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CHANGES IN $GABA_A$ RECEPTOR SUBUNIT EXPRESSION IN THE MIDBRAIN DURING THE OESTROUS CYCLE IN WISTAR RATS

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Abstract —In women, the late luteal phase or "premenstrual" period is commonly associated with psychological disturbances, which include mood changes and increased aggression. The underlying cause is unknown but one possibility is that fluctuations in levels of neuroactive steroids precipitate changes in expression of $GABA_A$ receptor subunits that result in functional changes in inhibitory control systems. The present study investigated the levels of expression of α4, β1 and δ $GABA_A$ receptor subunits in the periaqueductal gray matter (PAG) in rats and whether plasticity occurs during the oestrous cycle in females. In male rats α4, β1 and δ subunit immunoreactive neurones were present throughout the PAG in similar numbers. In female rats in proestrus, oestrus and early dioestrus, the density of α4, β1 and δ subunit immunoreactive cells was similar to males. However, in late dioestrus, the numbers increased significantly, especially in the dorsolateral PAG, a region which is particularly rich in GABAergic interneurones. These parallel changes may reflect an increase in expression of the α4β1δ $GABA_A$ receptor subtype. Recombinant α4β1δ receptors, expressed in *Xenopus* oocytes, exhibited and EC_{50} for GABA an order of magnitude lower (2.02±0.33 μM; mean±S.E.M.) than that found for the most ubiquitous α1β2γ2 $GABA_A$ receptor (32.8±2.5 μM). Increased expression of α4β1δ $GABA_A$ receptors in the interneurones of the PAG could render the panic circuitry abnormally excitable by disinhibiting the ongoing GABAergic inhibition. Similar changes in neuronal excitability within the PAG in women consequent to falling steroid levels in the late luteal phase of the menstrual cycle could contribute to the development of pre-menstrual dysphoria.

Key words: steroid hormones, immunocytochemistry, panic, $GABA_A$ receptor plasticity, periaqueductal gray matter, premenstrual dysphoric disorder.

Premenstrual symptoms are associated with falling progesterone levels during the late luteal phase of the menstrual cycle. Progesterone exhibits anxiolytic properties that are mediated by its metabolite, the neuroactive steroid allopregnanolone, the levels of which change in parallel with fluctuations in plasma progesterone concentration (Bitran et al., 1995). The effects of allopregnanolone are mediated, at least in part, by interaction with $GABA_A$ receptors (Majewska et al., 1986; Callachan et al., 1987), whereby responses to GABA are potentiated leading to increased neuronal inhibition (reviewed in Lambert et al., 2001).

In women, progesterone levels remain low during the follicular phase of the menstrual cycle and then rise during the luteal phase until, in the absence of a fertilised ovum, levels fall sharply prior to menstruation (McLachlan et al., 1987). During the premenstrual period, many women experience psychological changes, which include mood swings, irritability and increased susceptibility to development of anxiety states such as panic (Le Melledo et al., 2000). Female laboratory rats show a similar cyclical hormonal profile but over a shorter time course (4 days in rats compared with 28 days in women; Butcher et al., 1974). Moreover, the peak level of plasma progesterone achieved toward the end of the cycle is similar to women (Butcher et al., 1974; McLachlan et al., 1987) and like women, female Wistar rats also become more susceptible to panicogenic challenge in the non-receptive (dioestrus) phase of their cycle (Olsson et al., 2002).

The physiological changes that underlie the development of premenstrual symptoms are not understood. However, they may be linked to changes in expression of $GABA_A$ receptor subunits when progesterone levels fall. Several studies have reported that withdrawal from a progesterone dosing regimen in female rats but not in males, precipitated increased anxiety accompanied by a significant increase in the levels of $GABA_A$ receptor α4 and δ subunit protein in the hippocampus and amygdala (Gallo and Smith, 1993; Smith et al., 1998; Sundstrom-Poromaa et al., 2002; Gulinello et al., 2003). The effects were due to changes in levels of allopregnanolone, rather than the decline in the progesterone concentration per se (Smith et al., 1998).

At present it is not known whether the natural cyclical variation in progesterone, and hence allopregnanolone levels, would be sufficient to trigger changes in $GABA_A$ receptor subunit expression. To address this question, we have used immunocytochemical techniques to investigate plasticity of $GABA_A$ receptor expression during the oestrous cycle in female rats. The midbrain periaqueductal gray matter (PAG) is a potential target for modulation by endogenous neurosteroids since the output neurones are under powerful GABAergic control and stimulation in this

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Abbreviations: ANOVA, analysis of variance; $GABA_A$, GABA type A receptor; GAD, glutamic acid decarboxylase; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; I_{max}, maximal response; PAG, periaqueductal gray matter; PB, phosphate buffer; PMDD, premenstrual dysphoric disorder.

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region evokes a panic-like state both in humans and in rats (Lovick, 2000). Moreover, we have shown that local application of allopregnanolone to this region produces a decrease in functional excitability (Lovick, 2001).

$GABA_A$ receptors are pentameric oligomers that commonly comprise three different classes of subunit. The precise subunit composition defines the physiological and pharmacological characteristics of the receptor subtype (reviewed in Hevers and Luddens, 1998). However, $GABA_A$ receptor composition is not immutable. Withdrawal from an exogenous progesterone-dosing regimen, for example, led to increased co-expression of $\alpha4$ and δ subunit protein in hippocampus (Sundstrom-Poromaa et al., 2002). Withdrawal from anxiolytic benzodiazepines which, like progesterone-derived neurosteroids, also produce their effects by facilitating $GABA_A$ receptor-mediated inhibition, produced an increase in the steady state mRNA levels of $\alpha4$ and $\beta1$ subunit mRNA in both the cortex and hippocampus (Holt et al., 1996). The genes that encode the $\alpha4$ and $\beta1$ subunits are found, together with those for $\alpha2$ and $\gamma1$, on human chromosome four and exhibit parallel expression during embryonic neurogenesis in the rat spinal cord (Ma et al., 1993). These observations, together with the suggestion that the δ subunit, found on chromosome 1, is often associated with $GABA_A$ receptor subtypes containing the $\alpha4$ subunit (Bencsits et al., 1999; Sur et al., 1999) encouraged us to explore the concomitant changes in expression of $\alpha4$, $\beta1$ and δ subunits in the PAG during the oestrous cycle. Our finding of parallel increases in the expression of these subunits in late dioestrus suggested that expression of a receptor with an $\alpha4\beta1\delta$ composition may occur at this time. In order to investigate the possible functional consequences of such an event, we also studied the properties of recombinant $\alpha4\beta1\delta$ receptors expressed in oocytes of *Xenopus laevis* and showed that these receptors display unusual desensitization kinetics and an increased sensitivity to GABA. Some of the results from this study have been published in abstract form (Dunn et al., 2003; Griffiths et al., 2002, 2003).

EXPERIMENTAL PROCEDURES

Immunohistochemistry

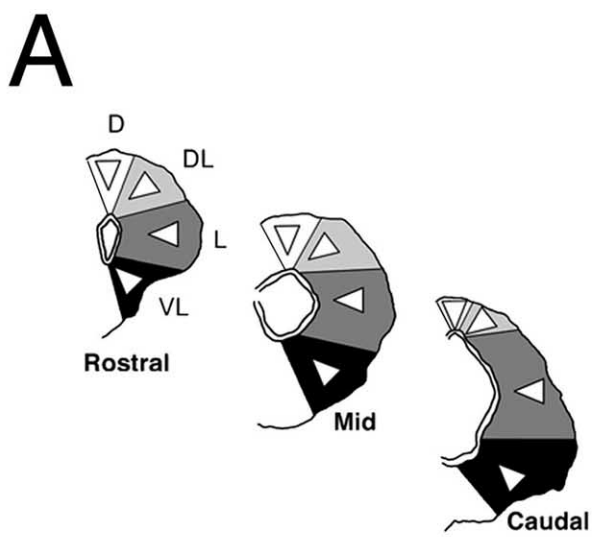
Adult male and female Wistar rats 200–250 g body weight were used. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986. The number of animals used in this study was the minimum necessary to give adequate statistical power to the results. Every effort was made to minimise any risk of suffering. In females, a vaginal smear was obtained immediately prior to starting each experiment. A staining kit (Diff-Quick; Dade Behring, Deerfield, IL, USA) was used to reveal the characteristic cytology of different stages of the oestrous cycle: proestrus, oestrus, early or late dioestrus (Bronson et al., 1966; Waynforth, 1980). Terminally anesthetized animals (urethane, 1–1.5 g kg^{-1} i.p.) were perfused with warm (30 °C) heparinized saline (100 U ml^{-1}) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 at room temperature. The brain was removed and post fixed for 1–2 h. Following cryoprotection overnight in 30% sucrose in PB at 4 °C, the midbrain was sectioned at 40 μm. Sections were incubated in primary antibodies directed against $\alpha4$, $\beta1$ or δ $GABA_A$ receptor subunits $\alpha4$ (N-19: sc-7355), $\beta1$ (N-19: sc-7361) and δ (C-20: sc-7368; Santa Cruz Biotechnology, USA), at a concentration of 10 μg ml^{-1} for 48–60 h, followed by a biotinylated anti-goat secondary antibody (0.75 μg ml^{-1} for 90 min) before immunoreactivity was revealed using a Vectastain ABC kit (Vector Laboratories) with diaminobenzidine as the chromogen. In order to minimize the effects of batch variation in processing, material from pairs of rats at different stages of the oestrous cycle was processed at the same time. Controls included pre-incubation of the primary antibody with blocking peptides containing the amino acid sequences against which the primary antibodies were raised (Santa Cruz Biotechnology; diluted one in five), or omission of either the primary or secondary antibody from the staining protocol. Immunostained sections were mounted on gelatinised slides, dehydrated, cleared and coverslipped using Hystomount (Hughes and Hughes, UK). The sections were viewed in an Olympus BH-2 microscope equipped with a drawing tube. Selected fields were photographed using an Olympus 3040 camera. Images were imported into Photoshop software for cropping but were not otherwise manipulated.

The PAG is organized functionally in terms of four longitudinal columns that are orientated dorsal, dorsolateral, lateral and ventrolateral to the aqueduct (Bandler and Shipley, 1994). For each rat, three sections were selected as representative of the rostral, mid and caudal PAG approximating to levels P5.8, P7.0 and P8.2 as defined in the atlas of Paxinos and Watson (1986; Fig. 1A). Using the drawing tube attachment on the microscope, the image of the PAG was projected onto a triangular shaped counting frame that represented an area of 50,000 μm^2 in the tissue observed using the $\times40$ objective. The counting frame was orientated with the apex toward the aqueduct (Fig. 1A) and the number of cells present within the frame in the dorsal, dorsolateral, lateral and ventrolateral PAG was counted. Counting was done by two observers, one of whom was blinded to the hormonal status of the animals. Material from rats at different stages of the oestrous cycle was assigned randomly to one or other counter. In order to check for consistency between counters selected sections were counted by both counters. The variation in cell counts was $<10\%$. For these sections the count entered into the database was the one from the observer initially assigned the section. The tissue density of immunostained cells within the dorsal, dorsolateral, lateral and ventrolateral PAG at rostral, mid and caudal levels of the PAG was measured in sections from five male rats and from four groups of female rats (five per group) at different stages of the oestrous cycle, i.e. proestrus, oestrus, early dioestrus and late dioestrus. Data were analyzed using one-way analysis of variance (ANOVA) followed by Fisher's PLSD post hoc test. Data were considered significant at the 5% level.

The intensity of immunostaining was quantified using ImageJ 1.29x software (NIH). In brief, pairs of fields from the same region of the PAG from brains that had been processed in parallel were photographed using the $\times40$ objective and the same camera settings and light intensity. A circular 6 μm^2 region of interest was selected on the digital image of each stained cell body and over an equal number of background regions. Stained cells were defined as discrete areas of immunoreaction product with a clearly defined boundary, >6 μm across the longest axis and with up to four tapering processes. The staining intensity of each cell was calculated by subtracting the mean gray value of all the background sample areas from the mean gray value for the cell.

Oocyte expression and two-electrode voltage clamp analysis

All cDNAs encoding the rat $GABA_A$ receptor subunits were subcloned into the expression vector pcDNA3.1 (Invitrogen, San Diego, CA, USA) and cRNA transcripts were prepared as described by Dunn et al. (1999). Oocytes from *X. laevis* (see Goldin, 1992) were injected with 50 nl of 1 mg/ml total cRNA. For each subunit combination used (Table 1), the transcripts were present



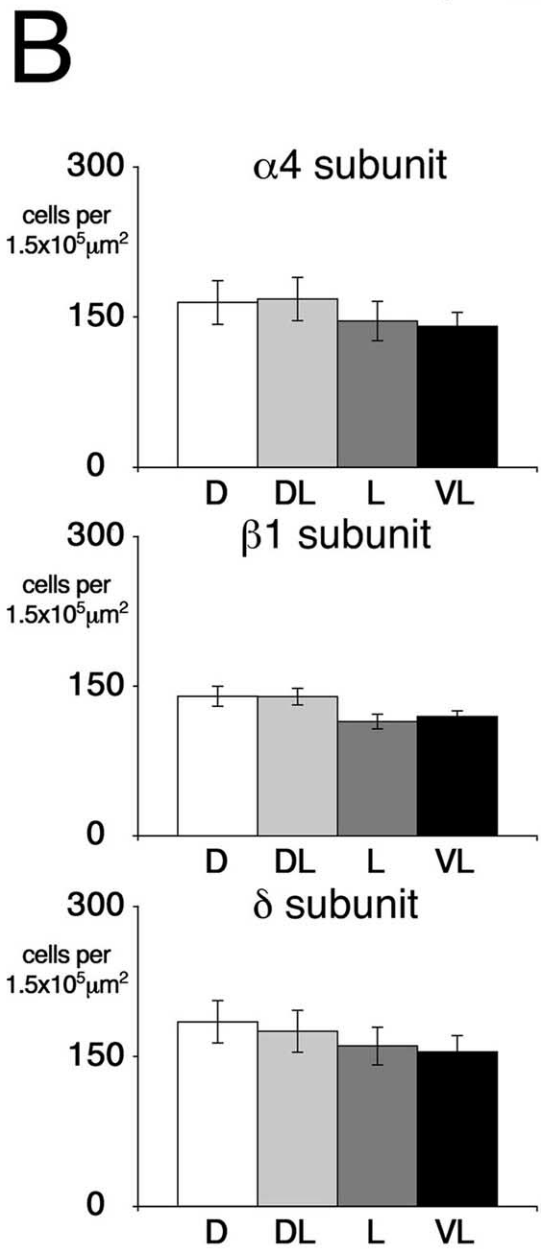


Table 1. The effect of changing subunit composition on the EC_{50} for GABA in recombinant $GABA_A$ receptors expressed in *Xenopus* oocytes; *n*=minimum of 5 for each data set.

Receptor subtype	EC_{50}±SEM (μM)
α1β2γ2L	32.8±2.5
α1β2γ3	13.7±3.5
α4β2γ3	~14.1 (I_{max} <15 nA)
α4β1γ3	27.3±3.2
α4β1δ	2.02±0.33

in a 1:1:1 stoichiometry. Following injection, the oocytes were maintained at 14 °C in Barth's solution (in mM): NaCl (88), KCl (1), $CaCl_2$ (0.5), $Ca(NO_3)_2$ (0.5), $MgSO_4$ (1), $NaHCO_3$ (2.4), HEPES (15), pH 7.4 supplemented with gentamicin (100 μg/ml). Standard two-electrode voltage clamp techniques were used to measure GABA-induced responses 2–7 days after cRNA injection. Currents were recorded using a GeneClamp 500 amplifier (Axon Instruments, Molecular Devices, Union City, CA, USA) and a holding potential of −60 mV. Data were collected and analyzed using pClamp6 software (Axon Instruments). Electrodes were filled with 3 M KCl and had resistances of 0.5–2.0 MΩ in frog Ringer's solution (in mM): NaCl (120), KCl (2), $CaCl_2$ (1.8) HEPES (5). During recordings, oocytes were continuously perfused at 5 ml/min with frog Ringer's. GABA EC_{50} values were obtained by agonist application for 30 s, at three concentrations per decade, appropriate time being allowed for recovery from desensitization. Experiments were repeated at least five times. Concentration-response curves appeared symmetrical about the EC_{50} suggesting that desensitization did not materially affect the results. Except where noted in Results, the maximal response (I_{max}) to a saturating concentration of GABA (3 mM) lay in the range of 100 nA-1 μA. Data were analyzed by nonlinear regression techniques using Prism3 (GraphPad Software, San Diego, CA, USA). The GABA concentration dependence of the observed current was fit by the equation:

$$I = (I_{max} \cdot [A]^n)/(EC_{50} + [A]^n)$$

where *I* is the measured amplitude of the evoked current, [*A*] is the GABA concentration, EC_{50} is the concentration of GABA producing 50% of the I_{max} and *n* is the Hill coefficient. In each experiment, the observed current was normalized to the I_{max} (100%), and these normalized values were used to construct the concentration-effect curves. Individual EC_{50} values were used to obtain the mean±S.E.M.

RESULTS

Diffuse immunoreaction product for α4, β1 or δ $GABA_A$ receptor subunits was present in the cytoplasm of neurones throughout the PAG in male and female rats. Staining was absent in the sections in which the primary antibody had been pre-incubated with blocking peptides and in sections in which the primary or secondary antibodies had been omitted from the staining protocol.

Fig. 1. (A) Diagrammatic representation of the PAG depicting the 12 triangular-shaped areas that were sampled to study the cellular expression of the $GABA_A$ receptor subunits. (B) Histograms show the mean density±S.E.M. of α4-, β1- and δ-immuno-positive cells present in the dorsal (D), dorsolateral (DL), lateral (L) and ventrolateral (VL) columns of the PAG in male rats; *n*=5 rats for each data set.

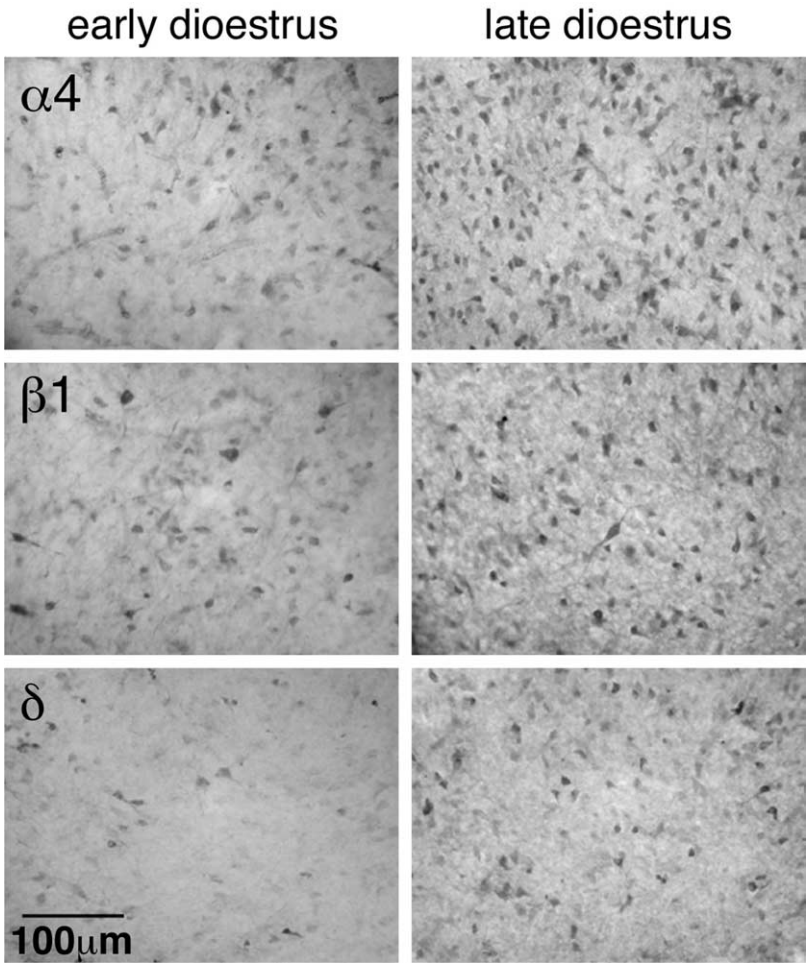


Fig. 2. Low power photomicrographs of immuno-positive cells containing α4-, β1- and δ- subunits within the PAG in female rats during early and late dioestrus. Note increased intensity of immunoreaction product in many cells during late dioestrus.

Immunostaining of $GABA_A$ receptor subunits in neurones in the PAG of male rats

The distribution of immunostained cells within the four columns of the PAG in male rats is illustrated in Fig. 1B. Initial analysis of the data revealed no significant differences between the numbers of stained cells present in sections taken from rostral, mid and caudal levels of the PAG. The numbers were therefore pooled for subsequent analyses. For each subunit, the density of immunostained cells also appeared similar in each of the four columns of the PAG, i.e. dorsal, dorsolateral, lateral and ventrolateral PAG.

$GABA_A$ receptor subunit immunostaining in the PAG at different stages of the oestrous cycle in female rats

Immunostaining for α4, β1 and δ receptor subunits was present in the PAG in female rats (Fig. 2). As in the males, there was no significant difference in the numbers of cells counted in different columns of the PAG in sections taken from the rostral, mid and caudal levels. The cell counts were therefore pooled. Subsequent analysis of the data for each of the receptor subunits studied revealed a significant effect of hormonal status and position within different columns of the PAG. Fig. 3 shows the density of α4, β1 and δ receptor subunit immunostained cells within the four sectors of the PAG for female rats at different stages of the oestrous cycle. In the early phases of the cycle, i.e. proestrus to early dioestrus, the numbers of α4 subunit immunoreactive cells present in each of the four columns of the PAG were similar, apart from oestrus when there was a transient but significant increase in the number of cells present in the dorsolateral column. However, in late dioestrus (equivalent to the late luteal phase in women), there was a significant increase in the density of α4 subunit positive cells in all columns. This effect was most pronounced in the dorsolateral column of the PAG where the number of immunoreactive cells almost doubled (Fig. 3).

Compared with α4 subunit-stained cells, β1 subunit-immunoreactive cells were less numerous. Moreover, in the early stages of the cycle, i.e. proestrus, oestrus and early dioestrus, the mean number of stained cells present in the dorsolateral column of the PAG was always lower than in the other columns (Fig. 3). However, as with the α4-immunostained material, late dioestrus was character-

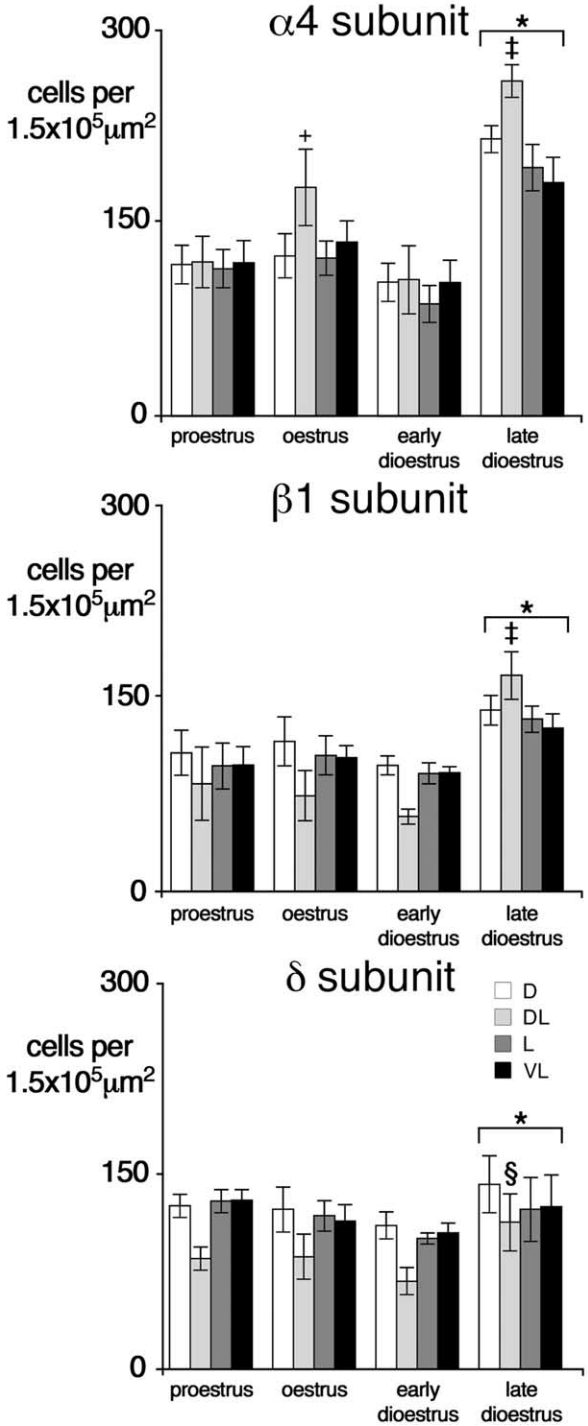


Fig. 3. Histograms show the mean density±S.E.M. of α4-, β1- and δ-immuno-positive cells present in the four columns of the PAG of female rats at each stage of the oestrous cycle; $n=5$ animals for each stage. * Total number of stained cells significantly greater than at other stages of the oestrous cycle. For the dorsolateral column: ‡ significantly greater number of α4 and β1 immunoreactive cells compared with all other stages of the cycle; + significantly greater number of α4 immunoreactive cells compared with proestrus and early dioestrus; § significantly greater number of δ subunit immunoreactive cells compared with early dioestrus. $P<0.05$ ANOVA followed by Fisher's PLSD post hoc test.

ized by a significant increase in the number of β1 subunit-immunostained cells in all four columns of the PAG, an effect that was most pronounced in the dorsolateral column of the PAG.

The distribution and density of δ subunit immunoreactive neurones was very similar to the β1 subunit-stained population. The total number of stained cells observed in late dioestrus was significantly greater than at the other stages of the oestrous cycle (Fig. 3). This effect was most pronounced in the dorsolateral column of the PAG. We also noted that the intensity of the immunostaining appeared greater in material obtained during late dioestrus for all three subunits (Fig. 2). This observation was confirmed by measuring the mean staining intensity per cell in the dorsolateral PAG in four rats in late dioestrus and comparing it to the staining intensity of cells in the four rats whose brains had been processed at the same time, two of which were in early dioestrus and two in proestrus. For each pair of rats, the mean intensity of immunostaining for each subunit in late dioestrus was increased. Immunostaining for α4 subunits was increased by 24–183%, β1 by 13–117% and δ by 42–183%.

Activation characteristics of recombinant receptors

The $GABA_A$ receptor consisting of α1β2γ2L subunits is the most common receptor subtype in the mammalian brain and this subtype exhibits an EC_{50} for GABA activation of 32.8±2.5 μM (Table 1). By sequentially changing a single subunit cRNA in the oocyte expression studies, the EC_{50}s for GABA-mediated responses could be determined for each of the receptor subtypes examined (Table 1). All the subunit combinations studied here exhibited robust currents with the exception of recombinant α4β2γ3 receptors where the maximum currents were insufficient (<15 nA) to allow an accurate estimation of the EC_{50}. The EC_{50} for GABA-induced currents for the α4β1δ receptor was some 10-fold lower than that for α1β2γ2L (Table 1). While the oocyte preparation is not amenable to the detailed study of agonist response kinetics, the α4β1δ combination exhibited obvious differences in current responses to GABA. Fig. 4 shows current responses for the α1β2γ2L and α4β1δ subunit combinations induced by a GABA concentration approximating its EC_{50} value for each receptor subtype. Although the other subunit combinations investigated exhibited similar behavior to the α1β2γ2L receptor, the α4β1δ receptor displayed much less desensitization during agonist application and noticeably slower deactivation upon removal of agonist.

DISCUSSION

The present study has demonstrated that plasticity of $GABA_A$ receptor subunit expression occurs during the oestrous cycle of the rat. Late dioestrus was associated with an increase in the expression of α4, β1 and δ subunit-immunopositive cells in the PAG. Not only did a larger number of neurones display immunoreactivity for α4, β1 or δ subunit protein but the mean density of the immunoreaction product in each stained cell was also increased. It is

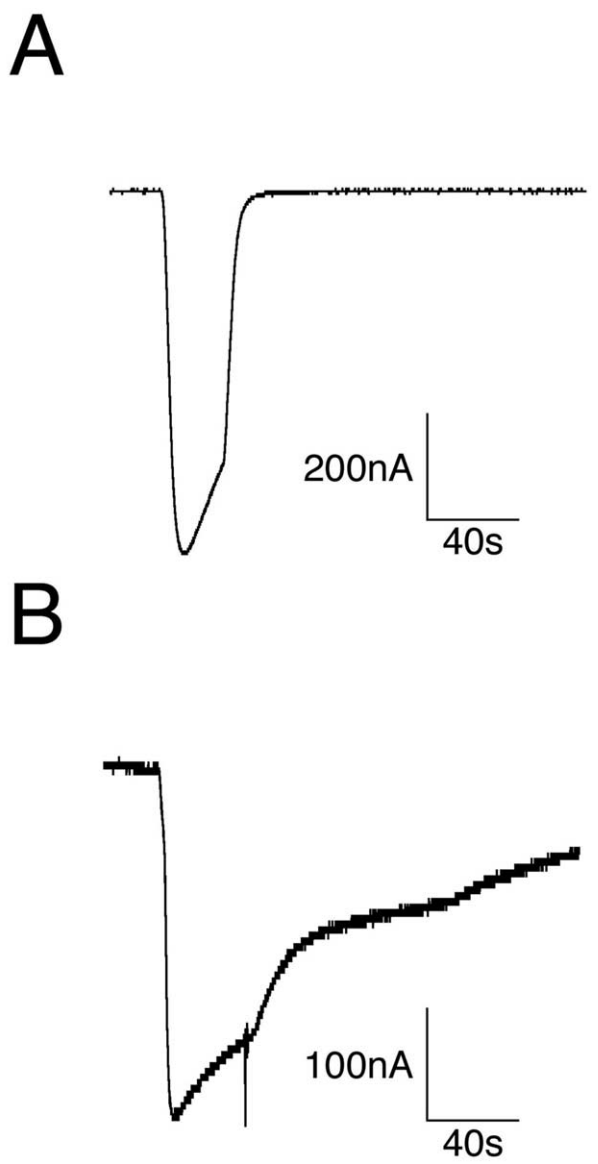


Fig. 4. Typical current traces recorded from *Xenopus* oocytes expressing the $\alpha1\beta2\gamma2L$ receptor (A) and $\alpha4\beta1\delta$ receptor (B) in response to a 30 s application of GABA at a concentration approximating its EC_{50} value in these receptor subtypes (30 μM and 3 μM respectively).

possible that new receptors were formed in cells that did not previously express the α4, β1 or δ subunits. Alternatively, the number of neurones in the PAG that express these subunits may remain constant throughout the cycle but the amount of immunoreaction product formed in some cells during the early phases of the cycle was too low to allow them to be visualized in the light microscope. Only when increased expression of receptor subunits occurred, leading to a greater receptor density per cell, would such neurones become visible. In the present study, immunoreaction product for each of the subunits investigated was present throughout the cytoplasm of the cells. Although the functional receptors would be located at the cell surface, the co-assembly of appropriate subunits into receptors is thought to take place initially in the endoplasmic reticulum before being transported to the Golgi apparatus and then exported to the surface membrane (Barnes, 2000). Thus immunoreaction product would be expected to be present throughout the cytoplasm. The increased numbers of cells displaying immunoreactivity in late dioestrus suggest that more receptors containing the α4, β1 and δ subunits were produced at this time and transported through the cytoplasm to the membrane. However, the disappearance of reaction product as the rats progressed to proestrus indicates that rather than being internalized or disassembled, the receptor subunit proteins are actually degraded or at least altered structurally, thus preventing antibody recognition. At the onset of early dioestrus plasma progesterone levels are high, but fall sharply over a period of about 8 h to stabilize at a low level in late dioestrus (Butcher et al., 1974). The increased numbers of α4, β1 and δ subunit-immunopositive cells present in late compared with early dioestrus indicate that significant changes in subunit receptor protein can occur within a matter of hours. This finding is in line with the rate at which $GABA_A$-receptor subunit expression can be dynamically regulated at the neuronal membrane (Barnes, 2000).

The coordinate increases in expression of α4, β1 and δ subunits in the PAG in late dioestrus are compatible with the notion that these subunits assemble to form a specific $GABA_A$ receptor subtype as they may do in other structures (Bencsits et al., 1999; Sur et al., 1999). In addition, the higher numbers of α4 compared with β1 and δ subunit immunoreactive neurones present in the PAG indicate that a significant number of receptors containing α4 subunit do not contain β1 or δ subunits (see also Bencsits et al., 1999). Parallel changes in expression of α4 and β1 subunits have been reported following chronic exposure to diazepam (Holt et al., 1996) suggesting that there may be coordinate expression of these two subunits. More recent studies using co-immunoprecipitation reported increases in α4 subunit and δ subunit expression in the hippocampus following withdrawal from an extended progesterone treatment regimen (Sundstrom-Poromaa et al., 2002). The finding of a concomitant increase in the expression of the α4, β1 and δ $GABA_A$-receptor subunits in the PAG at the time of the natural fall in progesterone during late dioestrus is consistent with these previous studies.

The present study focused only on α4, β1 and δ $GABA_A$-receptor subunits but it is possible that other subunits may undergo plastic changes as well. Interestingly, in δ-subunit knockout mice, immunoreactivity for γ2 but not α1, α2, α3 or α5-subunits increased in forebrain regions where δ-subunits were normally present (Peng et al., 2002). γ2-Subunit immunoreactive cells have been described in the PAG of rats (Pirker et al., 2000). Thus when the expression of δ-subunit containing receptors increases in the PAG, there may be a reduction in the density of receptors that contain the γ2-subunit. This would have considerable functional implications in terms of neuronal excitability as δ-subunit containing receptors are located extrasynaptically, whereas γ2-subunit containing receptors are found mainly at synaptic sites (Kneussel, 2002).

Our studies on recombinant $\alpha4\beta1\delta$ receptors in oocytes indicate that they display increased sensitivity to GABA. The EC_{50} for GABA was some 10-fold lower than at $\alpha1\beta2\gamma2L$ receptors. Interestingly, earlier reports suggested that $\alpha1\beta1\delta$ receptors, similar to the $\alpha4\beta1\delta$ receptor in this study, were not only about an order of magnitude more sensitive to GABA but also exhibited reduced desensitization during agonist application, and slower recovery after its removal (Saxena and Macdonald, 1994). The new receptors in the PAG that contain the δ subunit are likely to be located extrasynaptically (Nusser et al., 1998). In a double labelling study, immunoreactivity for $\alpha4$, $\beta1$ and δ subunits in the PAG co-localized with immunostaining for the GABA synthesizing enzyme glutamic acid decarboxylase (GAD) (Griffiths and Lovick, 2004). Moreover, the number of GAD-positive cells that were double labeled increased in late dioestrus when expression of $\alpha4$, $\beta1$ and δ subunits increased (Griffiths and Lovick, unpublished observations), suggesting that the new receptors were localized on GABAergic neurones. It is likely that many of the GABAergic cells are local interneurones, especially in the dorsolateral sector of the PAG. This region makes extensive intrinsic connections with other parts of the PAG (Jansen et al., 1998) but appears to contain few output neurones (Bandler and Shipley, 1994; Ennis et al., 1997; Krout et al., 1998; Reichling and Basbaum, 1991).

Receptors containing the δ subunit are thought to be responsible for mediating tonic inhibition (Nusser et al., 1998; Mody, 2001). They carry approximately four times the current carried by their synaptic counterparts (Nusser and Mody, 2002) and are thus likely to have significant importance in the control of neuronal excitability. An increase in the effectiveness of GABAergic tone at extrasynaptic $\alpha4\beta1\delta$ $GABA_A$ receptors on inhibitory interneurones in the PAG would lead to disinhibition of inhibitory tone and the overall excitability would increase.

$GABA_A$ receptors containing the δ subunit are also thought to be involved in mediating the effects of endogenous neurosteroids. A recent report has shown that the efficacy of a number of neuroactive steroids is markedly enhanced at $\alpha4\beta3\delta$ containing receptors compared with those comprising $\alpha4\beta3\gamma2$ (Brown et al., 2002). Moreover, δ knockout mice exhibit a marked reduction in sensitivity to the neurosteroids compared with their parent strain (Mihalek et al., 1999). The increase in expression of δ receptor subunits in the PAG during late dioestrus should lead to potentiation of the inhibitory actions of neurosteroids such as allopregnanolone (Lovick, 2001). However, the falling levels of allopregnanolone available to interact with the receptors during late dioestrus may offset this potential for increased neurosteroid modulation.

The presence of $\alpha4$ subunits confers insensitivity to benzodiazepines (Wafford et al., 1996). Thus increased expression of the $\alpha4$ subunit in late dioestrus may explain the variable sensitivity to the pharmacological effects of diazepam that is exhibited by mice at different stages of the oestrous cycle (Carey et al., 1992). Interestingly, women who suffer from premenstrual dysphoric disorder (PMDD) exhibit a reduced response to the benzodiazepines and symptom severity appears to be correlated with a greater blunting of the responses to the benzodiazepine, midazolam (Sundstrom et al., 1997). Other studies have reported that women who suffered from PMDD were particularly sensitive to the benzodiazepine antagonist flumazenil in the late luteal phase of their cycle, and responded with panic-like symptoms (Le Melledo et al., 2000). This finding is consistent with our preliminary observation that flumazenil acts as an inverse agonist at $\alpha4\beta1\delta$ $GABA_A$ receptors (Dunn et al., 2003).

In summary, the present study has demonstrated that neurones in the PAG show significant changes in $GABA_A$ receptor subunit expression as steroid hormone levels fluctuate during the oestrous cycle in female rats. In particular, the parallel rise in the number of neurones expressing $\alpha4$, $\beta1$ and δ $GABA_A$ receptor subunits during late dioestrus suggests increased expression of a receptor comprising these three subunits. In rats as in women, the increased anxiety levels which accompany falling progesterone and hence allopregnanolone levels, could result from decreased efficacy of GABAergic modulation in the PAG and other structures. However, falling progesterone was also associated with expression of new $\alpha4\beta1\delta$ receptors. When expressed in oocytes, recombinant $\alpha4\beta1\delta$ receptors were exceptionally sensitive to GABA. Since these receptors are likely to be located extrasynaptically, probably on GABAergic interneurones, the PAG would become more excitable as a consequence of inhibition of tonic activity in GABAergic interneurones. The extent to which the changes in receptor subunit expression in the PAG are replicated in other parts of the brain is not known. However, it seems reasonable to propose that similar changes in GABAergic function in other brain regions may contribute to the exacerbation of symptoms seen during the premenstrual period in a number of disparate syndromes e.g. catamenial epilepsy, postoperative nausea and vomiting, irritable bowel syndrome and panic disorder (Ensom, 2000).

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