Neonatal finasteride administration alters hippocampal $\alpha 4$ and $\delta$ GABA$_{\text{A}}$R 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 $\alpha 4$ and $\delta$ GABA$_{\text{A}}$ receptor (GABA$_{\text{A}}$R) subunits as a consequence of neonatal finasteride (a 5-$\alpha$ 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 these alterations.

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Key words: Finasteride, GABA$_{\text{A}}$R $\delta$ subunit, GABA$_{\text{A}}$R $\alpha 4$ 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 (3$\alpha$-hydroxy-5$\alpha$-pregnaone-20-one), is a 3$\alpha$-reduced progesterone metabolite (Rupprecht, 2003). Progesterone is readily metabolized in the brain to 5$\alpha$-dihydroprogesterone by 5$\alpha$-reductase enzymes; 5$\alpha$-dihydroprogesterone is...
then further reduced to allopregnanolone that mainly exerts its actions through the positive allosteric modulation of GABAA receptors (GABA\textsubscript{A}R) (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\textsubscript{A}R. GABA\textsubscript{A}R is a pentameric structure that mediates inhibition in the mature brain, and is typically composed of 2\textalpha, 2\beta and 1\gamma subunits. There are 19 different subtypes of GABA\textsubscript{A}R combinations indicating a high level of structural heterogeneity and function (Olsen and Sieghart, 2009). The combination of 2\alpha\beta\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). 2\alpha and 2\delta GABA\textsubscript{A}R 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 2\alpha2\beta2\delta GABA\textsubscript{A}R 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\textsubscript{A}R 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 5\alpha-reductase 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 2\alpha and 2\delta GABA\textsubscript{A}R 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 2\alpha and 2\delta subunits to elevation of allopregnanolone levels by progesterone administration in adulthood.

**Methods**

**Hippocampal GABA\textsubscript{A}R 2\alpha and 2\delta 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).
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 \(-80^\circ \)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 \( (10 \mu l/ml, \Sigma) \) and sodium orthovanadate \( (1 mM, \text{Roche, Switzerland}) \) 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 \( (\text{Pierce, USA}) \). Equal amounts of protein \( (30 \text{ or } 50 \mu g) \) were deposited by electrophoresis onto 10% SDS-polyacrylamide gels and transferred to a polyvinyl difluoride membrane \( (\text{Bio-Rad}) \).

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 \( \alpha_4 \), \( \delta \) GABA\(_A\)R subunits (rabbit anti-\( \alpha_4 \)GABAR: 1/1000 (Phosposolutions, Lucerne, Switzerland); rabbit anti-\( \delta \)GABAR: 1/800 (Phosposolutions, Switzerland)) or glyceraldehyde-3-phosphate dehydrogenase \( (\text{GAPDH, Sigma, 1/5000}) \) 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 \( (\text{Millipore, USA}) \) and the images were analysed by band densitometry with the Gene Snap and Gene Tools software in a Gene

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

<table>
<thead>
<tr>
<th>Neonatal treatment assignment and composition of groups in experiment 1</th>
<th>Postnatal day</th>
<th>Total</th>
</tr>
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<tr>
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<td>2</td>
</tr>
<tr>
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<td>6</td>
</tr>
<tr>
<td>Saline</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Vehicle</td>
<td>7</td>
<td>6</td>
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**Neonatal treatment assignment and composition of groups in experiment 3**

<table>
<thead>
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<th>Vehicle behaviour/ (WB)</th>
<th>Progesterone behaviour/ (WB)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pairs</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>NH</td>
<td>3</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Vehicle</td>
<td>7</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Finasteride</td>
<td>6</td>
<td>7</td>
<td>3</td>
</tr>
</tbody>
</table>

Animals used for Western blot (WB) analysis after behavioural evaluation are represented in parentheses.
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 μmol oligo(dT) and 0.5 μmol 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: Gabrd4 (F, 5′-AATGTGTCACCCACA-CCTCC-3′; R, 5′-TGCCCCAAATGTGACTGGAA-3′), Gabrd (F, 5′-AGGAACCGGGGTGTCAT-3′; R, 5′-CAGCACAGTGGATGCTA-3′), and Gapdh (F, 5′-AGGACCCGCAACTGGAA-3′; R, 5′-TACTCAGGCCAGACAT-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−ΔΔCT) 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 \(\beta\)-cyclodextrin administration effect on \(\alpha4\) and \(\delta\) GABA\(_{\alpha}R\) subunits expression, a preliminary two way ANOVA was performed with neonatal treatment (two levels: saline/\(\beta\)-cyclodextrin) 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/saline/\(\beta\)-cyclodextrin/saline/\(\alpha4\) nasteride and \(\beta\)-cyclodextrin/saline/\(\alpha4\) nasteride) 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/\(\alpha4\) nasteride) and adult administration (two levels: vehicle/progesterone). Data from hippocampal allopregnanolone levels were analysed using one way ANOVA for neonatal administration (three levels: NH/saline/\(\beta\)-cyclodextrin/\(\alpha4\) nasteride). Post-hoc Neuman–Keuls tests were used when necessary. Data are shown as mean \(\pm\) S.E.M.

Results

Hipocampal GABA\(_{\alpha}R\) \(\alpha4\) and \(\delta\) subunits expression during early development and \(\alpha4\) nasteride administration

The preliminary analysis performed in order to control the \(\beta\)-cyclodextrin administration, showed no differences between the neonatal vehicle (\(\beta\)-cyclodextrin and saline) administered groups in the analysis of \(\alpha4\) and \(\delta\) GABA\(_{\alpha}R\) subunits expression \((F_{4,33}=0.72;\ \text{N.S.; and } F_{4,33}=0.64;\ \text{N.S.},\ \text{respectively})\). 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 1a and 2a.

The analysis of the \(\alpha4\) subunit expression showed a significant interaction between neonatal treatment and postnatal day tested \((F_{6,86}=2.43;\ p<0.01)\) indicating that expression of the \(\alpha4\) subunit is different across postnatal days depending on the neonatal administration (Fig. 1). Separate analysis by neonatal treatment was then performed. The \(\alpha4\) GABA\(_{\alpha}R\) subunit showed an evolution pattern in both NH and Control groups during postnatal development \((F_{4,25}=4.99;\ p<0.01\ \text{and } F_{4,38}=3.78;\ p<0.01,\ \text{respectively})\). We observed that \(\alpha4\) 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 \((PD9/PD10/PD12/PD15;\ p<0.01\ \text{in NH group and } p<0.05\ \text{in Control group, for all comparisons; Fig. 1})\). In the neonatal finasteride administered groups, however, a different pattern of \(\alpha4\) GABA\(_{\alpha}R\) subunit expression was observed \((F_{4,24}=4.82;\ p<0.01)\). Although the \(\alpha4\) expression at PD6 was higher than at PD9 \((p<0.05)\) and PD10 \((p<0.01)\) and PD15 \((p<0.01)\), no differences were observed between PD6 and PD10 \((p>0.05)\), indicating that a second increase in \(\alpha4\) 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 \((F_{2,18}=4.38;\ p=0.02)\): \(\alpha4\) subunit expression was higher in animals treated with finasteride than in control animals \((p<0.05)\). Although an increase in \(\alpha4\) GABA\(_{\alpha}R\) 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 \((F_{2,45}=5.11;\ p=0.01\ \text{and } F_{4,45}=6.24;\ p<0.01,\ \text{respectively})\), 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. 3c).

Results of the \(\delta\) 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 \(\delta\) GABA\(_{\alpha}R\) subunit at PD12 decreasing at PD15 (PD12 vs. PD15: \(p<0.05)\). However, no differences were observed in control group in \(\delta\) expression \((F_{4,36}=1.88;\ \text{N.S.})\), indicating that \(\delta\) GABA\(_{\alpha}R\) remained stable along the
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$_A$R subunit levels at each time-point were

Fig. 1. Hippocampal α4 GABA$_A$R 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$_A$R subunit detected from hippocampus for each postnatal day. (b) Levels of α4 GABA$_A$R 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/PD10/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$_A$R subunit in controls groups: vehicle and saline ($F_{4,33}=0.72; N.S$). 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. 3b 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 GABAAR 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 GABAAR mRNA expression increases during early neonatal period (main effect \( F_{2,45}=5.10; \ p<0.01 \)). The lowest expression of α4 GABAAR mRNA is observed at PD6 (PD6 vs. PD9/PD12, § \ p<0.05 and PD6 vs. PD10/PD15; §§ \ p<0.01); finasteride administration globally increases α4 GABAAR 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 \( F_{2,45}=9.83; \ p<0.001 \). Partition analysis; neonatal administration: NH-PD6 vs. NH-PD10/PD15 and NH-PD9 vs. NH-PD10, # \ p<0.01; finasteride-PD10 vs. finasteride-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, Y \ \ Y \ \ 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, Y \ \ p<0.05. Data are shown as mean±S.E.M.

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

<table>
<thead>
<tr>
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<th>Neonatal neurosteroid treatment</th>
<th>NH</th>
<th>VEH</th>
<th>Finasteride</th>
</tr>
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<tr>
<td>TESTO</td>
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<td>0.29±0.13</td>
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<tr>
<td>ALLO</td>
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<td>THDOC</td>
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<tr>
<td>PREG</td>
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<td>1.98±0.73</td>
<td>1.45±0.39</td>
<td>1.21±0.11</td>
</tr>
<tr>
<td>EPALLO</td>
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<td>0.14±0.14</td>
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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. 3b). 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. 3b). 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. 3b).

**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 allopregnanolone 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...
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 \( (F_{2,21}=1.11; \text{N.S}) \) (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 \( (F_{2,42}=0.55; \text{N.S} \) and \( F_{1,42}=0.02; \text{N.S}, \) respectively) nor an interaction between neonatal and adult administration \( (F_{2,42}=0.75; \text{N.S}) \) 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 \( (F_{1,13}=3.11; \text{N.S} \) and \( F_{1,13}=2.34; \text{N.S}, \) respectively for vehicle group; and \( F_{1,15}=0.59; \text{N.S} \) and \( F_{1,15}=1.52; \text{N.S}, \) respectively, for NH group).

Hippocampal $\alpha_4$ and $\delta$ subunits expression in adulthood

When $\alpha_4$ 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 $\alpha_4$ expression in the hippocampus than the rest of the animals (see Fig. 5).

Regarding $\delta$ 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 $\delta$ 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 $\delta$ GABA$_A$R subunit expression were observed when the analysis was performed in animals administered with progesterone in adulthood \( (F_{2,17}=1.35; \text{N.S}) \). These results indicate a down regulation of $\delta$ expression as a consequence of adult progesterone administration only in animals that received finasteride (Fig. 5). Taken together, the hippocampal $\alpha_4$ and $\delta$ 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 $\alpha_4$ GABA$_A$R subunit expression at P6 that progressively decreased during early postnatal development in NH and control groups. The increase of $\alpha_4$ GABA$_A$R subunits could be related to a maternal
The disparity between α4 and δ GABA_A 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 α4 and γ2 subunit transcripts than of δ GABA\textsubscript{A}R subunit transcripts in the hippocampus by PD2 (Laurie et al., 1992; Didelon et al., 2000). Thus, the discrepancy between GABA\textsubscript{A}R α4 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\textsubscript{A}R subunit protein at PD12 which can be related to the increment of its transcript observed from PD10 to PD15, although no statistically significant at PD12, probably due to insufficient sensitivity in the technique. Gabra4 mRNA also increased during the second postnatal week, however, the α4 GABA\textsubscript{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 α4 and δ GABA\textsubscript{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 α4β2δ GABA\textsubscript{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\textsubscript{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\textsubscript{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 α4 GABA\textsubscript{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 α4 and δ (transcript and protein) GABA\textsubscript{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 α4 and δ GABA\textsubscript{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\textsubscript{A}R.
subunits in the hippocampus. Importantly, our results showed that the increase in α4 and δ GABAAR 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 α4βδ 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 GABAAR-gated current (hyperpolarizing response) only in those GABAAR expressing α4 and δ subunits, but not any other GABAAR. Interestingly, present results also show an increased expression of hippocampal α4 and δ GABAAR subunits as a consequence of acute allopregnanolone 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 α4 GABAAR subunit (Gulinello et al., 2001). Thus, changes in GABAAR 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 α4 and δ GABAAR 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 α4 and δ subunits GABAAR. These observations are consistent with the notion that changes in GABAAR 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 GABAAR 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 GABAAR 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 α4 and δ GABAAR 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 GABAAR subunit
is capable of rapid plastic changes, decreasing after short-term treatment with neurosteroids under conditions of altered maturation of GABAAR 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 GABAAR 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 GABAAR 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 GABAAR subunits, which is accompanied by an altered behavioural response to progesterone administration, even in adulthood.

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Statement of Interest

None.

References


Darbra S, Modol L, Vallee M, Pallarès M (2013) Neonatal neurosteroids levels are determinant in shaping adult


Follesa P, Mancuso L, Biggio G, Cagetti E, Franco M, Trapani F, Mancuso L, Biggio F, Cagetti E, Franco M, Trapani...


