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ScienceDirect
Pharmacology, Biochemistry and Behavior 90 (2008) 43 – 50
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
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GABA in the female brain — Oestrous cycle-related changes in GABAergic function in the periaqueductal grey matter

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Available online 23 December 2007

Abstract

In many women, aversive psychological and somatic symptoms develop during the late luteal phase of the menstrual cycle, when progesterone levels fall sharply. Following intravenous administration in anaesthetised rats, the progesterone metabolite allopregnanolone readily gained access to the periaqueductal grey (PAG), a region involved in generating panic-like anxiety, and inhibited neural activity via actions at $GABA_A$ receptors. Withdrawal of female rats from prolonged systemic dosing with progesterone leads to increased numbers of α4, β1 and δ $GABA_A$ receptor subunit-immunoreactive neurones in the PAG. In naturally cycling rats a similar upregulation occurred during late dioestrus, when progesterone levels fall. Functional experiments revealed that upregulation of α4β1δ receptor subunit expression was associated with a decrease in GABAergic tone in the PAG and increased responsiveness to a panicogenic CCK_2 receptor agonist. The oestrous cycle-linked plasticity of GABA receptors was absent in rats housed in quiet conditions in an isolated room suggesting that environmental factors may be able to influence the central response to hormonal changes. In susceptible animals, i.e. those housed in a communal animal holding room, oestrous cycle-related changes in GABAergic circuits may underlie the development of increased anxiety levels that represent a rodent counterpart to premenstrual syndrome in women.

Keywords: Periaqueductal grey matter; Progesterone; Allopregnanolone; $GABA_A$ receptor subunit plasticity; Oestrous cycle; Rat; Panic; Premenstrual syndrome

1. Introduction

The female brain operates in a constantly changing hormonal milieu that reflects the cyclical changes in the production of ovarian hormones during the menstrual cycle (oestrous cycle in animals). The major ovarian hormones progesterone and oestrogen are highly lipophilic, thus changes in plasma levels of these steroids are reflected by parallel changes in their concentration within the brain (Paul and Purdy, 2004). During the late luteal (premenstrual) phase of the menstrual cycle, when plasma and hence brain levels of oestrogen and progesterone fall, many women experience adverse psychological and physiological changes. These may include depression, irritability, anxiety, aggression, avoidance of social activity, breast tenderness and bloating: a constellation of symptoms known collectively as premenstrual syndrome (PMS) (Steiner, 1997). Sensitivity to most cutaneous pain stimulation procedures (the exception being electrical stimulation) develops during the luteal phase of the cycle (Fillingim and Ness, 2000; Riley et al., 1999) and patients with neuropathic pain or other anxiety-related diseases often report a worsening of their symptoms at this time (Ensom, 2000).

Premenstrual symptoms fail to develop during anovulatory cycles (Bäckström et al., 2003), indicating a causative link between fluctuating gonadal hormone levels and changes in brain function. However, there is considerable variation in the severity of the symptoms experienced by different women, despite similar changes in hormone levels (Schmidt et al., 1994). Clinical evidence indicates that the expression and severity of premenstrual symptoms may be influenced by environmental factors (Halbreich, 2003 for a review). This raises the interesting possibility that the environment may play a permissive role with respect to hormonal influences on neural function.

The late luteal (premenstrual) phase of the menstrual cycle is characterised by falling levels of progesterone (McLaughlin et al., 1987). Progesterone can influence neuronal activity in diverse brain regions via actions at specific nuclear bound receptors (Falkenstein et al., 2000). In addition to genomic effects, the steroid produces rapidly acting non-genomic effects via actions at the $GABA_A$ receptor. These effects are mediated not by the native steroid but via its neuroactive metabolite allopregnanolone (ALLO), which is a potent positive allosteric

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doi:10.1016/j.pbb.2007.12.014

modulator of the effects of GABA at $GABA_A$ receptors (Lambert et al., 2001). The steroid-sensitive isoforms of the GABA receptor — in particular those containing α4 and δ subunits, have been shown to exhibit remarkable plasticity in response to changing levels of ALLO in the extracellular environment. In the hippocampus and amygdala withdrawal from treatment with progesterone triggered an increase in levels of α4 and δ subunit mRNA, which was accompanied by changes in neuronal responsiveness to GABA (Gulinello et al., 2003a,b; Smith et al., 1998a,b).

More recently we have shown that a similar upregulation of α4, β1 and δ $GABA_A$ receptor subunit protein occurs in the periaqueductal grey matter in the midbrain (PAG) in response to withdrawal from progesterone (Griffiths and Lovick, 2005a). The PAG plays a pivotal role in the regulation of anxiety-related emotional behaviours as well as being a source of descending control on spinal nociceptive processing (Lovick, 1996 for a review). Upregulation of $GABA_A$ receptor subunit expression in the PAG when progesterone levels fall at the end of the menstrual/oestrous cycle could therefore lead to changes in the intrinsic excitability of the PAG circuits, predisposing to the development of increased anxiety and hyperalgesia during the premenstrual period. In male rats the intrinsic responsiveness of the PAG has been shown to vary according to the environmental context in which afferent stimuli are received (Walker and Carrive, 2003). This raises the interesting possibility that in females, the environment could be a factor that influences the magnitude of the PAG's response to oestrous cycle-linked changes in plasma progesterone levels.

2. Methods

All data were obtained from adult male and female Wistar rats, 239–300 g body weight. The animals were obtained from Charles River, UK and housed in Birmingham Biomedical Services Unit.

2.1. Electrophysiological studies

To determine whether neuroactive metabolites of progesterone can gain access to the PAG and influence its neurones directly, an initial series of electrophysiological studies was carried out on urethane-anaesthetised Wistar rats (1.0 g kg^{-1} (female); 1.5 g kg^{-1} (male) i.p.). Multibarrelled glass pipettes were used for extracellular recording and iontophoretic application of drugs to single neurones in the dorsal half of the PAG (Brack and Lovick, 2007). Ratemeter records were made of changes in neuronal firing rate evoked in response to intravenous injection of allopregnanolone (ALLO) or its synthetic water soluble analogue ORG20599 (Organon, UK). The role of GABAergic systems in mediating the effects of the steroid was probed in male rats by direct application of ORG20599, GABA and bicuculline (BIC, a $GABA_A$ receptor antagonist) to single neurones in the PAG by microiontophoresis. In female rats, oestrous cycle-linked changes in the level of ongoing GABAergic tone in the PAG were investigated by comparing responsiveness to BIC at different stages of the oestrous cycle. Oestrous cycle-linked changes in the functional responsiveness of the PAG circuitry were also investigated by measuring changes in neural activity in response to iontophoretic administration of the panicogenic CCK_2 receptor agonist pentagastrin at different stages of the cycle. In all studies, the location of recording sites was marked by iontophoretic deposition of pontamine sky blue dye (Brack and Lovick, 2007). Analysis was restricted to those cells in which the recording site was located in the dorsal half of the PAG.

2.2. Immunohistochemical studies

To investigate $GABA_A$ receptor subunit expression in the PAG, brains were obtained from terminally anaesthetised female rats that had been fixed by retrograde perfusion via the abdominal aorta with 100 ml heparinised saline followed by 200 ml 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. After post-fixation (2 h) and cryoprotection in 30% sucrose in PB, 40 μm thick frozen coronal sections were cut through the PAG. Immunoreactivity was revealed using well-characterised antibodies directed against the α4, β1 and δ $GABA_A$ receptor subunits (Griffiths and Lovick, 2005a) or the GABA synthesizing enzyme glutamic acid decarboxylase (GAD) (Griffiths and Lovick, 2005a,b). Controls incorporated into the procedure included omission of primary and secondary antibodies and pre-incubation of the antibodies with the peptide sequence against which the antibody had been raised. Estimations of the density of immunoreactive cells were made by counting the numbers of cells present within representative triangular-shaped regions of the PAG using a camera lucida arrangement (Lovick et al., 2005).

The stage of the oestrous cycle in female rats was determined by examination of vaginal smears (Brack et al., 2006). Smears were collected daily between 9–10 am Monday to Friday to establish normal cycling. Further smears were collected from individual rats immediately prior to sacrifice for immunohistochemistry and at intervals during electrophysiological experiments to check that the animals had remained in the same stage of their cycle throughout the experiment. In some rats a progesterone withdrawal regime (Smith et al., 1998a,b; Griffiths and Lovick, 2005a) was used to assess the effect that falling levels of the steroid might have on $GABA_A$ receptor subunit expression. Twice daily treatment of rats with progesterone (5 mg kg^{-1} i.p.) for 6 days was followed by a 24 h withdrawal period.

2.3. Environmental influences

On arrival from the supplier (Charles River) cohorts of rats were kept in one of two holding rooms within the animal facility. In the "standard" environment the animals were housed in pairs in cages furnished with sawdust, woodshavings and an adventure toy that consisted of an upturned plastic box with an entrance cut out of one wall. Standard rat chow and water were supplied ad libitum. The holding rooms were illuminated on a 12 h on 12 h off light cycle with lights on at 7 am. Male rats and other female rats of the same and different strains were housed in the same room. This communal holding room was visited

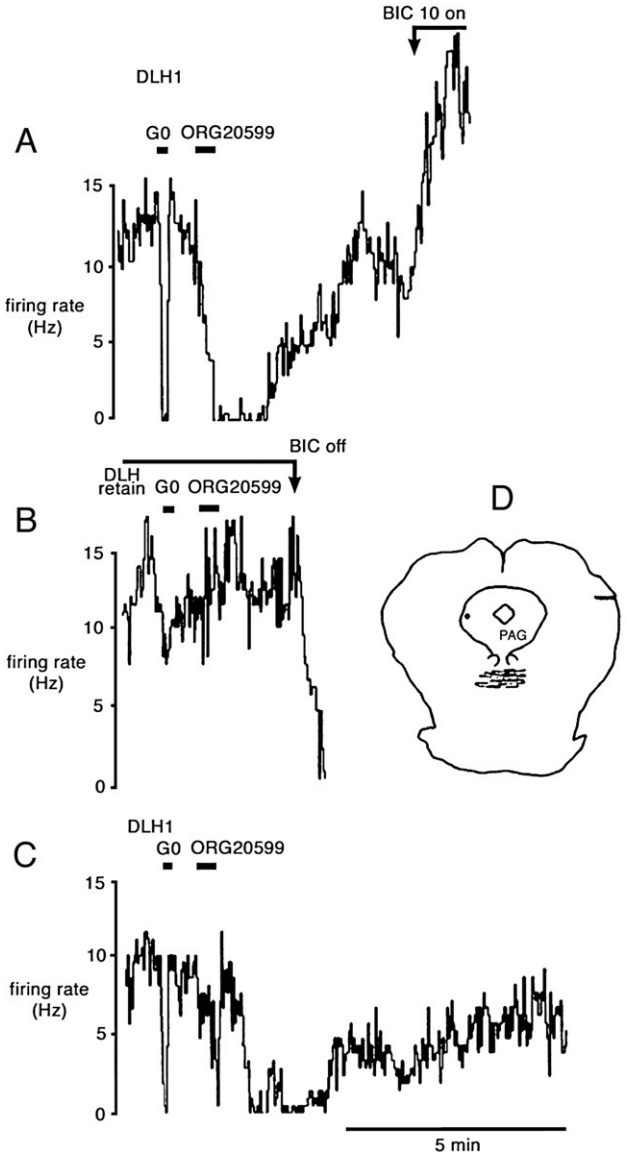

Fig. 1. Ratemeter records of the firing rate of a neurone in the PAG. A. Against a raised level of background activity induced by continuous application of D,L-homocysteic acid (DLH, 1 nA), GABA (G) and ORG20599 inhibited firing. Application of bicuculline (BIC, 10 nA) produced a dramatic increase in firing as GABAergic tone on the cell was blocked. B. To determine the effectiveness of BIC in blocking responses at $GABA_A$ receptors, basal firing was first returned to the pre-BIC level by stopping ejection of DLH. Under these conditions the responses to both GABA and ORG20599 were blocked. C. Recovery of the response. D. Location of the blue spot made to mark the recording site in the lateral PAG.

frequently by a wide range of laboratory personnel for cage cleaning, weighing, checking and issuing of animals etc. The radio played and was often interrupted by announcements over the PA system. For daily collection of vaginal smears the rats were taken in their cages into an adjacent room. Other rats were housed in pairs in the same type of cage as described above but in a quiet isolated room (no other animals in the room, no visitors, no talking, no radio). This "isolated" group was subjected to minimal disturbance from laboratory personnel except for the daily smears, which were carried out in the same room by the same laboratory personnel each day, who behaved in a subdued manner with minimal talking. Regardless of its housing environment, each rat was kept for at least 10 days after arrival from the supplier before being sacrificed for immunohistochemical processing of brain tissue. Selected rats were removed to a separate room for induction of anaesthesia in preparation for fixation of the tissue by vascular perfusion (see above). Midbrains from 4 cohorts of rats were examined: 2 from the "standard" environment and 2 from the "isolated" environment.

3. Results

3.1. GABAergic tone in the PAG in males

The majority of cells recorded in the PAG using multibarrelled micropipettes were either silent or showed only a low level of ongoing activity. To facilitate the study of inhibitory events, continuous iontophoretic application of D,L-homocysteic acid (DLH) was used to induce firing. Against this background activity, iontophoretic application of GABA (0–10 nA for 10 s) rapidly and reversibly produced a dose-related inhibition of activity in every cell tested ($n=12$, Figs. 1 and 2A). Application of BIC (10–20 nA) produced an increase in firing rate. In order to test the response to GABA against the same background firing rate as the control, the ejection of DLH was reduced during application of BIC. The response to GABA was blocked in the presence of bicuculline in all cells ($n=5$, Fig. 1).

3.2. Acute steroid modulation of GABA tone in the PAG

Intravenous bolus administration of allopregnanolone (ALLO, 0.1 mg kg^{-1} dissolved in 40% cyclodextran, $n=3$) or the synthetic water soluble analogue ORG20599 (Organon, UK,

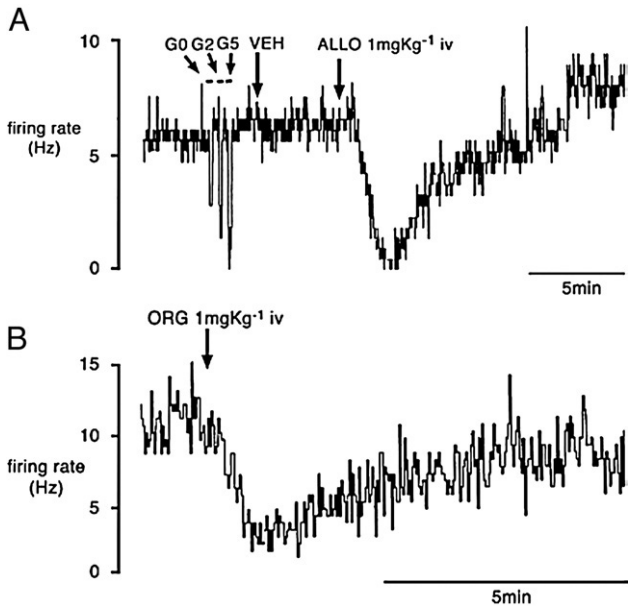

Fig. 2. A. Ratemeter record shows short-lasting dose-related inhibition of firing in response to iontophoretic application of GABA with increasing currents (G 0–5 nA) and longer-lasting response to intravenous injection of allopregnanolone (ALLO). B. Inhibition of firing evoked in response to i.v. injection of ORG20599 but not the vehicle (VEH, 40% cyclodextrin).

1 mg kg^{-1}, $n=3$) inhibited DLH-induced firing of neurones in the PAG (Fig. 2). In these cells the firing rate started to slow down 26–48 s after starting to inject the steroid and the rate remained suppressed for 360–374 s. When administered directly onto neurones in the PAG by iontophoresis (10–30 nA for 20 s, $n=8$) ORG20599 also produced an inhibition of firing (Fig. 1). In contrast to the brisk onset and offset of the response to GABA, the effect of ORG20599 was slow to build up and did not became maximal until 10–20 s after a 20 s period of drug ejection. Firing rate was depressed for 34–208 s. In 4 cells the response to ORG20599 was re-tested in the presence of BIC at a dose that blocked the inhibitory response of the same cell to GABA. During this time the total number of spikes fired was reduced to 49.5±3.1% of the number fired during the equivalent time period immediately prior to application of the drug. In the presence of BIC the inhibitory response to ORG20599 was completely suppressed and replaced instead by a 15–29% (mean 19.5±3.1%) increase in firing rate (Fig. 1).

3.3. GABAergic tone in the PAG in females at different stages of the oestrous cycle

As in males, most neurones recorded in the PAG in females were quiescent and a basal level of firing was induced by continuous application of DLH. Against this level of activity GABA (0–10 nA) produced a brisk reversible inhibition of activity. A further increase in firing was induced in the presence of BIC, revealing the presence of ongoing GABAergic tone, as in males. The responsiveness to BIC showed marked changes at different stages of the oestrous cycle. In response to stepwise increases in ejection current (0–30 nA) BIC evoked a dose-related increase in firing rate in all cells tested ($n=47$). Higher currents often induced changes in spike amplitude or an irregular firing pattern. Quantitative analysis of firing rate was therefore restricted to responses to ejection of BIC using currents of 30 nA or less. In response to standard ejecting currents of 20 nA and 30 nA the maximum firing rate achieved in the presence of BIC was significantly higher for cells recorded from rats in oestrous and late dioestrus compared to the other cycle stages (Fig. 3A) reflecting a lower level of ongoing GABAergic inhibition during oestrus and late dioestrus.

3.4. Changes in functional responsiveness to a CCK2 agonist during the oestrous cycle

To investigate whether oestrous cycle-linked changes in the level of ongoing GABAergic tone in the PAG could lead to changes in the functional responsiveness of anxiogenic and pro-nociceptive circuits, we investigated responses to the panicogenic CCK_2 receptor agonist pentagastrin (van Megan et al., 1994). At low ejecting currents (<30 nA), iontophoretic application of pentagastrin to neurones in the PAG produced an increase in firing rate that was reduced in the presence of the selective CCK_2 receptor antagonist CR2845 (Revel et al., 1998). The effectiveness of pentagastrin was found to be significantly enhanced in oestrus and late dioestrus compared to the other stages of the oestrous cycle (Fig. 3B).

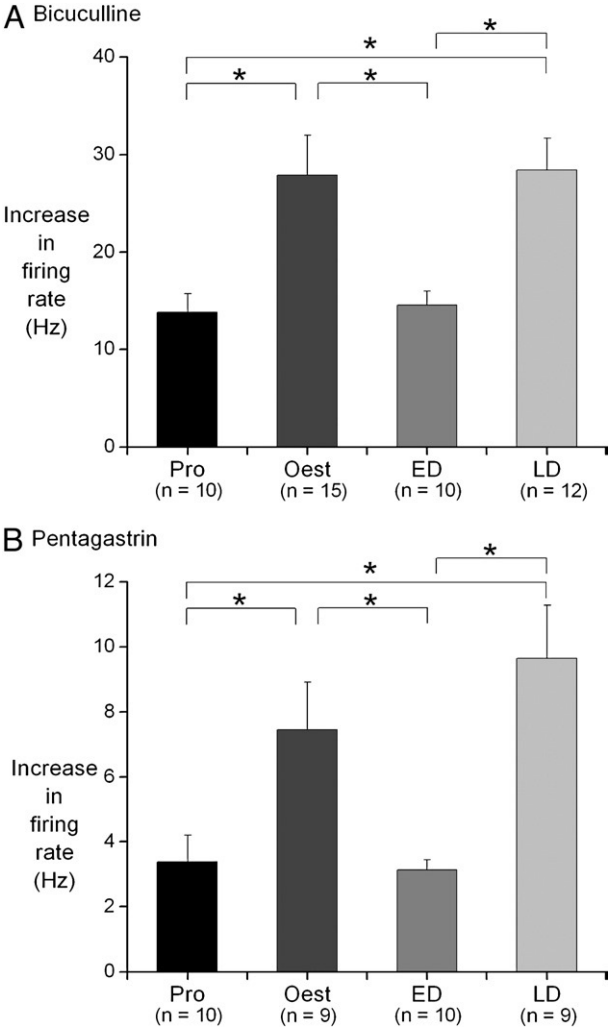

Fig. 3. A. Increase in firing rate evoked by iontophoretic application of A, bicuculline (BIC 20 nA) and B, pentagastrin (PG 30 nA) to neurones in the PAG at different stages of the oestrous cycle. B. Data show means±SEM. Pro: proestrus; Oest: oestrus; ED: early dioestrus; LD: late dioestrus. *$P<0.05$, ANOVA with Fisher's post hoc test. Data redrawn from Brack and Lovick (2007).

3.5. $GABA_A$ receptor subunit expression in the PAG

Diffuse immunoreaction product for α4, β1 and δ $GABA_A$ receptor subunits was present in the cytoplasm and proximal dendrites of neurones throughout the PAG in male and female rats. The central nuclear area of each cell was pale, indicating predominantly cytoplasmic and membrane locations of the subunit protein.

3.6. Plasticity of $GABA_A$ receptor subunit expression in female rats

The density of immunolabelled cells was estimated from counts of labelled cells made in representative 50,000 μm^2 areas in the dorsal, dorsolateral, lateral and ventrolateral sectors of the PAG at rostral, mid and caudal levels, i.e.12 areas sampled per rat. Progesterone withdrawal lead to increased numbers of α4, β1 and δ $GABA_A$ receptor subunit-immunoreactive neurones in the PAG (Fig. 4A). In spontaneously cycling rats a similar

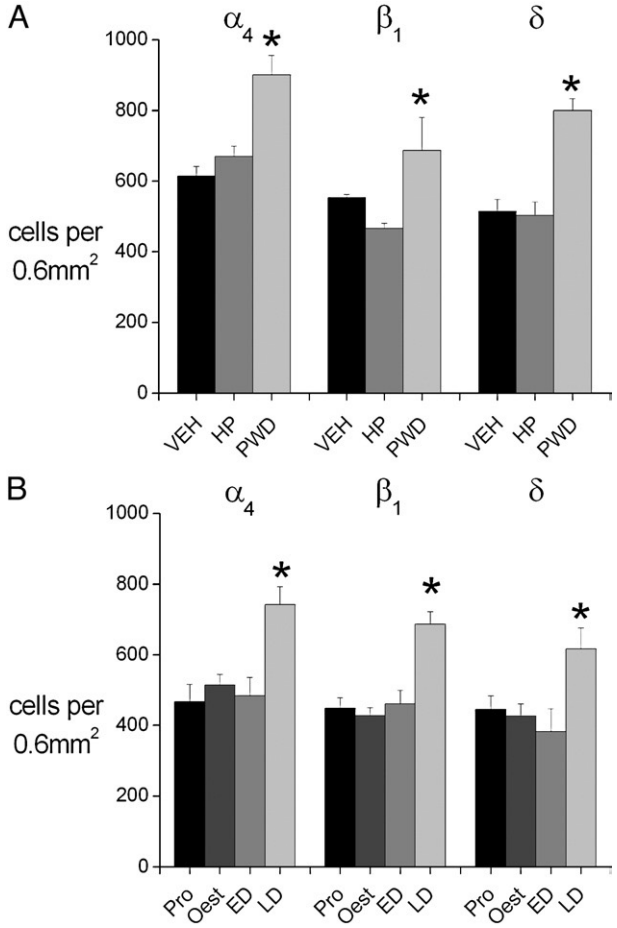

Fig. 4. Histograms show density of α4, β1 and δ $GABA_A$ receptor subunit-labelled cells A, following withdrawal from chronic treatment with progesterone and B, at different stages of the oestrus cycle. Abbreviations P: proestrus, O: oestrus; ED: early dioestrus, LD: late dioestrus; PWD: progesterone withdrawal, VEH: vehicle treatment; HP: high progesterone. *Significantly different from other stages of the cycle ($P<0.05$, ANOVA). Data redrawn from Griffiths and Lovick (2005a,b).

upregulation of these subunits also occurred during dioestrus (Fig. 4B), in line with the natural fall in progesterone levels that occurs at this time (Watanabe et al., 1990). Neurones that were immunopositive for the GABA synthesizing enzyme glutamic acid decarboxylase (GAD) were also present throughout the PAG. Double labelling studies were carried out to identify whether this cell population expressed receptors that contained α4, β1 or δ $GABA_A$ receptor subunits. More than 90% of the subunit-positive neurones co-localised GAD indicating that these subunits were expressed almost exclusively by GABAergic neurones (Fig. 5A). However, a significant minority the GAD-immunopositive population (almost 30% in the early stages of the oestrous cycle) did not co-localise α4, β1 or δ $GABA_A$ receptor subunits. The total number of GAD-positive cells remained constant during the oestrous cycle. However, when the expression of α4, β1 or δ $GABA_A$ receptor subunits increased in late dioestrus, the new subunit-positive cells were found to co-localise GAD. At this stage of the cycle, 90–92% of the GAD-immunopositive population expressed α4, β1 or δ $GABA_A$ receptor subunits (Fig. 5B).

3.7. Environmental influence on oestrous cycle-related plasticity of $GABA_A$ receptor subunit expression

In these studies $GABA_A$ receptor subunit expression in the PAG was compared in rats housed in either the standard conditions of a busy multiple user animal holding facility or in a quiet isolated environment. Tissue from two cohorts of "isolated" rats was obtained in early Spring 2006 and early Spring 2007 whilst tissue from two cohorts of "standard" rats was obtained in early Summer 2006 and early Spring 2007. Since there was no difference between the results obtained from the cohorts of rats that had been housed under the same conditions but 10–12 months apart, the data were pooled. Immunostaining was compared in rats at two different stages of the oestrous cycle: during proestrus, when α4, β1 or δ $GABA_A$ receptor subunit expression was expected to be low (see above) and during late dioestrus, when α4, β1 or δ $GABA_A$ receptor subunit expression was expected to be high (see above). The two cohorts of rats housed under standard conditions showed the expected increase in the numbers of subunit-immunoreactive cells in the PAG during late dioestrus (Fig. 6), in agreement with our previous findings (Lovick et al., 2005; Griffiths and Lovick 2005b). In contrast, in the two cohorts of animals housed in the isolated environment, the oestrous cycle-related upregulation of $GABA_A$ receptor subunit expression in the PAG

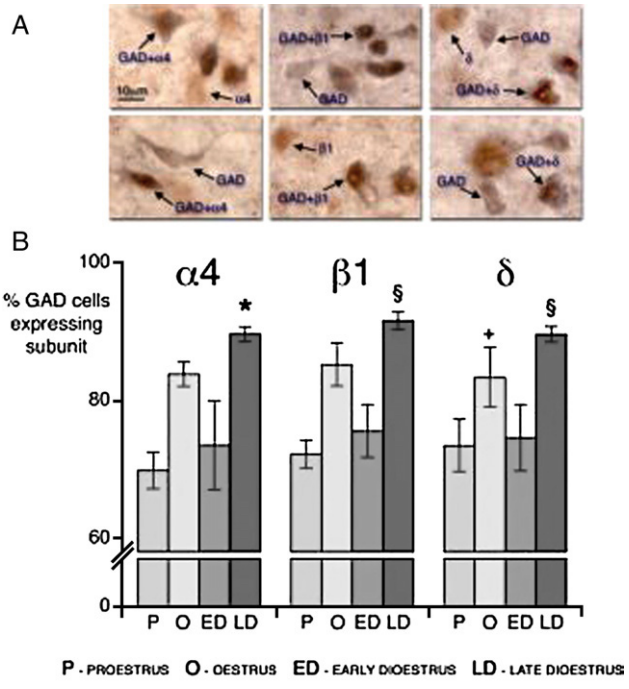

Fig. 5. A. Examples of neurones in the PAG immmunostained for GAD (blue/grey immunoreaction product) and α4, β1 or δ $GABA_A$ receptor subunits (red/brown immunoreaction product). Note presence of both reaction products in many cells. B. Histograms show percentage increase in number of double-labeled GAD-immunoreactive neurones at different stages of the oestrous cycle. *Significantly different from proestrus, oestrus and early dioestrus (α4) and from proestrus and early dioestrus (β1 and δ); § significantly different from proestrus and early dioestrus (δ). $P<0.05$ (ANOVA), Abbreviations as in Fig. 4. Data redrawn from Griffiths and Lovick (2005b).

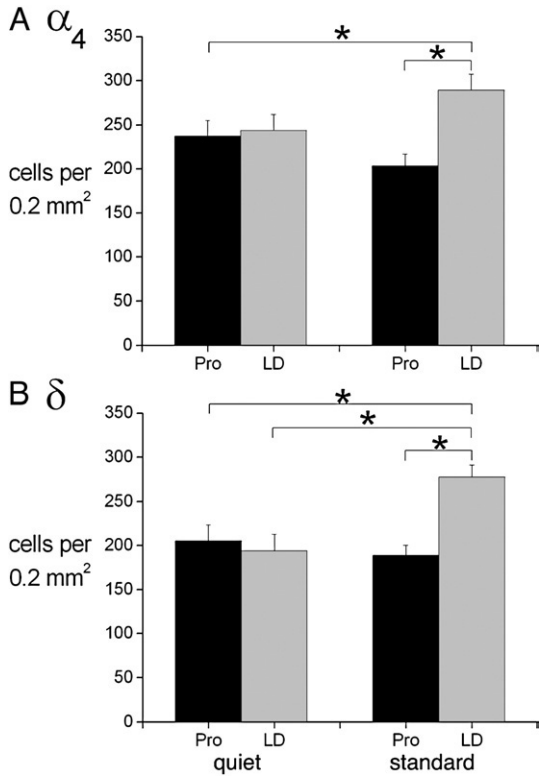

Fig. 6. Histograms show effect of housing rats under standard or quiet conditions on the density of α4 and δ $GABA_A$ receptor subunit-labelled cells in the PAG in rats in proestrus (Pro) and late dioestrus (LD). $n=8–11$ animals per group. *Significantly different ($P<0.05$, ANOVA with Fisher's post hoc test).

failed to occur (Fig. 6), even though the animals were cycling normally as judged from daily changes in vaginal cytology.

4. Discussion

The rapid onset, long lasting inhibition of neuronal activity following intravenous injection of allopregnanolone and its synthetic analogue ORG 20399 reflects the ready access of these steroids to the midbrain from the plasma. The effectiveness of iontophoretically applied ORG 20599 and its blockade in the presence of bicuculline indicates that this steroid can inhibit neuronal firing by a direct $GABA_A$ receptor-mediated action within the PAG. General anaesthetics are known to modulate the activity of GABAergic systems (Orser et al., 2002) and may therefore introduce a significant bias on the excitability of neural circuits *in vivo*. In the present study on anaesthetised preparations, the presence of inhibitory GABAergic tone in the PAG, which was revealed using the $GABA_A$ antagonist bicuculline, may therefore represent an unphysiological state. However, in studies on slices of PAG, which are unaffected by the presence of anaesthetics, we found that output neurones were quiescent or showed only low levels of activity (Lovick and Stezhka, 1999; Stezhka and Lovick, 1994). Moreover, the presence of ongoing GABAergic inhibition has also been demonstrated in the PAG of conscious rats (Schenberg et al., 1983). These findings suggest that urethane anaesthesia does not significantly compromise the functioning of the circuitry in the PAG.

Our experiments in male rats indicate that acute changes in plasma steroid levels can rapidly (within minutes) modulate the level of GABAergic inhibition in the PAG. In females, the situation is confounded by the longer term (day to day) changes in the expression of steroid-sensitive $GABA_A$ receptors in the PAG that occur in response to the cyclical change in expression of gonadal steroids during the oestrous cycle. During late dioestrus, a parallel upregulation of α4, β1 and δ $GABA_A$ receptor subunit expression occurred on the GABAergic interneurone population, which suggested that new receptors with the α4β1δ configuration had been formed. Since receptors containing the δ subunit are located extrasynaptically and carry tonic currents (Mody, 2001, 2005), increased numbers of α4β1δ receptors on the GABAergic interneurone population should lead to a *decrease* in the level of their ongoing activity. As a consequence of the reduced GABAergic tone, the intrinsic excitability of the PAG output neurones should *increase*. The results of our functional experiments indicated this to be the case.

One of the functional consequences of the reduced GABA tone was an increased responsiveness to pentagastrin. CCK_2 receptor agonists such as pentagastrin are panicogenic in humans and in animals and produce both anxiogenic and pro-nociceptive effects via actions within the PAG (Bertoglio and Zangrossi, 2005; Li and Han, 1989; Netto and Guimaraes 2004; van Megan et al., 1994; Zanoveli et al., 2004). Increased responsiveness of the CCK_2 receptor system during the late luteal phase in women could therefore contribute to the increase in anxiety and the hyperalgesia that develop at this time.

Whilst the above data provide a scenario that can explain how falling plasma and brain levels of progesterone during late dioestrus lead to changes in the excitability of the PAG circuitry, they cannot account for the functional changes that occur during oestrus. In oestrus, plasma levels of progesterone rise transiently. However, this surge does not appear to be long enough to induce upregulation of $GABA_A$ receptor subunits (see Brack and Lovick, 2007 for discussion of this point). During oestrus oestrogen levels are low, having fallen from the peak levels reached 24 h earlier in proestrus (Watanabe et al., 1990). In hippocampal tissue, where nuclear oestrogen receptor alpha (ERα) is present in a subset of GABAergic interneurones (Hart et al., 2001), withdrawal from oestrogen has been shown to be associated with a delayed (24 h) decrease in ongoing GABA tone at $GABA_A$ receptors, which corresponded with a decrease in the expression of GABA synthesizing enzymes (Rudick and Woolley, 2001). High concentrations of ERα are present within the PAG (VanderHorst et al., 2005). Thus by analogy with the findings in hippocampus, it is tempting to speculate that an oestrogen-triggered decrease in GABA synthesis could lead to reduced GABAergic inhibition in the PAG during oestrus, with a consequent increase in the excitability of the output neurone population. However, further experiments to examine the specific effects of fluctuations in oestrogen levels on GABAergic circuitry are clearly needed to resolve this question.

Falling levels of either progesterone or oestrogen during the oestrous cycle in the rat could at different times and via different mechanisms, lead to a reduction in the level of GABAergic inhibition. In women, the hormonal profile during the menstrual

cycle does not parallel exactly that of the rat in which the effects of withdrawal from oestrogen and progesterone on GABA tone, are temporally separated. During the late luteal phase in women, when premenstrual symptoms become worst, levels of progesterone are falling dramatically from a high level and plasma oestrogen levels are also undergoing a more modest fall (McLaughlin et al., 1987). The effects of these two events on GABAergic inhibition may therefore be compounded. In regions such as the PAG, the ensuing disinhibition of the anxiety and pain control circuitry could contribute to the menstrual cycle-related disorders.

Whilst cyclical changes in female gonadal hormone levels are clearly the primary factor that triggers changes in GABAergic control systems in the PAG, the response appears to be modified by environmental influences. In groups of rats housed in a quiet room isolated from other animals, oestrous cycle-evoked upregulation of expression of $GABA_A$ receptor subunit proteins was no longer detectable. This effect was reproduced in two cohorts of rats tested 12 months apart and is thus unlikely to represent an aberrant response of a single group of animals.

At present the reason for the lack of receptor subunit plasticity in animals housed in the "isolated" environment is not clear. The rats housed in quiet conditions still cycled as indicated from their vaginal smears. However, it is possible that absolute levels of steroid hormone levels were different in the groups of rats housed under different conditions. Previous studies reported that male rats raised in isolation showed a reduction in basal brain and plasma progesterone and allopregnanolone levels (Serra et al., 2000). In the present study in female rats brain and plasma levels of neuroactive steroids were not measured. Even so, absolute levels may not be important since clinical studies in women indicate that differences in plasma hormone levels between individuals do not correlate with the severity of premenstrual symptoms (Schmidt et al., 1994). The severity of premenstrual symptoms in women has however, been linked to levels of external stress (Halbreich, 2003). Young male rats subjected to social isolation stress after weaning (housing in individual cages as opposed to group housing) showed increased levels of α4 and δ $GABA_A$ receptor subunit immunoreactivity in the hippocampus compared to group-housed animals (Serra et al., in press). Differences in stress levels induced by housing conditions in the female rats used in the present study might therefore have been a significant contributory factor in determining receptor subunit plasticity. However, in proestrus there was no difference between the absolute numbers of subunit-labelled cells in the rats housed in "standard" or "isolated" conditions (Fig. 4). It was only at the later stage of the oestrous cycle, in late dioestrus, that differences in receptor subunit expression became apparent. Although it will be necessary to confirm these findings in non-cycling females and/or in males, it seems unlikely that the "isolated" housing environment in the present study influenced basal subunit expression. Rather, the environmental influence on receptor expression appeared to be specific to oestrous cycle-linked plasticity of $GABA_A$ receptor subunit expression in the rat. The functional characteristics of the GABAergic circuitry of the PAG in rats housed in the "isolated" condition have yet to be investigated. However, given the absence of changes in $GABA_A$ receptor subunit expression during the oestrous cycle in these rats, it seems unlikely that the functional responsiveness of the PAG circuitry will change significantly.

5. Summary

The present findings demonstrate that normal cyclical variations in plasma levels of gonadal hormones can produce significant changes in the functioning of GABAergic circuitry. In particular, falling levels of progesterone have the ability to trigger increased expression of extrasynaptic $GABA_A$ receptors and to increase the intrinsic level of excitability of neural circuitry within the PAG, which leads to functional changes that include increased responsiveness to panicogenic agents. These changes may contribute to the development of the aversive psychological symptoms that plague so many women during the late luteal phase of the menstrual cycle.

Acknowledgements

The work reported in this article was supported by grants from the Wellcome Trust, British Heart Foundation and Medical Research Council. ORG20599 was a generous gift from Organon UK.

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