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Role of brain neuroactive steroids in the functional interplay between the $GABA_A$ and the NPY-Y_1 receptor mediated signals in the amygdala

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Abstract

Various lines of evidence suggest a functional interaction between $GABA_A$ and Neuropeptide Y (NPY)-Y_1 receptor (Y_1R) mediated transmissions in various brain regions, which can be important in the regulation of sedation, feeding, anxious behaviour and neuronal excitability. By using a transgenic mouse model carrying the murine Y_1R gene promoter fused to the *lacZ* reporter gene (*Y_1R/LacZ* mice), we showed that prolonged pharmacologically or physiologically induced changes in the cerebrocortical concentrations of the neuroactive steroids 3alpha-hydroxy-5alpha-pregnan- 20-one (3alpha,5alpha TH PROG) and tetrahydrodeoxycorticosterone (3alpha,5alpha TH DOC) increases *Y_1R/LacZ* transgene expression in the central and medial amygdala, an effect similar to that induced by long-term treatment with positive modulators of the $GABA_A$ receptor complex (diazepam or abecarnil). We also demonstrated that fluctuations in the cerebrocortical concentrations of 3alpha,5alpha-TH PROG and 3alpha,5alpha TH DOC during voluntary ethanol consumption and ethanol withdrawal induces a marked increase in Y_1R gene expression that becomes apparent 48 h after withdrawal.

These data provide evidence that neuroactive steroids may play an important role in the functional interaction between the $GABA_A$ receptor and NPY-Y_1R mediated pathways in the amygdala, which might represent an important regulatory mechanism for modulation of several functions, including ethanol withdrawal.

Keywords: GABA; Neuroactive steroids; Neuropeptide Y; Y_1 receptors; Pregnancy; Ethanol; Stress; Amygdala; Transgenic mice

1. Introduction

Neuropeptide Y (NPY)[1] is one of the most abundant and widely distributed peptides in the central nervous system that belongs, together with the peptide YY (PYY) and the pancreatic polypeptide (PP), to the family of pancreatic peptides (Tatemoto et al., 1982). Immunohistochemical and *in situ* hybridization studies have shown that NPY is abundantly expressed in various brain regions of rodents and humans, including the cerebral cortex, amygdala, hypothalamus, hippocampus, locus coeruleus and nucleus accumbens (Allen et al., 1983; Chronwall et al., 1985; de Quidt and Emson, 1986; Dumont et al., 1992; Chronwall and Zukowska, 2004). In the brain, NPY participates in the regulation of several physiological functions that include emotional behaviour and stress response, neuronal excitability, feeding behaviour and energy homeostasis, ethanol consumption, learning and memory, GnRH secretion, sexual behaviour and circadian rhythms (Kalra and Crowley, 1992; Wahlestedt and Reis, 1993; Colmers and Bleakman, 1994; Baraban et al., 1997; Hokfelt et al., 1998; Vezzani et al., 1999).

NPY interacts with at least five different G-protein coupled receptors named Y_1, Y_2, Y_4, Y_5, and y6 subtypes (Blomqvist and Herzog, 1997; Michel et al., 1998). The Y_1 receptor (Y_1R), Y_2 receptor and Y_5 receptor bind preferentially to NPY and PYY whereas PP is the preferring ligand for the Y4 receptor. (Lundell et al., 1995; Rose et al., 1995; Gerald et al., 1996; Weinberg et al., 1996). Since the y6 receptor protein is truncated in most mammals and it is functional only in mouse and rabbit, its biological relevance remains to be clarified (Starback et al., 2000).

Abbreviations: NPY, neuropeptide Y; Y_1R, Y_1 receptor; GABA, γ-aminobutyric acid; $GABA_A$, GABA type A receptor; 3alpha,5alpha TH PROG, 3alpha-hydroxy-5alpha-pregnan-20-one; 3alpha,5alpha TH DOC, tetrahydrodeoxycorticosterone; MeA, medial amygdala; CeA, central amygdala; ARC, arcuate nucleus; PVN, paraventricular nucleus; IR, immunoreactive.

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The Y_1R subtype for NPY was the first NPY receptor to be cloned in the rat by us (Eva et al., 1990; Krause et al., 1992) and subsequently in human (Herzog et al., 1992) and mouse (Eva et al., 1992). Sequence analysis of mammalian and non-mammalian Y_1R demonstrated that it displays a high sequence homology in the transmembrane regions and it is highly conserved throughout evolution (Larhammar and Salaneck, 2004). Y_1R is coupled to the inhibition of adenylate cyclase, by the activation of a pertussis toxin sensitive GTP binding protein Gi/Go, and to the mobilization of Ca^{+2} from intracellular stores (Herzog et al., 1992). Moreover, Y_1R stimulates the mitogen activated protein kinase (MAPK) pathways, via extracellularly regulated kinase (ERK) phosphorylation, an effect that has been shown to be dependent on PI-3-kinase (Nie and Selbie, 1998; Mannon and Mele, 2000).

The distribution of Y_1R within the mammalian CNS was extensively analyzed using receptor autoradiography, immunohistochemistry and *in situ* hybridization. By using receptor autoradiography Y_1R-binding sites were found in the cerebral cortex, olfactory nuclei, dentate gyrus, thalamus, septum, cerebellum and in the paraventricular (PVN) and dorsomedial hypothalamic nuclei (Dumont et al., 1998). More recent studies in rat and mouse, using specific cRNA probes and *in situ* hybridization techniques, detected a larger amount of Y_1R mRNA positive neurons, in particular in the thalamus, in the limbic system (hippocampus, amygdala and bed nucleus of the stria terminalis) and in the hypothalamus (medial preoptic area, PVN, dorsomedial, ventromedial and arcuate (ARC) hypothalamic nuclei) (Kishi et al., 2005). The lack of localization of Y_1R mRNA with NPY mRNA observed in the human brain suggests that the Y_1R is mainly located postsynaptically (Caberlotto et al., 2000). However the presence of Y_1R has been identified in NPY-IR neurons in the nucleus accumbens, medial amygdala (MeA) and rat hippocampus suggesting that the Y_1R may also act as a presynaptic receptor (St-Pierre et al., 1998, 2000; Pickel et al., 1998; Oberto et al., 2001).

The physiological importance of the Y_1R was first discovered by pharmacological studies using selective agonists and antagonists showing high binding affinities for this receptor subtype. The functional contributions of Y_1R to a particular physiological process have also been provided by studies on genetically modified mice lacking the Y_1R subtype (Pedrazzini et al., 1998; Kushi et al., 1998; Kanatani et al., 2000; Naveilhan et al., 2001b; Howell et al., 2003) as well as by studies showing dynamic changes of this receptor subtype in response to different conditions, for instance stress and anxiety, convulsions or changes in feeding behaviour (Eva et al., submitted for publication).

In the brain, the Y_1R subtype has been involved in several NPY-induced responses, such as the anxiolytic effects and stress response (Broqua et al., 1995; Heilig, 1995; Sajdyk et al., 2004), ethanol drinking behaviour (Thiele et al., 2002; Schroeder et al., 2003; Thiele and Badia-Elder, 2003; Sparta et al., 2004), stimulation of food intake (Kask et al., 1998; Kanatani et al., 2000; Wisialowski et al., 2000; Mullins et al., 2001) and activation of neuroendocrine axis (Kalra et al., 1992; Besecke et al., 1994). Moreover, different lines of evidence have shown that Y_1R plays a permissive role in seizures (Gariboldi et al., 1998; Vezzani et al., 2000, 2002; Benmaamar et al., 2003).

2. NPY and γ-aminobutyric acid (GABA)

NPY has similar properties to those observed with positive modulators of the GABA type A ($GABA_A$) receptor complex, like benzodiazepines and neuroactive steroids, on sleep (Heilig and Murison, 1987; Yamada et al., 1996; Ehlers et al., 1997), anxiety, stress (Heilig et al., 1989, 1992; Broqua et al., 1995; Sajdyk et al., 1999; Bannon et al., 2000; Thorsell et al., 2000; Kask et al., 2001), convulsions (Mikkelsen et al., 1994; Erickson et al., 1996; Baraban et al., 1997; Woldbye et al., 1997; Bolwig et al., 1999), as well as feeding behaviour (Cowley et al., 2001; Pronchuk et al., 2002; cuna-Goycolea et al., 2005; Sato et al., 2005). Various lines of evidence suggest a functional interaction between GABA and NPY that can be important in the regulation of sedation, feeding, anxious behaviour and neuronal excitability (Kask et al., 1996; Oberto et al., 2000, 2002; Ferrara et al., 2001; Mikkelsen et al., 2001; Ovesjo et al., 2001; Zhang and Wang, 2005). For instance, GABA is coexpressed with NPY/Agouti-Related Protein in ARC neurons (Horvath et al., 1997; Pu et al., 1999) projecting into the PVN where they act synergistically to inhibit Corticotropin Releasing Factor neurons and to enhance feeding (Hahn et al., 1998; Broberger et al., 1998a,b; Pu et al., 1999). Moreover GABA is co-released from NPY/Agouti-Related Protein neurons in the ARC were it was shown to inhibit Proopiomelanocortin neurons by acting through different receptors (Horvath et al., 1997; Pu et al., 1999; Cowley et al., 2001).

Anatomical and functional evidence also suggests an interplay between hypothalamic GABAergic neurons and NPY (Pu et al., 1999; Cowley et al., 2001; Horvath et al., 2001; Pronchuk et al., 2002) in the regulation of both fertility and food intake (Kalra et al., 1991; Catzeflis et al., 1993; Glass et al., 1999; Raposinho et al., 2001). In particular, NPY has been suggested to inhibit fertility by decreasing the activity of GABAergic neurons afferent to Gonadotropin Releasing Hormone neurons, an effect that appears to be mediated via the NPY-Y_1R subtype (Sullivan and Moenter, 2004).

In the hippocampus and neocortex, NPY is made by neurons that almost all express GABA (Pu et al., 1999; Jinno and Kosaka, 2003). Results from several studies have indicated that neocortical and hippocampal neurons coexpressing NPY and GABA might be involved in the response to epileptogenesis. In the neocortex, NPY was found to increase GABAergic neurotransmission onto pyramidal neurons and to decrease inhibition on GABAergic interneurons (Bacci et al., 2002). Each of these NPY actions decreases excitability in cortical circuits, resulting in the potentiation of NPY and GABA inhibitory responses. NPY/GABA interneurons in the hippocampus are selectively ablated in rat epilepsy models *in vivo* (Sloviter, 1989, 1991) and in epileptic patients (de Lanerolle et al., 1989).

A functional interaction between the GABAergic and NPY-Y_1R mediated transmissions in the regulation of emotional behaviour was first demonstrated by Kask et al. (1996) that showed that the anxiolytic benzodiazepine diazepam blocks the anxiogenic effect of Y_1R antagonists. NPY was also reported to induce sedative effects through the GABAergic system by interacting with the Y_1R in the posterior hypothalamus (Naveilhan et al., 2001a).

On the other hand, modulation of $GABA_A$ receptor complex induces changes in the expression of NPY and NPY mRNA in various brain region. Acute, sub-chronic and chronic treatments with benzodiazepines affects the NPY immunoreactivity in the amygdala, cerebral cortex and locus coeruleus and hypothalamus in naive rats (Krysiak et al., 1999) and in rats with conditioned fear produced in the passive avoidance test (Krysiak et al., 2000), although these changes were dependent on the type of treatment and on the different brain nuclei. In addition, long-term treatment with flurazepam induces a decrease of NPY immunoreactivity in the hippocampus of tolerant and dependent rats that is associated with the decrease of Y_1R mRNA expression in tolerant rats but not during withdrawal (Zhang and Wang, 2005).

Moreover, intrahippocampal administration of antisense oligonucleotides against the $GABA_A$ receptor γ2 subunit, that induces spontaneous electrographic hippocampal seizures and elevates seizure threshold induced by electrical stimulation, increases NPY mRNA and receptor protein in the hippocampal region. (Mikkelsen et al., 2001). This induction of NPY has been suggested to be a part of compensatory mechanism acting in response to a reduction in functional hippocampal postsynaptic $GABA_A$ receptors.

By using a *Y_1R/LacZ* transgenic mouse model, we extensively investigated the functional interaction between GABAergic and NPY-Y_1R mediated transmission in the amygdala. We here summarize the results of several studies from our laboratory suggesting that changes in $GABA_A$ receptor function, induced by various pharmacological and physiological conditions, may affect NPY-Y_1R signal transmission by modulating the gene expression of the Y_1R subtype in the MeA and in the central amygdala (CeA).

3. The *Y_1R/LacZ* transgenic mouse model

We generated a transgenic mouse line (*Y_1R/LacZ* mice) bearing the 1.3 Kb 5′ flanking region of the murineY_1R gene promoter linked to the coding region of the *Escherichia coli LacZ* gene (Oberto et al., 1998). Analysis of *Y_1R/LacZ* transgene activity by histochemical staining of β-galactosidase with X-gal demonstrated that this construct contains sufficient information to replicate the expression pattern of the endogenous Y_1R gene in a CNS-restricted and developmental stage-specific manner. Four transgenic lines showed characteristic patterns of β-galactosidase activity in specific brain regions; moreover, the ontogenetic analysis indicated that the transgenic construct appears to be activated with the same timing of the endogenous Y_1R during the development of the embryonic nervous system.

The advantages of this transgenic model are that β-galactosidase staining with chromogenic substrate X-gal is more sensitive and more readily quantifiable than mRNA *in situ hybridization* and it can be usefully employed to quantitatively evaluate *in vivo* changes in transgene expression.

To study *in vivo* the regulation of Y_1R gene expression at the transcriptional level we employed computer-assisted morphometrical techniques to quantitatively evaluate changes in *Y_1R/LacZ* transgene expression by automatically quantifying the number of dye precipitates within a selected region, (Russ, 1995). These calculations allow a numerical measurement which is proportional to the histochemical activity of the expressed transgene and are extremely useful for statistical comparison of different treatments.

By using this approach, we have shown that chronic modulation of $GABA_A$ receptor function regulates Y_1R gene expression in the amygdala of *Y_1R/LacZ* transgenic mice. Furthermore we demonstrated that *Y_1R/LacZ* transgene expression in the CeA and MeA can be also modulated in response to a long-lasting increase in the cerebrocortical concentrations of neuroactive steroids induced by various pharmacological and physiological conditions.

4. Morphological evidence of a functional interplay between the GABAergic and NPY-Y_1R systems in the MeA

Using immunolabelling for NPY and GABA and histochemical staining of *Y_1R/LacZ* transgene expression we provided morphological evidence for a functional interaction between GABA and NPY-Y_1R mediated transmission in the MeA (Fig. 1) (Oberto et al., 2001). The confocal analysis of GABA and NPY-immunoreactive (IR) neurons in brain coronal sections from *Y_1R/LacZ* transgenic mice has shown that in the MeA the majority of NPY-IR cell bodies were positive for GABA and that, in some cases, GABA and NPY coexist within the same nerve fibers. Moreover, the simultaneous detection of β-galactosidase histochemical staining and of GABA and NPY immunostaining revealed a high degree of colocalization for GABA or NPY and the *Y_1R/LacZ* transgene. GABA-IR neurons coexpressing β-galactosidase histochemical staining were usually in close contact with NPY-IR fibers that were surrounding or covering the cell bodies, suggesting that NPY neurons innervate GABAergic neurons that also express the Y_1R gene and that NPY might inhibit GABA release by the activation of this receptor subtype. Few NPY-IR cell bodies also showed the β-galactosidase histochemical staining suggesting that the Y_1R may also act as an autoreceptor in this brain area. On the other hand, several GABA-IR elements, that do not display β-galactosidase positive staining, are surrounded by NPY positive fibers, indicating that not all GABAergic neurons are regulated by Y_1R. These findings could account for a complex response to NPY in the MeA and suggest that the interconnectivity between the NPY-Y_1R-mediated transmission and the GABAergic system may be an important regulatory mechanism within the MeA and may contribute to the effects of NPY on anxious behaviour.

5. Chronic modulation of $GABA_A$ receptor function regulates Y_1R gene expression in the amygdala

To provide functional evidence for interplay between GABA and NPY systems within the amygdala we first analyzed the effect of modulation of $GABA_A$ receptor complex on Y_1R gene expression in the MeA of *Y_1R/LacZ* transgenic mice.

Positive allosteric modulators of GABA receptor complex, such benzodiazepines, enhance $GABA_A$ receptor function and produce sedative, anxiolytic and anticonvulsant effects on behaviour (Costa et al., 1975; Haefely, 1986; Costa and Guidotti,

1996). Conversely negative allosteric modulators of the $GABA_A$ receptor complex, such as β-carbolines FG 7142, inhibit GABA-gated Cl-channels and elicit anxiogenic and proconvulsant activity (Dorow et al., 1983). Chronic administration of diazepam and other agonists of the benzodiazepine binding site decreases $GABA_A$ receptor function whereas repeated administration of β-carbolines increases sensitivity to its proconvulsant activity (Corda et al., 1985; Woods et al., 1992). These behavioural and physiological effects are associated with changes in the expression of $GABA_A$ receptor subunits mRNA (Chen et al., 1999).

We demonstrated that the prolonged treatment with positive (diazepam and abecarnil) or negative modulators (FG7142) of $GABA_A$ receptor function induces, respectively, a significant increase, or decrease, of the Y_1R gene expression in the MeA. These findings are consistent with the hypothesis that, in the amygdala, the NPY-Y_1 receptor-mediated transmission and

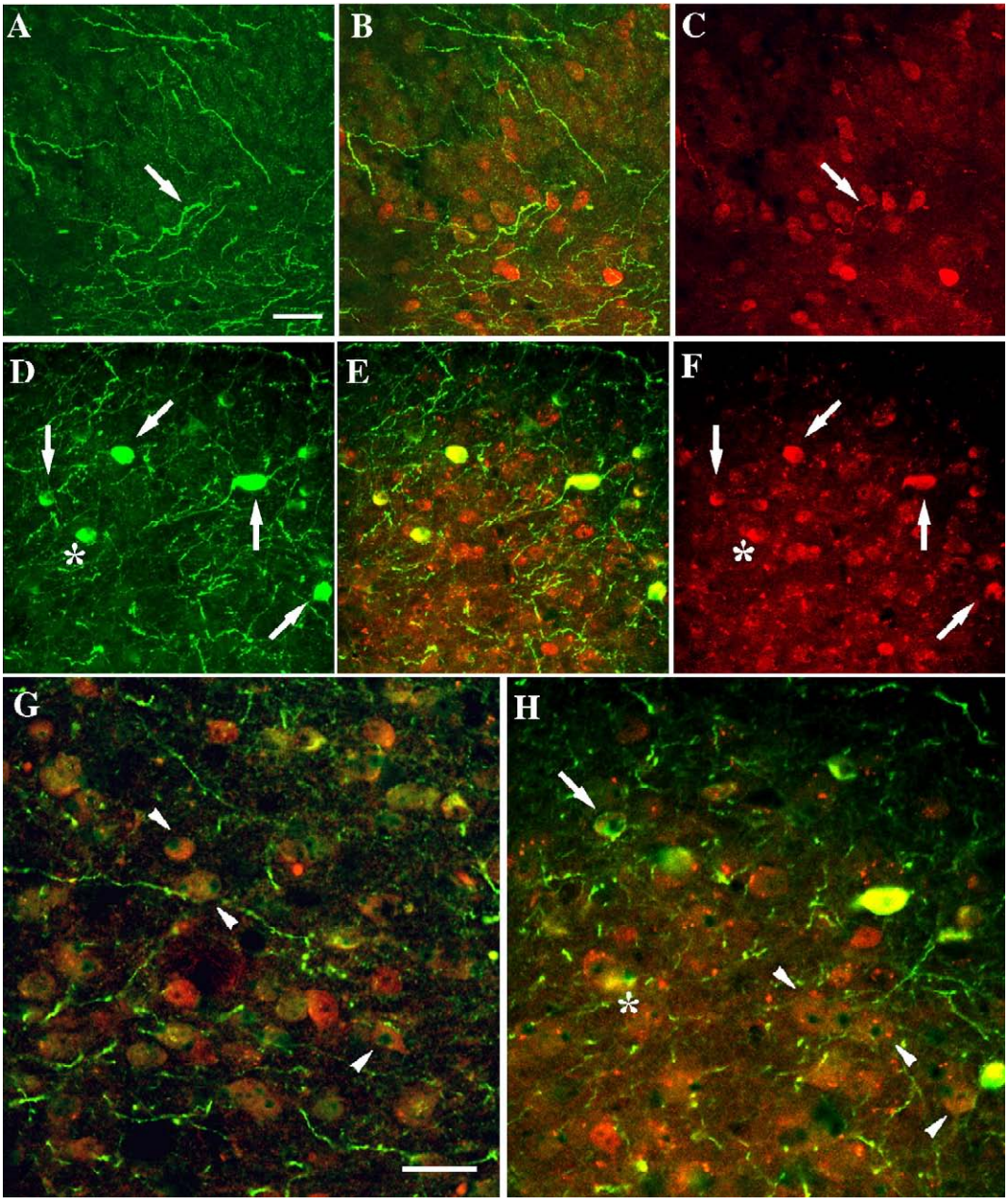

Fig. 1. Confocal images illustrating the coexistence and close relationships among NPY, GABA, and *Y_1R/LacZ* expressing elements. In green the immunolabeling for NPY, in red the immunolabelling for GABA, in dark blue the histochemical staining for β-galactosidase. Yellow cells or fibers are elements where NPY (green) and GABA (red) coexist. A–F: confocal images obtained merging several focus planes. Bar in A represents 30 μm. G, H: single, not merged confocal images. Bar in G represents 30 μm. In A and C, the arrow points to the same fiber recognisable for its characteristic shape showing NPY (A) and GABA (C) immunoreactivity. The same fiber is stained in yellow in B. In D and F the arrows point to numerous co-existent NPY- and GABA-immunoreactive cell bodies which are yellow in E. In G and H the arrowheads indicate a few of the numerous elements showing also the histochemical staining for β-galactosidase. In H the arrow point to NPY-positive cell bodies showing also β-galactosidase activity. The asterisk indicates one yellow cell body positive for β- galactosidase. The same cell is clearly identifiable in E (asterisk), the image reconstructed from the series to which H is belonging. Reprinted from Oberto et al., Neuropharmacology 2001;41: 639–642, with permission from Elsevier. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

GABA-ergic system are closely coupled and that NPY and GABA may functionally interact in the regulation of anxiety behaviour (Kask et al., 1996). The molecular mechanisms that underlie changes in *$Y_1R/LacZ$* transgene expression induced by prolonged administration with ligands of the BZ/ω binding site remain to be determined. Given that the NPY-Y_1R mediated transmission elicits behavioural effects that are indistinguishable from those induced by the activation of $GABA_A$ receptors (Heilig et al., 1989, 1992; Broqua et al., 1995; Sajdyk et al., 1999; Bannon et al., 2000; Thorsell et al., 2000; Kask et al., 2001) and that GABA and NPY coexist within the same neurons in the amygdala (Gustafson et al., 1986; McDonald and Pearson, 1989; Oberto et al., 2001), it is possible that changes in the function of $GABA_A$ receptors elicit compensatory responses in the firing rate of NPYcontaining neurons which, in turn, might be responsible of changes in Y_1 receptor gene transcriptional activity.

6. Role of neuroactive steroids in modulation of NPY-Y_1R transmission in the amygdala

6.1. Effects of a sustained increase in brain concentrations of neuroactive steroids on Y_1R gene expression

The 5-alpha-reducted progesterone (PROG) metabolites, 3alpha-hydroxy-5alpha-pregnan-20-one (3alpha,5alpha TH PROG) and tetrahydrodeoxycorticosterone (3alpha,5alpha TH DOC), are endogenous neuroactive steroids, which affect the excitability of central neurons in a manner independent of nuclear hormone receptors (Biggio and Purdy, 2001). These compounds are among the most potent positive allosteric modulators of the $GABA_A$ receptor complex (Majewska et al., 1986; Belelli and Lambert, 2005). Thus, their acute administration in pharmacological doses potentiate the $GABA_A$ receptor function and elicit anxiolytic, anticonvulsant and hypnotic/anesthetic effects similar to those produced by other positive allosteric modulators of $GABA_A$ receptors (Lambert et al., 2001; Rupprecht et al., 2001). In particular, the anxiolytic action of 3alpha,5alpha TH PROG has been demonstrated in various animal models of anxiety and the amygdaloid complex has recently been implicated as a brain region that plays an important role in mediating the anxiolytic action of 3alpha,5alpha TH PROG (Akwa et al., 1999).

Fluctuations in plasma or brain concentrations of 3alpha,5alpha TH PROG, occurring in association with specific physiological and pathological conditions, such as stress, pregnancy, the menstrual cycle, menopause, regulation of neuronal excitability, and a variety of neurological or psychiatric disorders (Barbaccia et al., 1996; Bicikova et al., 1998; Concas et al., 1998; Genazzani et al., 1998; Biggio and Purdy, 2001) result in changes in neuronal excitability and, in turn, in alteration of emotional state, sleep pattern, and seizure threshold (Guidotti and Costa, 1998; Engel and Grant, 2001; Wang et al., 2001). Moreover, physiological or pharmacologically induced changes in the levels of 3a,5a-TH PROG are associated, at the molecular level, with changes in the expression of $GABA_A$ receptor subunits (Biggio and Purdy, 2001; Biggio et al., 2001; Smith and Gong, 2004). Given that 3alpha,5alpha TH PROG and 3alpha,5alpha TH DOC are among the most potent positive modulators of $GABA_A$ receptor

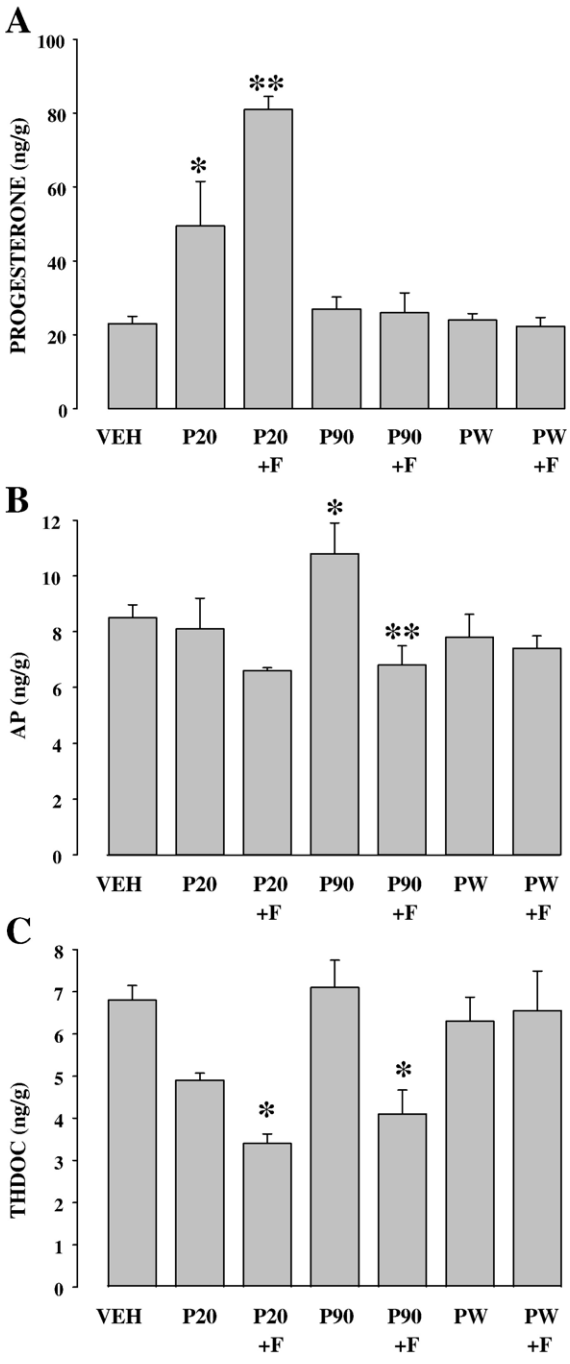

Fig. 2. Effects of long-term treatment with progesterone on the concentrations of progesterone (A), AP (B), and THDOC (C) in the cerebral cortex of Y_1R/LacZ transgenic mice. Mice were treated with vehicle (VEH), progesterone (P), or progesterone plus finasteride (P+F) according to the 3-week, single-injection, multiple-withdrawal paradigm, as previously described (Moran and Smith, 1998). They were killed 20 min (P20, P20+F), 90 min (VEH, P90, P90+F), or 24 h (PW, PW+F) after the last injection(W indicates withdrawal), and the concentrations of 41 progesterone, AP, and THDOC in cerebrocortical homogenates were determined. Data are means±SEM of values from 3 to 8 mice and are expressed as nanograms of steroid per gram of protein. Progesterone, *$p<0.01$ versus VEH, **$p<0.05$ versus P20; AP, *$p<0.05$ versus VEH, **$p<0.01$ versus P90; THDOC, *$p<0.05$ versus VEH, P90, PW, and PW+F (Newman–Keuls test). Reproduced from Ferrara et al., J. Neurochem. 2001;79: 417–425 with permission of Blackwell Publishing.

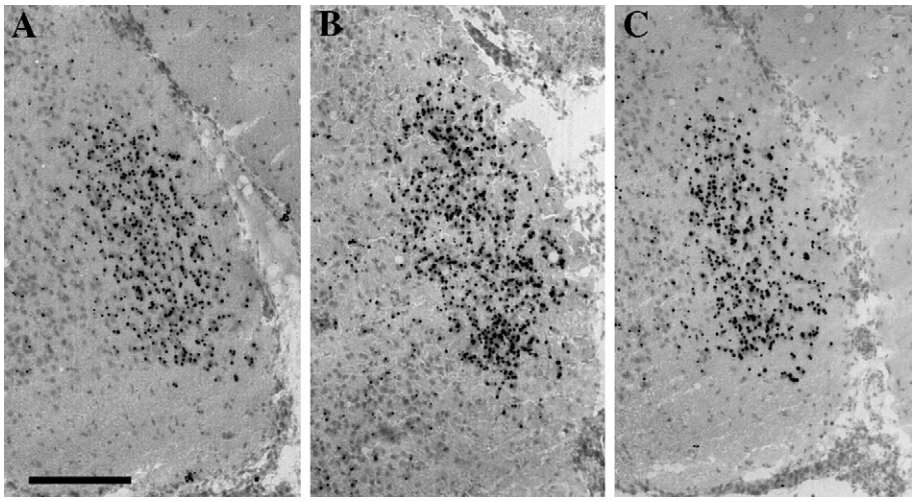

Fig. 3. Effect of long-term treatment with progesterone on *$Y_1R/LacZ$* transgene expression in the medial amygdala. Progesterone treatment was performed as described in Fig. 2. Histochemical staining of β-galactosidase activity in coronal sections of the medial amygdala 90 min after the last injection is shown for mice treated with vehicle (A) or with progesterone (5 mg/kg, i.p) (B), as well as for mice that received a single daily subcutaneous injection of finasteride (25 mg/kg) during the last week of treatment with progesterone (C). Scale bar, 100 μm. Reproduced from Ferrara et al., J. Neurochem. 2001;79: 417–425 with permission of Blackwell Publishing.

function known, we investigated whether fluctuation in the brain concentrations of these neuroactive steroids might also affect Y_1R gene expression in the amygdala.

Chronic treatment with progesterone or with 3alpha,5alpha TH PROG induces a significant augmentation of the cerebrocortical concentration of 3alpha,5alpha TH PROG and 3alpha,5alpha TH DOC (Fig. 2) that is associated with an increase in the expression of the *$Y_1R/LacZ$* transgene in the MeA

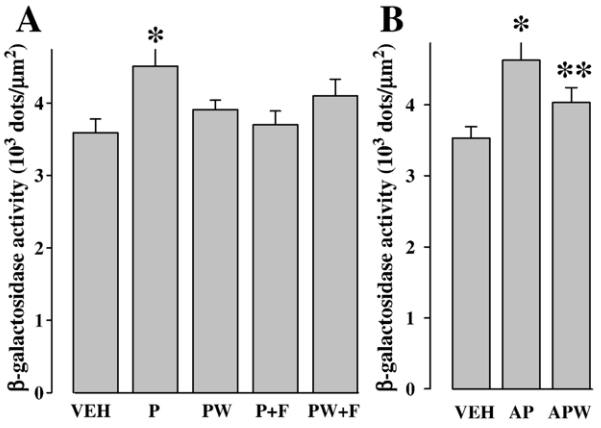

Fig. 4. Quantitation of *$Y_1R/LacZ$* transgene expression in the medial amygdala of mice subjected to longterm treatment with progesterone or AP. (A) Mice were subjected to prolonged treatment with vehicle (VEH), with progesterone (P), or with progesterone and finasteride (P+F) as described in Fig. 2, and were killed 90 min (VEH, P, P+F) or 24 h (PW, PW+F) after the last injection (W indicates withdrawal). Coronal sections of the medial amygdala were subjected to quantitative analysis of β-galactosidase histochemical staining. Data are expressed as the density of blue dots and are means±SEM of values from 5 to 15 mice. *$p<0.01$ versus VEH, PW, and P+F groups (Newman–Keuls test). (B) Mice were treated for 10 days with vehicle or AP (5 mg/kg, i.p.) and were killed 90 min (VEH, AP) or 24 h (APW) after the last injection. Data were analyzed as in (A) and are means±SEM of values from 5 to 10 mice. *$p<0.01$, **$p<0.05$ versus VEH (Newman–Keuls test). Reproduced from Ferrara et al., J. Neurochem. 2001;79: 417–425 with permission of Blackwell Publishing.

similar to that elicited by long-term administration of abecarnil or diazepam (Figs. 3A,B, 4) (Ferrara et al., 2001).

In addition, physiological fluctuations in the cerebrocortical concentrations of endogenous 3alpha,5alpha TH PROG and 3alpha,5alpha TH DOC during pregnancy (Table 1) are also associated with increased *$Y_1R/LacZ$* transgene expression in the MeA (Figs. 5A,B, 6) (Oberto et al., 2002). The levels of Y_1R mRNA in the MeA were also increased on day 18 of pregnancy, suggesting that changes in transgene expression reflect parallel changes in the endogenous Y_1R gene that are achieved, at least in part, via transcriptional mechanisms (Fig. 7). The increase in the Y_1R gene expression in the MeA induced by chronic treatment with progesterone or by physiological conditions, such as pregnancy, is likely to be mediated by 3alpha,5alpha TH PROG and 3alpha,5alpha TH DOC through their selective action at the $GABA_A$ receptor. In fact, pretreatment of *$Y_1R/LacZ$* transgenic mice with the 5α-reductase inhibitor finasteride, which inhibits the biosynthesis of 3alpha,5alpha TH PROG and

Table 1
Effects of finasteride treatment on the concentrations of progesterone, AP, and THDOC in the cerebral cortex of estrus and pregnant mice

	Steroid (ng/g)		
	Progesterone	AP	THDOC
Estrus	15.8±1.7	11.6±0.8	3.6±0.5
Estrus+finasteride	16.5±3.0	9.4±1.4	3.9±0.1
Pregnant	39.8±2.8*	24.2±2.2**	9.9±1.3**
Pregnant+finasteride	39.9±3.9*	15.8±1.9	4.5±0.4

Mice were treated with vehicle or finasteride (25 mg/kg, s.c.) from day 12 to day 17 of pregnancy and killed on day 18. Estrus mice were similarly treated with finasteride or vehicle for 6 days. Data are means±SEM of values from 4 to 10 mice and are expressed as nanograms of steroid per gram of cortical tissue. *$p<0.01$ versus estrus and estrus+finasteride; **$p<0.01$ versus estrus, estrus+finasteride, and pregnant+finasteride (Newman–Keuls test). Reproduced from Oberto et al., J. Neurochem. 2002;82: 1272-1281, with permission of Blackwell Publishing.

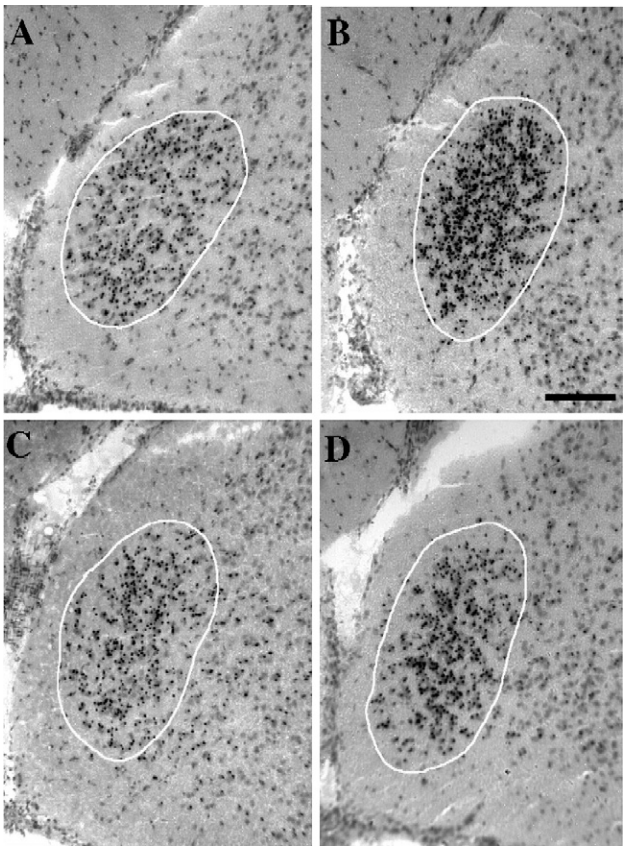

Fig. 5. Effects of pregnancy and delivery on *$Y_1R/LacZ$* transgene expression in the medial amygdala. Coronal sections of the medial amygdala of mice in estrus (A), on day 18 of pregnancy in the absence (B) or presence (C) of finasteride treatment (25 mg/kg, s.c. from day 12 to day 17), or on day 2 after delivery (D) were subjected to histochemical staining for β-galactosidase activity. Scale bar, 100 μm. Reproduced from Oberto et al., J. Neurochem. 2002;82: 1272–1281, with permission of Blackwell Publishing.

3alpha,5alpha TH DOC (Azzolina et al., 1997), prevented both the accumulation of 3alpha,5alpha TH PROG and 3alpha,5alpha TH DOC in the cerebral cortex as well as the increase in *$Y_1R/LacZ$* gene expression in the amygdala (Ferrara et al., 2001; Oberto et al., 2002). These findings therefore suggest that the high concentrations achieved by neuroactive steroids in the cerebral cortex of mice following chronic treatment with progesterone or during pregnancy might play a major role in modulation of both $GABA_A$ and Y_1R mediated transmissions.

Fluctuation in the levels of brain 3a,5a-TH PROG are associated with alterations of the subunit composition of the $GABA_A$ receptor as well as of $GABA_A$ receptor function (Biggio and Purdy, 2001; Biggio et al., 2001; Smith and Gong, 2004; Follesa et al., 2005; Serra et al., 2006); these changes might, in turn, produce adaptive responses in the NPY-mediated transmission and Y_1R gene expression. Indeed, the reduction in the brain concentrations of these steroids elicited either by a physiological condition (delivery) or by pharmacological treatment (finasteride) reversed or prevented both the increase in *$Y_1R/LacZ$* transgene expression (Ferrara et al., 2001; Oberto et al., 2002) as well as changes in the expression of $GABA_A$ receptor subunit genes (Biggio et al., 2001). The rapid decrease in the 3alpha,5alpha TH PROG and 3alpha,5alpha TH DOC cerebrocortical concentrations, following discontinuation of chronic progesterone administration (Fig. 2) as well as following delivery, induce withdrawal effects at the behavioural and molecular levels that are highly similar to those elicited by discontinuation of long-term treatment with positive allosteric modulators of $GABA_A$ receptor such as benzodiazepines (Follesa et al., 1998, 2001). However, the increase of *$Y_1R/LacZ$* transgene expression in the MeA was no longer apparent 24 h after the last hormone injection, or after delivery, indicating that changes in NPY-Y_1R signal do not play a role in progesterone-induced withdrawal syndrome (Figs. 4, 5D, 6A).

6.2. Effects of chronic voluntary alcohol consumption and ethanol withdrawal on Y_1R gene expression in the amygdala

Ethanol exerts anxiolytic, sedative/hypnotic, anticonvulsant, and muscle relaxant effects similar to those induced by different modulators of the $GABA_A$ receptor such as benzodiazepines, barbiturates, and neuroactive steroids (Brot et al., 1995; Grobin et al., 1998; Koob et al., 1998; VanDoren et al., 2000). Behavioural, neurochemical, and electrophysiological evidences indicate that $GABA_A$ receptors are indeed targets for the acute as well as chronic actions of ethanol. The acute action of ethanol is characterized by a facilitation of $GABA_A$ receptor function, whereas, on the contrary, its chronic exposure is associated to a diminished sensitivity to GABA, benzodiazepines and barbiturates, and to an enhanced sensitivity to neuroactive steroids and to the inverse agonists at the benzodiazepine receptors β-carbolines (Mhatre

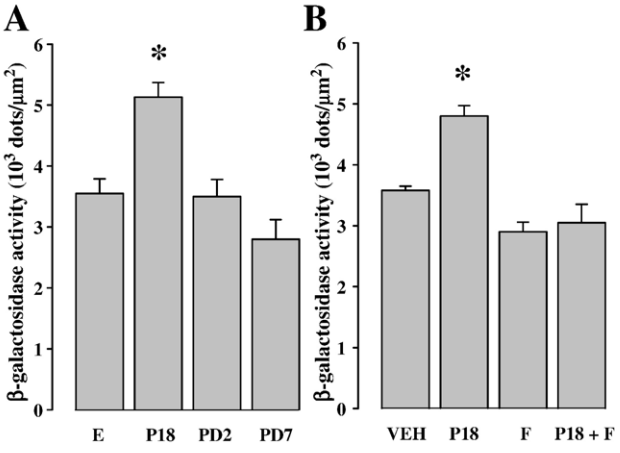

Fig. 6. Quantitation of *$Y_1R/LacZ$* transgene expression in the medial amygdala of mice during pregnancy and after delivery. (A) Coronal sections of the medial amygdala were subjected to quantitative analysis of β-galactosidase histochemical staining. Values are shown for mice in estrus (E), on day 18 of pregnancy (P18), and on days 2 (PD2) and 7 (PD7) after delivery. Data are expressed as the density of blue dots and are means±SEM of values from 5 to 9 mice. *$p<0.01$ versus E, PD2, and PD7 (Newman–Keuls test). (B) Pregnant mice were injected daily with vehicle or finasteride (25 mg/kg, s.c.) from day 12 to day 17 and then killed on day 18 (P18 and P18+F, respectively). Estrus mice were similarly treated with vehicle (VEH) or with finasteride (F) for 6 days. Coronal sections of the medial 43 amygdala were then analyzed for β-galactosidase activity as in (A). Data are means±SEM of values from 5 to 10 mice. *$p<0.01$ versus VEH, F, and P18+F (Newman–Keuls test). Reproduced from Oberto et al., J. Neurochem. 2002;82: 1272–1281, with permission of Blackwell Publishing.

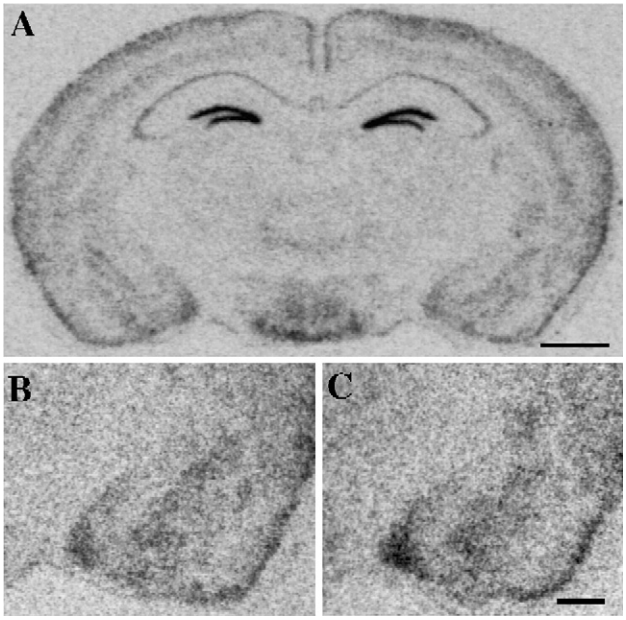

Fig. 7. Effects of pregnancy on Y_1 receptor mRNA expression in the medial amygdala. Low power magnification of an autoradiogram illustrating the neuroanatomical distribution of Y_1 receptor mRNA visualized by *in situ* hybridization on a coronal brain section from an estrous female mice (A). Scale bar, 1 mm. High power magnification of autoradiograms showing the right medial amygdala of female mice in estrus (B) and on day 18 of pregnancy (C). Original sections were hybridized with four oligonucleotide probes and exposed to X-ray film for 8 days. The figure is representative of results obtained from 4 to 6 mice in each group. Scale bar, 0.5 mm. Reproduced from Oberto et al., J. Neurochem. 2002;82: 1272–1281, with permission of Blackwell Publishing.

et al., 1988; Sanna et al., 1993; Becker and Jarvis, 1996; Devaud et al., 1996).

Moreover, long-term administration of ethanol and ethanol withdrawal elicit neurochemical and molecular effects similar to those induced by positive modulators of $GABA_A$ receptor function (Morrow et al., 1990; Mhatre et al., 1993; Devaud et al., 1997; Biggio et al., 2003). In particular, chronic ethanol administration differentially alters the expression of distinct $GABA_A$ receptor subunit mRNA and peptides in various brain regions (Mhatre and Ticku, 1992; Devaud et al., 1997; Sanna et al., 2003; Follesa et al., 2005; Olsen et al., 2005).

In addition to a direct action at the $GABA_A$ receptor, the acute action of ethanol at $GABA_A$ receptors was proposed to be mediated by neuroactive steroids produced in peripheral organs, or by peripheral precursors that are converted to neurosteroids by brain cells (Morrow et al., 1999). Acute ethanol administration increases the concentrations of allopregnanolone in the plasma, cerebral cortex, and hippocampus (Barbaccia et al., 1999; VanDoren et al., 2000; Morrow et al., 2001; Serra et al., 2003). Furthermore, pretreatment of animals with the 5α-reductase inhibitor finasteride, reduced the ethanol-induced increase in the cerebrocortical concentrations of 3alpha,5alpha TH PROG and prevented certain molecular, neurochemical, electrophysiological, and behavioural effects of ethanol (VanDoren et al., 2000; Khisti et al., 2002).

NPY is also involved with neurobiological responses to ethanol (Thiele and Badia-Elder, 2003; Pandey, 2003; Koob, 2003; Thiele et al., 2004). Voluntary alcohol consumption and resistance to the intoxicating effects of ethanol are inversely related to NPY levels in knockout and transgenic mice. NPY knockout mice consume significantly higher amounts of ethanol than do wild type strains, whereas transgenic mice over expressing NPY have a lower preference for ethanol than wild type mice (Thiele et al., 1998). Chronic ethanol administration and ethanol withdrawal also influence NPY signalling, although the effect of ethanol on NPY may vary with the duration and type of ethanol treatment, rodent strain and brain region. For instance, both investigator- and self-administered ethanol increases NPY levels in the hypothalamic structures and in ventrolateral medulla of Long Evans rats (Clark et al., 1998) whereas no differences in NPY protein content were observed in several brain regions of Wistar rats following 7 week exposure to ethanol vapours (Ehlers et al., 1998) or in Sprague Dawley rats fed for 15 days with ethanol diet (Roy and Pandey, 2002). Conversely, NPY immunoreactivity and NPY mRNA were shown to be decreased by single or repeated administrations of intoxicating doses of ethanol in distinct brain regions of rodents, including the hypothalamus, hippocampus and cerebral cortex (Kinoshita et al., 2000; Roy and Pandey, 2002; Bison and Crews, 2003).

On the other hand, consistent results have found that NPY levels and mRNA are significantly decreased 24 h after withdrawal in some brain regions involved in seizure activity and anxious behaviour, including CeA, MeA, cerebral cortex and hippocampus (Roy and Pandey, 2002; Zhang and Pandey, 2003; Bison and Crews, 2003), suggesting that a reduced activity of NPY neurons may represent one of the mechanisms implicated in ethanol withdrawal symptoms. This decrease is followed by a dramatic increase of NPY expression in the hippocampus that occurs 72 h after withdrawal which was suggested to represent a protective response against prolonged withdrawal seizure activity (Ehlers et al., 1998; Bison and Crews, 2003).

We demonstrated that Y_1R gene expression in the MeA and CeA of *Y_1R/LacZ* transgenic mice was not affected by voluntary alcohol consumption for 4 weeks, but it significantly increased 48 h following ethanol withdrawal (Mele et al., submitted for publication). This finding suggests the possibility that the decrease of NPY levels in the amygdaloid structures, that is associated with the behavioural manifestations of ethanol withdrawal, may induce the up-regulation of the Y_1R subtype.

To determine whether the increased biosynthesis of 3alpha,5alpha TH PROG induced by ethanol was responsible for its effect on Y_1R gene transcriptional activity we analyzed the effect of pretreatment with finasteride of *Y_1R/LacZ* transgenic mice on β-galactosidase expression in the CeA and MeA. Our findings demonstrated that finasteride administration prevented both the ethanolinduced increase of 3alpha,5alpha TH PROG in the cerebral cortex as well as the increase of *Y_1R/LacZ* gene expression in the amygdala induced by 24 h ethanol withdrawal in the amygdala (Mele et al., submitted for publication). These findings suggest that the ability of ethanol withdrawal to affect the NPY-Y_1R signal transmission involves fluctuation of brain concentrations of 3alpha,5alpha TH PROG and may possibly depend on changes of $GABA_A$ receptor function and subunit composition that occur during ethanol consumption and ethanol

withdrawal. Indeed, in addition to preventing the increase in *Y_1R/LacZ* transgene expression induced by ethanol withdrawal, finasteride pretreatment reduces ethanol withdrawal severity (Finn et al., 2004; Gorin et al., 2005).

6.3. Effect of a transient increase of cerebrocortical concentrations of neuroactive steroids on Y_1R gene expression in the amygdala of Y_1R/LacZ transgenic mice?

NPY and GABA may also interact to inhibit the excitatory effects of CRF in the amygdala and thereby contribute to the overall response to stressful or threatening stimuli (Heilig et al., 1994). Several acute stress paradigms, such as forced swimming, foot shock, and carbon dioxide inhalation, reduce $GABA_A$ receptor function in several brain regions, induce proconflict behavior (Corda and Biggio, 1986) and increase the concentrations of neuroactive steroids in plasma and the brain to levels that are high enough to activate $GABA_A$ receptors (Purdy et al., 1991; Barbaccia et al., 1996, 1997, 2001; Reddy and Rogawski, 2002). The rapid and persistent increase in neuroactive steroid concentrations induced by acute stress has been proposed to represent a homeostatic mechanism for the restoration of $GABA_A$ receptor function and to be physiologically relevant in protection against stress-induced seizures. On the other hand, short-term physical restraint, which induces anxiety, reduces NPY mRNA and protein concentrations in the amygdala and cortex of rats (Thorsell et al., 1998). In contrast, repeated exposure to restraint once daily for 10 days increases NPY expression in the amygdala and in the ARC (Thorsell et al., 1999).

Given that the sustained increase of cerebrocortical concentrations of neuroactive steroids that occurs during pregnancy or long-term administration of progesterone affect both $GABA_A$ receptor and Y_1R gene expression (Concas et al., 1998; Follesa et al., 1998; Ferrara et al., 2001; Oberto et al., 2002), we have examined the hypothesis that a rapid increase in the plasma and brain content of these compounds might also participate in the regulation of Y_1R plasticity in response to restraint stress.

Acute exposure to restraint induced a transient increase in the cerebrocortical concentrations of neuroactive steroids that returns to control values within 30 min. Acute restraint also increased *Y_1R/LacZ* transgene expression in the CeA and in the MeA of transgenic mice (Mele et al., 2004). Finasteride prevented the transient increase in the cerebrocortical concentrations of 3alpha,5alpha-TH PROG and 3alpha,5alpha-TH DOC, but failed to inhibit that in Y_1R gene expression in the amygdala of the stressed mice. Moreover, repeated exposure to restraint, that induces repetitive and transient elevation in the concentrations of neuroactive steroids in the cerebral cortex, failed to affect Y_1R gene (Mele et al., 2004). These finding, taken together, indicate that changes in the brain concentrations of neuroactive steroids and in Y_1R gene expression induced by restraint stress are achieved independently of each other and suggest that a sustained increase in the brain concentrations of these steroids may be required for modulation of Y_1R gene expression.

Accordingly, the exposure to 5 min foot shock stress as well as the acute treatment with ethanol, both of which transiently increase the concentrations of 3alpha,5alpha-TH PROG in the cerebral cortex, failed to affect Y_1R in transgenic mice (unpublished results).

7. Conclusions

Results reviewed above indicate that chronic administration of various modulators of the $GABA_A$ receptor complex as well as prolonged pharmacologically or physiologically induced changes in 3alpha,5alpha-TH PROG concentrations affect Y_1R gene expression in the MeA and CeA.

Molecular, physiological and neurochemical evidence demonstrated that long term exposure to these modulators triggers cellular adaptive mechanisms that involve alteration in the subunit composition of the $GABA_A$ receptor with regional differences which, in turn, might affect receptor function and altered pharmacological and behavioural sensitivity (Follesa et al., 2000, 2001, 2002).

Given the important role of the GABA/NPY interaction in regulation of several functions, including emotional behaviour and neuronal excitability, these findings suggest that altered GABA receptor function might trigger compensatory changes on NPY-containing neurons that, in turn, might be responsible of up-regulation of Y_1R gene. This hypothesis is supported by the observation that chronic treatment with benzodiazepines modulates the expression of NPY and NPY mRNA in various brain regions (Krysiak et al., 1999, 2000). Furthermore the decrease of $GABA_A$ receptor $\gamma 2$ subunit expression induced by the administration of antisense oligonucleotides is associated with a profound increase of NPY mRNA and receptor protein expression in rat hippocampus (Mikkelsen et al., 2001).

On the other hand, we have shown that fluctuations in the cerebrocortical concentration of 3alpha,5alpha- TH PROG during voluntary ethanol consumption and ethanol withdrawal induces a marked increase in Y_1 receptor gene expression that becomes apparent 48 h after withdrawal. This observation suggests that the increase of brain neuroactive steroid concentrations induced by voluntary ethanol consumption might produce changes on $GABA_A$ receptor function different from those induced by progesterone treatment or pregnancy. In this regard, ethanol has been shown to increase brain steroidogenesis by a local action independently on the activity of the hypothalamicpituitary-adrenal axis (Sanna et al., 2004). Given the diversity and heterogeneity of $GABA_A$ receptor expressed in different neurons the ethanol-induced changes in the expression of each subunit might differently affect the NPY-Y_1 pathway.

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