Universidade de Aveiro Departamento de Biologia
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Miguel Augusto Rodrigues de Amorim

Acção da progesterona em mecanismos de fosforilação proteica no sistema nervoso central

Progesterone actions in protein phosphorylation in the central nervous system

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Professor Doutor Edgar da Cruz e Silva, Professor Associado do Departamento de Biologia, da Universidade de Aveiro e do Professor Doutor Luis Miguel Garcia-Segura, Professor Investigador do Instituto Cajal, Consejo Superior de Investigaciones Científicas, Madrid.

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Dedico este trabalho à memória do Professor Doutor Edgar da Cruz e Silva.

o júri

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À Núria e seus pais.
À minha família, tendo, também, presentes aqueles que já não podem partilhar este momento.
Ao meu primo Artur Filipe.
Aos meus pais.

palavras-chave

Hormonas sexuais, esteróides neuroactivos, progesterona, dihidroprogesterona, tetrahidroprogesterona, cinases, fosfatases, proteína Tau

resumo

O presente trabalho propõe-se esclarecer o papel que a progesterona e os seus metabolitos exercem no sistema nervoso central. Nos últimos anos, com a descoberta da síntese local de esteróides no cérebro, a progesterona, assim como outras hormonas sexuais, ganharam uma relevância crescente em fenómenos tais como plasticidade neuronal e neuroprotecção. Ainda que já se comece a entender o papel de muitas hormonas no cérebro, tal como o estrogénio, o papel da progesterona continua menos conhecido. Deste modo, o nosso trabalho centrou-se na elucidação dos efeitos da progesterona em fenómenos de sobrevivência celular, plasticidade neuronal/sináptica.

Graças à colaboração com um grupo pioneiro em estudos sobre hormonas sexuais neuroactivas, o presente trabalho fornece uma importante contribuição ao entendimento do papel desta hormona no sistema nervoso central.

Este trabalho fornece novos dados, relativamente ao papel da progesterona e dos seus metabolitos reduzidos na regulação de vias de sinalização associadas com sobrevivência celular, tal como Akt/PI3K e ERK. Também é analisado o efeito do tratamento hormonal na expressão e estado de fosforilação da proteína Tau, sendo ainda motivo de estudo cinases e fosfatases envolvidas nestes mecanismos.

keywords

Sexual hormones, neuroactive steroids, progesterone, dihydroprogesterone, tetrahydroprogesterone, kinases, phosphatases, Tau protein

abstract

The present work has as its main aim, to unveil the role of progesterone and its reduced metabolites in the central nervous system. In the last years, with the discovery of local synthesis of steroids in the brain, progesterone, as well as other sexual hormones, acquired a new importance in brain phenomena like neuronal plasticity and neuroprotection. Although the role of many hormones in the brain, like the estrogen, is starting to be well known, the role of progesterone was less studied. Therefore, our work aim was to study the progesterone effects in the brain regarding cellular survival and neuronal/synaptic plasticity.

Thanks to the collaboration with a group pioneer in neuroactive sexual hormones studies, the present works provides an important contribution on the role of this hormone in the central nervous system.

This work offers new data regarding the role of progesterone and its reduced metabolites in the regulation of signaling pathways associated with cellular survival, like the Akt/PI3K and ERK. It is also studied the effect of hormonal treatment in the expression and phosphorylation state of Tau protein, as well as the kinases and phosphatases involved in these mechanisms.

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ABBREVIATIONS

3α-HSD	3α-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
AD	Alzheimer disease
Akt	protein kinase B
BAD	Bcl-2-associated death promoter
BDNF	brain derived neurotrophic factor
b.w.	body weight
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CREB	cAMP response element-binding protein
DHP	5α-dihydroprogesterone
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular-signal regulated kinase
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
GSK3	glycogen-synthase kinase-3
IGF-I	insulin-like growth factor-I
i.p.	intraperitoneal
IRS-1	insulin receptor substrate-1
LTD	long-term depression
LTP	long-term potentiation
MAPs	microtubule associated proteins
MAPK	mitogen-activated protein kinase
MEK	MAPK/ERK kinase or MKK
MTs	microtubules
mTOR	mammalian target of rapamycin
NaCl	sodium chloride
NF-KB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	nerve growth factor
NIH	National Institute of Health of United States of America
NP-40	tergitol-type NP-40, nonyl phenoxylpolyethoxylethanol
p42	ERK2

p44	ERK1
p85	PI3K regulatory subunit
p110	PI3K catalytic subunit
P450scc	cytochrome 450 side-chain cleavage
PDK1	3-phosphoinositide dependent kinase 1
PDZ	postsynaptic density protein, Drosophila disc large tumor suppressor, zonula occludens-1 protein structural domain
PI3K	phosphoinositide-3 kinase
PIP3	phosphatidylinositol (3,4,5) – trisphosphate/PtdIns-P3
PKC	protein kinase C
PNS	peripheral nervous system
PP2A	protein phosphatase 2
PP2AC	protein phosphatase 2 catalytic subunit
PR	progesterone receptor
PTEN	phosphatase and tensin homolog deleted on chromosome 10
Raf	proto-oncogene serine/threonine-protein kinase
Ras	protein subfamily of small GTPases/G-proteins
RTK	receptor of tyrosine kinase
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
THP	3α,5α-tetrahydroprogesterone
Tris-HCl	tris(hydroxymethyl)aminomethane-hydrochloric acid
Trk	tyrosine kinase-type receptor
Tween 20	polysorbate 20
VEGF	vascular endothelial growth factor

CHAPTER I

INTRODUCTION

I- INTRODUCTION

I.1 PROGESTERONE AND THE BRAIN

I.1.1 PROGESTERONE AND ITS METABOLITES

Progesterone is a product of cholesterol metabolism. Cholesterol is metabolized on pregnenolone by the cytochrome P450scc enzyme, located in the mitochondria. Pregnenolone is then metabolized to progesterone in the smooth endoplasmic reticulum by the enzyme 3-beta-hydroxysteroid dehydrogenase (Jung-Testas et al.,1989). In turn, progesterone can be reduced to 5α-dihydroprogesterone (DHP) by the enzyme 5α-reductase (Compagnone and Melon, 2000). DHP can be further reduced to 3α,5α-tetrahydroprogesterone (allopregnenolone or THP) by 3α-hydroxysteroid dehydrogenase (3α-HSD) or to 3β,5α-tetrahydroprogesterone (3β,5α-THP) by 3β-hydroxysteroid dehydrogenase (3β-HSD). The products of the DHP reduction are steroisomers (Compagnone and Melon, 2000). Furthermore, the conversion from DHP to THP is reversible, since THP is able to oxidize DHP in a reaction mediated by an isoform of the enzyme 3α-HSD (Melcangi et al., 1993; Dupont et al., 1994).

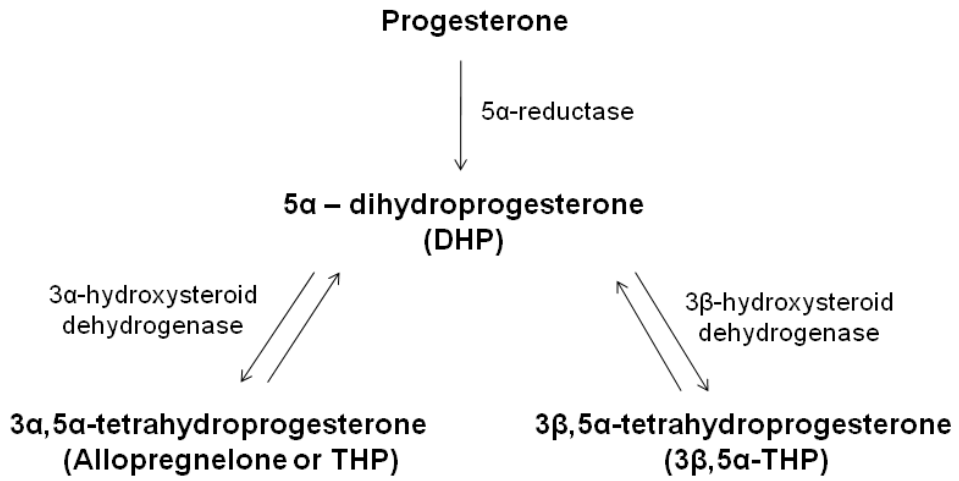

Figure I.1 - The metabolism of progesterone

I.1.2 PROGESTERONE SIGNALING IN THE CENTRAL NERVOUS SYSTEM

It is known that classical progesterone receptors control the actions exerted by the hormone and by its metabolite DHP in the brain (Brinton et al., 2008; Mani, 2008). Classical progesterone receptors are located intracellularly and are able to modulate the expression of genes that have progesterone response elements in their promoters (Roemer et al., 2006). For instance, progesterone regulates in some brain regions the expression of genes involved in the control of sexual behavior (Blaustein and Feder, 1979). There are two isoforms of the progesterone receptor, PR-A and PR-B, which although structurally similar are functionally different (Mani et al., 1994). PR-A that is a shorter form of PR-B, exerts a negative action in the transcription of PR-B, estrogen and glucocorticoids receptors. This negative regulation of estrogen receptors may explain why progestins antagonize some effects of estrogens (Bikle et al., 1992).

In addition, progesterone, as well as estradiol, can exert its actions through alternative mechanisms to the genomic actions mediated by classical nuclear receptors. A membrane progesterone receptor, with some features of the G-protein coupled receptors, has been recently discovered (Zhu et al., 2003b). There is also another membrane progesterone receptor, the 25Dx, which is expressed in some brain regions that are traditionally associated with sexual behavior (Krebs et al., 2000). Furthermore, the reduced metabolite of progesterone, THP, has high affinity for specific regions of the hydrophobic domain of the GABAA receptor, increasing the flux of chloride ions through the channel (Rupprecht et al., 1993; Belelli and Lambert, 2005). Therefore, the mechanisms of action of progesterone in the central nervous system involve genomic and non genomic actions which are in part mediated by its metabolites.

The membrane initiated signaling of progesterone may interact in the central nervous system with the signaling of growth factors and neurotrophins. For instance, there is evidence that progesterone may affect the activity of PI3K/Akt and ERK signaling in cortical explants (Singh, 2001), primary hippocampal cultures (Nilsen and Brinton, 2003) and retinal glial cells (Swiatek-De Lange et al., 2007). To further explore these interactions, in this study we have assessed the regulation of several kinases and phosphatases involved in signal transduction in the central nervous system.

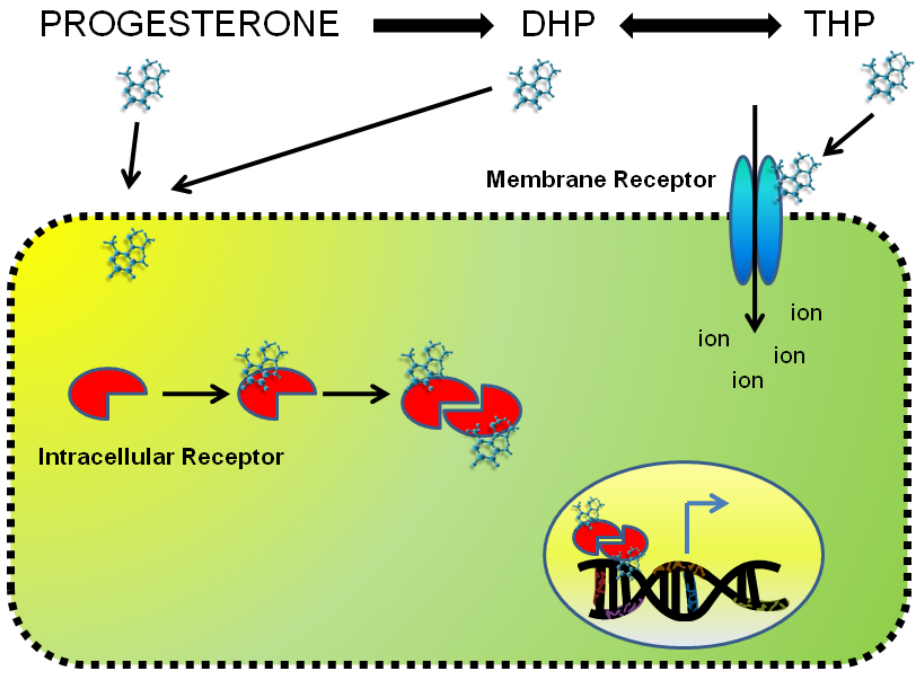

Figure I.2 - Mechanisms of action of the reduced metabolites of progesterone

I.1.3. ACTIONS OF PROGESTERONE IN THE CENTRAL NERVOUS SYSTEM

In addition to being a hormonal signal that acts in the brain to control neuroendocrine secretions and reproduction (Pfaff 1989; Etgen et al., 2006; Beyer, 2007), progesterone is a local paracrine factor synthesized by neural cells (Garcia-Segura and Melcangi, 2006). Progesterone and progesterone metabolites regulate synaptic development, plasticity and function in the cerebellum (Smith et al., 1987a,b,c; Smith, 1989, 1991; Sakamoto et al., 2001, 2002; Tsutsui et al., 2004), the hypothalamus (Perez et al., 1993; Garcia-Segura et al., 1994) and the hippocampus (Woolley and McEwen, 1993), among other brain regions, and are involved in the differentiation of neurons and glial cells and in the formation of myelin (Baulieu and Schumacher, 2000; Schumacher et al., 2004; Tsutsui et al., 2004; Ghoumari et al., 2005; Brinton et al., 2008). Progesterone is also a neuroprotective factor both in the peripheral and the central nervous system (Schumacher et al., 2007; Roglio et al., 2008; Cekic et al., 2009; DeNicola et al., 2009; Sayeed and Stein, 2009). In fact it has been shown the neuroprotective and anti-inflammatory properties of progesterone, in different experimental models of neurodegeneration (Ganter

et al., 1992; Garcia-Estrada et al., 1993; Drew and Chavis, 2000; Schumacher et al., 2004; Ciriza et al., 2004; Stein, 2005; Ciriza et al., 2006; Brinton et al., 2008). Finally, progesterone and its metabolites also affect adult neurogenesis in the hippocampus (Galea et al., 2006).

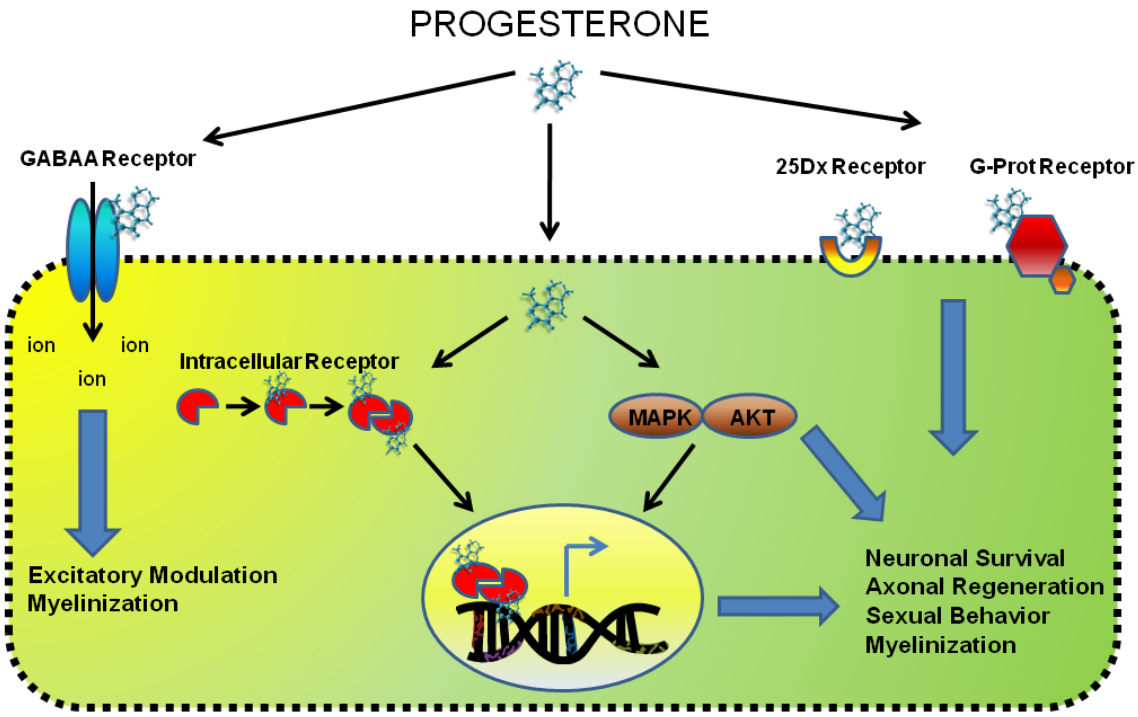

Figure I.3 – Neuroprotective actions of progesterone

I.2. KINASES AND PHOSPHATASES

I.2.1. PI3K/AKT SIGNALING

The phosphoinositide 3-kinase (PI3K) is member of a large family of PI3K-related kinases or PIKK, involved in cellular functions like cell growth, proliferation and survival. Another notable member of this family is the mammalian target of rapamycin (mTOR). All possess the characteristic PI3K homologous domain and a highly conserved carboxyl-terminal tail, however only PI3K is known to have an endogenous lipid substrate (Kuruvilla and Schreiber, 1999). PI3K is able to phosphorylate the 3 position hydroxyl group of the inositol ring of phosphatidylinositol (PtdIns), therefore these enzymes are also known as phosphatidylinositol-3 kinases. The PIK3 family is divided in three classes,

accordingly with its primary structure, regulation and *in vitro* lipid substrate specificity (Leevers et al., 1999). The majority of research on the PI3K has been focused on the Class I PI3K, that are heterodimeric molecules composed of a regulatory subunit known as p110 and catalytic subunit that can be related to either p85 or p101 (Songyang et al., 1993; Yoakim et al., 1994). The PI3K can be activated by G-protein coupled receptors and receptors of tyrosine kinase (RTK) (Leevers et al., 1999).

The serine threonine protein kinase Akt (also known as protein kinase B, PKB) mediates many of the effects downstream of the PI3K. Akt is an important protagonist in cell fate determination with many regulatory circuits operating in cell survival, migration, differentiation as well apoptosis (Kim and Chung, 2002; Song et al., 2005). Activation of Akt is a multistep process involving both membrane binding and phosphorylation. Upon PI3K activation and formation of phosphatydilinositol (3,4,5) – triphosphate (PtdnIns –P3 or PIP3), Akt is recruited to the plasma membrane where it binds to these phosphoinositides through its pH domain (Franke et al., 1997). Activation is then thought to involve a conformational change and two residues phosphorylation. One such phosphorylation site is contained in the kinase domain activation loop (Thr 308) and it is phosphorylated by another pH-domain containing protein, the 3-phosphoinositide dependent protein kinase 1 (PDK1) (Alessi, 2001). This is thought to be the major activating phosphorylation event. All in all, PI3K activation, induced by stimulation of growth factors, lead to Akt activation (Hooshmand-Rad et al., 2000; Thomas et al., 2002; Milburn et al., 2003). Conversely PI3K inactivation (i.e. using chemical inhibitors like wortmannin) leads to Akt inactivation. After activation, Akt can phosphorylate a variety of substrates involved in regulation of cellular key functions, like glycogen synthase kinase 3 (GSK3), Insulin Receptor Substrate-1 (IRS-1), BAD, human caspase 9, the mitogen stimulated Raf protein kinase, Forkhead and NF-KB transcription factors (Altiok et al., 1999; Datta et al., 1999; Galetic et al., 1999; Zhou et al., 2001).

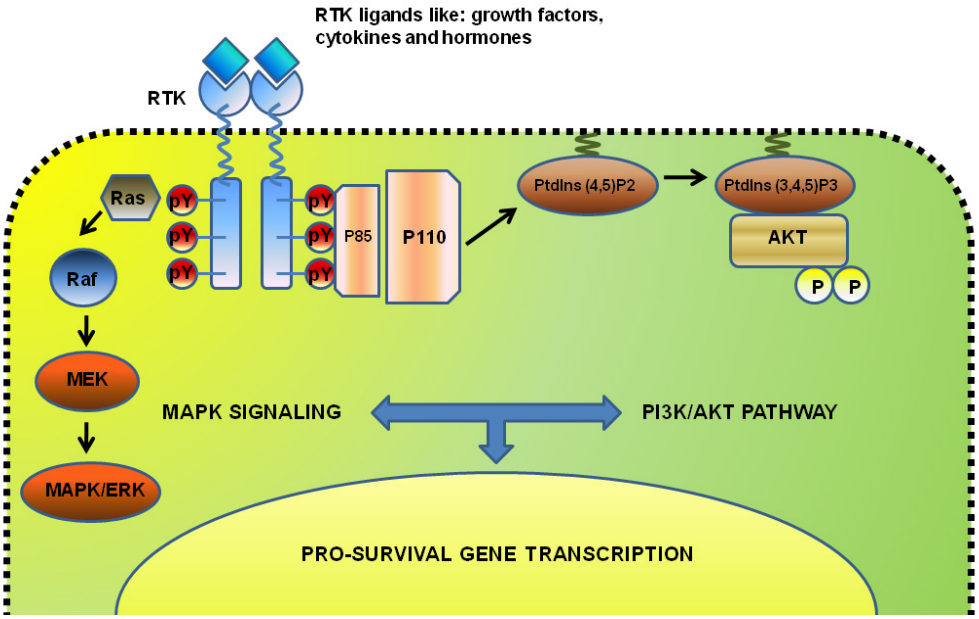

Figure I.4 – The PI3K/Akt signaling pathways

I.2.2. MAPK SIGNALING

Growth factors, through receptor tyrosine kinases, recruit cellular signaling proteins, to execute their cellular programs. The first of these signaling pathways to be discovered was the Ras-Raf-ERK signal transduction cascade, defined by extracellular-signal regulated kinases (ERKs) 1 and 2. (Seger and Krebs, 1995). The ERK cascade regulates cellular proliferation, differentiation and survival (Pagès et al., 1999). Also known as mitogen-activated protein kinases (MAPK) 3 and 1, the MAP kinases ERK1 and ERK2 are 44 and 42 KDa serine threonine kinases, respectively with 90% sequence identity in mammals. Initially isolated and cloned as kinases activated in response to insulin and nerve growth factor (NGF), ERK 1 and ERK2 are expressed in most tissues, with ERK2 levels generally higher than ERK1 (Boulton et al., 1990; Boulton et al., 1991). ERKs require both threonine and tyrosine phosphorylation, for full activity, and the finding that several phosphatases are able to inactivate ERKs, suggests that the duration and extent of activation is controlled by the balanced activites from MAPK/ERK kinases (MEKs or MKKs) and these phosphatases (Camps et al., 2000). Downstream, activated ERK regulates growth-factor responsive targets in the cytosol and also translocate to the nucleus where it phosphorylates several transcription factors, regulating gene expression. In fact

upon phosphorylation, ERK translocation to the nucleus is critical for both gene expression and DNA replication induced by growth factors (Brunet et al., 1999). Finally, cellular growth and proliferation require protein synthesis and the ERK cascade has been demonstrated to directly link growth-factor signaling to ribosome biogenesis (Stefanovsky et al., 2001).

I.2.3. PI3K/AKT AND MAPK SIGNALING IN THE CENTRAL NERVOUS SYSTEM

Many of the effects of neuroprotective compounds are mediated through the activation of the PI3K/Akt pathway and ERK signaling. For example, it has been shown that PI3K mediates the neuroprotective effects of estrogen in cultured cortical neurons (Honda et al., 2000). Furthermore, estrogen is able to attenuate ischemic oxidative damage in hippocampal neurons, in a process that involves Akt activation (Zhang et al., 2009). Insulin/PI3K signaling is able to protect dentate neurons from oxygen-glucose deprivation in organotypic slice cultures. (Sun et al., 2010). Astrocytes protect oligodendrocytes precursor cells via MEK/ERK and PI3K/Akt signaling (Arai and Lo, 2010) and stem cell factors protect against neuronal apoptosis by activating Akt/ERK in diabetic mice (Li et al., 2009).

Interestingly, inhibition of the receptor tyrosine kinases signaling inhibits the protective effects of progesterone against glutamate-induced toxicity in organotypic explants of the cerebral cortex (Kaur et al., 2007). Furthermore, the activation of PI3K and MAPK signaling are necessary for the manifestation of the neuroprotective effects of progesterone *in vitro* (Nilsen and Brinton, 2003; Kaur et al 2007; Brinton et al 2008). In fact, neuroprotective effects of progesterone against glutamate-induced toxicity in organotypic cultures of the cerebral cortex are inhibited by the MEK1/2 inhibitor UO126 (Kaur et al., 2007) and translocation of phosphorylated ERK to the cell nucleus appears to be an essential step in the neuroprotective mechanism of progesterone in primary hippocampal neurons (Nilsen and Brinton, 2003).

I.2.4. GSK3B AND TAU

Tau is a low molecular weight component of cytoskeletal structures and is known as one of the microtubule associated proteins (MAPs). Neuronal MAPs, which consist of tau and MAP2, regulate the assembly of microtubules (MTs). Although tau and MAP2 are thought to have similar functions, intracellular localization of tau largely differs from MAP2. The mRNAS encoding tau proteins are expressed predominantly in neurons, where tau proteins are localized mostly to axons of the central nervous system (CNS) and peripheral nervous system (PNS) under normal physiological conditions (Binder et al., 1985; Couchie et al., 1992). Tau is a phosphoprotein that can be phosphorylated at multiple sites, and under physiological conditions, changes in tau phosphorylation state are probably involved in the regulation of neuritic growth, synaptogenesis and synaptic plasticity. However, sustained phosphorylation of tau reduces its ability to bind and to stabilize microtubules, resulting in destabilization of the cytoskeleton and perturbation of axonal transport (Lindwall and Cole, 1984). Pathological hyperphosphorylation of Tau is associated to several neurodegenerative diseases (Buee et al., 2000; Avila et al., 2002, 2004). Several epitopes of phosphorylation of tau have been identified, like the PHF-1, that consists of the serine residues 396 and 404 (Otvos et al., 1994), the Tau-1 (Szendrei et al., 1993) and the serine 262 (Lauckner et al., 2003).

Glycogen synthase kinase 3 (GSK3) is a multifunctional serine/threonine protein kinase and key regulator of several cellular functions like glycogen metabolism, apoptosis, intracellular vesicular transport, transcription and cytoskeletal regulation. Two isoforms of GSK3 are reported in mammals: a 51 KDa GSK3a and a 47 KDa GSK3β. These two isoforms exhibit about 98% homology in their kinase domains, but only 36% homology in the last 36 aminoacid residues of the C-terminal (Woodgett, 1990; Cohen and Goedert, 2004). GSK3β is constitutively active in resting cells, and treatment of cells with an agent, such as insulin, is shown to cause GSK3β inactivation through a PI3K-dependent mechanism. PI3K induced activation of AKT results in phosphorylation of Ser9 on GSK3β which inhibits its activity (Cohen and Frame, 2001) Tau is one of the substrates of GSK3β (Ferrer et al., 2005), which is involved in the phosphorylation of Tau-1 and PHF-1 epitopes. GSK3β is highly expressed in the central nervous system (Takahashi et al., 1994), including the cerebellar cortex and the Purkinje cells of the cerebellum (Yao et al.,

2002).

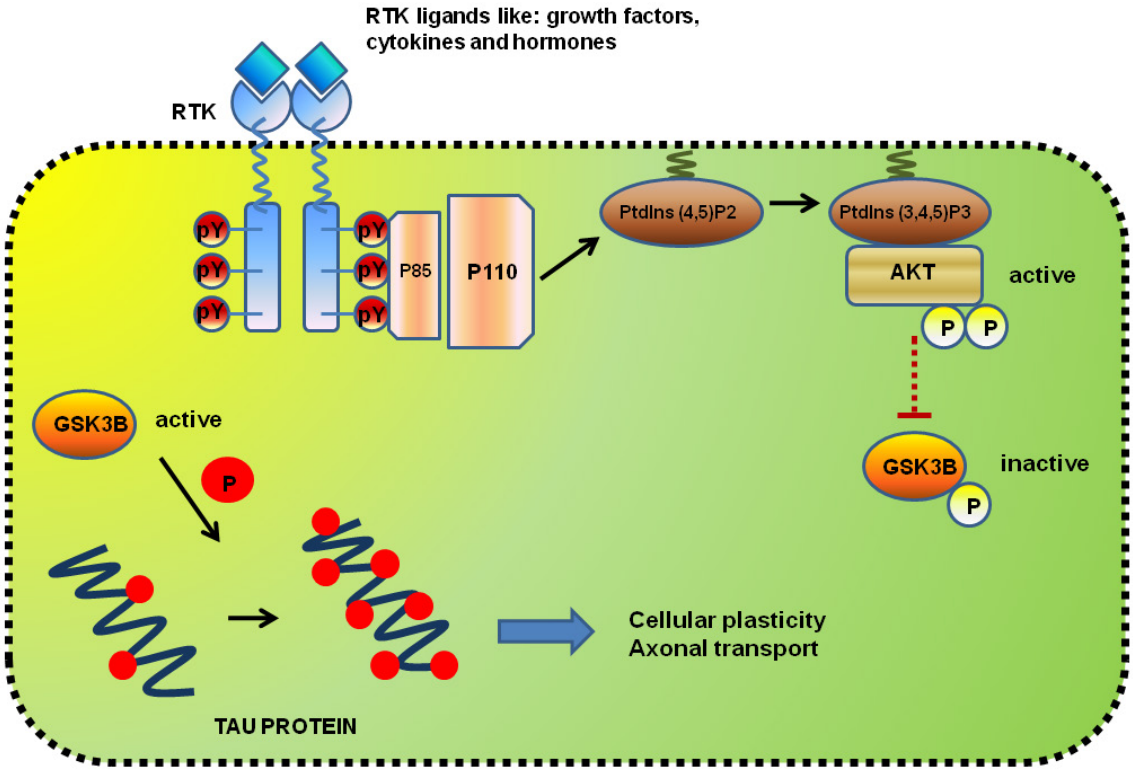

Figure I.5 – GSK3 and Tau signaling pathways

I.2.5. PROTEIN PHOSPHATASES: PP2A AND PTEN

PP2 or PP2A (Protein phosphatase 2) is a ubiquitous heterometric protein phosphatase. It is a well conserved serine/threonine phosphatase with broad substrate specificity and diverse cellular functions like DNA replication, translation, transcription, cell cycle division and apoptosis (Millward et al., 1999; Zolnierowicz, 2000; Janssens and Goris, 2001). For example, several of the targets of PP2A are proteins of signaling cascades such as MEK and AKT (Ory et al., 2003).The core enzyme consists of a catalytic C and a regulatory A, subunits (Janssens and Goris, 2001). In addition, there can be more regulatory subunits, that belong to four families of unrelated proteins, the regulatory subunits B, B'', B''' and B''''. These B regulatory subunits competitively bind to a shared binding site on the core A subunit (Janssens and Goris, 2001). The huge amount of holoenzyme components, especially B regulatory subunits, allows PP2A to exert several

functions. PP2A function can be regulated by expression, localization, set of subunits attached and post-translational modification. It has been reported that phosphorylation of PP2A at tyr307 occurs in response to epidermal growth factor (EGF) or insulin and results in substantial reduction of PP2A activity (Chen et al., 1992). Also, reversible methylation on the carboxyl group of Leu309 of PP2A has been observed (Turowski et al., 1995; Lee et al., 1996). Methylation alters the conformation of PP2A, as well its location and association with B regulatory subunits (Lee et al., 1996; Yu et al., 2001).

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a tumor suppressor gene (Li and Sun, 1997). The resulting protein is both a protein and lipid phosphatase (Vazquez and Sellers, 2000) and encompasses a phosphatase domain with homology to protein tyrosine phosphatases, dual specificity phosphatases and tensin and auxilin (Steck et al., 1997). This phosphatase is involved in the regulation of the cell cycle, preventing cells from growing and dividing too rapidly (Chu and Tarnawski, 2004). The lipid phosphatase activity of PTEN can dephosphorylate the PtdIns PIP2 and PIP3, the lipid products of PI3K lipid kinase activity (Maehama and Dixon, 1998). Thus PTEN antagonizes the classical PI3K/Akt signaling pathway. In fact, cells lacking PTEN function, exhibit two fold increase in PIP3 levels (Stambolic et al., 1998). PTEN has a C-terminal tail that contains a PDZ domain. PDZ domains are protein-protein interaction modules that play a critical role in the organization of diverse cell signaling complexes. The phosphorylation of three residues (S380, T382 and T383) within the C-terminal tail is necessary for maintaining PTEN stability and also acts inhibiting PTEN function (Georgescu et al., 2000; Vazquez et al., 2000). The unphosphorylated form of PTEN is in an “open” conformation” that allows PTEN recruitment to high molecular weight complexes (Vazquez et al., 2001). These complexes comprise PDZ-domain containing proteins and are important for PTEN localization to the plasma membrane (Wu et al., 2000; Vazquez et al., 2001) where it can exert its phospholipid phosphatase activity.

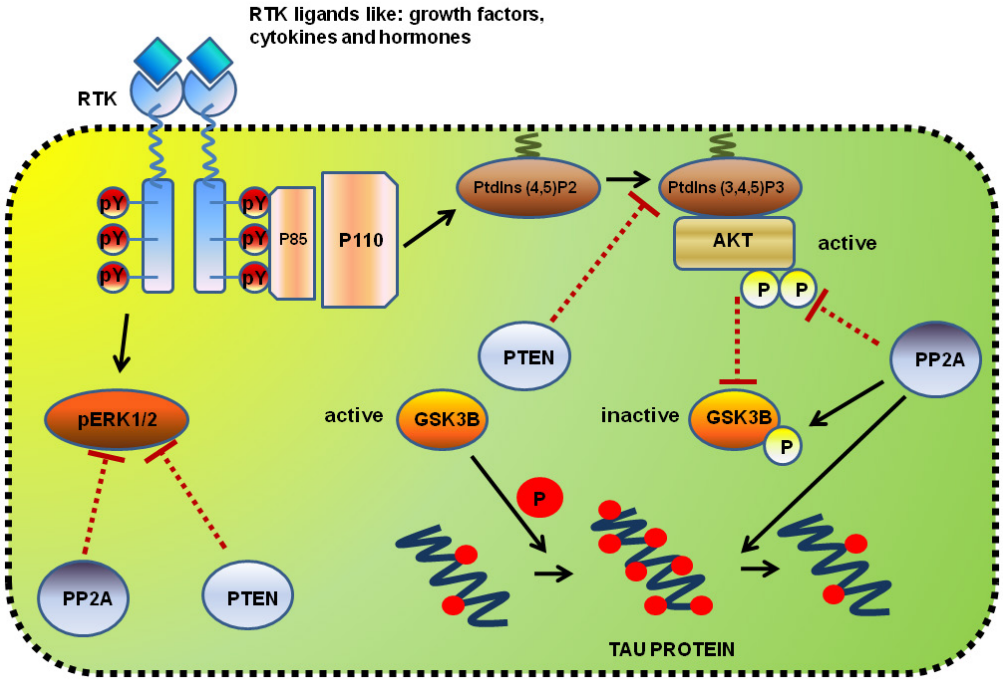

Figure I.6 – PP2A and PTEN signaling pathways

I.3. SPECIFIC AIMS

Our general aim was to analyze new signaling mechanism of progesterone in the central nervous system. In particular, our specific aims were:

1. To determine whether progesterone and its metabolites regulate MAPK and PI3K/Akt signaling in the central nervous system.
2. To determine whether progesterone and its metabolites regulate the state of phosphorylation of tau protein in the central nervous system, by the analysis of the phosphorylation of tau at epitopes Tau-1, PHF-1 and Ser262
3. To determine whether progesterone and its metabolites regulate the phosphorylation of GSK3 and therefore its activity in the central nervous system.
4. To determine whether progesterone and its metabolites regulate the phosphatases PP2A and PTEN expression and activity in the central nervous system.

CHAPTER II

REGULATION OF THE PHOSPHOINOSITIDE-3 KINASE AND MITOGEN-ACTIVATED PROTEIN KINASE SIGNALING PATHWAYS BY PROGESTERONE AND ITS REDUCED METABOLITES IN THE RAT BRAIN

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II - REGULATION OF THE PHOSPHOINOSITIDE-3 KINASE AND MITOGEN-ACTIVATED PROTEIN KINASE SIGNALING PATHWAYS BY PROGESTERONE AND ITS REDUCED METABOLITES IN THE RAT BRAIN

II.1 ABSTRACT

Several growth factors, such as vascular endothelial growth factor, brain derived neurotrophic factor and insulin-like growth factor-I are involved in the actions of progesterone in the central nervous system. Previous studies in neuronal and glial cultures have shown that progesterone may regulate growth factor signaling, increasing the phosphorylation of extracellular-signal regulated kinase (ERK) and the phosphorylation of Akt, components of the mitogen-activated protein kinase (MAPK) and the phosphoinositide-3 kinase (PI3K) signaling pathways, respectively. In this study we have evaluated whether progesterone and its reduced metabolites, dihydroprogesterone and tetrahydroprogesterone regulate PI3K and MAPK signaling in the brain of ovariectomized rats in vivo. Significant increases in the phosphorylation of ERK, in the expression of the catalytic (p110) and the regulatory (p85) subunits of PI3K and in the phosphorylation of Akt were observed in the hypothalamus, the hippocampus and the cerebellum, 24 h after progesterone administration. Progesterone metabolites partially mimicked the effect of progesterone and had a stronger effect on MAPK and PI3K signaling in the hypothalamus than in the other brain regions. These findings suggest that progesterone regulates MAPK and PI3K signaling pathways in the central nervous system in vivo by direct hormonal actions and by mechanisms involving progesterone metabolites.

II.2 INTRODUCTION

In addition to be a hormonal signal that acts in the brain to control neuroendocrine secretions and reproduction (Pfaff 1989; Etgen et al., 2006; Beyer, 2007), progesterone is a local paracrine factor synthesized by neural cells (Garcia-Segura and Melcangi, 2006). Furthermore, progesterone is metabolized in the nervous system to the neuroactive steroids dihydroprogesterone (DHP) and tetrahydroprogesterone (THP) via the enzymatic complex formed by the 5α-reductase and the 3α-hydroxysteroid dehydrogenase (Melcangi et al., 2008). Progesterone and progesterone metabolites regulate synaptic development, plasticity and function in the cerebellum (Smith et al., 1987a,b,c; Smith, 1989, 1991; Sakamoto et al., 2001, 2002; Tsutsui et al., 2004) the hypothalamus (Perez et al., 1993; Garcia-Segura et al., 1994) and the hippocampus (Woolley and McEwen, 1993), among other brain regions, and are involved in the differentiation of neurons and glial cells and in the formation of myelin (Baulieu and Schumacher, 2000; Schumacher et al., 2004; Tsutsui et al., 2004; Ghoumari et al., 2005; Brinton et al., 2008). Progesterone and its metabolites also affect adult neurogenesis in the hippocampus (Galea et al., 2006), modulate cognition and affection (Barbaccia et al., 2001; Vallee et al., 2001; Rupprecht, 2003; Birzniece et al., 2006; Frye, 2007; Brinton et al., 2008) and have neuroprotective and anti-inflammatory properties in different experimental models of neurodegeneration (Ganter et al., 1992; Garcia-Estrada et al., 1993; Drew and Chavis, 2000; Ciriza et al., 2004; Schumacher et al., 2004; Stein, 2005; Ciriza et al., 2006; Brinton et al., 2008). Progesterone and its metabolites have also anesthetic (Selye, 1941) and analgesic (Patte-Mensah et al., 2005) properties and influence sleep and feeding (Engel and Grant, 2001),

Growth factors, including vascular endothelial growth factor (VEGF) (Swiatek-De Lange et al., 2007), brain derived neurotrophic factor (BDNF) (Gonzalez et al., 2004, 2005; DeNicola et al., 2006; Gonzalez Deniselle et al., 2007; Kaur et al., 2007) and insulin/insulin-like growth factor-I (IGF-I) (Dueñas et al., 1994; Jung-Testas et al., 1994; Cardona-Gomez et al., 2000; Etgen, 2003; El-Bakri et al., 2004; Etgen et al., 2006) are involved in the mechanisms of action of progesterone in the central nervous system. Progesterone regulates the function of the retina by increasing the expression of VGEF by Muller glial cells (Swiatek-De Lange et al., 2007) and induces the expression of BDNF in ventral horn motoneurons from rats with spinal cord injury (Gonzalez et al., 2004, 2005;

DeNicola et al., 2006), in motoneurons of Wobbler mouse (Gonzalez Deniselle et al., 2007), a model of motoneuron degeneration, and in organotypic explants of the cerebral cortex (Kaur et al., 2007). IGF-I is involved in the regulation of gonadotropin release and sexual behavior by progesterone (Etgen, 2003; Etgen et al., 2006) and progesterone interacts with insulin in the induction of the expression of myelin basic protein by oligodendrocytes (Jung-Testas et al., 1994) and regulates IGF-I levels in the hypothalamus (Dueñas et al., 1994). Progesterone regulates also the expression of growth factor receptors, such as the IGF-I receptor (Cardona-Gomez et al., 2000; El-Bakri et al., 2004) and TrkB (DeNicola et al., 2006) in the central nervous system and inhibition of Trk signaling prevents the protective effects of progesterone against glutamate-induced toxicity in organotypic explants of the cerebral cortex (Kaur et al., 2007). In addition, progesterone regulates the intracellular signaling of growth factor receptors in vitro. In mouse cerebral cortical explants, progesterone increases the phosphorylation of Akt and the phosphorylation of extracellular-signal regulated kinase (ERK) (Singh, 2001), components of the phosphoinositide-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, respectively. In hippocampal neurons in culture progesterone promotes a rapid and transient activation of ERK and induces the translocation of phosphorylated ERK to the cell nucleus (Nilsen and Brinton, 2003). The activation of PI3K and MAPK signaling are necessary for the manifestation of the neuroprotective effects of progesterone in vitro (Nilsen and Brinton, 2003; Kaur et al., 2007; Brinton et al., 2008). Given the importance of growth factor signaling in the actions of progesterone in vitro, in this study we have explored whether progesterone affects PI3K and MAPK signaling in the brain in vivo and the possible role of progesterone metabolites in these effects.

II.3 MATERIALS AND METHODS

II.3.1 ANIMALS

Wistar albino female rats from our in-house colony were kept in a 12:12 h light-dark cycle and received food and water ad libitum. Animals were handled in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals, the principles presented in the Guidelines for the Use of Animals in Neuroscience Research by the Society for Neuroscience and following the European Union (86/609/EEC) legislation. Experimental procedures were approved by our Institutional Animal Use and Care Committee (Spanish National Research Council Animal Experimentation Committee). Special care was taken to minimize animal suffering and to reduce the number of animals used to the necessary minimum.

II.3.2 EXPERIMENTAL TREATMENTS

All the animals used in this study were bilaterally ovariectomized at the age of two months under 2,2,2-tribromoethanol anesthesia (0.2 g/kg body weight (b.w.), Fluka Chemika, Buchs, Switzerland). Rats were then housed in plastic cages and randomly assigned to the different treatments. Ten days after surgery, rats received one i.p. injection of progesterone (n=5) (2 mg/Kg b.w., Sigma, St. Louis, MO), DHP (n=5) (0.25 mg/kg b.w., Sigma), THP (n=5) (2 mg/kg b.w., Sigma) or vehicle (n=4) (0.2 ml of 20% [2-hydroxypropyl]-β-cyclodextrin, Fluka Chemika, Buchs, Switzerland). The selected doses of progesterone, DHP and THP were those showing optimal neuroprotective effects in previous studies in vivo (Ciriza et al., 2004, 2006) and result in physiological levels of these molecules in the plasma of ovariectomized rats (Ciriza et al., 2006). The animals were killed by decapitation 24 h after the administration of the steroids. The hippocampus, the hypothalamus and the cerebellum were quickly removed and immediately processed for protein extraction.

II.3.3 WESTERN BLOT ANALYSIS

Tissue samples were homogenized in lysis buffer (150 nM NaCl, 20 mM Tris-HCl, 10% glycerol, 5 mM EDTA, 1% NP-40, Roche, Mannhein, Germany) supplemented with protease and phosphatase inhibitors (50 μg/ml of phenyl methyl sulfonyl fluoride, 10 μg/ml aprotinin, 25 μg/ml leupeptin and 100 nM orthovanadate, all from Sigma). Proteins were obtained by centrifugation for 15 min at 15,000 rpm at 4°C and supernatant quantified by with a modified Bradford assay (BioRad, Munchen, Germany). Proteins (30 μg) were resolved using sodium dodecylsulphate-polyacrylamide gel electrophoresis (12% SDS-PAGE) with a Mini-Protean system (BioRad) and electrophoretically transferred to nitrocellulose membranes (GE Healthcare, formerly Amersham Bioscience, Piscataway, NJ, USA). The membranes were blocked with 5% non-fat dry milk diluted in 0.05% Tween-20 Tris-buffered saline and incubated overnight with the primary antibodies. Pre-stained markers (BioRad) were included for size determination.

II.3.4 ANTIBODIES

The following antibodies were used: rabbit polyclonal antibody against ERK-1/2 (Santa Cruz, diluted 1:1,000); mouse monoclonal antibody against phosphorylated ERK-1/2 (Santa Cruz, diluted 1:1,000); mouse monoclonal antibody against the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K) (Upstate, diluted 1:1,000); rabbit polyclonal antibody against the p110 catalytic subunit of PI3K (Santa Cruz, diluted 1:1,000); rabbit polyclonal antibody against Akt (Santa Cruz, diluted 1:1,000); rabbit polyclonal antibody against phosphorylated Akt (Cell Signaling, diluted 1:1,000); and mouse monoclonal antibody against anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, Chemicon, diluted 1:1,000). After incubation with the primary antibody, membranes were washed and incubated with horseradish peroxidase-coupled secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA; diluted 1:15,000). Immunoreactive bands were detected using an enhanced chemiluminescence system (GE Healthcare-Amersham). When needed, membranes were stripped using a commercial solution (Chemicon). Films were analyzed using the ImageQuant software version 3.22

(computing densitometer model 300A; Molecular Dynamics, Buckinghamshire, UK). The density of each band of different primary antibodies was normalized to its loading control (GAPDH). In order to minimize inter-assay variations, samples from all animals groups, in each experiment, were processed in parallel.

II.3.5 STATISTICAL ANALYSIS

Data were analyzed by using a one way analysis of variance (ANOVA) followed by a post hoc analysis with the Tukey's test. Prism 2.01 program (Graph Pad, CA) was used for calculating probability values. Values of $p<0.05$ were considered statistically significant.

II.4 RESULTS

II.4.1 ERK-1/2

Figure 1 shows examples of Western blots from total and phosphorylated ERK in the hypothalamus (Fig. 1A), the hippocampus (Fig. 1B) and the cerebellum (Fig. 1C).

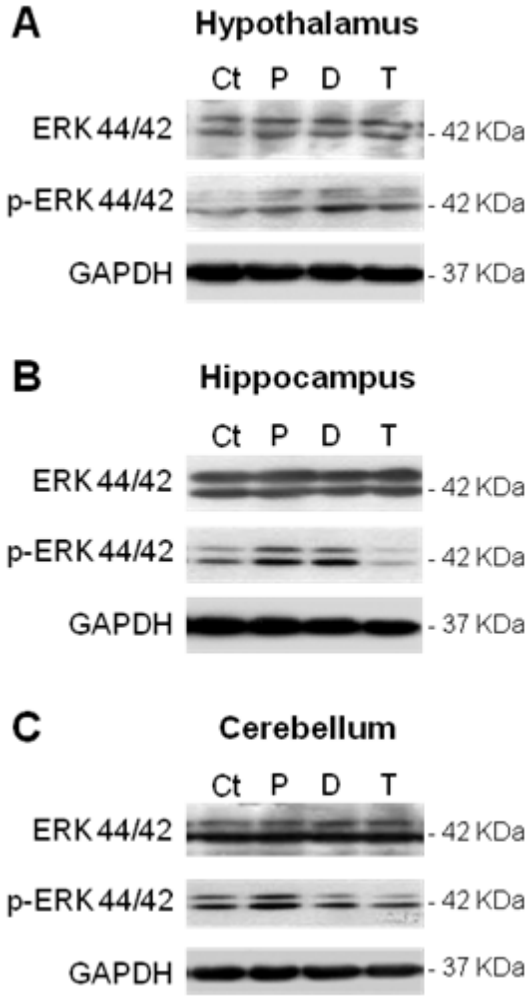

Figure II.1 - Examples of Western blots from (A), hypothalamic; (B), hippocampal and (C), cerebellar samples of ovariectomized rats treated with vehicle (Ct), progesterone (P), dihydroprogesterone (D) or tetrahydroprogesterone (T). The figure shows examples of bands immunodetected with a rabbit polyclonal antibody against ERK-1/2 (ERK 44/42), mouse monoclonal antibody against phosphorylated ERK-1/2 (p-ERK 44/42) and a mouse monoclonal antibody against anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), used as loading control.

The results of the densitometric analysis of the bands are shown in figures 2 to 4. The expression of ERK1/2 was unaffected by progesterone, DHP and THP in the three brain regions studied. In contrast, progesterone and its two metabolites, DHP and THP, significantly increased the phosphorylation of ERK-1 and ERK-2 in the hypothalamus (Fig. 2).

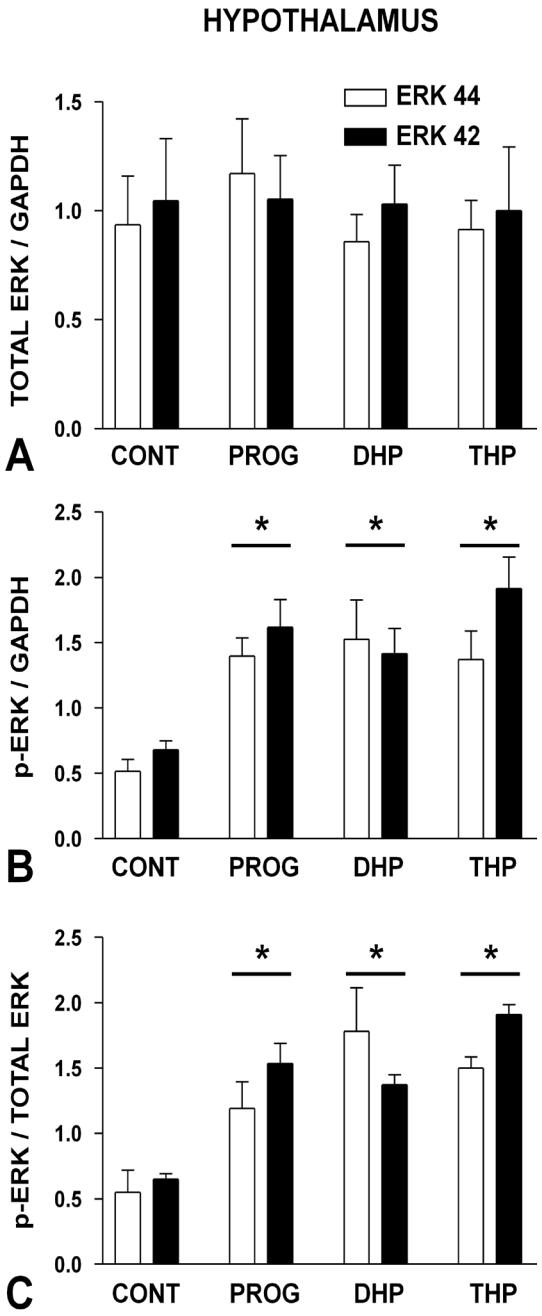

Figure II.2 - Effects of vehicle (CONT, n=4), progesterone (PROG, n=5), dihydroprogesterone (DHP, n=5) and tetrahydroprogesterone (THP, n=5) on the expression and the phosphorylation of ERK (ERK 44 and ERK 42) in the hypothalamus.

A) Values of total ERK normalized to GAPDH.

B) Values of phosphorylated ERK (p-ERK) normalized to GAPDH.

C) Values of phosphorylated ERK (p-ERK) normalized to total ERK.

Asterisk (*) - significant differences ($p<0.05$) versus the values of control rats.

Progesterone treatment resulted also in a significant increase in the phosphorylation of ERK-1 and ERK-2 in the hippocampus (Fig. 3). A similar effect was observed in the animals treated with DHP (Fig. 3). In contrast, the treatment with THP did not affect the phosphorylation of ERK-1/2 in the hippocampus (Fig. 3).

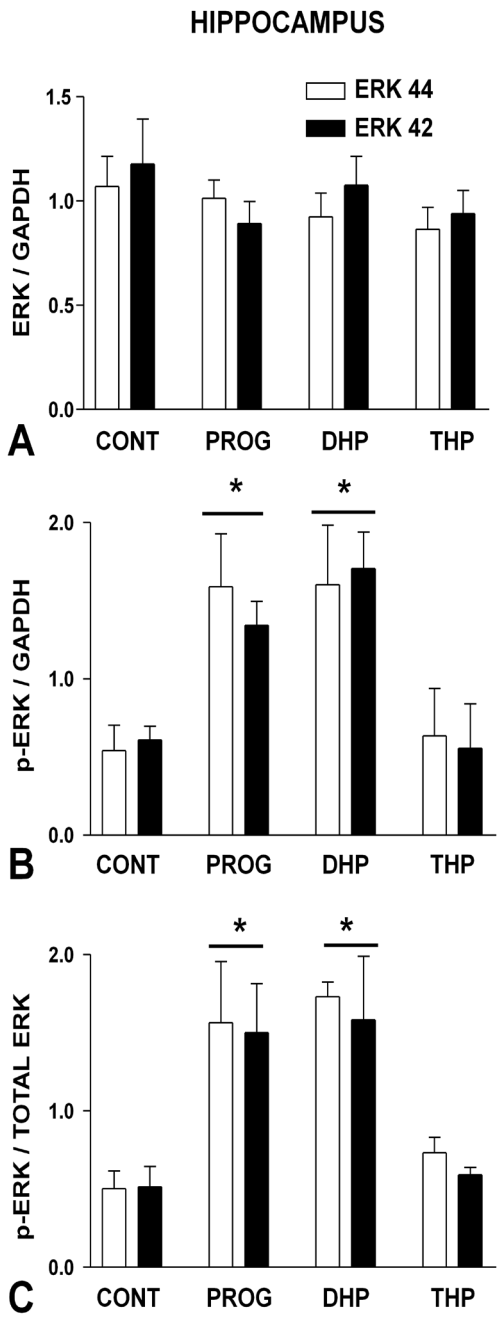

Figure II.3 - Effects of vehicle (CONT, n=4), progesterone (PROG, n=5), dihydroprogesterone (DHP, n=5) and tetrahydroprogesterone (THP, n=5) on the expression and the phosphorylation of ERK (ERK 44 and ERK 42) in the hippocampus.

A) Values of total ERK normalized to GAPDH.

B) Values of phosphorylated ERK (p-ERK) normalized to GAPDH.

C) Values of phosphorylated ERK (p-ERK) normalized to total ERK. **Asterisk (*)** - significant differences ($p<0.05$) versus the values of rats treated with vehicle or THP.

In the cerebellum, only progesterone resulted in a significant increase in the phosphorylation of ERK-1 and ERK-2 (Fig. 4).

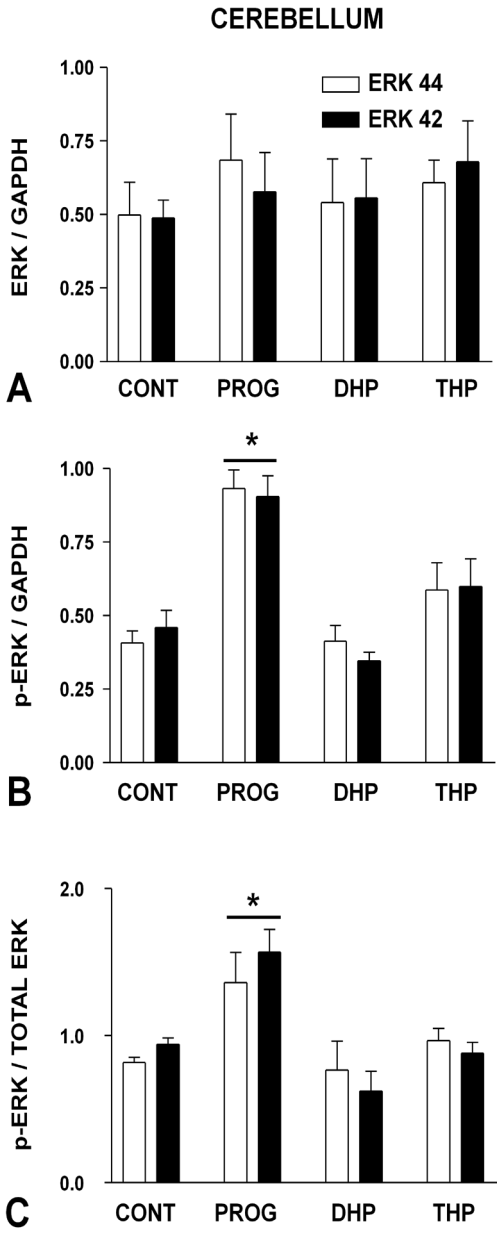

Figure II.4 - Effects of vehicle (CONT, n=4), progesterone (PROG, n=5), dihydroprogesterone (DHP, n=5) and tetrahydroprogesterone (THP, n=5) on the expression and the phosphorylation of ERK (ERK 44 and ERK 42) in the cerebellum.

A) Values of total ERK normalized to GAPDH.

B) Values of phosphorylated ERK (p-ERK) normalized to GAPDH.

C) Values of phosphorylated ERK (p-ERK) normalized to total ERK. **Asterisk (*)** - significant differences ($p<0.05$) versus the values of rats treated with vehicle, DHP or THP.

II.4.2 PI3K

Examples of Western blots for the subunits p110 and p85 of the PI3K in the hypothalamus, the hippocampus and the cerebellum are presented in figure 5.

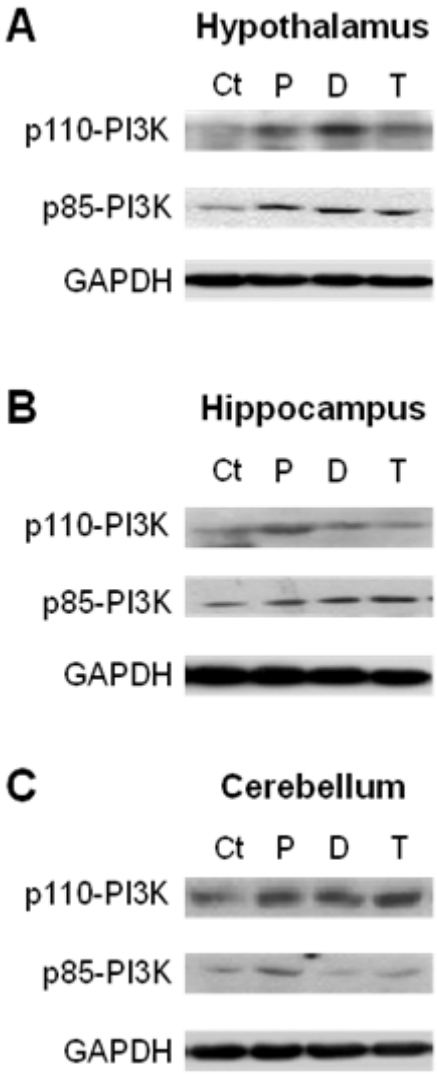

Figure II.5 - Examples of Western blots from (A), hypothalamic; (B), hippocampal and (C), cerebellar samples of ovariectomized rats treated with vehicle (Ct), progesterone (P), dihydroprogesterone (D) or tetrahydroprogesterone (T). The figure shows examples of bands immunodetected with a rabbit polyclonal antibody against the p110 catalytic subunit of PI3K (p110-PI3K), a mouse monoclonal antibody against the p85 regulatory subunit of PI3K (p85-PI3K) and a mouse monoclonal antibody against GAPDH, used as loading control.

The results of the densitometric analysis of the bands are shown in figures 6 and 7. Progesterone, DHP and THP induced a significant increase in the levels of p110-PI3K in the hypothalamus (Fig. 6A). The effect of DHP was significantly higher than the effect of THP (Fig. 6A). In the hippocampus progesterone treatment resulted in a significant increase in the levels of p110-PI3K (Fig. 6B). In contrast, DHP and THP did not affect the levels of p110-PI3K in this brain region. In the cerebellum, the levels of p110-PI3K showed a significant increase after the treatment with either progesterone, DHP or THP (Fig. 6C).

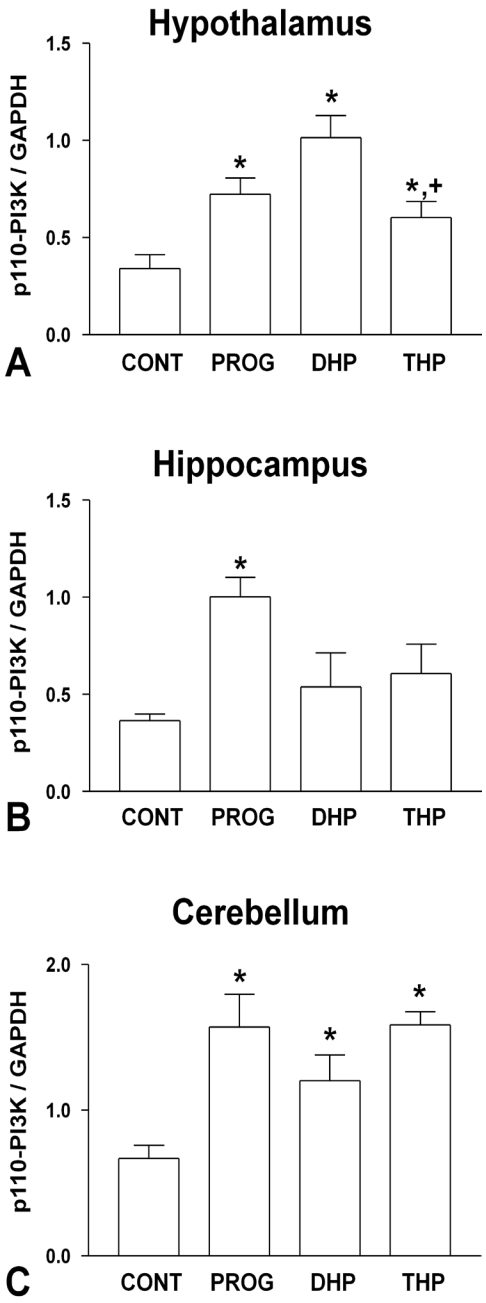

Figure II.6 - Effects of vehicle (CONT, n=4), progesterone (PROG, n=5), dihydroprogesterone (DHP, n=5) and tetrahydroprogesterone (THP, n=5) on the expression of p110-PI3K in the hypothalamus (A), the hippocampus (B) and the cerebellum (C). All values are normalized to GAPDH.

Asterisk (*) - significant differences ($p<0.05$) versus the values of control rats.

Plus (+) - significant difference ($p<0.05$) versus the value of the animals treated with DHP.

Treatment with progesterone also resulted in a significant increase in the expression of p85-PI3K in the hypothalamus, the hippocampus and the cerebellum (Fig. 7A, B, and C). In addition, progesterone metabolites increased the expression of p85-PI3K in the hypothalamus and the hippocampus, but not in the cerebellum (Fig. 7A, B and C).

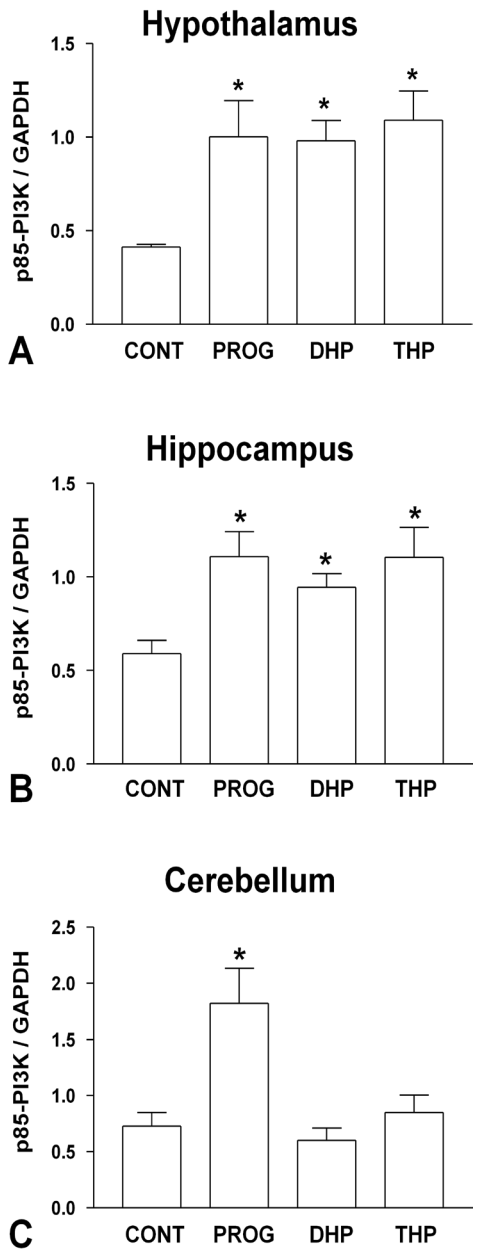

Figure II.7 - Effects of vehicle (CONT, n=4), progesterone (PROG, n=5), dihydroprogesterone (DHP, n=5) and tetrahydroprogesterone (THP, n=5) on the expression of p85-PI3K in the hypothalamus (A), the hippocampus (B) and the cerebellum (C). All values are normalized to GAPDH.

Asterisk (*) - significant differences ($p<0.05$) versus the values of control rats (in A and B) and versus the values of rats treated with vheicle, DHP or THP (in C).

II.4.3 AKT

The administration of progesterone also affected the phosphorylation of Akt in the hypothalamus, the hippocampus and the cerebellum. Examples of Western blots for total and phosphorylated Akt are shown in figure 8.

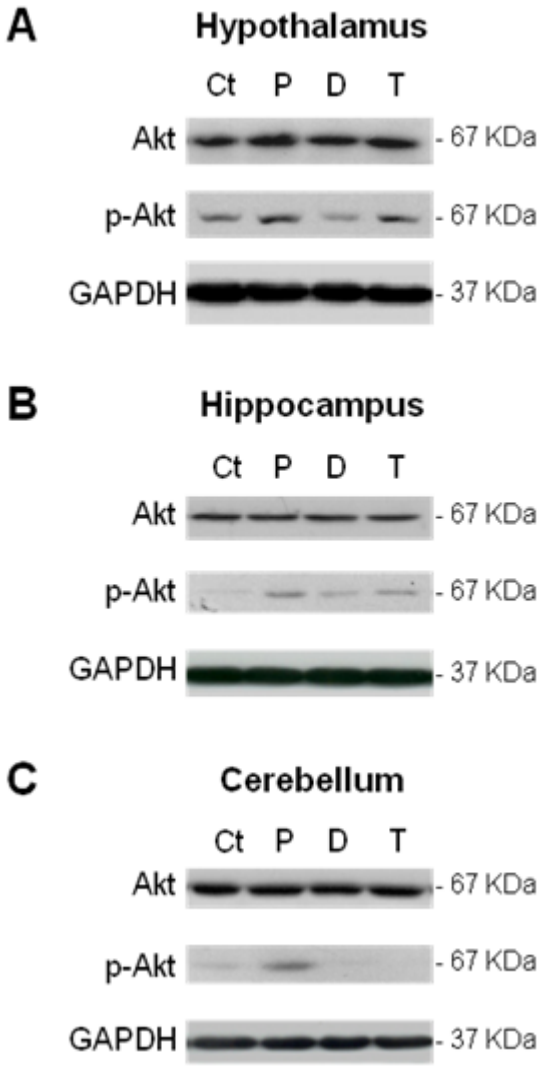

Figure II.8 - Examples of Western blots from (A), hypothalamic; (B), hippocampal and (C), cerebellar samples of ovariectomized rats treated with vehicle (Ct), progesterone (P), dihydroprogesterone (D) or tetrahydroprogesterone (T). The figure shows examples of bands immunodetected with a rabbit polyclonal antibody against Akt, a rabbit polyclonal antibody against phosphorylated Akt (p-Akt) and a mouse monoclonal antibody against GAPDH, used as loading control.

The densitometric data are shown in figures 9 to 11. As observed for basal ERK1/2 levels, neither progesterone nor its metabolites affected the basal levels of expression of Akt. However, both progesterone and THP resulted in a significant increase in the phosphorylation of Akt in the hypothalamus (Fig. 9).

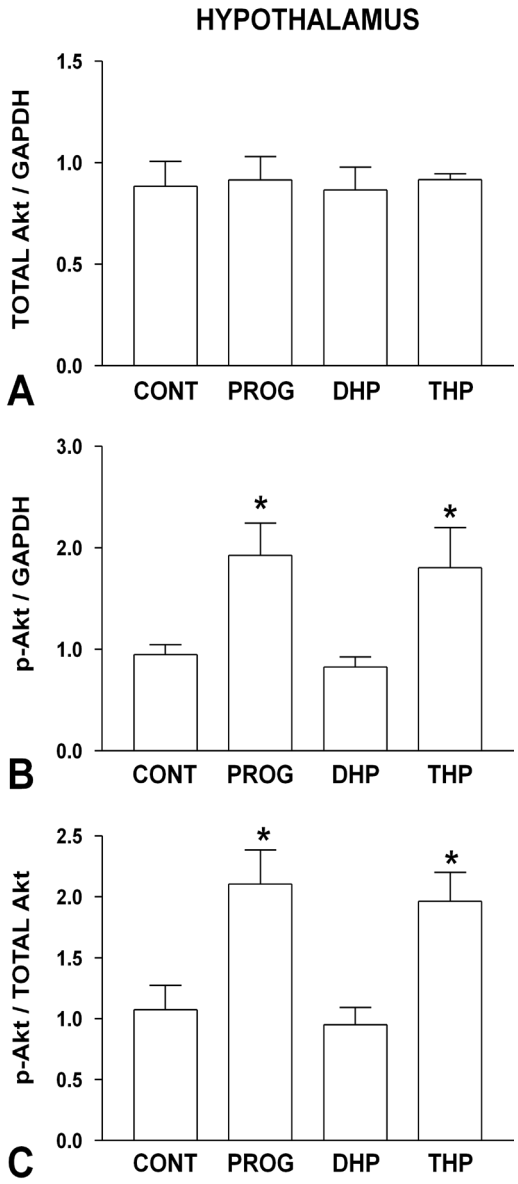

Figure II.9 - Effects of vehicle (CONT, n=4), progesterone (PROG, n=5), dihydroprogesterone (DHP, n=5) and tetrahydroprogesterone (THP, n=5) on the expression and the phosphorylation of Akt in the hypothalamus.

A) Values of total Akt normalized to GAPDH.

B) Values of phosphorylated Akt (p-Akt) normalized to GAPDH.

C) Values of phosphorylated Akt (p-Akt) normalized to total Akt.

Asterisk (*) - significant differences ($p<0.05$) versus the values of rats treated with vehicle or DHP.

The same behavior was observed in the hippocampus, with progesterone and THP treatment, increasing Akt phosphorylation. Again the basal levels of the total protein were unaffected with hormonal treatment (Fig.10).

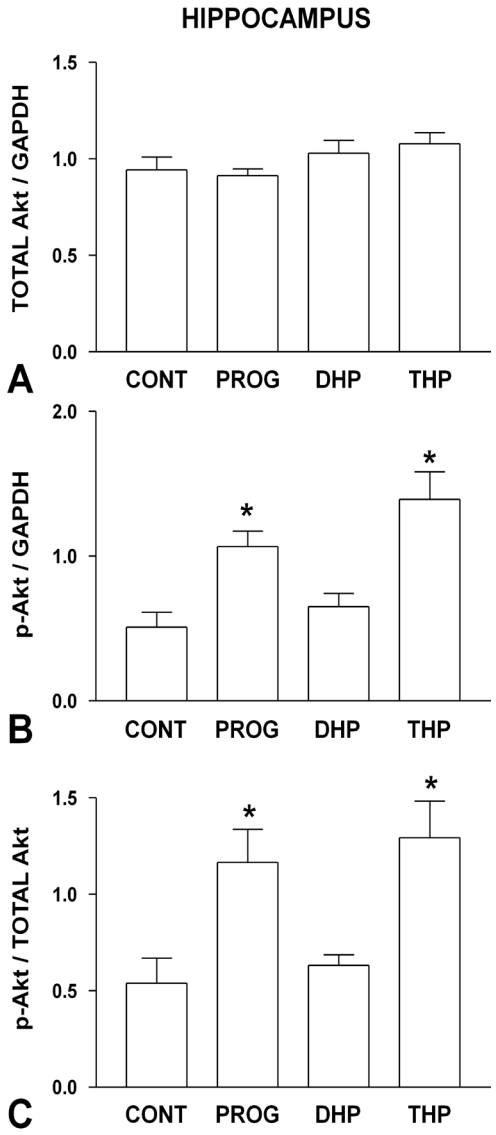

Figure II.10 - Effects of vehicle (CONT, n=4), progesterone (PROG, n=5), dihydroprogesterone (DHP, n=5) and tetrahydroprogesterone (THP, n=5) on the expression and the phosphorylation of Akt in the hippocampus.

A) Values of total Akt normalized to GAPDH.

B) Values of phosphorylated Akt (p-Akt) normalized to GAPDH.

C) Values of phosphorylated Akt (p-Akt) normalized to total Akt.

Asterisk (*) - significant differences ($p<0.05$) versus the values of rats treated with vehicle or DHP.

In contrast, only progesterone was able to increase the phosphorylation of Akt in the cerebellum. Again the basal levels of the total protein remained unaffected by hormonal treatment (Fig. 11).

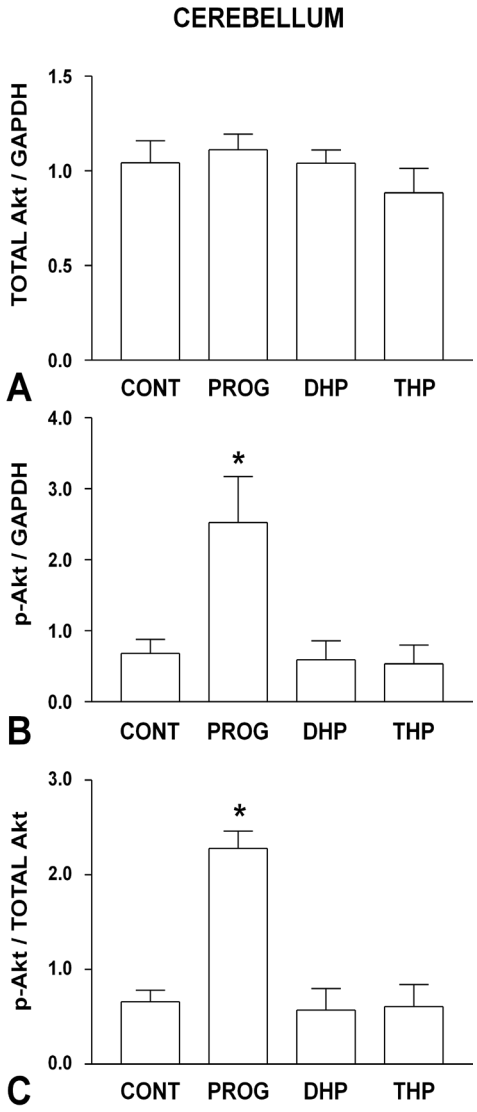

Figure II.11 - Effects of vehicle (CONT, n=4), progesterone (PROG, n=5), dihydroprogesterone (DHP, n=5) and tetrahydroprogesterone (THP, n=5) on the expression and the phosphorylation of Akt in the cerebellum.

A) Values of total Akt normalized to GAPDH.

B) Values of phosphorylated Akt (p-Akt) normalized to GAPDH.

C) Values of phosphorylated Akt (p-Akt) normalized to total Akt.

Asterisk (*) - significant differences ($p<0.05$) versus the values of rats treated with vehicle, DHP or THP.

II.5 DISCUSSION

The findings of the present study indicate that progesterone administration to young ovariectomized rats results in (i), an increased phosphorylation of ERK-1, ERK-2; (ii), an increased expression of the catalytic and regulatory subunits of PI3K and (iii), an increased phosphorylation of Akt. These effects were detected in the hippocampus, the hypothalamus and the cerebellum, assessed 24 h after the hormonal administration. Our results extend previous in vitro evidence of increased phosphorylation of ERK and/or Akt in cortical explants (Singh, 2001), primary hippocampal cultures (Nilsen and Brinton, 2003) and retinal glial cells (Swiatek-De Lange et al., 2007) treated with progesterone and suggest that the hormone may exert in the brain in vivo a sustained activation of the intracellular signaling of growth factor receptors, such as the IGF-I and BDNF (TrkB) receptors. In addition, our findings indicate that progesterone not only affects the phosphorylation of ERK and Akt, but also the expression of PI3K. Previous analyses have shown that the dose and pattern of administration of progesterone used in the present study results in plasma levels of the hormone that are within the ranges observed in diestrus and/or estrus rats and in hippocampal levels analogous to those observed during estrus (Ciriza et al., 2006). Therefore, the effects of progesterone on the phosphorylation of ERK-1, ERK-2 and Akt and on the expression of PI3K detected in the present study may represent a physiological action.

The finding that progesterone increases the phosphorylation of ERK-1, ERK-2 and Akt and the expression of PI3K in all brain regions studied, suggests that the MAPK and PI3K pathways are involved in widespread actions of the hormone through the brain. Given the important role of ERK 1/2 on the regulation of neuronal growth and survival, synaptic plasticity, learning and memory (Fukunaga and Miyamoto, 1998; Curtis and Finkbeiner, 1999; Grewal et al., 1999; Sweatt, 2001; Giovannini, 2006), it is conceivable that progesterone may affect these events in different brain regions by the activation of the MAPK signaling pathway. In fact, neuroprotective effects of progesterone against glutamate-induced toxicity in organotypic cultures of the cerebral cortex are inhibited by the MEK1/2 inhibitor UO126 (Kaur et al., 2007) and translocation of phosphorylated ERK to the cell nucleus appears to be an essential step in the neuroprotective mechanism of progesterone in primary hippocampal neurons (Nilsen and Brinton, 2003). In addition, the

MAPK pathway is involved in the effects of progesterone on sexual behavior, since the infusion of a MAPK inhibitor in the hypothalamus of estradiol-primed rats blocks progestin facilitation and sequential inhibition of lordosis and proceptive behaviors (Etgen et al., 2006). The PI3K signaling pathway is involved in the regulation of neuronal development, neuronal survival, synaptic plasticity, synaptic transmission and cognition (Skeberdis et al., 2001; Arimura and Kaibuchi, 2005; van der Heide et al., 2006; Zhao et al., 2006; Vetiska et al., 2007) and PI3K appears to mediate neuroprotective effects of progesterone in organotypic cultures of the cerebral cortex (Kaur et al., 2007). In the cerebellum, progesterone regulates the activity of glycogen synthase kinase-3 (GSK3) and the phosphorylation of the microtubule-associated protein Tau (Guerra-Araiza et al., 2007). These effects of progesterone, which could influence axonal transport and axonal growth and remodeling (Lindwall and Cole, 1984; Avila et al., 2004), may be in part mediated by the hormonal modulation of the PI3K/Akt signaling pathway, since Akt is upstream of GSK3, which, in turn, regulates the phosphorylation of Tau (Guerra-Araiza et al., 2007).

Progesterone is rapidly metabolized in the brain by the enzyme 5α-reductase into its reduced derivative dihydroprogesterone (DHP), which subsequently is further reduced to tetrahydroprogesterone (THP, also known as allopregnanolone) by the enzyme 3α-hydroxysteroid dehydrogenase (Garcia-Segura and Melcangi, 2006). Progesterone administration at the dose used in the present study results in a significant increase in the brain levels of DHP and THP (Ciriza et al., 2006). The reduced metabolites of progesterone mediate several of the effects of the hormone in the brain, including affective, cognitive and neuroprotective actions (Frye, 1995; Frank and Sagratella, 2000; Frye and Scalise, 2000; Ciriza et al., 2004; Djebaili et al., 2004; He et al., 2004; Rhodes and Frye, 2004; Rhodes et al., 2004; Djebaili et al., 2005; Ciriza et al., 2006). Our findings, showing that the reduced derivatives of progesterone affect the phosphorylation of ERK-1/2 and Akt and the expression of PI3K in some brain regions, suggest that progesterone metabolism may be involved in the hormonal effects on the MAPK and PI3K signaling pathways. Thus, DHP mimicked the effect of progesterone on the phosphorylation of ERK-1/2 in the hypothalamus and the hippocampus, while THP mimicked the effect of progesterone in the phosphorylation of ERK-1/2 in the hypothalamus (Fig. 12 A,B). However, none of the metabolites mimicked the effect of progesterone on the phosphorylation of ERK-1/2 in the cerebellum (Fig. 12 C). Concerning the PI3K pathway,

both progesterone metabolites increased the expression of p110-PI3K in the hypothalamus and cerebellum and the expression of p85-PI3K in the hypothalamus and the hippocampus (Fig. 12). However, DHP and THP did not affect the expression of p110-PI3K in the hippocampus and the expression of p85-PI3K in the cerebellum (Fig. 12).

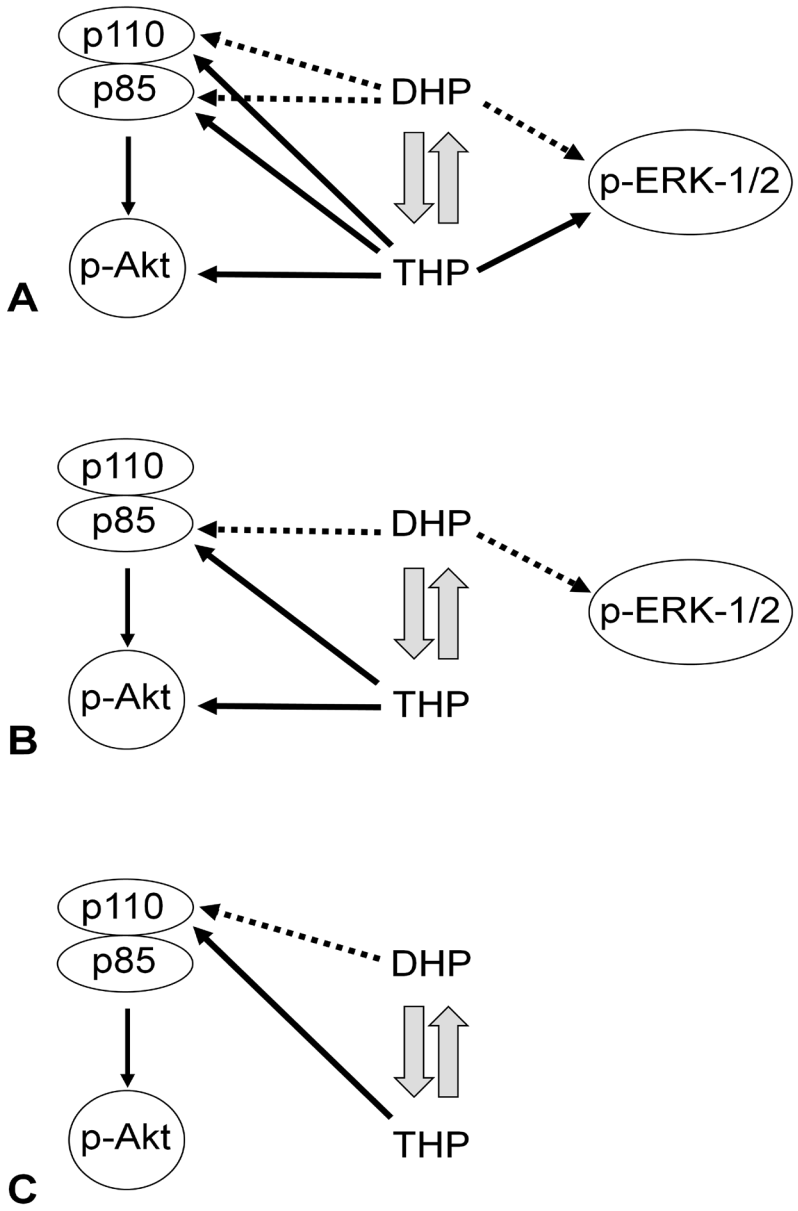

Figure II.12 - Summary of the effects of the metabolites of progesterone on the expression of p110-PI3K (p110) and p85-PI3K (p85) and on the phosphorylation of ERK (p-ERK-1/2) and Akt (p-Akt) in the hypothalamus (A), the hippocampus (B) and the cerebellum (C).

Furthermore, Akt phosphorylation was unaffected by DHP and was increased by THP in the hypothalamus and hippocampus but not in the cerebellum (Fig. 12). Therefore, DHP and THP did not fully mimic the effect of the hormone on MAPK and PI3K signaling pathways. Our findings do not exclude that progesterone metabolites may affect ERK and Akt phosphorylation in some brain regions at shorter times than progesterone does. However, our results suggest that acute administration of DHP and THP is not able to maintain a sustained activation of ERK and Akt in some brain regions, at least for a period of 24 h after the administration of the steroids. The functional significance of the regional differences in the effects of progesterone metabolites on the regulation of the MAPK and PI3K signaling pathways is unknown. However, it is of interest to note that progesterone metabolites seem to exert a stronger regulation of the MAPK and PI3K signaling pathways in the hypothalamus, a target for hormonal (Pfaff 1989; Ronnekleiv and Kelly, 2005; Etgen et al., 2006; Beyer, 2007) and paracrine (Micevych and Sinchak, 2008) reproductive actions of progesterone, than in the hippocampus and the cerebellum, brain regions in which non-reproductive actions of progesterone predominate.

Progesterone, DHP and THP act by complementary mechanisms in the brain. THP is an allosteric agonist of $GABA_A$ receptors (Puia et al., 1990; Majewska, 1992; Follesa et al., 2001; Lambert et al., 2003; Belelli and Lambert, 2005; Belelli et al., 2006) and may increase the phosphorylation of ERK-1/2 and Akt in the hypothalamus and the phosphorylation of Akt in the hippocampus via membrane effects. Brain regional differences in the subunit composition of $GABA_A$ receptors (Heldt and Ressler, 2007), which is known to affect the response to THP (Belelli and Lambert, 2005; Belelli et al., 2006), may explain the regional specific effects of THP on the MAPK and PI3K pathway. At difference to THP, progesterone and DHP bind to classical nuclear progesterone receptors (Rupprecht et al., 1993; Melcangi et al., 1999). Therefore, regulation of progesterone receptor mediated transcription may potentially be involved on the effects of DHP on the phosphorylation of ERK-1/2 in the hippocampus and the hypothalamus. Thus, the different regional effects of DHP and progesterone may be in part the consequence of the different regional expression of nuclear progesterone receptor isoforms within the brain (Guerra-Araiza et al., 2003). Furthermore, progesterone and DHP may also exert their effects by acting at the plasma membrane or the cytoplasm. Indeed, previous studies have shown that the classical progesterone receptor may be associated with p42 MAPK and

PI3K in Xenopus oocytes (Bagowski et al., 2001) and that a membrane-impermeable progesterone conjugate induces calcium influx and subsequent PI3K-mediated phosphorylation of PKC and ERK-1/2 in retinal Muller glial cells (Swiatek-De Lange et al., 2007). Classical progesterone receptors located at extranuclear sites are also involved in the regulation of the activity of the MAPK pathway (Boonyaratanakornkit et al., 2008). In addition, membrane effects may be mediated by the receptor 25-Dx (Krebs et al., 2000; Sakamoto et al., 2004; Meffre et al., 2005; Swiatek-De Lange et al., 2007; Brinton et al., 2008; Guennoun et al., 2008), a steroid binding protein that is also known as membrane-associated progesterone receptor component 1, ratp28 and inner zone antigen (Swiatek-De Lange et al., 2007). Another target of progesterone that is involved in rapid signaling mechanisms is the σ1 receptor, a cytoplasmic molecule expressed in the nervous system and that binds several steroids, including progesterone (Maurice et al., 1999; Alonso et al., 2000; Waterhouse et al., 2007). Actions of progesterone and its metabolites may also be mediated by a recently discovered family of membrane progesterone receptors (Zhu et al., 2003a,b; Hanna et al., 2006; Thomas, 2008). Regional differences in the expression of membrane progesterone receptors (Krebs et al., 2000; Guennoun et al., 2008) may also explain the regional differences in the effects of progesterone and its metabolites in the PI3K and MAPK pathways. In addition, since the enzyme 3α-hydroxysteroid dehydrogenase can either reduce DHP to THP or oxidize THP back to DHP (Garcia-Segura and Melcangi, 2006) differences in the inter-conversion of DHP and THP may also contribute to the regional specific effects of progesterone metabolites (Fig. 12). Finally, since both neurons and glial cells are targets for progesterone and its metabolites (Brinton et al., 2008; Guennoun et al., 2008; Melcangi et al., 2008), the final effect of these steroids on MAPK and PI3K signaling in the brain may result from the integration of actions elicited by different molecular mechanisms on different cell types.

The effects of progesterone and its metabolites on the MAPK and PI3K signaling pathways may have important functional implications for the regulation of the actions of growth factors in the brain. The interaction of progesterone and its metabolites with the intracellular signaling of growth factors, such as VEGF, BDNF and IGF-I, may modulate the actions of these factors on glial and neuronal development, physiological function and response to brain injury. In addition, progesterone and its metabolites may interact with other regulators of growth factor signaling, including the ovarian hormone estradiol.

Estradiol is known to regulate the activity of MAPK and PI3K signaling pathways in the brain (Etgen and Acosta-Martinez, 2003; Marin et al., 2005; Mannella and Brinton, 2006) and to affect the actions of different growth factors, such as nerve growth factor, basic fibroblast growth factor, glial cell line-derived neurotrophic factor, BDNF and IGF-I (Etgen and Garcia-Segura, 2009). Therefore, acting on MAPK and PI3K, progesterone and estradiol may potentially interact in the regulation of growth factor signaling in the brain and this may be part of the mechanisms involved in the coordinated regulation of brain function, brain plasticity, neuroendocrine and behavioral events by ovarian hormones.

In conclusion, our findings indicate that physiological levels of progesterone increase the phosphorylation of ERK-1/2 and Akt and the expression of the p85 regulatory subunit and of the p110 catalytic subunit of PI3K in all brain regions explored in ovariectomized rats: the hypothalamus, the hippocampus and the cerebellum. These results suggest that activation of MAPK and PI3K signaling is involved in the effects of progesterone in the central nervous system in vivo. Furthermore, progesterone metabolites exert a regulation of the MAPK and PI3K signaling with regional specificity, with marked differences between the hypothalamus, a target for the reproductive actions of progesterone and the other brain regions. This suggests that the final regulatory action of progesterone on MAPK and PI3K signaling in the brain is the result of a combination of mechanism directly activated by progesterone and mechanisms activated by its metabolites.

CHAPTER III

EFFECTS OF PROGESTERONE AND ITS REDUCED METABOLITES, DIHYDROPROGESTERONE AND TETRAHYDROPROGESTERONE, ON THE EXPRESSION AND PHOSPHORYLATION OF GLYCOGEN SYNTHASE KINASE-3 AND THE MICROTUBULE-ASSOCIATED PROTEIN TAU IN THE RAT CEREBELLUM

Christian Guerra-Araiza[1,2], Miguel A.R. Amorim[1], Ignacio Camacho-Arroyo[2]and Luis M. Garcia-Segura[1] (2007). Effects of progesterone and its reduced metabolites, dihydroprogesterone and tetrahydroprogesterone, on the expression and phosphorylation of glycogen synthase kinase-3 and the microtubule-associated protein Tau in the rat cerebellum. Dev Neurobiol. 67:510-20.

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III - EFFECTS OF PROGESTERONE AND ITS REDUCED METABOLITES, DIHYDROPROGESTERONE AND TETRAHYDROPROGESTERONE, ON THE EXPRESSION AND PHOSPHORYLATION OF GLYCOGEN SYNTHASE KINASE-3 AND THE MICROTUBULE-ASSOCIATED PROTEIN TAU IN THE RAT CEREBELLUM

III.1 ABSTRACT

Progesterone exerts a variety of actions in the brain, where it is rapidly metabolized to 5α-dihydroprogesterone (DHP) and 3α,5α-tetrahydroprogesterone (THP). The effect of progesterone and its metabolites on the expression and phosphorylation of the microtubule-associated protein Tau and glycogen synthase kinase-3β (GSK3β), a kinase involved in Tau phosphorylation, were assessed in two progesterone-sensitive brain areas: the hypothalamus and the cerebellum. Administration of progesterone, DHP and THP to ovariectomized rats did not affect Tau and GSK3β assessed in whole hypothalamic homogenates. In contrast, progesterone and its metabolites resulted in a significant decrease in the expression of Tau and GSK3β in the cerebellum. Furthermore, progesterone administration resulted in an increase in the phosphorylation of two epitopes of Tau (Tau-1 and PHF-1) phosphorylated by GSK3β, but did not affect the phosphorylation of an epitope of Tau (Ser262) that is GSK3β insensitive. These effects were accompanied by a decrease in the phosphorylation of GSK3β in serine, which is associated to an increase in its activity, suggesting that the effect of progesterone on Tau-1 and PHF-1 phosphorylation in the cerebellum is mediated by GSK3β. The regulation of Tau expression and phosphorylation by progesterone may contribute to the hormonal regulation of cerebellar function by the modification of neuronal cytoskeleton.

III.2 INTRODUCTION

Tau is a microtubule-associated protein that regulates the assembly and stabilization of neuronal and glial microtubules and influences axonal growth, axonal shape and microtubule-associated axonal transport (Buee et al., 2000; Avila et al., 2002, 2004). Under physiological conditions, changes in the phosphorylation of Tau are probably involved in the regulation of neuritic growth, synaptogenesis and synaptic plasticity. However, sustained phosphorylation of Tau reduces its ability to bind and to stabilize microtubules, resulting in destabilization of the cytoskeleton and perturbation of axonal transport (Lindwall and Cole, 1984). Pathological hyperphosphorylation of Tau is associated to several neurodegenerative diseases (Buee et al., 2000; Avila et al., 2002, 2004).

Gonadal hormones promote axonal growth and neuronal plasticity (Garcia-Segura et al., 1994; Parducz et al., 2006) and the regulation of Tau expression and phosphorylation may be involved in these hormonal actions. Estradiol increases the expression of Tau in primary neuronal cultures (Ferreira and Caceres, 1991; Alvarez-de-la-Rosa et al., 2005) an effect that is associated to an increase in axonal growth (Ferreira and Caceres, 1991). In addition, estradiol decreases Tau phosphorylation in primary cortical and hippocampal neurons, in hippocampal slice cultures and in the hippocampus in vivo (Cardona-Gomez et al., 2004; Alvarez-de-la-Rosa et al., 2005; Goodenough et al., 2005). All these findings suggest that estradiol may affect axonal growth and synaptic plasticity regulating Tau expression and phosphorylation. Furthermore, estradiol decreases total and phosphorylated Tau in the entorhinal cortex of Ts65Dn mice, an animal model of Down's syndrome (Hunter et al., 2004), suggesting that the hormone may also decrease the pathological hyperphosphorylation of Tau that is associated to several neurodegenerative diseases (Buee et al., 2000; Avila et al., 2002, 2004). Testosterone also decreases the hyperphosphorylation of Tau induced by heat shock in the rat brain (Papasozomenos, 1997; Papasozomenos and Papasozomenos, 1999; Papasozomenos and Shanavas, 2002). While these data indicate that testosterone and estradiol may affect the expression and/or the activity of Tau, it is unknown whether progesterone, another neuroactive gonadal steroid may exert similar effects.

The effects of progesterone in the central nervous system are not limited to the control of neuroendocrine regulation and reproduction (Baulieu and Scumacher, 2000;

Birzniece et al., 2006). Progesterone regulates synaptic function (Smith et al., 1987a), promotes neuronal and glial differentiation (Tsutsui et al., 2004; Ghoumari et al., 2005) and has neuroprotective properties in different experimental models of neurodegeneration (Schumacher et al., 2004; Stein, 2005; De Nicola et al., 2006). In the central nervous system, progesterone is rapidly metabolized to 5α-dihydroprogesterone (DHP) by the enzyme 5α-reductase, and DHP is further reduced to 3α,5α-tetrahydroprogesterone (THP) by the enzyme 3α-hydroxysteroid dehydrogenase (Stoffel-Wagner et al., 1998; Melcangi et al., 2001; Mellon et al., 2001). Progesterone metabolites are involved in the effects of progesterone in the central nervous system (Melcangi et al., 2001; Giachino et al., 2003: Rhodes et al., 2004; Matsumoto et al., 2005; Patte-Mensah et al., 2005; Ciriza et al., 2006).

In this study we have assessed whether progesterone and its metabolites DHP and THP may affect Tau expression and phosphorylation in the rat brain. We have selected two different brain areas for analysis: the hypothalamus, a classical target for progesterone neuroendocrine and reproductive actions (Ronnekleiv and Kelly, 2005) and the cerebellum a brain area involved in motor control that is also affected by the hormone (Smith et al., 1987a,c; Smith, 1989, 1991; Sakamoto et al., 2001, 2002; Tsutsui et al., 2004). Since glycogen synthase kinase-3β (GSK3β) is one of the kinases that regulate Tau phosphorylation (Ferrer et al., 2005) and appears to be involved in the effects of testosterone and estrogen on Tau phosphorylation in the rat brain (Papasozomenos and Shanavas, 2002; Cardona-Gomez et al., 2004), we have also assessed the effects of progesterone and its metabolites on the phosphorylation of this kinase.

III.3 MATERIAL AND METHODS

III.3.1 ANIMALS

Wistar albino female rats from our in-house colony were kept in a 12:12 h light-dark cycle and received food and water ad libitum. Animals were handled in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals, the principles presented in the Guidelines for the Use of Animals in Neuroscience Research by the Society for Neuroscience and following the European Union (86/609/EEC) legislation. Experimental procedures were approved by our Institutional Animal Use and Care Committee (Spanish National Research Council Animal Experimentation Committee). Special care was taken to minimize animal suffering and to reduce the number of animals used to the necessary minimum. Two month-old rats were bilaterally ovariectomized under 2,2,2-tribromoethanol anesthesia (0.2 g/kg body weight (b.w.), Fluka Chemika, Buchs, Switzerland). Rats were then housed in plastic cages and randomly assigned to the different treatments.

III.3.2 EXPERIMENTAL TREATMENTS

Ten days after surgery, rats received one i.p. injection of progesterone (n=5) (2 mg/kg b.w., Sigma, St. Louis, MO), DHP (n=5) (0.25 mg/kg b.w., Sigma), THP (n=5) (2 mg/kg b.w., Sigma) or vehicle (n=4) (0.2 ml of 20% [2-hydroxypropyl]-β-cyclodextrin, Fluka Chemika, Buchs, Switzerland). These doses of progesterone, DHP and THP result in physiological levels of these molecules in the plasma of ovariectomized rats (Ciriza et al., 2006). The animals were killed by decapitation 24 h after the administration of the steroids. The cerebellum and the whole hypothalamus, limited by the optic chiasm and the caudal portion of the mammillary bodies, were quickly removed and immediately processed for protein extraction.

III.3.3 WESTERN BLOT ANALYSIS

Hypothalamic and cerebellar samples were homogenized in lysis buffer (150 nM NaCl, 20 mM Tris-HCl, 10% glycerol, 5 mM EDTA, 1% NP-40, Roche, Mannhein, Germany) supplemented with protease and phosphatase inhibitors (50 μg/ml of phenyl methyl sulfonyl fluoride, 10 μg/ml aprotinin, 25 μg/ml leupeptin and 100 nM orthovanadate, all from Sigma). Proteins were obtained by centrifugation for 15 min at 15,000 rpm at 4°C and supernatant quantified by with a modified Bradford assay (BioRad, Munchen, Germany). Proteins (30 μg) were resolved using sodium dodecylsulphate-polyacrylamide gel electrophoresis (12% SDS-PAGE) with a Mini-Protean system (BioRad) and electrophoretically transferred to nitrocellulose membranes (GE Healthcare, formerly Amersham Bioscience, Piscataway, NJ, USA). The membranes were blocked with 5% non-fat dry milk diluted in 0.05% Tween-20 Tris-buffered saline and incubated overnight with the primary antibodies (diluted 1:1000). Pre-stained broad range markers (BioRad) were included for size determination.

III.3.4 ANTIBODIES

The following antibodies were used: rabbit anti-Tau polyclonal antibody (H-150, Santa Cruz Biotechnology, Santa Cruz, CA), which recognizes six Tau isoforms of 46-80 kDa molecular weight; rabbit polyclonal antibody that recognizes phosphorylation in serine 262 of Tau (p-Tau Ser 262, Santa Cruz Biotechnology); mouse monoclonal antibody that recognized the anti-dephosphorylated Tau-1 epitope of Tau (TAU-1, Chemicon, Temecula, CA); rabbit polyclonal antibody that recognizes phosphorylation in the PHF-1 epitope of Tau (a gift of Dr. Peter Davies, Albert Einstein College of Medicine, Bronx, NY, USA); mouse anti-GSK3β monoclonal antibody (BD Biosciences Pharmingen, San Diego, CA); mouse anti-phosphorylated GSK3β monoclonal antibody ($pSer^9$ GSK3β, Sigma), mouse anti-βIII tubulin monoclonal antibody (Promega, Madison, WI) and mouse anti- glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, Chemicon). After incubation with the primary antibody, membranes were washed and incubated with horseradish peroxidase-coupled secondary antibodies (Jackson ImmunoResearch Laboratories Inc.,

West Grove, PA; diluted 1:15,000). Immunoreactive bands were detected using an enhanced chemiluminescence system (GE Healthcare-Amersham). When needed, membranes were stripped using a commercial solution (Chemicon). Films were analyzed using the ImageQuant software version 3.22 (computing densitometer model 300A; Molecular Dynamics, Buckinghamshire, UK). The density of each band of different primary antibodies was normalized to its respective loading control. Two different molecules were used as loading controls: βIII tubulin and GAPDH. Identical results were obtained with both controls. The numerical data presented in the figures correspond to values normalized with GAPDH. In order to minimize inter-assay variations, samples from all animals groups, in each experiment, were processed in parallel.

III.3.5 STATISTICAL ANALYSIS

Data were analysed by using a one way analysis of variance (ANOVA) followed by a post hoc analysis with the Tukey's test. Prism 2.01 program (Graph Pad, CA) was used for calculating probability values. Values of $p<0.05$ were considered statistically significant.

III.4 RESULTS

III.4.1 TAU

Figure 1 shows examples of Western blots from hypothalamic samples. The expression of total Tau and the amount of phosphorylated Tau at epitopes Ser262, Tau-1 and PHF-1 were unaffected by progesterone, DHP and THP in the hypothalamus.

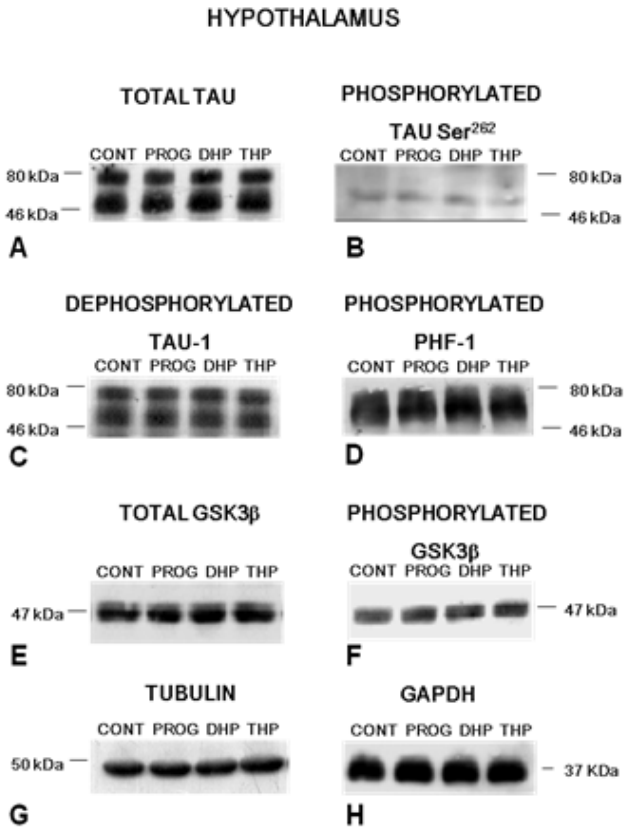

Figure III.1 - Examples of Western blots from hypothalamic samples of ovariectomized rats treated with progesterone (PROG), dihydroprogesterone (DHP), tetrahydroprogesterone (THP) or vehicle (CONT). The figure shows examples of bands immunodetected with the following antibodies: A, anti-Tau antibody; B, antibody that recognizes phosphorylation in serine 262 of Tau; C, antibody that recognized the anti-dephosphorylated Tau-1 epitope of Tau; D, antibody that recognizes phosphorylation in the PHF-1 epitope of Tau; E, anti-GSK3β antibody; F, antibody that recognizes GSK3β phosphorylated in serine 9; G, antibody that recognizes βIII tubulin and H, antibody that recognizes GAPDH. βIII tubulin and GAPDH were used as loading controls. The two bands shown in panels A and C are specific and were both included in the densitometric analysis.

In contrast, progesterone treatment resulted in a significant decrease in the expression of Tau in the cerebellum (Figs. 2 and 3A). This effect was also observed after the administration of DHP and THP (Figs. 2 and 3A). The amount of phosphorylated Tau at epitope Ser262 was not affected by the steroid treatments in the cerebellum (Fig. 2).

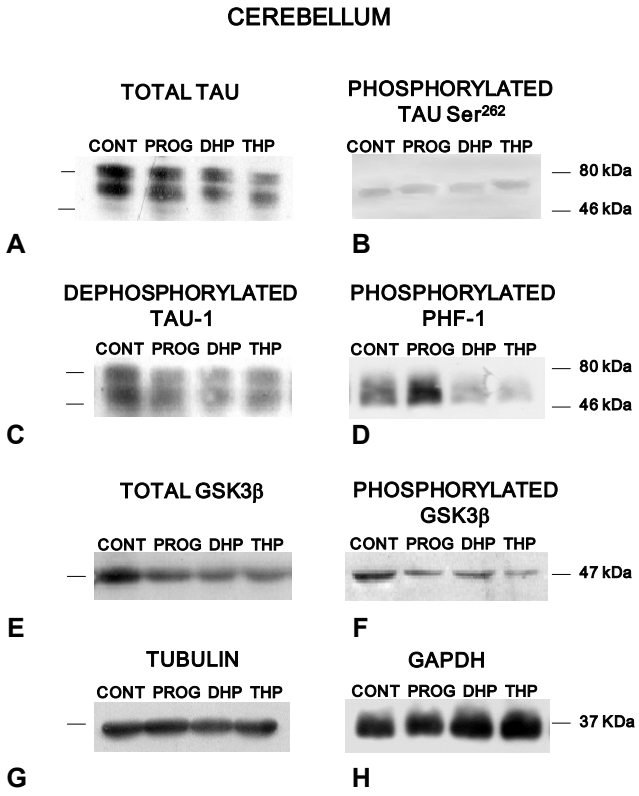

Figure III.2 - Examples of Western blots from cerebellar samples of ovariectomized rats treated with progesterone (PROG), dihydroprogesterone (DHP), tetrahydroprogesterone (THP) or vehicle (CONT). The figure shows examples of bands immunodetected with the following antibodies: A, anti-Tau antibody; B, antibody that recognizes phosphorylation in serine 262 of Tau; C, antibody that recognized the anti-dephosphorylated Tau-1 epitope of Tau; D, antibody that recognizes phosphorylation in the PHF-1 epitope of Tau; E, anti-GSK3β antibody; F, antibody that recognizes GSK3β phosphorylated in serine 9; G, antibody that recognizes βIII tubulin and H, antibody that recognizes GAPDH. βIII tubulin and GAPDH were used as loading controls. The two bands shown in panels A and C are specific and were both included in the densitometric analysis.

However, the amount of Tau dephosphorylated at the Tau-1 epitope in the cerebellum was decreased after the treatment with progesterone, DHP and THP (Figs. 2 and 3B). Furthermore, the ratio of Tau dephosphorylated at the Tau-1 epitope versus total Tau showed a significant decrease in the cerebellum of rats treated with progesterone (Fig. 3C).

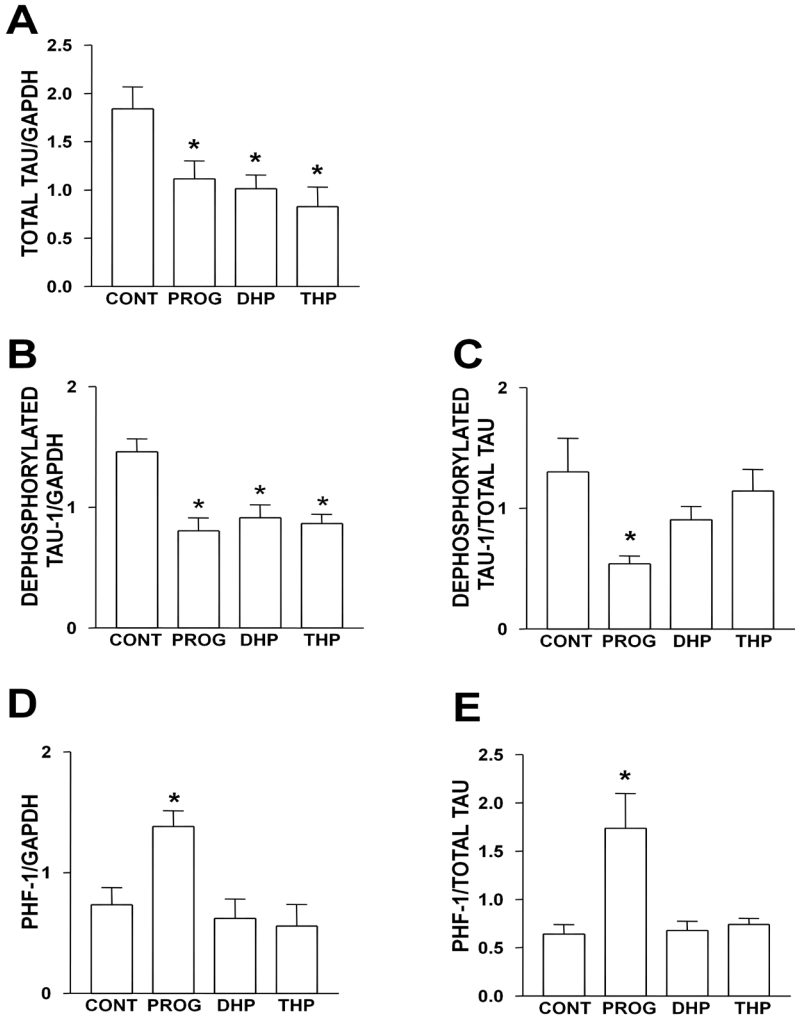

Figure III.3 - Effects of progesterone (PROG, n=5), dihydroprogesterone (DHP, n=5) and tetrahydroprogesterone (THP, n=5) on the expression of Tau and on its phosphorylation at epitopes Tau-1 and PHF-1 in the cerebellum of ovariectomized rats. A, levels of total Tau; B, dephosphorylated Tau-1 epitope; C, the ratio of dephosphorylated Tau-1 epitope versus total Tau; D, phosphorylation of the PHF-1 epitope; E, the ratio of phosphorylated PHF-1 epitope versus total Tau. Control rats (CONT, n=4) were treated with vehicle. Data were normalized to GADPH values and are expressed as the mean±SEM. Asterisk, significant differences ($p<0.05$) versus the values of control rats.

This ratio was not affected by DHP or THP (Fig. 3C), indicating that the effects of these metabolites were limited to the expression of Tau while progesterone regulated both the expression of Tau and its phosphorylation at the Tau-1 epitope. Progesterone also increased the phosphorylation of Tau at the PHF-1 epitope (Fig. 3D) and the ratio of phosphorylation of Tau at the PHF-1 epitope versus total Tau (Fig. 3E). DHP and THP did not affect phosphorylation at the PHF-1 epitope.

IV.4.2 GSK3β

GSK3β is the major kinase that regulates Tau phosphorylation. Tau-1 and PHF-1 epitopes of Tau are phosphorylated by GSK3β. In contrast, GSK3β does not affect the phosphorylation of Ser262 epitope. Therefore, the expression of GSK3β and the levels of phosphorylated GSK3β were assessed to determine whether changes in Tau-1 and PHF-1 phosphorylation were associated to modifications in the phosphorylation of GSK3β.

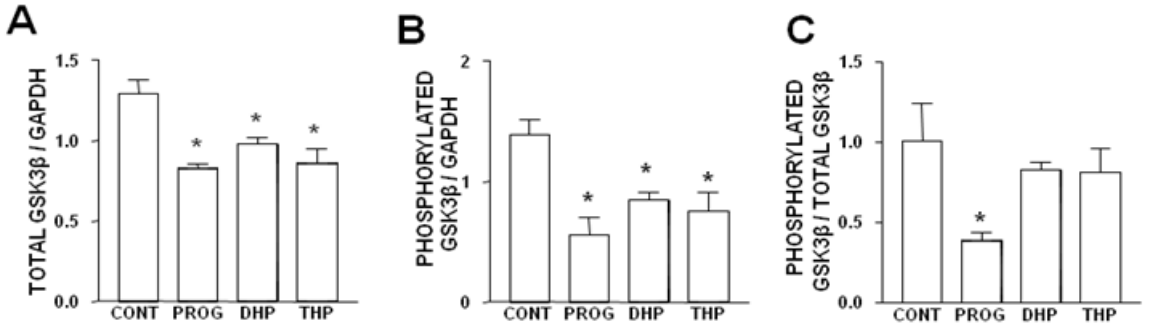

Figure III.4 - Effects of progesterone (PROG, n=5), dihydroprogesterone (DHP, n=5) and tetrahydroprogesterone (THP, n=5) on the expression of GSK3β and on its phosphorylation at serine 9 in the cerebellum of ovariectomized rats. A, levels of total GSK3β; B, phosphorylated GSK3β at serine 9; D, ratio of phosphorylated versus total GSK3β. Control rats (CONT, n=4) were treated with vehicle. Data were normalized to GADPH values and are expressed as the mean±SEM. Asterisk, significant differences ($p<0.05$) versus the values of control rats.

As it was observed for Tau, the expression and the phosphorylation of GSK3β were not affected in the hypothalamus by the treatments with progesterone, DHP or THP (Fig. 1). In contrast, progesterone, DHP and THP resulted in a significant decrease in the

expression of GSK3β in the cerebellum (Figs. 2 and 4A). This effect was associated to a significant decrease in the levels of phosphorylated GSK3β in serine in the animals treated with these steroids (Figs. 2 and 4B). The ratio of phosphorylated versus total GSK3β was significantly decreased in the cerebellum of the animals treated with progesterone and was not affected by the treatments with DHP and THP (Fig. 4C). Therefore, as it was observed for Tau, the effects of DHP and THP were limited to the expression of GSK3β while progesterone regulated both the expression and the phosphorylation of this molecule.

III.5 DISCUSSION

Morphological and electrophysiological actions of progesterone have been characterized in Purkinje cells of the cerebellar cortex. Progesterone administration significantly enhances inhibitory responses of Purkinje cells to GABA and suppressed glutamate excitation (Smith et al., 1987a,c; Smith, 1989, 1991) an effect that may be in part mediated by progesterone metabolites (Smith et al., 1987b; Follesa et al., 2000). Furthermore, progesterone promotes dose-dependent outgrowth of Purkinje cell dendrites and dendritic spines, and an increase in the density of Purkinje spine synapses during cerebellar development (Sakamoto et al., 2001, 2002; Tsutsui et al., 2004). In addition, progesterone may affect cerebellar glial cells and increases myelin basic protein expression by cerebellar oligodendrocytes (Ghoumari et al., 2003) and the proliferation of cerebellar oligodendrocyte precursors (Ghoumari et al., 2005).

Our findings, showing that progesterone treatment results in a decrease in the expression of Tau in the cerebellum, extend the results of previous studies showing that this brain area is a target for progesterone. The effect of progesterone on Tau expression seems to have anatomical specificity, since it was not observed in the hypothalamus and previous studies have shown that progesterone does not alter Tau expression in the hippocampus (Reyna-Neyra et al., 2002). In addition, progesterone administration resulted in an increase in the phosphorylation of Tau, at the Tau-1 and PHF-1 epitopes, in the cerebellum. Once more, this effect was not observed in the hypothalamus. These data suggest that progesterone may have specific effects in Tau expression and phosphorylation in the cerebellum. However, it cannot be excluded that progesterone might affect Tau

expression and/or phosphorylation in specific regions of the hypothalamus and that these possible regional changes would be undetectable by the analysis of homogenates from the whole hypothalamus. Thus, the differences between hypothalamus and cerebellum may be quantitative rather than qualitative. In addition, many of the progesterone effects on the hypothalamus require prior estrogen treatment, which was not done in the present experiments carried out in estrogen-depleted female rats.

The different effects of progesterone in the cerebellum and the hypothalamus may be in part due to differences in the expression of progesterone receptors. Classical progesterone receptors are expressed in the hypothalamus (Warembourg, 1978; Warembourg et al., 1986; Olster and Blaustein, 1990; Guerra-Araiza et al., 2001, 2002; Lonstein and Blaustein, 2004) and the cerebellum (Kato et al., 1993, 1994; Sakamoto et al., 2001; Curran-Rauhut and Petersen, 2002; Guerra-Araiza et al., 2001, 2002; Sakamoto et al., 2003). However, the expression of the isoforms A (PR-A) and B (PR-B) of the progesterone receptor differs between these two brain areas, at least in male rats, where in adults PR-A predominates in the hypothalamus and PR-B predominates in the cerebellum (Guerra-Araiza et al., 2001). Another target of progesterone that is expressed at different levels in the hypothalamus and cerebellum is the σ1 receptor, a cytoplasmic molecule involved in rapid signaling that is antagonized by progesterone (Maurice et al., 1999; Alonso et al., 2000). The effects of progesterone may also be mediated by 25-Dx, a putative membrane progesterone receptor expressed in the hypothalamus and cerebellum (Krebs et al., 2000; Sakamoto et al., 2004; Meffre et al., 2005) or by mPRβ, a membrane protein expressed in the brain that is homologous to a fish membrane progestin receptor (Zhu et al., 2003a).

The effect of progesterone on the phosphorylation of the Tau-1 and PHF-1 epitopes in the cerebellum was not observed when another Tau epitope, Ser 262, was assessed. Tau is one of the substrates of GSK3β (Ferrer et al., 2005), which is involved in the phosphorylation of Tau-1 and PHF-1 epitopes, while the phosphorylation of the Ser262 epitope, involved in Tau binding to microtubules (Biernat et al., 1993), is GSK3β insensitive. GSK3β is highly expressed in the central nervous system (Takahashi et al., 1994), including the cerebellar cortex and the Purkinje cells of the cerebellum (Yao et al., 2002). Therefore, one of the possible mediators involved in the effect of progesterone on cerebellar Tau phosphorylation at the Tau-1 and PHF-1 epitopes is GSK3β. Our findings

indicate that the increase in Tau-1 and PHF-1 phosphorylation in the cerebellum as a result of progesterone administration is accompanied by a decrease in the phosphorylation of GSK3β in serine, which is associated to an increase in its activity (Cohen and Frame, 2001). In contrast, in the hypothalamus, where progesterone was unable to affect Tau-1 and PHF-1 phosphorylation, GSK3β phosphorylation was also unaffected by the hormone. Therefore, our findings suggest that progesterone increases Tau-1 and PHF-1 phosphorylation in the cerebellum by decreasing the phosphorylation of GSK3β in serine.

Progesterone metabolism may be involved in the effects of the hormone on Tau and GSK3β. Progesterone is converted within the central nervous system to DHP, by the enzyme 5α-reductase and DHP is metabolized to THP by the enzyme 3α-hydroxysteroid dehydrogenase. DHP is a ligand of progesterone receptors and THP an allosteric agonist of $GABA_A$ receptors (Puia et al., 1990; Majewska, 1992; Melcangi et al., 2001; Lambert et al., 2003). Progesterone metabolites may in part mediate the effect of progesterone on the expression of Tau and GSK3β in the cerebellum since both DHP and THP had similar effects to progesterone on these parameters and since the enzymes 5α-reductase and 3α-hydroxysteroid dehydrogenase are expressed by Purkinje cells (Agis-Balboa et al., 2006). However, only progesterone regulated the phosphorylation of Tau and GSK3β, suggesting that the final effect of progesterone on these molecules may be the result of a combination of effects directly mediated by progesterone and effects mediated by its metabolites. Progesterone and DHP may regulate the expression of Tau and GSK3β in the cerebellum acting on progesterone receptors. According to this hypothesis, the effect of THP may be explained by a retro-conversion into DHP by the reversible enzyme 3α-hydroxysteroid dehydrogenase. Alternatively, progesterone may be metabolized to DHP and this steroid in THP. Then, THP may modulate the expression of Tau and GSK3β by a mechanism mediated by its interaction with the $GABA_A$ receptor. Thus, both progesterone receptors and $GABA_A$ receptors may potentially mediate the effects of progesterone, DHP and THP on the expression of Tau and GSK3β. However, another mechanism should be responsible for the action of progesterone on the phosphorylation of Tau and GSK3β, since in this case the lack of the effect of DHP and THP seem to discard the involvement of progesterone and $GABA_A$ receptors, respectively. Therefore, alternative mechanisms of action of progesterone, including the interaction with putative membrane steroid receptors, such as 25-Dx or mPRβ, may be postulated for the effects of the hormone on the phosphorylation

of Tau and GSK3β in the cerebellum.

The effect of progesterone on the expression and phosphorylation of GSK3β and Tau may be highly relevant for cerebellar function. Progesterone may affect GSK3β and Tau by paracrine or autocrine actions, since the rat cerebellum expresses cytochrome P450 side-chain cleavage (P450scc), the enzyme that transforms cholesterol in pregnenolone (Sanne and Krueger, 1995; Ukena et al., 1998; Tsutsui et al., 2004; Lavaque et al., 2006) and 3beta-hydroxysteroid dehydrogenase/delta5-delta4-isomerase (3betaHSD), the enzyme that transforms pregnenolone in progesterone (Guennoun et al., 1995; Ukena et al., 1999). Locally produced or hormonal progesterone may affect the cytoskeleton in cerebellar cells by regulating Tau expression and phosphorylation, since Tau regulates the dynamic instability of microtubules, its growth and bundling (Paglini et al., 2000). In addition, Tau is involved in the regulation of vesicle transport, the interaction of microtubules with the plasma membrane and the intracellular localization of proteins such fyn, 14-3-3 and protein phosphatase 2A (Stoothoff and Johnson, 2005). By its actions on Tau, progesterone may alter the function of cerebellar neurons and oligodendrocytes, since both cell types express Tau (LoPresti et al., 1995; Stoothoff and Johnson, 2005) and both cell types are a target for the actions of the steroid (Tsutsui et al., 2004; Ghoumari et al., 2005). The expression and subcellular distribution of Tau in cerebellar neurons in culture is highly correlated with the morphological development of axons (Paglini et al., 2000) and cerebellar neurons failed to extend axon-like processes when Tau expression is blocked (Caceres and Kosik, 1990). Furthermore, one of the most important alterations detected in the brain of Tau-deficient mice is a reduction in the number and density of microtubules in cerebellar parallel fibers (Harada et al., 1994). Progesterone may increase phosphorylation of Tau-1 and PHF-1 in the cerebellum by decreasing GSK3β phosphorylation, which results in an increased activity of the kinase. In turn, phosphorylation of Tau by GSK3β alters Tau distribution between axon and cell body, reduces its binding to microtubules, promotes its aggregation and increases its susceptibility to proteolysis. All these changes could influence axonal transport and axonal growth and remodeling in cerebellar cells (Lindwall and Cole, 1984; Avila et al., 2004) and may contribute to the physiological regulation of synaptic cerebellar function by progesterone (Tsutsui, 2006).

CHAPTER IV

PROGESTERONE REGULATES THE PHOSPHORYLATION OF PROTEIN PHOSPHATASES IN THE BRAIN

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IV- PROGESTERONE REGULATES THE PHOSPHORYLATION OF PROTEIN PHOSPHATASES IN THE BRAIN

IV.1 ABSTRACT

Previous studies have shown that progesterone modulates the activity of different kinases and the phosphorylation of Tau in the brain. These actions of progesterone may be involved in the hormonal regulation of neuronal differentiation, neuronal function, and neuroprotection. However, the action of progesterone on protein phosphatases in the nervous system has not been explored previously. In this study we have assessed the effect of the administration of progesterone to adult ovariectomized rats on protein phosphatase 2A (PP2A) and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) in the hypothalamus, the hippocampus, and the cerebellum. Total levels of PP2A, the state of methylation of PP2A, and total levels of PTEN were unaffected by the hormone in the three brain regions studied. In contrast, progesterone significantly increased the levels of PP2A phosphorylated in tyrosine 307 in the hippocampus and the cerebellum and significantly decreased the levels of PTEN phosphorylated in serine 380 in the hypothalamus and in the hippocampus compared with control values. Estradiol priming blocked the effect of progesterone on PP2A phosphorylation in the hippocampus and on PTEN phosphorylation in the hypothalamus and the hippocampus. In contrast, the action of progesterone on PP2A phosphorylation in the cerebellum was not modified by estradiol priming. These findings suggest that the regulation of the phosphorylation of PP2A and PTEN may be involved in the effects of progesterone on the phosphorylation of Tau and on the activity of phophoinositide-3 kinase and mitogen-activated protein kinase in the brain.

IV.2 INTRODUCTION

Progesterone acts in the nervous system as a hormone and as a locally produced neuroactive steroid (Melcangi et al., 2008; Micevych and Sinchak, 2008). Neural actions of progesterone participate in a variety of events, including the control of reproduction (Micevych and Sinchak, 2008; Mani and Portillo, 2010), the regulation of neuronal and glial development and function (Melcangi et al., 2008; Tsutsui, 2008), the release of neurotransmitters (Zheng, 2009), and the modulation of nonreproductive behaviors (Frye, 2009). Progesterone is also a neuroprotective factor in both the peripheral and the central nervous systems (Schumacher et al., 2007; Roglio et al., 2008; Cekic et al., 2009; DeNicola et al., 2009; Sayeed and Stein, 2009).

Progesterone acts in the nervous system in part through classical steroid nuclear receptors (Brinton et al., 2008; Mani, 2008). Ligand binding to nuclear steroid receptors results in their activation, allowing the recruitment of transcriptional coregulators to control the transcription of target genes. In addition, progesterone activates membrane progesterone receptors PRα, PRβ, and PRγ (Brinton et al., 2008; Guennoun et al., 2008; Sakamoto et al., 2008; Thomas, 2008), which are involved in the regulation of membrane/cytoplasmic signaling. Therefore, progesterone mdulates the phosphorylation of kinases such as extracellular signal-regulated kinase (ERK), phosphoinositide 3-kinases (PI3K), Akt, and glycogen synthase kinase 3 (GSK3) (Singh, 2001; Nilsen and Brinton, 2003; Ballaré et al., 2006; Guerra-Araiza et al., 2007, 2009; Dressing et al., 2009). These kinases participate in the actions of progesterone to control neuronal differentiation, neuronal function, reproductive behavior, and neuroprotection (Acosta-Martínez et al., 2006; Kaur et al., 2007; Cai et al., 2008; Koulen et al., 2008; Hwang et al., 2009; Liu et al., 2009; Mani and Portillo, 2010; Zhang et al., 2010). However, the mechanisms involved in the regulation of ERK and PI3K/Akt/GSK3 signaling by progesterone in the nervous system remain unidentified. Potential targets of the hormone on which to exert this regulation are protein phosphatases.

Protein phosphatase 2 (PP2A) is an antagonist of ERK activity (Haccard et al., 1990; Alessi et al., 1995; Silverstein et al., 2002; Yu et al., 2004; Junttila et al., 2008). Indeed, treatment of different cell types with okadaic acid, a well-known selective inhibitor of PP2A, causes activation of ERK (Gause et al., 1993; Sonoda et al., 1997). PP2A is also

capable of dephosphorylating Akt at T308 (Millward et al., 1999) and regulates Tau phosphorylation in the nervous tissue directly and via GSK3 β (Quian et al., 2010). PP2A is also an essential modulator of long-term potentiation (LTP) and long-term depression (LTD) (Jouvenceau et al., 2003; Sun et al., 2003) and is involved in the mechanisms of learning and memory (Bennet et al., 2001; Woo and Nguyen, 2002; Yamashita et al., 2006). Rats with reduced levels of PP2A, resulting from chronic pharmacological treatments, show memory impairments (Arendt et al., 1995). Furthermore, immunoblotting and immunohistochemical analyses have revealed that there is a significant reduction in the amount of PP2A in the frontal and temporal cortex of Alzheimer's disease (AD) patients that is correlated with a decreased PP2A activity (Sontag et al., 2004). In addition, PP2A inactivation by phosphorylation in Y307 has been associated with AD neurofibrillary pathology and Tau hyperphosphorylation (Liu et al., 2008).

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a tumor suppressor protein with phosphatase properties. PTEN regulates the PI3K/Akt/GSK3 canonical pathway, where PTEN dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and promotes its inhibition (Myers et al., 1998; Wu et al., 1998; Vazquez and Sellers, 2000). PTEN is expressed in neurons in human, mouse, and rat brains (Kyrilenko et al., 1999; Sano et al., 1999; Lachyankar et al., 2000). PTEN is involved in the regulation of neuronal migration (Marino et al., 2002), differentiation (Lachyankar et al., 2000), neuronal volume (Backman et al., 2001; Kwon et al., 2001), and apoptosis (Kyrilenko et al., 1999). Furthermore, PTEN, like PP2A, regulates Tau phosphorylation, in a mechanism independent of the classical PI3K/Akt pathway, possibly by reducing ERK1/2 activity (Kerr et al., 2006). There is evidence that phosphorylation of PTEN at its carboxyl-terminal results in loss of phosphatase activity (Vazquez et al., 2000; Torres and Pulido, 2001; Guzeloglu-Kayisli et al., 2003; Ning et al., 2006; Odriozola et al., 2007).

Although the effect of progesterone on the phosphorylation of different kinases (Singh, 2001; Nilsen and Brinton, 2003; Guerra-Araiza et al., 2007, 2009) and on the phosphorylation of Tau (Carroll et al., 2007; Guerra-Araiza et al., 2007) has been previously characterized, it is unknown whether progesterone modulates PP2A and PTEN in the brain. Therefore, in this study, we have assessed whether progesterone regulates the expression, phosphorylation, and methylation of PP2A and the expression and phosphorylation of PTEN in three different brain regions, the hippocampus, the hypothalamus, and the cerebellum.

IV.3 MATERIALS AND METHODS

IV.3.1 ANIMALS

Wistar albino female rats from our in-house colony were kept on a 12:12-hr light-dark cycle and received food and water ad libitum. Animals were handled in accordance with the guidelines published in the NIH Guide for the care and use of laboratory animals, the principles presented in the Guidelines for the use of animals in neuroscience research by the Society for Neuroscience, and following the European Union (86/609/EEC) legislation. Experimental procedures were approved by our Institutional Animal Use and Care Committee. Special care was taken to minimize animal suffering and to reduce the number of animals used to the minimum necessary.

IV.3.2 EXPERIMENTAL TREATMENTS

All the animals used in this study were bilaterally ovariectomized at the age of 2 months under 2,2,2-tribromoethanol anesthesia [0.2 g/kg body weight (b.w.); Fluka Chemika, Buchs, Switzerland]. Rats were then housed in plastic cages and randomly assigned to the different treatments. Nine days after surgery, rats received one i.p. injection of estradiol (n = 4; 50 μg/Kg b.w.) or vehicle (n = 4; 0.2 ml of 20% [2-hydroxypropyl]-β-cyclodextrin; Fluka Chemika). Twenty-four hours after the administration of estradiol or vehicle, animals received one i.p. injection of progesterone (n = 4; 2 mg/kg b.w.; Sigma, St. Louis, MO) or one injection of vehicle. The selected dose of progesterone results in physiological levels of this molecule in the plasma of ovariectomized rats (Ciriza et al., 2006). The animals were killed by decapitation 24 hr after the second injection. The hippocampus, hypothalamus, and cerebellum were quickly removed and immediately processed for protein extraction.

IV.3.3 WESTERN BLOT ANALYSIS

Tissue samples were homogenized in lysis buffer (150 nM NaCl, 20 mM Tris-HCl, 10% glycerol, 5 mM EDTA, 1% NP-40; Roche, Mannhein, Germany) supplemented with protease and phosphatase inhibitors (50 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 25 μg/ml leupeptin, and 100 nM orthovanadate; all from Sigma). Proteins were obtained by centrifugation for 15 min at 15,000 rpm at 4°C, and the supernatant was quantified with a modified Bradford assay (Bio-Rad, Munchen, Germany). Proteins (30 μg) were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% SDS-PAGE) with a Mini-Protean system (Bio-Rad) and electrophoretically transferred to nitrocellulose membranes (GE Healthcare; formerly Amersham Bioscience, Piscataway, NJ). The membranes were blocked with 5% nonfat dry milk diluted in 0.05% Tween-20 Tris-buffered saline and incubated overnight with the primary antibodies. Prestained markers (Bio-Rad) were included for size determination.

IV.3.4 ANTIBODIES

The following antibodies were used: mouse monoclonal antibody against PP2A Cα (BD Transduction Laboratories, San Diego, CA; diluted 1:1,000), rabbit polyclonal antibody against phosphorylated PP2Ay307 (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:500), mouse monoclonal antibody against the nonmethylated PP2A C (Cell Signaling, Beverly, MA; diluted 1:1,000), mouse monoclonal antibody against the methylated PP2A C (Upstate Biotechnology, Lake Placid, NY; diluted 1:100), mouse monoclonal antibody against PTEN (Santa Cruz Biotechnology; diluted 1:500), rabbit polyclonal antibody against the phosphorylated PTENser380 (Cell Signaling, Beverly, MA; diluted 1:2,000), mouse monoclonal antibody against βIII-tubulin (Promega, Madison, WI; diluted 1:10,000), and mouse monoclonal antibody against glyceraldehyde-3-phosphate-dehydrogenase (GAPDH; Chemicon, Temecula, CA; diluted 1:3,000). After incubation with the primary antibody, membranes were washed and incubated with horseradish peroxidase-coupled secondary antibodies (Jackson Immunoresearch, West

Grove, PA; diluted 1:15,000). Immunoreactive bands were detected by using an enhanced chemiluminescence system (GE Healthcare-Amersham). The density of each band of different primary antibodies was normalized to its loading control (tubulin or GAPDH). Different loading controls were used to avoid coincidence of their molecular weights with those of the analyzed proteins. Thus GAPDH was used when the analyzed protein and tubulin had a similar molecular weight. Conversely, tubulin was used when the analyzed protein and GAPDH had a similar molecular weight. Phosphorylated forms of PP2A and PTEN were first normalized to their loading controls and then to total protein values of PP2A and PTEN, respectively. To minimize interassay variations, samples from all animals groups, in each experiment, were processed in parallel.

IV.3.5 STATISTICAL ANALYSIS

The N used for statistical analysis was the number of animals. Data were analyzed via one-way ANOVA, followed by a post hoc analysis with the Tukey's test. The Prism 2.01 program (GraphPad, San Diego, CA) was used for calculating probability values. $P < 0.05$ was considered statistically significant.

IV.4 RESULTS

IV.4.1 PP2A

The levels of PP2A were unaffected by the treatments with progesterone, estradiol, or estradiol and progesterone in the three brain regions studied, the hypothalamus, hippocampus, and cerebellum (Figs. 1, 2A,C,E). In contrast, treatments with progesterone alone significantly increased the levels of PP2A phosphorylated in tyrosine 307 in the hippocampus (Figs. 1, 2D) and the cerebellum (Figs. 1, 2F) compared with control values. The levels of PP2A phosphorylated in tyrosine 307 were not significantly affected in the hypothalamus (Figs. 1, 2B) or hippocampus (Figs. 1, 2D) of the animals treated with estradiol alone or with progesterone after estradiol priming. However, in these animals, a significant increase in the levels of PP2A phosphorylated in tyrosine 307 was detected in the cerebellum (Figs. 1, 2F) in comparison with control values. The state of methylation of PP2A was unaffected by the treatment with progesterone, estradiol, or estradiol and progesterone in the three brain regions studied (data not shown).

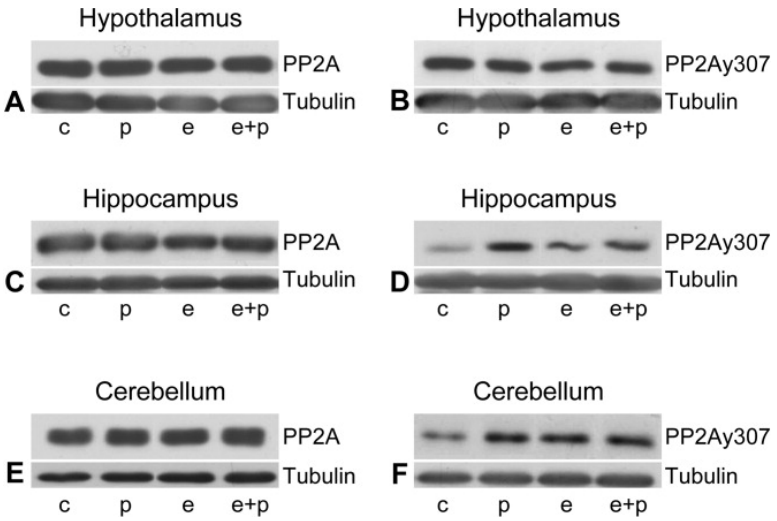

Figure IV.1 - Examples of Western blots from hypothalamic (A,B), hippocampal (C,D), and cerebellar (E,F) samples of ovariectomized rats treated with vehicle (c), progesterone (p), estradiol (e), or estradiol and progesterone (e + p). Examples of bands immunodetected with a mouse monoclonal antibody against PP2A (A,C,E), a rabbit polyclonal antibody against PP2A phosphorylated in tyrosine 307 (PP2Ay307; B,D,F), and a mouse monoclonal antibody against βIII-tubulin, used as loading control.

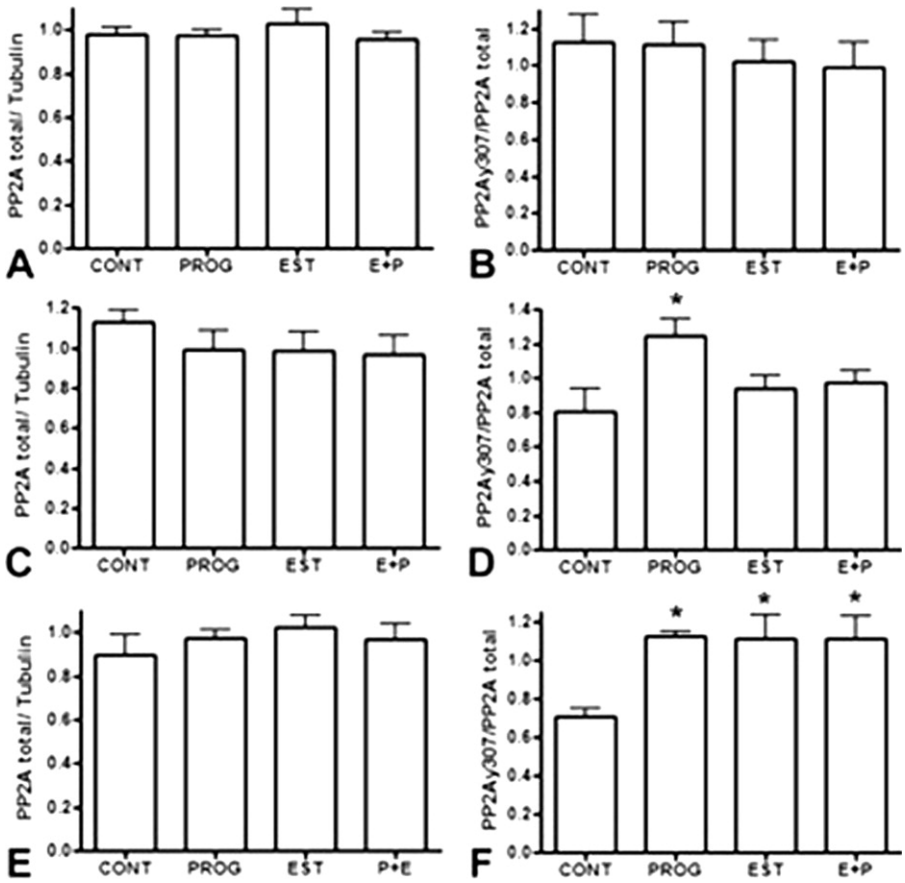

Figure IV.2 - Results of the densitometytic analysis from PP2A in the hypothalamus (A,B), hippocampus (C,D), and cerebellum (E,F). A,C,E: Total PP2A, normalized to tubulin. B,D,F: PP2A phosphorylated in tyrosine 307 (PP2Ay307), first normalized to tubulin and then normalized to total PP2A. CONT, control animals; PROG, animals injected with progesterone; EST, estradiol; E + P, animals treated with estradiol and progesterone. The number of animals was 4 (N = 4) in all experimental groups. *Significant difference vs. control values at $P < 0.05$.

IV.5.2 PTEN

The levels of PTEN were unaffected by the treatment with progesterone, estradiol, or estradiol and progesterone compared with control values in the three brain regions studied (Figs. 3, 4A,C,E). In contrast, progesterone administration in the absence of estradiol priming significantly decreased the levels of PTEN phosphorylated in serine 380 in the hypothalamus and in the hippocampus compared with control values (Figs. 3, 4B,D). Administration of estradiol alone also resulted in a significant decrease in PTEN phosphorylation in the hypothalamus but did not affect PTEN phosphorylation in the hippocampus and the cerebellum compared with control values. In the animals treated with estradiol and progesterone, the levels of PTEN phosphorylated in serine 380 were not significantly different from control values in the brain regions studied (Figs. 3, 4B,D,F).

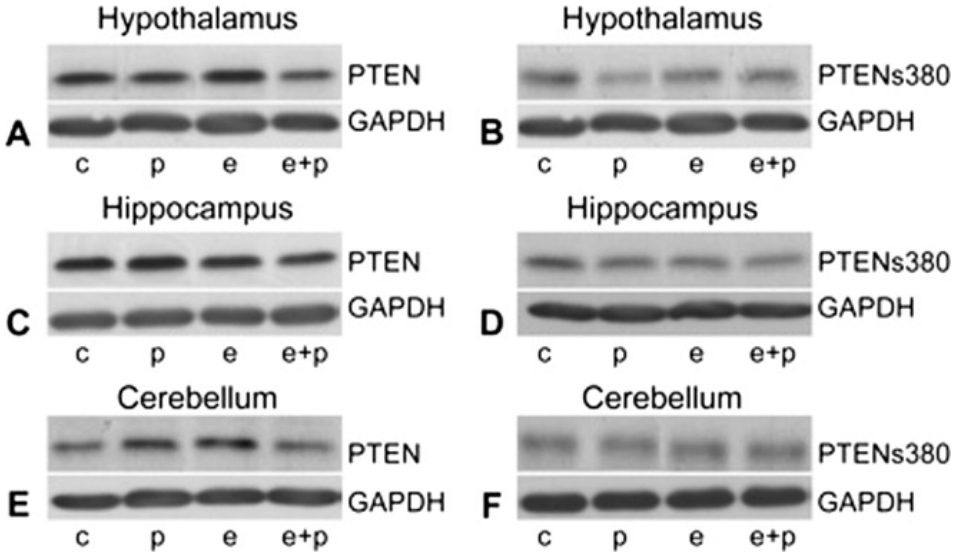

Figure IV.3 - Examples of Western blots from hypothalamic (A,B), hippocampal (C,D), and cerebellar (E,F) samples of ovariectomized rats treated with vehicle (c), progesterone (p), estradiol (e), or estradiol and progesterone (e + p). Examples of bands immunodetected with a mouse monoclonal antibody against PTEN (A,C,E), a rabbit polyclonal antibody against PTEN phosphorylated in serine 380 (PTENs380; B,D,F), and a mouse monoclonal antibody against glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), used as loading control.

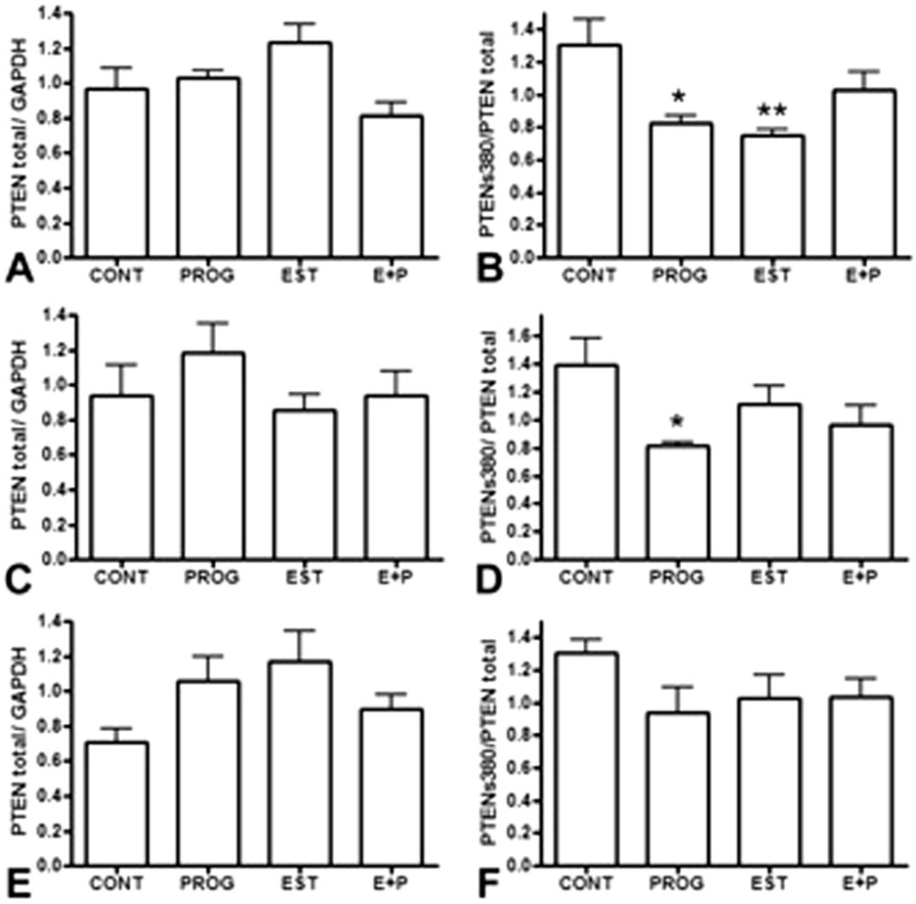

Figure IV.4 - Results of the densitomeytic analysis from PTEN in the hypothalamus (A,B), the hippocampus (C,D), and the cerebellum (E,F). A,C,E: Total PTEN, normalized to GAPDH. B, D,F: PTEN phosphorylated in serine 380 (PTENs380), first notmalized to GAPDH and then normalized to total PTEN. CONT, control animals; PROG, animals injected with progesterone; EST, estradiol; E + P, animals treated with estradiol and progesterone. The number of animals was 4 (N = 4) in all experimental groups. *Significant difference vs. control values at $P < 0.05$. **Significant difference vs. control values at $P < 0.01$.

IV.5 DISCUSSION

The findings of the present study indicate that progesterone regulates the phosphorylation of PP2A and PTEN in a regionally specific manner in the brain of young ovariectomized rats. Progesterone administration results in an increased phosphorylation of PP2A in tyrosine 307 in the hippocampus and cerebellum and in a decrease in the phosphorylation of PTEN in serine 380 in the hypothalamus and in the hippocampus. In contrast, the state of methylation of PP2A and the basal levels of PP2A and PTEN were unaffected by the administration of progesterone. These effects were detected 24 hr after the hormonal treatment. Insofar as progesterone is metabolized in the nervous system to the neuroactive steroids dihydroprogesterone and tetrahydroprogesterone (Melcangi et al., 2008), it is conceivable that some of the effects of the hormone on PP2A and PTEN phosphorylation are mediated by its metabolites.

Our findings provide novel information regarding PP2A modulation in the brain and extend previous evidences of the regulation of PTEN by progesterone in other tissues, such as the human endometrium (Guzeloglu-Kayisli et al., 2003). Previous analyses have shown that the dose and pattern of progesterone treatment used in this study result in hormonal levels that are within the ranges observed in the plasma and the hippocampus of estrus rats (Ciriza et al., 2006). Therefore, the effects of progesterone on the phosphorylation of PP2A and PTEN may represent a physiological action, suggesting that some of the neural actions of the hormone may be mediated by the regulation of the mechanisms of dephosphorylation in which these two protein phosphatases are involved.

Progesterone regulates in the brain many of the functions controlled by PP2A and PTEN. For instance, progesterone regulates in the brain the phophoinositide-3 kinase and the mitogen-activated protein kinase pathways (Guerra-Araiza et al., 2009), which are targets for PP2A and PTEN. PP2A and PTEN may also be involved in the regulation of Tau phosphorylation by progesterone (Guerra-Araiza et al., 2007). Interestingly, our present findings indicate that PP2A phosphorylation is down-regulated by progesterone in the cerebellum and unchanged in the hypothalamus, which is in accordance with previous observations of an increase in Tau phophorylation in the cerebellum but not in the hypothalamus of progesterone-treated rats (Guerra-Araiza et al., 2007).

The effects of progesterone on PP2A and PTEN might also have important

functional implications for the regulation of the actions of growth factors in the brain. The interaction of progesterone with the intracellular signaling of growth factors, such as VEGF, BDNF and IGF-I, via the regulation of PP2A and PTEN, may modulate the actions of these factors on glial and neuronal development, physiology, and pathology. In addition, progesterone may interact with other regulators of growth factor signaling, including the ovarian hormone estradiol. Estradiol is known to exert several actions in the brain that are mediated by PP2A and PTEN (Yi et al., 2005, 2009; Yi and Simpkins, 2008) and to affect the actions of several growth factors, such as nerve growth factor, glial cell line-derived neurotrophic factor, BDNF, and IGF-I (Etgen and Garcia-Segura, 2009). Therefore, acting on PP2A and PTEN, progesterone may potentially interact with estradiol in the control of neuroendocrine and behavioral events by the regulation of growth factor signaling. In this regard, the regulation exerted by estradiol on the actions of progesterone on PP2A and PTEN in the brain is of interest. Our findings indicate that estradiol priming prevents the effect of progesterone on PP2A phosphorylation in the hippocampus and on PTEN phosphorylation in the hypothalamus and the hippocampus. In contrast, the action of progesterone on PP2A phosphorylation in the cerebellum is unaffected by estradiol priming. This suggests that the changes in the levels of estradiol and progesterone associated with different endocrine conditions, such as puberty, reproductive cycles, pregnancy, or menopause, may contribute to regulate phosphatase activity in the brain with regional specificity.

CHAPTER V

DISCUSSION

V- GENERAL DISCUSSION: NEW SIGNALING MECHANISMS OF PROGESTERONE IN THE CNS

The studies presented here have explored new molecular mechanisms of progesterone signaling in the central nervous system. We will now discuss our findings in the context of previous knowledge on the actions of progesterone in the brain.

V.1 PROGESTERONE REGULATION OF THE PI3K/AKT AND MAPK SIGNALING IN THE RAT BRAIN

Our findings indicate that a physiological dose of progesterone regulates PI3K/Akt and MAPK signaling in the central nervous system. Progesterone administration to young ovariectomized rats results in increased phosphorylation of ERK1/2, in increased expression of the catalytic and regulatory subunits of PI3K and in increased phosphorylation, and therefore activation of Akt (Chapter II). Progesterone effects were detected in the hippocampus, the hypothalamus and the cerebellum, assessed 24 h after the hormonal administration. These findings extend previous in vitro evidence of increased phosphorylation of ERK and/or Akt in cortical explants (Singh, 2001), primary hippocampal cultures (Nilsen and Brinton, 2003) and retinal glial cells (Swiatek-De Lange et al., 2007) treated with progesterone and suggest that the hormone may exert in the brain in vivo a sustained activation of the intracellular signaling of growth factor receptors, such as the IGF-I and BDNF (TrkB) receptors.

We have also observed that reduced derivatives of progesterone affect the phosphorylation of ERK-1/2 and Akt and the expression of PI3K in some brain regions, suggesting that progesterone metabolism may be involved in the hormonal effects on the MAPK and PI3K signaling pathways. For example, DHP mimicked the effect of progesterone on the phosphorylation of ERK-1/2 in the hypothalamus and the hippocampus, while THP mimicked the effect of progesterone in the phosphorylation of ERK-1/2 in the hypothalamus. However, none of the metabolites mimicked the effect of progesterone on the phosphorylation of ERK-1/2 in the cerebellum. Concerning the PI3K pathway, both progesterone metabolites increased the expression of p110-PI3K in the hypothalamus and cerebellum and the expression of p85-PI3K in the hypothalamus and the hippocampus. However, DHP and THP did not affect the expression of p110-PI3K in the hippocampus

and the expression of p85-PI3K in the cerebellum. Furthermore, Akt phosphorylation was unaffected by DHP and was increased by THP in the hypothalamus and hippocampus but not in the cerebellum. Therefore, DHP and THP did not fully mimic the effect of the hormone on MAPK and PI3K signaling pathways (Chapter II).

V.2 PROGESTERONE REGULATION OF TAU AND GSK3B IN THE RAT BRAIN

Our findings indicate that a physiological dose of progesterone results in a decrease in the expression of Tau and in an increase in the phosphorylation of Tau, at the Tau-1 and PHF-1 epitopes but not of Ser262 epitope (Chapter III) The increase in Tau-1 and PHF-1 phosphorylation as a result of progesterone administration is accompanied by a decrease in the phosphorylation of GSK3β in serine, which is associated to an increase in its activity (Chapter III). Progesterone effects were detected only in the cerebellum and assessed 24 h after the hormonal administration. Our findings provide novel information regarding regulation of Tau protein by progesterone, after other studies showed the importance of another gonadal hormone, the estradiol, in the regulation of Tau. In fact, estradiol increases the expression of Tau in primary neuronal cultures (Ferreira and Caceres, 1991; Alvarez-de-la-Rosa et al., 2005) an effect that is associated to an increase in axonal growth (Ferreira and Caceres, 1991). In addition, estradiol decreases Tau phosphorylation in primary cortical and hippocampal neurons, in hippocampal slice cultures and in the hippocampus in vivo (Cardona-Gomez et al., 2004; Alvarez-de-la-Rosa et al., 2005; Goodenough et al., 2005) Therefore our findings suggest that progesterone may be highly relevant for cerebellar function by regulating Tau expression and phosphorylation, since Tau regulates the dynamic instability of microtubules, its growth and bundling (Paglini et al., 2000) which ultimately leads to regulation of axonal transport, axonal growth and synaptic plasticity.

We have also observed that reduced derivatives of progesterone affect the expression of Tau in the cerebellum, suggesting that progesterone metabolism may be involved in the hormonal effects on the regulation of Tau protein (Chapter III). However, only progesterone regulated the phosphorylation of Tau and GSK3β, suggesting that the final effect of progesterone on these molecules may be the result of a combination of

effects directly mediated by progesterone and effects mediated by its metabolites (Chapter III).

V.3 PROGESTERONE REGULATION OF PP2A AND PTEN IN THE RAT BRAIN

Our findings indicate that a physiological dose of progesterone regulates the phosphorylation of PP2A and PTEN in the brain of young ovariectomized rats. Progesterone administration results in an increased phosphorylation of PP2A in tyrosine 307 in the hippocampus and cerebellum and in a decrease in the phosphorylation of PTEN in serine 380 in the hypothalamus and in the hippocampus (Chapter IV). In contrast, the state of methylation of PP2A and the basal levels of PP2A and PTEN were unaffected by the administration of progesterone. These effects were observed 24 h after the hormonal treatment and provide novel information regarding PP2A modulation in the brain by progesterone and extend previous evidences of the regulation of PTEN by progesterone in other tissues, such as the human endometrium (Guzeloglu-Kayisli et al., 2003). The effects of progesterone on PP2A and PTEN, suggest that the interaction of progesterone with the intracellular signaling of growth factors, such as VEGF, BDNF and IGF-I and their actions in glial and neuronal development, physiology and pathology may be mediated via the regulation of PP2A and PTEN. In addition, progesterone may interact with other regulators of growth factor signaling, including the ovarian hormone estradiol which is known to exert several actions in the brain that are mediated by PP2A and PTEN (Yi et al., 2005, 2008, 2009), and to affect the actions of several growth factors, such as nerve growth factor, glial cell line-derived neurotrophic factor, BDNF and IGF-I (Etgen and Garcia-Segura, 2009).

We also observed that estradiol priming prevents the effect of progesterone on PP2A phosphorylation in the hippocampus and on PTEN phosphorylation in the hypothalamus and the hippocampus. In contrast, the action of progesterone on PP2A phosphorylation in the cerebellum is unaffected by estradiol priming (Chapter IV). Furthermore, reduced derivatives of progesterone were unable to affect the state of phosphorylation of these phosphatases, with the exception of the phosphorylation of PP2A in the cerebellum, where THP was able to mimic progesterone effects (data not shown).

V.4 SUMMARY AND CONCLUSIONS

Considering the previous data regarding the progesterone effect in the MAPK and the PI3K signaling pathways, we expected that PTEN, like PP2A, would be downregulated by progesterone administration. However, we observed exactly the opposite. Progesterone not only did not downregulate PTEN, but upregulated its activity, by reducing the phosphorylated (inactive) form of PTEN in its protein pool. Although the meaning of this event is not known yet, our findings suggest that the upregulation of Akt by progesterone is independent of PTEN. It can to be excluded, however, that PTEN activity may be unable to antagonize another more potent kinase activity acting in parallel to Akt. One possible explanation on how progesterone may upregulate Akt and PTEN activities at the same time, may reside in a PI3K independent mechanism, by which Akt is activated. For instance, it has been shown that dopamine is able to promote Akt activation, even when PI3K inhibitor wortmannin is used. Furthermore the overexpression of a mutant Akt that cannot be phosphorylated, impaired the cAMP Response Element-Binding Protein (CREB) phosphorylation induced by dopamine (Brami-Cherrier et al., 2002). Interestingly steroid hormone treatment has been shown to promote phosphorylation of CREB in the anteroventral periventricular nucleus (Gu et al., 1996).

Our findings suggest that GSK3 activation and tau phosphorylation by progesterone are also independent from PTEN. PTEN overexpression was shown to reduce tau phosphorylation (Kerr et al., 2006), however progesterone administration resulted in higher levels of active PTEN in our study and at the same time increased GSK3 activity and tau phosphorylation (Guerra-Araiza et al., 2007). Furthermore, it was shown that PTEN, acting as classical PI3K pathway suppressor, promotes GSK3 activation by Akt inhibition. This is not the case in our study, since higher levels of active PTEN promoted by progesterone treatment did not result in the inactivation of Akt but in its activation (Guerra-Araiza et al., 2009). Therefore, our findings suggest that in the brain progesterone induces GSK3 activation and tau phosphorylation by a mechanism that is independent of the canonical PI3K/Akt pathway. An interesting alternative route is the Wnt pathway, that could explain how progesterone is able to promote Akt phosphorylation and at the same time GSK3 activation. Previous work showed that in the inhibition of Wnt pathway, as the inhibition of PI3K signaling increases GSK3 activity and tau phosphorylation (Mercado-Gomez et al., 2008) and that the two pathways are independent (Ng et al., 2009). Supporting this

hypothesis, it was shown that Dickkopf-1, an inhibitor of Wnt signaling is upregulated by progesterone treatment in human endometrial stromal cells (Tulac et al., 2006). Further studies should determine whether Wnt pathway is involved in the effects of progesterone in the brain.

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