Allopregnanolone and ganaxolone increase the firing activity of dorsal raphe nucleus serotonergic neurons in female rats

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Abstract
Accumulating evidence suggest a reciprocal interaction between neurosteroids, especially 5α-pregnan-3α,5α-THP, allopregnanolone, and the serotonergic (5-HT) system. Both 5-HT and neurosteroids seem to play an important role in the pathophysiology of major depression. We have previously shown that a 7-d treatment with 3α,5α-THP drastically increases the spontaneous firing activity of dorsal raphe nucleus (DRN) 5-HT neurons in female rats. This study was thus undertaken to better characterize this modulation and to assess the effects of ganaxolone, a synthetic analogue of 3α,5α-THP. Female rats received 50 mg/kg . d of 3α,5α-THP or ganaxolone for 3 and 7 days. Others received 3α,5α-THP concomitantly with the antiprogestin RU486 (50 mg/kg . d, each), which was also administered alone. Acute experiments were also carried out with a single injection of 3α,5α-THP (1 mg/kg). Finally, both 3α,5α-THP and ganaxolone (50 mg/kg . d) were administered along with the selective serotonin reuptake inhibitor (SSRI) citalopram (10 mg/kg . d). In-vivo extracellular unitary recordings of 5-HT neurons from the DRN, revealed that 3α,5α-THP and ganaxolone increased their firing activity after 3 and 7 d of treatment. A 7-d treatment with RU486 had the same effect. Furthermore, an increase could be seen as soon as after 30–60 min following a single injection with 3α,5α-THP. Interestingly, both 3α,5α-THP and ganaxolone prevented the citalopram-induced reduction in firing activity after 3-d treatments. These data demonstrate the ability of 3α,5α-THP and ganaxolone to positively modulate the firing activity of DRN 5-HT neurons in female rats. Moreover, these results suggest that these neuroactive steroids might represent interesting adjuvants in the treatment of mood disorders in female patients.

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Introduction
Women are twice as likely to suffer from major depression than men (Angst et al., 2002; Bijl et al., 2002; Blazer et al., 2001; Breslau et al., 1995; Kessler et al., 1993, 1994; Kessler and Walters, 1998; Maes et al., 1986; Regier et al., 1988; Robins et al., 1984). Furthermore, onset or exacerbation of depressive episodes are more frequent during periods of hormonal fluctuations such as puberty, menstrual cycles, the postpartum period and menopause (Burt and Stein, 2002; Endicott, 1993; Endicott and Halbreich, 1988; Hamilton, 1993; Kornstein, 1997; Weissman and Klerman, 1985; Yonkers and White, 1992). Ovarian hormones have thus been hypothesized to play an important role in women's mood disorders (Endicott, 1993; Eriksson, 1999; Joffe and Cohen, 1998; Pajer, 1995; Parry, 1989). Serotonin (5-HT) has also long been implicated in the pathophysiology of depression (Coppen, 1967; Lapin and Oxenkrug, 1969), the most compelling evidence being probably the enhancement of 5-HT neurotransmission seen following all anti-depressant treatments (Blier and de Montigny, 1999; Racagni and Brunello, 1999).

Ovarian steroids modulate the expression of different proteins of the 5-HT system, including 5-HT receptors (see review by Bethea et al., 1999). Moreover, plasma and cerebrospinal fluid (CSF) levels of neuroactive steroids, such as 5α-pregnan-3α-ol,20-one...
(3α,5α-THP, allopregnanolone) seem altered during depressive episodes and normalized by antidepressant treatments, mood improvements being correlated with the increase of 3α,5α-THP levels in the CSF (Romeo et al., 1998; Ströhle et al., 1999, 2000; Uzunova et al., 1999). This could suggest, in addition to their respective implication in depression, a functional interrelationship between ovarian steroids and the 5-HT system.

Studies in rodents also support these observations as animal models of depression decrease cerebral levels of 3α,5α-THP in several brain areas (Dong et al., 2001; Serra et al., 2000; Uzunova et al., 2003). Moreover, treatments with various antidepressants increase 3α,5α-THP levels (Uzunov et al., 1996; Uzunova et al., 2004), while 3α,5α-THP produced an antidepressant-like effects in the Porsolt forced swimming test (Khisti et al., 2000; Khisti and Chopde, 2000). Conversely, in the same paradigm, inhibition of its formation, using finasteride (a selective inhibitor of 5α-reductase), leads to depressive-like symptoms (Azzolina et al., 1997).

We have previously shown, using in-vivo electrophysiological extracellular recordings, that a 7-d treatment with 3α,5α-THP drastically increased the spontaneous firing activity of dorsal raphe nucleus (DRN) 5-HT neurons in female rats, thus offering a biological basis for the putative antidepressant properties of 3α,5α-THP. The present study was undertaken to better characterize this modulation in terms of time-frame and mechanism of action. Similarly, the effects of ganaxolone, the 3β-methylated synthetic analogue of 3α,5α-THP (Monaghan et al., 1997), were also assessed. Ganaxolone presents a pharmacological profile (Carter et al., 1997), very similar to that of 3α,5α-THP, and was used to assess whether it could potentially be used as a substitute for 3α,5α-THP in this paradigm. Finally, the potential therapeutic use of these two steroids as adjuvants to a selective serotonin reuptake inhibitor (SSRI), in the treatment of depression, was also investigated.

Method

Animals

Freely cycling female Sprague–Dawley rats (Charles River, St Constant, Québec, Canada), weighing between 250 and 325 g and kept under standard laboratory conditions (12:12 light/dark cycle with access to food and water ad libitum), were used for the experiments. Ethical committee approval was obtained from the McGill University Animal Ethical Care Committee. Animals were treated following the Canadian Council on Animal Care (CCAC) guidelines.

Treatments with steroids

All steroids were dissolved in 3% (v/v) ethanol/distilled water and administered intracerebroventricularly (i.c.v.) by means of a cannulae (Alza, Palo Alto, CA, USA), which was implanted in the left lateral ventricle of the rat brain. For acute administrations, the cannulae was attached to a 5 μl Hamilton syringe, while for chronic treatments it was connected to a subcutaneous osmotic minipump (Alza), which continuously delivered the steroids. Surgeries were performed as described by the manufacturer (Alza) and under chloral hydrate anaesthesia. The doses of steroid used during chronic administrations were 50 μg/kg . d, whereas the acute dose of 3α,5α-THP was 1 g/kg.

Females received either a single acute injection, or a 3- or 7-d treatment with 3α,5α-THP. This steroid was also administered concomitantly with the progesterone receptor antagonist RU486 for 7 d. Some females received only RU486 for 7 d. Ganaxolone was administered for 3 or 7 d. Controls were treated with the vehicle (3% ethanol) for the appropriate period of time. Finally, 3α,5α-THP, ganaxolone or the vehicle were co-administered with citalopram (a SSRI, 10 mg/kg . d) for 3 d. Citalopram was dissolved in distilled water and also administered by means of a subcutaneous osmotic minipump (Alza). One osmotic minipump contained only one drug or one steroid. In the cases of concomitant administrations, two minipumps were used. Dosages of neurosteroids and durations of the treatments were chosen according to results obtained in previous studies, using the same electrophysiological model (Robichaud and Debonnel, 2004; Robichaud et al., 2002).

Electrophysiological experiments

All rats were anaesthetized by an intraperitoneal injection of chloral hydrate (400 mg/kg). Additional doses of 100 mg/kg were administered when needed. Rats were immobilized in a stereotaxic apparatus and their body temperature was maintained at ~37 °C throughout the experiment by a thermistor-controlled heating pad.

Extracellular unitary recording of serotonergic neurons were obtained with single-barreled glass micropipettes pulled in a conventional manner, filled with a 1 M NaCl solution and of final impedance ranging between 2 and 6 MΩ. A 4-mm-diameter hole was drilled in the skull of the rat ~1 mm anterior of
lambda and centred with respect to the midline. The unitary activity of DRN 5-HT neurons was recorded by lowering the micropipette on the midline, along descents covering the antero-posterior length of the nucleus from 300 to 1500 m anterior of lambda.

Spontaneously active DRN 5-HT neurons were encountered over a depth of 1 mm starting immediately below the ventral border of the Sylvius aqueduct. They were identified according to the criteria of Aghajanian: a slow and regular rhythmical firing rate and a shape of action potential with a large initial positive spike of 1–2 ms duration and a post-spike hyperpolarization (Aghajanian et al., 1978; Aghajanian and Vandermaelen, 1982).

For each experimental group, the basal firing rate of 5-HT neurons was calculated by averaging the firing rate of each neuron measured. This was achieved by recording, for at least 60 s, each 5-HT neuron encountered in complete descents in the DRN of at least five rats.

In the acute experiments, however, at least 10 rats were used in each experimental group. At least one descent was performed prior to the i.c.v. acute injection and one descent was performed during each subsequent 30-min period.

**Statistics**

Statistical analyses were performed with the software SigmaStat for Windows Version 2.0 (Jandel Corporation, San Rafael, CA, USA). Average values are expressed as mean ± S.E.M. One-way ANOVA, with $\alpha = 0.05$, followed by a post-hoc analysis using Tukey’s method of comparison vs. control were used for evaluating statistical significance. Results ($F$) of statistical analysis are expressed in terms of degrees of freedom.
freedom between groups and number of groups compared. Significance was considered as $p < 0.05$.

**Drugs**

Steroids used were: 5α-pregnan-3α-ol,20-one, RU486 (purchased from Steraloids, Newport RI, USA) and 

3α-hydroxy-3β-methyl-5α-pregn-20-one (ganaxolone, a generous gift from Dr Purdy, University of California, San Diego, CA, USA). Citalopram was kindly provided by Lundbeck (Copenhagen, Denmark).
As shown in Figure 1, a 7-d treatment with 3α,5α-THP increased significantly the firing activity of 5-HT neurons in female rats. In order to assess the potential role of progesterone receptor (PR) in mediating this effect, some rats were concomitantly treated with the PR antagonist RU486. RU486 did not prevent the effect of 3α,5α-THP as an elevated firing activity of the same magnitude was still present after this combined treatment (Figure 1b). Moreover, RU486 had an unexpected effect on its own and enhanced the firing activity of 5-HT neurons (Figure 1c).

A similar effect on 5-HT neuronal firing rate, which was increased from 0.98 to 1.92 Hz, could also be observed following only 3 d of administration of 3α,5α-THP (Figure 2a). Furthermore, acute experiments showed an increase in firing activity as early as 30–60 min following a single injection of 3α,5α-THP (1 μg/kg i.c.v.) (Figure 2b). Before, and up to 30 min following the injection, no statistically significant difference in 5-HT neuronal firing rate was observed between treated rats and controls (Figure 2b). However, in the following time-frames (i.e. 30–59 and 60–90 min post-injection), this difference became statistically significant from ~1.2 to 1.8 Hz (Figure 2b).

Citalopram, administered for 3 d, reduced the basal firing activity of 5-HT neurons by ~50%, (Figure 3). Because of its ability to increase the firing activity of DRN neurons, it was hypothesized that 3α,5α-THP might prevent this initial reduction in firing activity. Indeed, the 5-HT neuronal firing rate of rats co-treated with 3α,5α-THP and citalopram did not differ from that of controls (Figure 3a).

The effect of ganaxolone (the synthetic analogue of 3α,5α-THP) was then investigated. Both 3- and 7-d administrations of ganaxolone increased the firing activity of 5-HT neurons when compared to the appropriate controls (Figure 4). Similarly to 3α,5α-THP, ganaxolone was also able to partially prevent the citalopram-induced decrease of the firing activity of 5-HT neurons. Their firing rate remained significantly lower in citalopram-treated rats compared to controls but this difference no longer reached statistical significance when ganaxolone was co-administered with citalopram (Figure 3b).

Discussion

The first finding of this study is the enhanced firing activity of 5-HT neurons observed following a treatment with either 3α,5α-THP or ganaxolone. A greater firing activity of 5-HT neurons, previously shown in female rats after 7 d of 3α,5α-THP administration, was confirmed by the present experiments. Furthermore, this increase was already present after 3 d of treatment.
and 30–60 min following an acute i.c.v. administration. Similarly, both 3- and 7-d administrations of the synthetic analogue ganaxolone enhanced the firing activity of 5-HT neurons.

We have previously shown that during pregnancy the spontaneous firing rate of 5-HT neurons increases in parallel with plasmatic progesterone levels, to finally reach an enhancement > 100% in late pregnancy (Klink et al., 2002). However, a chronic treatment with a daily dose of 50 g/kg progesterone failed to modify the firing activity of DRN 5-HT neurons (Robichaud and Debonnel, 2004). In rats, the principal metabolic pathway for cerebral progesterone seems to be its sequential reduction into 5α-DHP and 3α,5α-THP (Karavolas and Hodges, 1991; Korneyev et al., 1993). Furthermore, 3α,5α-THP has often been shown to be responsible for various effects observed with progesterone on neuronal activity (Costa et al., 1995; Gulinello et al., 2001; Smith et al., 1998a,b). It seemed, therefore, possible that 3α,5α-THP is mediating the modulation of 5-HT neuronal activity observed during pregnancy (Klink et al., 2002). The present data and those obtained in previous experiments (Robichaud and Debonnel, 2004) therefore suggest that the increased firing rate observed during pregnancy could be the results of the additional effects of several neurosteroids including 5α-DHP and 3α,5α-THP.

The mechanism(s) by which 3α,5α-THP and ganaxolone increase the 5-HT neuronal firing activity remains unclear. The fact that 3α,5α-THP induces a relatively rapid (within 30 min) enhancement of the firing activity of 5-HT neurons suggests that a genomic mechanism of action, such as mediated via PR, is not likely. This is also supported by the lack of effect of progesterone itself (Robichaud and Debonnel, 2004). However, to completely rule out a potential role of PR in the effects of 3α,5α-THP, the PR antagonist RU486 was used. RU486 was not only unable to prevent the increase in 5-HT neuronal firing activity induced by 3α,5α-THP, but RU486 by itself also enhanced the basal firing activity of 5-HT neurons. RU486 is not selective to PR and also binds glucocorticoid receptors (GR) (Nordeen et al., 1995). However, the antagonistic effects on GR are unlikely to underlie this increase of 5-HT neuronal firing activity since corticosterone itself was ineffective in this regard. The present results thus remain puzzling and other experiments will be required to explain this phenomenon.

The 5-HT1A somatodendritic autoreceptor and the GABA_A receptor exert an important role in the control of the firing activity of 5-HT neurons. During pregnancy, we have shown that 5-HT1A autoreceptors are partially desensitized (Robichaud et al., 2002). If this desensitization was brought about by 3α,5α-THP, levels of which rise dramatically during pregnancy, intracerebral administration of this steroid would also be expected to reduce the function of 5-HT1A autoreceptors. This could explain, in part, the enhanced 5-HT neuronal firing activity reported by the present study. However, it cannot constitute the only reason as, in anaesthetized animals, there is no tonic activation of the 5-HT1A receptor.

The modulation of 5-HT neuronal activity by 3α,5α-THP and ganaxolone could also result from their interaction with GABA_A receptors since both of them are potent positive allosteric modulators (Carter et al., 1997; Mascia et al., 2002). In rats, DRN 5-HT neurons are under a tonic GABAergic inhibition, which is mostly mediated by GABA_A receptors (Gervasoni et al., 2000; Innis and Aghajanian, 1987). Interestingly, the GABAergic tonic inhibition of 5-HT neurons was dramatically reduced during pregnancy compared to cycling females (Robichaud et al., 2002). Again, if 3α,5α-THP was responsible for the reduced GABAergic tonic inhibition of the 5-HT neurons, it would probably occur in the present protocol. Furthermore, it is plausible that ganaxolone, having a very similar pharmacological profile at GABA_A receptors (Carter et al., 1997), might have a comparable effect. Accumulating evidence also suggests that sustained high levels of neuroactive steroids reduce GABA_A receptor responsiveness (Concas et al., 1998; Friedman et al., 1993; Gulinello et al., 2001; Robichaud and Debonnel, 2002; Yu et al., 1996; Yu and Ticku 1995a,b). Therefore, the combination of a reduced sensitivity of 5-HT1A autoreceptors, a reduced sensitivity of GABA_A receptors, and a reduced tonic inhibition by endogenous GABA, could constitute, at least, part of the explanation for the increase in firing activity of DRN 5-HT neurons observed in the present experiments.

The third and most interesting finding of this study is the ability of both 3α,5α-THP and ganaxolone to prevent the SSRI citalopram to induce a reduction of the firing activity of 5-HT neurons. It has been established for many years that treatments with SSRI...
cause an initial decrease in the firing activity of 5-HT neurons in male rats (Chaput et al., 1986; Romero et al., 1996). SSRIs, by blocking reuptake, increase the amount of extracellular 5-HT, which activates somatodendritic 5-HT_{1A} autoreceptors, leading, initially to an inhibition of the firing activity of 5-HT neurons and, later, to a gradual desensitisation of these receptors. However, to our knowledge, this is the first report confirming that it is also true concerning females. Furthermore, the present report indicates that a 3-d treatment with either 3α,5α-THP or ganaxolone, administered concomitantly with the SSRI citalopram, can prevent this initial reduction of the firing activity of 5-HT neurons in females. If the delay in therapeutic onset of action of SSRIs is indeed due to the time that 5-HT neurons recover from the initial reduction in firing activity, then preventing this reduction might be very helpful in accelerating the beneficial effects of these antidepressants.

An inverse correlation between CSF and plasma levels of 3α,5α-THP and the intensity of major depression in humans has been reported (Romeo et al., 1998; Ströhle et al., 1999; Uzunova et al., 1998). Moreover, animal models have shown that reduced cerebral levels of 3α,5α-THP are associated with depressive-like behaviour (Dong et al., 2001; Frye and Walf, 2002; Uzunova et al., 2003, 2004) while administration of this steroid leads to antidepressant-like effects (Khisti et al., 2000; Khisti and Chopde, 2000). No study has yet assessed the antidepressant-like effect of ganaxolone. However, the antidepressant-like effect of 3α,5α-THP in the Porsolt forced swimming test is potentiated by the GABA_A agonist muscimol and prevented by the antagonist bicuculline (Khisti et al., 2000; Khisti and Chopde, 2000). Ganaxolone, having similar pharmacological characteristics at GABA_A receptors as 3α,5α-THP (Carter et al., 1997), may potentially lead to similar behavioural effects.

The efficacy and potency of 3α,5α-THP and ganaxolone are very similar (Carter et al., 1997). However, an important distinction between them is found in the fact that ganaxolone is not metabolized into a hormonally active compound (Monaghan et al., 1999). For this reason, ganaxolone was expected to be a good candidate for treating epilepsy (Monaghan et al., 1999). Indeed, it was found to efficiently protect against a variety of seizure types in rodents (Carter et al., 1997; Gasior et al., 2000; Reddy and Rogawski, 2000a,b). Moreover, based on experiments assessing changes in the GABA_A receptor subunit following withdrawal from long-term exposure to these steroids, ganaxolone is expected to induce less withdrawal effects than those observed after discontinuation of chronic treatment with 3α,5α-THP or other GABA_A-receptor-positive modulators (Mascia et al., 2002). Finally, ganaxolone had anti-epileptic activity in humans (Laxer et al., 2000) and has an interesting pharmacokinetic profile and is safe and well tolerated, at up to relatively high doses, in both men and women (Monaghan et al., 1997). Combined with the present results, the fact that ganaxolone has already been used safely in humans in a therapeutic context suggests that this synthetic steroid could possibly be useful in the treatment of depression.

In conclusion, the present experiments show that both 3α,5α-THP and ganaxolone increase the firing activity of 5-HT neurons, and that they both can prevent the citalopram-induced reduction of this activity. This not only offers a biological basis for the antidepressant-like effect of 3α,5α-THP but also supports the hypothesis that ganaxolone might have such beneficial properties. Furthermore, our data suggest that 3α,5α-THP or ganaxolone could constitute good candidates as adjuvants to reduce the delay before therapeutic onset, seen with SSRIs. Since naturally occurring neuroactive steroids might not be suitable for chronic treatments (due to their very short half-life and side-effects profile) (Gasior et al., 2000), if ganaxolone had antidepressant properties, it could be especially promising as a treatment or as an adjuvant in the treatment of mood disorders in female depressed patients.

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Statement of Interest
None.

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