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Hormone-dependent regulation of $GABA_A$ receptor γ subunit mRNAs in sexually dimorphic regions of the rat brain

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Transmission mediated by gamma-aminobutyric acid type A ($GABA_A$) receptors expressed within the medial preoptic area (mPOA) and the ventromedial nucleus (VMN) of the hypothalamus is known to play critical, but contrasting, roles in regulating steroid-dependent sexual behaviours in rats. Previous studies have demonstrated a striking dichotomy in receptor composition between the two regions with regard to γ, but not α or β, subunit expression. To test if gonadal steroids regulate the expression of the γ subunit genes within the mPOA and the VMN, *in situ* hybridization analysis for messenger RNAs encoding the γ1, γ2Short (γ2S) and γ2Long (γ2L) subunits was done in gonadectomized male and female rats and in gonadally intact females over the oestrous cycle. No significant differences in the expression of the γ subunit mRNAs were observed in gonadectomized male versus female rats. Significant effects of gonadal state in female rats were observed for γ1 mRNA levels in the mPOA and γ2L levels in the VMN. These data demonstrate that gonadal hormones exert activational control of expression of $GABA_A$ receptor γ subunit mRNAs and suggest that differences in receptor structure may contribute to the functional modulation of female sexual behaviours mediated by GABAergic transmission in these regions.

Keywords: $GABA_A$ receptors; hypothalamus; *in situ* hybridization; gonadal hormones

1. INTRODUCTION

The medial preoptic area (mPOA) and the ventromedial nucleus of the hypothalamus (VMN) are regions that are critical for the production of sexual behaviours in both male and female rats (McGinnis *et al*. 1996; for a review, see Meisel & Sachs 1994; Pfaff *et al*. 1994). Consistent with the ability of steroid hormones to induce and modulate sexual behaviours by acting on neural substrates in the mPOA and the VMN, high levels of mRNAs encoding the androgen and oestrogen receptors are detected in both regions (Simerly *et al*. 1990; MacLusky *et al*. 1997). Moreover, sexual dimorphisms in neural structure and function that arise from the perinatal action of gonadal steroids have been described extensively for both sites (for a review, see McEwen 1992; Madeira & Lieberman 1995).

The expression of sexual behaviours is regulated by a rich array of neurotransmitters and neuromodulators, including γ-aminobutyric acid (GABA), in the mPOA and the VMN (Kow *et al*. 1994; for a review, see Meisel & Sachs 1994; Pfaff *et al*. 1994). Many aspects of GABAergic transmission, including the levels of GABA (for a review, see Herbison 1997), the number of $GABA_A$ receptors (Schumacher *et al*. 1989), the expression of $GABA_A$ receptor subunit genes (Herbison & Fénelon 1995) and $GABA_A$ receptor function (Smith *et al*. 1996), have been shown to be regulated by gonadal steroids and to be sexually dimorphic. Although GABAergic transmission is important in both the mPOA and the VMN, the effects of $GABA_A$ receptor activation on sexual behaviour are quite different between the two regions. Specifically, in female rats, $GABA_A$ receptor agonists will inhibit lordosis when infused into the mPOA, but facilitate this behaviour when injected into the VMN (McCarthy 1995). Differential modulation of sexual behaviours by the GABAergic systems in the VMN and the mPOA may reflect not only inherent differences in the connectivity of the two regions, but also differences in the functional modulation of these circuits by region-specific and structurally distinct $GABA_A$ receptors.

The $GABA_A$ receptor is a hetero-oligomeric protein for which five families of mammalian subunit genes (α1–6, β1–3, γ1–3, δ and ε) have been identified (Whiting *et al*. 1997; for a review, see Sieghart 1995). Alternative splicing of specific transcripts, including the γ2 subunit mRNA (γ2S and γ2L), further enhances the structural and functional diversity of these receptors (for a review, see Sieghart 1995). Previous studies using both *in situ* hybridization analysis of mRNA levels and immunocytochemical analysis of subunit protein expression have demonstrated that in both the mPOA and the VMN, α2

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and β3 transcripts and subunits are the predominant members expressed from their respective gene families. However, the regions differ markedly in expression of γ subunits. In the VMN, γ2 expression is high, whereas levels of γ1 are low. In contrast, although levels of γ2 are barely detectable in the mPOA, high levels of γ1 subunit mRNA and protein are restricted to the mPOA and a limited number of forebrain regions including the medial amygdala (MeA), the lateral septal nuclei (LS), and the bed nucleus of the stria terminalis (ST) (Ymer *et al*. 1990; Araki *et al*. 1992, 1993; Wisden *et al*. 1992; Herbison & Fénelon 1995). Expression of γ3 mRNA is negligible in both regions (Wisden *et al*. 1992).

Subunit composition is known to affect a host of functional properties of the receptor. In particular, γ subunit composition determines the affinity of the receptor for GABA, its mean channel-open time, single-channel conductance, rate of desensitization and sensitivity to allosteric modulators (for a review, see Sieghart 1995). Immunocytochemical and *in situ* hybridization studies suggest that structurally distinct $GABA_A$ receptors are expressed in the mPOA and the VMN. Region-specific differences in receptor type, in turn, may subserve differences in functional GABAergic transmission in the mPOA versus the VMN, which will influence the production of sexual behaviours. To test if gonadal steroids regulate the expression of the γ subunit gene within these regions, we have performed *in situ* hybridization analysis of γ1, γ2S and γ2L subunit-specific mRNAs in (i) gonadectomized adult male and female rats to assess permanent (organizational) differences in expression of γ subunit mRNAs between the sexes, and (ii) gonadally intact females to assess transient (activational) changes in γ subunit expression over the oestrous cycle.

2. MATERIALS AND METHODS

Gonadally intact, adult Long–Evans rats derived from stock initially obtained from Harlan Sprague–Dawley (Indianapolis, IN) were maintained in the animal colony of the Psychology Department of Dartmouth College. Animals were housed in groups of two or three under temperature-controlled conditions and maintained on a 12:12 h light/dark cycle (lights switched off at 12.00) with food and water *ad libitum*. Stages of the oestrous cycle were determined from the analysis of cell types recovered from a vaginal lavage collected daily at approximately 12.00 (Long & Evans 1922). All subjects were 60–90 days old when killed. Twelve animals (four for each stage of the cycle) were used in experiments examining changes of γ subunit expression across the oestrous cycle. Eight animals (four of each sex) were used for assessment of sex-specific differences. Animals that were used to assess organizational differences in expression of γ subunit mRNAs between the sexes were gonadectomized seven days before being killed.

Oligonucleotides corresponding to subunit residues 341–354 of the γ1 complementary DNA (Ymer *et al*. 1990), nucleotides 1162–1176 of the γ2L cDNA, which incorporate the nucleotide sequence encoding the eight amino-acid insert between nucleotides 1167 and 1168 of this subunit (Whiting *et al*. 1990; Miralles *et al*. 1994), and nucleotides 1147–1185 of the γ2S cDNA (Shivers *et al*. 1989) were synthesized by Operon Technologies, Inc. (Alameda, CA). They were 3′ end-labelled using a 10:1 molar ratio of α-^{35}S-dATP (Dupont/New England Nuclear, Boston, MA; 1000–1270 Ci mmol^{-1}) to oligonucleotide. Unincorporated free nucleotides were removed using Centri-Sep columns (Princeton Separations, Inc., Adelphia, NJ). A labelled probe (specific activity $0.5–1 \times 10^9$ c.p.m. per μg) was resuspended in a hybridization mixture (Wisden *et al*. 1992) at a concentration of 0.5×10^6 c.p.m. per 100 μl. Frozen sections (20 μm) were cut, mounted on poly-L-lysine-coated slides, fixed with 4% paraformaldehyde (Fisher Scientific, Springfield, NJ) and dehydrated in serial ethanol (EtOH). Before hybridization, slides were treated sequentially with 0.1 M glycine in 1X PBS, 0.1 M triethanolamine/0.25% acetic anhydride in 1X PBS, and 2X SSC, and then dehydrated in serial EtOH and air-dried. Hybridization was done at 42 °C overnight. After hybridization, sections were washed for 5 min at room temperature in 1X SSC, followed by three 30 min washes at 55 °C in 1X SSC, and a final 5 min wash in 1X SSC at room temperature. Sections were dehydrated in 30% EtOH/0.6 M NaCl, 60% EtOH/0.6 M NaCl, 80% EtOH, 95% EtOH and 100% EtOH, air-dried for *ca.* 1 h, and apposed to Biomax MR film (Eastman Kodak Co., New Haven, CT) for three weeks. Included in each film cassette was a slide mounted with ^{14}C microscales (Amersham Corp., Arlington Heights, IL), which was used to quantify densitometric signals (Huntsman *et al*. 1994), and slides incubated with the labelled probe and a 50-fold excess of unlabelled oligonucleotide to assess non-specific hybridization. Slides were dipped in emulsion (Kodak NTB2 diluted 1:1 with water), stored at 4 °C for two months, and stained through emulsion with cresyl violet. Quantitative analysis of the autoradiograms was done using the MCID system (Imaging Research, St Catherines, Ontario). The density values are expressed as mean ±s.e.m. Grain counts through emulsion were done for images captured on an Olympus BX50 microscope and a Dage VE1000 CCD camera system (Optical Analysis Corp., Nashua, NH) using NIH Image software. Cells with grain counts that were three times that of cells in sections hybridized in the presence of 50-fold excess unlabelled oligonucleotide, were judged to be 'positive' for a specific mRNA (Herbison *et al*. 1995). Percentages of positive cells were estimated from the analysis of 50 cells from each side of the brain, and estimates of grain counts were made for 50 cells that had counts above background from each side of the brain (Herbison *et al*. 1995). Data were analysed using a one-way analysis of variance followed by post hoc comparisons using the Scheffé test.

3. RESULTS

In adult rats, both *in situ* hybridization and immunocytochemical analyses strongly support the hypothesis that α2, β3 and γ1 subunits comprise the predominant isoform of the $GABA_A$ receptor expressed in the mPOA, whereas α2, β3 and γ2 mRNAs predominate in the VMN (Wisden *et al*. 1992; Herbison & Fénelon 1995). As expected from previous experiments (Ymer *et al*. 1990; Wisden *et al*. 1992; Herbison & Fénelon 1995), we found that high levels of signal corresponding to the γ1 subunit mRNA were restricted in the adult brain to the mPOA, LS and ST (figure 1). Although it has been established that the γ2 subunit mRNA is expressed in the VMN (Wisden *et al*. 1992), the oligonucleotide probe used in this previous study did not distinguish between the γ2S and γ2L splice variants. Hybridizations performed with oligonucleotides specific for the γ2S and γ2L mRNAs revealed barely detectable labelling for the γ2S subunit

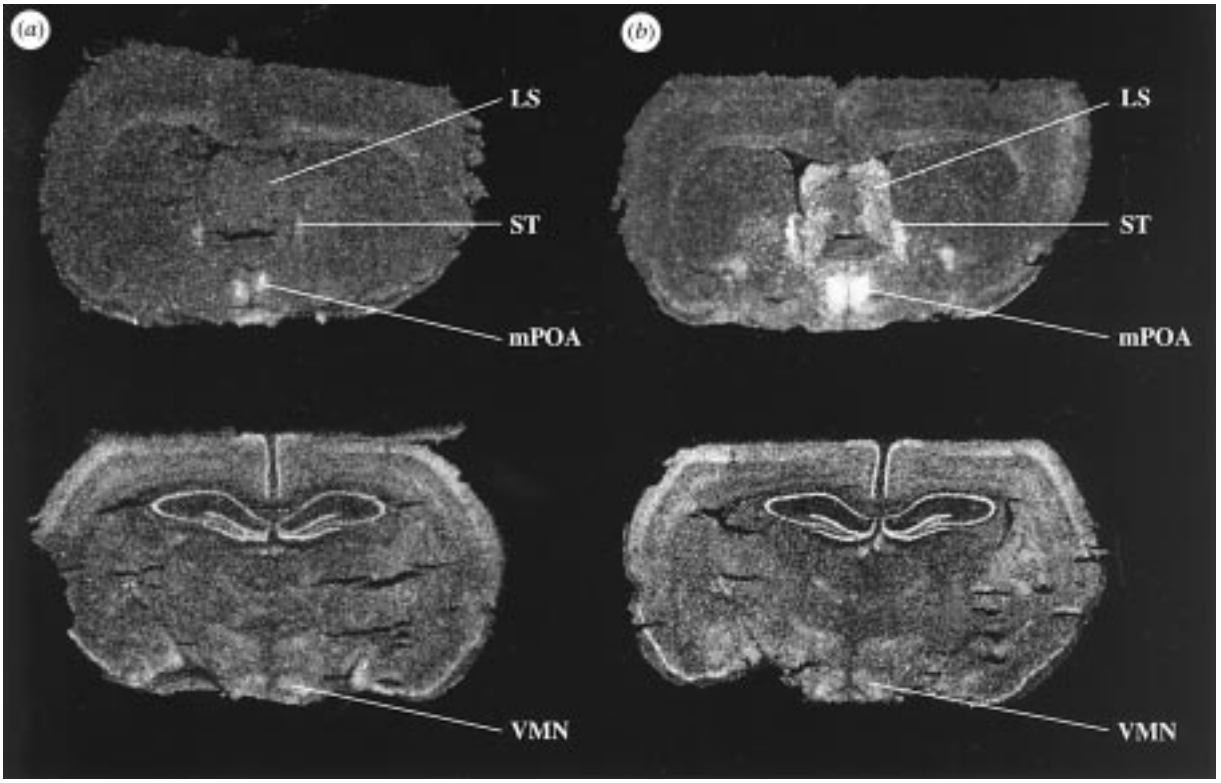

Figure 1. Distribution of γ1 subunit mRNA in the mPOA, and γ2L subunit mRNA in the VMN, of (*a*) dioestrous and (*b*) oestrous females. Darkfield autoradiograms indicating hybridization of ^{35}S-labelled oligonucleotides specific for the γ1 subunit mRNA in the mPOA, LS and ST (top), or for the γ2L subunit mRNA in the VMN (bottom), in representative coronal sections of brains from dioestrous and oestrous female rats.

mRNA within the VMN (data not shown), but marked signal corresponding to the γ2L transcript, which was highest in the ventrolateral, hormone-sensitive region of this nucleus (Pfaff & Keiner 1973; MacLusky *et al*. 1997) (figure 2). No appreciable signal for the γ2 subunit mRNA was detected in the mPOA, nor was signal for the γ1 mRNA detected in the VMN (data not shown).

It has been shown that oestrogen administered to gonadectomized female rats increases the levels of γ1 mRNA in the mPOA (Herbison & Fénelon 1995). To test if activational changes in γ subunit expression occur in gonadally intact females in response to cyclical changes in endogenous hormones, the expression of γ1 subunit mRNA in the mPOA and of γ2L subunit mRNA in the VMN was assessed for dioestrous, pro-oestrous and oestrous female rats.

Levels of γ1 subunit mRNA within the mPOA were appreciably lower in dioestrous than in pro-oestrous or oestrous females (1.3-fold and 1.7-fold, respectively) (figure 1), although densitometric analysis of autoradiographic films indicated that these differences were not statistically significant. Densitometric analysis of expression of γ1 mRNA in the LS indicated that levels of this transcript were also appreciably, but not significantly, lower in dioestrus females (data not shown). In the VMN, levels of γ2L subunit mRNA were also lowest during dioestrus (figure 1) although again, the differences were not significant. A secondary assessment of autoradiographic films limited to a region approximating the hormone-sensitive medial preoptic nucleus (MPN) (Gorski *et al*. 1978) within the mPOA and the hormone-sensitive ventrolateral region of the VMN (vlVMN) revealed a similar pattern of expression of γ subunit mRNAs across the oestrous cycle (figure 2). In agreement with densitometric analysis, subsequent emulsion autoradiography indicated that the number of grains per neuron in the MPN was lowest in dioestrous females (figure 2). The overall main effect of day on the number of grains per neuron, approached significance ($p < 0.08$), and a comparison of grains per neuron in dioestrous versus oestrous females, strongly suggests that with a larger sample size, this difference would attain statistical significance (figure 2). The percentage of neurons expressing the γ1 mRNA, however, was significantly lower ($p < 0.006$) in dioestrous versus oestrous females (figure 2). In the vlVMN, the number of grains per neuron was lower for animals in dioestrus than in pro-oestrus, and this difference was statistically significant ($p = 0.01$) (figure 2). Taken together, our analysis indicates that activational changes in expression of γ subunit mRNA were evident in both regions, with levels of γ1 mRNA in the MPN and γ2L mRNA in the vlVMN being lowest in dioestrus.

To determine if organizational actions of gonadal steroids result in permanent, sex-specific differences in γ subunit mRNA levels in the VMN and the mPOA, *in situ* hybridizations were done for adult male and female animals that had been gonadectomized seven days before being killed. Neither densitometric analysis nor assessment of grain counts revealed any appreciable differences in the expression of either γ1 subunit mRNA in the mPOA or of γ2L subunit mRNA in the

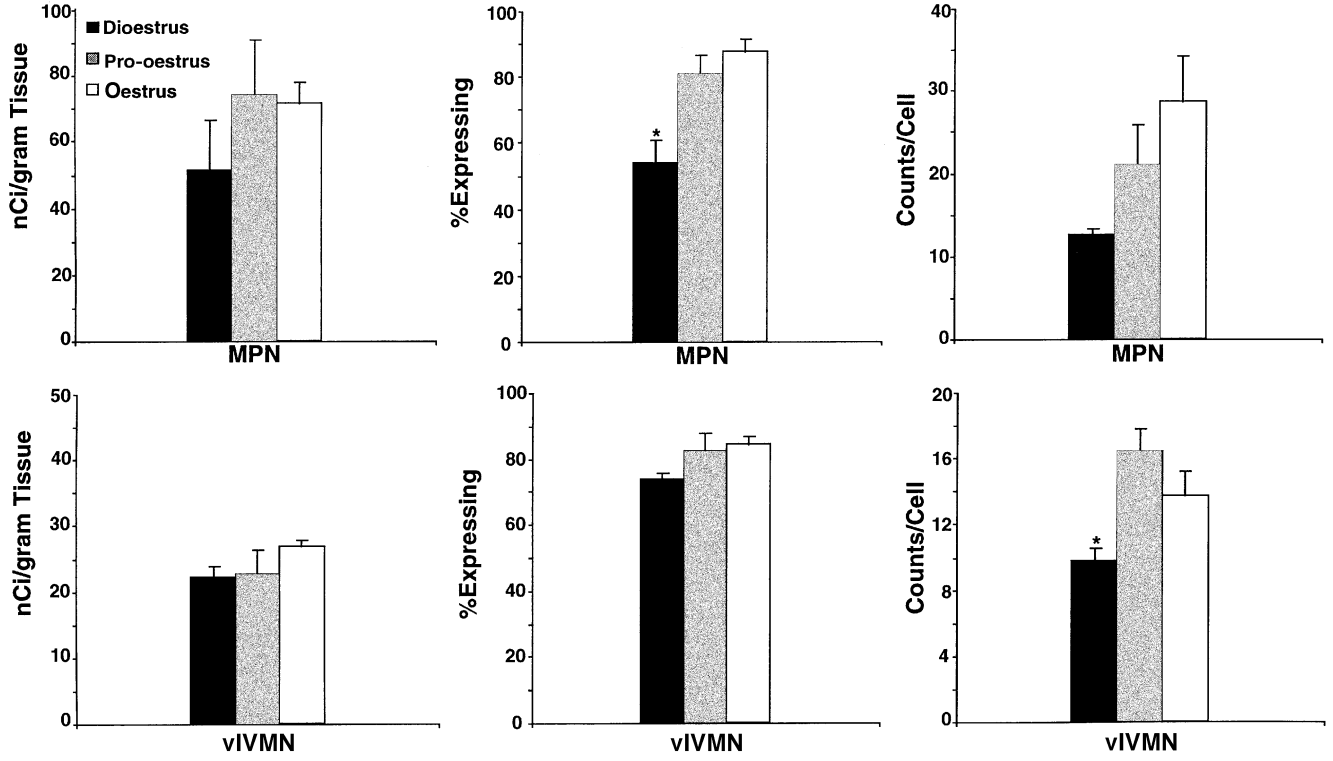

Figure 2. Assessment of activational changes in γ subunit mRNA over the oestrous cycle. Data shown in the left panels indicate quantification of signals by densitometry of autoradiographic films corresponding to the γ1 subunit mRNA probe in the MPN and the γ2L subunit mRNA probe in the vlVMN. Data shown in the middle panels indicate the percentage of cells judged to be expressing the respective γ subunit mRNA (i.e. cells judged as 'positive': see § 2). Data shown in the right panels indicate the number of silver grains (counts) per neuron in those cells that expressed the specific mRNA. Data are from four animals for each phase of the oestrous cycle. Error bars indicate s.e.m. An asterisk indicates that the percentage of expressing neurons was significantly lower for animals in dioestrus versus oestrus in the MPN, and that the number of counts per neuron was significantly lower for animals in dioestrus versus pro-oestrus in the vlVMN.

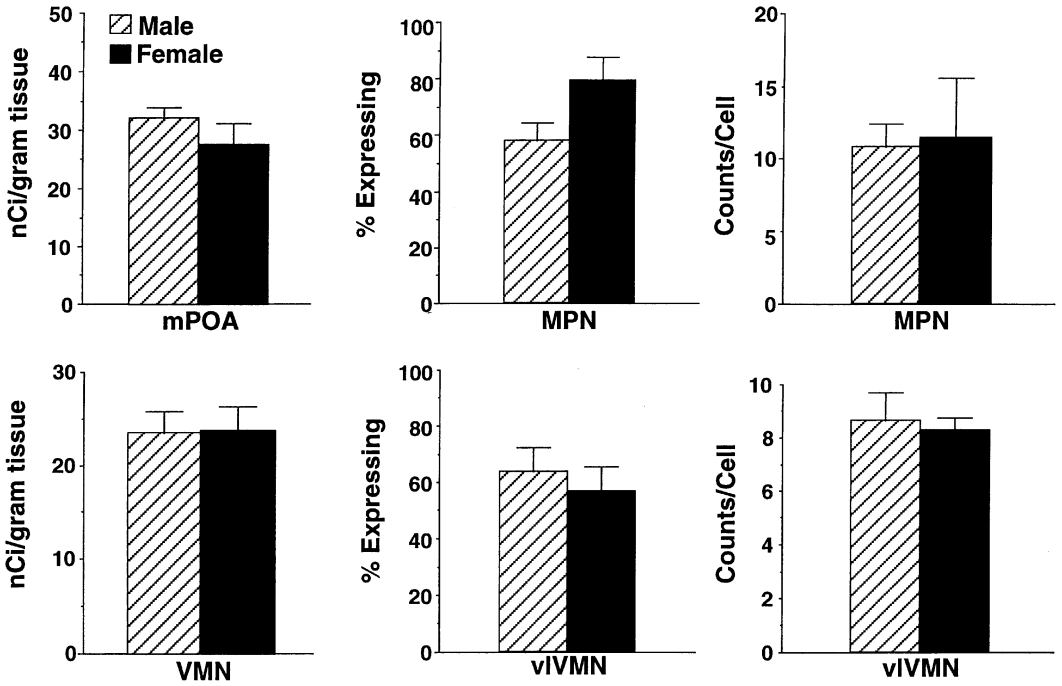

Figure 3. Assessment of organizational sex differences in γ subunit mRNA. Data shown in the left panels indicate quantification of signals by densitometry of autoradiographic films corresponding to the γ1 subunit mRNA probe in the mPOA and the γ2L subunit mRNA probe in the VMN. Data shown in the middle panels indicate the percentage of expressing cells in the MPN and in the vlVMN. Data in the right-hand panels indicate the number of silver grains (counts) per neuron in those cells that expressed the specific mRNA. Data are from four animals of each sex that were gonadectomized seven days before being killed. Error bars indicate s.e.m.

VMN in gonadectomized male versus female animals (figure 3).

4. DISCUSSION

The VMN is an essential site for steroid-dependent induction of sexual behaviours in rats (McGinnis *et al*. 1996; for review, see Pfaff *et al*. 1994). The mPOA plays a critical role in the modulation of these behaviours by inhibiting feminine sexual behaviour and facilitating masculine sexual behaviour patterns (for a review, see Pfaff *et al*. 1994; Meisel & Sachs 1994). Neurotransmission mediated by $GABA_A$ receptors in the VMN and the mPOA has significant, but contrasting, effects on the expression of sexual behaviours (Fernández-Guasti *et al*. 1986; for a review, see McCarthy 1995). Although both regions express predominantly α2 and β3 subunit transcripts, they have diametrically opposing patterns of expression of the γ1 and the γ2 subunit mRNAs (Wisden *et al*. 1992; Herbison & Fénelon 1995). Here, we report that levels of γ1 subunit mRNA in the mPOA and γ2L subunit mRNA in the VMN fluctuate over the oestrous cycle in female rats, but are not different between gonadectomized male and female rats. These results suggest that organizational differences in expression of γ1 subunit mRNA do not contribute to the expression of sex-specific behaviours between adult male and female rats, but that activational changes in the levels of $GABA_A$ receptor subunit mRNAs may modulate the expression of female sexual behaviours. Although both densitometric analysis and emulsion autoradiography revealed a consistent trend that γ subunit mRNAs were lower in dioestrus than at other stages of the oestrous cycle, for most of the assessments made, these differences did not attain significance. We believe that the lack of statistical significance may be attributed, in part, to the variability between animals. In addition, for densitometric analysis of films, it is possible that the expression of γ mRNAs in glial cells may provide a level of background which, if not hormone regulated, could mask significant changes in the neurons themselves, which would have been detected in subsequent analysis of single cells.

Previous studies suggest that the increases in γ subunit mRNAs detected in the mPOA and the VMN do not arise simply as a result of a non-specific hormone-induced increase in the overall level of $GABA_A$ receptors. Specifically, using ^{3}H-muscimol autoradiography (O'Connor *et al*. 1988; Schumacher *et al*. 1989; McCarthy *et al*. 1992), it has been demonstrated in gonadectomized female rats that oestrogen alone decreases the levels of ^{3}H-muscimol detected in the mPOA and the VMN. Oestrogen plus progesterone treatment reverses the effects of oestrogen alone, but only to control levels (Schumacher *et al*. 1989). The assumption that activational changes in $GABA_A$ receptor expression is specific for individual subunit mRNAs is further supported by experiments indicating that oestrogen treatment of gonadectomized females causes increased expression of α2 and γ1, but not β3, subunit mRNAs in the mPOA (Herbison & Fénelon 1995). Finally, gonadal state does not influence expression of the γ2 subunit mRNA in the hippocampus (Orchinik *et al*. 1995), suggesting that steroid-dependent regulation of γ subunit mRNA levels is not a ubiquitous phenomenon throughout the brain.

Gonadal steroid regulation of expression of $GABA_A$ receptor subunit genes has been shown to have significant effects on synaptic integration and the expression of hormone-dependent behaviours. Regulation of both α and γ subunit mRNAs by naturally occurring fluctuations in gonadal steroids has been demonstrated in the supraoptic nucleus (SON) of the hypothalamus of rats during parturition and lactation (Fénelon & Herbison 1996; Brussaard *et al*. 1997). The steroid-induced changes in α subunit expression in the SON have been shown to diminish the time course of synaptic inhibition of oxytocin neurons, thus facilitating firing of these neurons and the downstream reproductive behaviours they regulate.

In addition to inducing changes in receptor kinetics, steroid-dependent changes in $GABA_A$ receptor subunit expression have been shown to alter the sensitivity of $GABA_A$ receptors to specific allosteric modulators, including both steroid derivatives (Brussaard *et al*. 1997) and benzodiazepines (Smith *et al*. 1998). These changes in the sensitivity of the $GABA_A$ receptor to allosteric modulators are believed to underlie changes in the production of reproductive behaviours (Brussaard *et al*. 1997), and to contribute to hormone-dependent cognitive dysfunction (Smith *et al*. 1998). In the mPOA and the VMN, regulation of γ subunit expression may exert important regulatory control of behaviours by altering the interplay between $GABA_A$ receptors and compounds that modulate them allosterically (for a review, see Sieghart 1995). Of particular interest, we have found that both androgenic steroids and benzodiazepine-like compounds have diametrically opposite effects, facilitating $GABA_A$ receptor-mediated synaptic currents in the VMN, while inhibiting these responses in the mPOA (Jorge-Rivera & Henderson 1998; Nett *et al*. 1998).

It is known that both benzodiazepine and steroid allosteric modulators can alter hormone-dependent sexual behaviours when infused into the VMN and the mPOA (McCarthy *et al*. 1995; Frye *et al*. 1996*a*,*b*). Moreover, levels of endogenous modulators (for a review, see Majewska 1992) and behavioural sensitivity to the action of modulators (Fernández-Guasti & Picazo 1990) vary across the oestrous cycle. Collectively, these studies suggest that the GABAergic circuitry within the hypothalamus and forebrain, which is responsible for modulating the expression of sexual behaviours, is highly plastic. Hormonal state influences both $GABA_A$ receptor composition (via regulation of subunit gene expression) and the levels of allosteric modulators. Receptor subunit composition will determine the sensitivity of receptors to allosteric modulation, and thus influence the efficacy of synaptic transmission and the resultant behavioural output from these neural networks. In this context, gonadal steroids, $GABA_A$ receptors and allosteric modulators may act in concert to generate sexual and reproductive behaviours appropriate to specific hormonal conditions.

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