Effects of neonatal Allopregnanolone alteration levels on adult behaviour

Role of dorsal hippocampus

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“Science might set limits to knowledge,
but should not set limits to imagination
”

Bertrand Russell

Table of contents

Abbreviations

AMPA: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AT: Acquisition trial

A1: Adenosine receptor 1

A2: Adenosine receptor 2

BO: Boissier test

CA1: Cornus Amonis 1

CA3: Cornus Amonis 3

CNS: Central neurvous system

Cl^-: Chloride

DHEA: Dehiepiandrosterone

EPM: Elevated plus maze

$GABA_AR$/ $GABA_ARs$: γ-aminobutyric acid receptor/s

GDPs: Giant depolarizing potentials

GFAP: Glial fibrillary acidic protein

KCC2: Potassium chloride co-transporter 2

MAP2: Microtubule-associated protein 2

MBR: Mitochondria benzodiazepine receptor

nAch: Nicotinic acetylcholine receptor

NBM: Nucleus basalis magnocellularis

NH: No handled

NKCC1: Sodium potassium chloride co-transporter 1

NMDA: *N*-Methyl-D-aspartic acid

NS/Ns: Neurosteroid/s

PREG: Pregnenolone

PREGS: Pregnenolone sulphate

PXR: Pregnane xenobiotic receptor

P450scc: P450 side-chain-cleavage

RT: Retention trial

StAR: Steroidogenic acute-regulatory protein

THDOC: Tetrahydrodeoxycorticosterone

TSPO: Translocator protein

3α,5α-THPROG: 3α-5α-tetrahydroprogesterone

3βHSD: 3β hydroxysteroid

5-HT3: 5-hydroxytryptamine receptor 3

Introduction

1. General overview

Allopregnanolone or 3α-5α-tetrahydroprogesterone is a α-reduced progesterone metabolite that belongs to the family of neurosteroids (Ns) (Paul and Purdy, 1992; Robel and Baulieu, 1994). The term neurosteroid (NS) refers to steroids that can be synthesized *de novo* by the nervous tissue independently of peripheral sources such as ovaries or adrenal glands and are capable to modulate neural excitability (Baulieu et al. 1981; Baulieu, 1998). Baulieu and colleagues observed levels of steroids such as dehydroepiandrosterone (DHEA) and pregnenolone (PREG) in the nervous system, after gonad or adrenalectomy (the main steroidogenic glands) (Baulieu et al., 1981). Later on, other studies demonstrated the presence in the brain of enzymes that are also involved in classic steroidogenesis and confirmed the fact that steroids are capable to be synthesized *in situ* by the nervous tissue (reviewed in Mensah-Nyagan et al., 1999; Compagnone and Mellon, 2000). In the last three decades many studies have emerged stressing the relevance of Ns multiple roles on pathologic behaviour, aging processes or degenerative diseases, and regeneration of damaged tissue (Rupprecht et al., 2001; Dubrovsky, 2005; Mellon, 2007; Kussius et al., 2009). Controlled local synthesis of Ns has been described to fluctuate during life due to physiological conditions such as menstrual cycle and pregnancy (Gidler et al., 2001; Maguire et al., 2005; reviewed in Frye et al., 2011 and Brunton et al., 2014), aging (Bernardi et al., 1998), and to participate in neural development, cognitive processes and mood control (Kussius et al., 2009; Mellon, 2007). Nowadays it is well accepted that Ns are an important target concerning pathologic behaviour (Barbaccia et al., 1998; Rupprecht et al. 2001; Dubrovsky, 2005; Marx et al., 2006; Mellon, 2007; Kussius et al. 2009; Frye, 2009; Marx et al., 2009, 2011).

1.1 Ns biosynthesis

Biosynthesis of Ns begins with the enzymatic conversion of cholesterol to pregnenolone. Cholesterol is delivered into the inner mitochondrial membrane through the steroidogenic acute regulatory protein (StAR) (Sierra, 2004). The rate-limiting step on the Ns synthesis is the movement into the inner mitochondrial membrane mediated by the translocator protein (TSPO) as known as mitochondrial benzodiazepine receptor (MBR) (Papadopoulos et al., 2006; Rone et al., 2009). Once into the mitochondria, cholesterol is catalysed to pregnenolone through several enzymatic reactions mediated both by the P450 side-chain-cleavage (P450scc) and non-P450scc enzymes (reviewed in Mellon and Griffin, 2002), see Fig 1. Then, progesterone is formed from pregnenolone by the 3β hydroxysteroid (3βHSD) enzymes. Thus, the NS Allopregnanolone is synthesized from progesterone via two enzymatic steps: first one, the 5α-reductase reduced progesterone yielding dihydroprogesterone and the second one, is the 3α reduction of the C3 ketone, mediated by 3αHSD and yielding Allopregnanolone (Mellon et al., 2001; Mellon and Griffin,

2002; Belelli and Lambert, 2005; Mellon, 2007, see Fig 1). The presence of enzymes responsible of Allopregnanolone synthesis has been described in the nervous tissue (Mensah-Nyagan et al., 1999; Compagnone and Mellon, 2000; Mellon and Griffin, 2002; Agis-Balboa et al., 2006). For instance co-localization of 3αHSD and 5α reductase have been demonstrated in pyramidal neurons of the cortex and the glutamatergic granular cells of the dentate gyrus, glutamatergic (dorsomedial) and GABAergic (reticular) cells of the thalamus, GABAergic cells of the striatum, glutamatergic neurons of the amygdala, and GABAergic Purkinje neurons of the cerebellum (Agis-Balboa et al., 2006). Indeed, recent findings indicate that glutamatergic neurons could be the main source for Ns synthesis acting as paracrine or autocrine regulators of somatodendritic activity (Chisari et al., 2010; Chisari et al., 2011; Chisiari et al., 2012) and as a paracrine regulator of membrane receptors located on dendrites and cell bodies of glutamatergic neurons (Akk et al., 2005). However, previous observations also pointed out that glial cell: Schwann cells in the PNS and oligodendrocytes and astrocytes in the CNS, could also participate in Ns synthesis (Baulieu, 1998).

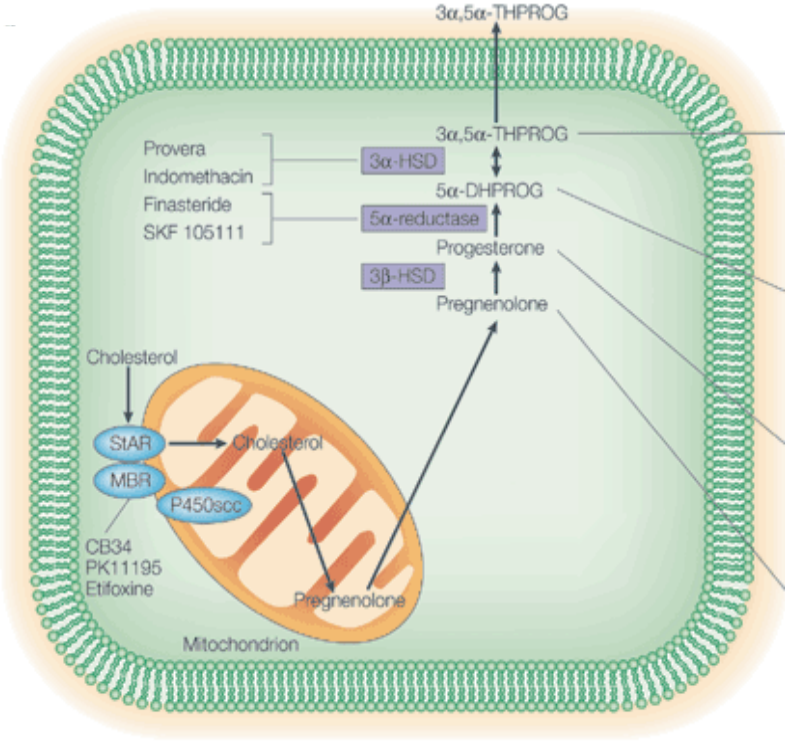

Fig 1: Structure and synthesis of Ns: Biosynthesis of pregnenolone derived Ns. The pathway for the synthesis of Allopregnanolone (3**α**,5**α**-THPROG) from cholesterol is shown. Steroidogenic acute-regulatory protein (StAR) interacts with the mitochondrial benzodiazepine receptor (MBR) to facilitate the transport of cholesterol across the mitochondrial membrane (Figure extracted from Belelli and Lambert, 2005).

1.2 Ns mechanisms of action

1.2.1 Genomic effects

Steroid hormones exert their classical actions by passing through the cell membrane and binding to their respective cytoplasmic intracellular receptors. The receptor bound steroid hormone and then translocate to the nucleus were they can modulate gene expression. Allopregnanolone and ring A reduced pregnanes derived from progesterone have also been reported to regulate gene expression (reviewed in Rupprecht and Holsboer, 1999; Rupprecht, 2003; Frye et al., 2012), see Fig 2 (right). It has been suggested that differentially from progesterone, Allopregnanolone is inactive at progesterone receptor and that its effect on gene expression could be mediated by its modulation of the pregnane xenobiotic receptor (PXR) (Lamba, et al., 2004; Langmade, 2006; reviewed in Frye et al., 2012).

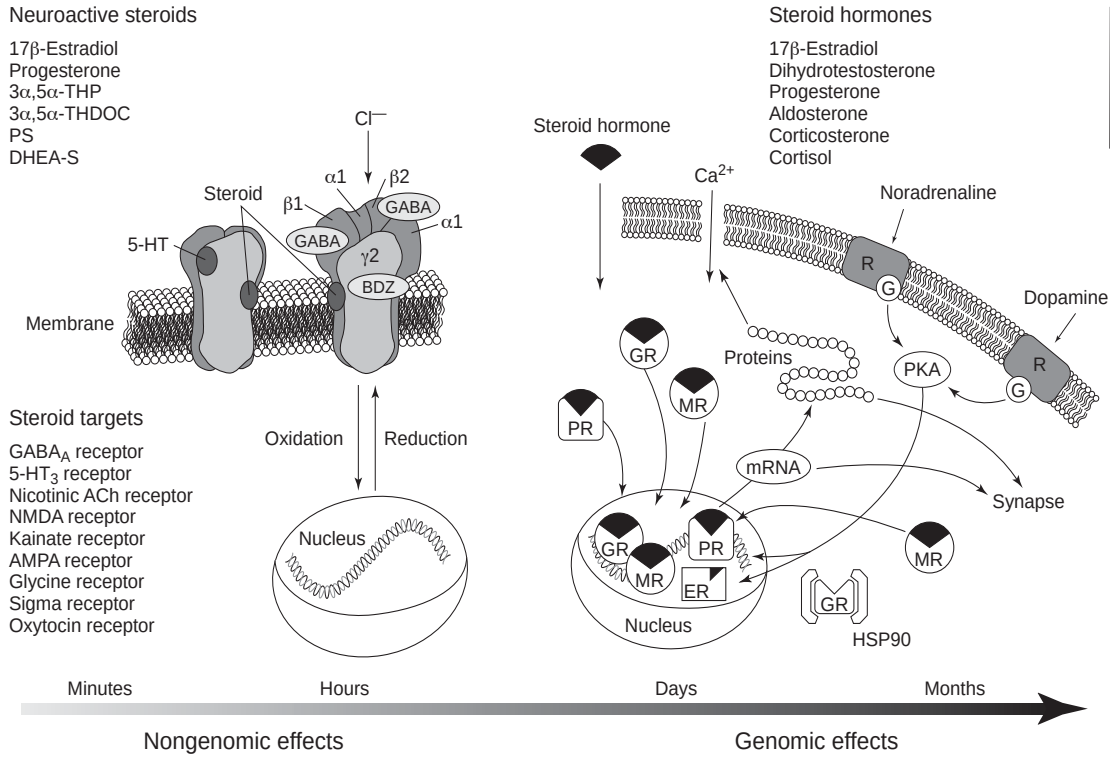

Fig 2 Ns mechanisms of non-genomic vs. genomic actions: Ns are capable to modulate neural excitability by binding directly to specific membrane receptors located in the plasmatic membrane of neurons (left). Whereas “classical view” of steroid hormones actions, involved their binding to intracellular receptors promoting the gene expression changes (right). Figure extracted from Rupprech and Holsboer, 1999.

1.2.1 Modulation of ionotropic receptors

Besides their classical steroids actions, an important mechanism of action of Ns is their ability to modulate neural excitability by acting through membrane receptors. The fact that Ns could interact with membrane receptors, was firstly described in 1986 by Majewska and co-workers (Majewska et al., 1986), when they observed that the NS Allopregnanolone exerted its actions, mainly through the modulation of the GABAA receptor ($GABA_AR$) (Majewska et al., 1986). This "rapid" effect of NS involved that, while the classical steroids actions require longer time period to exert their effect and are limited by the rate of protein biosynthesis, this "new view" implied that Ns effects occurred rapidly from milliseconds to seconds (Majewska et al., 1986), see Fig 2 (left). In this sense, 5α-reduced pregnanes such as Allopregnanolone have been described to modulate several ionotropic receptors such as $GABA_AR$, NMDA, nAch or 5-HT3 (Majewska et al., 1986; Rupprecht and Holsboer 1999; Belelli and Lambert, 2005; Lambet et al., 2009; Zhung 2009), see Table I.

Although Allopregnanolone can act on several ionotropic receptors its main effect is mediated through the allosteric positive modulation of the $GABA_ARs$, by increasing the aperture time and frequency opening of the Cl^- channel (Majewska et al., 1986). Allopregnanolone effects on $GABA_ARs$ have been described to act via two discrete binding sites, one located between the transmembrane domains M1 and M4 of the α subunits of the receptor, and the other, in the interface between α and β subunits (Hosie et al. 2006), see Fig 3a. The $GABA_AR$ is a pentameric structure typically composed by 2α 2β and 1γ or δ subunits (reviewed in Jacob et al., 2008 and Olsen and Sieghart, 2009), see Fig 3b. The aperture of the $GABA_AR$ channel induces an increase in the membrane permeability to Cl^-, leading to a postsynaptic hyperpolarizing response (Staley and Proctor, 1999). There are 19 different $GABA_ARs$ isoforms indicating a high level of structural heterogeneity and function (Olsen and Sieghart, 2009). Those can be classified between receptors that are present at synapses and participate in phasic inhibition, such as $GABA_ARs$ containing α1, α2, α3, γ1, γ2 or γ3 subunits (Essrich et al., 1998; Farrant and Nusser, 2005), and $GABA_ARs$ related to the modulation of tonic inhibition, which preferably contain α4, α5, α6 or δ subunits and that are extrasynaptically and perisynaptically located in somatic, dendritic or axonal regions of the neuron membrane (Belelli et al., 2009; Glykys and Mody, 2007), see Fig 3c. Phasic inhibition is characterized by a short inhibitory postsynaptic response during the presence of GABA (Farrant and Nusser, 2005), while persistent or tonic inhibition rather than mediate a rapid inhibitory postsynaptic response, has been proposed to act as a paracrine regulation of the neuron tone (for review see Semyanov, et al., 2004; Kullmann et al., 2005; Farrant and Nusser, 2005; Glykys and Mody, 2007; Beleli et al., 2009). Although Allopregnanolone is able to interact with all $GABA_ARs$ subtypes, $GABA_ARs$ containing the δ subunit are preferred substrates for Allopregnanolone (Mihalek et al., 1999; Belelli et al., 2002; Stell et al., 2003; Spigelman et al., 2003; reviewed in

Smith et al., 2007; Herd et al., 2007; Shen and Smith, 2009). Moreover, it has been shown that knocked out mice lacking δ subunit have reduced sensitivity to hippocampal Allopregnanolone effects (Mihalek et al., 1999; Spigelman et al., 2003). Concretely, the combination of the α4β2δ $GABA_AR$ has been demonstrated to be especially sensitive to the Allopregnanolone fluctuation levels (Smith et al., 1998; Gulinello et al., 2001; Follesa et al., 2002; Gulinello et al., 2002; Shen et al., 2005; Biggio et al., 2006; Gangisetty and Reddy, 2010).

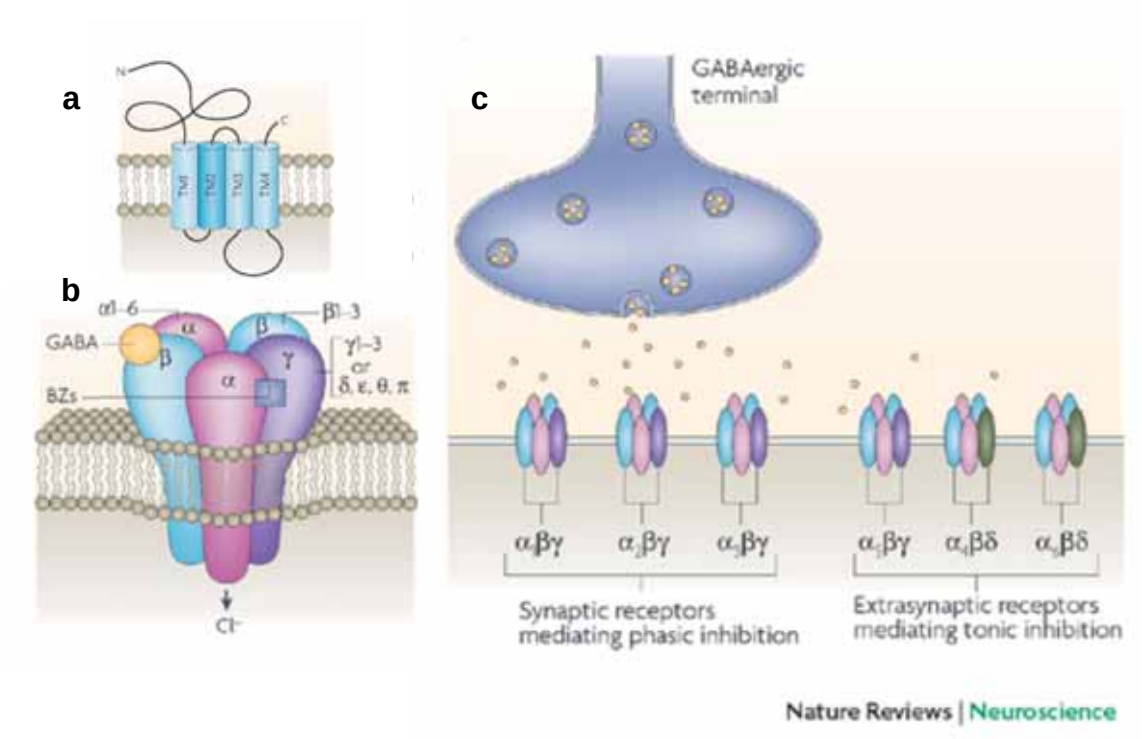

Fig 3: $GABA_AR$ composition and localization: **a)** $GABA_AR$ subunits consist of four hydrophobic transmembrane domains (TM1-4). **b)** Five subunits from seven subunit subfamilies (α, β, γ, δ, ε, θ and π) assemble to form a heteropentameric Cl- permeable channel. **c**) $GABA_AR$ compose of α (1,2,3) subunits together with a β and a γ are thought to be preferably synaptically located, whereas receptors containing α5βγ or α4βσ are mainly localized at extrasynaptic sites. Figure extracted from Jacob et al., 2008.

On the other hand, sulphated Ns such as pregnenolone sulphate (PREGS) has been described to negatively modulate the $GABA_ARs$ (Majewska and Schwartz, 1987; Akk et al., 2001). Although they do not show the same selectivity for $GABA_ARs$ such as other Ns, a specific binding sites in the M1 and between M2-M3 transmembrane domains of the α1 and β2 subunits of the $GABA_ARs$ have been described to be necessary for the negative modulatory effect of PREGS (Wardell et al., 2006; Wang et al., 2007). A relevant mechanism of action of PREGS effect is acting as a positive modulator of the NMDA receptors (Bowly et al., 1993; Horak et al., 2004; Wang et al., 2007; Schumacher et a., 2008; Kussius et al. 2009). The extracellular M3-M4 loop of the NR2

subunit has been shown to be a key determinant for the PREGS actions on the NMDA receptors. This hydrophobic pocket at the NR1/NR2B interface has been named “Steroid Modulatory Domain 1 (Horak et al., 2006; Schumacher et al., 2008). Within the NMDA receptors family, the negative modulation of AMPA and Kainate receptors by PREGS has also been reported (Mameli et al., 2005; Shirakana et al., 2005; Mellon et al., 2007). Furthermore, the sigma type 1 receptor is another relevant target of PREGS modulation (Monnet and Maurice, 2006; Schumacher et al., 2008). While other Ns such as DHEAS have been shown to be agonist of σ1 receptor, PREGS acts as an inverse agonist (Monnet and Maurice, 2006), see Table 1.

Table I: Ns mechanisms of action: Modulation of ionotropic receptors (Table adapted from Dubrovsky, 2005).

Neurosteroid	Receptor	Modulation
Progesterone	nAChR	Negative (Valera et al., 1992)
	Sigma 1	Negative (Monnet and Maurice, 2006)
	5-HT3	Negative (Wetzel et al., 1998)
Allopregnanolone	GABAA	Positive (Majewska et al., 1986)
	nAChR	Negative (Dazzi et al., 1996)
	5-HT3	Negative (Wetzel et al., 1998)
PREGS	NMDA	Positive (Wu et al., 1991)
	GABAA	Negative (Akk et al., 2001)
	AMPA/Kainate	Negative (Wu et al., 1991;Yaghoubi et al., 1998)
	nAChR	Positive (Dubrovsky, 2005)
	Sigma 1	Negative (Monnet and Maurice, 2006)
	5-HT3	No effects (Wetzel et al., 1998)

1.3 Ns behavioural profile

Similar to other $GABA_AR$ positive modulators such as benzodiazepines or barbiturates, Ns that act as positive modulators of $GABA_ARs$ have been demonstrated to exert an anxiolytic (Bitran et al., 1993; 1995; 2000), hypnotic (Damianisch et al., 2001) and anticonvulsive effect (Landgren et al., 1997). In fact, the role that Allopregnanolone plays on behaviour when is systemically injected, has been well established. However, the implication of the brain structures that have a role in the behavioural effects of Ns still needs to be clarified. In this sense, the amygdala has been postulated as a relevant area. It has been hypothesised that this nucleus can modulate the

anxiolytic effects of Ns, as microinjections of Allopregnanolone into the central nucleus of the amygdala showed anxiolytic effects in the elevated plus maze (EPM) and conflict test (Akwa et al., 1999). Another area postulated to be relevant regarding Ns effect on behaviour, is the hippocampus. Previous studies demonstrated that systemic administration of finasteride (a 5α reductase inhibitor) decreases hippocampal levels of Allopregnanolone and increases depressive behaviour in pregnant rats (Frye and Walf, 2002; Frye and Walf, 2004b). In this line, increased hippocampal Allopregnanolone levels have also been demonstrated to increases exploratory and decreases anxiety-like behaviour (Frye and Rhodes, 2007). Furthermore, other studies also showed that microinjection of pregnenolone into the dorsal hippocampus induced a decrease in anxiety scores on the EPM (Bitran et al. 1999). However, other authors showed no effects of hippocampal Allopregnanolone administration in the EPM or shock-probe burying test (Engin and Treit, 2007), see Table II. Concerning learning and memory, Allopregnanolone administration has been reported to exert a detrimental profile, similarly to other $GABA_AR$ positive modulators (Mayo et al., 1993; Ladurelle et al., 2000; Matthews et al., 2002). Previous studies showed that Allopregnanolone deteriorates spatial learning in the Morris test (Johansson et al., 2002; Matthews et al., 2002; Turkmen et al., 2006), and also non-associative learning tasks in the "Y" maze (Ladurelle et al., 2000), when administered systemic or intraventricularly. Furthermore, it has been postulated that the increase in Allopregnanolone levels induced by ethanol administration, could be participating in the detrimental effects of alcohol on learning (Silvers et al., 2003).

The promnesic effect of other Ns such as PREGS or DHEA has been well-documented (Vallée et al., 1997; Darnaudéry et al., 2000; Johanson et al., 2002). For instance, improvement in memory retention when PREGS was systemically administered (Isaacson et al., 1995) has been demonstrated, but also a reversal of the memory impairment induced by alcohol administration (Melchior and Ritzmann, 1996) or scopolamine (Meziane et al., 1996; Mathis et al., 1996; Vallée et al., 2001) and in old cognitively impaired rats (Vallée et al., 1997). Moreover, PREGS promnesic effects have also been described when injected into the amygdala (Flood et al., 1995), into the hippocampus (Darnaudéry et al., 2000), intraventricularly (Flood et al., 1992) or into the nucleus basalis magnocellularis (NBM) (Pallarès et al., 1998) in several learning tests. However, PREGS effects on passive avoidance in a previous study carried out in our laboratory showed a detrimental effect of PREGS in the passive avoidance when administered intrahippocampally but after the application of environmental stress (Martin-Garcia and Pallarès, 2008). The promnesic effect of PREGS has been postulated to take place through the potentiation of NMDA receptors located in the pyramidal neurons of the hippocampus (Bowly, 1993). However, it has also been suggested that PREGS enhancing profile could be done through the potentiation of the cholinergic neurons (Pallarès et al., 1998; Darnaudéry et al., 2000). Table II show anxiety effects of Ns administration

in several brain structures further experiments are detailed in the Annex 1.

Table II: Effects of Allopregnanolone, progesterone and pregnenolone administration on anxiety-like behaviour

Neurosteroid	Administration	Test	Effect	Ref.
Allopregnanolone	Amygdala	EPM	Anxiolytic	Akwa et al., 1999
	Amygdala	Conflict test	Anxiolytic	Akwa et al., 1999
	Hippocampus	EPM	No response	Engin & Treit, 2007
	Hippocampus	Shock-probe burying test	No response	Engin & Treit, 2007
	Ventricular	Y maze	Detrimental	Ladurelle et al., 2000
Progesterone	Amygdala	EPM	Anxiolytic	Frye and Walf, 2004a
	Amygdala	Open Field	Anxiolytic	Frye and Walf, 2004a
Pregnenolone	Hippocampus	EPM	Anxiolytic	Bitran et al., 1999

2. Ns and postnatal development

Ns synthesis has been demonstrated in early stages of foetal development of the rat brain (Pomata et al., 2000). During the last pregnancy period Allopregnanolone levels highly increase and decline prior to parturition in the forebrain of embryonic rats (Grobin and Morrow, 2001), see Fig 4a. This increase in Allopregnanolone levels during pregnancy has been proposed to be part of a protective mechanism against gestational stress. In this sense, it has been described that Allopregnanolone induces central opioid inhibition of neuroendocrine stress responses during pregnancy (Brunton et al., 2009; Hirst et al., 2009). Furthermore, Allopregnanolone has also been described to be particularly relevant during developmental stages. Although Allopregnanolone is present in foetal brain in similar levels to adults (Kellogs et al., 2006), its synthesis has been described to fluctuate during the postnatal developmental period (Grobin and Morrow, 2001; Grobin et al., 2003; Griffin et al., 2004). From the day of birth and in the two first weeks of life, cortical Allopregnanolone levels show important fluctuations, as showed by an initial elevation in the day of birth and a progressive decrease in the first week followed by a secondary elevation during the second week, reaching maximum values between postnatal days 10-14 in rats (P10-P14) (Grobin and Morrow, 2001; Grobin et al., 2003). The finding of the secondary peak in Allopregnanolone present during at time of a remarkable change in $GABA_AR$ signalling (from excitatory to inhibitory) suggests that GABAergic Ns modulation may participate in the normal developmental of GABAergic neurotransmission (Grobin and Morrow, 2001), see Fig 4b.

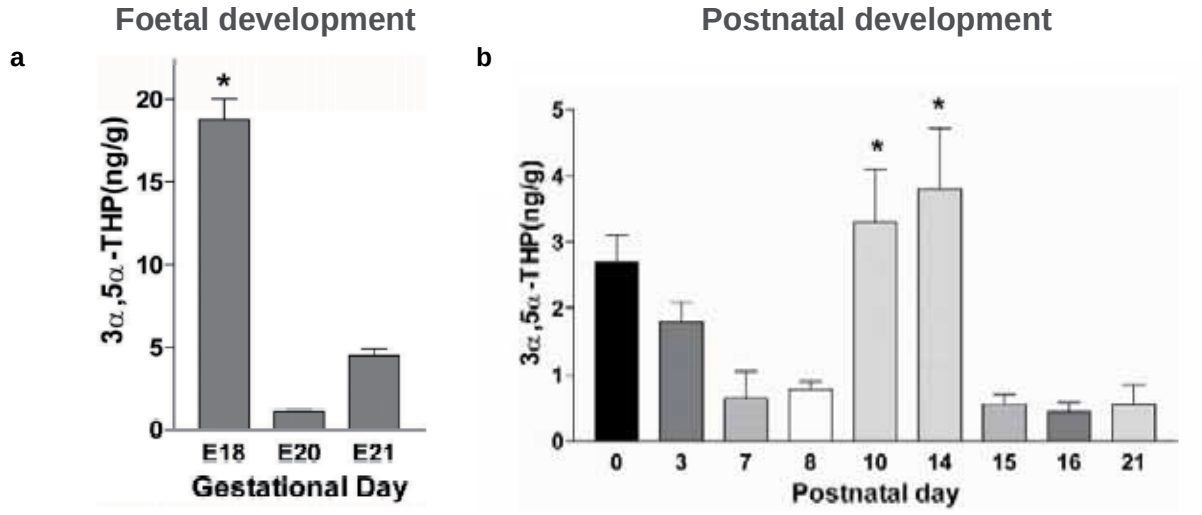

Fig 4. Allopregnanolone levels during postnatal development. **a)** Allopregnanolone levels in the forebrain of embryonic rats fall prior to partiturion (P21). **b)** Fluctuations of cortical Alloprenganolone levels after birth (P0) and during postnatal development. Figure from Grobin and Morrow, 2001.

2.1 Neonatal Ns levels and CNS maturation: Effects on hippocampal development

It has been documented that the alteration of the physiological levels of Ns in early neonatal phases alters the maturation of certain cerebral structures such as the meso-cortical and meso-striatal dopaminergic pathways (Muneoka et al., 2002; Muneoka and Takigawa, 2002), the adenosinergic systems throughout A1 (Muneoka et al., 2002) or A2A receptors (Shirayama et al., 2001) or the GABAergic thalamic-cortical system (Grobin et al., 2003; Gizerian et al., 2004). Moreover, exogenous administration of Ns has also been described to alter hippocampal maturation (Shirayama et al., 2005). Other studies also indicated that the NS tetrahydrodeoxycorticosterone (THDOC) (that shares profile of action with Allopregnanolone as $GABA_AR$ allosteric positive modulator) has important modulatory effects in hippocampal GABAergic synapsis during development at concentrations that occur naturally in the brain (Cooper et al., 1999). Neonatal administrations of pregnenolone (from postnatal days 3-7), the main Allopregnanolone precursor by its conversion to progesterone, or the NS DHEA, increased the expression of microtubule-associated protein 2 (MAP2) in the granule cell layer of dentate gyrus and in the pyramidal cell layer of CA3 region at post-puberty (7 weeks of age) (Iwata et al., 2005). MAP2 protein is a cytoskeleton member detected mainly in dendrites that affects the shape, polarity and plasticity of neurons by controlling microtubule assembly. Thus, it has been proposed that exogenous NS during the neonatal period can bind to MAP2 and directly affect its expression and dendritic arborisation, and that this MAP2 increased expression might be an interesting phenotype involving stress and motivation because CA3 region of the hippocampus is vulnerable to stressful conditions, including elevated levels of glucocorticoids (Iwata et al., 2005). Other studies have also shown post-pubertal alterations in the hippocampal expression of the synaptic vesicle membrane-associated protein synapsin I, and also an increase in the number of neuropeptide Y-positive cells, in animals that were administered neonatally with pregnenolone or DHEA (Shirayama et al., 2005). Other changes such as an increase in the number and size of glial fibrillary acidic protein (GFAP) immunoreactive astrocytes or an increase in the extension of arborisation was seen in the overall hippocampus at both pre-puberty and post-puberty ages in animals that were neonatally injected with pregnenolone or DHEA from P3 to P7 (Shirayama et al., 2005). It is important to remark that some of the reported changes induced by gestational and perinatal Ns administration such as glial cell abnormalities (Cotter et al., 2001; García-Segura and Melcangi, 2006), changes in neuropeptide Y function (Redrobe et al., 2002) and alterations of synaptic proteins in the hippocampus have been related to psychiatric diseases such as emotional disorders, depression and schizophrenia (Vawter et al., 2002).

Regarding the effects of Allopregnanolone administration during early development stages, *in vitro* studies have shown that this NS induces cytoarchitectural regression in cultured fetal hippocampal neurons (Brinton, 1994). Other studies demonstrated that its administration in granule

cells of the dentate gyrus during postnatal period increases GABAergic conductance in hippocampal slices of rat pups (Mtchedlishvili et al., 2003). Studies carried out during late gestation of foetal sheep also reported that Allopregnanolone influences the rates of cellular apoptosis and proliferation in the hippocampus. In this way, finasteride treatment increased apoptosis in CA1 and CA3 hippocampal regions as well as astrocytes proliferation (Yawno et al., 2009). As these effects can be prevented by the co-administration of the Allopregnanolone analogous alfaxalone, it has been proposed that Allopregnanolone (and homologs) provide protection to the foetal brain against hypoxia and excitatory stress in late gestation and also have an important role in the modelling of the brain throughout de last stages of gestation (Yawno et al., 2009). Besides the impact of Allopregnanolone manipulation levels in the hippocampal maturation, other studies demonstrated that maintenance of this NS levels is also relevant for the maturation of other brain areas. Indeed, Grobin et al., (2003) showed an altered localization of cortical parvalbumin-positive interneurons of adult rats that were neontally administered with Allopregnanolone (10mg/kg) (Grobin et al., 2003) and a decrease in the number of total neurons in the medial dorsal thalamus (Gizerian et al., 2004), further indicating that neonatal Ns have a relevant role during developmental stages and participate in the foetal and postnatal development of the hippocampus and other brain structures.

2.2 Neonatal Ns and behaviour

Manipulation of neonatal Ns levels throughout early development has been implicated in the alteration of adolescent and adult behaviours (Martín-García et al., 2008; Darbra and Pallarès, 2009; 2010; 2011; 2012). In this sense, previous results of our laboratory demonstrated that Allopregnanolone administration (10 mg/kg) at P5 increases novelty-directed locomotion in the open field and decreases the anxiolytic-like profile of the benzodiazepine lorazepam in the EPM in adult male rats (Darbra and Pallarès, 2009). This dose of Allopregnanolone was chosen as a similar dose (8 mg/kg) in adult animals raises cortical Allopregnanolone levels to the range observed with swim stress (Vallée et al., 2000). Moreover, the habituation of activity in the open field test in adulthood was slowed down by the neonatal Allopregnanolone administration (at the same dose) (Darbra and Pallarès, 2009). In this way, it has been shown that neonatal stress also increased the locomotor activity and slowed down its intra-session habituation in the open field test in adult male rats (Duvovicky et al., 1999), suggesting a possible relation between neonatal stress and endogenous Allopregnanolone levels. Also, other authors have documented an increase in the adult locomotor response to amphetamine as a consequence of neonatal administration of Allopregnanolone (Gizerian et al., 2006). It has been reported that neonatal pregnenolone administration (10 µg/g from P3 to P7), induced hyper-locomotion in rats in the open field at pre and post-puberty, an increase that was more persistent in females than in males (Muneoka et al.,

2002). The reported increase in novelty-directed locomotion seems indicate a possible reduction in the environmental stress related to the novelty exposition.

On the other hand, neonatal finasteride administration (50 mg/kg, from P5 to P9) increases emotional reactivity in situations of stress or conflict in the adolescent age, as reflected by the reduction in exploration of a novelty situation (decreasing novelty-directed activity and holes exploration in the Boissier test) (Darbra and Pallarès, 2010). Also, it has been documented an anxiogenic-like profile of neonatal finasteride administration when the EPM was tested in adult animals that were intrahippocampally infused with Allopregnanolone, PREGS or vehicle (Martín-García et al., 2008). This anxiogenic profile could be related to a decrease of adult endogenous Allopregnanolone levels induced by neonatal finasteride administration during neonatal stages, as it has been reported by other authors (Paris et al., 2011). In agreement, termination of pseudo-pregnancy state (characterized by an abrupt decrease in progesterone and Allopregnanolone levels) induced an anxiogenic-like profile in the EPM (Bitran and Smith, 2005). Taken together, this data point out that fluctuations of NS levels during this developmental period modify adult behaviour resulting in a changes in anxiety-like behaviours.

3. GABA$_A$R and development

GABA is the major inhibitory neurotransmitter in the adult brain, but early in development GABA$_A$R actions are excitatory (reviewed in Ben-Ari et al., 2007). During brain development there is a progressive shift in the pattern of network activity toward an adult form (from excitatory to inhibitory) that is sustained by a sequence of gradual changes in voltage and transmitter gated currents. Depolarizing GABA during development and the subsequent shift to inhibitory transmission are widely accepted as key events in the proper development of neuronal networks and brain structures (reviewed in Ben-Ari et al., 2007). Indeed, the progressive reduction of intracellular chloride in neurons and the associated switch in GABA polarity has been confirmed in several structures widespread along the CNS and in a wide range of animal species (from worms to higher mammals) *in vitro* and *in vivo* studies (Obata et al., 1978; Ben-Ari et al., 1989, 1994; Owens et al., 1996; Ben-Ari, 2002; Owens and Kriegstein, 2002; Tyzio et al., 2008; and reviewed in Ben-Ari et al., 2007 and Blaesse et al., 2009). Furthermore, this change has been demonstrated to participate in postnatal neurogenesis, neuronal migration, synaptogenesis and prunning (Groc et al., 2002; Groc et al., 2003; Manet et al., 2005; Manet et al., 2006; Akermand and Cline, 2007), and necessary to accomplish the formation of neuronal circuitry.

Activation of GABA$_A$Rs during postnatal development produces membrane depolarization sufficient (in some cases) to reach spike threshold and to generate sodium action potentials (Dzhala and Staley, 2003; Sipila et al., 2006) or activation of voltage gated calcium (Ca2+) channels (Leinekugel et al., 1995, 1997; Bray and Myenlieff, 2009). Furthermore, depolarization induced by the activation of GABA$_A$Rs have been reported to be sufficient to remove the voltage-dependent magnesium blockade from NMDA channels operating in synergy with NMDA and AMPA receptors in the developing circuit (Ben-Ari et al., 1997), see Fig 5. This synergistic action is a key factor that enhances neuronal activity and facilitates the generation of synchronized patterns that make the neurons to fire together, the so-called Giant depolarizing potentials (GDPs) (Ben-Ari et a., 1989; Ben-Ari et al., 1997; Ben-Ari, 2002; Ben-Ari et al., 2007). GDPs are synchronized events that engage large numbers of neurons to fire together (and remain together) (Leinekugel et al., 1998; Ben-Ari et al., 2007; Dehorter et al., 2012) as a result of depolarizing actions of GABA$_A$Rs that occur only during developmental stages (Ben-Ari et al., 1989). This pattern of activity is orchestrated by a subset of GABAergic hippocampo-septal interneurons described as “Hub neuronal generators“ (Bonifazi et al., 2009). Although it has been extensively studied in the hippocampus, it has also been confirmed in a wide range of other brain structures such as the entorhinal cortex, the neocortex or the striatum, as a consequence of depolarizing actions of GABA$_A$R (Ben-Ari et al., 1989). Those synchronized events have been reported to occur between the end of the first and the second postnatal week when GABA$_A$R switches from excitatory to

inhibitory in the hippocampus (reviewed in Ben-Ari et al., 2007). Developmental changes in GABA signalling are determined by a reduction of intracellular chloride concentration. Several chloride co-transporters control neuronal chloride homeostasis. Among them the NKCC1 (accumulate chloride) and KCC2 (chloride extruder) play a pivotal role in GABA$_A$Rs actions (Riviera et al., 1999; Ganguly et al., 2001; Stein et al., 2004; Chudotvorova et al., 2005; Ikeda et al., 2005; Ben-Ari et al., 2007; Bray and Myenlieff, 2009), see Fig 7. The KCC2 is the principal chloride extruder expressed in adult neurons. Immature neurons maintain intracellular Cl^- concentration at a high level and exhibit a shunting or membrane depolarization upon activation of GABA$_A$Rs due to the increased expression of NKCC1 and the low expression of KCC2. After birth hippocampal KCC2 is barely detectable and increases progressively until reaching an adult profile. At maturity, KCC2 is up regulated whereas NKCC1 down-regulates and maintains low intracellular Cl^- concentrations resulting in GABAergic inhibitory responses (Payne et al., 2003; Ben-Ari et al., 2007), see Fig 6-7. Several studies have provided a detailed description of embryonic and postnatal maturation in the hippocampus (and other structures) (reviewed in Ben-Ari et al., 2007 and Dehorter et al., 2012). In this sense, it has been reported that GABA$_A$Rs remain excitatory until P6-P8 (Cherubini et al., 1991; Riviera et al., 1999; Gubellinin et al., 2001), whereas other studies indicated that GABA$_A$Rs switch occur later (P8-P10) *(*Tyzio et al., 2006; Sipila et al., 2006). However, the intrinsic signalling responsible for KCC2 up-regulation remains to be elucidated. Previous studies have reported the relevance of increased GABAergic activity (when still is excitatory) as an autocrine way to increase KCC2 expression rather than other signals (Khirug et al., 2010). However, the impact in the CNS of early expression of the KCC2 is controversial. Some in vitro studies reported that induced expression of KCC2 increases the number of functional synapses (Chudotvorova et al., 2005) and is involved in the dendritic spine formation (Hong et a., 2007). However, other studies reported that early expression of KCC2 stops neuronal migration and cortical connectivity *in vitro* (Bortone and Polleux, 2009) and that KCC2 interacts with cytoskeleton proteins to promote spine development (Li et al., 2007). Further experiments also demonstrated that reduction of KCC2 expression during embryonic development alters morphological maturation of neonatal cortical neurons in vivo (Cancedda et al., 2007).

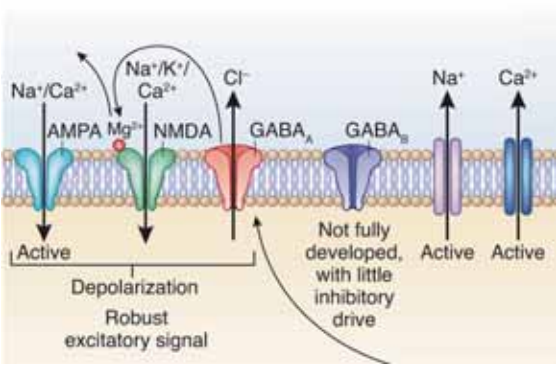

Fig 5. Excitatory actions of GABAAR are capable to activate other receptors such as NMDA or AMPA: In the immature brain, GABAA-mediated excitation serves to depolarize the membrane, resulting in activation of the NMDA and AMPA channel and depolarization. (Figure extracted from Scott and Holmes, 2012).

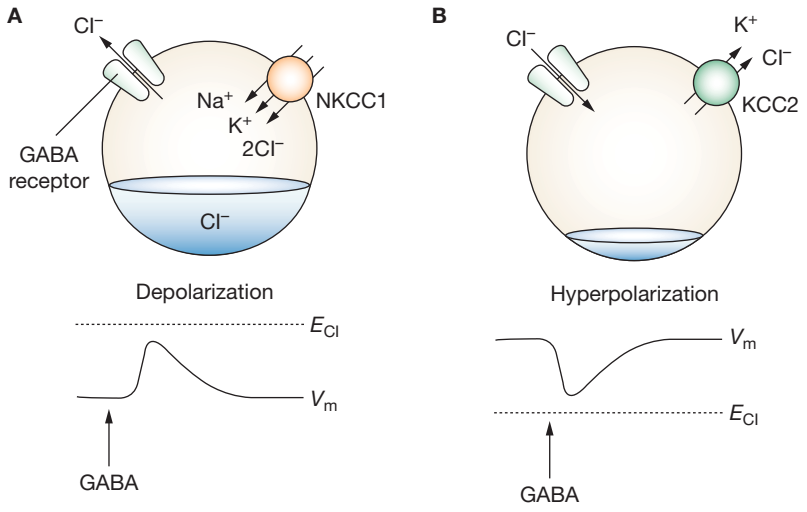

Fig 6: Changes of intracellular Cl- concentration during development and at maturity. a) Schematic representation of GABA excitatory actions postnatal during development. **b)** Schematic representation of GABA switch in mature neurons around second postnatal week of the rat life. (Figure extracted from Kahle et al., 2008).

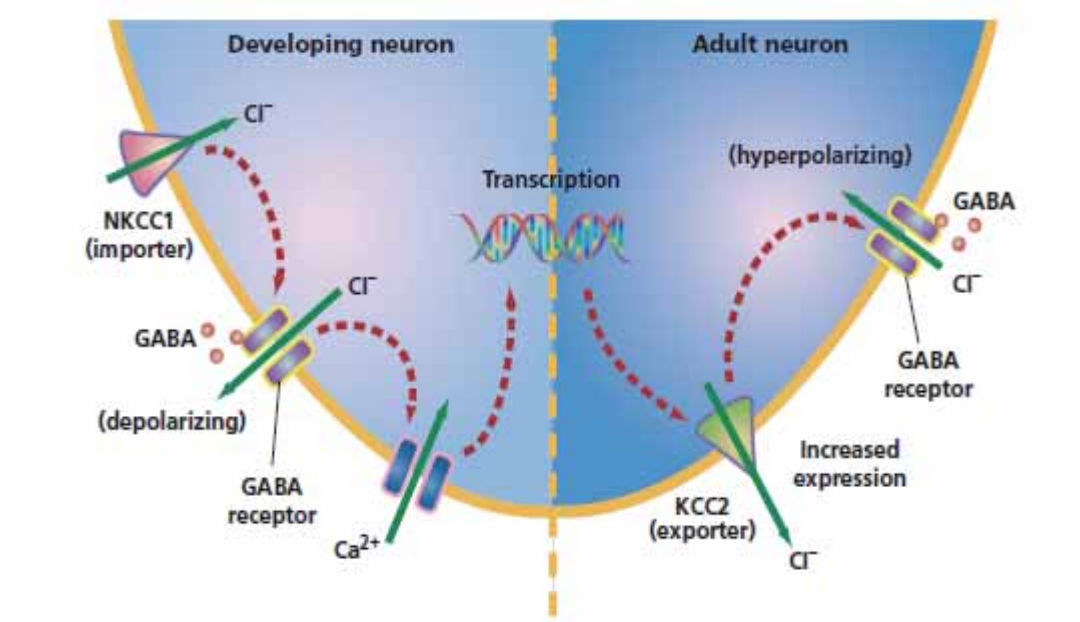

Fig 7: Changes in NKCC1 and KCC2 expression during development and at maturity. (Left) NKCC1 is mostly expressed during developmental stages, while KCC2 is highly expressed at maturity **(Right).** The increase in the neuronal expression of KCC2 turns GABA Rs polarity into hyperpolarizing and thus, inhibitory. (Figure extracted from Staley and Smith, 2001).

Changes between GABA$_A$R subunits have also been reported during early development and throughout life, showing a temporal and regional specific expression (Laurie et al., 1992). In this sense, previous studies demonstrated that GABA$_A$R subunits: A) Differ substantially from embryonic to neonatal brain; B) Some population persist throughout development and change in

adult brain; C) Other do not change and express as in the adulthood (Laurie et al., 1992). In the hippocampus, α2 and α5 subunits increase from embryonic day 17 until adulthood, α1 increases progressively being strongly expressed in the adulthood, while the α4 and δ do not increase its expression until P12 (Laurie et al., 1992). Changes in the expression of GABA$_A$R subunits could result in different effects of Allopregnanolone during development, due to differences in the sensitivity of the GABA$_A$R to Allopregnanolone binding. *In vitro* studies in hippocampal cells showed an increase in GABA$_A$R sensitivity to Allopregnanolone modulation from P7 to P14 (Mtchedlishvili et al., 2003), coincident with the increase in the expression of α4 and δ subunits around P12 (Laurie et al., 1992). Other studies demonstrated that tonic inhibition in dentate granule cells is larger during second postnatal week (Holter et al., 2010). Recent findings also pointed out an increase in cell surface expression of α4ß2δ GABA$_A$R due to Allopregnanolone administration *in vitro,* supporting the hypothesis that this receptor is highly responsive to the presence of the NS (Kuver et al., 2012). Concerning Allopregnanolone effects on other GABA$_A$R subunits, previous study showed that neonatal Allopregnanolone prevented the increase of α2 subunit in cortical cultures (Poulter et al., 1997), suggesting that local synthesis of Allopregnanolone may alter the time of GABA$_A$R subunits increase during early stages of development (Mellon et al., 2007). Thus, modulation of GABA$_A$Rs functioning during development arises in part from changes in the chloride ion reversal potential (mediated by changes in the expression of chloride co-transporters) and different expression of GABA$_A$Rs subtypes.

Objectives & hypothesis

Objectives and hypothesis

The main objective of the present work is to assess the effects of neonatal manipulation of NS levels on behavioural response to intrahippocampal Ns administration and the participation of hippocampal $GABA_A$Rs.

Specific objectives

1. To study the role of dorsal hippocampus in the Ns modulation on exploration, anxiety-like behaviour and aversive learning.

2. To study the effects of neonatal manipulation of Ns levels in the modulation on exploration, anxiety and aversive learning in response to intrahippocampal administration of Ns.

3. To study the mechanisms underlying hippocampal and behavioural changes due to neonatal manipulation of Ns levels by assessing:

    - Exploration and anxiety-like behaviour in response to elevation of Allopregnanolone levels (progesterone administration)..

    - Hippocampal $GABA_A$R subunits: Focused on α4 and δ subunits expression.

    - Hippocampal KCC2 expression.

Hypothesis

1rts hypothesis: Hippocampus participates in the effects of Ns on exploratory, anxiety-like and aversive learning behaviour.

2nd hypothesis: Modulation of Ns levels during postnatal development affects hippocampal maturation and behavioural response to intrahippocampal Ns administration.

3rd hypothesis: Manipulation of neonatal Ns levels alters hippocampal $GABA_A$Rs containing α4 and δ subunits.

4rd hypothesis: Neonatal manipulation of Ns can alter the hippocampal expression of KCC2.

Experiment 1

Research article
"Neurosteroids infusions into CA1 hippocampal region on exploration, anxiety-like behaviour and aversive learning"

Research article
"Alteration of neonatal Allopregnanolone levels affects exploration, anxiety, aversive learning and adult behavioural response to intrahippocampal neurosteroids"

Poster presentation (Annex 2)
"Effects of neurosteroids into CA1 hippocampal region on exploration, anxiety-like behaviour and aversive learning" Societat Catalana de Biologia (2010)

)

Poster presentation (Annex 3)
"Neonatal disturbed Allop levels affect adult performance of the passive avoidance and alter the adult CA1 hippocampal response to neurosteroids" Behavioural Brain Research Society (2011)

In the present experiments we investigated the role of dorsal hippocampus in the modulatory effects of Ns on exploratory, anxiety-like and aversive learning responses. For that purpose animals were intrahippocampally administered with Allopregnanolone (2ug/0.5ul), PREGS (5ng/0.5ul) or vehicle in each hippocampus (80-90 days old) and then tested in the Boissier (exploratory behaviour), EPM test (anxiety-like behaviour) and passive avoidance (aversive learning) in the adult age.

In addition, we tested whether manipulation of Ns during development changed exploratory, anxiety-like and aversive learning responses to intrahippocampal administration of Ns in the adulthood. For that purpose animals were administered with Allopregnanolone (20mg/kg), finasteride (50mg/kg) or vehicle from P5 to P9. To control manipulation effects during early stages a no handled group (NH) was also added to the experiment. In adult age, animals were intrahippocampally administered with Allopregnanolone (2ug/0.5ul), PREGS (5ng/0.5ul) or vehicle in each hippocampus and then tested in the Boissier (exploratory behaviour), EPM test (anxiety-like behaviour) and passive avoidance (aversive learning) in the adult age.
An schematic representation of the experimental design can be observed in the following page.

Specific objectives

-To study the role of dorsal CA1 hippocampus on exploration, anxiety and aversive learning in response to Allopregnanolone or PREGS administration.

-To study if neonatal Ns manipulation modify behavioural response to intrahippocampal administration of Allopregnanolone or PREGS.

Experiment 1a: Neurosteroids infusion into CA1 hippocampal region on exploration, anxiety-like behaviour and aversive learning

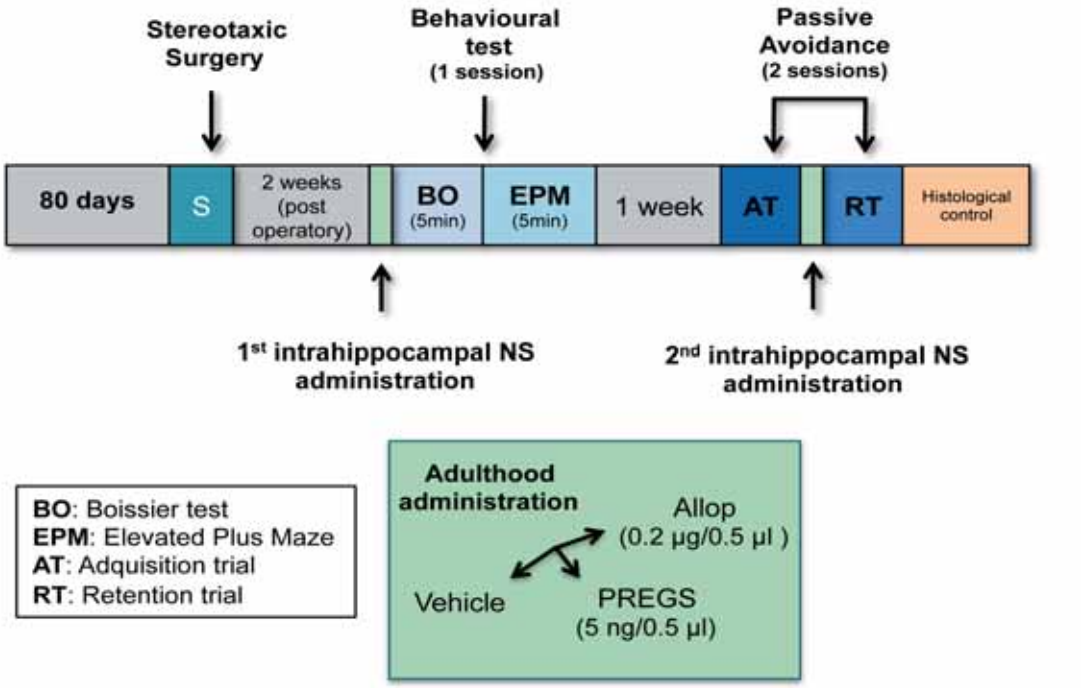

Fig 8: Experimental design

Experiment 1b: Alteration of neonatal Allopregnanolone levels affects exploration, anxiety, aversive learning and behavioural response to intrahippocampal neurosteroids

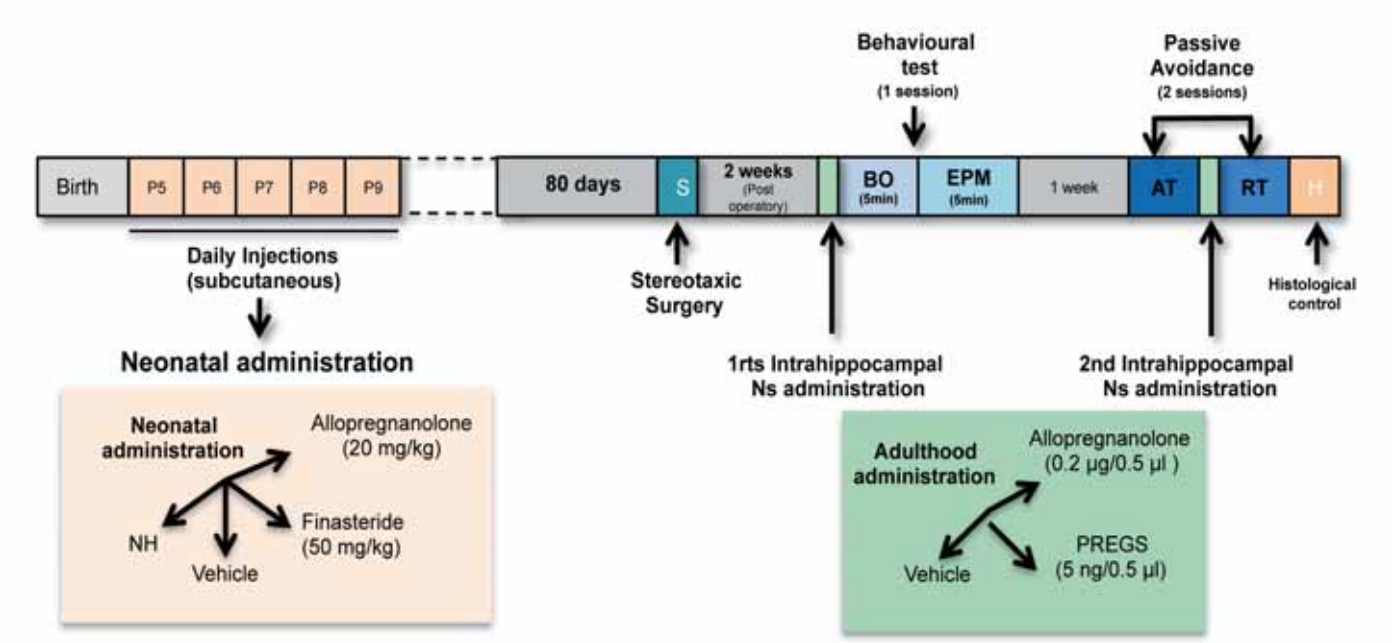

Fig 9: Experimental design

Experiment 1a:
Hippocampus and Ns modulation of behaviour

Research article

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Research report

Neurosteroids infusion into the CA1 hippocampal region on exploration, anxiety-like behaviour and aversive learning

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ABSTRACT

Neurosteroids (NS) are substances synthesised *de novo* in the brain that have rapid modulatory effects on ionotropic receptors. Specifically, NS can act as positive allosteric modulators of GABAA receptors as pregnanolone or allopregnanolone (Allop), or GABAA negative modulators and NMDA positive modulators as pregnenolone (PREG) or dehydroepiandrosterone (DHEA) and their sulphate esters (PREGS and DHEAS). Given this, their role in anxiety and emotional disturbances has been suggested. In addition, NS such as PREGS or DHEAS have demonstrated a promnesic role in several learning tests. The aim of the present work is to highlight the role that the dorsal (CA1) hippocampus plays in the behavioural profile of NS such as Allop and PREGS in tests assessing exploration, anxiety and aversive learning in rats. For this purpose, animals were administered intrahippocampally with Allop (0.2 μg/0.5 μl), PREGS (5 ng/0.5 μl) or vehicle in each hippocampus, and tested in the Boissier and elevated plus maze (EPM) tests. For learning test we have chosen the passive avoidance paradigm. Results indicate that intrahippocampal administration of Allop enhances exploration, reflected in an increase in the total and the inner number of head-dips. Allop-injected animals also showed an increase in the percentage of entries into the open arms of the EPM, suggesting an anxiolytic-like profile. In addition, post-acquisition PREGS administration enhanced passive avoidance retention, while post-acquisition Allop administration had no effects on aversive learning retention. These results point out the important role of the dorsal (CA1) hippocampus in several NS behavioural effects, such as exploration, anxiety, learning and memory.

1. Introduction

The term neurosteroid (NS) refers to steroids which can be synthesized *de novo* by the nervous tissue such as pregnenolone, progesterone, allopregnenolone (Allop) and their sulphate esters [1,2]. In the last three decades many studies have emerged stressing the relevance of their multiple roles in normal and pathological behaviour, ageing processes and regeneration of damaged tissue [2–4]. NS levels are susceptible to fluctuation depending on physiological or psychopathological states such as age, stress, pregnancy, menstruation or the alcohol ingestion. Therefore NS fluctuations can constitute an important target related to social, cognitive and emotional impairment as well as age-related diseases [2,5,6].

Ring A reduced pregnanes like Allop can, after oxidation, regulate gene expression via the P receptor [7]. However, besides their genomic effects, NS can also alter neuronal excitability via the cell surface by interacting with specific neurotransmitter receptors [8]. Concretely, NS as Allop binds to GABAA receptors increasing the time and frequency of the opening Cl-channel [9]. Instead, NS such as pregnenolone sulphate (PREGS) has been described as both a positive NMDA receptor modulator [10] and GABAA negative modulator [11]. Previous behavioural studies have shown that the increase of progesterone or progesterone metabolites (such as Allop) in proestrous female rats increases the anxiolytic profile in anxiety test as the EPM or the open field [12]. In addition, systemically injected Allop showed an anxiolytic profile in the mirrored chamber test [13] similar to benzodiazepines or barbiturates. In this sense, NS that act as positive allosteric modulators of GABAA have been described as anxiolytic, sedative and anticonvulsive substances [14]. On the other hand, systemically administered PREGS showed a biphasic effect on anxiety responses depending on the dosage, in the mirrored chamber [13] but also in the EPM [15]. In fact, the role that NS play in behaviour when systemically injected, has been well established. However, the relevance of the structures that have a role in the behavioural effects of NS, generate contradictory results and hence, needs to be clarified. In this sense, the amygdala has been postulated as a region of importance [16–18]. In fact, it has been hypothesised that this nucleus can modulate the anxiolytic effects of NS [16]. At this respect, microinjections of Allop into the central nucleus of the amygdala showed anxiolytic effects in the EPM and conflict test [16]. In addition, the administration of progesterone into the medial nucleus of the amygdala also

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showed anxiolytic-like profile in the open field and in the EPM [17]. Moreover, as a part of the limbic system and thus, as an important area for the modulation of affective behaviours, hippocampus has also been described to play a role in emotional processes. Instead, the role it plays in the NS modulation seems to be contradictory. Previous studies have shown that systemic administration of finasteride (5-alpha reductase inhibitor that impedes the first step of the conversion from progesterone to Allop) decreases hippocampal levels of Allop and increases depressive behaviour in pregnant rats [19,20]. Moreover, intrahippocampal administrations of Allop or pregnanolone (also a GABAA positive modulator) have also shown an anxiolytic-like profile in the open field test [21] and in the EPM, respectively [22]. However, Allop administration into the hippocampus has failed to elicit any anxiolytic effect in the EPM or the in the shock-probe test [23]. Given this, further studies focusing on the role of Allop into the hippocampus are necessary to highlight the importance that this limbic structure plays in the NS modulation of anxiety.

Regardless of learning and memory, the role of the hippocampus in the effects of NS is well-documented [24–26]. Several experiments indicate that the hippocampus is related to learning and memory facilitation induced by PREGS or dehydroepiandrosterone (DHEA) administration, for instance in old cognitively impaired rats in maze tests [5] or in alcohol-drinking rats in lever-press conditioning [27]. In addition, intrahippocampal PREGS administration has also shown to improve the active avoidance retention in a T-maze [28]. However, PREGS effects on passive avoidance are not so well-documented. At this respect, one previous study carried out in our laboratory showed a detrimental effect of PREGS in the passive avoidance when administered intrahippocampally but after the application of environmental stress [29]. Instead, other studies have shown an improvement in memory retention when PREGS was systemically administered [30] but also a reversal of the memory impairment induced pharmacologically [31]. Thus, new experiments are needed to assess the involvement of hippocampus on NS performance in aversive learning. On the contrary, the observed profile of GABA-positive NS such as Allop on learning and memory has been well established to be detrimental [32–34]. Several studies showed that Allop deteriorates spatial learning in the Morris test [26], and also non-associative learning tasks in the "Y" maze [34], especially after its pre-acquisition administration.

Since the role that the dorsal (CA1) hippocampus plays on Allop modulation of anxiety and PREGS in passive avoidance is controversial, the aim of the present work is to point out such role on novelty exploration, anxiety and aversive learning. Based on the GABAA positive modulation profile, we hypothesise that intrahippocampal Allop administration will increase the novelty exploration in the Boissier test and also reduce the anxiety responses in the EPM. In addition, based on the hippocampus implication on PREGS promnesic profile showed in several learning tests and the potentiation of memory retention in the passive avoidance when PREGS was injected systemically, we hypothesise that retention of passive avoidance will be increased in subjects injected with intrahippocampal PREGS and decreased in Allop subjects.

2. Methods

2.1. Animals

37 male Wistar rats raised at in-house colony (Laboratori de Psicobiologia, Universitat Autònoma de Barcelona, Barcelona, Spain) and allowed with food and water *ad libitum* were used. Rats were housed in a temperature controlled animal room (22–24 °C) on a 12-h light/dark cycle. All animals were obtained, housed, and sacrificed in accordance with the protocol approved by the Committee of the Universitat Autònoma de Barcelona for Care and Use of Experimental Animals and the Department of Environment from Generalitat de Catalunya (Regional Government). This protocol follows the guidelines approved by the European Council Directive (86/609/ECC) for care and use of laboratory animals.

2.2. Surgery

Surgery was carried out at 80-day-old animals. For permanent implantation of cannula, animals were anesthetized with ketamine (120 mg/kg) and xylazine (10 mg/kg). Using standard stereotaxic techniques, bilateral 21-gauge stainless steel double guide cannula (model C232G-3.8; Plastics One, Brilaney, Dusserldorf, Germany) was implanted into the CA1 region of the dorsal hippocampus (anteroposterior, 3.6 mm; mediolateral, 1.8 mm; dorsoventral, 1.8 mm from bregma). Each guide cannula was fitted with dummy cannula with no projection. Double guide cannulae were permanently mounted to the skull with four screws and dental cement.

2.3. Neurosteroid administration

After recovery from surgery the day of behavioural testing, freely moving rats were injected with Allop (0.2 μg/0.5 μl), PREGS (5 ng/0.5 μl) or vehicle (20% cyclodextrin((2-hydroxypropyl)-β-cyclodextrin)) in each hippocampus. NS doses were determined based on previous studies carried out in our laboratory, according to be previously found effective in altering anxiety behaviours and also learning and memory [21,29,35,36]. Allop and PREGS were dissolved in 0.9% NaCl by sonication for 10 min and suspended in 20% cyclodextrin ((2-hydroxypropyl)-β-cyclodextrin). For injections, double internal 28-gauge stainless steel cannula (model C232I; Plastics One) was inserted extending 1 mm below the guide cannula to a final depth of 2.8 mm from the skull (see Fig. 1). Injection needles were connected with polyethylene tubing to a microsyringe (10 μl) driven by the infusion pump (Harvard 22). Solutions were infused during 1 min at a constant rate of 0.5 μl/min. Control rats received the same volume (0.5 μl) of vehicle at the same rate of infusion. Injection needles were removed from the guide cannula 2 min after infusions in order to prevent drug reflux.

2.4. Experimental procedures

After surgery, males were randomly assigned to different intrahippocampal groups of injection. Animals were allowed 10–15 days to recover from surgery before injections and the behavioural evaluation. Animals were injected twice with the same NS: immediately before the Boissier test and immediately after the acquisition trial of the passive avoidance. The EPM was carried out immediately after the Boissier test (5 min after the first injection). The passive avoidance acquisition was performed one week after the Boissier and EPM tests. Retention of passive avoidance was performed 24 h after acquisition.

2.5. Boissier exploration test

A square wooden arena (58 cm × 58 cm × 58 cm) with 16 equidistant holes (5 cm in diameter) was used for the Boissier test. The apparatus was situated in a room lit by a bright light (300 lx mean). This test measures activity and provides a relatively reliable measurement of stimulus-directed exploratory behaviour [37,38]. It was tested for 5 min and was evaluated by means of an activity monitoring system (SMART, Letica, Barcelona, Spain). This system is based on the automated analysis of real-time video-images, recorded by a video camera that is suspended from the ceiling over the arena. The distance moved was recorded for the total arena as locomotor activity, as well as for a virtual 29 cm × 29 cm centre zone. In addition, the number of entries into, and the time spent in the centre zone was also measured as anxiety relevant scores. Moreover, the number of head-dips (into the holes up to the eye line) was recorded as an exploratory measure. After each trial, the apparatus was cleaned with a water solution containing ethanol (20%, v/v) in order to prevent any olfactory-induced behavioural modifications.

2.6. Elevated plus maze

The EPM consisted of two open arms and two closed arms (10 cm wide × 50 cm long) perpendicular to each other, and elevated 50 cm from the floor. The walls of the closed arms were 40 cm high. The arms were connected by a central square measuring 10 cm × 10 cm. The animal was placed in the centre of the apparatus facing an open arm and its behaviour was recorded for 5 min. The maze was situated in a room lit by a dim light (36 lx mean) and it was cleaned between animals to minimize olfactory cues between trials. The following variables were recorded: number of open and closed arm entries; number of total entries; time spent in the open and closed arms and in the centre of the apparatus; and distance travelled across the open and closed arms by mean of a SMART (see above). The percentage of time spent in the open arms (% OAT) and the percentage of open arms entries (% OAE) were used as anxiety measures. The number of open arms plus closed arms entries was used as a measure of activity. An entry was counted whenever the animal crossed with all four pawns into an arm. Increased % OAT or % OAE is indicative of a reduced anxiety state in the EPM [39]. In addition the percentage of time spent in the centre of the maze was used as an independent measure of decision making [40,41]. The presence of differences between activity and decision making measures would suggest that any difference observed for anxiety measures could be part of a non-selective behavioural effect of NS administration. Tests were videotaped, monitored and scored at the time by an observer sited in the next room blinded of the NS

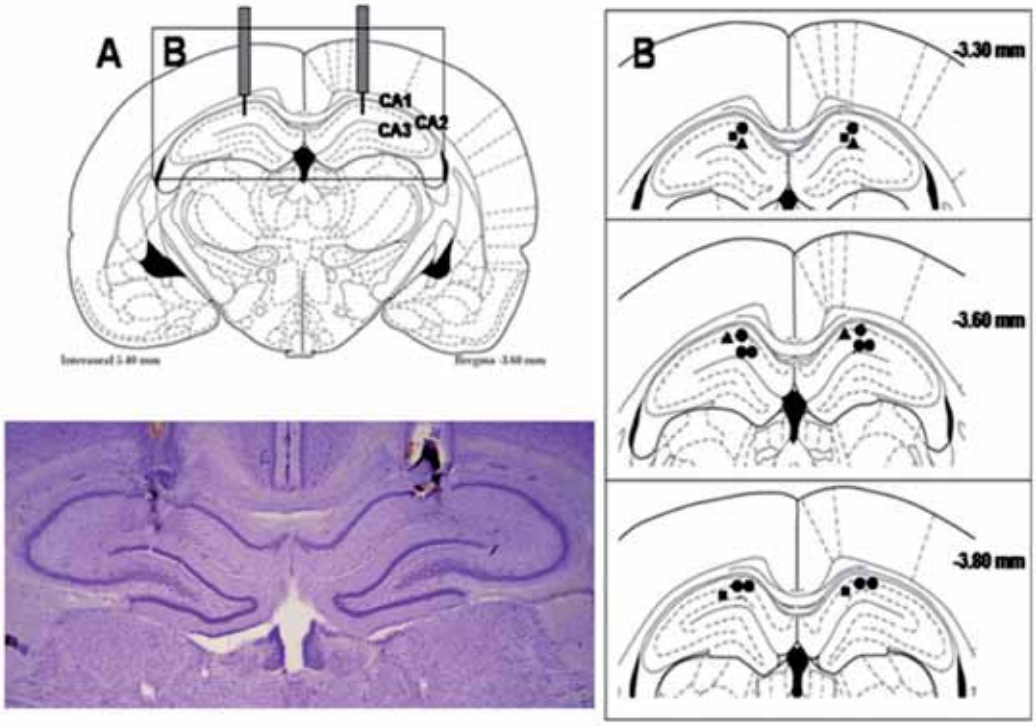

Fig. 1. (A) Schematic representation of the point of injection illustrating cannulae localization into the hippocampus, section 3.60 mm posterior to bregma. The marked area indicates the area shown in (B). (B) Bilateral cannula tip placements throughout the rostral-caudal extent of the CA1 area of the hippocampus (from 3.30 to 3.80 mm posterior to bregma). Each symbol refers to the number of cannula tip placements at the site indicated in the section (▲: $n = 2$; ■: $n = 1$; ●: $n = 4$,). (Photomicrograph of cresyl violet-stained coronal section showing cannula placement (magnification ×2).

administration. EPM was carried out during 5 min immediately after Boissier test, as it has been previously described [39].

2.7. Passive avoidance test

Rats were trained in a computerized one-trial-passive avoidance (Letica-Panlab, Barcelona, Spain) following the same order as in the Boissier and EPM tests. The experimental apparatus consisted of a squared white compartment (32 cm × 32 cm × 25 cm) communicated with a closed dark compartment (19 cm × 11 cm × 13 cm) through a motorized sliding door. In the acquisition trial, the subjects were placed in the illuminated compartment and were allowed to freely explore the apparatus. As soon as the animals entered the dark compartment (with the four pawns), the sliding door was automatically closed and the subject received an unavoidable scrambled high foot-shock of 0.5 mA during 3 s. These parameters were used based on previous works [29,36]. The latency to enter the dark compartment (acquisition latency (AL)) was measured. Animals were intrahippocampally injected with NS immediately after acquisition, and returned to their home cages. Retention of passive avoidance response was measured 24 h after the acquisition. For retention, animals were placed again in the white compartment, and the latency to enter the dark compartment was measured (RL). Animals were free of drugs during retention trial and no shock was administered. When the rat did not enter the dark compartment for 600 s, it was removed from the box and assigned a ceiling score of 600 s. Animals were weighed before each trail. Passive avoidance learning was measured as the difference between RL and AL.

2.8. Histological control

Animals were sacrificed by deep anaesthesia with i.p. sodium pentobarbital (200 mg/kg body weight at a concentration of 60 mg/ml), and their brains were removed and stored in 10% formalin. Brains were vobrosliced in 100 μ coronal sections, mounted and cresyl violet stained. Localization of the guide cannula and infusion sites was confirmed histologically for each rat. The diffusion of injection was controlled throughout a previous pilot study ($n = 3$) in which subjects were injected with the same volume (0.5 μl) of a tionine solution, to ensure that the drug was not diffused to other hippocampal or brain areas. Only animals in which histology confirmed that the infusion cannula was located within the CA1 area of the hippocampus were included in the analysis. Thus, 7 animals were excluded from statistical analysis because of placement errors: vehicle ($n = 1$), Allop ($n = 4$) and PREGS ($n = 2$). The final experimental groups were the following: vehicle = 11 Allop = 9, PREGS = 10. See Fig. 1 for correct cannula placements.

2.9. Statistical analysis

We used the STATISTICA package (StatSoft, Tulsa, USA) for data analyses. The normality of the data was assessed by means of the Kolmogorov–Smirnov test. Data from the Boissier test, EPM and passive avoidance were analyzed using an analysis of variance (ANOVA) with NS (neurosteroid administration, three levels: Allop, PREGS or control) as between factor. Moreover, a mixed analysis of variance with NS (Allop, PREGS or control) as between-subject factor and passive avoidance learning (RL–AL) as within-subject factor in order to analyze body weight influence on passive avoidance retention. *Post hoc* Duncan tests were used when necessary.

3. Results

3.1. Body weight

At behavioural testing, animals were completely recovered from surgery as reveal body weight evolution (data not shown). Clinical exploration guided by standard welfare protocol, excluded any neurological damage in subjects used on the behavioural tests.

3.2. Boissier test

The analyses of locomotor activity measured as distance travelled in the total arena and in the inner zone, show no significant effects of NS administration [$F(2,27) = 1.7959$, $P = 0.1852$ and $F(2,27) = 1.4717$, $P = 0.2473$, respectively], see Fig. 2A. In addition the analysis of the ratio between the inner and global activity (data not shown), show no differences between groups ($F(2,27) = 0.2425$, $P = 0.25$). Regarding exploratory behaviour, the analysis of variance revealed a NS effect on the number of head dips [$F(2,27) = 4.7$,

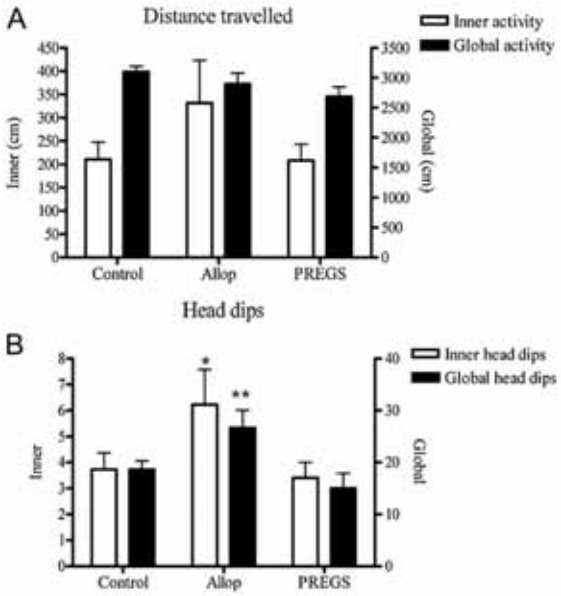

Fig. 2. (A) Locomotor activity in the Boissier exploration test. Activity is expressed as distance travelled in total and inner arena (distance expressed in cm). (B) Total and Inner head dips in the arena ($^{*}P<0.05$, Allop vs PREGS and Allop vs control/$^{**}P<0.01$, Allop vs PREGS and Allop vs control).

$P<0.01$]. Allop treated animals showed a significant increase in the head dipping scores, compared to both PREGS treated animals and controls (Duncan, $P<0.01$ and $P=0.05$, respectively). Similar results were obtained when only the head dips of the virtual central zone were analyzed (see Fig. 2B).

3.3. Elevated plus maze

A NS effect was found in the % OAE [$F(2,27)=3.98$, $P<0.05$]. *Post hoc* analyses showed that the number of entries in the open arms was higher in the Allop administered subjects than in PREGS or control groups (Duncan, $P<0.05$ for Allop vs PREGS and control) as indicated in Fig. 3A. Although animals treated with Allop spent more time in the open arms than the rest of the animals, this difference did not reach statistical significance [$F(2,27)=1.271$, $P=0.296$], see Fig. 3B. In addition, intrahippocampal NS administration altered the open arms activity, although this effect just failed to reach statistical significance [$F(2,27)=2.96$, $P=0.068$]. Subsequent *post hoc* analysis showed an increase in the distance travelled across the open arms in Allop injected animals compared to controls (Duncan, $P<0.05$). Also, no effect was observed on closed arms activity [$F(2,27)=1.284$, $P=0.293$]. Distance travelled across the open and closed arms is showed in Fig. 3C. Moreover, neither the number of total arms entries nor the time spent in the centre of the apparatus, were affected by NS infusion [$F(2,27)=0.885$, $P=0.424$ and $F(2,27)=0.905$, $P=0.416$, respectively].

3.4. Aversive learning in passive avoidance test

Results showed no differences between groups in the latency of acquisition [$F(2,27)=0.111$, $P>0.05$], see Fig. 4B. However, as shown in Fig. 4B, the latency of retention was significantly different depending on the injection group [$F(2,27)=3.9530$, $P<0.05$]. Also, the analysis of passive avoidance learning (RL–AL) showed a significant effect of NS [$F(2,27)=4.0201$, $P<0.05$]. The ANOVA showed that passive avoidance learning (RL–AL values) was significantly different depending on the intrahippocampal NS injection

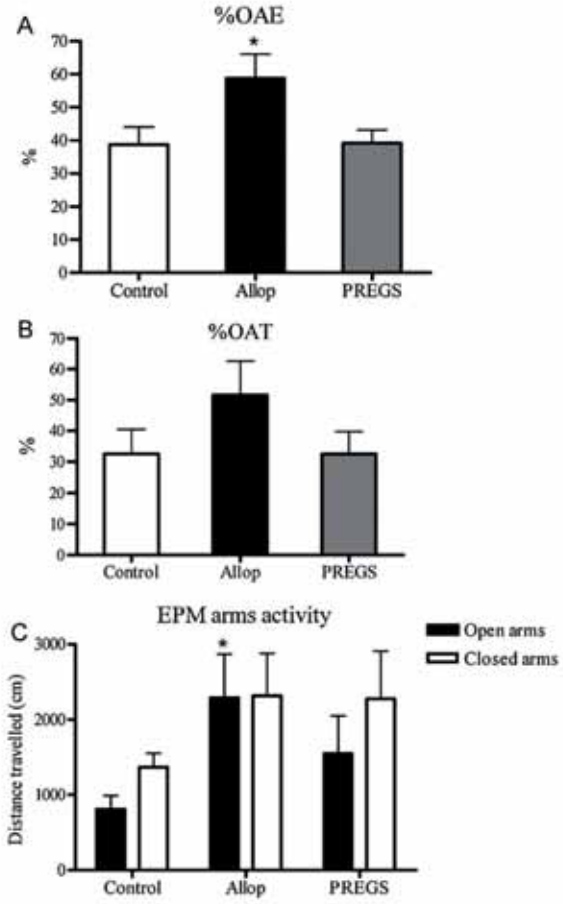

Fig. 3. Effects of intrahippocampal NS injection in the EPM test. (A) Percentage of entries in open arms. (B) Percentage of time spent in the open arms. (C) Distance travelled across the open and closed arms ($^{*}P<0.05$, Allop vs control).

treatment. Subsequent *post hoc* Duncan's tests indicated that RL–AL scores were higher in the PREGS group than in the control ($P<0.05$), without differences between Allop and PREGS nor Allop and control, see Fig. 4A. Concerning, the percentage of animals reaching learning criterion (not entering to the dark compartment in 10 min) chi-square test showed significant differences between PREGS (90%) and control (22.3%) groups [Chi-square = 10.98; $P<0.01$]. In addition, body weight remained steady across the two behaviour-testing days, and there were no differences between groups [$F(2,27)=0.521$; $P=0.599$].

4. Discussion

4.1. Exploration, locomotion and anxiety behaviour

The present results support our previous hypothesis and suggest that the CA1 hippocampal region is involved in the Allop modulation of exploration as shown by the enhancement of head-dipping behaviour in the Boissier test. Together with head-dipping scores, the analysis of locomotion can also be considered as a novelty-directed exploration indicator. However, several studies have reported that increased locomotion is not necessarily related to an enhancement in exploration [42]. For instance, an increase in locomotor activity in the Boissier test due to the stimulating effects of a drug could not be related to an increase in exploration,

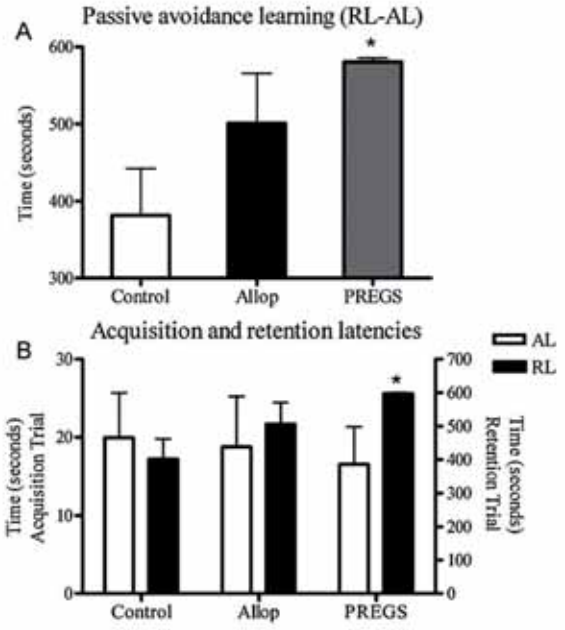

Fig. 4. Effects of intrahippocampal NS administration on passive avoidance. (A) Passive avoidance learning measured as the difference between RL and AL. (B) Latencies to enter in the dark compartment in the Acquisition Trial (AL (acquisition latency) (Allop = PREGS = control)) and in the retention trial (RL(Retention latency): PREGS < (Allop and control) (*$P < 0.05$).

but rather to the animal choosing to escape [42,43]. In the present experiment, the increase in head dipping scores induced by Allop cannot be explained by changes in the animal's general activity, since locomotion was not affected in any group in the Boissier test. Moreover, this behavioural profile is in line with the previous described profile for GABAA positive modulators such as benzodiazepines. In this sense, systemically administered diazepam or chlordiazepoxide also has been shown to increase head-dipping scores without affecting locomotion [44].

On the other hand, results show no effect of intrahippocampal PREGS administration on head dipping or locomotion scores. As mentioned in the introduction section, it would be expected to find a decrease in novelty-exploration according to the documented anxiogenic-like profile of PREGS [13,15]. However, in the present study intrahippocampal PREGS dose was chosen in accordance with its promnesic profile described in previous studies [32,35] in order to assess the hippocampal role in PREGS modulation of memory consolidation processes. Thus, the lack of effect could be attributed to the dose administered. Moreover, it has to be considered that molecular targets of PREGS include AMPA, kainite, glycine, sigma Type I and nicotinic acetylcholine receptors, in addition to the aforementioned GABAA or NMDA modulation [2], and all these receptors are located in the hippocampus [45]. Furthermore, results on the EPM show a lack of effect of intrahippocampal PREGS administration. Thus, the anxiogenic-like profile described for PREGS when administered systemically [13,15] has not been found. A lack of PREGS effect has also been observed in the novelty exploration test of Boissier (see above), a neophobia test. The lack of effect in the EPM could be attributed to the same reasons argued for the Boissier test, so further studies are necessary to determine the dose–response of PREGS on anxiety when administered directly into the hippocampus.

The present findings also support our hypothesis about the role of dorsal hippocampus in the modulation of the anxiolytic profile shown by Allop. Results in the EPM test showed an increase in the percentage of entries into the open arms compared to both control and PREGS groups. This data is supported by the enhancement of distance travelled in the open arms seen in Allop injected subjects. However, although it seems that the activity in the closed arms of the EPM is lower in the control group than in the Allop or PREGS groups, the differences are not statistically significant. Thus, we can assume there are no relevant differences between groups in the closed arms activity. The present finding is also consistent with previous data obtained when Allop is systemically administered in rodents [46] and with the results obtained in novelty-exploration in the present study. Previous studies have suggested that Allop anxiolytic effects may occur via GABAA receptors increasing the GABAA receptor-gated Cl-channel conductance in hippocampal neurons [7,14,22]. As mentioned, present data support the hypothesis about the role of the dorsal (CA1) hippocampus plays in emotional processes and NS modulation of anxiety and exploration. At this respect, it has been postulated that the dorsal aspect of the hippocampus is more involved in memory-related functions, whereas the ventral aspect of the hippocampus mediates fear and anxiety responses [47]. However, in contrast to these anatomical and functional considerations, the results of the present study demonstrate that dorsal the (CA1) aspect of the hippocampus is involved in the modulation of anxiety-related behaviours. This result is also consistent with previous studies carried out in our laboratory, showing that dorsal intrahippocampal Allop administration induced a rapid habituation to the environmental stress measured in a circular open field over five days of testing [28]. Moreover, the results are also in accordance with previous findings obtained with benzodiazepine infusions (also positive modulators of GABAA receptors) on the dorsal hippocampus [48]. In this regard, the stimulation of Allop synthesis in the dorsal (CA1) hippocampus with FGIN 1-27 has also showed to produce anxiolytic-like effects in the EPM [49]. Coherently, it has been reported that dorsal intrahippocampal [19,50] administration of finasteride showed an anxiogenic-like profile in the open field [19,50] and EPM test [50], decreasing locomotion and the time spent in the central zone, and decreasing the time spent in the open arms, respectively. Thus, the present results support the hypothesis about the role that the dorsal hippocampus plays in the NS modulation of exploration and anxiety, and is among the first to demonstrate that hippocampal Allop causes an increase in exploratory behaviour, and also an increase in the anxiolysis in the EPM. However, complementary studies assessing the role of ventral hippocampus in the NS modulation of anxiety, learning and memory would be necessary to clarify the specific involvement of each hippocampus aspect in NS modulation of emotional processes. Present results point out the importance CA1 hippocampal region in the modulation of emotional behaviours, but also are in accordance with previous studies administering other NS which have observed the same described profile, suggesting that together with other brain structures such as the amygdala or the medial septum, the dorsal hippocampus could be an important target for explaining the effects of NS in emotional behaviour.

4.2. Passive avoidance retention

Results showed that intrahippocampal PREGS administration increased passive avoidance retention after its post-acquisition administration. In accordance with this data, the promnesic effects of PREGS have been documented in active and passive avoidance, and in spatial learning, with PREGS being injected systemically [30], intrahippocampally [28] or into the nucleus basalis magnocellularis (NBM) [32,35]. Other experiments have revealed that intrahippocampal [24] PREGS administration reverses the memory impairments induced in cognitively impaired (aged) rats, but also induced by co-administration of alcohol and nicotine [27]. One

postulated mechanism underlying the memory enhancing actions of PREGS is the potentiating of NMDA receptors. In this sense, results in the active avoidance and lever-pressing tasks [51] showed the involvement of NMDA receptors in the hippocampus that are located in hippocampal pyramidal neurons [52]. Moreover, another proposed mechanism is the potentiation of cholinergic systems [25]. In this sense, it has been observed that PREGS administered into the NBM increases not only spatial memory recognition but also the release of acetylcholine (ACh) in the frontal cortex and amygdala [35]. It is plausible that the increase in cortical and amygdaline ACh could be mediated by basal forebrain GABAergic inhibition. However, PREGS administration into the medial septum, the main cholinergic nucleus of the basal forebrain together with the NBM, also increases ACh release in the hippocampus [25]. Thus the two described mechanisms (i.e. modulation of hippocampal NMDA receptors and increase in ACh levels) could be complementary and probably related. Although the actions of PREGS on hippocampal σ1 receptors [53] could also be involved, it has been described that the antiamnesic effect of PREGS is more related to a direct interaction with NMDA and GABAA receptors than to an interaction with the σ1 receptors [54].

As mentioned in the introduction, a previous study carried out in our laboratory indicated that a post-training intrahippocampal PREGS administration (at the same doses as in the present experiment) produced an impairment of passive avoidance retention [29]. However, in this experiment animals were exposed to a non-familiar open field (20 min) immediately after intrahippocampal infusions. Thus it seems that the stress induced by the major acute stressor (high foot-shock punishment) at the end of the training session could have interacted with the environmental stress related to novelty open field exposure to produce deterioration in passive avoidance retention. In this sense, it has been reported that stress can have detrimental effects on passive avoidance retention without affecting spatial memory in senescence-accelerated mice [55]. The present study has evaluated the PREGS post-acquisition administration effect, with no interference of additional environmental stressors (open field) on passive avoidance retention, and demonstrates the promnesic role of PREGS in this aversive memory task and the importance of the dorsal hippocampus in explaining this effect. Moreover, comparing the present study with the above-mentioned earlier work, the results point out the role that stress might play in learning and memory processes.

On the other hand, it seems that the detrimental effects of Allop are produced by pre-acquisition administration. In this sense, impairment by Allop in associative [26] and non-associative [32] learning has been reported, systemically or directly into the NMB, respectively. In addition, in Y mazes, Allop led to memory deterioration when was pre-acquisition administered [34]. It has been suggested that detrimental effect of Allop could be specific on learning rather than on memory processes [26] and this fact may explain the lack of Allop intrahippocampal effects when it is post-acquisition administered.

5. Conclusions

In summary, the results obtained in the present study indicate that the intrahippocampal administration of Allop causes an anxiolytic-like profile in the EPM but also produces an increase in the exploratory behaviour, suggesting that together with other brain structures such as the amygdala or the medial septum, the dorsal (CA1) hippocampus could be an important target for explaining the effects of NS on emotional behaviour. In addition, post-training intrahippocampal PREGS injection produced an enhancement of passive avoidance task in accordance with its previous described promnesic profile. Taken together, these results suggest the relevance of the dorsal hippocampus (CA1 zone) in explaining the relationship between NS, emotional behaviour, aversive learning and memory, and support the important role that NS may play in emotional and cognitive processes.

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Experiment 1b:
Neonatal Ns & behavioural response to intrahippocampal NS

Research article

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Research report

Alteration of neonatal Allopregnanolone levels affects exploration, anxiety, aversive learning and adult behavioural response to intrahippocampal neurosteroids

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HIGHLIGHTS

- Neonatal Allop is important for the adult intrahippocampal axiolytic profile of NS.
- Neonatal alteration of Allop levels alters adult exploratory and anxiety behaviour.
- Neonatal alteration of Allop levels affects adult avoidance learning performance.

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ABSTRACT

Neurosteroids (NS) are well known to exert modulatory effects on ionotropic receptors. Recent findings indicate that NS could also act as important factors during development. In this sense, neonatal modifications of Allopregnanolone (Allop) levels during critical periods have been demonstrate to alter the morphology of the hippocampus but also other brain structures. The aim of the present work is to screen whether the alterations of Allop levels modify adult CA1 hippocampal response to NS administration. For this purpose, pups were injected with Allop (20 mg/kg s.c.), Finasteride (5α-reductase inhibitor that impedes Allop synthesis) (50 mg/kg s.c.) or Vehicle from postnatal day 5 (P5) to postnatal day 9 (P9). NS levels were tested at P5. To test the behavioural hippocampal response to NS in adulthood, animals were implanted with a bilateral cannula into the CA1 hippocampus at 80 days old and injected with Allop (0.2 μg/0.5 μl), Pregnenolone sulphate (5 ng/0.5 μl) or Vehicle in each hippocampus. After injections animals were tested in the Boisser test to assess exploratory behaviour, the elevated plus maze to assess anxiety and the passive avoidance to test aversive learning. Results indicate that alteration of neonatal Allop or pregnenolone levels (by Allop and Finasteride administration, respectively) suppressed intrahippocampal Allop anxiolytic effect in the EPM. Moreover our results also indicate that manipulation of neonatal Allop levels (Allop and Finast administration) alters exploratory and anxiety-like behaviour and impairs aversive learning in the adulthood. These data point out the role of Allop in the maturation of hippocampal function and behaviour.

1. Introduction

Neurosteroids (NS) are steroids that are synthesized de novo by the nervous tissue and are well known for exerting modulatory actions on neurons excitability through the modulation of ionotropic receptors [1,2]. In this sense, NS acting as positive modulators of GABAA receptors such as Allopregnanolone (Allop or 3α,5α-tetrahydroprogesterone), have been described to show anticonvulsive [3] and anxiolytic effects when injected systemically [4], into the amygdala [5] or into the hippocampus [6]. Furthermore, ring A reduced pregnanes like Allop have also been described to have a detrimental learning profile when injected systemically [7], directly into the nucleus basalis magnocelularis [8], intraventricularly [9], or into the amygdala [10] in adult animals. On the other hand, NS that act as negative modulators of GABAA receptors such as pregnenolone (PREG), dehydroepiandrosterone (DHEA) and their sulphated esters (PREGS and DHEAS) have been described to act as proconvulsive [11], anxiogenic [4,12] and also to improve memory in several learning tests when injected systemically [7], into the amygdala [10], into the nucleus basalis magnocellularis [13] and also into the hippocampus [6,14]. In this sense, this promnesic effect has been postulated to take place through the potentiation of NMDA receptors located in the pyramidal neurons of the hippocampus [15]. However, it has also been

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suggested that PREGS enhancing profile could be done through the potentiation of the cholinergic neurons via GABAergic inhibition [13,16].

Recent findings indicate NS acting as GABAA positive modulators, such as Allop are important keys during brain development [17]. In fact, it has been described that cortical Allop peak appears before birth and a second peak of Allop occurs during the second postnatal week [18,19]. The specific role that NS play in development, however, has not been yet elucidated. It has been postulated that increased levels of neonatal Allop promote the establishment of neuronal circuitry and supports the survival of developing neurons [20]. Also, previous studies demonstrated that alteration of neonatal Allop levels has a profound effect on the morphology and the structure of several brain areas such as the cortex and the thalamus [19,21,22] but also alters critically the normal development of the hippocampus [23–26]. Accordingly, alterations in adult behaviour have also been reported. In this sense, previous results in our laboratory have shown that manipulation of neonatal Allop levels alters the performance of the elevated plus maze (EPM) and the aversive learning in the passive avoidance test [27]. In addition, Allop administration (from postnatal day 5 (P5) to postnatal day 9 (P9)) has been shown to deteriorate PPI in the adulthood [28], to alter adulthood responses to GABAA modulators (such as benzodiazepines) in the EPM [29], to modify the CA1 response to NS in the open field test [30] and to induce an anxiolytic profile in the EPM [31]. These previous data seem to suggest that alteration of NS during critical developmental periods have important consequences in adult behaviour, however, no studies have been performed to assess the interaction between adult intrahippocampal administration of NS and neonatal injection of an Allop dose (20 mg/kg), that induces anxiolytic-like profile in the adult age [31].

Therefore, given that neonatal Allop levels have been described to play a crucial role in the correct maturation of central CNS, and concretely to the correct formation of hippocampal circuitry, the aims of the present work are to assess the NS hippocampal levels (Allop, THDOC, testosterone, epiallopregnanolone, PREG) at P5 in response to the neonatal treatment (Allop, Finasteride (Finast), Vehicle and No handled (NH)) and to screen whether the alteration of developmental NS levels modifies the effect of adult NS intrahippocampal administration (Allop, PREGS, Vehicle) on exploratory, anxiety and aversive learning behaviour. We have chosen the Boissier test, the EPM and the passive avoidance to test several aspects of the emotional behaviour. We hypothesize that the alteration of the physiological neonatal Allop levels (Allop or Finast administration) can alter adult response to exploration, anxiety and to aversive learning and also, adult behavioural response to NS such as PREGS or Allop when they are administered into the hippocampus.

2. Material and methods

2.1. Animals and neonatal NS administration

A total number of one hundred eighty seven animals were used in the experiment (see Table 1). Twenty-five animals were used for NS quantification and one hundred sixty-two were used for adult intrahippocampal administration and behavioural evaluation. All animals used were male Wistar rats raised at in-house colony (Laboratori de Psicobiologia, Universitat Autònoma de Barcelona, Barcelona, Spain) allowed with food and water ad libitum. Rats were housed in a temperature-controlled animal room (22–24 °C) on a 12-h light/dark cycle. Experimental sessions were run during the light portion of the cycle (lights on at 08:00 h). The male breeders were separated from the females after 48 h, pregnant females were closely watched and on the day of birth (designed day 0), mothers were removed from the cage and litters were culled to 10 pups. In order to avoid any cohort effects, each litter of the same colony was assigned to different neonatal treatment groups. Pups (males and females) were subcutaneously (s.c) injected with: Finasteride (Finast) (50 mg/kg, $n = 41$), Allop (20 mg/kg, $n = 42$) or Vehicle ($n = 42$), once per day from the fifth to the ninth day alter birth (P5–P9). All products were obtained from SIGMA (Deisenhofen, Germany). In addition, a non-handled group (NH) ($n = 37$) was included in order to avoid the possible effects of the drugs administration (see Table 1 for final experimental groups and number of pairs and litters). Drugs (Allop, Finasteride and Vehicle) were dissolved in 0.9% NaCl by sonication for 10 min and suspended in 10% cyclodextrin ((2-hydroxypropyl)-β-cyclodextrin). As Vehicle, 10%-cyclodextrin dissolved in 0.9% NaCl was used. Injection volume was 0.1 ml/10 g body weight. After injections, pups were immediately returned to the home cage with their mother. After weaning (P21) males were separated into groups of brothers (with a maximum of five subjects per cage), and females were sacrificed. After recovery from surgery, males of each neonatal condition were randomly assigned to each group and they were behaviourally evaluated. All animals were obtained, housed, and sacrificed in accordance with the protocol approved by the Committee of the UniversitatAutònoma de Barcelona for Care and Use of Experimental Animals and the Department of Environment from Generalitat de Catalunya (Regional Government). This protocol follows the guidelines approved by the European Council Directive (86/609/ECC) for care and use of laboratory animals.

2.2. Hippocampal NS quantification

The twenty-five animals were sacrificed by decapitation at postnatal day 5 (P5), after 1 h of neonatal administration. Postnatal day 5 was chosen to ensure that NS hippocampal levels were at least altered during the first postnatal injection given that an adaptation effect could result from the repeated NS treatment. Brains were removed and the hippocampus were harvested and frozen in dry ice. Brains were kept stored at −80 °C until they were used for the steroid quantification. Pregnenolone (PREG), Allop, epiallopregnanolone, THDOC and testosterone were determined by gas chromatography/mass spectrometry according to Vallee et al. [32].

2.3. Surgery

Surgery was carried out at 80-day-old animals. For permanent implantation of cannula, animals were anaesthetized with ketamine (120 mg/kg) and xylazine (10 mg/kg). Using standard stereotaxic techniques, bilateral 21-gauge stainless steel double guide cannula (model C232G-3.8; Plastics One, Bilaney, Dusserldorf, Germany) was implanted into the CA1 region of the dorsal hippocampus (anteroposterior, 3.6 mm; mediolateral, 1.8 mm; dorsoventral, 1.8 mm from bregma). Each guide cannula was fitted with dummy cannula with no projection. Double guide cannulae were permanently mounted to the skull with four screws and dental cement.

2.4. Adult NS administration

After recovery from surgery (2 weeks), the day of behavioural testing freely moving rats were injected with Allop (0.2 μg/0.5 μl), pregnenolone sulphate (PREGS) (5 ng/0.5 μl) (SIGMA, Deisenhofen (Germany)) or Vehicle (10% cyclodextrin) in each hippocampus. See Table 1 for final experimental groups composition. NS doses were determined based on previous studies carried out in our laboratory, according to affect anxiety, learning and memory responses [13,14,27,33]. Allop and PREGS were dissolved in 0.9% NaCl by sonication for 10 min and suspended in 10% cyclodextrin. For injections, double internal 28-gauge stainless steel cannula (model C232I; Plastics One) was inserted extending 1 mm below the guide cannula to a final depth of 2.8 mm from the skull (see Fig. 1). Injection needles were connected with polyethylene tubing to a microsyringe (10 μl) driven by the infusion pump (Harvard 22). Solutions were infused during 1 min at a constant rate of 0.5 μl/min. Control rats received the same volume (0.5 μl) of Vehicle at the same rate of infusion. Injection needles were removed from the guide cannula 2 min after infusions in order to prevent drug reflux. Animals were injected twice: before Boissier test and after passive avoidance acquisition one week later, with the same substance.

2.5. Boissier exploration test

A square wooden arena (58 cm × 58 cm × 58 cm) with 16 equidistant holes (5 cm in diameter) was used for the Boissier test. The apparatus was situated in a room lit by a bright light (300 lx mean). This test measures activity and provides a relatively reliable measurement of stimulus-directed exploratory behaviour [34,35]. It was tested for 5 min and was evaluated by means of an activity monitoring system (SMART, Letica, Barcelona, Spain). This system is based on the automated analysis of real-time video-images, recorded by a video camera that is suspended from the ceiling over the arena. The distance moved was recorded for the total arena as locomotor activity, as well as for a virtual 29 cm × 29 cm centre zone. In addition, the number of entries into, and the time spent in the centre zone was also measured as anxiety relevant scores. Moreover, the number of head-dips (into the holes up to the eye line) was recorded as an exploratory measure. After each trial, the apparatus was cleaned with a water solution containing ethanol (20%, v/v) in order to prevent any olfactory-induced behavioural modifications. Rats were tested immediately after intrahippocampal NS infusions (between 09:00 and 11:00 h). Five animals were excluded from the distance-travelled analysis because of detection problems.

2.6. Elevated plus maze

The EPM consisted of two open and two closed arms (10 cm wide × 50 cm long) perpendicular to each other and elevated 50 cm from the floor. The walls of the

Table 1
Neonatal treatment assignments for behavioural test and adult intrahippocamapal administration, excluding animals with errors in cannula placements ($n = 32$).

Pair	1		2		3		4		5		6		7		8		9		10		11		12		Adult intrahippocampal administration			
Litter	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	Total	Allop	PREGS	Vehicle
Allop			5		3				6						4			7			3			5	33	12	11	10
Finasteride	6					5						5				4	6						7		33	11	11	11
Vehicle				7				6			7			6						9					35	10	13	12
NH		5					6			4			3						6			5			29	9	10	10

Neonatal administered animals for hippocampal NS quantification

Pair	1	2	3	4	Final groups
Litter	1	1	1	1	Total
Allop		5			5
Finasteride			5		5
Vehicle				5	5
NH	5				5

closed arms were 40 cm high. The arms were connected by a central square measuring 10 × 10 cm. The animal was placed in the centre of the apparatus facing an open arm and its behaviour was recorded for 5 min. The maze was situated in a room lit by a dim light (36 lx mean) and it was cleaned between animals to minimize olfactory cues between trials. The following variables were recorded: number of open and closed arm entries; number of total entries; time spent in the open and closed arms and in the centre of the apparatus; and distance travelled across the open and closed arms by mean of a SMART (see above). The percentage of time spent in the open arms (%OAT) and the percentage of open arms entries (%OAE) were used as anxiety measures. The number of open arms plus closed arms entries was used as a measure of activity. An entry was counted whenever the animal crossed with all four pawns into an arm. Increased %OAT or %OAE is indicative of a reduced anxiety state in the EPM [36]. In addition the percentage of time spent in the centre of the maze was used as an independent measure of decision-making [37,38]. The presence of differences between activity and decision making measures would suggest that any differences observed for anxiety measures could be part of a non-selective behavioural effect of NS administration. Tests were videotaped, monitored and scored at the time by an observer sited in the next room blinded of the NS administration. EPM was carried out immediately after Boissier test, as it has been previously described [36].

2.7. Passive avoidance test

Rats were trained in a computerized one-trial-passive avoidance (Letica-Panlab, Barcelona, Spain). The experimental apparatus consisted of a squared white compartment (32 × 32 × 25 cm) communicated with a closed dark compartment (19 × 11 × 13 cm) through a motorized sliding door. In the acquisition trial, the subjects were placed in the illuminated compartment and were allowed to freely explore the apparatus. As soon as the animals entered the dark compartment (with the four pawns), the sliding door was automatically closed and the subject received an unavoidable scrambled high foot-shock of 0.5 mA during 3 s. These parameters were used based on previous works [6,27]. The latency to enter the dark compartment (acquisition latency (AL)) was measured. Animals that did not enter in the dark compartment during acquisition trial were excluded from the analysis ($n = 8$). Subjects were intrahippocampally injected with NS immediately after acquisition, and returned to their home cages. Retention of passive avoidance response was measured 24 h after the acquisition. For retention, animals were placed in the white compartment and the latency to enter the dark compartment was measured (RL). Animals were free of drugs during retention trial and no shock was administered. When the rat did not enter the dark compartment for 600 s, it was removed from the box and assigned a ceiling score of 600 s. Animals were weighed before each

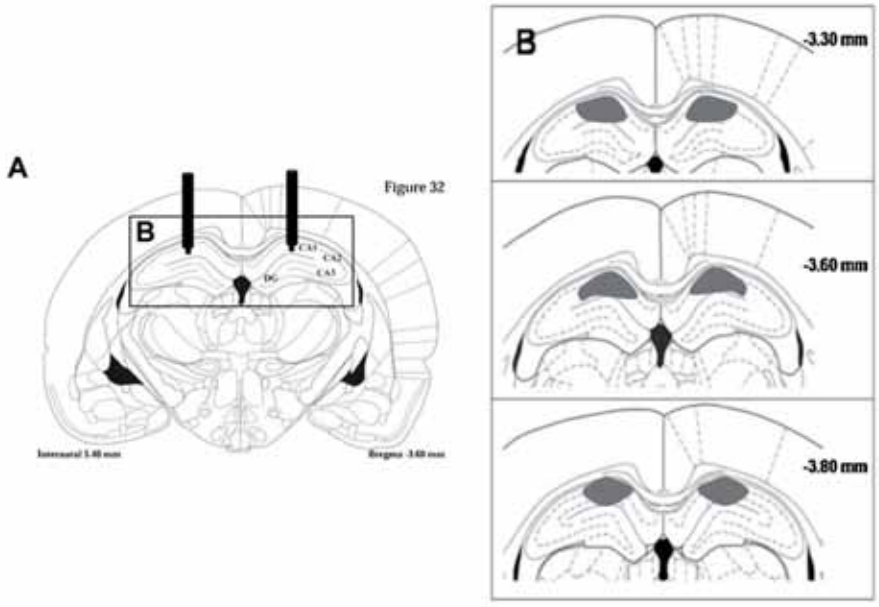

Fig. 1. (A) Schematic representation of the point of injection illustrating cannulae localization into the hippocampus, section 3.60 mm posterior to bregma. The marked area indicates the area shown in B. (B) Representative image of the global area of cannula placements.

trail. Passive avoidance learning was measured as the difference between RL and AL.

2.8. Histological control

Animals were sacrificed by deep anaesthesia with i.p. sodium pentobarbital (200 mg/kg body weight at a concentration of 60 mg/ml), and their brains were removed and stored in 10% formalin. Brains were vibrosliced in 100 μ coronal sections, mounted and cresyl violet stained. Localization of the guide cannula and infusion sites was confirmed histologically for each rat. The diffusion of injection was controlled throughout a previous pilot study ($n = 3$) in which subjects were injected with the same volume (0.5 μl) of a tionine solution, to ensure that the drug was not diffused to other hippocampal or brain areas. Only animals in which histology confirmed that the infusion cannula was located within the CA1 area of the hippocampus were included in the analysis. Thus, 32 animals were excluded from statistical analysis because of placement errors (NH = 8; Vehicle = 7; Allop = 9; Finast = 8). The final experimental groups are shown in Table 1. See Fig. 1 for correct cannula placements.

2.9. Statistical analysis

The statistical analysis was performed using the STATISTICA package (StatSoft, Tulsa, USA). The normality of the data was assessed by means of the Kolmogorov–Smirnov test. Data from passive avoidance were analyzed using a two-way ANOVA with neonatal treatment (four levels: NH/Vehicle/Finast/Allop) and adulthood administration (three levels: Vehicle/PREGS/Allop) as between subject factors. Subsequent partition analyses and post hoc Duncan tests were used when necessary. Data are shown as mean ± SEM.

3. Results

3.1. Neonatal NS levels

The analysis of the NS levels at first neonatal administration day (P5), showed differences between groups in the hippocampal Allop levels [$F_{(4,20)} = 79.95$, $p < 0.001$]. The post hoc test Duncan showed that Allop levels were significantly increased in those subjects injected with Allop compared to the rest of the groups (Allop vs. NH/Vehicle/Finast) ($p < 0.001$), see Table 2. In addition, differences were also found in the analysis of the Testosterone and Epiallopregnanolone levels [$F_{(4,20)} = 130.1$, $p < 0.01$ and $F_{(4,20)} = 58.79$, $p < 0.001$, respectively]. Post hoc Duncan test showed that Testosterone and Epiallopregnanolone levels were increased in subjects neonatally injected with Allop compared to the rest of the groups (Allop vs. NH/Vehicle/Finast: $p < 0.001$), see Table 2. Besides, differences were also found in the analysis of the PREG levels [$F_{(4,20)} = 119.7$, $p < 0.001$]. In this sense, subsequent post hoc Duncan test showed an increase of PREG levels in Finast injected animals compared to the rest of the groups (Finast vs. Allop/NH/Vehicle = $p < 0.001$). No differences between groups were found in the analysis of the THDOC hippocampal levels.

3.2. Boissier test

3.2.1. Locomotor activity

The analyses of the global distance travelled (in cm) in the Boissier test showed a significant main effect of the neonatal NS administration [$F_{(3,113)} = 5.79$; $p = 0.001$]. Post hoc Duncan test showed a decrease in activity in neonatal Allop injected animals (2384.35 ± 99.08) compared to the rest of the groups (Allop vs. NH (2934.17 ± 86.74), $p < 0.001$; Allop vs. Vehicle (2758.05 ± 90.04), $p < 0.01$; Allop vs. Finast (2860.07 ± 123.32), $p < 0.01$). On the other hand, no significant effect was found in the analysis of the adult intrahippocamapal administration [$F_{(2,113)} = 0.45$; N.S.], neither its interaction [$F_{(6,113)} = 1.39$; N.S.]. The analysis of inner activity also showed neonatal main effect [$F_{(3,113)} = 2.72$; $p < 0.05$]. Post hoc test (Duncan) showed a decrease in inner activity in animals neonatally injected (Allop and Finast) compared to the NH group (248.80 ± 34.22) (NH vs. Allop (163.49 ± 15.13), $p < 0.01$ and NH vs. Finast (179.37 ± 18.65), $p < 0.05$). No significant effect was found in the analysis of the adult intrahippocamapal administration [$F_{(2,113)} = 1.43$; N.S.], neither its interaction [$F_{(6,114)} = 0.82$; N.S.]. The analyses of the number of entries in the inner zone did not show any differences between groups (data not shown).

3.2.2. Head dipping

Regarding exploratory behaviour, a main neonatal effect was found [$F_{(3,118)} = 5.64$; $p < 0.001$], see Fig. 2A. In this sense, post hoc Duncan test showed a decrease in the number of global head dips in Finast and Vehicle groups compared to the NH and Allop neonatal injected animals (NH vs. Finast $p < 0.005$; NH vs. Vehicle $p < 0.01$; Allop vs. Finast $p < 0.01$; Allop vs. Vehicle $p < 0.05$). Furthermore, an intrahippocampal effect was found in animals injected with Allop in the adulthood [$F_{(2,118)} = 4.32$; $p = 0.01$], Fig. 2A′. In this sense, an increase in the number of global head dips in the Boissier was observed in intrahippocampal Allop administered subjects. Post hoc Duncan test showed differences between Allop and PREGS ($p < 0.01$) and a tendency between the Allop and Vehicle groups ($p = 0.07$). No significant effects were found in the interaction between neonatal and adult intrahippocampal administration [$F_{(6,118)} = 1.53$; N.S.].

In addition, the analysis of the inner head dips showed similar results to the global head dips [$F_{(3,118)} = 5.84$; $p < 0.001$], see Fig. 2B. In this sense, a decrease in the inner head dips explored was observed in Finast and Vehicle groups compared to Allop (Duncan's: Allop vs. Finast $p < 0.001$; Allop vs. Vehicle $p < 0.04$) and also between the NH and the Finast groups ($p < 0.01$), see Fig. 2B. Although differences did not reach statistical significance, a tendency to increase the number of inner head by Allop was also found in the analyses of the adult intrahippocampal administration [$F_{(2,118)} = 12.53$; $p = 0.08$]. No differences were observed in the interaction between neonatal and adult intrahippocampal administration [$F_{(6,118)} = 1.60$; N.S.].

3.3. Elevated plus maze

3.3.1. Distance travelled

The analyses of distance travelled in the open arms showed significant differences between neonatal administration groups [$F_{(3,118)} = 9.97$; $p < 0.001$], see Fig. 3A. Post hoc Duncan analyses showed that NH groups differed from all the neonatal administered subjects ($p < 0.001$ for all groups). Also, an adult intrahippocampal administration effect was found [$F_{(2,118)} = 4.38$; $p = 0.01$], see Fig. 3A′. An increase in the distance travelled across the open arms in Allop intrahippocampal injected animals was observed compared to the control animals (Vehicle adult) (Duncan's Allop vs. Vehicle: $p < 0.01$) and Allop compared to PREGS intrahippocampal administered animals (Duncan's Allop vs. PREGS: $p < 0.05$). No interaction effect was observed between neonatal and adult NS administration [$F_{(6,118)} = 1.75$; N.S.].

3.3.2. %OAE and %TOA

The analyses of the %OAE showed a significant interaction between neonatal treatment and adult intrahippocampal administration [$F_{(6,118)} = 2.78$; $p = 0.01$]. Thus, we performed a partition analysis in order to analyze differences between groups. In this sense, the analysis of NH and Vehicle animals showed a main effect of intrahippocampal administration [$F_{(2,26)} = 3.90$; $p < 0.05$ and $F_{(2,32)} = 4.23$; $p < 0.05$, respectively]. Post hoc Duncan test indicated an increase in % of entries in the open arms in animals intrahippocampally administered with Allop compared to adult Vehicle and PREGS (NH: Allop vs. Vehicle/PREGS ($p < 0.05$) and Vehicle (Neo): Allop vs. Vehicle (adult) ($p < 0.01$) and Allop vs. PREGS ($p < 0.05$)). The analysis of Allop and Finast neonatal administered animals did not showed any differences between

Table 2
Hippocampal NS levels at P5.

	Neonatal treatment			
	NH	Vehicle	Allop	Finast
Testosterone	0.36 ± 0.21	0.39 ± 0.23	3.82 ± 0.59**	0.43 ± 0.08
Allopregnanolone	0.41 ± 0.17	4.35 ± 2.47	322.45 ± 76.43***	40.00 ± 12.10
THDOC	17.13 ± 6.37	12.67 ± 5.87	24.66 ± 3.63	1898 ± 3.33
Pregnenolone	3.52 ± 0.95	6.59 ± 1.76	3.19 ± 0.60	3488 ± 5.89***
Epiallopregnanolone	0.02 ± 0.01	0.30 ± 0.26	11.13 ± 3.20#	0.05 ± 0.04

NS levels expressed in ng/g tissue (mean ± sem).
** $p < 0.001$.
*** $p < 0.0001$.
# $p < 0.005$.

adult intrahippocampal administration [$F_{(2,31)} = 0.58$; N.S. and $F_{(2,29)} = 1.25$; N.S., respectively]. See Fig. 3B.

On the other hand, a neonatal affect was also found in the analysis of %TOA [$F_{(3,118)} = 3.51$; $p < 0.01$], see Fig. 4C. Post hoc Duncan test showed a decrease in the time spend in the open arms in neonatal Finast and Vehicle administered animals, when compared to NH and Allop (NH vs. Finast, $p < 0.05$; NH vs. Vehicle, $p < 0.05$; Allop vs. Finast, $p < 0.05$; Allop vs. Vehicle, $p < 0.05$). No differences were found in the adult intrahippocampal NS administration [$F_{(2,118)} = 1.35$; N.S.], neither its interaction [$F_{(6,118)} = 1.17$; N.S.], see Fig. 3C.

3.4. Aversive learning of the passive avoidance

3.4.1. Acquisition latency

The analysis of the acquisition latency showed no differences between neonatal injected groups [$F_{(3,110)} = 1.19$; N.S.], adult intrahippocampal NS administration [$F_{(2,110)} = 0.63$; N.S.], nor its interaction [$F_{(6,110)} = 0.88$; N.S.] (data not shown).

3.4.2. Aversive learning: retention latency (RL)–acquisition latency (AL)

Results of RL–AL, showed differences between groups depending on the neonatal treatment [$F_{(3,110)} = 2.70$; $p < 0.05$] (see Fig. 4A). Post hoc Duncan indicated that neonatal injected animals with Allop and Finast, differed from NH group (NH vs. Allop, $p < 0.01$ and NH vs. Finast, $p < 0.09$). Also, the analysis of the control groups showed no differences between them (NH and Vehicle subjects) ($p = 0.32$). Furthermore, results of adult intrahippocampal treatment also showed an Allop enhancing effect of the passive avoidance retention latency [$F_{(2,110)} = 4.00$; $p < 0.05$], see Fig. 4A′. Post hoc Duncan test showed that Allop intrahippocampally administered animals increased the latency to enter in the dark compartment compared to the control (Vehicle) group ($p < 0.01$). On the other hand, no significant interaction was found between neonatal treatment and adult intrahippocampal administration [$F_{(6,110)} = 1.19$; N.S.]. See Fig. 4A.

Given that Allop obtained data is not in accordance to several previous studies [39–42], we performed a separated analysis of NH animals. The analysis of the NH group showed differences between

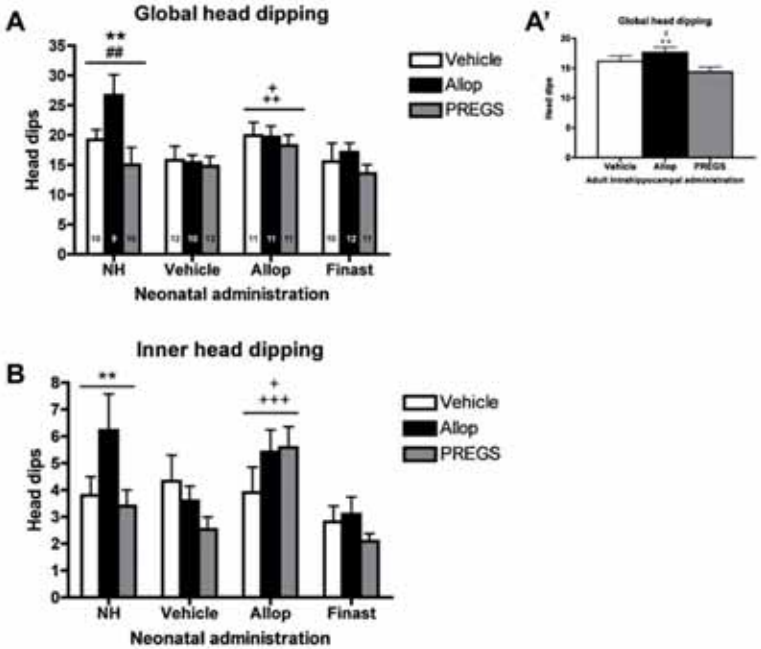

Fig. 2. Head dips exploration in the Boissier test. (A) Effects of neonatal and adult intrahippocampal administration in the global head dips. Neonatal administration effect: **NH vs. Finast ($p < 0.01$); ##NH vs. Vehicle ($p < 0.01$); ++Allop vs. Finast ($p < 0.01$); +Allop vs. Vehicle ($p < 0.05$). (A′) Adult intrahippocampal administration effect: **Allop vs. PREGS ($p < 0.01$); #Allop vs. Vehicle ($p = 0.07$). (B) Effects of neonatal and adult intrahippocampal administration in the inner head dips. Neonatal administration effect: **NH vs. Finast ($p < 0.01$); +++Allop vs. Finast ($p < 0.001$); +Allop vs. vehicle ($p < 0.05$). The final number of subjects are represented in bars.

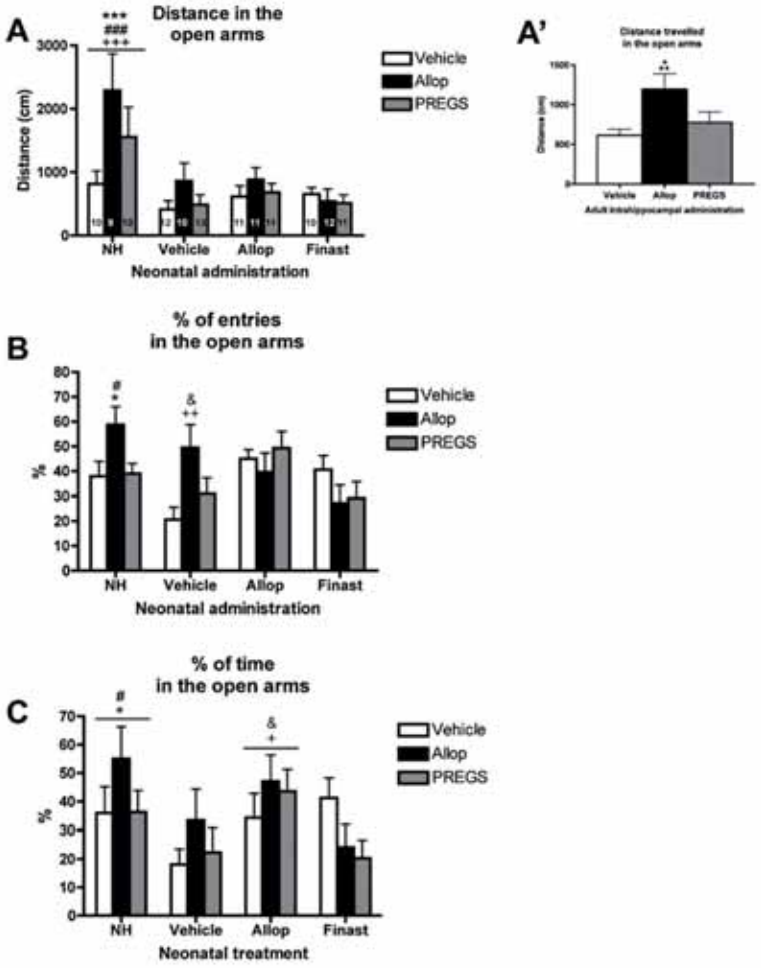

Fig. 3. Anxiety-like behaviour in the elevated plus maze. (A) Neonatal and adult intrahippocampal administration in the activity in the open arms. Neonatal effect: ***NH vs. Vehicle ($p < 0.001$); ###NH vs. Allop ($p < 0.001$); +++NH vs. Finast ($p < 0.001$). (A') Adult intrahippocampal administration effect. **Allop vs. Vehicle ($p < 0.01$); *Allop vs. PREGS ($p < 0.05$). B) Neonatal and adult intrahippocampal administration in the %OAE. The increase in the %OAE in Allop intrahippocampal administered animals [intrahippocampal Allop > (vehicle, PREGS)] is observed in NH (#Allop vs. Vehicle ($p < 0.05$) and *Allop vs. PREGS ($p < 0.05$)) and Vehicle animals (++Allop vs. Vehicle ($p < 0.01$); &Allop vs. PREGS ($p < 0.05$)) but not in neonatal administered Allop and Fianst groups. (C) Neonatal and adult intrahippocampal administration in the %TOA. Neonatal administration effect: *NH vs. Finast ($p < 0.05$); #NH vs. Vehicle ($p < 0.05$); &Allop vs. Finast ($p < 0.05$); +Allop vs. Vehicle ($p < 0.05$). The final number of subjects are represented in bars.

the injected groups in the RL-AL [$F_{(2,24)} = 3.38$; $p < 0.05$] see Fig. 4B. Post hoc Duncan test showed that aversive learning was increased in subjects intrahippocampally injected with PREGS compared to the control (Vehicle adult) group ($p < 0.05$). In addition the analysis of the retention latency showed similar results as described for the difference between retention and acquisition latency (data not shown).

4. Discussion

4.1. Neonatal NS levels

Results of the present study showed that hippocampal Allop levels were highly increased (300-fold times than in NH and Vehicle groups) in animals neonatally injected with Allop at P5. In addition, an increase in Epiallopregnanolone and Testosterone levels was also observed in these animals. The enhancement of hippocampal Epiallopregnanolone is consistent with the metabolic pathway of both NS (Allopregnanolone and Epiallopregnanolone). A previous study has shown that the increase in the Allop levels can also lead to an increase of the NS Epiallopregnanolone given that these both NS share a part of its steroidogenic route [43]. On the other hand, the increase in hippocampal Testosterone levels was unexpected (given that these both NS do not share the same metabolic pathway). Testosterone is synthesized from androstenediol by the enzyme 3-β-hydroxy-steroid dehydrogenase (3βHSD) [44] and together with its metabolite (dehydrotestosterone (DHT)) is the major male gonadial hormone responsible for the male sexual characteristics. In contrast, Allop is synthesized from Progesterone (which is well known for its effects on reproductive female behaviour and gonadial function) by the enzyme 5α-reductase and 3α-hydroxysteroid dehydrogenase (3αHSD) [45]. It is plausible that the high increase in Allop levels could be affecting other endocrines (directly or indirectly), given that one hormone never works alone and its effect may depend and affects its interaction with other hormones.

In addition, results obtained in the analysis of the Finast group did not show a decrease in the Allop levels compared to the control groups (Vehicle and NH). However, Finast injected subjects showed an increase in pregnenolone (PREG) levels at P5. As mentioned,

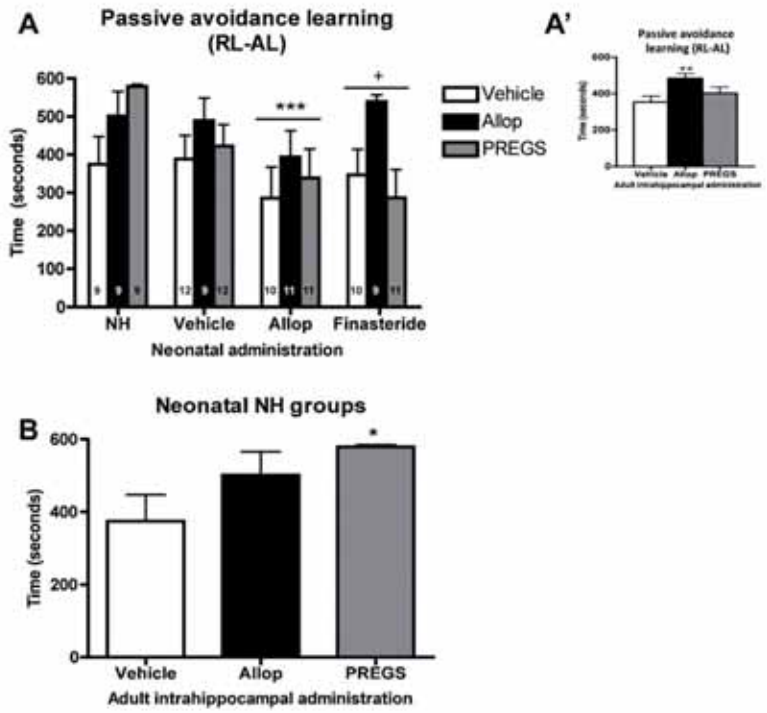

Fig. 4. Aversive learning in the passive avoidance test. (A) Neonatal and adult intrahippocampal administration and aversive learning (retention latency (RL)–acquisition latency (AL)). Neonatal administration effect: ***NH vs. Allop ($p < 0.001$); +Finast vs. NH ($p = 0.09$). (A') Adult intrahippocampal administration effect. **Allop vs. Vehicle ($p < 0.01$). B) Neonatal NH groups. Intrahippocampal administration effect: *PREGS vs. Vehicle ($p < 0.05$). The final number of subjects are represented in bars. Eight subjects were excluded from the analysis (see Section 2).

in adult animals, Finast is a potent inhibitor of the 5α-reductase and impedes the conversion from progesterone to dihydroprogesterone (DHP) and thus, impedes Allop synthesis [46]. It has been described that there are two main types of 5α reductase (Type 1 and Type 2) [47]. Both isozymes are able to 5α-reduce all steroids, but with different kinetics and specificity. Androgens, progestogens and corticosteroids can all be 5α reduced; their affinity for the type I isoform is in the micromolar range, much lower than that for the type II isoform, which is in the nanomolar range [48], however, for both isozymes, Progesterone is the preferred substrate, followed by Testosterone and at a considerable distance corticosterone. Thus, it is plausible that the inhibition of the type II isoform could increase the activity of the other in a dose-dependent way, explaining the inability of Finast to decrease the Allop levels as such in the control groups (NH and Vehicle). Nevertheless, differences in metabolic turnover between neonatal pups and adult rats are also plausible and cannot be discarded. Further studies would be of interest, to clarify Finast actions on Allop levels at early stages. On the other hand, inhibition of the 5α-reductase metabolite leads to an increase of the main precursor molecule PREG. PREG is synthesized from cholesterol by the cholesterol side-chain cleavage enzyme (P450scc) and then is metabolized into different neuroactive steroids [49]. In this sense, PREG and its sulphated form PREGS have been widely described to modulate several receptors such as GABAA, NMDA, AMPA, kainite, nicotinic, and also sigma I type receptor [1,50,51]. Thus, the behavioural intrahippocampal results of the present study in the Finast injected animals can be attributed to the increase in the NS PREG rather than in the decrease of the Allop levels. Globally, it is also necessary to keep in mind that the effects reported in the analysis of the behavioural data could be related not only to the observed changes in hippocampal Allop or PREG levels but also to changes in these NS and other NS in different brain structures.

4.2. Neonatal and adult intrahippocampal NS administration effects and exploration and anxiety-like behaviour

As hypothesized (see Section 1), results of the present experiment demonstrate that neonatal Allop levels manipulations alter the hippocampal response to NS. In this sense, the increase in the percentage of entries in the open arms of the EPM in adult animals after the intrahippocampal Allop administration (indicating an anxiolytic effect) was only observed in control animals (NH and Vehicle). In fact, animals that received neonatal Allop or Finast showed a different behavioural response to intrahippocampal Allop administration compared to those that did not receive it.

Furthermore, present data also indicate that the neonatal administration of Allop affect adult exploratory and anxiety-like behaviour. In this sense, the analysis of the exploratory behaviour (head dipping (global and inner)) and the anxiolytic like behaviour, showed that the neonatal administration of Allop increases exploration and induces an anxiolytic-like profile when comparing to the neonatal vehicle administered animals as showed by the increase in the number of global and inner head dips and the increase in the percentage of entries and time spent in the open arms, respectively. Moreover, results of intrahippocampal administration, showed an increase in the exploratory behaviour in adult animals intrahippocampally administered with Allop as reflected in the increase in the head dips. These data is in accordance with the Allop anxiolytic GABAA positive modulator profile previously described (see Section 1) [45] and with in previous studies of our lab in the open field test [30], in the EPM [6,27,30] and with neonatal Allop profile on

adult anxiety scores, when administered at lower doses (10 mg/kg) [31]. However, Allop neonatal results have to be taken carefully and separately from Allop adult pharmacological effect, given that an increase in the exploratory behaviour and a decrease in anxiety when exposed to novel environments could indicate an impulsive and risking behaviour.

Interestingly, results of neonatal Finast administration showed a decrease in the exploratory behaviour together with the Vehicle animals without effect on the EPM test. In this sense, previous experiments in our lab have shown that neonatal administration of Finast induces an anxiogenic profile in the EPM [27]. However in this previous experiment, the anxiety-assessing test (EPM) was performed alone without a previous Boissier. It has been reported, that the Boissier test before the EPM is optimal to avoid the well-known neophobia effect on animals [34–36]. It is plausible that these differences in the procedures could affect the behavioural performance of the animals, given that different aspects of anxiety-like behaviour is evaluated in each procedure.

In addition, it is also important to remark that in the present experiment differences were observed between NH and neonatal Vehicle administrated animals. For instance, a decrease in head dips explored and an increase in anxiety scores were observed in neonatal administered Vehicle animals. However, this effect seems to be reversed by the neonatal administration of Allop. In this sense, it has been previously reported that early-life environmental events can induce profound long-lasting changes in several behavioural and neuroendocrine systems in the adulthood [54,55]. Although postnatal manipulation carried out in this experiment is minimized to 5 days of injection after that pups are quickly returned to the mother in order to avoid interferences with the neonatal treatment, results on Vehicle animals demonstrate that neonatal manipulation of pups also affects the adult behaviour.

Globally, present data highlight the importance of Allop levels during a critical developmental window in which the formation of several structures such as the hippocampus occurs. Furthermore, it is also necessary to keep in mind that GABAA receptor exerts excitatory actions during development, and the switch from excitatory to inhibitory forms takes place around second week of neonatal rat pups [52,53]. Thus, it is plausible that the administration of Allop during the first postnatal week alters the CNS excitability through the activation of GABAA receptor and has important consequences in the formation of the adult CNS.

4.3. Neonatal and adult intrahippocampal NS administration effects and aversive learning performance

Results of the present experiment indicate that neonatal administration of Allop alters passive avoidance learning. Although the neonatal Allop administration (a dose of 10 mg/kg from P5 to P9), did not affect the passive avoidance performance in the adulthood in previous experiments [27], present data indicate that neonatal Allop administered at higher doses (20 mg/kg) decreases the aversive learning in the adult age. Present result is in accordance with the previous described detrimental Allop adult profile (see Section 1), but also with the effects of other GABAA positive modulators such as ethanol [14,56]. In fact, it has also been demonstrated that neonatal ethanol administration (from P4 to P9) deteriorates spatial learning in the adulthood [57] and that effect is not mediated through its effect on NMDA and AMPA receptors [58] but through the ethanol action on GABAA receptors [59]. In accordance, previous studies demonstrated that ethanol administration induced a dose and time-dependent increase in brain concentration of Allop which is in part also involved in the alteration of hippocampal CA1 neurophysiology and learning impairments in the adulthood [60–62]. On the other hand, neonatal Finast administration also showed an altered adult aversive learning performance. In this sense, accordingly to a previous experiment [27] a decrease in aversive learning when compared to NH was also observed in neonatal Finast administered animals. As observed in NS quantification results, neonatal Finast administration importantly increased the levels of PREG. Thus, it is plausible that the detrimental effect in aversive learning could be due to the actions of PREG onto plenty of receptors such as GABAA, NMDA, AMPA, kainite, nicotinic, and also sigma I type [1,50,51], that are present in the neonatal brain.

In addition and on the contrary of what was expected, results of adult NS administration showed that intrahippocampal Allop increases the passive avoidance learning. This surprising result does not agree with the previous mentioned Allop profile (see Section 1). However, the partition analysis performed only with NH animals showed an increase in aversive learning in subjects administered with PREGS when compared to the intrahippocampally administered with Vehicle, without effects of intrahippocampal Allop, in agreement with previous data of our laboratory [6]. In this sense, although no significant interaction was found between neonatal and adult administration in the aversive learning performance, present data could indicate that neonatal administration modifies the behavioural effect of PREGS infused into hippocampus in the adulthood.

5. Conclusions

The present study demonstrates that neonatal alteration of Allop levels during the first postnatal week is important for exploratory behaviour, anxiety relevant scores and avoidance learning performance in the adulthood. Moreover, present data also indicates that neonatal alteration of Allop and PREG levels can modify adult hippocampal response to adult Allop administration and behavioural performance as reflected in the EPM test. This is the first time to our knowledge that effect is documented in the literature at the doses tested, and supports the importance of neonatal Allop levels for the maturation and adult response of the hippocampus. However, further studies would be necessary to asses the mechanisms underlying these changes for a completely understanding of the NS role in the CNS maturation and in the aetiology of psychopathologies.

Acknowledgements

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Experiment 2

Research article

"Neonatal finasteride administration alters hippocampal α4 and δ $GABA_AR$ subunits expression and behavioural responses to progesterone in adult rats"

Poster presentation (Annex 4)

" Alteration of neonatal Allopregnanolone levels affects α4 and δ $GABA_AR$ subunits expression and adult behavioural hippocampal response to neurosteroids"

In the present experiment we studied the changes in hippocampal α4 and δ $GABA_AR$ subunits during early developmental stages (from P6 until P15) and in the adulthood, induced by neonatal NS manipulation levels. For that purpose animals were neonatally administered as described in the Experiment 1: with finasteride (50mg/kg), vehicle or saline from P5 to P9. A NH group was also included.

In addition, we also tested whether changes in hippocampal α4 and δ $GABA_AR$ subunits induced by neonatal NS administration were accompanied by an altered behavioural response to increased Allopregnanolone levels (progesterone administration). For that purpose adult (80-90 days) animals were administrated with progesterone (25mg/kg) during 48h in order to increase Allopregnanolone fluctuating levels and exploratory (Boissier) and anxiety-like behaviour (EPM) was tested 20min after the last progesterone administration. An schematic representation of the experimental design can be observed in the following page.

Specific objectives

• To study hippocampal α4 and δ $GABA_AR$ subunits expression during early development as a consequence of neonatal fluctuations of Ns levels.

• To study whether neonatal manipulation of Ns levels modifies adult behavioural response to increased Allopregnanolone levels (induced by progesterone administration).

• To study hippocamapal α4 and δ $GABA_AR$ subunits expression in the adulthood as a consequence of neonatal administration and adult progesterone administration.

Effects of neonatal manipulation of Ns and hippocampal expression of α4 and δ $GABA_AR$ subunits and behavioural response to progesterone

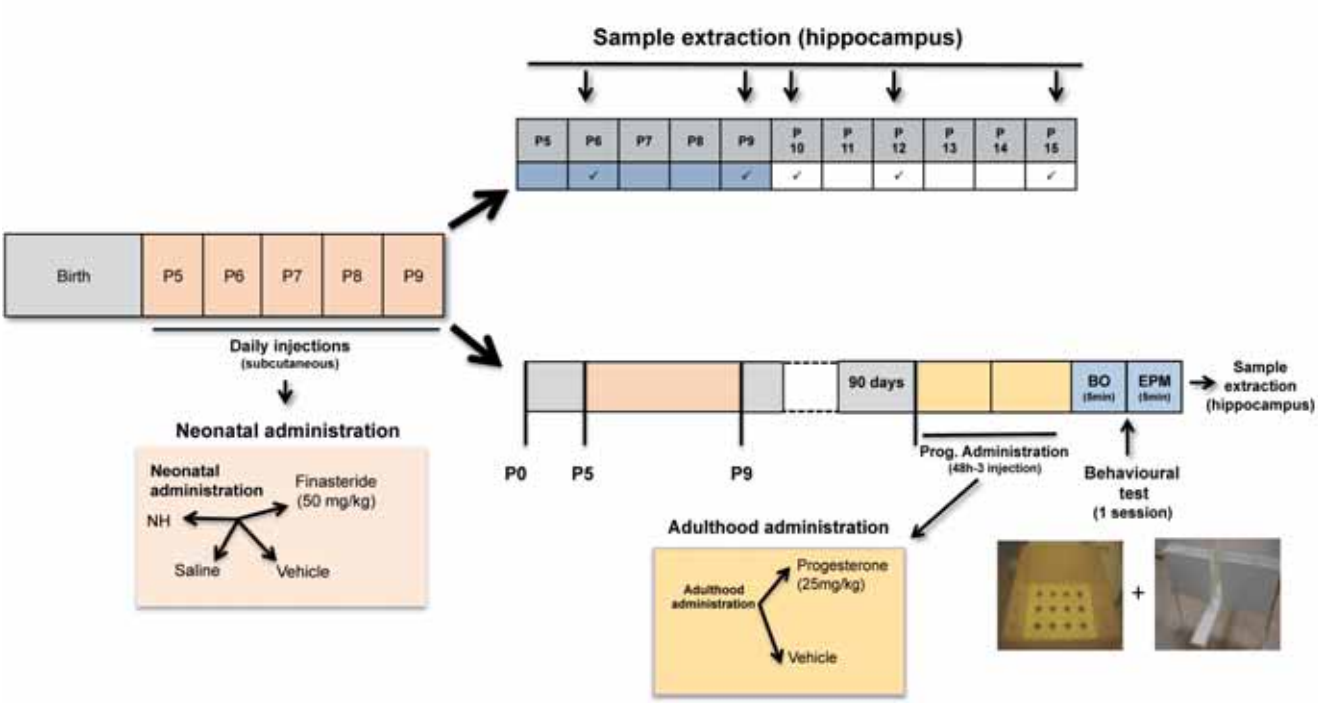

Fig 10: Experimental design

Experiment 2:
Neonatal finasteride, hippocampal $GABA_A$Rs & behaviour

Research article

Mòdol L, Casas C, Llidó A, Vallée M, Navarro X, Pallarès M, Darbra S. Neonatal finasteride administration alters hippocampal α4 and δ $GABA_AR$ subunits expression and behavioural responses to progesterone in adult rats. Int J Neuropshychophamacology, 2014,

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ARTICLE

Neonatal finasteride administration alters hippocampal α4 and δ $GABA_AR$ subunits expression and behavioural responses to progesterone in adult rats

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Abstract

Allopregnanolone is a neurosteroid that has been reported to fluctuate during early developmental stages. Previous experiments reported the importance of neonatal endogenous allopregnanolone levels for the maturation of the central nervous system and particularly for the hippocampus. Changes in neonatal allopregnanolone levels have been related to altered adult behaviour and with psychopathological susceptibility, including anxiety disorders, schizophrenia and drug abuse. However, the mechanism underlying these changes remains to be elucidated. In the present study we assessed changes in hippocampal expression of α4 and δ $GABA_A$ receptor ($GABA_AR$) subunits as a consequence of neonatal finasteride (a 5-α reductase inhibitor) administration during early development (PD6 to PD15) in male rats. We observed that the treatment altered the temporal window of the natural peak in the expression of these subunits during development. Additionally, the level of these subunits were higher than in non-handled and control animals in the adult hippocampus. We observed that in adulthood, neonatal finasteride-treated animals presented an anxiogenic-like profile in response to progesterone administration which was absent in the rest of the groups. In conclusion, these results corroborate the relevance of neonatal maintenance of neurosteroid levels for behavioural anxiety responses in the adult, and point to some of the mechanisms involved in this alterations.

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Key words: Finasteride, $GABA_AR$ δ subunit, $GABA_AR$ α4 subunit, hippocampus, neurodevelopment.

Introduction

Neurosteroids are steroids that can be synthesized *de novo* in the nervous tissue from cholesterol (Baulieu et al., 1981). Although neurosteroids can act by binding on nuclear receptors, they can also modulate neuronal excitability by the allosteric modulation of ionotropic receptors (Majewska et al., 1986; Rupprecht, 2003). In the brain, neurosteroid concentrations vary regionally, depending on environmental and behavioural circumstances. In fact, the fluctuation of neurosteroid levels has generated an increasing interest given its relation with psychiatric conditions, including anxiety, depression, schizophrenia and other cognitive and mood disorders (Dubrovsky, 2005; Marx et al., 2011).

The neurosteroid allopregnanolone (3α-hydroxy-5α-pregnaone-20-one), is a 3α-reduced progesterone metabolite (Rupprecht, 2003). Progesterone is readily metabolized in the brain to 5α-dihydroprogesterone by 5α-reductase enzymes; 5α-dihydroprogesterone is

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then further reduced to allopregnanolone that mainly exerts its actions through the positive allosteric modulation of GABAA receptors ($GABA_AR$) (Majewska et al., 1986). In the rat, the cortical levels of allopregnanolone fluctuate greatly during development, showing a first prenatal peak followed by low levels during birth and the first week of life (from PD0 to PD8), these levels are similar to those found in adult brain. During the second week of life (from PD10 to PD14) a second peak is also observed (Grobin and Morrow, 2001; Grobin et al., 2006).

Previous studies have shown the relevance of neonatal allopregnanolone levels for brain maturation (Grobin et al., 2003, 2006; Gizerian et al., 2004) and for adult behaviour (Martin-García et al., 2008; Darbra and Pallarès, 2010, 2011, 2012), supporting the relevance of postnatal allopregnanolone levels for brain development and for adult affective behaviour. Concretely, previous studies in our laboratory showed that alteration of neonatal allopregnanolone or pregnenolone levels (by allopregnanolone and finasteride administration, respectively, from PD5 to PD9) suppressed intrahippocampal allopregnanolone anxiolytic effects in the elevated plus maze-test (Modol et al., 2013). We have also reported that animals which, as neonates suffered, subchronic increases in allopregnanolone levels (by allopregnanolone administration, from PD5 to PD9) or pregnenolone and testosterone levels (by finasteride administration, from PD5 to PD9) did not show the improvement of the prepulse inhibition response observed in control animals following intrahippocampal allopregnanolone administration (Darbra et al., 2013). However, the mechanisms by which these developmental changes take place are still unknown.

One possible mechanism could be through the main allopregnanolone modulation ionotropic target, the $GABA_AR$. $GABA_AR$ is a pentameric structure that mediates inhibition in the mature brain, and is typically composed of 2α, 2β and 1γ subunits. There are 19 different subtypes of $GABA_AR$ combinations indicating a high level of structural heterogeneity and function (Olsen and Sieghart, 2009). The combination of $\alpha4\beta2\delta$ has been described to be important for tonic inhibition in dentate granule cells of the hippocampus (Stell et al., 2003) and in CA1 pyramidal neurons (Mangan et al., 2005). $\alpha4$ and δ $GABA_AR$ subunits have been reported to be insensitive to benzodiazepines modulation but especially sensitive to fluctuating allopregnanolone levels as a consequence of physiological (Follesa et al., 2002) and pathological conditions, such as anxiety (Gulinello et al., 2001; Shen et al., 2005), epileptic seizures (Brooks-Kayal et al., 1998) or alcohol intake (Sundstrom-Poromaa, et al., 2002). Changes in steroid sensitivity corresponding to plastic changes have been reported as a consequence of changes in progesterone metabolites *in vitro* (Biggio et al., 2006; Shen et al., 2007) and *in vivo* studies in pregnant and pseudopregnant rats (Concas et al., 1998; Smith et al., 1998).

Given that alterations of allopregnanolone levels during a critical developmental stage have been shown to alter behavioural response to intrahippocampal allopregnanolone administration, and that $\alpha4\beta2\delta$ $GABA_AR$ plays an important role in the allopregnanolone effect, we hypothesized that changes in allopregnanolone biosynthesis during development and in the early neonatal period could affect $GABA_AR$ subunit expression in the hippocampus. We conducted three different experiments in order to study the hippocampal mechanisms underlying manipulation of neonatal allopregnanolone levels by finasteride administration, a 5α-reductasse inhibitor that impedes the synthesis from progesterone to dihydroprogesterone, i.e. an inhibitor of allopregnanolone synthesis (Azzolina et al., 1997; Mukai et al., 2008). We have previously shown that neonatal administration of finasteride (from PD5 to PD9) induces an anxiogenic-like profile in the elevated plus maze test (Martin-Garcia et al., 2008) and decreases the novelty-induced locomotor activity both in open field and in the Boissier test (Darbra and Pallarès, 2010). We assessed the changes in hippocampal $\alpha4$ and δ $GABA_AR$ subunits expression during early development (from PD6 to PD15), as a consequence of finasteride administration and hippocampal neurosteroids levels were analysed at PD10, 24 h after the last injection of finasteride. We further studied the effects of neonatal allopregnanolone manipulation on anxiety-like behaviour and the expression of hippocampal $\alpha4$ and δ subunits to elevation of allopregnanolone levels by progesterone administration in adulthood.

Methods

Hippocampal $GABA_AR$ α4 and δ subunits expression during early development and finasteride administration

Animals

One hundred and two male Wistar rats derived from 21 pairings raised at an in-house colony (Laboratori de Psicobiologia, Universitat Autònoma de Barcelona, Spain) were used for Western blot analysis (see Table 1 for a detailed neonatal treatment assignment according pairs and groups composition). Rats were housed in a temperature-controlled room (22–24 °C)

Table 1 Neonatal treatment assignments and compositions of groups in experiments 1 and 3

Neonatal treatment assignment and composition of groups in experiment 1																						Postnatal day					
Pairings	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	PD6	PD9	PD10	PD12	PD15	Total
NH	5			6		6	5					4	4									5	8	6	5	6	30
Saline		4			5					4				4			4					5	4	4	4	4	21
Vehicle									4						5	5		4			4	4	5	5	4	4	22
Finasteride			7					6			5								5	6		7	6	6	5	5	29

Neonatal treatment assignment and composition of groups in experiment 3											Adult administration		
Pairings	1	2	3	4	5	6	7	8	9	10	Vehicle behaviour/ (WB)	Progesterone behaviour/ (WB)	Total
NH	3					4		7		3	8/(7)	9/(7)	17
Vehicle		7		4			4				7/(7)	8/(6)	15
Finasteride			6		7				3		9/(6)	7/(6)	16

Animals used for Western blot (WB) analysis after behavioural evaluation are represented in parenthesses.

on a 12 h light/dark cycle. Male breeders were separated from the females after 48 h, pregnant females were closely watched and on the day of birth (designed day 0) litters were culled to 10 pups.

Neonatal administration

Pups were injected s.c. with: finasteride (50 mg/kg) (n=29), β-cyclodextrin vehicle (n=22) or saline (n=21), once per day from PD5 to PD9. In addition, a non-handled (NH) group (n=30) was included in order to avoid the possible effects of neonatal manipulation (see Table 1). Finasteride, obtained from Sigma (Germany), was dissolved in vehicle solution 20% cyclodextrin ((2-hydroxypropyl)-β-cyclodextrin, also from Sigma). Complete dissolution of finasteride and cyclodextrin was achieved by sonication. The injection volume was 0.1 ml/10 g body weight. After injections, pups were immediately returned to the home cage with their mother. All animals were obtained, housed and sacrificed in accordance with the protocol approved by the Committee for Care and Use of Experimental Animals of the Universitat Autònoma de Barcelona and Generalitat de Catalunya (Regional Government) and follows the guidelines approved by the European Council Directive (86/609/ECC).

Sample extraction and Western blot analysis

Male rats were sacrificed by decapitation at PD6 (n=21), PD9 (n=23), PD10 (n=21), PD12 (n=18) and PD15 (n=19). At PD6 and PD9 they were sacrificed 1 h after the last administration. Brains were removed and half of their hippocampus was dissected out, immediately frozen in dry ice and stored at −80 °C until needed. For protein extraction, the hippocampus was homogenized with a Mixer Mill MM 400 (Retsch Gmbh, Germany) in 10 mM HEPES (pH 7.4), 2% Triton X-100, 0.3 M KCL, 300 mM NaCl, 1 mM EDTA containing protease inhibitor cocktail (10 μl/ml, Sigma) and sodium orthovanadate (1 mM, Roche, Switzerland) and cleared by centrifuging at 13000 g for 20 min at 4 °C. Supernatant was used for protein content quantification by the bicinchoninic acid (BCA) protein assay (Pierce, USA). Equal amounts of protein (30 or 50 μg) were deposited by electrophoresis onto 10% SDS-polyacrylamide gels and transferred to a polyvinyl difluoride membrane (Bio-Rad). Membranes were blocked with 5% skimmed milk powder in TBS (100 mM Tris, 0.9%. NaCl, pH 7.6), 0.05% Tween-20 (TBS-Tween) for 1 h and incubated with primary antibodies against α4, δ $GABA_AR$ subunits (rabbit anti-α4GABAR: 1/1000 (Phosposolutions, Lucerne, Switzerland); rabbit anti-δGABAR: 1/800 (Phosposolutions, Switzerland)) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Sigma, 1/5000) overnight at 4 °C. Horseradish peroxidase coupled antibody was used for secondary incubation in TBS-Tween for 90 min at room temperature. After washing with TBS-Tween, blots were developed using an ECL Plus detection kit (Millipore, USA) and the images were analysed by band densitometry with the Gene Snap and Gene Tools software in a Gene

Genome apparatus (Syngene, UK). Probing with the δ antibody yielded a double band in adult samples, as reported for other GABA subunits (Kern and Sieghart, 1994) and so both were considered for densitometry. The results were standardized to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (36 kDa band) control protein. The same control subject was included in all blots and so densitometry for each individual was relative to its GAPDH values and this control subject.

RNA and protein extraction

The other half of the hippocampus from the same animals used for protein extraction (n=4 for each group) was immerse in RLTβ buffer from the RNA extraction kit (Qiagen, Germany) to obtain total RNA following the manufacturer's instructions. Two micrograms of RNA was reverse-transcribed using 10 mmol/l, DTT, 200 U superscript II RNase H reverse transcriptase (Invitrogen, USA), 10 U RNase out ribonuclease inhibitor (Invitrogen) and 0.5 M oligo(dT) and 0.5 M of random hexamers (BioLabs, USA). The reverse transcription cycle conditions were 25 °C for 10 min, 42 °C for 1 h and 72 °C for 10 min. The primers used for real-time PCR were: *Gabra4* (F, 5′-AATGTGTCAGCCACACCTCC-3′; R, 5′-TGCCCCAAATGTGACTGGAA-3′), *Gabrd* (F, 5′-AGGAACCGGGGTGTCTACAT-3′; R, 5′-CAGCACAGTGGTGATGCCTA-3′) and *Gapdh* (F, 5′-AGTTCAACGGCACAGTCAAG-3′; R, 5′-TACTCAGCACCAGCATCACC-3′). Real-time PCR (iCycler iQ5 Real-Time PCR Detection System, USA) was performed using Brilliant III Ultra-Fast SYBR® Green qPCR master mix (Agilent Technologies, USA). We previously fixed the optimal concentration of the cDNA to be used as the template for each gene analysis to obtain reliable CT (threshold cycle) values for relative quantification. Four samples were used per condition and each sample was run in duplicate. The thermal cycling conditions were: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s, 60 °C for 1 min. CT values were obtained and analysed with the iCycler iQ5 Software. Fold change in gene expression was estimated using the CT comparative method (2^{-DDCT}) normalizing to Gapdh CT values and relative to vehicle and saline control samples, which presented no differences between them at all times analysed.

Hippocampal neurosteroids levels at PD10

Given the results observed at PD10, hippocampal neurosteroids levels were determined at PD10. Fourteen male Wistar rats, derived from three pairings raised at the in-house colony, were administered β-cyclodextrin (n=4) or finasteride (n=5) as described in experiment 1. Five more animals were used as NH group (n=5). Animals were sacrificed by decapitation at postnatal day 10 (PD10). Brains were removed and the hippocampus was harvested and frozen in dry ice. Samples were stored at −80 °C until used for the steroid quantification. Each specimen included two weighed hippocampi. Pregnenolone, allopregnanolone, epiallopregnanolone, THDOC, and testosterone were determined by isotope dilution combined with GC/MS according to the protocol previously described (Vallée et al., 2000; George et al., 2010).

Effects of neonatal allopregnanolone levels on the behavioural response and the expression of hippocampal α4 and δ subunits to progesterone administration in the adult rat

Animals and neonatal administration

Forty-eight male Wistar rats derived from 10 pairings raised at the in-house colony were used. Drug solution and neonatal administration procedures were conducted as described in experiment 1 (see Table 1).

Adult administration

In order to increase allopregnanolone levels, animals were injected i.p. with progesterone (progesterone, 25 mg/kg) (n=24) or vehicle (n=24) at 90 days of age (87–92 days). These steroid administration paradigms have been shown to result in physiological levels of circulating steroids (Moran and Smith, 1998). Animals were administered daily (once per day) for three consecutive days and control rats received the same volume of vehicle (20% cyclodextrin). At 20 min after the third administration, adult animals were behaviourally evaluated and then sacrificed.

Elevated plus maze test

Elevated plus maze test was carried out as described previously (Pellow et al., 1985; Darbra and Pallarès, 2012). Briefly, animal was placed in the centre of the apparatus facing an open arm and recorded for 5 min. The number of entries into open and closed arms, number of total entries and time spent in the open and closed arms and in the centre of the apparatus were recorded. Increased percentage of time in or entries into open arms is indicative of a reduced anxiety state in the elevated plus maze test (Pellow et al., 1985). The number of open arms plus closed arms entries was used as a measure of activity. The percentage of time spent in the centre of the maze

was used as an independent measure of decision-making (Manhaes et al., 2008).

Sample extraction and Western blot analysis

Thirty-nine male rats were used for Western blot studies. They were sacrificed by decapitation immediately after the behavioural tests and 30 min after the last injection of progesterone (n=19) or vehicle (n=20). Samples and protein extraction and Western blot procedures were conducted as described previously for early development studies (see above).

Statistical analyses

The statistical analyses were performed using the STATISTICA package (StatSoft, USA). In order to control the possible β-cyclodextrine administration effect on α4 and δ GABA$_A$R subunits expression, a preliminary two way ANOVA was performed with neonatal treatment (two levels: saline/β-cyclodextrine) and postnatal day (five levels: PD6/PD9/PD10/PD12/PD15) as factors. Data from the Western blots were analysed using two way ANOVA with neonatal treatment (three or four levels: NH/β-cyclodextrin/saline/finasteride) and postnatal day (five levels: PD6/PD9/PD10/PD12/PD15). Data from adult Western blots, RT-PCR, and EPM test were analysed using two way ANOVA with neonatal treatment (three levels: NH/control/finasteride) and adult administration (two levels: vehicle/progesterone). Data from hippocampal allopregnanolone levels were analysed using one way ANOVA for neonatal administration (three levels: NH/β-cyclodextrin/finasteride). *Post-hoc* Neuman–Keuls tests were used when necessary. Data are shown as mean ± S.E.M.

Results

Hippocampal GABA$_A$R α4 and δ subunits expression during early development and finasteride administration

The preliminary analysis performed in order to control the consequences of the β-cyclodextrin administration, showed no differences between the neonatal vehicle (β-cyclodextrin and saline) administered groups in the analysis of α4 and δ GABA$_A$R subunits expression ($F_{4,33}$=0.72; N.S; and $F_{4,33}$=0.64; N.S, respectively). Therefore, we pooled the results in a single group as control (i.e. vehicle and saline animals) for comparisons with the other neonatal treatment. See Figs 1*a* and 2*a*.

The analysis of the α4 subunit expression showed a significant interaction between neonatal treatment and postnatal day tested ($F_{8,87}$=2, 43; p<0.01) indicating that expression of the α4 subunit is different across postnatal days depending on the neonatal administration (Fig. 1). Separate analysis by neonatal treatment was then performed. The α4 GABA$_A$R subunit showed an evolution pattern in both NH and Control groups during postnatal development ($F_{4,25}$=4.99; p<0.01 and $F_{4,38}$=3.78; p<0.01, respectively). We observed that α4 expression was high at PD6 in both groups and down regulated progressively along early postnatal development. *Post-hoc* analysis showed differences when comparing the values at PD6 with those at later postnatal days (PD9/PD10/PD12/PD15; p<0.01 in NH group and p<0.05 in Control group, for all comparisons; Fig. 1). In the neonatal finasteride administered groups, however, a different pattern of α4 GABA$_A$R subunit expression was observed ($F_{4,24}$=4.82; p<0.01). Although the α4 expression at PD6 was higher than at PD9 (p<0.05), PD12 (p<0.01) and PD15 (p< 0.01), no differences were observed between PD6 and PD10 (p>0.05), indicating that a second increase in α4 subunit expression occurs at PD10 due to neonatal finasteride administration (Fig. 1). Complementary analyses at each time-point were performed. Significant differences at PD10 were observed ($F_{2,18}$=4.38; p=0.02): α4 subunit expression was higher in animals treated with finasteride than in control animals (p< 0.05). Although an increase in α4 GABA$_A$R subunit expression in NH animals was observed at PD12, no statistical differences among groups $F_{2,15}$=2.77; p=0.09) (Fig. 1).

We also analysed the relative expression of the subunits at transcript level using Real-time PCR. Regarding *Gabra4* mRNAs expression, significant effects of both neonatal treatment and postnatal day tested were observed ($F_{2,45}$=5.11; p=0.01 and $F_{4,45}$=6.24; p<0.01, respectively), while no significant interaction effect was observed. Consecutive *post-hoc* analysis showed that neonatal finasteride administration globally increased *Gabra4* mRNA expression compared to control animals (i.e. NH and vehicle +saline groups, N-K p<0.01 and p<0.05, respectively). Moreover, *Gabra4* mRNA expression showed an increased through early neonatal period (i.e. PD6 to PD15), being the lowest at PD6 (see Fig. 3*a*).

Results of the δ subunit expression showed significant interaction between neonatal administration and postnatal day ($F_{8,87}$=3.82; p<0.001) (Fig. 2). Separate analysis by neonatal treatment was then performed. Results of NH animals showed a peak of δ GABA$_A$R subunit at PD12 decreasing at PD15 (PD12 *vs.* PD15: p<0.05). However, no differences were observed in control group in δ expression ($F_{4,36}$=1.88; N.S), indicating that δ GABA$_A$R remained stable along the

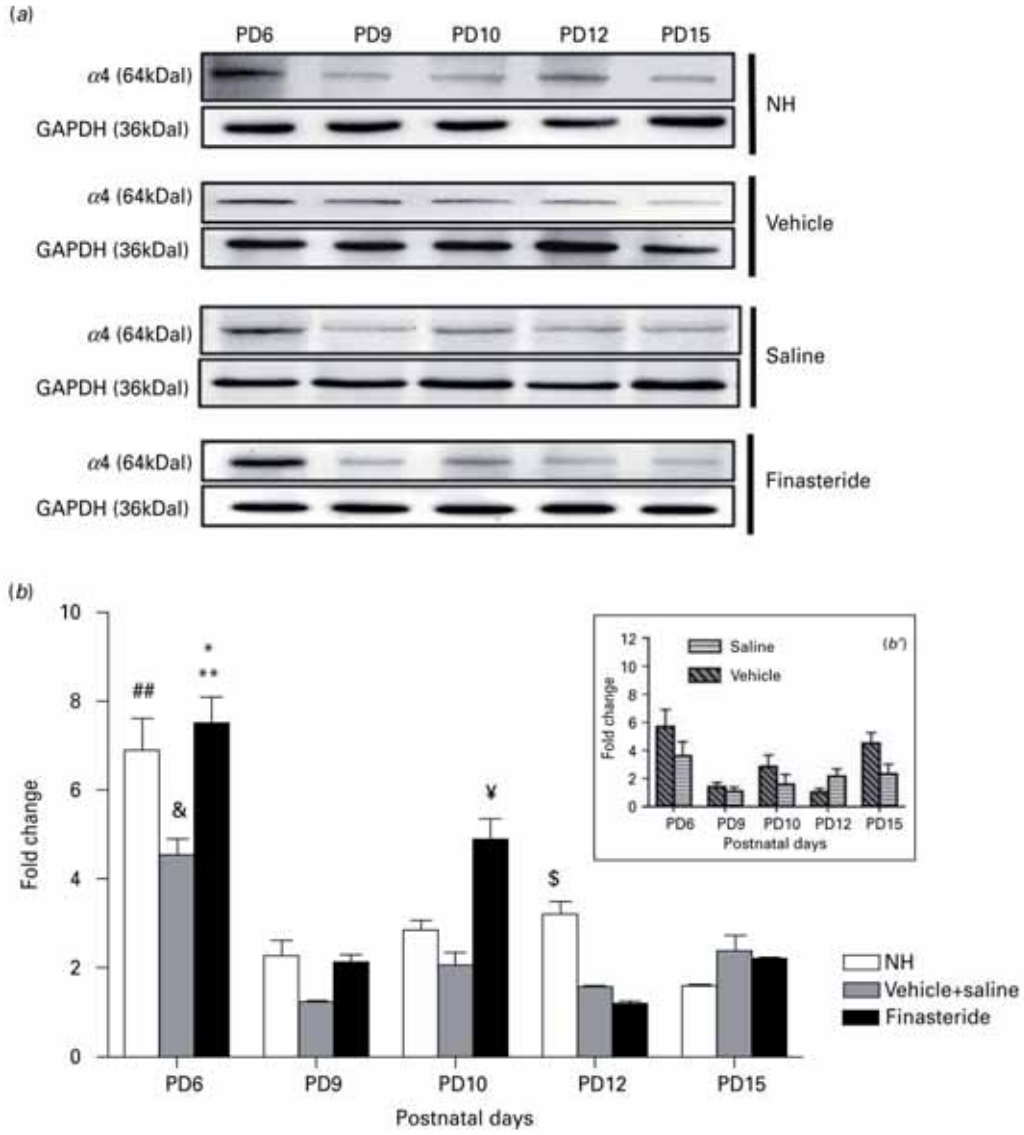

Fig. 1. Hippocampal α4 $GABA_AR$ subunit expression during postnatal development and neonatal administration normalized to GAPDH levels and to the same control subject was included in all blots. Bars represent the average±S.E.M. fold changes of protein expression in the hippocampus of non-handled (NH) (open bars), vehicle and saline (control) (grey bars) and finasteride treated (closed bars) animals at the chosen neonatal age (from PD6 to PD15) (rats/condition/age, see Table 1). (*a*) Representative Western blots of α4 $GABA_AR$ subunit detected from hippocampus for each postnatal day. (*b*) Levels of α4 $GABA_AR$ subunit in the three experimental groups. Interaction effect ($F_{8,87}$=2,43; p=0.01). Partition analysis; *Neonatal administration*: NH-PD6 *vs.* NH-PD9/PD10/PD12/PD15, ##p<0.01; vehicle+saline-PD6 *vs.* vehicle+saline-PD9/PD10/PD12/PD15, & p<0.05; finasteride-PD6 *vs.* finasteride-PD9/PD15, *p<0.05 and finasteride-PD6 *vs.* finasteride-PD12, **p<0.01. *Postnatal day*: at PD10, finasteride *vs.* vehicle+saline, ¥ p<0.05; at PD12, NH *vs.* finasteride, $. (*b'*) Levels of α4 $GABA_AR$ subunit in controls groups: vehicle and saline ($F_{4,33}$=0.72; N.S). Data are shown as mean±S.E.M.

postnatal days tested. These differences may indicate a neonatal manipulation effect (Fig. 2). On the other hand, in the group with neonatal finasteride administration, in accordance with results of the α4 subunit, an increase of δ subunit expression was observed at PD10 ($F_{4,27}$=9.84; p<0.0001). Thus, the naturally occurring increased in δ subunit expression observed in NH group had been shifted to the left in finasteride-treated animals: higher levels of δ subunit expression were observed at PD10 than at PD6, PD9, PD12 and PD15 (see Fig. 2). Additional comparisons of δ $GABA_AR$ subunit levels at each time-point were

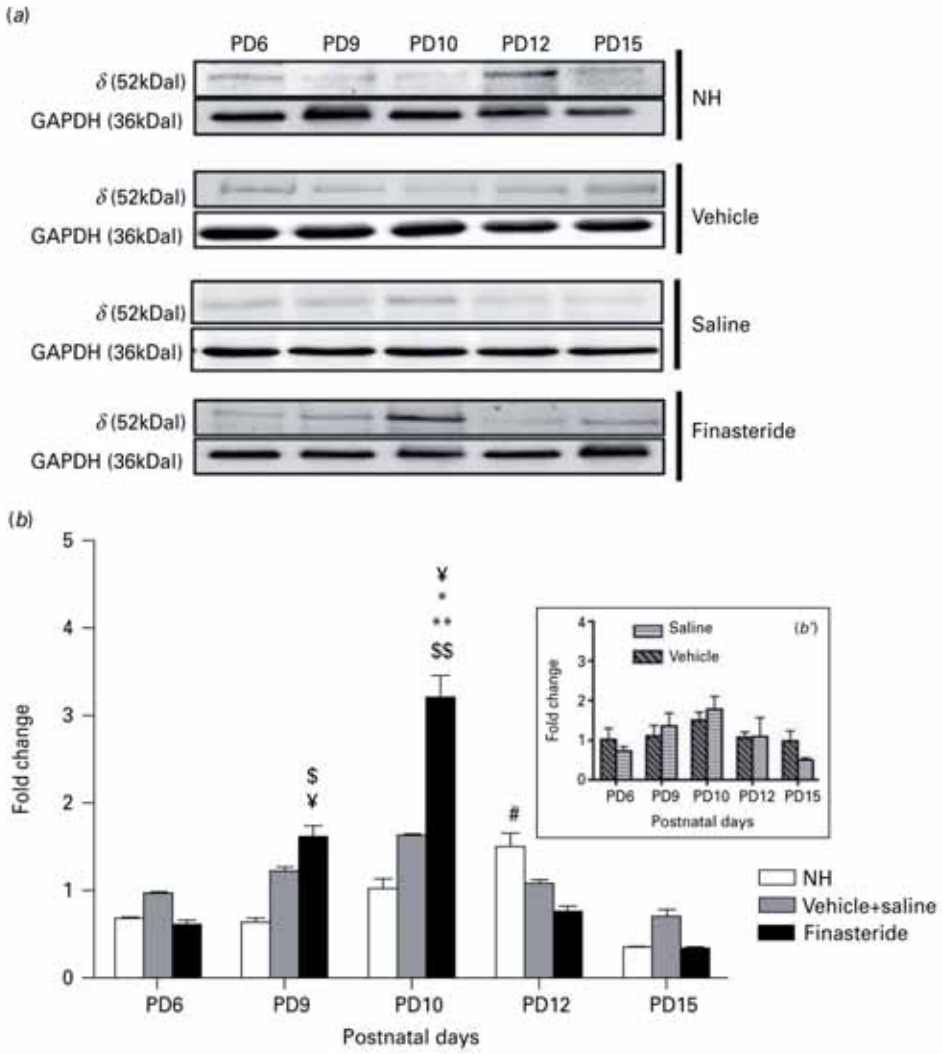

Fig. 2. Hippocampal δ $GABA_AR$ subunit expression during postnatal development and neonatal administration normalized to GAPDH levels and to the same control subject was included in all blots. Bars represent the average±S.E.M. fold changes of protein expression in the hippocampus of non-handled (NH) (open bars), vehicle and saline (control) (grey bars) and finasteride treated (closed bars) animals at the chosen neonatal age (from PD6 to PD15). (*a*) Representative Western blots of δ $GABA_AR$ subunit detected from hippocampus for each postnatal day. (*b*) Levels of δ $GABA_AR$ subunit in the three experimental groups. Interaction effect ($F_{8,87}$=3.82; p<0.001). Partition analysis; *Neonatal administration*: NH-PD12 *vs.* NH-PD15, #p<0.05; Finasteride-PD10 *vs.* Finasteride-PD9, *p<0.05; Finasteride-PD10 *vs.* Finasteride-PD6/PD12/PD15, **p<0.01. *Postnatal day*: at PD9, NH *vs.* finasteride, $$p$<0.05 and vehicle+saline *vs.* finasteride, ¥ p<0.05; at PD10, NH *vs.* finasteride, $$$p$<0.01 and vehicle+saline *vs.* finasteride, ¥ p<0.05. (*b'*) Levels of δ $GABA_AR$ subunit in controls groups: vehicle and saline ($F_{4,33}$=0.64; N.S). Data are shown as mean±S.E.M.

performed. Differences between neonatal treatments were found at PD9 ($F_{2,20}$=4.95; p<0.01) and PD10 ($F_{2,18}$=7.70; p<0.005). An increase in δ expression was observed at PD9 and PD10 in finasteride administered animals compared to NH (p<0.01 and p<0.001, respectively) and Control groups (p<0.05 for both) (Fig. 2).

ANOVA analysis showed significant interaction between neonatal administration and postnatal day in *Gabrd* mRNA levels ($F_{8,45}$=9.83; p<0.001) (Fig. 3). Separate analysis by neonatal treatment was then performed. In NH animals, higher levels of *Gabrd* mRNA were observed at PD10 and at PD15 than at PD6 and PD9 (N-K p<0.01 for PD10 and p<0.05 for PD15; see Fig. 3*b* for a detailed *post-hoc* analysis). In the control group, an increase of the transcript was only observed at PD15 (N-K: p<0.05 from all). In contrast, in the finasteride neonatal-administered group, an increase of

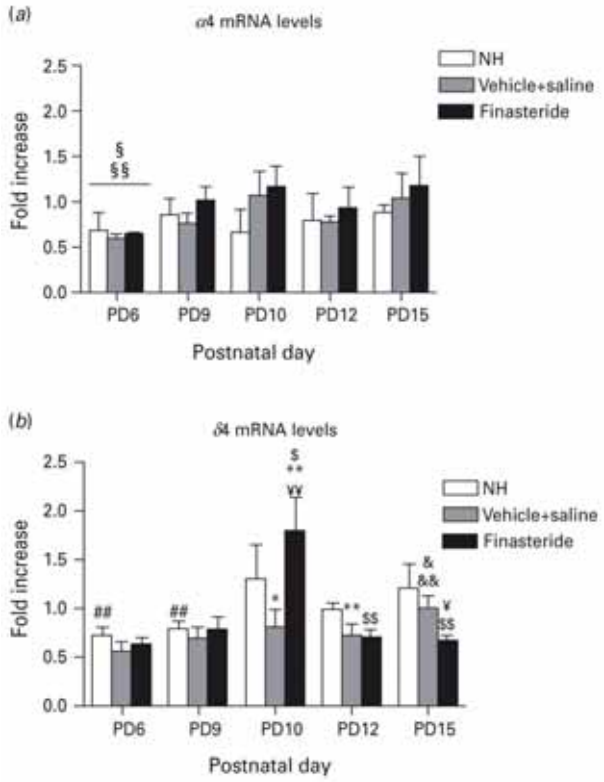

Fig. 3. Real-time PCR relative quantification of (*a*) α4 and (*b*) δ $GABA_A$R mRNAs, normalized to the GAPDH levels and to average value of control PD15 rats to evaluate developmental changes. Bars represent the average±S.E.M. fold changes of gene expression in the hippocampus of non-handled (NH) (open bars), vehicle and saline (control) (gray bars) and finasteride treated (closed bars) animals at the chosen neonatal age (from PD6 to PD15) (n=4 rats/condition/age). (*a*) α4 $GABA_A$R mRNA expression increases during early neonatal period (main effect $F_{2,45}$=5.10; p=0.01). The lowest expression of α4 $GABA_A$R mRNA is observed at PD6 (PD6 *vs.* PD9/PD12, § p<0.05 and PD6 *vs.* PD10/PD15; §§ p<0.01).; finasteride adminsitration globally increases α4 $GABA_A$R mRNA expression (main effect $F_{1,45}$=6.24; p<0.001; NH *vs.* finasteride p<0.01 and vehicle+saline *vs.* finasteride p<0.05). (*b*) Hippocampal δ GABAAR mRNA expression during postnatal development in the three experimental groups. Interaction effect ($F_{8,45}$=9.83; p<0.001). Partition analysis; neonatal administration: NH-PD6 *vs.* NH-PD10/PD15 and NH-PD9 *vs.* NH-PD10, ## p<0.01; finasteride-PD10 *vs.* finasteride-PD6/PD9/PD12/PD15, **p<0.01;vehicle +saline-PD15 *vs.* vehicle+saline-PD9/PD10/PD12, & p<0.05 and vehicle+saline-PD15 *vs.* vehicle+saline-PD6 && p<0.01. Postnatal day: at PD10, NH *vs.* finasteride, $ p<0.05; * NH *vs.* vehicle+saline, p<0.05 and vehicle+saline *vs.* finasteride, ¥ ¥ p<0.01; at PD12, NH *vs.* finasteride, $$ p<0.01 and NH *vs.* vehicle+saline, **p<0.01; at PD15 NH *vs.* finasteride, $$$p$<0.01 and vehicle+saline *vs.* finasteride, ¥ p<0.05. Data are shown as mean±S.E.M.

Table 2. Hippocampal neurosteroids levels at PD10 (expressed as ng/g tissue)

	Neonatal neurosteroid treatment		
	NH	VEH	Finasteride
TESTO	0.29±0.13	0.10±0.09	0.23±0.11
ALLO	56.23±31.25	0.77±0.20	1.22±0.58
THDOC	0.03±0.01	–	0.02±0.01
PREG	1.98±0.73	1.45±0.39	1.21±0.11
EPIALLO	0.14±0.14	0.006±0.002	0.006±0.002

Gabrd mRNA expression was observed at PD10 ($F_{4,15}$=35.16; p<0.001), in accordance with the results observed for protein expression (see Fig. 3*b*). Regarding effects of treatments at each time-point, we found relative differences at PD10 ($F_{2,9}$=11.55; p<0.01), PD12 ($F_{2,9}$=12.03; p<0.01) and PD15 ($F_{2,9}$=10.94; p< 0.01). An increase in *Gabrd* mRNA levels (about two-fold above control) was observed at PD10 in finasteride administered animals compared to NH and control groups (p<0.05 for both, see Fig. 3*b*). This level drop by PD12 was observed in the NH group which presented higher relative levels compared to the others (p<0.01 for both). Later on, by PD15, transcript were higher in both NH and control animals than in finasteride-treated animals (p<0.01 for both, see Fig. 3*b*)

Hippocampal neurosteroids levels at PD10

The analysis of hippocampal neurosteroid levels only showed a significant neonatal administration effect on allopregnanolone levels ($F_{2,11}$=4.08, p<0.05). Table 2 shows a detailed description of the hippocampal neurosteroid levels. Hippocampal allopregnanolone concentration was higher in NH than in the other groups (i.e. vehicle and finasteride groups, p<0.05 for both) while no differences between vehicle and finasteride groups were observed. Thus, an allopreganaonolone peak at PD10 was only observed in NH animals.

Effects of neonatal finasteride and progesterone administration on anxiety-like behaviour in adulthood

The analysis of anxiety-like behaviour showed an interaction effect between neonatal and adult administration in both the percentage of entries and the percentage of time in the open arms ($F_{2,42}$=3.71; p<0.05 and $F_{2,42}$=3.35; p<0.05, respectively), indicating that the behavioural response to allopregnanolone

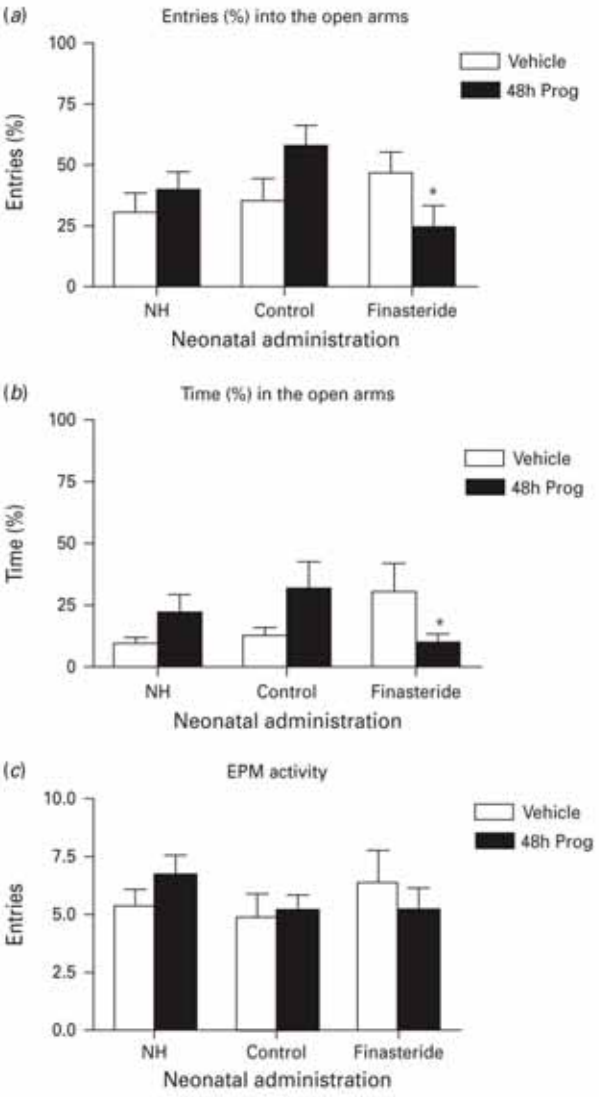

Fig. 4. Effects of neonatal and adult administration on anxiety-like behaviour. (*a*) Percentage of entries in the open arms. Interaction effect ($F_{2,42}$=3.71; p=0.03). Progesterone administration effect: *(p<0.05) finasteride *vs.* control. (*b*) Percentage of time in the open arms of the elevated plus maze. Interaction effect ($F_{2,42}$=3.35; p=0.04). Progesterone administration effect: *(p<0.05) finasteride *vs.* control. (*c*) Locomotor activity (open plus closed arms entries). Data are shown as mean±S.E.M.

increased levels (i.e. progesterone administration) depends on the previous neonatal treatment. Separate analysis for each adult treatment were performed. In vehicle animals, no behavioural differences were observed as a consequence of neonatal administration ($F_{2,21}$=1.11; N.S) (Fig. 4). However, progesterone treatment decreased the percentage of time in the open arms (entries and time) only in the finasteride-treated group, reflecting an increase in anxiety induced by the synergetic action of both neonatal finasteride and adult progesterone administration ($F_{2,21}$=3.73; p<0.05 and $F_{2,42}$=3.35; p<0.05, respectively) (see Fig. 4). Moreover, neither an effect of neonatal or adult administration ($F_{2,42}$=0.55; N.S and $F_{1,42}$=0.02; N.S, respectively) nor an interaction between neonatal and adult administration ($F_{2,42}$=0.75; N.S) were found in the analysis of activity in the maze (Fig. 4).

In order to analyse the effects of progesterone effects on anxiety-like behaviour, additional analyses in NH and vehicle groups were performed. No significant effects of progesterone were observed in both the percentage of entries and the percentage of time in the open arms in vehicle or NH groups ($F_{1,13}$=3.11; NS and $F_{1,13}$=2.34; NS, respectively for vehicle group; and $F_{1,15}$=0.59; NS and $F_{1,15}$=1.52; NS, respectively, for NH group).

Hippocampal $GABA_A$R α4 and δ subunits expression in adulthood

When α4 $GABA_A$R subunit expression was analysed, a significant neonatal effect was only observed ($F_{2,33}$=3.88; p<0.05). Animals that received finasteride had significantly higher levels of α4 expression in the hippocampus than the rest of the animals (see Fig. 5).

Regarding δ $GABA_A$R subunit expression, a significant effect of neonatal treatment ($F_{2,33}$=9.78; p<0.001) along with a significant interaction between neonatal and adult treatment were observed ($F_{2,33}$=3.75; p<0.05). In vehicle animals, an increase in δ expression was only observed in animals that were administered with finasteride compared to the other groups ($F_{2,16}$=10.50; p<0.001; see Fig. 5). Instead, no differences in δ $GABA_A$R subunit expression were observed when the analysis was performed in animals administered with progesterone in adulthood ($F_{2,17}$=1.35; N.S). These results indicate a down regulation of δ expression as a consequence of adult progesterone administration only in animals that received finasteride (Fig. 5). Taken together, the hippocampal α4 and δ $GABA_A$R subunit expression showed an increase only in animals that were administered with finasteride as neonates.

Discussion

Results in experiment 1 showed an important increase in hippocampal α4 $GABA_A$R subunit expression at P6 that progressively decreased during early postnatal development in NH and control groups. The increase of α4 $GABA_A$R subunits could be related to a maternal

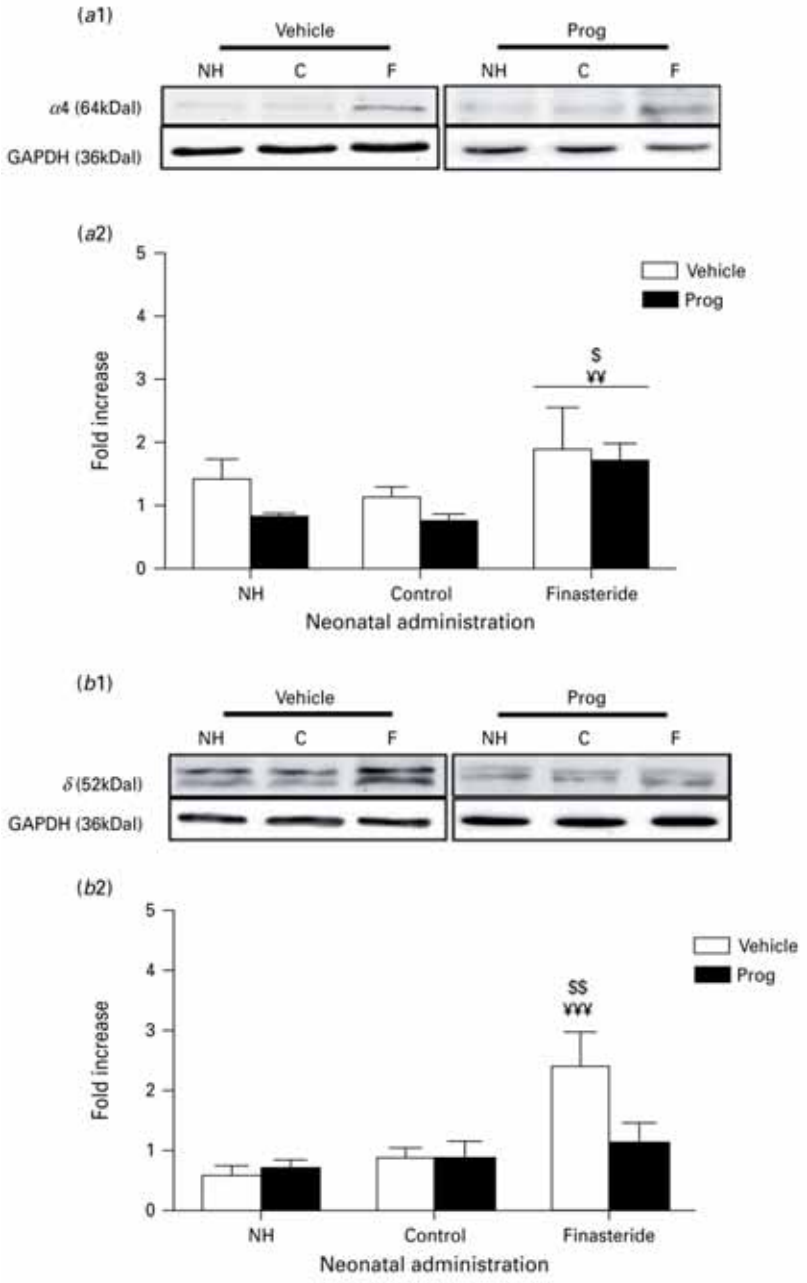

Fig. 5. Adult hippocampal α4 $GABA_AR$ subunit expression normalized to GAPDH levels and to the same control subject was included in all blots. Neonatal and adult administration. (*a*1) Representative Western blots of α4 $GABA_AR$ subunit detected from adult hippocampus. Neonatal groups *vs.* adult administration. (*a*2) Neonatal effect ($F_{2,33}=3.88$; $p=0.03$). Finasteride *vs.* NH, \$ $p<0.05$; finasteride *vs.* control, ¥¥ <0.01. (*b*1) Representative Western blots of δ $GABA_AR$ subunit detected from adult hippocampus normalized to GAPDH levels and to the same control subject was included in all blots. Neonatal groups *vs.* adult administration. (*b*2) Interaction effect ($F_{2,33}=3.75$; $p=0.03$). In adult vehicle adminsitrated groups, finasteride *vs.* NH, \$\$ ($p<0.01$) and finasteride *vs.* control ¥¥¥ $p<0.001$. Data are shown as mean ± S.E.M.

effect and the huge amount of allopregnanolone levels present before delivery (Grobin and Morrow, 2001). This α4 $GABA_AR$ subunit expression pattern, however, was not observed for the δ subunit expression at P6. The disparity between α4 and δ $GABA_AR$ subunits was not expected and could indicate that α4 subunits have different co-expression patterns other than the δ subunit during early development. In fact, previous

studies reported higher amounts of $\alpha4$ and $\gamma2$ subunit transcripts than of δ $GABA_A$R subunit transcripts in the hippocampus by PD2 (Laurie et al., 1992; Didelon et al., 2000). Thus, the discrepancy between $GABA_A$R $\alpha4$ and δ subunit expression at very early stages suggests differential roles and communicating signals under developmental conditions. In the second postnatal week, results obtained from NH animals showed an increase of hippocampal δ $GABA_A$R subunit protein at PD12 which can be related to the increment of its transcript observed from PD10 to PD15, although not statistically significant at PD12, probably due to insufficient sensitivity in the technique. *Gabra4* mRNA also increased during the second postnatal week, however, the $\alpha4$ $GABA_A$R subunit was increased but not significantly at PD12 ($p=0.09$). Indeed, this observation is in agreement with the results reported by Laurie and collaborators using *in situ* hybridization (Laurie et al., 1992). The relevance of the $\alpha4$ and δ $GABA_A$R subunit increase at PD12 remains to be elucidated. However, it could be related to the endogenous increase of hippocampal allopregnanolone levels observed at PD10. This naturally occurring peak is in accordance with results reported by others (i.e. PD10–PD14; Grobin and Morrow, 2001), and results mainly from endogenous neurosteroidogenesis, as we can exclude, at these ages, the persistence of maternal steroids in the cerebral tissue (Ibanez et al., 2003). Recent results reported by Kuver et al., 2012 showing that cell surface expression of $\alpha4ß2\delta$ $GABA_A$R is increased by allopregnanolone *in vitro* (Kuver et al., 2012) also support the hypothesis that this receptor is highly responsive to the presence of neurosteroids. Indeed, at PD10 we did not observe any increase of allopregnanolone levels or any later δ subunit expression increase (at PD12) in neonatal vehicle groups. This could be due to a neonatal manipulation effect on the vehicle (Modol et al., 2013). Importantly, the $GABA_A$R have been postulated to be one of the first sources of activity in the neonatal brain according to their excitatory profile at early stages (Leinekugel et al., 1995; Ben-Ari, 2002; Owens and Kriegstein, 2002; Ben-Ari et al., 2007). Neonatal activation of $GABA_A$R is important, being necessary for maturation of interneurons and pyramidal neurons in the hippocampus (Ben-Ari, 2002; Ben-Ari et al., 2007), providing important communication signals during development.

Regarding finasteride administration effects, we found an increase at PD6 in hippocampal $\alpha4$ $GABA_A$R subunit expression, followed by a second peak occurring at PD10. It is plausible that the early increase may be related to a maternal effect and the huge amount of allopregnanolone levels present before delivery (see above NH results). Finasteride administration would affect *de novo* synthesized allopregnanolone by the pup and would probably be linked to the increase of both $\alpha4$ and δ (transcript and protein) $GABA_A$R subunit. The fact that finasteride administration increased *Gabrd* mRNA and the corresponding peptide in a similar way, while the *Gabra4* mRNA expression pattern differs from the peptide, could indicate that the mechanisms by which finasteride regulates the expression of these subunits is different.

The results of this study indicate an increase in hippocampal allopregnanolone levels at PD10 in NH but not in finasteride-treated animals, while no differences between vehicle and finasteride groups were observed. In a previous experiment, however, we found an increase in hippocampal allopregnanolone levels as a consequence of neonatal finasteride administration at PD9, i.e. at the end of finasteride treatment (Darbra et al., 2013). This discrepancy between results obtained at PD9 and PD10 may be related to the time elapsed since the last injection of finasteride (i.e. 24 h). Other authors reported a single finasteride administration effect on allopregnanolone and THDOC production which lasted longer than 19 h, although at this time the brain allopregnanolone and THDOC concentration had begun to return to pretreatment values in female rats (Concas et al., 1998). In this sense, hippocampal THDOC and testosterone levels that we have detected at PD10 also showed no differences among groups. Thus, the increase in $\alpha4$ and δ $GABA_A$R subunits expression observed in these animals could be attributed to the fluctuations in hippocampal allopregnanolone levels due to finasteride administration. However, effects mediated via progesterone and testosterone are also possible. Allopregnanolone can be converted back to 5α-dihydroprogesterone and potentially interact with the progesterone receptor. Inhibition of 5α-reductase activity would reduce the conversion of testosterone to 5α-dihydrotestosterone. In fact, we have previously reported that finasteride treatment increased hippocampal levels of testosterone and pregnenolone at PD9, i.e. at the end of neonatal treatment (Darbra et al., 2013). It must be outlined that pregnenolone is known to be rapidly metabolized in other steroids, including excitatory steroids and inhibitory steroids like progesterone and its reduced metabolites. Thus, the treatment with finasteride may induce alternative metabolic pathways, such as 3α-reduction, 20α-reduction and 21-hydroxylation in the progesterone metabolism (Mukai et al., 2008); some effects may arise from changes in these steroids in the neonatal brain.

The present results highlight the role of neonatal neurosteroids levels in the expression of $GABA_A$R

subunits in the hippocampus. Importantly, our results showed that the increase in $\alpha4$ and δ $GABA_AR$ subunits expression is maintained in the adult in neonatal finasteride-treated animals (see Fig. 5). Our results also demonstrated that neonatal finasteride-treated animals spent less time and entered less frequently into the open arms, without affecting locomotor activity, than control animals when they were administered progesterone but not vehicle, indicating an anxiogenic-like profile induced by progesterone administration in the adult only in those animals administered with finasteride. In agreement with the present results, an anxiogenic-like profile in the elevated plus maze test was also observed following progesterone withdrawal in a rodent model of premenstrual anxiety (Smith et al., 2006), and as a consequence of acute allopregnanolone administration at a physiological dose after the onset of puberty in mice (Shen et al., 2007). This paradoxical increase of anxiety as a consequence of allopregnanolone or its precursor progesterone is dependent upon the increased expression of $\alpha4\beta\delta$ GABA receptors in hippocampal area CA1, as shown by the inability of allopregnanolone to induce an anxiogenic profile in δ knock-out mice (Smith et al, 1998; Shen et al., 2007). As reported earlier by Shen et al. (2007), one possible mechanism for this observed increase of anxiety score can be attributed to the ability of allopregnanolone to reverse its classic effect of enhancing $GABA_AR$-gated current (hyperpolarizing response) only in those $GABA_AR$ expressing $\alpha4$ and δ subunits, but not any other $GABA_AR$. Interestingly, present results also show an increased expression of hippocampal $\alpha4$ and δ $GABA_AR$ subunits as a consequence of neonatal finasteride administration, which are responsible for the anxiogenic-like behaviour (see Fig. 4). However, other mechanisms induced by neonatal finasteride administration cannot be ruled out and need to be further studied.

Regarding the effects of progesterone administration, some previous studies reported that acute treatment decreases anxiety in the elevated plus maze test (Bitran et al., 1993; Reddy et al., 2005), however, no other effects were reported (Frye et al., 2006; Starkey and Bridges, 2010). A dose-dependent behavioural pattern of progesterone administration has been suggested with an inverted U-shaped profile effect on elevated plus maze test; low (1 mg/kg) and high (100 mg/kg) doses significantly decreased the time spent in the open arms, whereas an intermediate dose (10 mg/kg) significantly increased this parameter in male mice (Gomez et al., 2002). Our results for progesterone treatment in NH and control groups are not consistent with the elevated anxiety levels after 48 h administration of progesterone shown in other studies (Gulinello and Smith, 2003). These contradictory results may be explained mainly due to gender differences. While the present study is performed on males the other one used females (Gulinello and Smith, 2003). Also, other differences such as the dose of progesterone (5 mg/kg *vs.* 25 mg/kg) or the time of observation (4–5 h *vs.* 20 min after the last injection) may also be relevant.

In female rats, an increase in anxiety scores after 48 h exposure to progesterone (three injections over a 48 h period, 5 mg/kg i.p.) has also been reported, accompanied by an increase in the hippocampal $\alpha4$ $GABA_AR$ subunit (Gulinello et al., 2001). Thus, changes in $GABA_AR$ expression and function due to hormone exposure have been postulated to underlie the increased anxiety evident after 48 h exposure to elevated allopregnanolone levels (Gulinello and Smith, 2003). Our results indicate that progesterone administration: (1) had no effects on elevated plus maze test and $\alpha4$ and δ $GABA_AR$ subunit expression in NH and control groups; and (2) induced an anxiogenic-like behaviour only in neonatal finasteride-treated animals, accompanied by an increased expression of the hippocampal $\alpha4$ and δ subunits $GABA_AR$. These observations are consistent with the notion that changes in $GABA_AR$ expression and function occur due to fluctuating levels of allopregnanolone. We previously reported an increase in hippocampal allopregnanolone levels as a consequence of neonatal finasteride administration at PD9, i.e. at the end of finasteride treatment (Darbra et al., 2013). Thus, our results highlight the importance of neonatal allopregnanolone manipulation and its impact on $GABA_AR$ expression, which can lead to an altered adult system that responds differently to environmental cues. However, the functional mechanism by which finasteride administration in neonatal pups induces the observed increase in $GABA_AR$ subunits remain to be elucidated.

Our data also shown that progesterone administration down regulates the δ subunit expression only in neonatal finasteride-treated animals. In fact, neonatal finasteride administration resulted in an increase of hippocampal levels of $\alpha4$ and δ $GABA_AR$ subunit immunoreactivity by two- to threefold above control levels in the adulthood (see Fig. 5). Importantly, δ subunit expression returned to control values after progesterone administration. Extrasynaptically localized δ subunit-containing receptors mediate tonic GABAergic inhibition in many brain regions and confer neurosteroid sensitivity (Belelli et al., 2002; Spigelman et al., 2003). Our findings indicated that δ $GABA_AR$ subunit

is capable of rapid plastic changes, decreasing after short-term treatment with neurosteroids under conditions of altered maturation of $GABA_A$R expression (i.e. neonatal finasteride administration).

It has been previously reported that neonatal alteration of allopregnanolone levels during the postnatal period could have severe consequences on the maturation of inhibitory hippocampal circuitry and also in other brain areas (Grobin et al., 2003, 2006; Gizerian et al., 2004). Previous results from our laboratory have shown the relevance of neonatal allopregnanolone levels for adult behaviour and the behavioural response to intrahippocampal neurosteroids administration (Darbra and Pallarès, 2009, 2010, 2011, 2012). An increase in hippocampal *Gabra4* and *Gabrd* mRNA in adulthood has been related to seizures (Brooks-Kayal et al., 1998; Cohen et al., 2003; Maguire et al., 2005). Importantly, neurosteroids action through δ subunit-containing $GABA_A$R is required for the physiological response to stress and stress-induced anxiety-like behaviour (Sarkar et al., 2011). Moreover, a reduction in the *Gabrd* mRNA expression after chronic stress has also been reported (Verkuyl et al., 2004). Taken together, all these previous data suggest the involvement of neonatal neurosteroids in the etiology of several psychiatric conditions, including anxiety-related disorders, schizophrenia and epilepsy (Brooks-Kayal et al., 1998; Cohen et al., 2003; Dubrovsky, 2005).

In summary, the results of the present study demonstrate the importance of neonatal neurosteroid levels for the maturation of the hippocampal $GABA_A$R system. This is the first study demonstrating that neonatal finasteride administration (from PD5 to PD9) modifies neonatal and adult expression of the α4 and δ $GABA_A$R subunits, which is accompanied by an altered behavioural response to progesterone administration, even in adulthood.

Acknowledgments

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Statement of Interest

None.

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Experiment 3

Research article

"Neonatal allopregnanolone or finasteride administration modifies hippocampal K^+ Cl^- co-transporter expression during early development in male rats"

In Experiment 3 we investigated the effects of neonatal manipulation of Ns levels on the expression KCC2. During development $GABA_AR$ exerts depolarizing action instead of its inhibitory profile at adult age. Cation-chloride cotransporters that modify intracellular chloride concentration through changes in their expression mediate the polarity of $GABA_AR$. Among them, KCC2 has been described to change during developmental stages and related to the switch of the $GABA_AR$ from inhibitory to excitatory. Thus, as results of Experiment 2 we observed that neonatal alteration of Allopregnanolone levels modifies the expression of $GABA_ARs$, in the present experiment we assessed the changes in KCC2. Animals were neonatally administered as described in Experiment 1: with Allopregnanolone (20mg/kg), finasteride (50mg/kg) or vehicle. An schematic representation of the experimental design can be observed in the following page.

Specific objectives

• To study the effects of the neonatal Allopregnanolone or finasteride administration in the KCC2 expression during early stages of development.

Neonatal allopregnanolone or finasteride administration modifies hippocampal K^+ Cl^- co-transporter expression during early development in male rats

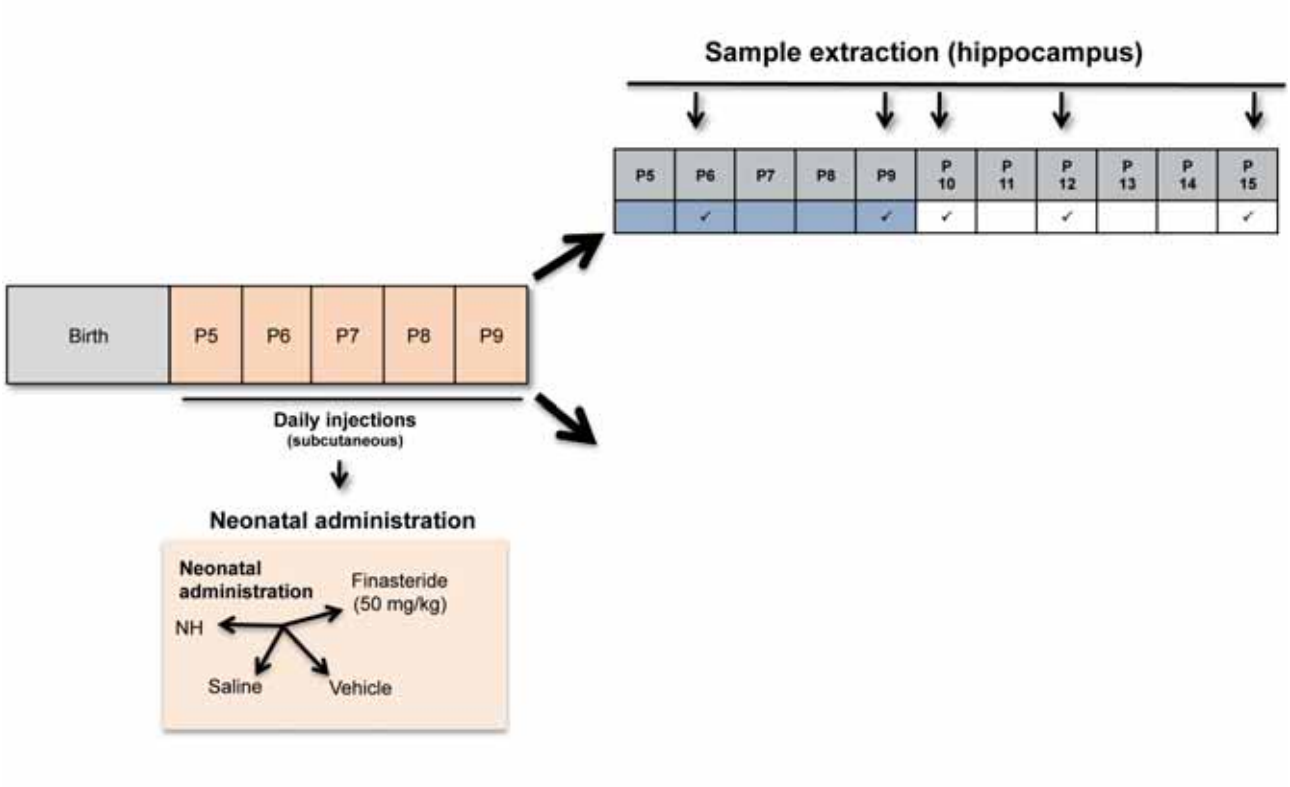

Fig 11: Experimental design

Experiment 3:
Neonatal Ns & hippocampal KCC2

Research article

Mòdol L, Casas C, Navarro X, Llidó A, Pallarès M, Darbra S. Neonatal allopregnanolone or finasteride administration modifies hippocampal K^+ Cl^- co-transporter expression during early development in male rats. *Under review*

Neonatal allopregnanolone or finasteride administration modifies hippocampal K^+ Cl^- co-transporter expression during early development in male rats

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ABSTRACT

The maintenance of levels of endogenous neurosteroids (NS) across early postnatal development of the brain, particularly to the hippocampus, is crucial for their maturation. Allopregnanolone (Allop) is a NS that exerts its effect mainly through the modulation of the GABAA receptor (GABAAR). During early development, GABA, acting through GABAAR, that predominantly produces depolarization shifts to hyperpolarization in mature neurons, around the second postnatal week in rats. Several factors contribute to this change including the progressive increase of the neuron-specific K+ /Cl- co-transporter 2 (KCC2) (a chloride exporter) levels. Thus, we aimed to analyse whether a different profile of NS levels during development is critical and can alter this natural progression of KCC2 stages. We administrated sustained Allop (20mg/kg) or Finasteride (5α-reductase inhibitor, 50mg/kg) from the 5th postnatal day (PD5) to PD9 and assessed changes in the hippocampal expression of KCC2 at transcript and protein levels as well as its active phosphorylated state in male rats. Taken together data indicated that manipulation of NS levels during early development influence KCC2 levels and point out the importance of neonatal NS levels for the hippocampal development.

Keywords: neurosteroids, development, hippocampus, intra cellular chloride, GABAA receptor, rat

Introduction

The maintenance of endogenous neurosteroids (NS) levels has been postulated to be of importance for the maturation of the CNS and particularly for the hippocampus (Mellon, 2007). Previous results from our laboratory have shown the relevance of neonatal NS levels (from postnatal days 5 to 9, PD5 to PD9) for adult behaviour such as anxiety, exploration and sensorimotor gating evaluated by means of the prepulse inhibition of the acoustic startle response (Darbra and Pallarès 2010; 2012) and for the behavioural response to intrahippocampal NS administration (Darbra et al, 2013b). Previous results from our laboratory showed that alteration of neonatal allopregnanolone (Allop) or pregnenolone levels was capable to suppress the typical anxiolytic effects provoked by intrahippocampal Allop administration in the adult (Mòdol et al., 2013). Besides, animals which suffered subchronic increases in Allop levels or pregnenolone and testosterone levels during neonatal period, did not show the improvement of the prepulse inhibition response due to the intrahippocampal Allop administration (Darbra et al., 2013a). Although the mechanisms underlying these alterations are still unexplained, we recently reported data showing that neonatal NS levels affected both neonatal and adult hippocampal expression of the α4 and δ gamma-aminobutyric acid A receptor (GABAAR) subunits, which was accompanied by an altered behavioural response to progesterone administration in adulthood (Mòdol et al., 2014).

During development, GABAAR activation produces neuronal depolarization instead of hyperpolarization which is characteristic of the adult period. Depolarizing GABAAR endows the system the necessary signalling to accomplish postnatal neurogenesis, neuronal migration, synaptogenesis and prunning (Ben-Ari et al., 2007; Bortone and Polleux, 2009). It is known that this discrepancy between inmature and mature neuronal behaviour is due to opposite sign of Cl^- intra and extracellular gradients. This change in the sign of Cl^- gradients occurs around PD5–PD7, depending on the up regulation of the neuron-specific K^+ /Cl^- cotransporter 2 (KCC2) expression among other signals (Ben-Ari et al., 2007). In particular, the phosphorylation of the residue S940 in the intracellular C-terminal domain of KCC2, mediated by protein kinase C, stabilizes KCC2 on the neuronal cell surface and increases its co-transporter activity (Lee et al., 2007; Lee et al., 2010). Thus, early expression of KCC2 contribute to the shift of GABA actions and impacts neuronal maturation as well as the formation of GABAergic synapses (Dehorter et al., 2012) (for review see Fiumelli and Woodin, 2007). Of note, Indeed, chronic GABAAR blockade delayed both the GABA switch and the developmental increase in the expression of KCC2 (Ganguly et al., 2001; Leitch et al., 2005).

Taking into account the importance of NS for the maturation of hippocampus , particularly its GABAAR system, it is reasonable to think that neonatal NS levels contribute to developmental expression of KCC2. So, the aim of the present study is to test whether the neonatal Allop level alteration modifies KCC2 expression in the hippocampus. We hypothesize that alteration of Allop levels is capable to modify KCC2 expression pattern due to its positive allosteric GABAAR modulator profile. For that purpose, we have altered neonatal Allop levels and we have analysed KCC2 gene and protein expression as well as the status of its active phosphorylated form during postnatal period.

Methods

Animals and neonatal Allop levels alteration

One hundred and twenty-five male Wistar rats were used and housed in a temperature-controlled animal room (22–24 °C) on a 12-h light/dark cycle. (Laboratori de Psicobiologia, Universitat Autònoma de Barcelona). The male breeders were separated from the females after 48h, pregnant females were closely watched and on the day of birth (designed PD0) litters were culled to 10 pups. Pups were subcutaneously (s.c.) injected with: Allop (3α-hydroxy-5α-pregnan-20-one; 20 mg/kg, *n*=33), finasteride (a 5α-reductase inhibitor that impedes the conversion from progesterone to dihydroprogesterone; 50 mg/kg, *n*=28), vehicle (10%-cyclodextrine as control, *n*=27) and saline (n=24) once per day from PD5 to PD9). A non-handled group (NH; *n*=30) was included. Drugs were dissolved in 10% cyclodextrin ((2-Hydroxypropyl)-β-cyclodextrin) in 0.9% NaCl. The injection volume was 0.1 ml/10 g body weight. All animals were obtained, housed, and sacrificed in accordance with the protocol approved by the Committee of the Universitat Autònoma de Barcelona for Care and Use of Experimental Animals and the Department of Environment from Generalitat de Catalunya. This protocol follows the guidelines approved by the European Council Directive (2010/63/EU).

KCC2 expression during early development

KCC2 transcript and protein abundance was determined using Real-time PCR and western blot respectively Male rats were sacrificed by decapitation at PD6 (n=27), PD9 (n=25), PD10 (n=22), PD12 (n=23), and PD15 (n=28). At PD6 and PD9, animals were sacrificed 1 hour after the injection. Their hippocampus was dissected out, immediately frozen in dry ice, and half of it was homogenized in 10 mM HEPES (pH 7.4), 2% Triton X-100, 0.3 M KCL, 300 mM NaCl, 1 mM EDTA, protease inhibitor cocktail (10 µl/ml, Sigma St. Louis, MO, USA) and sodium orthovanadate (1 mM, Roche, Basel, Switzerland) for protein extraction as described in Mòdol et al 2014. Protein concentration was measured by BCA protein assay (Pierce, Rockford, IL) and equal amounts (30 µg) were used for western blotting. Membranes were blocked for 1 h in TBST (100mM Tris, 0.9%. NaCl, 0.05% Tween-20, pH 7.6) with 5 % BSA and primary antibodies against KCC2 (rabbit anti-KCC2,

Millipore, 1/1000), phosphorylated KCC2 (rabbit anti-phospho-^{Ser940} KCC2, Phosphosolutions, 1/1000) or glyceraldehyde-3-phophate dehydrogenase (GAPDH, Sigma, 1:5000) were used for overnight incubation (4°C). Horseradish peroxidase coupled antibodies (Pierce) were used for secondary incubation and blots were developed with the ECL Plus detection kit (Millipore). Images were analysed by band densitometry (Gene Tools software, Gene Genome apparatus, Syngene, Cambridge, UK). The same control animal was used in each membrane performed to relativize the results and GAPDH band was used as a loading control. The other half of the hippocampus was immerse in RLTβ buffer and total RNA was obtained using the EasyRNA extraction kit (Qiagen, Hilden, Germany) following manufacturer instructions. Two micrograms of RNA was reverse-transcribed as described in Mòdol et al 2013b. Real-Time PCR (iQ5, BioRad Foster City, CA, USA) using Brilliant III Ultra-Fast SYBR® Green qPCR master mix (Agilent Technologies, Santa Clara, CA, USA) and the following primers: *KCC2* (F,5'-CTTCACCCGAAACAATGTCACAGAG-3';R,5'-CAGGGTGAAGTAGGAGGTCATATCAC-3') and *Gapdh* (F, 5'-AGTTCAACGGCACAGTCAAG-3'; R, 5'-TACTCAGCACCAGCATCACC-3'). Three-four samples were used per condition and each sample was run in duplicate. The thermal cycling conditions were: 50 ºC for 2 min, 95 ºC for 10 min and 40 cycles of 95 ºC for 15 s, 60 ºC for 1 min. Fold change in gene expression was estimated using the CT comparative method (2 $^{-DDCT}$) normalizing to *Gapdh* CT values and relative to control samples at each time point. Data was analyzed using two-way analysis of variance (ANOVA) with neonatal treatment (NEO, 5 levels: NH/Saline/Veh/Finasteride/Allop) and postnatal day (DAY, 5 levels) using STATISTICA package (StatSoft, Tulsa, USA). In order to control the possible β-cyclodextrine administration effect on KCC2 expression, a preliminary two way ANOVA was performed with neonatal treatment (two levels: Saline/VEH) and postnatal day (five levels: PD6/PD9/PD10/PD12/PD15) as factors. Post hoc polynomial contrasts were used when necessary.

Results

In order to analyse the effects of the β-cyclodextrine administration, an additional ANOVA with NEO (2 levels, Saline and VEH) and DAY was also performed. No differences in KCC2 and pKCC2 protein and *KCC2* mRNA between the neonatal vehicle (β-cyclodextrin and saline) administered groups were observed [$F_{(1,42)}$=0.72; N.S; $F_{(1,33)}$=0.01; N.S; and $F_{(1,35)}$=3. 35; N.S, respectively]. A significant main effect of DAY [$F_{(4,42)}$=11.72, P< 0.001; $F_{(4,33)}$=14.17, P< 0.001; and $F_{(4,35)}$=2.76, P<0.05 respectively] was also found reflecting the expected progressive increase in KCC3 expression. Because of NEOX DAY interaction effect was not observed, the normal KCC2 increase across

the values was not affected by the cyclodextrine. Thus, the neonatal NS manipulation on hippocampal KCC2 expression could be unlikely attributable to the use of cyclodextrine as vehicle.

The analysis of the *KCC2* mRNA levels across early postnatal development showed a significant NEO effect [$F_{(4,107)}$=4.39;p<0.01] and a significant DAY effect [$F_{(4,107)}$=3.98;p<0.01] while no significant interaction effect NEO X DAY [$F_{(16,107)}$=1.25; NS] was observed. That is, a natural progressive increase in KCC2 transcript levels from PD6 onwards was observed in all groups. NH animals, however, showed higher *KCC2* mRNA levels than the rest of the groups (N-K; P<0.05 vs all). No significant differences among others groups were observed (see fig 1)

The analysis of the KCC2 protein abundance across early postnatal development showed a significant NEO effect [F(4,118)=5.40;p<0.001], a significant DAY effect [F(4,118)=12.99;P<0.0001] and a significant interaction effect NEO X DAY [F(16,118)=2,47;p<0.01]. In NH, VEH and Saline animals, a significant effect of DAY was observed [F(4,25)=5.71, p<0.005; F(4,22)=1.61, p<0.01 and F(4,20)=6.24, p<0.01, respectively]: A lineal increase of

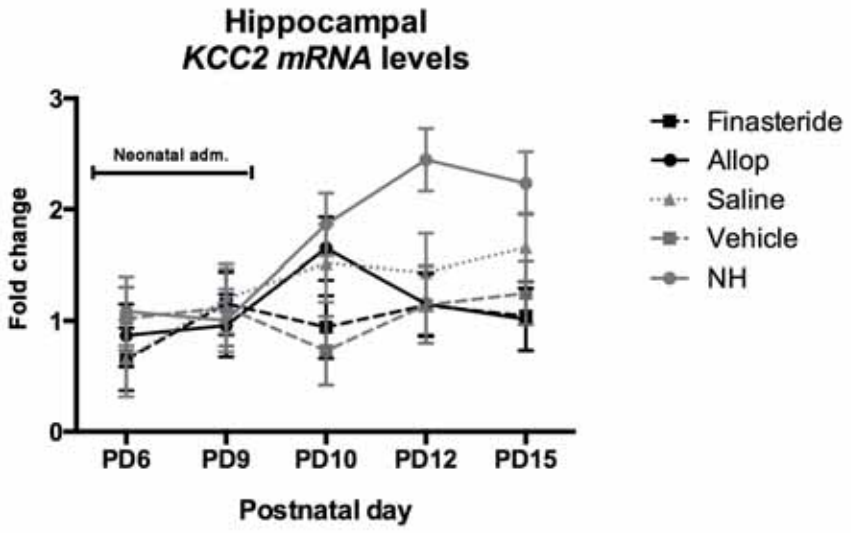

Figure 1: Real-time PCR relative quantification of *KCC2* mRNAs, normalized to the GAPDH levels and to average value of control PD15 rats to evaluate developmental changes (rats/condition/age, n=4-6). Globally, *KCC2* mRNA levels increased in all experimental groups (polynomial contrast; t=--2.41 p<0.01). A neonatal administration effect was also observed [$F_{(4, 107)}$=4.39;p<0.01]. Globally, *KCC2* mRNA levels in NH animals were higher than the rest (N-K p<0.05 vs all) . Lines represent the average ± SEM fold changes of gene expression in the hippocampus of no handled (NH) (gray circle and gray line), vehicle (gray square and dashed gray line), saline (gray triangle and dashed gray line), Allop (black circle and black line) and finasteride treated (black squared and dashed black line) animals at the chosen neonatal age (from PD6 to PD15).Data are shown as mean ± SEM

protein levels along postnatal days P6 to P15 was observed in control groups (t=4.54, p<0.001; t=4.82, p<0.001 and t=4.95, p<0.001, respectively), indicating a developmental increase in hippocampal KCC2 levels (see Fig 2). In Allop treated animals a DAY effect was also observed in the hippocampal KCC2 expression [F(4,28)=6.56, p<0.001], however, a significant cubic trend was found by polynomial post hoc contrast (t=-3.22, p<0.01), indicating two inflection points, which is characteristic of a cubic trend. That is, an inverted U-shaped profile from PD6 to PD10 and a progressive decrease from PD10 to PD15. Note that there is a significant cubic trend, but not a significant linear or quadratic trend. In contrast, results of neonatal finasteride administered animals showed no significant changes of the KCC2 protein abundance during early postnatal development [$F_{(4,23)}$=1.52; N.S]. Thus, non-lineal increase of protein levels along postnatal days was observed in finasteride-treated animals.

Expression of phosphorylated form of the KCC2 (pKCC2) was also analysed. Results also showed a significant NEO effect [$F_{(4,102)}$=9.02;p<0.001], DAY [$F_{(4,102)}$=14.00;p<0.001] and a significant interaction between NEO X DAY [$F_{(16,102)}$=3.85;p<0.001]. Similar to what we observed in the KCC2 results, a developmental increase in the active (phosphorylated) form of the KCC2 occurred from PD6 to PD15 in control groups (NH, VEH and Saline), and a significant cubic trend was also found in samples from Allop-treated animals while developmental lineal increase in pKCC2 was not observed in finasteride group (see fig 2A and 2B for detailed post-hoc analysis).

Discussion

Results of the present study indicated that alterations in the neonatal NS levels by exogenous Allop administration or by finasteride administration change the developmental hippocampal expression and protein abundance profile of the KCC2 during neonatal period *in vivo*.

We have observed that developmental KCC2 expression and levels can be modulated by Allop administration to obtain a markedly different profile from that of the natural developmental increase observed in control animals (NH and control groups) from PD6 onwards. Neonatal Allop administration promoted an overexpression of KCC2 to achieve supranatural protein levels around PD10 which in turn can contribute to feedback down-regulate its own gene expression later on. However, an excess of protein is not followed by a quick phosphorylation of KCC2 which seems to be different-regulated event that account posteriorly, from PD12, but may depend on protein availability at that time. Thus, a precocious excess of KCC2 protein levels

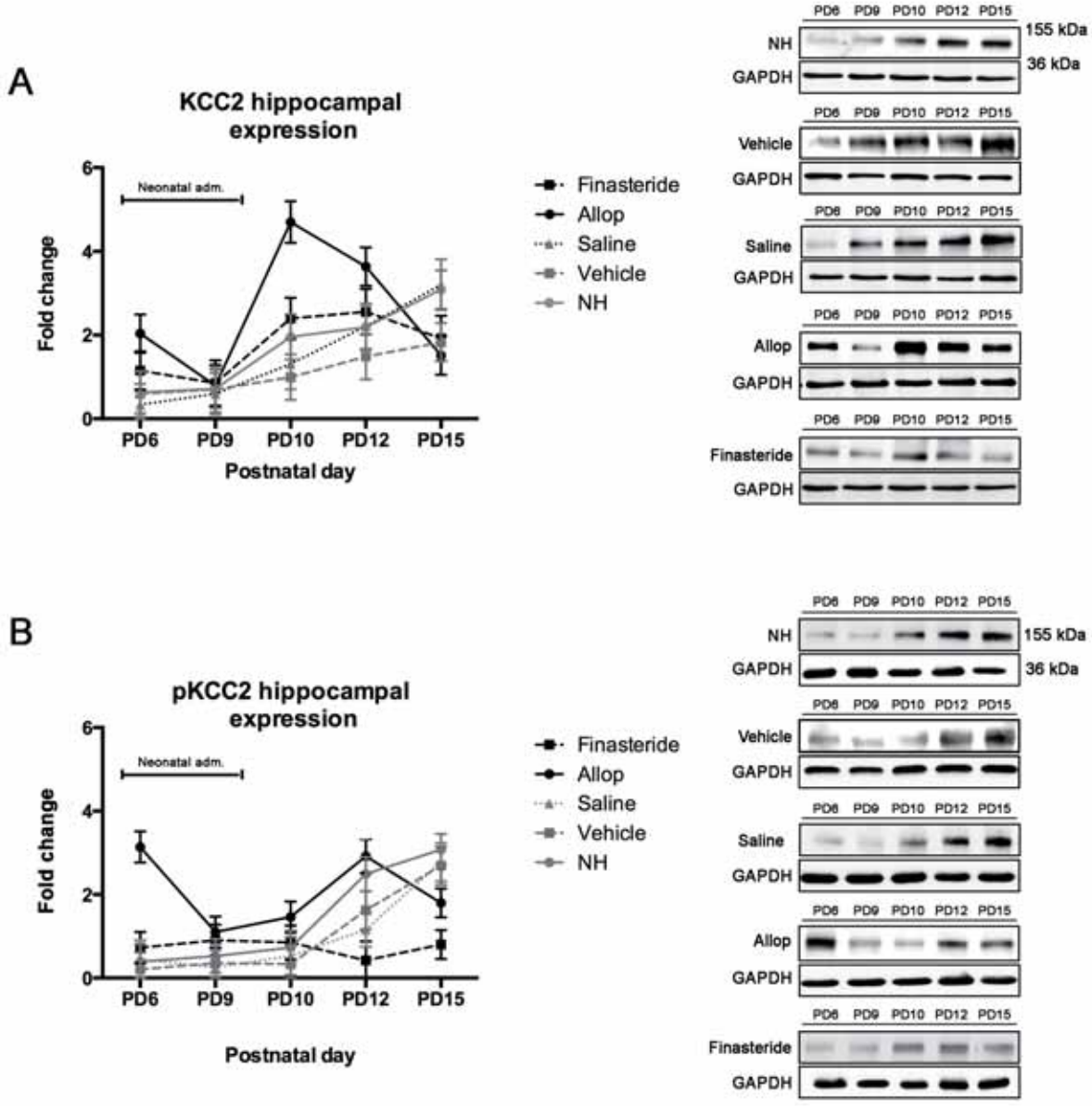

Figure 2: Hippocampal KCC2 abundance determined by protein semi-quantification during postnatal development and neonatal administration **A)**. KCC2 protein abundance in the five experimental groups detected from hippocampus for each postnatal day (rats/condition/age, n=4-7). Interaction effect [$F_{(12,105)}$=2,22;p<0.01]. A lineal increase of protein levels along postnatal days PD6 to PD15 was observed in all control groups (polynomial contrast; NH t=4.54, p<0.001; Vehicle t=4.82, p<0.001 and Saline t=4.95, p<0.001), while in Allop group a significant U-shaped profile from PD6 to PD10 and a progressive decrease from PD10 to PD15 (polynomial contrast; t=-3.22, p<0.01) was observed. No significant changes of the KCC2 abundance during early postnatal development were observed in finasteride group. **B)** Similar expression patterns were observed in the phosphorylated form of the KCC2 (pKCC2; rats/condition/age, n=4-7). Interaction effect [$F_{(12,86)}$=3.67;p<0.01]. A developmental increase in pKCC2 occurred from PD6 to PD15 in all control groups (polynomial contrast; NH t=5.88, p<0.001; Vehicle t=4.51, p<0.001 and Saline t=3.93, p<0.001), a significant cubic trend was also found in Allop group (polynomial contrast; t=-3.07, p<0.01) while developmental lineal increase in pKCC2 was not observed in finasteride animals. Representative Western blots of KCC2 and pKCC2 accompanied the plots. KCC2 abundance normalized to GAPDH levels and to the same control subject was included in all blots. Lines represent the average ± SEM fold changes of protein abundance in the hippocampus of no handled (NH) (gray circle and gray line), vehicle (gray square and dashed gray line), saline (gray triangle and dashed gray line), Allop (black circle and black line) and finasteride treated (black squared and dashed black line) animals at the chosen neonatal age (from PD6 to PD15). Data are shown as mean ± SEM.

may not lead to an advanced disposal of its functional form. Taking into account that Allop exposure partially overlies the developmental window when GABA's depolarising action becomes hyperpolarizing from PD7 to PD14 (Wang and Kriengstein, 2011), it is conceivable that the increase of KCC2 expression was related to a prompted elevation of intracellular chloride associated with Allop-induced GABAAR activation. Indeed, high concentrations of NS positive GABAAR modulators, as those obtained in the hippocampus by neonatal Allop treatment (Darbra et al., 2013a), can directly gate the GABAAR (Belelli and Lambert, 2005). This functional up regulation is particularly notably at PD6 at the beginning of Allop administration (see fig 2B).

Interestingly, developmental up regulation of KCC2 protein and *KCC2* mRNA levels fail to occur in finasteride-treated animals. Recently reported data emphasize the role of intracellular chloride concentration in regulating the α3-α1 and δ GABAAR subunits expression *in vitro* and therefore the decay kinetics of GABAergic postsynaptic currents and tonic inhibition (Succol et al., 2012). Extra-synaptically localized δ contained $GABA_AR$ receptors mediated persistent tonic inhibition currents (Belelli and Lambert, 2005). It has been shown that the effects of the neurosteroid Allop can reverse from enhancing GABA-gated current to inhibiting current at α4βδ GABAAR in a Cl^- dependent manner. The expression of these receptors increased in the CA1 region of the hippocampus at the onset of puberty, where they generated an outward current (Shen et al., 2007). In fact, Allop is capable to reverse its classic effect of enhancing GABAAR-gated current (hyperpolarizing response) only in those GABAAR expressing α4 and δ subunits, but not any other GABAAR (Shen et al., 2007). An up regulation of the expression of α4 and δ subunits is also observed in the adult hippocampus when circulating levels of Allop decrease (Allop withdrawal) (Shen et al., 2007). In that sense, we have previously reported that finasteride treatment increased hippocampal levels of Allop, testosterone and pregnenolone at PD9, i.e. at the end of neonatal treatment (Darbra et al., 2013a), and upregulated the expression of neonatal and adult hippocampal α4 and δ subunits, which was accompanied by an altered behavioural response to progesterone administration, even in adulthood (Mòdol et al., 2014). Thus, the KCC2 down –regulation observed in finasteride-treated animals could be contributing to maintain a high level of intracellular chloride concentration which may lead to depolarizing actions observed at α4βδ GABAAR receptors.

Accumulated evidence suggests that the GABAergic system is associated with neurodevelopmental psychiatric disorders such as schizophrenia or depression (for review see Hines et al., 2012). Disturbed GABA neurotransmission in schizophrenia might arise in part from alterations on the nature of GABA neurotransmission, which

can be hyperpolarizing, depolarizing, or shunting depending on the flow of chloride ions when GABAAR are activated (Bartos et al., 2007). Recent studies implicate KCC2 in both the genetic and neurodevelopmental etiologies of schizophrenia (Hyde et al., 2011) and changes in the KCC2 hippocampal and cortical expression have been also recently described in schizophrenia (Tao et al., 2012; Arion and Lewis, 2011).

Extensive evidences indicate an activity-dependent regulation of both the normal developmental switch in GABAergic polarity from excitation to inhibition and the efficacy of GABAergic transmission through its action on KCC2-mediated chloride homeostasis (Fiumelli and Woodin, 2007). In this line, it has been speculated that GABAAR subtypes composition could be regulated by intracellular chloride concentration in a synaptic-specific fashion (Succol et al., 2013). Thus, changes in the KCC2 hippocampal expression during early stages could be related with changes in the α4 and δ GABAAR subunits expression in both neonatal and adult hippocampus observed as a consequence of neonatal NS levels alterations (Mòdol et al, 2014).

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Discussion

Discussion

Role of neonatal Ns fluctuations in the behavioural response to intrahippocampal Ns administration

In the Experiment 1 we studied the effects of neonatal manipulation of Ns levels on the behavioural response to intrahippocampal administration of Ns. For this purpose we first assessed the implication of dorsal hippocampus in the Ns behavioural effects on exploration, anxiety and aversive learning as the hippocampus has been implicated in the modulation of these behaviours (Bitran et al., 1999; Zhang et al., 2002; Howland et al., 2004; Adams and Van den Buuse, 2011; Mineur et al., 2013). Accordingly to its previously described $GABA_AR$ allosteric positive modulator profile (Belelli and Lambert, 2005; Lambert et al., 2003, 2009), intrahippocampal Allopregnanolone induced an increase in the exploratory behaviour in the Boissier test and exerted an anxiolytic-like profile in the EPM (Experiment 1). Similar results have also been obtained with other Ns such as pregnenolone into the dorsal hippocampus (Bitran et al., 1999). Also, we studied the role of the hippocampus in the modulation of Ns effects in the passive avoidance learning, an aversive learning with an important emotional component. In this paradigm, the animal must inhibit the natural tendency to enter into a dark compartment in order to avoid an electric foot-shock. Thus, passive avoidance is considered as a negative reinforced learning. Our results showed that intrahippocampal PREGS administration increased the aversive learning (Experiment 1) in accordance with the previously described promnesic profile of PREGS (Pallarès et al., 1998; Vallée et al., 1997; Mayo et al., 2003). It has been suggested that PREGS promnesic profile can be mediated by the potentiation of NMDA receptors in pyramidal neurons (Bowly, 1993), but also through the potentiation of cholinergic neurons (Pallarès et al., 1998; Darnaudéry et al., 2000). Thereby, these findings suggest that together with other postulated brain structures such as the amygdala (Akwa et al., 1999; Engin and Treit, 2007) or the lateral septum (Bitran et al., 1999), the dorsal hippocampus could be relevant for explaining the effects of Ns on exploratory, anxiety-like or aversive learning behaviour.

It is well established that Ns participate in shaping and organising the CNS during development, having influence in brain function and in a variety of behaviours (see Introduction). Thus, it could be expected that if neonatal alteration of Ns affects the development of the hippocampus, consequently the behavioural response to intrahippocampal Ns administration could be also modified. For that purpose, we studied if neonatal manipulation of Ns levels alters exploration, anxiety and aversive learning responses to intrahippocampal Ns administration. Our results showed that neonatal Allopregnanolone administration abolished the anxiolytic profile induced by hippocampal Allopregnanolone (Experiment 1). Moreover, results of aversive learning response showed that the previously described PREGS promnesic profile when administered into

the hippocampus is not observed in animals neonatally administered with Allopregnanolone or finasteride. Nevertheless, results of exploratory behaviour showed that neonatal manipulation of Ns did not affect the behavioural response to intrahippocampal Allopregnanolone administration (Experiment 1). These data indicate that neonatal manipulation of Ns modifies the intrahippocampal Allopregnanolone anxiolytic and PREGS promnesic profile (Experiment 1). In this line, previous studies of our laboratory have also demonstrated that neonatal alteration of Ns levels abolish the decrease in locomotor activity induced by intrahippocampal Allopregnanolone administration tested in the open field (Darbra and Pallarès, 2011). Thus, present results support our hypothesis concerning that alteration of Ns levels during early developmental affects the behavioural response to intrahippocampal Ns administration; and point out the role of neonatal Ns for the hippocampal development.

Mechanisms underlying hippocampal and behavioural changes due to neonatal manipulation of Ns levels: Focused on the hippocampal expression of $GABA_AR$ containing α4 and δ subunits

The α4 and δ subunits combination has been described to be the most sensitive to Ns (reviewed in Shen and Smith, 2009). Thus, given the obtained results in the Experiment 1, we hypothesised that some of the hippocampal mechanisms underlying the behavioural alterations that we observed due to neonatal alteration of Ns levels could be mediated through changes in $GABA_AR$ containing α4 and δ subunits. Our results indicated that neonatal finasteride administration increases hippocampal expression pattern of α4 and δ $GABA_AR$ subunits during postnatal development (from P6 to P15) mainly at P10 (Experiment 2), thereby corroborating our hypothesis concerning that neonatal manipulation of Ns levels modifies hippocampal α4 and δ $GABA_AR$ subunits. Other authors reported that changes in the expression of α4 and δ $GABA_AR$ subunits could be induced by the fluctuation of Allopregnanolone or its precursor progesterone (Smith et al., 1998; Gulinello et al., 2001; Gulinello et al., 2002; Shen et al., 2005; Gangisetty and Reddy, 2010). In this sense, we have also observed fluctuation of Ns levels induced by finasteride administration (i.e. testosterone, pregnenolone, Allopregnanolone) (Darbra et al., 2013; Experiment 2). Thus, the reported hippocampal changes observed in α4 and δ $GABA_AR$ subunits expression might be related to the fluctuations on hippocampal Ns levels induced by the administration of finasteride. Nevertheless, the functional mechanism by which finasteride administration in neonatal pups could induce the observed increase in $GABA_AR$ subunits expression remains to be elucidated. Indeed, neonatal activation of the $GABA_ARs$ is an important source of activity, necessary for the GABA-glutamate sequential maturation of interneurons and

pyramidal neurons in the hippocampus (Ben-Ari, 2002; Ben-Ari et al., 2007). Thereby, alterations in physiological levels of GABA-positive NS during these periods can alter the physiological maturation of GABAergic systems, also affecting other neurotransmitters and modifying the expression of other $GABA_ARs$ in the hippocampus.

Interestingly, neonatal finasteride administration increased the expression of hippocampal α4 and δ $GABA_AR$ subunits not only during postnatal period but also in the adulthood (Experiment 2). Furthermore, our results showed that increased hippocampal α4 and δ $GABA_AR$ subunits expression was also accompanied by an altered anxiety response to progesterone administration in the adulthood (Experiment 2). A possible mechanism to explain the increase of anxiety scores after progesterone administrations can be attributed to the ability of Allopregnanolone to reverse its classic inhibitory effect of enhancing $GABA_AR$-gated currents (hyperpolarizing response) only in those $GABA_ARs$ expressing α4 and δ subunits, but not any other $GABA_ARs$ as showed by other authors (Shen et al., 2007). However, the involvement of other mechanisms or other $GABA_AR$ subunits cannot be discarded. Thus, our data indicate that changes in hippocampal α4 and δ subunits of the $GABA_AR$ induced by finasteride administration are related to the altered anxiety-like behaviour in response to progesterone administration in the adulthood. It is important to remark that an increase in α4 and δ $GABA_AR$ subunits mRNA in adulthood has been related to epileptic seizures (Brooks- Kayal et al., 1998; Cohen et al., 2003; Maguire et al., 2005), and that NS action through δ subunit-containing $GABA_AR$ is required for the physiological response to stress and stress-induced anxiety-like behaviour (Sarkar et al., 2011). In agreement with the above-presented data, other studies demonstrated changes in cortical $GABA_AR$ subunits expression in adulthood as a consequence of neonatal steroids administration (Calza et al., 2010). Taken together, results of Experiment 2 suggest that neonatal Ns fluctuation alter the hippocampal expression of α4 and δ subunits of the $GABA_AR$. Concretely, finasteride administration induced changes in hippocampal α4 and δ subunits related to the altered adult anxiety-like behaviour in response to progesterone administration. Thus, present results indicate that early life NS manipulations are important for the maturation of the $GABA_AR$, and can lead to an altered adult system that responds differently to Ns in the adulthood (Brooks-Kayal et al., 2001; Calza et al., 2010).

Mechanisms underlying hippocampal and behavioural changes due to neonatal manipulation of Ns levels: Changes in the hippocampal expression of KCC2

Our results of Experiment 3 indicated that neonatal alteration of Ns by both Allopregnanolone or finasteride administration alters the developmental expression of KCC2 in the hippocampus. In one hand, neonatal Allopregnanolone administration induced a marked up regulation of hippocampal KCC2 at earlier stages in comparison with NH and Control groups, while neonatal finasteride administration impaired the normal progressive up regulation of KCC2 from P6 until P15 (Experiment 3). Thus, the hypothesis that neonatal Ns fluctuation alters the hippocampal expression of KCC2 during postnatal stages was verified.

Taking into account that Allopregnanolone exposure partially overlies the developmental window when GABA is depolarising and that it became hyperpolarizing from P7 to P14 (Wang and Kriengstein, 2011), it is conceivable that the KCC2 up regulation could be related to an elevation of intracellular chloride associated with activation of $GABA_A$Rs by Allopregnanolone administration. As mentioned, NS that act as an allosteric positive modulators of $GABA_A$R, can also directly gate the $GABA_A$R when administered at high concentrations (Belelli and Lambert, 2005). Other authors showed that early KCC2 up regulation affects morphology of cortical neurons *in vivo* (Li et al., 2007) and is sufficient to reduce interneuron migration by rendering $GABA_A$Rs activation hyperpolarizing (Li et al., 2007; Bortone and Polleux, 2009). Moreover, previous studies demonstrated that neonatal Allopregnanolone administration to hippocampal cultures decreases neurite length (Brinton, 1994). Thus, is possible that changes in the KCC2 expression induced by the administration of NS during a relevant developmental window could affect structurally and morphologically hippocampal interneurons. In this sense, other studies reported a decrease in thalamic and cortical interneurons induced by Allopregnanolone administration at P5 (Gizerian et al., 2004; Grobin et al., 2006). Changes in the timing pattern of KCC2 expression has also been shown to alter the excitatory driving force of Cl^-, shifting from excitatory to inhibitory signalling in the hippocampus and other neural structures (Ben-Ari et al., 1989; Ikeda et al., 2005; Payne et al., 2003; Ben-Ari et al., 2007; Khirug et al., 2010) related to an altered morphology and migration of neurons (Li et al., 2007; Bortone and Polleux, 2009). Thereby, it is plausible that behavioural alterations observed in the present work could also be related to the reported changes in the timing pattern expression of hippocampal KCC2 induced by the alteration of neonatal NS levels. Indeed, the relation between KCC2 alteration and adult behavioural disturbances is beginning to generate an increasing interest in the study of the aetiology of some psychopathologies, such as schizophrenia and affective disorders (Arion and Lewis, 2011; Hyde et al., 2011; Tao et al., 2012). In this sense, extensive evidences indicate an activity-dependent regulation of both the normal developmental switch in GABAergic polarity from excitation to inhibition and the efficacy of

GABAergic transmission through its action on KCC2-mediated chloride homeostasis (Fiumelli and Woodin, 2007). In this line, it has been speculated that $GABA_AR$ subtypes composition could also be regulated by intracellular chloride concentration in a synaptic-specific fashion (Succol et al., 2013).

In summary, present results demonstrate that Ns fluctuation during postnatal development affect the hippocampal expression of KCC2. These changes could also be related with changes in the α4 and δ $GABA_AR$ subunits expression in both neonatal and adult hippocampus observed as a consequence of neonatal Ns levels alterations.

Conclusions

Findings highlights

Experiment 1

A) Dorsal hippocampus is a relevant structure related to the Ns effects on exploration, anxiety-like behaviour and aversive learning.

B) Neonatal Allopregnanolone administration alters the anxiolytic profile induced by intrahippocampal Allopregnanolone administration

C) Alteration of neonatal Ns levels modify the promnesic profile induced by intrahippocampal PREGS administration

Experiment 2

A) Neonatal finasteride administration increases hippocampal α4 and δ $GABA_AR$ subunits expression during postnatal development and in the adulthood.

B) Neonatal finasteride increases anxiety-like behaviour in response to progesterone administration accompanied by a decrease in hippocampal δ $GABA_AR$ subunit.

Experiment 3

A) Neonatal administration of Allopregnanolone induces an early up regulation of hippocampal KCC2.

B) Neonatal administration of finasteride prevented the hippocampal increase of KCC2 during postnatal development.

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Annex

Annex 1

Research article

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Neonatal allopregnanolone levels alteration: Effects on behavior and role of the hippocampus

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ABSTRACT

Several works have pointed out the importance of the neurosteroid allopregnanolone for the maturation of the central nervous system and for adult behavior. The alteration of neonatal allopregnanolone levels in the first weeks of life alters emotional adult behavior and sensory gating processes. Without ruling out brain structures, some of these behavioral alterations seem to be related to a different functioning of the hippocampus in adult age. We focus here on the different behavioral studies that have revealed the importance of neonatal allopregnanolone levels for the adult response to novel environmental stimuli, anxiety-related behaviors and processing of sensory inputs (prepulse inhibition). An increase in neonatal physiological allopregnanolone levels decreases anxiety and increases novelty responses in adult age, thus affecting the individual response to environmental cues. These effects are also accompanied by a decrease in prepulse inhibition, indicating alterations in sensory gating that have been related to that present in disorders, such as schizophrenia. Moreover, behavioral studies have shown that some of these effects are related to a different functioning of the dorsal hippocampus, as the behavioral effects (decrease in anxiety and locomotion or increase in prepulse inhibition) of intrahippocampal allopregnanolone infusions in adult age are not present in those subjects in whom neonatal allopregnanolone levels were altered. Recent data indicated that this hippocampal involvement may be related to alterations in the expression of gamma-aminobutyric-acid receptors containing α4 and δ subunits, molecular alterations that can persist into adult age and that can, in part, explain the reported behavioral disturbances.

Contents

Abbreviations: AlloP, allopregnanolone; KCC2, cotransporter K^+–Cl^- 2; DHEA, dehydroepiandrosterone; EMS, early maternal separation; GABA, gamma amino-butyric acid; NMDA, glutamate N-methyl-D-aspartate; MAP2, microtubule-associated protein 2; PN, postnatal day; PPI, prepulse inhibition; THDOC, tetrahydrodeoxycorticosterone; $GABA_AR$, type A gamma amino-butyric acid receptors.

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1. Introduction

Allopregnanolone (AlloP) or 3α-hydroxy-5α-pregnane-20-one is a 3α-reduced progesterone metabolite (Baulieu, 1998; Robel and Baulieu, 1994; Rupprecht, 2003) that acts as positive allosteric modulator of the type A gamma amino-butyric acid receptors ($GABA_AR$) (Farrant and Nusser, 2005; Hosie et al., 2006; Majewska et al., 1986; Puia et al., 1990, 1991). Previous works have shown the importance of endogenous AlloP levels during development for adolescent and adult behavior and for nervous system maturation. It has been demonstrated that the inhibiting the formation of 5α-reduced steroids during late gestation in rats reduces gestational length, the number of viable pups per litter, and impairs cognitive (object recognition task) and neuroendocrine function (corticosterone levels) in the juvenile offspring (Paris et al., 2011a,b). Moreover, rat cortical levels of AlloP in the forebrain of embryonic rats vary widely throughout development. During the last pregnancy period AlloP levels sharply increase, and decline prior to parturition (Grobin and Morrow, 2001). These high AlloP levels during pregnancy could be part of a protective mechanism against gestational stress, as it has been described that the central opioid inhibition of neuroendocrine stress responses in pregnancy in the rat is induced by the neurosteroid AlloP (Brunton et al., 2009). From the day of birth and during the two first weeks of life, cortical AlloP levels show important fluctuations, as indicated by an initial elevation on the day of birth and a progressive decrease in the first week, followed by a secondary elevation during the second week, reaching maximum values between postnatal days (PN) 10–14 (Grobin and Morrow, 2001; Grobin et al., 2003). Moreover, AlloP levels show sexual dimorphism at PN25. Female cerebral cortical AlloP levels at 21–33 days following birth are higher than males (Grobin and Morrow, 2001).

Although GABA is the main inhibitory neurotransmitter in the mammalian adult encephalon, it exerts excitatory actions in several brain structures during early development (Ben-Ari et al., 1994, 1997; Obrietan and Van den Pol, 1995; Owens and Kriegstein, 2002). Interestingly, the secondary peak in AlloP levels is coincident with the period of transition from excitation to inhibition in the function of $GABA_AR$ (Grobin and Morrow, 2001; Staley et al., 1995). The presence of this AlloP peak coinciding with this important change in GABA signaling suggests that GABAergic neurosteroids may participate in the development of GABAergic neurotransmission. GABA may have a major role in brain maturation, since in immature cells GABA participates critically in the formation of synapses and activity in neuronal networks (Ben-Ari et al., 2007). It is possible that the elevation in cortical AlloP levels influences the function of $GABA_AR$ during the second week of life, and the timing of increased AlloP levels may differentially impact particular brain regions (Grobin and Morrow, 2001), including the hippocampus (Mellon, 2007). Thus, variations in the maturation of GABA neurotransmission in several brain regions can influence adult behaviors that are mediated or related to this neurotransmitter and to these brain regions.

In this article, we review the main published data related to the effects of neonatal AlloP levels alteration on adult and adolescent behavior, and the role that the hippocampal GABAergic system plays in these effects (see Fig. 1 for the summary diagram).

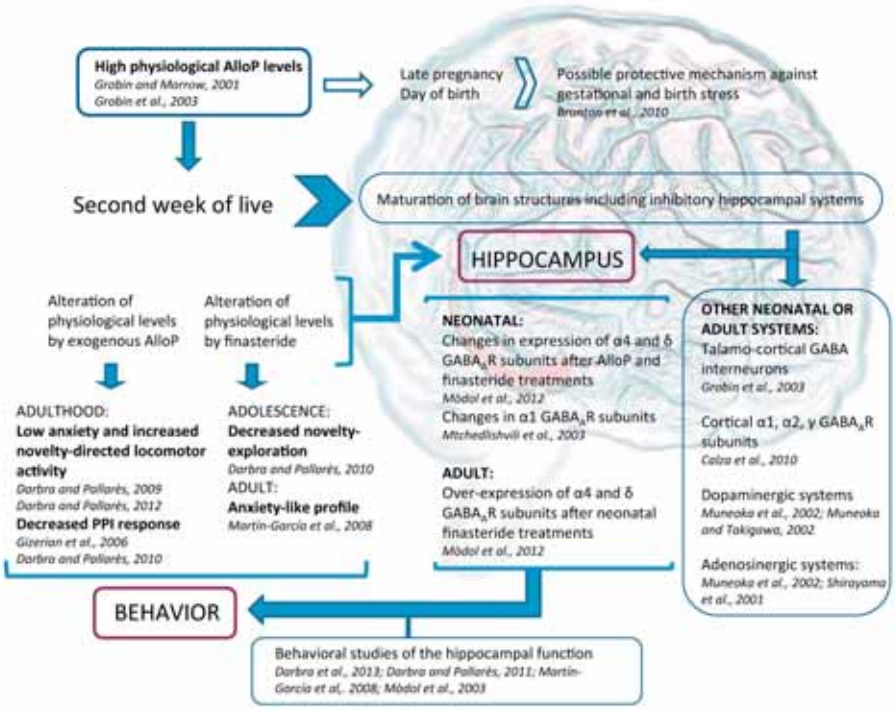

Fig. 1. Summary diagram indicating the most studied relationships between neonatal AlloP, adult behavior and hippocampal GABAergic systems.

2. Alteration of neonatal AlloP levels affects adult and adolescent behavior

Neonatal manipulation of AlloP levels, by means of systemic administration of AlloP, and its synthesis inhibitors finasteride (Azzolina et al., 1997; Mukai et al., 2008) or SKF105,111 (Guidotti et al., 2001; Matsumoto et al., 1999; Pibiri et al., 2008; Pinna et al., 2000) alters adolescent and adult behaviors. For instance, it has been described that AlloP administration (10 mg/kg) at PN5 increases novelty-directed locomotion in the open field and decreases the anxiolytic-like profile of the benzodiazepine lorazepam in the elevated plus-maze test (EPM) in adult male rats (Darbra and Pallarès, 2009). This dose of AlloP was chosen, as a similar dose (8 mg/kg) in adult animals raises cortical AlloP levels to the range observed with swim stress (Vallée et al., 2000). Moreover, the habituation of activity in the open field test in adulthood was slowed down by the neonatal AlloP administration (Darbra and Pallarès, 2009). In this way, it has been shown that neonatal stress also increased the locomotor activity and slowed down its intra-session habituation in the open field test in adult male rats (Dubovický et al., 1999), suggesting a possible relationship between neonatal stress and endogenous AlloP levels. Other authors have documented an increase in the adult locomotor response to amphetamine as a consequence of the neonatal administration of AlloP (Gizerian et al., 2006). It has been proposed that excitatory amino acids projections originating in the prefrontal cortex may play a particularly important role in the development of progressive augmentation of behavioral responses to psychomotor stimulants (behavioral sensitization), perhaps via their regulatory effects on midbrain dopamine neurons (Wolf, 1998). According to these data, it has been reported that neonatal pregnenolone administration (10 μg/g from PN3 to PN7), an AlloP precursor via its conversion to progesterone, induced hyper-locomotion in rats in the open field at pre- and post-puberty, an increase that was more persistent in females than in males (Muneoka et al., 2002). The reported increase in novelty-directed locomotion as a consequence of neonatal 10 mg/kg of AlloP administration (Darbra and Pallarès, 2009) seems to indicate a possible reduction in the behavioral response to a novel environment exposition. However, an anxiolytic-like profile in the EPM test was only obtained with higher neonatal AlloP doses (20 mg/kg from PN5 to PN9) (Darbra and Pallarès, 2012). Thus, the animals that were treated in the neonatal period with AlloP showed more entries and time in the open arms than controls in adulthood. Other authors have reported an increase in the time spent in the open arms in adult male rats that were administered lower AlloP doses in the neonatal period (5 mg/kg from PN2 to PN6), but unfortunately the results on the number or percentage of entries in the open arms were not reported (Zimmerberg and Kajunski, 2004). These results are in accordance with others indicating that prenatal AlloP administration (during the last week of gestation) decreased anxious behavior in the plus maze in the male adult offspring (Zimmerberg et al., 2010). Taken together, these data indicate a possible reduction in stress responses to novel environmental experiences and an anxiolytic-like profile in adult rats that were injected with AlloP in the neonatal period. However, AlloP neonatal results have to be taken carefully given that an increase in the exploratory behavior and a decrease in anxiety when exposed to novel environments could indicate an impulsive and risking behavior (Mòdol et al., 2013). In this sense, it has been described that a sensation seeking pattern of behavior, impulsivity and novelty preference can increase the vulnerability to psycho-stimulant drug abuse in humans (Kelly et al., 2006) and in animals (Molander et al., 2011).

On the other hand, some results obtained with neonatal administration of the 5α-reductase inhibitor finasteride are consistent with neonatal AlloP results. For instance, neonatal finasteride administration (50 mg/kg from PN5 to PN9) increases emotional reactivity in situations of stress or conflict in the adolescent age, as reflected by the reduction in exploration of a novelty situation (Darbra and Pallarès, 2010), decreasing novelty-directed activity and holes exploration in the Boissier test (Boissier and Simon, 1967). Thus, these data indicate that the alteration of neonatal AlloP levels can modify the animal response to different environmental stressors in adult age, and suggest that neonatal AlloP levels can be crucial for determining individual differences in emotional behaviors.

Other experiments have shown that the alteration of neonatal AlloP levels can affect the processing of sensory inputs to the brain. It has been reported that AlloP administration (10 mg/kg) at PN2 or PN5 (Gizerian et al., 2006), or between PN5 and PN9 (Darbra and Pallarès, 2010) decreased prepulse inhibition (PPI) in adult age. PPI measures the ability to inhibit startle responses after low prepulse stimuli, involving sensory gating processes. PPI behavior is considered as a prefrontal cortex-dependent behavior (Gizerian et al., 2006; Swerdlow et al., 2001), but the hippocampus (both dorsal and ventral aspects) also seems to be an important brain structure for the PPI, since it appears to contribute to sensorimotor gating (Zhang et al., 2002). The deterioration of PPI ability is one of the most widely accepted symptoms of schizophrenia in humans and animal models (Swerdlow et al., 2008). Thus, the results obtained in these studies indicate the importance of neonatal neurosteroid levels in the development of hippocampal and prefrontal cortex function, and their relevance in a behavioral phenotype that some have likened to that present in schizophrenia (Swerdlow et al., 2008). Consistently, the decrease in adult PPI observed in animals that were injected with AlloP at PN2 was reversed by means of the administration of the antipsychotic drug clozapine 15 min prior to PPI testing (Gizerian et al., 2006). Clozapine is an atypical antipsychotic drug that in addition to its actions on monoaminergic pathways also alters GABAergic systems in several brain structures, including the limbic cortex (Deutch, 1995; Zink et al., 2004). Taken together, the presented data demonstrate the relevance of neonatal AlloP levels for emotional and sensory gating behavior in adult age, and suggest that some alterations in neonatal AlloP levels can be a determinant factor for adult individual differences in the behavioral response to novel environmental stimuli, and for the vulnerability to psychopathologies such as the schizophrenia.

3. Role of the hippocampus in the effects of neonatal AlloP levels on adult and adolescent behavior

As mentioned above, neonatal AlloP levels are involved in adult behaviors such as the response to a novel environment, the emotional processing and the sensorial gating. In our laboratory, we have tested the possible role of the hippocampus in the effects of neonatal AlloP levels alteration on adult behavior, since the functioning of this brain structure seems to be related to spatial exploration (Xu et al., 1998), anxiety-related behaviors (Bitran et al., 1999; Mineur et al., 2013; Mòdol et al., 2011) and sensory gating (Adams and Van den Buuse, 2011; Howland et al., 2004; Zhang et al., 2002). One can hypothesize that if neonatal AlloP administration affects the development of the hippocampus, consequently, the behavioral response to adult intrahippocampal neurosteroids administration would be different depending on neonatal AlloP manipulation (see Fig. 2 for the main results).

3.1. Anxiety-related and environmental novelty-related behaviors

Previous results show that the adult infusions of AlloP (0.2 μg/0.5 μl in each hemisphere) into the dorsal hippocampus (CA1

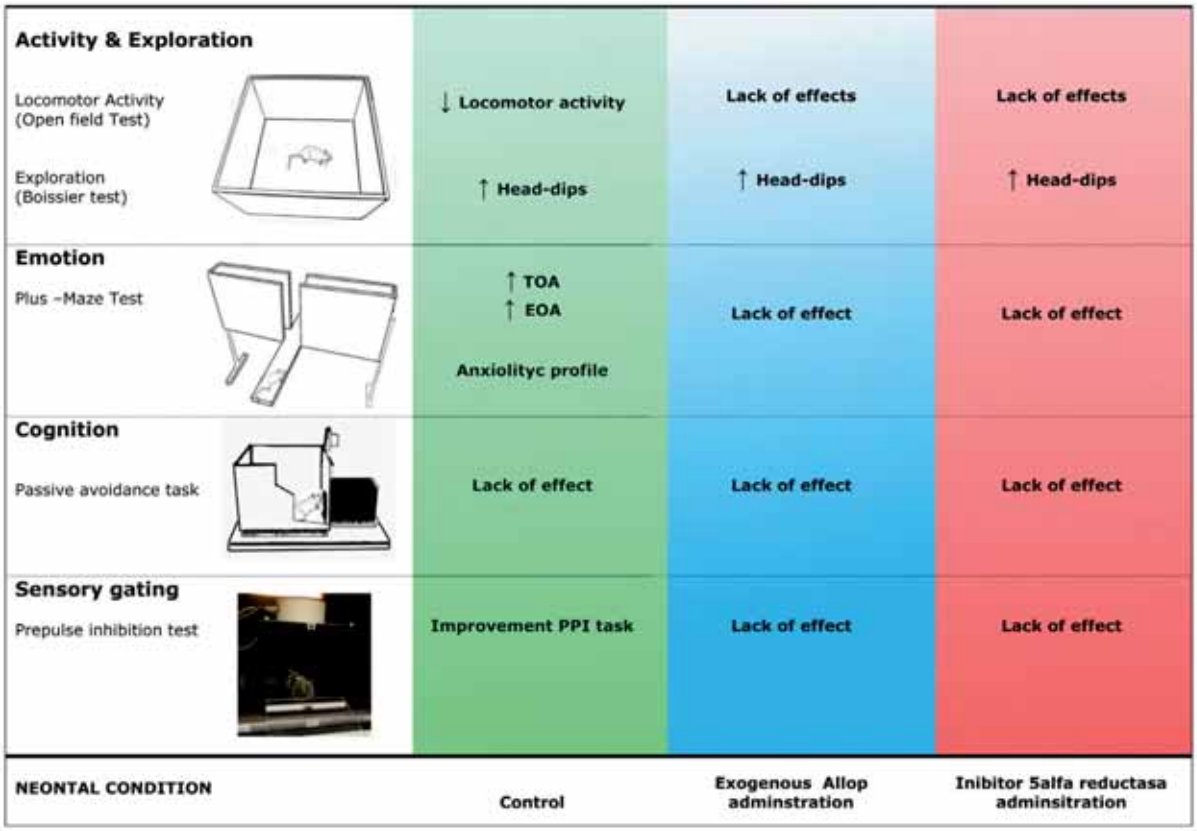

Fig. 2. The behavioral response to the intrahippocampal AlloP depends on early neonatal neurosteroid levels. AlloP (0.2 μg/0.5 μl per side) was centrally administered by a bilateral 21 gauge stainless steel double guide cannula implanted into the CA1 region of the dorsal hippocampus (anteroposterior: −3.6 mm; mediolateral: −1.8 mm; dorsoventral: −1.8 mm) from bregma according to the Paxinos and Watson atlas (Paxinos and Watson, 1997) and activity and exploration behaviors, emotion, learning and sensory gating were evaluated. AlloP (10 or 20 mg/kg) and finasteride (50 mg/kg) neonatal administration (PN5–PN9) alter the intrahippocampal AlloP effects on locomotor activity, anxiety-related behavior (EPM test) and sensory gating (PPI). TOA, time in open arms; EOA, entries into open arms. For more details see text.

region) induce a robust anxiolytic-like profile in the EPM test (Mòdol et al., 2011), indicated by a significant increase in the percentage of entries, time and activity in the open arms. A similar effect was obtained after infusions of pregnenolone into the dorsal hippocampus at doses of 2.5 and 5 mg/kg (Bitran et al., 1999). These interesting data suggest that, together with other brain structures such as the amygdala (Akwa et al., 1999; Engin and Treit, 2007) or the lateral septum (Bitran et al., 1999), the dorsal hippocampus could be an important target for explaining the effects of neurosteroids on emotional behavior. However, intrahippocampal AlloP infusions did not induce anxiolytic-like effects in those animals that were injected with AlloP in the neonatal period (Mòdol et al., 2013). This result is in agreement with previous data indicating that neonatal AlloP administration decreased the anxiolytic-like profile of the systemic administration of the benzodiazepine lorazepam in adult rats (Darbra and Pallarès, 2009). Thus the functioning of the dorsal hippocampus in adult age, related to emotional behavior and in response to the AlloP infusion, is different in the animals that were treated with AlloP in the neonatal period.

On the other hand, interactions between neonatal alterations of AlloP levels and exploration and locomotion in a new environment (Boissier test) after intrahippocampal neurosteroids infusions were not detected (Darbra et al., 2013; Mòdol et al., 2013). The increase in head-dipping behavior induced by intrahippocampal AlloP administration was not affected by neonatal alterations of AlloP levels (Mòdol et al., 2013). However, when analyzing the locomotor activity recorded in an open field (during 20 min), dorsal intrahippocampal infusions of AlloP (0.2 μg) presented sedative and motor depressing effects, as reflected by a decrease in locomotor activity (Martín-García and Pallarès, 2005a,b; Darbra and Pallarès, 2011). This motor-depressing effect seems to be related to the well known action of AlloP as allosteric positive modulator of the $GABA_AR$ (Belelli and Lambert, 2005; Lambert et al., 2003, 2009). Similarly to anxiety data, the locomotor depressing effects of intrahippocampal AlloP administration in male rats were not present in those subjects that were injected with 10 mg/kg of AlloP from PN5 to PN9 (Darbra and Pallarès, 2011).

3.2. Aversive learning

Moreover, we have studied the passive avoidance learning, an aversive learning with important emotional component that is, at least in part, hippocampus-dependent (Mòdol et al., 2011). In this paradigm, the animal must inhibit the natural tendency to enter a dark compartment in order to avoid an electric foot-shock. Thus, passive avoidance is considered as a negative reinforced learning. In adult animals, dorsal intrahippocampal infusions of pregnenolone sulphate (5 ng/0.5 μl in each hemisphere), an allosteric negative $GABA_AR$ modulator (Malayev et al., 2002; Schumacher et al., 2008) and a positive glutamate N-methyl-D-aspartate (NMDA) receptor modulator (Gibbs et al., 2006) that shows promnesic profile in several behavioral tests (Mayo et al., 2003;

Martín-García and Pallarès, 2005c; Pallarès et al., 1998; Vallée et al., 1997), may instead produce an impairment of passive avoidance in animals that were exposed to environmental stress (Martín-García and Pallarès, 2008). This impairment in passive avoidance task seems to be specific for this negative reinforced learning and limited to animals previously submitted to environmental stress (Martín-García and Pallarès, 2008). However, the passive avoidance impairment was not present in the animals that were injected with AlloP in the neonatal period (10 mg/kg from PN5 to PN9) (Martín-García et al., 2008). To explain these results, it is important to consider previous data indicating that neonatal AlloP administration decreases anxiety in adult age (Darbra and Pallarès, 2012). Therefore, neonatal AlloP-induced anxiety reduction in adulthood could have reversed the negative effects on the retention of the passive avoidance task related to the combination of intrahippocampal pregnenolone sulphate administration and environmental stress, taken into account that the important emotional component of this kind of learning can influence its performance. Nevertheless, intrahippocampal AlloP infusions (0.2 μg/0.5 μl in each hemisphere), did not affect passive avoidance retention latency, and this lack of effects was not modified by neonatal AlloP levels alteration (Martín-García et al., 2008; Mòdol et al., 2013).

3.3. Sensory-motor gating

Recent results from our laboratory on sensory gating have shown that the administration of AlloP (0.2 μg) into the dorsal hippocampus (CA1) of adult male rats increases the percentage of PPI without affecting basal startle amplitudes (Darbra et al., 2012). Taking into account that a decrease in PPI can be considered as a symptom of schizophrenia in humans and in animal models (Kilts, 2001), an increase in PPI induced by AlloP administration may have relevant therapeutic implications. In this way, it has been reported that the treatment with clozapine or olanzapine increases brain and plasma levels of AlloP and pregnenolone, particularly in the hippocampus (Marx et al., 2006). It has even been postulated that the administration of pregnenolone may contribute to clinical actions of these antipsychotics (Marx et al., 2006), especially reducing negative symptoms and cognitive impairment in schizophrenia (Marx et al., 2009). Thus, this effect of pregnenolone could be related to its conversion to AlloP, which in turn could reduce, possibly via positive $GABA_AR$ modulation, the dysfunctional hippocampus hyperactivity that has been related to several symptoms of schizophrenia (Zierhut et al., 2010). In the same way as the data presented above, neonatal administration of Allop (20 mg/kg from PN5 to PN9) suppressed the increase in PPI after intrahippocampal AlloP administration in adult male rats (Darbra et al., 2013). These data are in accordance with previous works indicating that neonatal AlloP administration (10 mg/kg) decreased PPI in adult age (Darbra and Pallarès, 2010). Thus, it is reasonable to expect not to find the facilitating effect of intrahippocampal AlloP infusions in PPI task in neonatal AlloP injected rats.

3.4. Neonatal finasteride effects on behavioral responses to intrahippocampal AlloP infusions

Interestingly, the effects of adult administration of AlloP in the dorsal hippocampus on anxiety (EPM test) and sensory gating (PPI test), as well as the infusions of pregnenolone sulphate in an emotional aversive learning (passive avoidance test), were not present in those animals that were injected with the AlloP synthesis inhibitor finasteride in the neonatal period (50 mg/kg from PN5 to PN9) (Darbra and Pallarès, 2011; Darbra et al., 2013; Martín-García et al., 2008; Mòdol et al., 2013). Obtaining a similar neutralization of adult intrahippocampal neurosteroids effects in neonatal AlloP and finasteride treated animals was unexpected. In order to clear this concern, we analyzed the neonatal levels of several neurosteroids including AlloP in the hippocampus, by means of the gas chromatography/mass spectroscopy technique. As expected, neonatal subcutaneous injections of AlloP (20 mg/kg between PN5 and PN9) caused very high concentrations of AlloP in the hippocampus at PN5 (Mòdol et al., 2013) and PN9 (Darbra et al., 2013), which was three hundred times higher than that seen in controls. The increase of AlloP concentrations was accompanied by increased epiallopregnanolone levels, in agreement with previous data that reported an increase of both neurosteroids in frontal cortex as a consequence of a single AlloP administration in adult rats (Vallée et al., 2000). These results are consistent with the metabolic pathway of these neurosteroids, specifically with their interconversion catalyzed by 3α- and 3β-hydroxy steroid oxidoreductases in brain (Compagnone and Mellon, 2000). Unexpectedly, finasteride not only failed to reduce, but also tended to increase, hippocampal AlloP levels in PN5 (Mòdol et al., 2013) and PN9 (Darbra et al., 2013). This different effect between adults and neonates in finasteride effects on AlloP levels remains unclear. It could be related to maturational changes in the 5α-reductase, as the changes in AlloP synthesis during postnatal development (Grobin et al., 2003) have been attributed (Mellon, 2007) to changes in expression of 5α-reductase and 3α-hydroxysteroid-descarboxylase, the enzyme that converts dehydroprogesterone to AlloP (Agís-Balboa et al., 2006). Also, changes in the 17β-hydroxysteroid dehydrogenase type 10, which is essential to the oxidative metabolism of AlloP and THDOC (He et al., 2005; Yang et al., 2007) cannot be discarded. One of these changes could be related to differences in the metabolic neurosteroids turnover between adulthood and neonatal periods, taking into account that brain dissections were made 1 h after finasteride injections. Important reductions of AlloP levels 1 h after a single finasteride administration (10 mg/kg intraperitoneal) have been described in 7 weeks-old rats exposed to immobilization (Mukai et al., 2008). Although the brain AlloP levels are extremely low under normal conditions, they are significantly elevated after acute stress application in several stress paradigms, such as immobilization (Higashi et al., 2007), forced swimming (Vallée et al., 2000) and carbon dioxide inhalation (Barbaccia et al., 1994). However, the effects of finasteride on AlloP levels have always been measured in adult animals. Therefore, there are no data on the effects of finasteride in neonatal period other than those obtained in our laboratory. Also, it is important to mention that AlloP levels are very low in the first week of life, and that the maximum values of that described as the second maturational AlloP peak does not appear until PN10–14 (Grobin and Morrow, 2001). Anyway, this moderate increase in hippocampal levels of AlloP in PN5 (Mòdol et al., 2013) and PN9 (Darbra et al., 2013) induced by subcutaneous finasteride administration could explain the similarities observed in the hippocampal behavioral studies.

Overall, the results presented above seem to indicate that a wide range of behavioral responses to neurochemical modulation of the hippocampus are altered depending on the manipulation of neonatal AlloP levels, suggesting that neonatal manipulation of AlloP levels alters the normal functioning of the hippocampus.

4. Neonatal neurosteroids levels and hippocampus maturation

The hippocampal $GABA_AR$ have an increased sensitivity to AlloP during postnatal development (Mtchedlishvili et al., 2003). Thus, it has been suggested that the enhancement of GABA evoked currents after AlloP administration into the granule cells that is increased during the postnatal development of the dentate gyrus can be related to the increased expression of $GABA_AR$ α1 subunit in

the neonatal period (Mtchedlishvili et al., 2003). Moreover, other studies indicate that the neurosteroid tetrahydrodeoxycorticosterone (THDOC), which shares a profile of action with AlloP as a $GABA_AR$ allosteric positive modulator, has important modulatory effects in hippocampal GABAergic synapsis during development at concentrations which occur naturally in the brain (Cooper et al., 1999). Thus, the hippocampal increase in the sensitivity to AlloP and the important role of other α-reduced neurosteroids as THDOC in the modulation of hippocampal synapses during development suggests that the administration of exogenous neurosteroids during neonatal development can dramatically alter the maturation of the hippocampus. In fact, it has been documented that the alteration of the physiological levels of neurosteroids in early neonatal phases provokes alterations in the maturation of certain cerebral structures such as the hippocampus (Iwata et al., 2005; Shirayama et al., 2005), the GABAergic thalamic–cortical system (Grobin et al., 2003), the meso-cortical and meso-striatal dopaminergic pathways (Muneoka et al., 2002; Muneoka and Takigawa, 2002) or the adenosinergic systems throughout A1 (Muneoka et al., 2002) or A2A receptors (Shirayama et al., 2001).

In vitro studies on the hippocampus have shown that the neurosteroid AlloP induces cytoarchitectural regression in cultured fetal hippocampal neurons (Brinton, 1994). Moreover, the neonatal administrations of pregnenolone (from postnatal days 3 to 7), the main AlloP precursor by its conversion to progesterone, or of the neurosteroid dehydroepiandrosterone (DHEA), increased the expression of microtubule-associated protein 2 (MAP2) in the granule cell layer of dentate gyrus and in the pyramidal cell layer of CA3 region at post-puberty (7 weeks of age) (Iwata et al., 2005). MAP2 protein is a cytoskeleton member detected mainly in dendrites that affects the shape, polarity and plasticity of neurons by controlling microtubule assembly. Thus, it has been proposed that exogenous neurosteroids during the neonatal period can bind to MAP2 and directly affect its expression and dendritic arborization, and that this MAP2 increased expression might be an interesting phenotype involving stress and motivation, as the CA3 region of the hippocampus is vulnerable to stressful conditions, including elevated levels of glucocorticoids (Iwata et al., 2005). Other studies have also shown post-pubertal alterations in the hippocampal expression of the synaptic vesicle membrane-associated protein synapsin I, and also an increase in the number of neuropeptide Y-positive cells, in animals that were administered with pregnenolone or DHEA in the neonatal period (Shirayama et al., 2005). Other changes such as an increase in the number and size of glial fibrillary acidic protein immunoreactive astrocytes or an increase in the extension of arborization was seen in the overall hippocampus at both pre-puberty and post-puberty ages in animals that were injected with pregnenolone or DHEA from PN3 to PN7 (Shirayama et al., 2005). It should be mentioned that glial cell abnormalities (Cotter et al., 2001), changes in neuropeptide Y function (Redrobe et al., 2002) and alterations of synaptic proteins in the hippocampus (Vawter et al., 2002) seem to be related to psychiatric diseases such as emotional disorders, depression and schizophrenia. It is also important to note that neuroactive steroids regulate the development of glial cells (Melcangi et al., 2011). Indeed, the morphology, immunoreactivity, enzymatic activity and gene expression of astroglia can be modified by postnatal actions of neuroactive steroids (Garcia-Segura and Melcangi, 2006), effects that may be highly relevant for the neuronal connectivity.

On the other hand, studies carried out in late gestation of fetal sheep suggest that neurosteroids, such as AlloP, influence the rates of cellular apoptosis and proliferation in the hippocampus. In this way, finasteride treatment increased apoptosis in CA1 and CA3 hippocampal regions as well as astrocytes proliferation (Yawno et al., 2009). As these effects can be prevented by the co-administration of the AlloP analogous alfaxalone, it has been proposed that 5α-reduced neurosteroids including AlloP provide protection to the fetal brain against hypoxia and excitatory stress in late gestation, and also have an important role in the modeling of the brain throughout the last stages of gestation (Yawno et al., 2009). Gestational neuroactive steroid concentrations are needed for normal cell proliferation and cell death in the late gestation brain, and a loss of these steroids at preterm birth may adversely affect development and vulnerability to injury (Hirst et al., 2009).

Summarizing, both behavioral and molecular studies indicate that neurosteroids, such as AlloP, participate in the fetal and postnatal development of the hippocampus. Thus, the alteration of its physiological levels can produce developmental alterations that can be reflected in future adolescent or adult behaviors.

5. Neonatal AlloP levels and $GABA_A$ receptors

The $GABA_AR$ is a pentameric structure that mediates inhibition in the adult brain, and is typically composed of 2α, 2β and 1γ subunits. There are 19 different subtypes of $GABA_AR$ combinations, indicating a high level of structural heterogeneity and function (Olsen and Sieghart, 2009). $GABA_AR$ can be classified between those present at synapses and that participate in phasic inhibition, such as those containing α1, α2, α3, γ1, γ2 or γ3 subunits, and those related to the modulation of tonic inhibition, which contain α4, α5, α6 or δ subunits and that are extrasynaptically and perisynaptically located (Belelli et al., 2009; Glykys and Mody, 2007). The combination of the α4β2δ has been described to be important for tonic inhibition in dentate granule cells of the hippocampus (Stell et al., 2003) and into the CA1 pyramidal neurons (Mangan et al., 2005). $GABA_AR$ containing α4 and δ subunits have been reported to be insensitive to benzodiazepines modulation but especially sensitive to fluctuating AlloP levels, due to physiological and pathological conditions, such as anxiety (Gulinello et al., 2001; Shen et al., 2005), epileptic disease (Brooks-Kayal et al., 1998), alcohol intake (Sundstrom-Poromaa et al., 2002) and schizophrenia (Hashimoto et al., 2008).

Recent experiments from our laboratory indicate that the pattern of expression of α4 and δ $GABA_AR$ subunits is different when analyzing between PN6 and PN12 (Mòdol et al., 2012), probably related to the α4 co-expression with subunits other than δ during early development (Laurie et al., 1992; Didelon et al., 2000). Moreover, neonatal AlloP administration (20 mg/kg from PN5 to PN9) globally decreases the neonatal expression α4 and δ $GABA_AR$ subunits (Mòdol et al., 2012). This effect seems to be related to a pharmacological down-regulation, as the increase in hippocampal AlloP levels after systemic AlloP administration is very high in relation to controls (Darbra et al., 2013; Mòdol et al., 2013). On the other hand, neonatal finasteride administration increases α4 and δ $GABA_AR$ subunits expression in the hippocampus during early development (Mòdol et al., 2012). In particular, finasteride administration (50 mg/kg) from PN5 to PN9 increased α4 and δ expression at PN10, an effect that can be attributed to the fluctuations in hippocampal AlloP levels (moderate increase) recorded at PN5 (Mòdol et al., 2013) and PN9 (Darbra et al., 2013) due to finasteride administration. Moreover, finasteride administration anticipated the natural expression peaks (observed in non-treated animals) from PN12 to PN10 in α4 and from PN12 to PN9 in δ subunits (Mòdol et al., 2013). The fact that δ $GABA_AR$ subunit expression is increased from PN9 in finasteride-treated animals suggests that this subunit is highly sensitive to fluctuationg AlloP levels around the second postnatal week of rat life. In this way, it has been proposed that the insertion of the δ subunit into functional $GABA_AR$ in the first days of life (PN0–PN2) may enhance the efficacy of GABA in the immediate postnatal periods when this

amino acid is still exerting a depolarizing and excitatory action (Didelon et al., 2000), thus it is coherent to expect an increased sensitivity to AlloP fluctuating levels. In general, long-term changes in GABAergic synaptic activity can be induced during restricted development periods by a conditioning protocol relevant to the physiological condition, and such activity-induced modifications may represent a physiological mechanism for the functional maturation of GABAergic synaptic transmission (Gubellini et al., 2001). Moreover, neonatal activation of the $GABA_AR$ is an important source of activity, necessary for the GABA-glutamate sequential maturation of interneurons and pyramidal neurons in the hippocampus (Ben-Ari, 2002; Ben-Ari et al., 2007). Thus, alterations in physiological levels of GABA-positive neurosteroids during these periods can alter the physiological maturation of GABAergic systems, including the expression of some $GABA_AR$ in several brain structures, such as the hippocampus. However, the functional mechanism by which finasteride administration in neonatal pups could induce the observed increase in $GABA_AR$ subunits expression remains to be elucidated.

Interestingly, the over-expression of the α4 and δ $GABA_AR$ subunits in the hippocampus is maintained in adult age in neonatal finasteride treated animals (Mòdol et al., 2012). In this sense, it is important to note that an increase in α4 and δ $GABA_AR$ subunits mRNA in adulthood has been related to epileptic seizures (Brooks-Kayal et al., 1998; Cohen et al., 2003; Maguire et al., 2005), and that neurosteroids action through δ subunit-containing $GABA_AR$ is required for the physiological response to stress and stress-induced anxiety-like behavior (Sarkar et al., 2011). Also, an up-regulation of α4 and δ $GABA_AR$ subunits has recently been reported in women with psychosocial stress (burnout syndrome) (Bäckström et al., 2012). It has been documented that not only short-term steroid treatment (3α-5β-tetrahydroprogesterone or 17β-estradiol + progesterone) (Shen et al., 2005), but also AlloP (Smith et al., 2006) or progesterone withdrawal (Gulinello et al., 2002; Smith et al., 1998), increase δ $GABA_AR$ subunit expression in CA1 hippocampus of female rats. Withdrawal from the GABA-modulatory neurosteroid AlloP after 21 days exposure to its precursor, progesterone, also increased anxiety in male rats in conjunction with up-regulation of the α4 subunit of the $GABA_AR$ in the hippocampus (Gulinello et al., 2002). Thus, fluctuations in the circulating levels of these neurosteroids across endogenous ovarian cycles, at puberty, during pregnancy, or following chronic stress produce periods of prolonged exposure and withdrawal, where changes in $GABA_AR$ subunit composition may occur as compensatory responses to sustained levels of inhibition (Smith et al., 2007). Although the GABAergic inhibition in limbic circuits, which contributes to the regulation of anxiety, is increased by AlloP administration before puberty and in adulthood, during puberty AlloP reduces the tonic inhibition of pyramidal cells in CA1 hippocampal region of female rodents, leading to increased excitability (Shen et al., 2007). Therefore, the inhibition of α4β2δ $GABA_AR$ by AlloP provides a mechanism to explain the generation of anxiety that has been documented at puberty in female rodents after the administration of AlloP or progesterone (Gulinello et al., 2001; Shen et al., 2007). This possible mechanism to explain the increase of anxiety scores after AlloP or progesterone administrations can be attributed to the ability of AlloP to reverse its classic effect of enhancing $GABA_AR$-gated currents (hyperpolarizing response) only in those $GABA_AR$ expressing α4 and δ subunits, but not any other $GABA_AR$.

Molecular changes induced by manipulation of neonatal AlloP levels that are maintained in adult age could be related, at least in part, to the well documented alterations in behavior. In agreement with the data presented above, other studies have shown changes in cortical $GABA_AR$ subunits expression in adulthood as a consequence of neonatal estradiol administration (Calza et al., 2010). In this sense, neonatal administration of beta-estradiol 3-benzoate on the day of birth resulted in marked decreases in the concentrations of progesterone and AlloP in the cerebral cortex at 21, 60, and 180 days after birth, increased the cerebro-cortical abundance of α1, α2, and γ2 subunits of the $GABA_AR$ without affecting that of α3, α4, α5, or δ subunits, and potentiated the reduction in locomotor activity, as well as the anxiolytic-like effect in the EPM test of the benzodiazepine diazepam (Calza et al., 2010). Thus, as shown by the mentioned studies, early life AlloP manipulations are important for the maturation of the $GABA_AR$, and can lead to an altered adult system that responds differently to the environment (Brooks-Kayal et al., 2001; Calza et al., 2010). Furthermore, as $GABA_AR$ exert excitatory actions on neurons excitability that provides an important mechanism in the formation of synapses and activity in neuronal networks during the first week of postnatal period (Ben-Ari et al., 2007), it is reasonable to think that an alteration in neonatal AlloP levels in the first week of life can have severe consequences on the maturation of inhibitory hippocampal circuitry and in other brain areas (Gizerian et al., 2004; Grobin et al., 2006; Iwata et al., 2005; Shirayama et al., 2005). Thus, the results of hippocampal $GABA_AR$ studies are in accordance with the behavioral results presented above.

6. Relationships between neonatal AlloP levels and neonatal stress

Similarly to the alterations of neonatal AlloP levels, the neurodevelopment changes caused by applying perinatal stress have been related to adolescent and adult behavioral alterations. Animal studies have clearly indicated that exposure to several types of stressors during development produces persistent behavioral disturbances that are associated with hormonal, neurotransmitter and functional changes, and resemble an array of psychopathological conditions (Fumagalli et al., 2007). For instance, prenatal stress induced by immobilization causes alterations in the morphology of microglia and the inflammatory response of the hippocampus of adult female mice (Diz-Chaves et al., 2012), and induces long-term effects in cell turnover in the hippocampus–hypothalamus–pituitary axis in adult male rats (Baquedano et al., 2011). Also, immune stress in late pregnant rats decreases length of gestation and fecundity, and alters later cognitive (object recognition task) and anxiety-like behavior of surviving pre-adolescent offspring (Paris et al., 2011a,b). Early maternal separation (EMS) has been characterized as a good model of neonatal stress. Although a variety of procedures have been described depending on the time of separation and the day/days that it is done (Lehmann et al., 2002; Slotten et al., 2006), EMS causes a delay in neurodevelopment (Ellenbroek et al., 2005) and reduces the expression of brain-derived neurotrophic factor and NMDA receptors in the hippocampus (Roceri et al., 2002). This model, in addition to causing stress to the offspring, alters the behavior of the mother, providing less care and rejecting the litter when they meet again (Champagne and Meaney, 2001). Moreover, EMS affects the correct maturation of the hypothalamus–pituitary axis and determines its response to stress in adulthood (Ellenbroek and Cools, 2002; Lehmann et al., 2002). As with the effects in adolescent or adult behavior of neonatal AlloP administered during the neonatal period, EMS also has been associated with increased susceptibility to psychopathologies related to neurodevelopment disorders, such as schizophrenia (Ellenbroek et al., 1998; Fumagalli et al., 2007). It is worthwhile mentioning, that although AlloP levels are typically increased in response to acute stress in adult animals (Barbaccia et al., 1998) by different neurochemical mechanisms (Gunn et al., 2011), an early postnatal stimulation, which consisted of intraperitoneal saline injections and several short episodes of EMS, decreased adult hippocampal AlloP levels

(Frye et al., 2006). On the other hand, pups that had been repeatedly isolated (PN2–PN8) had significantly greater whole brain AlloP levels compared to controls in the neonatal age (Kehoe et al., 2000). Thus, the biosocial stress of isolation in neonatal rats alters central pregnane steroids in neonatal and adult ages. Therefore, the study of the possible interactions between both interventions (neonatal AlloP administration and EMS) is of relevance. In a recent study, we performed a single separation of 24 h on PN9, which has been shown to be enough to cause disruptions in adolescent and adult behaviors (Ellenbroek et al., 1998; Lehmann et al., 2002; Marco et al., 2007), in animals that were previously injected with AlloP (10 mg/kg from PN5 to PN9). EMS caused an increase in locomotor activity, as well as a decrease in the head-dipping behavior in the holes exploration test of Boissier during adolescence, which could be indicating an increased anxiety when confronting new environments (Llidó et al., 2013), results in agreement with others obtained in adult rats (Rentesi et al., 2010). Interestingly, prior neonatal AlloP administration seems to have a protective effect against the effects of EMS on locomotor activity and exploration in adolescence, reversing the decrease in novelty-exploration (Llidó et al., 2013). In the PPI test, PPI disruption in maternally deprived rats occurs only after the puberty (Ellenbroek et al., 1998). Thus, in adult age, both neonatal AlloP administration and EMS caused a disruption in sensorimotor gating (Llidó et al., 2013), according to previous studies (Darbra and Pallarès, 2010; Ellenbroek et al., 1998; Ellenbroek and Cools, 2002). Although neonatal AlloP administration neutralized the effects of EMS on novelty-exploration behaviors in the adolescence, it seems that neonatal AlloP administration is not protective against the deleterious effects of EMS on PPI behavior. In general, these results indicate that some effects of neonatal stress on adolescent behavior, especially concerning the increase in stress responses when animals are submitted to novel environments, can be reversed by the previous neonatal administration of AlloP, suggesting that the increase in brain AlloP levels could be related to a mechanism that counteracts the deleterious effects of stress. In this way, it has been hypothesized that GABAergic 3α-reduced neuroactive steroids may serve as an endogenous counter-regulatory mechanisms against the occurrence of some anxiety disorders, such as spontaneous panic attacks (Rupprecht, 2003; Schüle et al., 2011) or neuropathic pain when these are synthesized in the spinal cord (Mensah-Nyagan et al., 2008). In contrast, behaviors that imply sensory gating are not sensitive to this modulation and are deteriorated by the two neonatal manipulations (neonatal stress and AlloP levels alteration). Finally, it is important to consider the possible role of the hippocampus in the effects of perinatal stress on adult behavior. For instance, anxiety-like behavior of prenatally stressed rats is associated with a selective reduction of glutamate release in the ventral hippocampus (Marrocco et al., 2012). Also, maternal deprivation has important cellular and biochemical effects in the developmental hippocampus, which can be dependent on gender (Llorente et al., 2008), and alters exploratory behavior and corticotropin releasing factor expression in neurons in the amygdala and hippocampus in post-weaning rats (Becker et al., 2007). Thus, changes in the development of the hippocampus that seem be related to neonatal stress, and also to alterations in neonatal AlloP levels can be related to some of the behavioral alterations detected in adult age as a consequence of neonatal manipulations.

7. Perspectives

New perspectives in this interesting field include the in-depth study of the neurochemical and cellular basis involved in mediating the effects of alterations in neonatal AlloP levels on adolescent and adult behavior, and especially the molecular and cellular changes that can be maintained until adolescence or adult ages. To date, a few molecular targets have been described as regards this, mainly involving GABA systems, such as the distribution of GABA interneurons in the thalamic–cortical system (Grobin et al., 2003) or alterations in the expression of α4 and δ $GABA_AR$ subunits in the hippocampus (Mòdol et al., 2012). Also, other studies have implicated the adenosinergic and dopaminergic pathways (Muneoka et al., 2002) that seem to be altered in pre- and post-pubertal rats. However, it is of great interest to study other brain structures and other possible neurochemical pathways; since the main behaviors studied (emotional response, motor activity or sensory gating) involve the participation of several brain nuclei. For instance, there is great interest in studying the role of other limbic structures, such as the amygdala or the cholinergic basal forebrain, brain structures that have been related to several behavioral effects of neurosteroids, such as those on anxiety (Akwa et al., 1999), spatial learning (Mayo et al., 1993; Pallarès et al., 1998) or paradoxical sleep (Darnaudéry et al., 1999). It would be also worthwhile studying the possible morphological changes in the adult hippocampus as well as other related structures. At a neonatal level, the second peak in physiological AlloP levels (with maximum values between PN10 and PN14) is coincident with the period of transition from excitation to inhibition in the function of $GABA_AR$. It has been documented that the cotransporter K^+–Cl^- 2 (KCC2) seems to be implicated in this transition (Rivera et al., 1999). Thus, in our laboratory we are testing whether neonatal AlloP levels changes alter the expression of KCC2 protein in neonatal and adult ages, as the increase in AlloP production in the second week of life could trigger the $GABA_AR$ transition from excitatory to inhibitory forms through alterations in KCC2 function.

Finally, the fact that neonatal AlloP administration decreases anxiety and increases exploration in novel environments in adult age is opening new interesting perspectives of research into behavior. It has been described that a sensation seeking pattern of behavior, impulsivity and novelty preference can increase the vulnerability to psycho-stimulant drug abuse in humans (Kelly et al., 2006) and in animals (Molander et al., 2011). Thus, new experiments are being carried out in order to elucidate if neonatal AlloP administration increases the vulnerability to drug abuse in adolescent and adult animals.

8. Conclusions

In conclusion, the maintenance of physiological neonatal AlloP levels plays an important role for the correct maturation of the hippocampus and for related behavioral responses to environmental stress, such as emotional processing and sensorial gating. In general, an increase in neonatal physiological AlloP levels decreases anxiety and increases novelty responses in adult age, thus affecting the individual coping with environmental cues. AlloP-induced changes in anxiety-like behavior and sensory-motor gating are related to the functioning of the dorsal hippocampus occurring during neonatal period, when it is sensitive to manipulations of endogenous AlloP levels, since the behavioral effects (decrease in anxiety and locomotion or increase in prepulse inhibition) of intrahippocampal AlloP infusions in adult age are not present in those subjects where their neonatal AlloP levels were altered. Moreover, neonatal AlloP manipulations have a drastic impact on hippocampal $GABA_AR$ expression that can lead to an adult altered inhibitory system that responds differently to environmental cues. Finally, although some adolescent disturbances in emotional and novelty seeking behaviors induced by neonatal stress can be neutralized by increasing the neonatal levels of AlloP, acting as a protective mechanism against environmental stress, some other behaviors as the prepulse inhibition of the

acoustic startle response or anxiety responses in adulthood can be significantly affected by the alteration of physiological neonatal levels of AlloP.

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Annex 2

Poster presentation:
Societat Catalana de Biologia
Barcelona (2010)

Effects of neurosteroids infusion into CA1 hippocampal region on exploration, anxiety-like behaviour and aversive learning

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INTRODUCTION

The term neurosteroids (NS) firstly described by Baulieu (Baulieu et al. 1981), refers to substances derived from cholesterol that can be synthesised de novo by the nervous tissue such as pregnenolone, progesterone, allopregnenolone (Allop) and their sulphate esters (Dubrovsky 2005). NS can modulate plenty of receptors (Rupprecht and Holsboer 1999), but mainly their action focuses on GABA A and NMDA receptors. NS as Allop act as a positive allosteric modulator of GABAA receptors, increasing the opening time and frequency of the Cl- channel (Majewska et al. 1986). On the other hand NS as pregnenolone, dehydroepiandrosterone (DHEA) and their sulphate esters (PPREGS and DHEAS) have been described as GABAA negative modulators and NMDA positive modulators (Kussius et al., 2009). Given that, its role on anxiety and emotional disturbances has been suggested. In addition, NS as PREGS or DHEAS have shown promnesic profile in several learning tests (Flood et al., 1992; Mayo et al., 1993). Together with the amygdala, hippocampus is one of the most important structures responsible for the NS modulation (Weill-Engerer et al.,2002) but also as a part of the limbic system it has been widely related with the modulation of emotional behaviours, learning and memory procedures (Flood et al., 1995; Engin and Treit, 2007). The present study focused on the effect of NS infused into CA1 of the hippocampus region and its implication in learning, memory and emotional behaviour in rats.

METHODS

Stereotaxic surgery

- 37 male Wistar rats.
- Surgery at 90 days old.
- Permanent, bilateral, intrahippocampal cannula implanted.

NS administration

Animals injected bilaterally with:
- Allop (0.2µg/0.5µl).
- PREGS (5ng/0.5µl).
- Saline.

Exploration and anxiety tests (1rts week)

Boissier:
- Global and inner locomotor activity and exploration (head dipping).

EPM:
- Number of entries and time in open and closed arms.

Passive avoidance memory test (2nd week)

- 2 trials (AT and RT 24 hours later).
- NS administered post-acquisition trial.
- Differences between AT and RT latencies was analised.

Histology

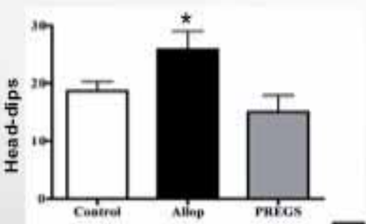
6 animals excluded:
- 1 control / 3 Allop / 2 PREGS.

Final experimental groups:
- 11 control.
- 10 Allop.
- 10 PREGS.

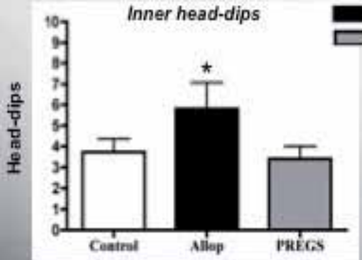
RESULTS

Boissier exploration test

Global head-dips

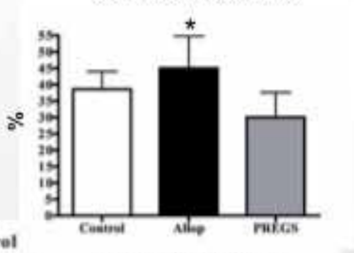


Inner head-dips

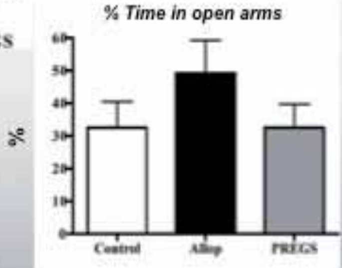


EPM

% Entries in open arms

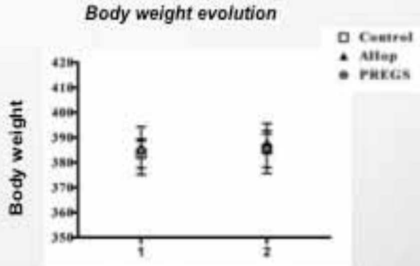


% Time in open arms

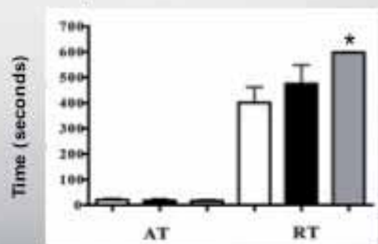


Passive avoidance

Body weight evolution



Adcquisition-retention latencies



CONCLUSIONS

1) Intrahippocampal (CA1) Allop administration enhances exploratory behaviour in the Boissier test.

2)Intrahippocampal (CA1) Allop administration induces anxiolitic-like profile (increased in % of OAE) in the EPM.

3) Intrahippocampal PREGS administration at the dose tested, has no effects neither in exploration nor in anxiety behaviour.

4) Post-trainning intrahippocampal PREGS administration enhances passive avoidance retention

5) Post-trainning intrahippocampal Allop administration, at the dose tested, had no effects on passive avoidance retention.

6) CA1 hippocampal region plays a role in the effects of NS on emotional behaviour but also on aversive learning and memory

REFERENCES

Annex 3

Poster presentation:
Behavioural Brain Research Society
Sevilla (2011)

Neonatal disturbed Allop levels affect adult performance of the passive avoidance and alter the adult CA1 hippocampal response to neurosteroids

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INTRODUCTION

Neurosteroids (NS) are steroids that can be found in the brain (since they can be synthesized periferically from gonads or *in situ* by the nervous tissue) and are well known for exerting modulatory effects through their actions on ionotropic receptors [1]. Although NS such Allop has been widely related with several psychopatologies and stress responses [2, 3, 4], recent finding indicate that NS could also act as an important factors during development [5]. In this sense, Allop altered levels have been demonstrate to alter the morphology of several structures in the brain [6], such as the hipoccampus [7, 8, 9] , to cause an alteration of the adult hippocampal response to other GABAA positive modulators such as benzodiazepines [10] and to modify the CA1 response to NS in the open field [11]. Thus, although NS role during development have not been yet elucidated, all this previous data seems to suggest that alteration of NS during critical developmental period can lead to the alteration of the morphology and function of hippocampus, which is reflected in adult behavioural disturbances. Given that, the aims of the present work are to assess the NS hippocampal levels (Allop, THDOC, epiallopregnaniolone, pregnenolone (PREG)) at PS in response to the neonatal treatment (Alllop, Finast, Vehicle and NH) and also to screen whether the alteration of developmental NS levels influences the learning responses to intrahippocampal NS administration in the adulthood.

METHODS

Neonatal administration, adult intrahippocampal administration and behavioural testing

Neonatal administration
- Allop (20 mg/kg).
- Finasteride (50 mg/kg).
- Vehicle (10% cyclodextrine).
- No handled (NH).

Diary subcutaneous injections consecutively from postnatal day 5 (PS) to postnatal day 9 (P9).

Stereotaxic surgery and NS administration

Animals injected bilaterally with:
- Allop (0.2µg/0.5µl).
- PREGS (5ng/0.5µl).
- Control (Saline).

- 160 male Wistar rats.
- Surgery at 80 days old.
- Permanent, bilateral, intrahippocampal cannula implanted.
- After 2 weeks of recovery animals were injected.

Passive avoidance test

-2 trials (AT and RT 24 hours later).
-NS administered post-acquisition trial.
-Differences between AT and RT latencies were analyzed.

Histology

Final experimental groups:
-Allop: Allop=12, PREGS=12,Control=10.
-Fin: Allop=9, PREGS=11, Control=11.
-Vehicle: Allop=9, PREGS=13, Control=12.
-NH: Allop=7, PREGS=10, Control=10.

Determination of neonatal NS levels

Mass spectrometry of NS hippocampal levels at PS
- -NH= 5 subjets
- -Vehicle= 5 subjects
- -Allop= 5 subjects
- - Finasteride= 5 subjects

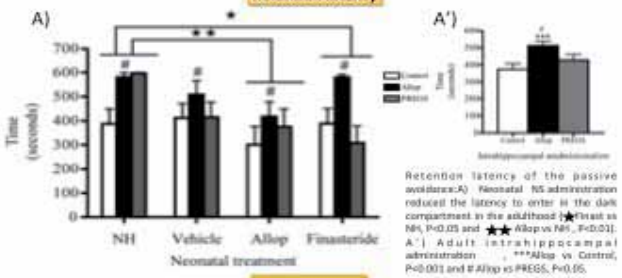
Pups were sacrified and hippocampus was removed at postnatal day 5 (PS). Brains were kept stored at -80 °C until they were used for the steroid quantification by means of mass spectrometry.

CONCLUSIONS

1-. Alteration of physiological Allop levels during the first postnatal week deteriorates the passive avoidance learning performance in the adulthood.
2-. Alteration of endogenous Allop levels can modify the adult hippocampal response to Allop or PREGS in avoidance learning tasks.
3-. Neonatal Finasteride induced detrimental effect in passive avoidance retention can be reversed by the hippocampal Allop administration or exacerbated through the hippocampal PREGS administration in the adulthood .
4-. The detrimental learning effect induced by the neonatal administration of Allop cannot be reversed through any of the NS intrahippocampally administered in the adulthood at the doses tested.
5-. Neonatal Allop administration increased the hippocamcampal Allop levels

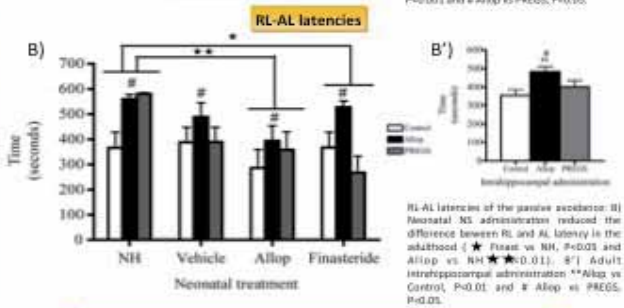
RESULTS

Retention latency



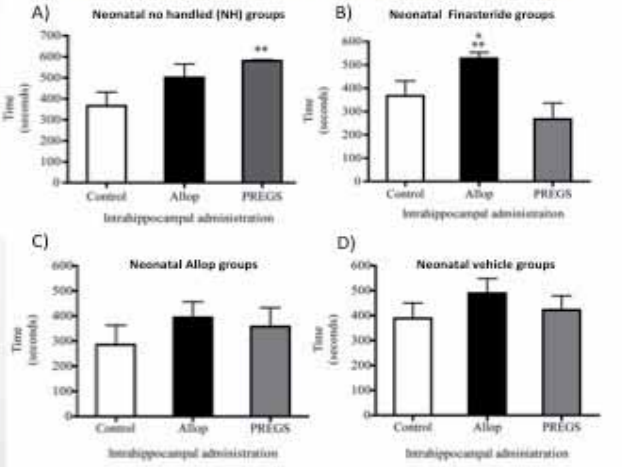
Retention latency of the passive avoidance:A) Neonatal NS administration reduced the latency to enter in the dark compartment in the adulthood (★Finast vs NH, P<0.05 and ★★ Allop vs NH , P<0.01). A') Adult intrahippocampal administration , ***Allop vs Control, P<0.001 and # Allop vs PREGS, P<0.05.

RL-AL latencies



RL-AL latencies of the passive avoidance: B) Neonatal NS administration reduced the difference between RL and AL latency in the adulthood (★ Finast vs NH, P<0.05 and Allop vs NH ★★P<0.01). B') Adult intrahippocampal administration **Allop vs Control, P<0.01 and # Allop vs PREGS, P<0.05.

SEPARATED ANALYSIS OF NEONATAL INJECTED GROUPS: RL –AL latencies



Separated analisys of neonatal treatment : RL-AL latencies for each group. A) NH groups: PREGS vs Control (P<0.01). B) Finasteride groups: Allop vs Control P<0.05 and Allop vs PREGS P<0.01. No differences were observed between groups on the intrahippocampal administration of neonatal Allop and Vehicle groups (Figures C and D respectively). Similar results were observed on the analisys of the retention latency (RL).

HIPPOCAMPAL NS LEVELS AT POSTNATAL DAY 5

	Neonatal treatment			
	NH	Vehicle	Allop	Finasteride
Allop	0,41±0,17	4,35±2,47	**322,45±76,43*** **	40,00±12,10
THDOC	17,13±6,37	12,67±5,87	24,66±3,63	18,98±3,33
PREG	3,52±0,95	6,59±1,76	3,19±0,60	**34,88±5,89*** **
EpiAllop	0,02±0,01	0,30±0,26	**11,11±3,20 #**	0,05±0,04

NS levels expressed in ng/g tissue (mean ± sem). ***P<0,0001; # P<0,01.

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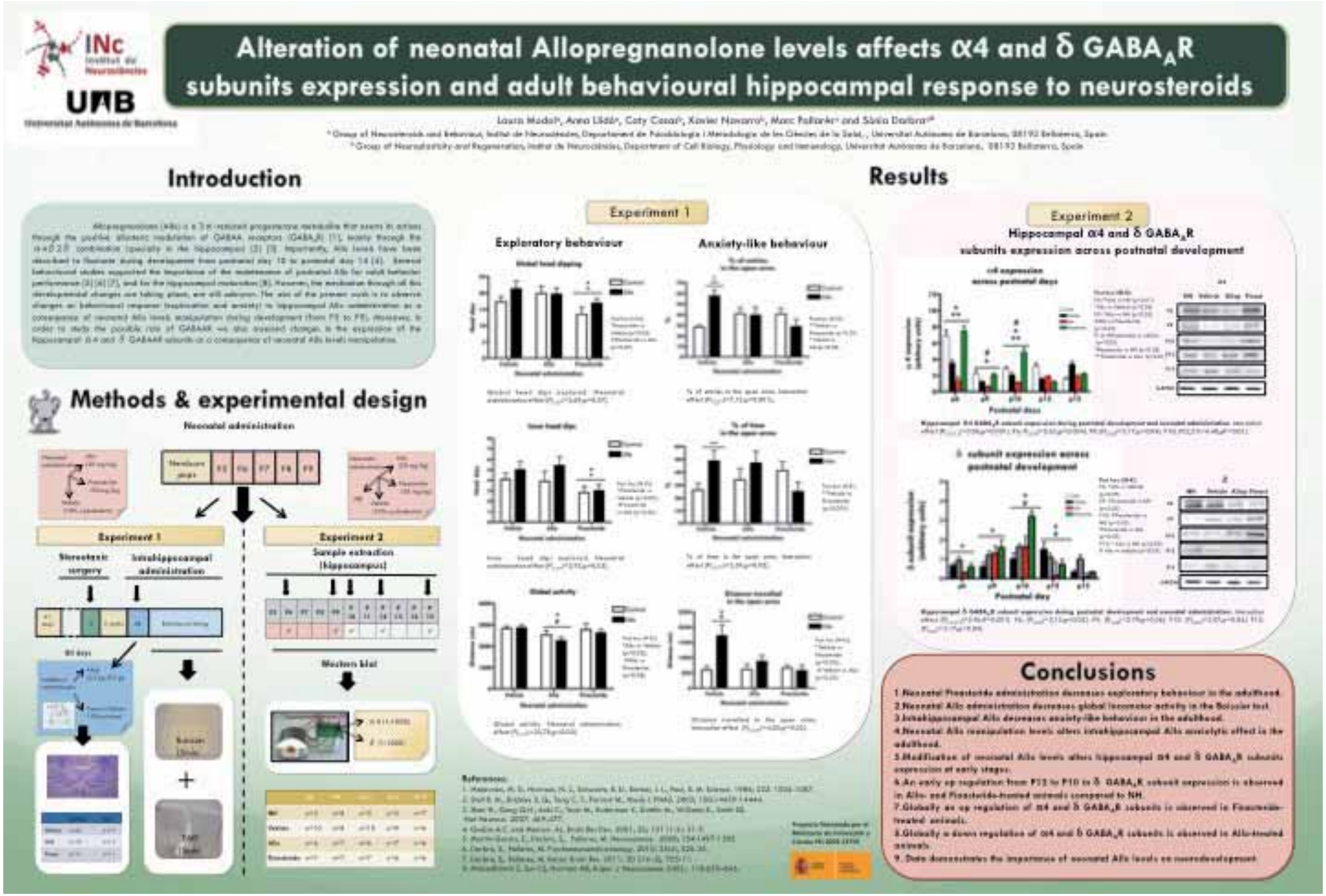
Annex 4

Poster presentation:
Federation of European Neuroscience Society
Barcelona (2012)


Alteration of neonatal Allopregnanolone levels affects α4 and δ GABAAR subunits expression and adult behavioural hippocampal response to neurosteroids
Introduction
Methods & experimental design
Experiment 1
Experiment 2
Western blot
Results
Experiment 1
Exploratory behaviour
Anxiety-like behaviour
Experiment 2
Hippocampal α4 and δ GABAAR subunits expression across postnatal development
Conclusions
References

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