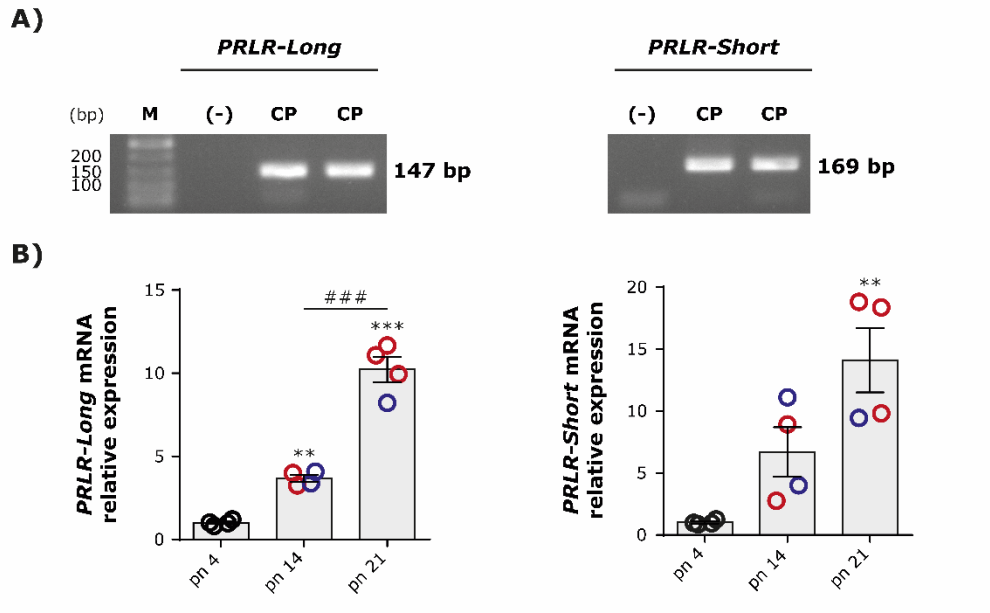


Figure 6.1. Expression of prolactin receptors (PRLR) in the postnatal rat choroid plexus (CP). (A) Gel electrophoresis of RT-PCR products showing the presence of the *PRLR-Long* (left) and *PRLR-Short* (right) isoforms in pools of four CPs collected from postnatal (pn) rats with 4—6 days old. (B) The expression of *PRLR-Long* (left) and *PRLR-Short* (right) mRNA in the CP of postnatal rats of different ages was analyzed by RT-qPCR. Scattered plots with bar represent the means ± SEM (n = 4). Individual values of each sample are shown by circles (Black: pools of CPs collected from 5 animals; Red: CPs collected from female rats; Blue: CPs collected from male rats). *PRLR-Long* and *PRLR-Short* expression levels between groups were compared using one-way ANOVA followed by Tukey's post hoc test (** $p < 0.01$, *** $p < 0.001$ compared with pn4; ### $p < 0.001$ compared with pn14). M: Molecular weight marker.

6.3.2. Effects of prolactin in the postnatal rat CP transcriptome

To explore the influence of prolactin in the postnatal rat CP, CP explants collected from 4—6 days old animals were exposed to prolactin. To investigate if PRLR were responsive to prolactin in the postnatal rat CP, the levels of pSTAT5 were assessed thirty minutes after exposure to different concentrations of prolactin. All tested prolactin concentrations seemed to increase the levels of pSTAT5 (Figure 6.2.A), suggesting that at this stage of development PRLR are

responsive to this hormone. Furthermore, neither of the tested prolactin concentrations was associated with decreased tissue viability (Figure 6.2.B). Taking into account these results, a higher dose of prolactin (100 ηg/mL) was chosen for the further *ex vivo* experiments.

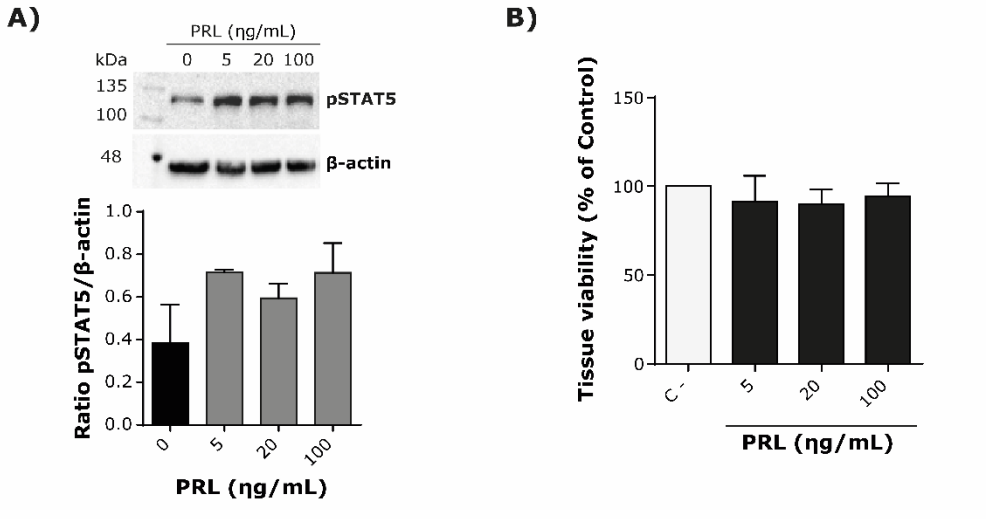


Figure 6.2. Prolactin stimulus increases signal transducer and activator of transcription 5 (STAT5) phosphorylation in the postnatal rat choroid plexus and is not associated with decreased tissue viability. (A) Western blot analysis of pSTAT5 levels in CP explants untreated (C -) or treated with different prolactin (PRL) concentrations (5, 20 or 100 ηg/mL). pSTAT5 levels were normalized by β-actin. The bar graph represents means ± SEM from two independent experiments. (B) CP explants tissue viability was assessed at the end of *ex vivo* experiments (n = 5). Results are presented as the means ± SEM. Tissue viability between groups was compared using one-way ANOVA followed by Tukey's post hoc test. No statistically significant differences were observed.

Prolactin exposure was not associated with alterations in the expression of either *PRLR-Long* (Prolactin: 1.05 ± 0.23 versus Control: 1.19 ± 0.22, $p = 0.846$) or *PRLR-Short* (Prolactin: 1.43 ± 0.16 versus Control: 1.06 ± 0.17, $p = 0.138$) isoforms in the postnatal rat CP (Figure 6.3), at least at the concentration and time of exposure used in the *ex vivo* experiments.

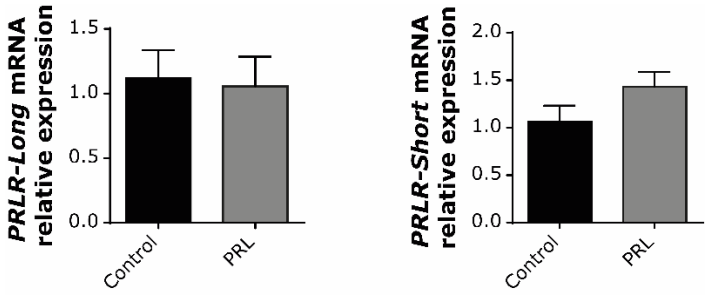


Figure 6.3. Prolactin exposure does not influence the expression levels of prolactin receptors (PRLR) in the rat postnatal choroid plexus (CP). The expression of *PRLR-Long* and *PRLR-Short* was analyzed by RT-qPCR in CP explants collected from 4—6 days old rats left untreated (Control) or treated with prolactin (PRL, 100 ηg/mL) for eight hours. Bar graphs represent the means ± SEM (n ≥ 5). The expression levels of Control and PRL-treated groups were compared using Student's two-tailed unpaired *t*-test.

To investigate if prolactin exposure modulates the expression of genes involved in cell growth and neurogenesis, the expression of *Igf2*, *Otx2* and *Spp1* was analyzed in the postnatal rat CPs

using RT-qPCR (Figure 6.4). Although it was possible to observe a trend towards the decrease of *Igf2* expression in the postnatal rat CP after incubation with prolactin (Prolactin: 0.68 ± 0.15 versus Control: 1.05 ± 0.14, p = 0.092), this reduction was not statistically significant. No statistically significant difference was observed in the expression levels of *Otx2* mRNA either (Prolactin: 1.06 ± 0.14 versus Control: 1.04 ± 0.14, p = 0.918). In turn, prolactin treatment significantly reduced the expression of *Spp1* mRNA by 1.8-fold (Prolactin: 0.57 ± 0.07 versus Control: 1.07 ± 0.17, p = 0.033).

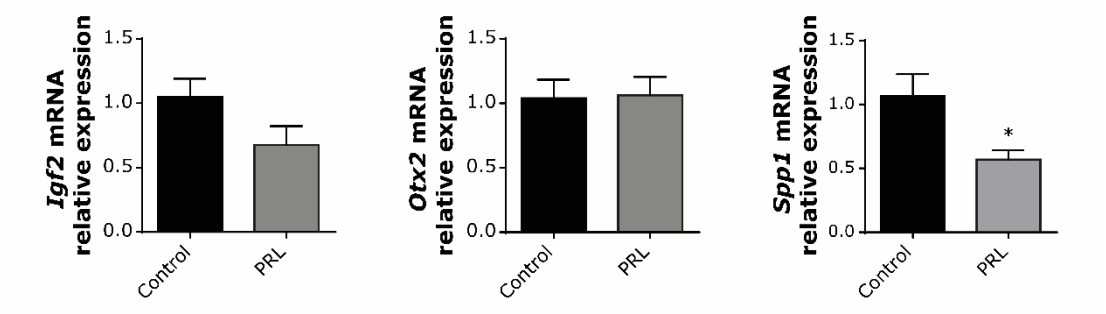


Figure 6.4. Effect of prolactin in the expression of growth and neurogenesis-associated factors in the rat postnatal choroid plexus (CP). The expression of insulin-like growth factor II (*Igf2*), homeobox protein OTX2 (*Otx2*) and osteopontin (*Spp1*) was analyzed by RT-qPCR in CP explants collected from 4—6 days old rats left untreated (Control) or treated with prolactin (PRL, 100 ηg/mL) for eight hours. Bar graphs represent the means ± SEM (n ≥ 5). The expression levels of Control and PRL-treated groups were compared using Student's two-tailed unpaired *t*-test (* $p < 0.05$).

To investigate if prolactin treatment influences the permeability of the blood-cerebrospinal fluid barrier, the expression of genes associated with barrier proteins was also assessed by RT-qPCR (Figure 6.5). No significant difference was observed in *Ocln* expression between the groups, despite the levels of *Ocln* mRNA tended to be lower in postnatal CPs treated with prolactin (Prolactin: 0.65 ± 0.15 versus Control: 1.06 ± 0.16, p = 0.086). On the other hand, postnatal prolactin exposure significantly decreased the expression of *Cldn5* by 2.2-fold (Prolactin: 0.46 ±0.14 versus 1.09 ± 0.20, p = 0.037). The same reduction was present in *Ccdn1*, a gene that encodes a protein associated with cell cycle progression. In this case, prolactin-treatment induced a 2.8-fold significant decrease in the expression of *Ccdn1* mRNA (Prolactin: 0.36 ± 0.11 versus Control: 1.13 ± 0.20, p = 0.011).

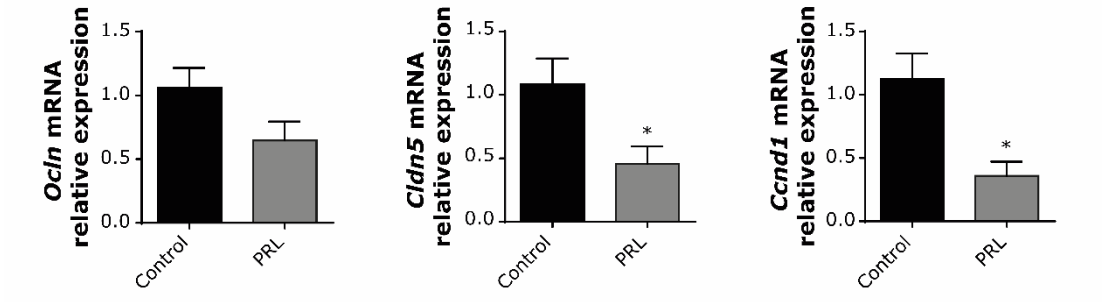


Figure 6.5. Influence of prolactin in the expression of barrier- and proliferation-related factors in the rat postnatal choroid plexus (CP). The expression of occludin (*Ocln*), claudin 5 (*Cldn5*) and cyclin D1 (*Ccnd1*) was analyzed by RT-qPCR in CP explants collected from 4—6 days old rats

(legend continued on next page)

left untreated (Control) or treated with prolactin (PRL, 100 ηg/mL) for eight hours. Bar graphs represent the means ± SEM (n ≥ 5). The expression levels of Control and PRL-treated groups were compared using Student's two-tailed unpaired *t*-test (* $p < 0.05$).

Finally, the effect of postnatal prolactin exposure on the expression of proinflammatory *Il1b* and *Il6* genes in the CP, was also evaluated (Figure 6.6). Prolactin-treatment was associated with a 3.3-fold significant increase in the expression of *Il1b* mRNA in the postnatal rat CP (Prolactin: 3.28 ± 0.79 versus Control: 1.03 ± 0.11, $p = 0.018$). However, no statistically significant difference was observed in the expression levels of *Il6* (Prolactin: 1.07 ± 0.06 versus Control: 1.03 ± 0.12, $p = 0.731$).

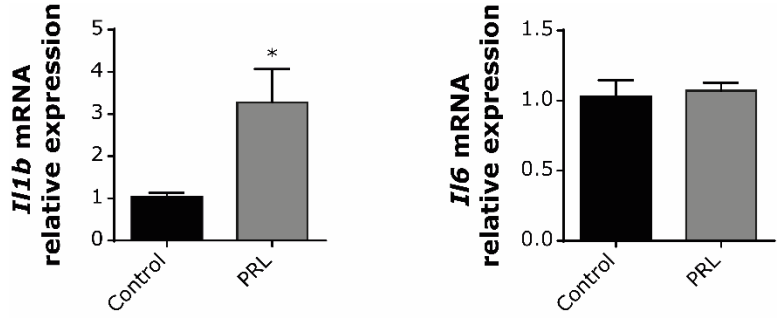


Figure 6.6. Effect of prolactin in the expression of inflammation factors in the rat postnatal choroid plexus (CP). The expression of Interleukin-1 beta (*Il1b*) and Interleukin-6 (*Il6*) was analyzed by RT-qPCR in CP explants collected from 4—6 days old rats left untreated (Control) or treated with prolactin (PRL, 100 ηg/mL) for eight hours. Bar graphs represent the means ± SEM (n ≥ 5). The expression levels of Control and PRL-treated groups were compared using Student's two-tailed unpaired *t*-test (* $p < 0.05$).

6.4. Discussion

Evidence supports that prolactin is available in maternal milk and transported to the offspring circulation during suckling [63, 64]. The presence of both PRLR-Long and -Short isoforms mRNA was described previously in the adult rat CP [65–67]. In rodents, the expression of PRLR in the CP seems to be influenced by factors such as reproductive stage [45, 66, 68], estrogen treatment [67], pup contact [69], or exposure to stress [70]. Although the high expression of PRLR had been associated with prolactin transport into the brain at the CP in the past [49], it is currently believed that its transportation is not mediated by a receptor-mediated mechanism present in this brain structure [50]. The role of prolactin in the CP is still not fully understood, despite the high levels of PRLR observed. As so, we performed a study to identify the effects of prolactin exposure in the postnatal rat CP barrier, neurogenic and proinflammatory genes.

As expected, we present evidence that both the PRLR-Long and -Short isoforms are expressed in the CP of postnatal rats. Furthermore, the expression of both isoforms seems to increase with postnatal age. These results are in accordance with previous studies, which report the presence of PRLR mRNA in the CP of fetal and postnatal rats [71, 72]. Although Hirai and colleagues had analyzed the expression of PRLR in the CPs collected from both male and female rats with different postnatal ages (2-, 4- and 8-weeks old) than those chosen in our study (4-, 14 and 21-days old), they also described an increase of PRLR expression with postnatal age [72].

Furthermore, this increase was accompanied by the rise of prolactin plasma concentration throughout postnatal life, leading the authors to suggest that the increase of PRLR in the CP during postnatal development increased the response to prolactin and the development of "maternal-like behavior" in juvenile rats [72]. Besides PRLR mRNA expression, the presence and localization of immunoreactive PRLR protein, coincident with PRLR expression and placental lactogen binding, was also described in fetal rat CP, suggesting that PRLR are functional at this stage and that placental lactogen and prolactin may be involved in neonatal development [71]. The apparent increase of pSTAT5 levels, the main signaling mediator activated by PRLR activation in the brain [60], in the postnatal CP after prolactin treatment observed in this work seems to support this theory.

Contrary to previous works, reporting that prolactin upregulates the expression of PRLR in rodents CP [72, 73], postnatal rat CP exposure to prolactin was not associated with the increase of any of the PRLR isoforms in our study. These differences may be explained by the distinct experimental models, prolactin concentrations and prolactin origin used to evaluate the expression of PRLR in the CP. For instance, while Hirai and colleagues investigated PRLR expression by RT-qPCR in CPs collected from postnatal rats that were only exposed to endogenous prolactin levels [72], in the work of Tabata and colleagues, PRLR expression was studied in the CP of prolactin-deficient mice injected twice a day with human prolactin throughout the total of three days [73]. In our work, CP explants were incubated for only eight hours with a high dose of rat prolactin (100 ηg/mL). As so, the hypothesis that prolonged exposure to prolactin may result in the increase of PRLR in the postnatal CP cannot be excluded. The time of exposure is a factor that should be considered regarding prolactin effects in postnatal rat CP. As reviewed before, exposure time modulates the action of prolactin [74]. Another explanation for the lack of prolactin-induction of *PRLR* expression could be the fact that we opted for an exogenous source of prolactin to perform the *ex vivo* incubations. In an earlier report conducted in a human mammary epithelial tumor cell line manipulated to overexpress prolactin, endogenously produced prolactin was associated with increased *PRLR-Long* isoform expression, while in turn treatment with exogenous human prolactin produced the opposite effect [75].

Based on the evidence that associated prolactin with the induction of SVZ neurogenesis during early pregnancy and lactation in mice [4, 5], and that the CP is part of the SVZ niche, contributing with the secretion of factors that modulate the generation of new neurons in this neurogenic region [53], we investigate the effects of prolactin exposure in the expression of neurogenesis-associated factors in the postnatal rat CP. Prolactin was formerly linked with the modulation of *Igf2* expression in the adult female mice CP during lactation. It was speculated that this increase may be associated with the increase in SVZ neurogenesis observed during lactation [51]. In our work, no statistically significant alterations were observed in the expression of *Igf2* in the postnatal CP after exposure to prolactin, although a decreased trend was visible regarding the expression of this growth factor. Similarly, prolactin exposure seems to

not affect the expression of *Otx2*, a homeoprotein produced and secreted by the CP that was also associated with ventricular-SVZ neurogenesis modulation [76]. In turn, prolactin treatment led to a reduction of *Spp1* expression in the postnatal CP. Together with other factors including endostatin and transforming growth factor beta-2, SPP1 secreted by mice CP was formerly purported as a factor that promotes the colony formation and proliferation of neural stem cells isolated from mice ventricular-SVZ [53]. As reported previously, the role of prolactin in the CP is possibly mediated by reproductive status. While during lactation, prolactin seems to induce the upregulation of several genes, a mainly inhibitory effect was observed in the CP transcriptome of female mice during diestrus [51]. Our results suggest that prolactin may also negatively influence the expression of growth- and neurogenesis factors during the postnatal stage. The administration of high doses of prolactin to rats in early postnatal life (continuous treatment with ovine prolactin from postnatal day 1 to postnatal day 14) was previously associated with reduced levels of dentate gyrus and olfactory bulb neurogenesis, leading to development of anxiety behaviors in adulthood [77]. However, further studies are necessary to understand if prolactin has a comparable inhibitory effect in the expression of other neurogenesis modulators and if CP could be involved in the decreased neurogenesis associated with in rat exposure to high doses of prolactin during postnatal life.

Prolactin was previously linked with the modulation of barrier permeability of the mammary and kidney epithelium [78, 79], as well as the blood-brain barrier [80]. In an *in vitro* model of the bovine blood-brain barrier (BBB) prolactin treatment-induced endothelial cells proliferation and decreased barrier permeability [80]. In addition, bromocriptine treatment, an inhibitor of prolactin secretion, increased rat BBB permeability [81]. Based on these data, we investigated if prolactin may also influence the expression of junction proteins in the rat CP. In line with the reported in bovine BBB cells [80], prolactin did not influence CP tissue viability. However, the treatment of postnatal rat CP explants with prolactin decreased the expression of *Cldn5* and *Ocln*, even though in the last this reduction did not reach statistical significance. These observations conflict with preceding results reporting the prolactin-induced increase of CLDN5 and OCLN protein levels in bovine BBB cells [80]. Prolactin addition to cultures of mouse mammary and canine kidney cell lines was also linked with increased levels of OCLN [78, 79]. Since CP constitutes the blood-cerebrospinal barrier, these results may suggest that prolactin exposure during the postnatal life may increase the permeability of this brain barrier.

Prolactin has been described as a proliferation and differentiation promotor in target tissues like the mammary gland [38], liver [39], or immune cells [82]. In these tissues, the promoter of *Ccnd1*, a regulator of cell cycle progression, is a known target of prolactin action and PRLR activation culminates in the increase of *Ccnd1* expression [28, 38]. However, prolactin was also associated with the induction of apoptosis and decreased proliferation in rat lactotrophs [43]. More surprisingly, in postnatal rat liver, prolactin action seems to be development stage-dependent, promoting liver growth during the majority of postnatal life but having an inhibitory action at 2 weeks old [28]. To investigate if prolactin influenced CP proliferation, we evaluate its

effect on the levels of *Ccnd1*. We observed that *Ccnd1* expression of was significantly reduced in prolactin treated postnatal CPs. Since prolactin exposure was not associated with a decrease in CP tissue viability, we speculate that prolactin may have an inhibitory effect on the CP during early postnatal life, at least at the concentration used in this study.

Among the many biological functions attributed to prolactin, this hormone is also acknowledged as a modulator of the immune system and inflammation. Prolactin has been associated with pro- and anti-inflammatory properties, including in the central nervous system (reviewed by [74, 82, 83]). For example, prolactin has a protective role against excitotoxicity-induced neuroinflammation by reducing the levels of pro-inflammatory and increasing the levels of anti-inflammatory cytokines in rat neurons [84]. On the other hand, extrapituitary prolactin produced by antigen-presenting cells in a mice model for multiple sclerosis was associated with the increase of a specific group of immune cells responsible for neuroinflammation in this disease [85]. In this work, prolactin exposure increased the expression of proinflammatory *Il1b* while no significant difference was observed in *Il6* expression levels. We also intended to ascertain if prolactin influenced the expression of other inflammation-associated genes in the CP, such as *Tnfa* and interferon-gamma. However, the expression levels of these genes were very low in the CP at this development stage, and it was not possible to optimize the experimental conditions to perform the RT-qPCR technique (data not shown). Conflicting results regarding prolactin-mediated induction of Il1b levels have also been described. While in bovine mammary epithelial cells prolactin exposure led to an increase in IL1B levels [86], prolactin treatment had the opposite effect on the levels of IL1B in the joints of mice with induced inflammatory arthritis [87] and human placental cells exposed to lipopolysaccharide-induced inflammation [88].

In summary, our results present evidence that the exposure of CP to high concentrations of prolactin during early postnatal life decreased the expression of neurogenesis- and barrier-associated factors while in turn increased the expression of proinflammatory *Il1b*. Prolactin action in the CP may likely be development stage-dependent as in other target tissues. Further studies are necessary to uncover the possible brain impact of the reported prolactin-induced alterations in the transcriptome of early postnatal CP. Based on the alterations in the development of maternal behaviors [89] and on the tendency to develop depressive-like behaviors reported in rodents exposed to elevated levels of prolactin during early life [77], we hypothesize that CP could be involved in some of these brain alterations, including reduced neurogenesis.

6.5. References

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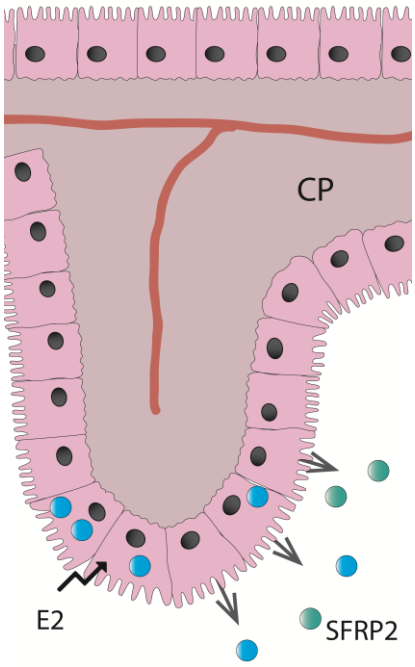
Chapter 7

Secreted frizzled-related protein 2: Another choroid plexus-derived peptide with putative neurogenic potential



7.1. Introduction

Secreted frizzled-related protein 2 (SFRP2), also known as secreted apoptosis-related protein 1, is a 33.4 kDa secreted glycoprotein involved in the regulation of the wingless (Wnt) signaling pathway [1, 2]. Structurally, SFRP2 is composed of an N-terminal signal peptide, responsible for SFRP2 secretion, a frizzled-like cysteine-rich domain (CRD), and a C-terminal netrin-related motif (NRT) [1, 3]. As a consequence of the high similarity of the SFRP2 frizzled-like CRD and the extracellular domain of frizzled receptors, it is believed that SFRP2 competes with these receptors by binding to soluble Wnt factors, thus interfering with Wnt signaling [1, 3, 4]. Wnt signaling program is cell type-dependent and has been implicated in a wide range of biological actions, particularly significant during embryogenesis, but equally relevant in adult life, including regulation of cell proliferation and tissue homeostasis [5–8]. Wnt signaling can be mediated by different pathways, including the canonical Wnt/β-catenin pathway, and the noncanonical Wnt/Ca^{2+} and planar cell polarity (PCP) pathways (Figure 7.1) [1, 8–10]. Although being considered an antagonist of the Wnt pathway [11–13], SFRP2 is also acknowledged as an agonist of this signaling pathway and its action depends on factors such as concentration and cell context [1, 14–16].

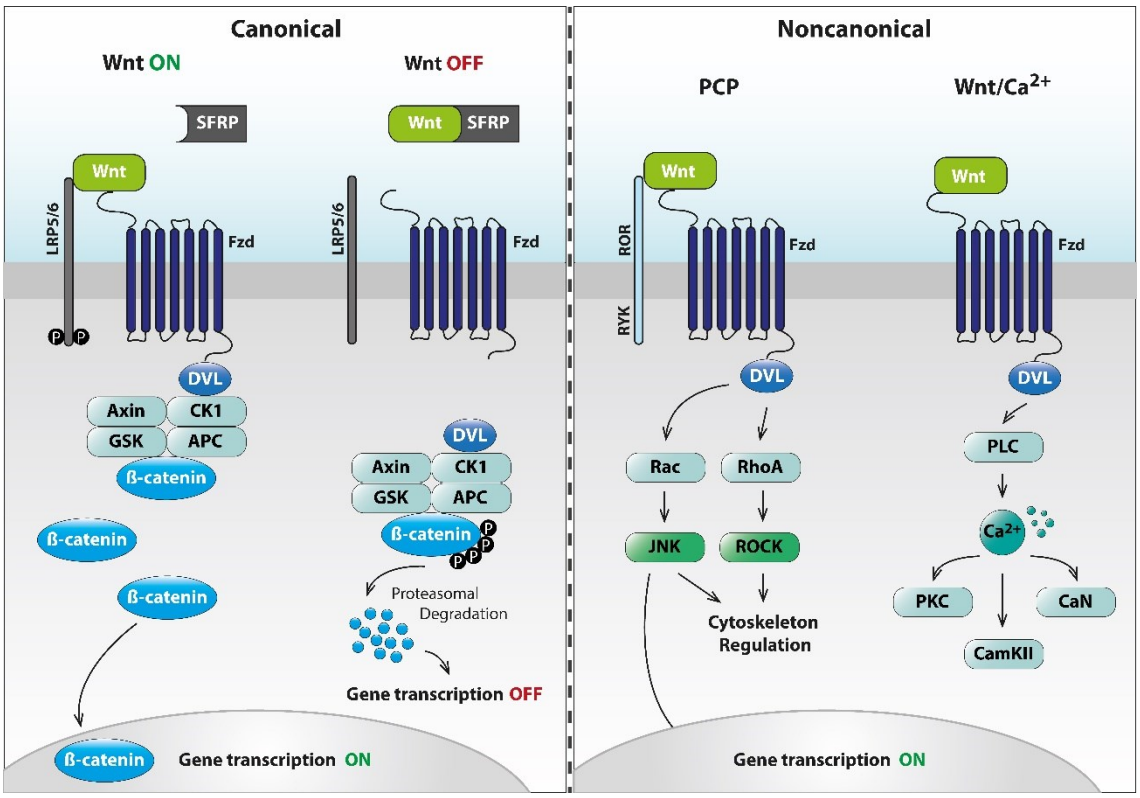


Figure 7.1. Overview of Wnt signaling pathways. In the canonical pathway (left panel), the Wnt ligand binds to frizzled receptor (Fzd) and low-density lipoprotein receptor-related protein 5/6 (LRP6/6), recruiting the destruction complex and preventing the degradation of β-catenin. Then, β-catenin translocates to the nucleus where it activates the transcription of Wnt target genes. In the absence of Wnt or in the presence of secreted frizzled-related proteins (SFRP) that compete with Fzd by binding to Wnt ligands, the destruction complex is activated and leads to β-catenin ubiquitin and consequent proteasomal degradation. In the noncanonical Wnt/Ca^{2+} pathway, Wnt binding induces the activation of trimeric G proteins. Consequently, phospholipase C (PLC) induces Ca^{2+} release and activation of protein kinase C

(legend continued on next page)

(PKC), calcium/calmodulin-dependent protein kinase II (CamKII), calcineurin (CaN). In the noncanonical planar cell polarity (PCP) pathway, Wnt binding triggers the activation of the GTPases Rac, RhoA, Rho-associated protein kinase (ROCK) and Jun N-terminal kinase (JNK) that regulate cytoskeletal rearrangement. Adapted from [8, 9, 17]. APC: adenomatous polyposis coli; CK1: casein kinase 1, DVL: dishevelled; GSK: glycogen synthase kinase; ROR: receptor tyrosine kinase; RYK: receptor tyrosine kinase.

The Wnt signaling pathway, Wnt ligands, and Wnt modulators are recognized for their fundamental role in the development of the central nervous system during embryogenesis (reviewed by [6, 18–20]). However, Wnt related factors and modulators are also expressed in the postnatal and adult brain [21–24] and were involved in the regulation of neural stem cells proliferation, differentiation and homeostasis [7, 9, 10, 24, 25]. Induced SFRP2 over-expression in mouse embryonic cells and mesenchymal stem cells isolated from human apical papilla seems to promote neural differentiation, possibly by Wnt pathway inhibition [26, 27]. Moreover, together with SFRP1, SFRP2 is necessary for the normal development of the ventral midbrain in mice and can increase the number of dopaminergic neurons when added to mouse embryonic stem cells cultures [28]. The Wnt pathway has also been implicated in the postnatal modulation of entorhinal cortex and hippocampus synaptic activity in mice, and SFRP2, as an inhibitor of the Wnt pathway, may be involved in this process [29]. Beyond the putative role in neural stem cells proliferation, SFPR2 also modulates the migration and cell fate of intestinal epithelial progenitor cells in mice [30] and regulates self-renewal and apoptosis of mice mesenchymal stem cells [31]. In contrast, treatment of mice retinal stem cells with SFRP2 was associated with decreased retinal neurosphere number, by promoting progenitor cell quiescence [32]. The same neurosphere number reduction effect was observed in secondary passages of SFRP2-treated mice primary subependymal zone cultures [33].

Wnt signaling dysregulation, mainly associated with synaptic activity alterations, has been related to the development of neurodegenerative and mental disorders like schizophrenia, epilepsy, bipolar disorder, Alzheimer's disease and behavioral abnormalities (reviewed by [9, 22, 34, 35]). SFRP2 alterations, seem to be implicated in some of the neurological disorders associated with Wnt deficiencies. In rats continuously treated with morphine, SFRP2 intracerebral injection reduced morphine withdrawal symptoms, preventing the activation of the Wnt pathway and decreasing the loss of dendritic spine density induced by morphine withdrawal [36]. In contrast, SFRP2 exposure was associated with increased levels of tau hyperphosphorylation in rat-derived cortical neural cultures, a pathological characteristic observed in Alzheimer’s disease, suggesting that SFRP2 may be implicated in this pathology [37]. In fact, in *Octodon degus*, a natural rodent model used to study neurodegenerative disorders, altered Wnt signaling was associated with altered expression of Wnt ligands and increased levels of SFRP2 in the hippocampus of aged animals [38].

In addition to neurological disorders, increasing evidence support that SFRP2-mediated modulation of the Wnt signaling may be relevant in several pathological conditions like cardiac diseases, inflammation-induced muscular atrophy, and brain cancers [2, 39–43]. SFRP2 seems to have a protective effect on glioblastoma, by inhibiting Wnt pathway activation and

consequently decreasing glioblastoma cells' invasion and proliferation ability [44, 45]. In addition, higher methylation levels of the *SFRP2* gene promoter were found in samples of brain tumor patients [46]. The same protective effect of SFRP2 was described in pituitary adenomas [39, 47], choriocarcinoma [43], cervical cancer [48], and esophageal carcinoma [49]. SFRP2 is often described as a tumor suppressor gene [1, 15, 39, 50], although a pro-tumorigenic effect of SFPR2 has also been reported [1, 51–54]. On the other hand, SFRP2 seems to have a potential therapeutic action in the treatment of cardiac diseases, such as ischemic injury, cardiac atrophy, and myocardial infarction [42, 55–57].

The presence of SFRP2 transcripts was reported previously in both rat and human choroid plexus (CP) tissues using microarray and RNAseq transcriptomic analysis techniques [58, 59], suggesting that this brain structure may contribute to the production of this glycoprotein in the brain. Furthermore, differential expression of the SFRP2 gene in the CPs collected from male and female rats could be an indication that SFRP2 expression is possibly modulated by sex hormones in this intraventricular brain structure [58, 60]. Considering the potential of SFRP2 as a modulator of neural stem cell hemostasis and the proximity of the CP to the subventricular zone (SVZ), one of the neurogenic niches in the adult brain [25, 61, 62], this work aimed to investigate if SFRP2 is expressed and secreted by the rat CP and to assess if its expression is regulated by sex hormones.

7.2. Material and Methods

7.2.1. Animals

The CP tissues were collected from the lateral brain ventricles of Wistar Han rats aged between 3—21 postnatal days. Animals were housed in proper cages, at constant room temperature in a 12 h light/12 h dark photoperiod and given standard laboratory chow and water *ad libitum*. Animal tissue collection was approved by the Animal Welfare and Ethics Committee of the Health Science Research Centre of the University of Beira Interior, in compliance with National (Decree-law 113/2013, corrected by the Decree-law nº 1/2019) and European Union rules (Directive 2010/63/EU) for the care and handling of laboratory animals. No further licensing was required since the study did not involve animal experimentation. Before decapitation, Wistar Han rats up to 6 days old were anesthetized in ice, while older animals were anesthetized with isoflurane. All efforts were made to minimize the number of animals used as well as their suffering.

7.2.2. Primary CP epithelial cell cultures and *ex vivo* studies

Primary CP epithelial cell cultures (CPEC) were established from CPs dissected from 3—6 days old postnatal rats only, following the protocol previously described by Gonçalves and colleagues [63]. CPs were collected from the lateral brain ventricles to cold phosphate-buffered saline (PBS) immediately after decapitation. CPs were digested in a solution of 0.2% pronase (Fluka, Seelze, Germany), diluted in PBS, for 5 minutes at 37 °C. Dissociated cells were washed twice

with high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, ThermoFisher Scientific, USA, Cat# 12800017) supplemented with 100 U/mL antibiotics and 10% FBS and seeded into 12 (four CPs per well) or 24 (two CPs per well) wells culture plates. The growth culture medium, consisting of high-glucose DMEM culture medium supplemented with 100 U/mL antibiotics, 10% FBS, 10 ηg/mL human epidermal growth factor (Sigma-Aldrich, Germany, Cat# E9644), 5 μg/mL insulin (Sigma-Aldrich, Germany, Cat# I9278) and 30 μM cytosine arabinoside (Sigma-Aldrich, Germany, Cat# C1768), was replaced 1 day after seeding, and every 2 days thereafter. All studies were performed using cultures established for at least 4—5 days.

For the *ex vivo* experiments, CP tissues (CP explants) were collected from 3—6 days old postnatal rats and placed directly in high-glucose DMEM without phenol red (Gibco, ThermoFisher Scientific, USA, Cat# 12800017) supplemented with 10% charcoal-stripped fetal bovine serum (FBS; Capricorn Scientific, Germany, Cat# FBS-12B) and 100 U/mL of penicillin/streptomycin (Sigma-Aldrich, Germany, Cat# P4333) in 24-wells culture plates (sixteen CPs per well). To assess the influence of sex hormones in the regulation of SFRP2 expression, two hours after collection and the initial incubation in culture medium, CP explants were stimulated with two different concentrations of 17β-estradiol (E2; Sigma-Aldrich, Germany, Cat# E8875: 0.1 and 1 ηM), progesterone (P4; Calbiochem; 10 and 100 ηM), 4,5α-dihydrotestosterone (DHT; Sigma-Aldrich, Germany, Cat# A8380: 1 and 10 ηM) or vehicle (0.0001—0.01% ethanol) and incubated for additional 24 hours. All sex hormone concentrations included in this study were close to the reported physiological levels in rats [64, 65]. All cultures were maintained in a humidified incubator in 95% air—5% CO_2 at 37 °C.

7.2.3. RNA extraction and PCR reverse transcription (RT-PCR)

Total RNA was extracted from CPEC, and CP tissues. These tissues were collected from 3—21 days old animals. Except for 4 days old rats, where total RNA was extracted from pools containing the CPs of 4 animals from the same litter, total RNA was extracted from the CPs of individual animals. Kidney tissue collected from an adult female rat was used as a positive control. Total RNA was extracted from all samples using TRIzol (TripleXtractor, Grisp, Portugal, Cat# GB23.0100), according to the manufacturer's instructions. RNA was quantified by spectrophotometry (NanoPhotometer™, Implen, Germany) and RNA integrity was assessed by agarose (1.5 %) gel electrophoresis containing *GreenSafe* staining (NZYTech Ltd., Portugal, Cat# MB13201). To prevent possible contaminations with genomic DNA, samples were treated with DNAse I (Sigma-Aldrich, Germany, Cat# AMPD1) before complementary DNA synthesis. 500 ηg of CPEC and 1 μg of CPs and kidney total RNA were transcribed into complementary DNA using M-MuLV Reverse Transcriptase (NZYTech Ltd., Portugal, Cat# MB08301, 20 μL reaction), following the manufacturer's instructions. PCR reactions were carried out in a final volume of 10 μL using NZYTaq II 2x Green Master Mix (NZYTech Ltd., Portugal, Cat# MB35801) and 0.4 μM of each primer (Table 7.1), on a MultiGene™ Optimax Thermal Cycler (Labnet International, USA). The following PCR cycling protocol was used: an initial 3-minute

denaturing reaction at 95 °C, followed by 30 seconds at 94 °C, 30 seconds at 60 °C, and 30 seconds at 72°C for 40 cycles with a final 10 minutes extension at 72°C. SFRP2 full-length PCR products were analyzed in a 1 % agarose gel electrophoresis, visualized under UV with *GreenSafe* staining (NZYTech Ltd., Portugal, Cat# MB13201) and Sanger sequenced (STAB VIDA, Portugal) to confirm the gene identity. A negative control consisting of a PCR reaction without template was included in all the assays.

7.2.4. Real-time quantitative PCR

To evaluate the SFRP2 mRNA expression levels in the CPs of animals at different postnatal ages, real-time quantitative PCR (RT-qPCR) was performed using Xpert Fast SYBR 2X Mastermix (Grisp, Portugal, Cat# GE20.5100) on CFX Connect Real-Time PCR Detection System (Bio-Rad, USA). For each RT-qPCR, 1 µL of the synthesized cDNA, in a total volume of 10 µL reactions, was used. The following cycling program was used for RT-qPCR: Initial denaturation at 95 °C for 3 minutes, followed by 40 cycles of denaturation at 95 °C, annealing at 60 °C for 30 seconds and extension at 72 °C for 10 seconds. Specific oligonucleotide primers sequences used for RT-qPCR are described in Table 1. Fluorescence was measured at the end of each cycle and the threshold amplification number (Ct) for each gene was determined using the CFX Manager™ Software (Bio-Rad, USA). The ΔCt was calculated as the difference between the Ct of SFRP2 and the Ct of the reference gene Cyclophilin A (CypA). The relative expression of the SFRP2 gene was calculated using the $2^{-(\Delta\Delta Ct)}$ method [66, 67]. Four samples were used per group and all samples were assayed in duplicate. To ensure the specificity of the amplification, a melting curve analysis was performed at the end of the final cycle.

Table 7.1. Sequences of the oligonucleotide primers used in RT-PCR and RT-qPCR.

Gene	RefSeq	Primer sequence (5' – 3')	Amplicon size (bp)	AT (°C)
SFRP2*	NM_001100700.1	Fw: GTCGCTAGTCCACGATGCT RV: TGAGACTGTAGCTCTCCCGA	1013	60
SFRP2†	NM_001100700.1	Fw: AGAACGAGGATGACAACGACA RV: ACACCCCGTTCAGCTTGTAA	150	60
CypA†	NM_017101.1	FW: GTCGCTAGTCCACGATGCT RV: TGAGACTGTAGCTCTCCCGA	163	60

*Primers used in RT-PCR; †Primers used in RT-qPCR. AT: Annealing temperature; FW: Forward, RV: Reverse.

7.2.5. Whole-mount immunofluorescence

To evaluate the localization of SFRP2 in the rat CP tissue, the whole-mount immunofluorescence technique was performed, as previously described [63]. CPs were collected from 6-day old rats and placed into a 48-wells culture plate with cold 4 % paraformaldehyde and incubated for 30 minutes at room temperature, to fix the tissues. All the incubations and washing steps were performed using a plate shaker. After 3 washes with PBS, CPs were

incubated for 2 hours with a solution of 30% sucrose diluted in PBS, until the tissues sank in each well. Then, CPs were washed three more times with PBS and incubated for 1 hour at room temperature with monoclonal mouse anti-occludin Alexa Fluor 594® antibody (1:200; ThermoFisher Scientific, USA, Cat# 331594, RRID:AB_2532186). Afterwards, CPs were washed three times with PBS incubated for 4 hours at room temperature with blocking solution (2.5 % bovine serum albumin, BSA, in PBS with 0.2% Triton X-100) and washed three more times with PBS with 0.1 % Tween-20 (PBS-T), before overnight incubation at 4 °C with rabbit anti-rat SFRP2 polyclonal primary antibody (1:100; Bioworld Technology, Inc., USA, Cat# BS7759). Next, CPs were washed five times with PBS-T and incubated with secondary Alexa Fluor 488® goat anti-rabbit IgG conjugated antibody (1:1,000; Molecular Probes, ThermoFisher Scientific, USA, Cat# A-11008, RRID:AB_143165A-11008) for 3 hours at room temperature. CP explants were then washed five times with PBS-T, incubated for 20 minutes at room temperature with Hoechst 33342 (1:1,000; Thermo Fisher Scientific, USA, Cat# I34406) and washed three more times with PBS-T. With the help of forceps and a paintbrush, CP tissues were mounted onto microscope slides with Dako Fluorescence Mounting Medium (Dako, USA, Cat# S3023) and covered with a coverslip. CP explants incubated without the SFRP2 primary antibody were used as negative controls. Slides were visualized under a confocal microscope LSM 710 (Carl Zeiss, Germany) using a magnification of 63x (Plan-Apochromat 63x/1.4 Oil DIC M27) and analyzed with ZEN lite software (Carl Zeiss, Germany).

7.2.6. Immunocytochemistry

For immunocytochemistry experiments, CPEC were cultured and grown to 70-80 % confluence on glass coverslips. Cells were washed with PBS and fixed with cold 4 % paraformaldehyde for 10 minutes at room temperature, using a plate shaker. Then cells were washed with PBS and incubated for 1 hour at room temperature with monoclonal mouse anti-occludin Alexa Fluor 594® antibody (1:200). After washing with PBS three times, cells were incubated with 1 % Triton X-100 in PBS for 5 minutes at room temperature. Next, cells were washed with PBS and incubated with blocking solution (20 % FBS in PBS-T 0.1 %) for 1 hour at room temperature. Afterward, cells were incubated overnight at 4 °C rabbit anti-rat SFRP2 polyclonal primary antibody (1:100) and incubated for 1 hour at room temperature with Alexa Fluor 488® goat anti-rabbit IgG conjugate (1:1,000). Cells nuclei were stained for 10 min with Hoechst 33342 (1:1,000). Lastly, coverslips were washed several times with PBS-T and mounted onto microscope slides with Dako Fluorescence Mounting Medium (Dako, USA, Cat# S3023). Confocal images of the cell's preparations were acquired using an LSM 710 confocal laser scanning microscope (Carl Zeiss, Germany) under a 63x magnification (Plan-Apochromat 63x/1.4 Oil DIC M27) and analyzed with ZEN lite software (Carl Zeiss, Germany). Cells incubated in the absence of SFRP2 primary antibody were used as negative controls.

7.2.7. Protein extraction and Western Blot

To evaluate the presence and the levels of SFRP2 protein in CPEC and CPs, tissues and primary cells were homogenized, on ice, using radioimmunoprecipitation assay buffer (RIPA) (150 mM

sodium chloride, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 50 mM Tris pH 8.0) completed with 1 mM phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate and 40 µL/mL cOmplete™ EDTA Free protease inhibitor cocktail (Roche, Sigma-Aldrich, Germany, 25x stock, Cat# 11873580001). Lysates were centrifuged at 10,000 *g* for 10 minutes at 4 °C and supernatants were collected and stored at -20 °C until use. Culture medium supernatants collected from the *ex vivo* experiments were concentrated to a final volume of approximately 50 µL using Vivaspin® 500 µL spin columns (Sartorius, Germany, Cat# VS0112). Total protein quantification was performed using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA, Cat# 23225). Protein extracts (50 µg) and concentrated culture supernatants (15 µL) were diluted in 4X sample buffer containing freshly added 10 % β-mercaptoethanol, denatured at 100 °C for 5 minutes and separated by 12.5% SDS-PAGE. Then, proteins were transferred to polyvinylidene difluoride membranes (Amersham™ Hybond™ 0.45 µm, GE Healthcare, UK, Cat# 10600023) and membranes were blocked for 1 hour with 5 % skimmed milk in Tris-buffered saline with 0.1 % Tween 20 (TBS-T 0.1%), before being incubated overnight at 4 °C with rabbit anti-rat SFRP2 polyclonal primary antibody (1:1,000). After being washed with TBS-T 0.1 %, membranes were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:20,000; Thermo Fisher Scientific Cat# 31466, RRID:AB_10960844). Protein bands were visualized by chemiluminescence using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, USA, Cat# 34577) and detected using a ChemiDoc™ MP Imaging System (Bio-Rad, USA) and Image Lab 5.1 software (Bio-Rad, USA, RRID:SCR_014210). In quantitative experiments, densitometry of each protein band was measured using Image Lab software and the relative intensity of each SFRP2 band was normalized against α-tubulin levels (1:10,000; Sigma-Aldrich Cat# T9026, RRID:AB_477593) and compared between groups. In the case of the culture medium supernatants, densitometry values were normalized by the total protein previously quantified. At least four independent samples within each group were quantified.

7.2.8. Statistical analysis

Values are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, CA, RRID:SCR_002798). Statistical significance of differences between two groups was analyzed with Student's two-tailed unpaired *t*-test, while one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to compare more than two groups. Significant differences between groups were set at $p < 0.05$.

7.3. Results

7.3.1. SFRP2 is expressed in the rat CP

To investigate if the rat CP could be a source of SFRP2 in the brain, we first evaluate the expression of *Sfrp2* in the CP of postnatal animals by RT-PCR. The amplicon corresponding to the full-length *Sfrp2* mRNA (1013 bp) was detected in samples of RNA from postnatal animals

CPs and CPEC cells (Figure 7.2.A). The treatment of total RNA samples with DNAse before cDNA synthesis excluded possible genomic contaminations of the tested samples. The absence of amplicons in the negative controls used in RT-PCR and qPCR, and the high identity between the Sanger sequenced RT-PCR products and the published sequence of *Sfrp2* rat mRNA (accession number NM_001100700.1), further confirmed the specificity of the primers used. The highest expression level of *Sfrp2* in the CP of postnatal rats was found at 4 days old (Figure 7.1.B). However, the differences in the expression of *Sfrp2* throughout postnatal life were not statistically significant.

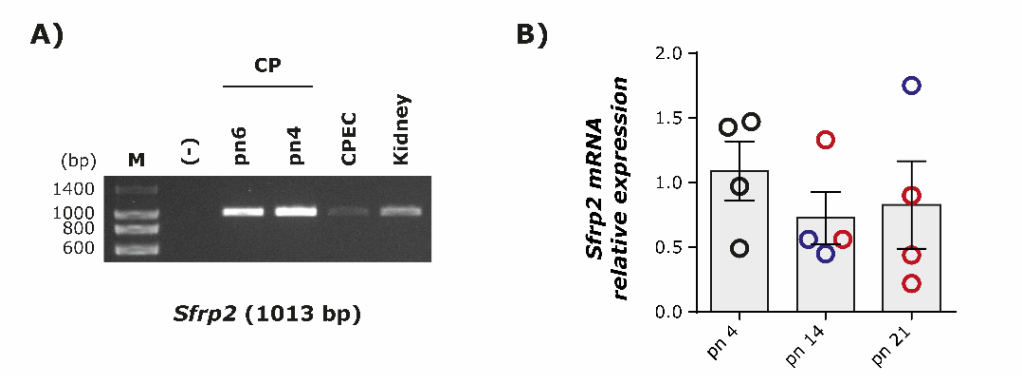


Figure 7.2. SFRP2 is expressed in the rat choroid plexus (CP). (A) Gel electrophoresis of RT-PCR products shows the presence of full-length SFRP2 transcripts in the CP of postnatal rats (pn) with 4 and 6 days old and CP epithelial cells (CPEC). Total RNA extracted from the kidney tissue of an adult female rat was used as a positive control. (B) The expression of *Sfrp2* mRNA in the CP of postnatal rats of different ages was analyzed by RT-qPCR. Scattered plots with bar represent the means ± SEM (n = 4). Individual values of each sample are shown by circles (Black: pools of CPs collected from 5 animals; Red: CPs collected from females; Blue: CPs collected from male rats). *Sfrp2* expression levels between groups were compared using one-way ANOVA followed by Tukey's post hoc test. No statistically significant differences were observed. M: Molecular weight marker NZYDNA Ladder VIII (NZYTech Ltd., Portugal); (-) Negative control.

The presence of SFRP2 protein was evaluated by Western blot (Figure 7.3). A 26 kDa protein, corresponding to the SFRP2 protein according to the primary antibody manufacturer's datasheet, was visible in the lysates of CP tissues collected from the lateral brain ventricles of postnatal animals, as well as in CP protein extracts dissected from 3-month-old female and male rats. No band corresponding to SFRP2 protein was detected in CPEC total protein lysates. SFRP2 immunoreactive protein was also present in the concentrated culture medium supernatants collected at the end of the *ex vivo* experiments, suggesting that CP explants are able to secrete SFRP2. SFRP2 immunoreactive protein was absent in concentrated culture medium supernatants that were not incubated with CP explants (data not shown).

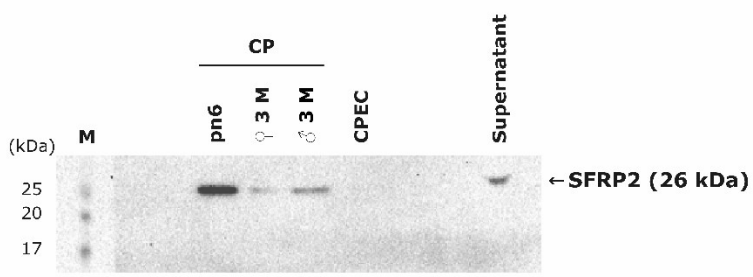


Figure 7.3. SFRP2 protein is present in rat choroid plexus (CP). Protein extracts (50 µg) of CP tissues collected from 6 days old animals (pn6), a 3-month-old female rat (♀ 3 M), a 3-month-old male rat (♂ 3 M), primary cultures of rat CP epithelial cells (CPEC) and concentrated culture medium supernatants (15 µL) collected at the end of *ex vivo* experiments were resolved by 12.5 % SDS-PAGE followed by blotting using anti-rat SFRP2 polyclonal primary antibody (1:1000) and horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:20,000). A protein band with the expected molecular weight (26 kDa) was present in CP lysates and culture medium supernatant. M: GRS Protein Marker Multicolor (Grisp, Portugal).

7.3.2. SFRP2 protein is localized in the cytoplasm and the cell membrane of epithelial CP cells

In addition to confirming the presence of SFRP2 protein in the rat CP, the cellular localization of SFRP2 in this brain structure was further examined using both whole-mount and immunocytochemistry techniques. Positive immunostaining was observed in the cytoplasm of the epithelial cells in the rat CP tissues (Figure 7.4.A). Furthermore, the presence of SFRP2 immunoreactivity was also observed in primary cultures of CPEC. Within CPEC, positive immunostaining was found in the cytoplasm but also in the cellular membrane of the cells, overlapping the occludin staining (Figure 7.4.B). No positive staining was observed when the SFRP2 polyclonal primary antibody was omitted from the incubations, reinforcing the results obtained.

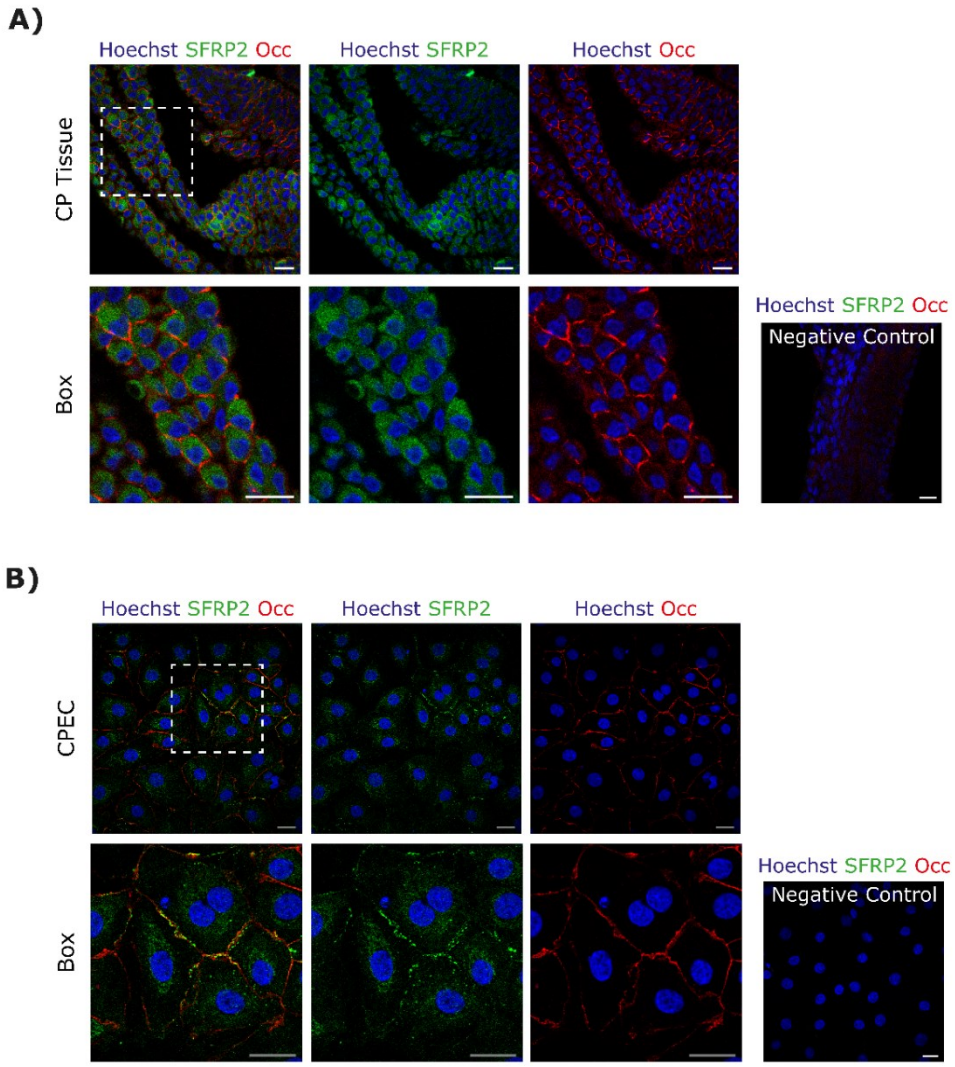


Figure 7.4. Cellular location of SFRP2 protein in the rat choroid plexus (CP). (A) Confocal images of SFRP2 protein presence in tissues of postnatal rats (6 days old) and (B) CPEC cells. CP explants (A) and CPEC cells (B) were incubated with anti-occludin Alexa Fluor 594® conjugated antibody and anti-rat SFRP2 polyclonal primary antibody (1:100), followed by incubation secondary antibody Alexa Fluor® 488 goat anti-rabbit (dilution 1:1,000). Nuclei were stained with Hoechst 33342 (dilution 1:1,000). Boxed areas present in (A) and (B) are shown in the respective lower panels. Merged images of coverslips incubated without anti-rat SFRP2 polyclonal primary antibody are shown (Negative Control). Images were obtained on an LSM 710 confocal laser scanning microscope under 63x magnification. Scale bar, 20 µm. Occ: occludin.

7.3.3. Influence of sex hormones in the production and secretion of SFRP2 in the rat CP

To assess the influence of sex hormones on the production and secretion of SFRP2 in the rat CP, CP explants were incubated with E2, P4, or DHT. After 24 hours of incubation, SFRP2 protein levels in CP lysates and culture medium supernatants of the *ex vivo* experiments were analyzed by Western blot. In this regard, except for the E2 incubation that was able to increase SFRP2 levels in the CP, no further statistically significant differences were observed with sex hormones (Figure 7.5). However, despite the rise of SFRP2 protein amount observed in CP tissues after the incubation with the higher concentration of E2 (E2 1 ηM: 199.6 % ± 29.4 versus Vehicle, $p <$

0.05), no differences were observed in the levels of SFRP2 protein secreted into the culture medium supernatants.

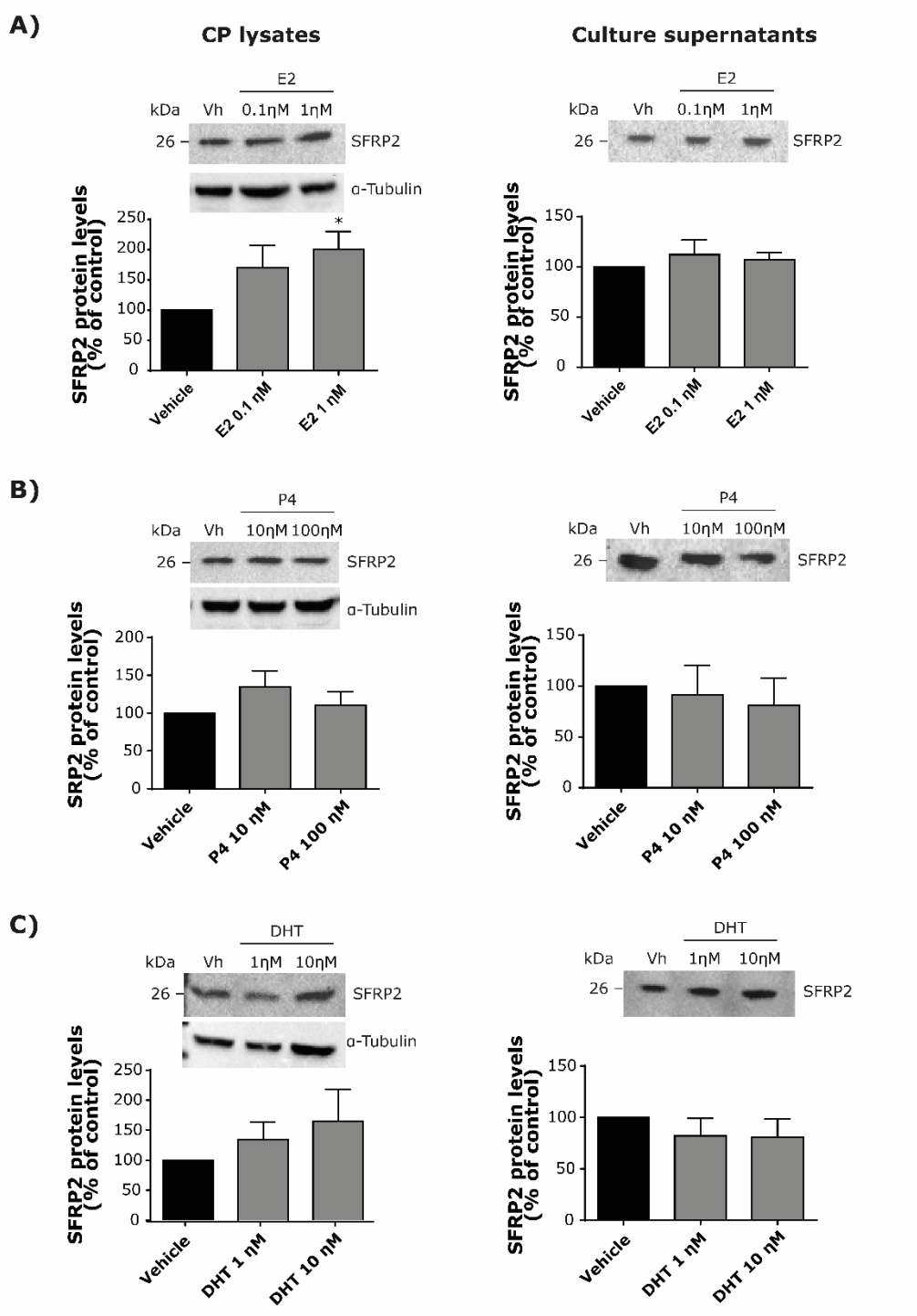


Figure 7.5. Effects of 17β-estradiol (E2), progesterone (P4), and 4,5α-dihydrotestosterone (DHT) on SFRP2 protein levels in the choroid plexus and culture medium supernatants. In *ex vivo* experiments, CP explants were incubated for 24 hours with either vehicle (Vh), or 0.1 or 1 ηM of E2 (A), 10 or 100 ηM of P4 (B), or 1 or 10 ηM of DHT (C). After incubation, SFRP2 protein levels were analyzed by Western blot in both CP lysates (50 µg; left panel) and concentrated culture medium supernatants (right panel). SFRP2 levels were normalized by α-tubulin in the case of CP lysates and by total protein in the case of culture medium supernatants. Bar graphs represent means ± SEM from at least four independent experiments (n ≥ 4). Statistically significant differences to control were determined using Student's two-tailed unpaired *t*-test (* $p < 0.05$).

7.4. Discussion

The Wnt canonical signaling pathway plays a crucial role in the development of the central nervous system (reviewed by [9, 10, 68]). Within the brain, SFRP2, a regulator of the Wnt signaling cascade, has been implicated in the maintenance of neural stem cells homeostasis and the disruption of its balance has been involved in neurological pathologies [26, 28, 37]. In the adult brain, neural stem cells are vital to brain plasticity and brain repair (reviewed by [68–70]). Therefore, it is important to disclose how endogenous factors with the potential to modulate neurogenesis are produced and regulated within the brain. In this regard, the CP, a brain structure that is in close contact with the SVZ, one of the adult neurogenic niches, contributes to the production of several signaling molecules that regulate neurogenesis [71, 72]. In this work, we present evidence that CP may also be a source of SFRP2 in the brain.

The presence of *Sfrp2* transcripts had been previously reported in human and rat CP tissues [58, 59]. However, none of the earlier transcriptomic analysis studies has validated the results of SFRP2 expression in follow-up studies. In this work, we show that *Sfrp2* mRNA is expressed in CP tissues of postnatal rats and CPEC by RT-PCR reinforcing the results previously obtained in the aforementioned microarray and RNAseq transcriptomic studies [58, 59]. The presence of the same size transcript in rat kidney tissue, together with the identical amplicon identity between the Sanger sequenced RT-PCR product and the known sequence of rat *Sfrp2* mRNA sequence, further support the results obtained. Postnatal and adult expression of *Sfrp2* has been formerly reported in several regions of the rodents brain, including the hippocampus, the olfactory ventricle, the arcuate nucleus and the cortex [23, 38, 73], suggesting that the expression of *Sfrp2* in the brain is not restricted to development stages and that SFRP2 is also relevant in adult brain function. Nevertheless, information regarding the role of this Wnt modulator in the adult brain is limited.

Western blot analysis revealed the presence of a 26 kDa protein, corresponding to SFRP2, in the postnatal rat CP and CP lysates collected from both adult females and males (3-month-old). However, despite the positive immunostaining observed in the immunocytochemistry experiments, SFRP2 protein could not be detected in CPEC lysates by Western blot, suggesting that the amount of SFRP2 protein in CPEC may be below the detection limits of the technique. The visualization of SFRP2 positive immunostaining in postnatal rat CP tissues strengthened the results obtained by Western blot. It was also possible to conclude that SFRP2 protein is mainly localized in the cytoplasm of the epithelial cells of the rat CP tissues, but SFRP2 positive immunostaining was also present in the cell membrane and at the perinuclear region of CPEC. Similar SFRP2 immunostaining patterns results have been described earlier in human melanocytes, keratinocytes, fibroblast, and skeletal muscle cells [41, 74] and mice intestine cells [30].

In addition to synthesizing SFRP2, the rat CP is also able to secret this glycoprotein as suggested by the presence of SFRP2 protein in the CP conditioned medium. The presence of higher

expression levels of *Sfrp2* in CPs collected from female than male rats in previous transcriptomic studies [58], together with evidence that supports that CP function is influenced by sex hormones [60], led us to initially hypothesize that SFRP2 production by the CP could be regulated by sex hormones. However, in our *ex vivo* experiments, only the higher concentration of E2 was able to positively modulate the levels of SFRP2 in rat CP explants. Although the incubation with E2 led to an increase in SFRP2 protein levels in the rat CP, no alterations were observed in the levels of SFRP2 secreted by this brain structure, suggesting that despite increasing the SFRP2 content within CP, E2 does not regulate the secretion process of this protein. Neither P4 nor DHT stimulus influenced the secretion or the SFRP2 protein levels in the rat CP. These results may indicate that the synthesis and secretion of SFRP2 in the rat CP are possibly regulated at the post-transcriptional level as well. Increased expression of *Sfrp2* mediated by E2 was reported in the iliac artery of monkeys with established atherosclerosis and this increase was associated with a putative protective role of E2 in this condition [75]. Higher levels of circulating SFRP2 in the serum of females had also been described in humans. However, this study included samples collected from healthy subjects and individuals with abnormal glucose tolerance, and SFRP2 levels were also positively correlated with other cofactors such as insulin levels or body mass index [76]. In contrast with our results, E2 stimulus was associated with decreased *Sfrp2* mRNA levels, independent of E2 receptor α, in mice uterus cells of transgenic mice [77]. The discrepant results may be a consequence of the pathway activated by E2 since in our study neither of the E2 receptors present in the CP was blocked. Nevertheless, in similarity with the Wnt pathway and its modulators, the regulation and function of CP-derived SFRP2 may be cell type and cell context-dependent.

Another possible mediator of *Sfrp2* expression in the CP could be age. Although we have studied the expression of Sfrp2 in the CPs collected from animals of different ages, this study was limited to the postnatal phase. At this stage, no differences in the expression of *Sfrp2* mRNA were observed, despite *Sfrp2* expression seeming to be higher in the early postnatal phase. Besides the analysis of the presence of SFRP2 protein in rats at 3 months old, the assessment of *Sfrp2* mRNA in older animals was not performed in this work. This additional study might be relevant since age-associated differences in SFRP2 levels were described in both humans and rodents. In a recent transcriptomic study, the expression of Sfrp2 mRNA in the human CP seems to decrease with age [59]. However, these results conflict with those described in rodents, where an age-associated increase in brain SFRP2 levels was found [38]. Nonetheless, it is important to notice that SFRP2 levels were analyzed in the whole brain in the last study rather than just in the CP as in the former [38, 59]. In addition to age, photoperiod may be another factor contributing to the modulation of *Sfrp2* expression in the CP. Photoperiod regulation of *Sfrp2* mRNA levels has been reported in the hypothalamus of rodents [73, 78]. Moreover, CP itself harbors an internal circadian clock that regulates several CP functions [79, 80], which may indicate that the circadian clock may influence *Sfrp2* expression.

In the adult SVZ, the activation of the canonical Wnt signaling promotes the generation of new neurons, especially after injury [25, 68, 81–83]. Furthermore, alterations in the Wnt signaling at the SVZ of mice with impaired neurogenesis were also reported [84]. As a classical Wnt pathway modulator, SFRP2 was associated with the *in vitro* induction of neural differentiation of mouse embryonic stem cells [26, 28]. The same ability to induce neural differentiation was also observed when SFRP2 overexpression was induced in stem cells collected from the human apical papilla [27]. In addition, the SOX2-induced increase of SFRP2 prompts the neural differentiation of human neural progenitor cells [85]. SFRP2 is currently being investigated for its potential to modulate mesenchymal stem cells, which are the base of modern stem cell tissue regeneration therapies [11, 27, 86, 87]. Moreover, despite having no effect on the number of primary subependymal zone-derived neurospheres, the passage of SPFR2-treated neurospheres resulted in reduced secondary neurospheres [33]. Since SVZ harbors stem cells in adult life and are in direct contact with the CP secretome, we hypothesize that SFRP2 produced and secreted by the CP may influence the generation of new neurons in this neurogenic niche. However, further studies are necessary to clarify the role of CP-derived SFRP2 in the rat brain and to investigate if SFRP2 has any therapeutic potential in neurodegenerative disorders, by modulating SVZ neurogenesis. Another possible brain function of SPRP2 produced by the CP that should also be considered in additional studies is the modulation of synaptic activity [33, 88].

In summary, these findings support the hypothesis that the rat CP is a source of SFRP2 in the brain and that SFRP2 with origin in the CP is possibly secreted. Although initially postulated that sex hormones may influence the production and secretion of SFRP2 in the CP, only E2 seems to positively regulate SFRP2 levels. For its location within the lateral brain ventricles, we speculate that CP-derived SFRP2 may be involved in the regulation of SVZ neurogenesis. Additional studies are necessary to understand how the production of SFRP2 is locally regulated in the rat CP and what is the role of SFRP2 secreted by the CP in the brain and the central nervous system.

7.5. References

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Chapter 8

Concluding remarks and future perspectives

8.1. Concluding remarks and future perspectives

The CPs establish the blood-cerebrospinal fluid (CSF) barrier (BCSFB). Aside from the main role in the production and clearance of CSF several other functions have been attributed to the CP [1–3]. Over the past decades, CPs have been assigned with immunosurveillance and chemical surveillance functions and are a recognized source of numerous factors that reach the central nervous system and modulate neurogenesis [3–6]. The CPs, lining within the brain lateral ventricles, are in close contact with the SVZ, and the CP itself is recognized as a constituent of this neurogenic niche. In addition, CP-derived factors may also reach the SGZ through CSF-mediated signaling [7].

Based on a microarray transcriptomic analysis study previously conducted by our research group, using CPs from rats, one of the CP-derived factors identified with potential to induce SVZ neurogenesis is prolactin [8]. Along with an essential role in reproduction and lactation [9, 10], prolactin modulates several brain functions and has been described as a peptide able to induce SVZ neurogenesis during pregnancy [11–13].

The first aim of this work was to analyze the expression, synthesis, and secretion of prolactin by the rat CP. In Chapter 4, we present evidence that the lateral ventricles of CP can synthesize and secrete PRL. In addition, the assays performed using an *in vitro* model to replicate the *in vivo* structure of the rat CP provided evidence that CP epithelial cells (CPEC) are able to secrete PRL both through the apical membrane facing the CSF and the basal membrane side facing the blood. Using the same *in vitro* model it was possible to observe that PRLR expression appears to be mainly located in the CPEC cytoplasm and partially translocate to the cell membrane in response to PRL stimulus. Based on these data, our results suggest that rather than just being a probable gateway for prolactin transport into the CNS, the rat CP itself may be an alternative source of prolactin to the brain. Furthermore, we hypothesize that the CP may detect peripheral PRL changes and induce PRL secretion to balance these alterations. Further studies are necessary to assess the relevance of CP-derived prolactin in the brain, with a focus on the evaluation of the impact of prolactin with origin in the CP in the SVZ neurogenesis. For that, it would be relevant to conduct studies to investigate the impact of prolactin CP knockdown in SVZ neurogenesis, using *in vitro* (short interfering RNA gene silencing) and *in vivo* (injection with prolactin morpholino-antisense oligonucleotides) approaches. These types of assays would make it possible to understand if SVZ neurogenesis is mediated by CP-derived prolactin or prolactin from other biological sources.

Since the microarray transcriptomic study previously performed also reported gender differences regarding prolactin expression in the rat CP [8], another of the objectives of this thesis was to investigate if prolactin synthesis and secretion by rat CP were regulated by sex hormones. Based on evidence collected using an *ex vivo* model of the CP (CP explants), the findings reported in Chapter 5 of this work suggest that unlike in adult rat CP, the production of prolactin in the postnatal CP may not be regulated by sex hormones. Yet, these results are

limited to the sex hormone concentrations and incubation periods used in our experiments and it is not possible to conclude if longer incubation periods or exposure to other sex hormones concentrations would modulate prolactin synthesis in the rat CP. The influence of factors associated with the modulation of prolactin expression in the pituitary like hypoxia, exposure to other hormones or growth factors like thyrotropin-releasing hormone, epidermal growth factor, or kisspeptin, should be investigated in future works [14–17].

The CP is the structure that presents the higher expression levels of PRLR within the mouse brain [18, 19]. However, recent evidence support that prolactin transport into the brain is not mediated by the PRLR present in the CP, opposing the primary hypothesis that PRLR present in this brain structure are involved in prolactin transport [20]. The exact function of prolactin in the rat CP is still poorly studied, particularly during postnatal stages. As so, another aim of this work was to assess the effects of prolactin exposure in the postnatal rat CP. For that, we evaluated the effect of prolactin exposure in the transcriptome of CPs collected from early postanal rats incubated with a high concentration of prolactin for 8 hours. Real-time quantitative PCR results reported in Chapter 6, suggest that prolactin exposure during early postnatal life reduced the expression of neurogenesis- and barrier-associated factors, and increased the expression of proinflammatory *Il1b*. Since early life exposure to elevated levels of prolactin has been associated with alterations in the development of maternal behaviors and the development of depressive-like behaviors in rodents [21], we believe that CP could be involved in some of these brain alterations, including reduced neurogenesis. On the other hand, higher levels of prolactin during pregnancy were also associated with neurogenesis induction in rodents. Thus, we also hypothesized that the action of prolactin on the CP may be development stage-dependent like in other target tissues. To confirm this hypothesis, additional studies should be performed to assess if transcriptomic alterations induced by prolactin exposure are also associated with proteins levels variations. Furthermore, *in vivo* studies should also be carried out to unveil the full biological action of prolactin in the postnatal CP and to assess its impact on brain function.

Finally, the last aim of this work was to identify additional factors with the potential to induce neurogenesis possibly synthesized and secreted by the CP. As so, we investigated if the rat CP could be a source of SFRP2, a peptide involved in the regulation of the Wnt signaling pathway, that has also been implicated in neural stem cell proliferation [22–24]. The presence of SFRP2 mRNA in the CP and the observation of SFRP2 protein in total protein extracts of CPs and culture supernatants described throughout Chapter 7 support this hypothesis. Alike prolactin, it was also initially postulated that sex hormones may influence the production and secretion of SFRP2 in the CP. However, only E2 exposure was associated with increased levels of SFRP2 in CP lysates. None of the other sex hormones tested was related to the modulation of SFRP2 secretion. Once again, due to the location of CPs within the lateral brain ventricles, we speculate that CP-derived SFRP2 may be involved in the regulation of SVZ neurogenesis. Once again, additional studies are necessary to test this hypothesis and to understand the role of SFRP2

produced by the CP in the brain. Similar to prolactin, the effect of SFPR2 CP knockdown on SVZ neurogenesis should be assessed in future *in vitro* and *in vivo* assays. Furthermore, further research work should be enrolled on how the production of SFRP2 is regulated in the rat CP. Factors such as age and the influence of photoperiod and circadian rhythm [25, 26] should be considered in future studies focused on the investigation of modulators of the expression of SFRP2 in the CP.

Altogether, the evidence reported throughout this work supports the relevance of the CP as a source of peptides with the potential to modulate brain function. In this perspective, due to the proximity of the CP to neurogenic niches both prolactin and SFRP2 have been described as neurogenic factors that may be responsible for the induction of progenitor stem cell proliferation. Although initially hypothesized that sex hormones could modulate the production and secretion of the two peptides investigated in this work, only estradiol seems to be associated with SFRP2 production in the CP. In this context, further investigation is necessary to fully understand the exact roles of prolactin and SFRP2 originated by the CP. Considering the worldwide increasing aging rate and the consequent rise of age-associated disorders including neurodegenerative diseases, CP-derived peptides may be considered for future therapeutic approaches. As a vital regulator of the central nervous system microenvironment and homeostasis, the CP and, more specifically alterations of the CP and CP-derived peptides, have been implicated in the progress of neurological conditions such as Alzheimer's disease, multiple sclerosis, or schizophrenia [27–30]. Based on this evidence, the potential of CPEC cells and CPEC secretome is currently been investigated as a therapeutic approach in brain disorders such as Parkinson's disease, stroke, Huntington's disease, sensorineural hearing loss, and Alzheimer's disease, with positive outcomes in all the disorders [31–35]. In sum, fully understanding the impact of CP-derived peptides, including prolactin and SFPR2, as identified in this work, would be of utmost relevance to our understanding of brain function.

8.2. References

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