NOTE TO USERS

MODULATION OF DORSAL RAPHE NUCLEUS SEROTONERGIC NEURONS BY NEUROACTIVE STEROIDS IN RELATION TO MOOD DISORDERS

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STATEMENT OF CONTRIBUTIONS

I contributed to some of the experiments as well as to the writing of Chapter 2 but Dr. R. Klink did most of the experiments and writing. I did most of the experiments and wrote most of Chapter 3, which was complemented by Dr. R. Klink's work. I did all the experiment for all the other chapters and I wrote them all with the helpful guidance of my supervisor. With the exception of parts of Chapter 2 and 3, I was responsible for data collection, statistical analysis and manuscript writing.

ABSTRACT

Important gender differences exist regarding affective disorders, and depression affects women two times more than men. Accumulating evidence suggests a functional interrelationship between ovarian steroids, the serotonergic (5-HT) system and depression. The objective of this thesis was thus to study the modulation of the 5-HT neuronal firing activity by neuroactive steroids and compare it between genders. It was achieved by means of *in vivo* extracellular unitary recordings of dorsal raphe nucleus (DRN) 5-HT neurons in anesthetized rats.

The basal firing rate of DRN 5-HT neurons was significantly higher in males (M) and pregnant females (P17) as compared to freely cycling females (F). During pregnancy, 5-HT_{1A} autoreceptors were partially desensitized, which is consistent with the higher 5-HT neuronal firing activity. The GABAergic tonic inhibition of 5-HT neurons was lower in both M and P17 as compared to F, which is also in agreement with their greater 5-HT neuronal firing rate.

In F, 5β-pregnane-3,20-dione (5β-DHP), 5α-pregnane-3α-ol,20-one (3α,5α-THP), dehydroepiandrosterone (DHEA), its sulfated form DHEAS, testosterone (T), 17β-estradiol (17β-E) and ganaxolone (a synthetic analog to 3α,5α-THP) significantly increased the firing activity of 5-HT neurons. Of those, only DHEAS, T and 17β-E were also effective in M. The effect of 3α,5α-

THP and ganaxolone in F, as well as of DHEAS in both M and F, could be seen as early as after a 3-day treatment. Furthermore, 3α,5α-THP and ganaxolone prevented the initial decrease in firing activity caused by citalopram (a selective 5-HT reuptake inhibitor), which is responsible for its delay of therapeutic action. However, DHEAS could prevent it only partially in both genders.

These results constitute strong evidence of gender differences in both the basal firing activity of 5-HT neurons and in their modulation by neuroactive steroids. They also present some mechanisms of action by which gender and hormonal fluctuations influence the 5-HT neuronal function. Finally, the results of this thesis offer a cellular basis for the putative antidepressant effects of neurosteroids, which may prove important particularly for affective disorders in women.

RÉSUMÉ

La propension à développer une dépression majeure est deux fois plus importante chez la femme que chez l'homme, suggérant un rôle des hormones stéroïdes dans cette pathologie. L'augmentation de la neurotransmission sérotoninergique (5-HT) observée lors des traitements par des antidépresseurs souligne également l'implication du système 5-HT.

Pour étudier la modulation de l'activité 5-HT par les neurostéroïdes ainsi que son effet différentiel relativement au sexe, l'activité extracellulaire unitaire des neurones du raphé dorsal a été enregistrée *in vivo*, chez le rat anesthésié. Le taux de décharge de base des neurones 5-HT est plus élevé chez les mâles (M) et les femelles gestantes (P17) que chez les femelles contrôles (F). Ce taux de décharge plus rapide durant la gestation pourrait s'expliquer par la désensibilisation partielle des autorécepteurs 5-HT_{1A}. L'inhibition tonique GABAergique des neurones 5-HT est moindre chez les M et les P17 que chez les F, ce qui peut également contribuer aux taux de décharge accrus des neurones 5-HT.

Chez les F, la 5β-pregnane-3,20-dione (5β-DHP), la 5α-pregnane-3α-ol,20-one (3α,5α-THP), la dehydroepiandrosterone (DHEA), son homologue sulfatée la DHEAS, la testosterone (T), le 17β-estradiol (17β-E) et la ganaxolone (un analogue synthétique de la 3α,5α-THP) induisent une augmentation du taux

de décharge des neurones 5-HT. Parmi ceux–ci, seule la DHEAS, la T et la 17β-E ont un effet notable chez les M. L'effet de la 3α,5α-THP et de la ganaxolone chez les F ainsi que de la DHEAS chez les deux sexes, est observé dès 3 jours de traitement. Par ailleurs, la 3α,5α-THP et la ganaxolone préviennent le ralentissement initial de la décharge des neurones 5-HT causé par le citalopram (inhibiteur sélectif de la recapture de la 5-HT) qui retarde son effet thérapeutique. En revanche, la DHEAS ne prévient que partiellement ce ralentissement chez les deux sexes.

Ces résultats montrent des différences sexuelles tant sur le taux de décharge basal des neurones 5-HT que sur leur réponse à une modulation par des stéroïdes neuroactifs. Enfin, les données de cette thèse offrent une base cellulaire à l'effet antidépresseur potentiel des neurosteroïdes qui pourraient s'avérer important pour le traitement des troubles de l'humeur féminins.

ABBREVIATIONS

AC - adenylyl cyclase
ADX/CX – adrenalectomy/castration, adrenalectomized/castrated
AMPA – α-amino-3-hydroxy-methylisoxazole-4-propionic acid
APP - amyloid β precursor protein
ATP - adenosine triphosphate
BDZ – benzodiazepine
B-raf - serine/threonine protein kinase B
BSA - bovine serum albumin
cAMP – cyclic adenosine monophosphate
CNS - central nervous system
CREB - cAMP response element-binding protein
CSF - cerebrospinal fluid
5α-DHDOC - 5α-pregnan-21-ol-3, 20-dione
DHEA - dehydroepiandrosterone
DHEAS - dehydroepiandrosterone sulfate
3α-diol - 5α-androstane-3α,17β-diol
5α-DHP - 5α-pregnane-3,20-dione
5β-DHP - 5β-pregnane-3,20-dione
5α-DHT – dihydrotestosterone
DNA – deoxyribonucleic acid
DOC - 21-hydroxyprogesterone, deoxycorticosterone
DRN - dorsal raphe nuleus
DTG – 1,3-di-(2-tolyl)guanidine
17α-E - 17α-estradiol
17β-E - 17β-estradiol
E - estrogen
EBF - emopamil binding protein
E-BSA - bovine serum albumin conjugated estrogen
ER – estrogen receptor
ERα - estrogen receptor α
ERβ - estrogen receptor β
ERE - estrogen responsive element
ERK - extracellular signal-regulated kinase
ERαKO - estrogen receptor α knockout
GABA – γ-aminubutyric acid
GIRK - G protein-activated inwardly rectifying K^+ channel
GTP – guanosine triphosphate
GTPγS - guanosine 5 -[γ-thio]triphosphate
5-HIAA – 5-hydroxyindole acetic acid
3α-HSD - 3α-hydroxysteroid dehydrogenase
3β-HSD - 3β-hydroxysteroid dehydrogenase
17β-HSD - 17β-hydroxysteroid dehydrogenase
HST - hydroxysteroid sulfotransferase
5-HT – serotonin

4-IBP – 4-(N-benzylpiperidin-4-yl)-4-iodobenzamide
i.c.v. - intracerebroventricularly
i.p. - intraperitoneally
IPSC - inhibitory postsynaptic current
i.v. – intravenously
JO-1784 – igmesine, (+)-N-cyclopropylmethyl-N-methyl-1,4-diphenyl-1-1-ethyl-but-3-en-1-ylamine hydrochloride
kDa – kilodalton
δ KO – $GABA_A$ receptor subunit δ knock out
LC - locus coeruleus
LTP - long-term potentiation
MAO - monoamine oxidase
MAO-A - monoamine oxidase A
MAOI - monoamine oxidase inhibitor
MAPK - mitogen-activated protein kinase
2-Me-5-HT - 2-methyl-5-hydroxytryptamine
mER - estrogen membrane receptor
μM - micromolar
mM - millimolar
MPOA-AH - medial preoptic area-anterior hypothalamus
mPFC – medial prefrontal cortex
MPPF - 4,2-(methoxyphenyl)-1-[2-(N-2-pyridinyl)-p-fluorobenzamido]ethylpiperazine
mPR - progesterone membrane receptor
mRNA – messenger ribonucleic acid
NE – norepinephrine
nM - nanomolar
NMDA - *N*-methyl-D-aspartate
6-OHDA – 6-hydroxydopamine
8-OH-DPAT - 8-hydroxy-2-(di-n-propylamino)tetralin
OVX – ovariectomy, ovariectomized
P – progesterone
PBR - peripheral-type benzodiazepine receptor
P-BSA - bovine serum albumin conjugated progesterone
pCREB – phosphorylated cAMP response element-binding protein
PET - positron emission tomography
PKA - protein kinase A
PKCδ - protein kinase C δ
PLC – phospholipase C
PMDD - premenstrual dysphoric disorder, premenstrual dysphoria
PMS - premenstrual syndrome
POA - preoptic area
PP – postpartum period
PR – progesterone receptor
PREG – pregnenolone
PRKO - progestereon receptor knockout

PS - pregnenolone sulfate
P450aro - cytochrome P450 aromatase
P450c17 - cytochrome P450 17α-hydroxylase
P450scc - cytochrome P450 cholesterol side-chain cleavage
PTX - pertussis toxin
RU58668 - (11β-[4-[5-[(4,4,5,5,5-pentafluoropentyl)sulfonyl]pentyloxy]phenyl]-estra-1,3,5(10)-triene-3,17β-diol;
SERT - serotonin transporter
SKF-10,047 – N-allylnormetazocine
SNRI - selective norepinephrine reuptake inhibitor
SSRI - selective serotonin reuptake inhibitor
STS - steroid sulfatase
T – testosterone
TBPS - *t*-butylbicyclophosphorothionate
TCA - tricyclic antidepressant
TH - tyrosine hydroxylase
3α,5α-THDOC - 5α-pregnan-3α,21-diol,20-one
3α,5β-THDOC - 5β-pregnan-3α,21-diol,20-one
3α,5α-THP - 5α-pregnan-3α-ol,20-one, allopregnanolone
3β,5α-THP - 5α-pregnan-3β-ol,20-one
3α,5β-THP - 5β-pregnan-3α-ol,20-one
3β,5β-THP - 5β-pregnan-3β-ol,20-one
TPH - tryptophan hydroxylase
WAY100635 – N-[2-(4-[2-methoxyphenyl]-1-piperazinyl)ethyl]-N-2-pyridinylcyclohexanecarboxamide
WT - wildtype

Chapter 1

INTRODUCTION

1.1 Serotonin and depression

Serotonin (5-HT) was first hypothesized to play a role in the pathophysiology of depression in the late 1960s (105,278). Since then, a large body of evidence has been accumulating to implicate the 5-HT system in mood disorders (424). Most of it derives from the observation that every antidepressant treatment enhances 5-HT neurotransmission.

1.1.1 Dorsal raphe nuleus

The dorsal raphe nucleus (DRN) is located in the brainstem, in the ventromedial region of the midbrain periaqueductal gray (281,555) below the cerebral aqueduct (235). It is one of the brain regions most densely populated with 5-HT neuronal cell bodies (235,281,500,555). Depending on the species, 5-HT neurons constitute between 30% (rat) (4,555) and 70% (cat) (555) of the total number of DRN cells. The organization and types of cells forming the DRN as well as their projection networks are very similar across mammalian species (4,235). The DRN is also one of the nuclei (along with the median raphe nucleus) from which originate the majority of 5-HT neurons innervating the whole brain (4,235). It is noteworthy that DRN 5-HT neurons project extensively to limbic areas (235), which are involved in the control of emotions (113).

1.1.2 Basics of 5-HT neurotransmission

DRN 5-HT neurons have a spontaneous firing activity, which can be modulated by different auto- and hetero-receptors located on the cell body and dendrites (87,235,410). The release of 5-HT by nerve terminals is directly influenced by the neuronal firing activity (4,410) and can be modulated by 5-$HT_{1B/1D}$ receptors located on the axon terminal (4,223,312,410). The quantity of 5-HT in the synaptic cleft is also dependent on the rate of its reuptake by 5-HT transporter (SERT) and its degradation by monoamine oxidase (MAO) (387,410). Finally, different postsynaptic 5-HT receptors mediate a variety of effects characteristic of 5-HT neurotransmission.

1.1.3 Mechanisms of action of antidepressants

Antidepressant treatments can act through different mechanisms and at various points along this system to increase 5-HT neurotransmission (63,64). For instance tricyclic antidepressant (TCA) drugs and electroconvulsive therapy seem to increase the sensitivity of postsynaptic 5-HT_{1A} receptors (63,64,94,114,115). Monoamine oxidase inhibitors (MAOIs) and selective serotonin reuptake inhibitors (SSRIs) both increase the amount of 5-HT available in the synaptic cleft, the former by preventing its degradation and the latter by blocking its reuptake by the presynaptic cell (59,64,65,93,387). This increased extracellular amount of 5-HT is also present in the somatodendritic area where it activates 5-HT_{1A} autoreceptors (63,64,199,222,424). This leads to an initial inhibition of the

5-HT neuronal firing rate but also to a subsequent gradual desensitization of these receptors (59,63,64,93,199). The 5-HT neurons, eventually free from autoregulation, recover their initial frequency of action potential firing, and the drug-induced increase in synaptic 5-HT concentration can finally be expressed as enhanced 5-HT neurotransmission (59,62,64,93,387,424). Indeed, it has been shown that chronic treatments with different types of antidepressants result in greater tonic activation of postsynaptic 5-HT_{1A} receptors, indicating a net increase in 5-HT neurotransmission (198). This desensitization process takes about two to three weeks and is consistent with the delayed therapeutic onset of antidepressant action (59,63,64,93). The particular importance of 5-HT_{1A} receptors in the neurobiology of depression is thus underscored.

1.1.4 5-HT_{1A} receptor levels in depression

Postmortem studies have shown increased binding levels of 5-HT_{1A} receptors in the DRN (21,505), but not prefrontal cortex or hippocampus (504), of suicide victims with major depression as compared to controls. There is new evidence showing that a gene polymorphism in the 5-HT_{1A} promoter is associated with major depression and suicide (284). The biological consequence of this polymorphism is an impaired repression of the 5-HT_{1A} autoreceptor gene expression (284), which would explain the above mentioned observation. However, this was contradicted by another postmortem study in suicide victims with major depression showing reduced number of DRN 5-HT_{1A} receptors as compared to controls (21). Furthermore, other studies, using positron emission

tomography (PET) imaging, also reported lower 5-HT_{1A} receptor binding potential in raphe nuclei as well as in different cortical regions in depressed patients as compared to healthy controls (123,458). A lesser number of cortical 5-HT_{1A} receptors would be consistent with reduced 5-HT neurotransmission in depressed patients. On the other hand, a reduction in 5-HT_{1A} autoreceptor would expectedly lead to enhanced 5-HT neuronal firing activity rather than to depression. However, this might represent a homeostatic adaptation to compensate for the reduced neurotransmission, as reflected by lower postsynaptic 5-HT_{1A} receptor binding levels, or vice versa (21). Nevertheless, taken together, these studies indicate differences in 5-HT_{1A} receptors between depressed and non-depressed people and support an important role for this receptor in the pathophysiology of depression.

1.2 Women and depression

Gender differences in mood disorders have been extensively documented. It is well established that major depression affects women twice more often than men (19,53,57,73,250-252,301,434,439). The lifetime prevalence has been estimated to be around 21-23% for women and 11-14% for men (19,57,73,250,252). This 2:1 ratio appears to be constant across cultures (19,566,567). For seasonal affective disorder the difference was reported to be even greater, with a women: men ratio of 7:2 (283). Differences in prevalence for major depression between sexes first appear at female puberty, around 12-14 years old (73,478). Since puberty is the time when ovarian hormones start to

fluctuate, these hormones have long been hypothesized to play an important role in women's mood disorders (129,131,239,388,394).

Furthermore, in female patients, depressive episodes seem to develop or exacerbate during periods associated with hormonal fluctuations such as puberty (208,394), menstrual cycles (129,130,208,394,567,585), the postpartum period (208,262,394,567) and menopause (75,262,567). For instance, during the luteal phase of the menstrual cycle (or premenstrual period) up to 70% of women complain of lowered mood and of emotional distress, varying in severity (203,207,291,449), while 2% to 10% meet the criteria for prememenstrual dysphoric disorder (PMDD) (48,131,291,520). Again, this seems to be true cross-culturally (291). During the postpartum period, 10% to 22% of women suffer from major depression (378,379,381) while up to 85% experience mild to moderate depressive symptoms or "postpartum blues" (48,267,379,381). Women with a history of depression have higher risks of developing premenstrual depressive symptoms (130,201,585) as well as postpartum (378) and peri-menopause depressions (397).

It has been proposed that some women might be more vulnerable to depressive illnesses and that normal hormonal fluctuations, and their subsequent effects on the central nervous system, may be enough to trigger mood disturbances (129,204,208,239,388,394,464,501). This greater susceptibility of women to depression, especially during periods of hormonal variations, suggests a role for ovarian hormones in the pathophysiology of female affective disorders.

For clarity purposes, the hormonal fluctuations during the menstrual cycle, pregnancy and menopause will be briefly summarized. The menstrual cycle begins with the onset of menses, which last about 5 days (310). This menstrual phase is characterized by low plasmatic levels of estrogen (E) and progesterone (P) (310). E levels increase during the follicular phase, which usually spans from the 5^{th} to 14^{th} day of the cycle, and then suddenly drop just before ovulation (around the 14^{th} day) (310). The last phase of the menstrual cycle is the luteal phase (day 14 to 28), during which both E and P levels increase to peak in mid-luteal phase (310). It should be noted that, during this phase, E levels do not rise as high as during the follicular phase and that the levels of P are greater than those of E (310). During the late luteal phase, which represents the last 7 to 10 days of the cycle, the levels of both E and P drop dramatically, thus triggering the menses (310). Throughout pregnancy, there is a constant and important increase in plasmatic levels of both E and P (219). Just before parturition, P levels drop drastically (310) and during the postpartum period, there is an approximate 100-fold and 10-fold decrease in plasmatic P and E levels, respectively (214). Menopause is also associated with decreased levels of E and P (177).

1.2.1. Gender differences in human 5-HT system and in response to antidepressants

The gender differences in depression could result, at least in part, from anatomical and functional dissimilarities along the 5-HT system of men and

women. For instance, tryptophan depletion was shown to exacerbate depressive symptoms in women suffering from premenstrual syndrome (339) and to significantly lower the mood of healthy women (128,288) but not men (34,128). Furthermore, women seem to have a lower rate of 5-HT synthesis than men (372), which might make them more vulnerable to depression if there is greater need for 5-HT, in which cases synthesis might not be sufficient to compensate for the reduction in cerebral 5-HT levels (372). Interestingly, women also seem to have higher 5-HT_{1A} binding potential in several regions of the brain, including the DRN (395). All these differences could contribute to women's greater vulnerability to mood disorders.

5-HT system gender differences are also reflected by the distinctive response of men and women to various antidepressants. In the treatment of major depression, women were shown to better respond to sertraline (an SSRI) than to imipramine (a tricyclic) and the opposite was true for men (263). Fluoxetine (an SSRI) was also shown to be more effective than maprotiline (a selective norepinephrine reuptake inhibitor, SNRI) for treating premenopausal women suffering from major depression, while no such difference between these two drugs were found in men or in older women (311). It thus appears that SSRIs are better suited for the treatment of major depression in women. It could suggest a greater sensitivity of their 5HT system as compared to that of postmenopausal women and men, who are not exposed to hormonal fluctuations. All this evidence emphasizes the intricate relationship between ovarian hormones, 5-HT system and women mood disorders.

1.2.2 5-HT and female mood disorders

The premenstrual syndrome (PMS) or premenstrual dysphoric disorder (PMDD) is characterized by recurring depressive symptoms, which appear during the last week of the luteal phase of the menstrual cycle and remit shortly after the onset of menses (18). The time frame for the symptoms in PMS is different from that of major depression (18). However, since the depressive symptoms correlate with the hormonal fluctuations, the study of this condition could provide insight into the role of ovarian steroids in depressive symptoms and in mood disorders in general.

As mentioned above, SSRIs are efficient treatments for major depression. Furthermore, they seem to have greater beneficial effects than other non-serotonergic antidepressants in treating PMS (132,153,398). Interestingly, their beneficial effects are usually seen within a shorter timeframe in PMS than in major depression (132,153,503,517,570,584). Administration of SSRIs limited to the luteal phase of the menstrual cycle has also proven effective in reducing premenstrual psychological symptoms (205,237,516,586), and even more so than continuous administration (152,570). Taken together, these observations could suggest a different mechanism of action than in major depression.

It was also suggested that SSRIs' therapeutic effects were mediated, at least in part, by their enhancement of the cerebral levels of 5α-pregnane-3α-ol,20-

one (allopregnanolone, 3α,5α-THP), a metabolite of P (see figure 1 for partial metabolic pathway) (180,187). Similarly, L-tryptophan infusions were shown to induce greater increases in 3α,5α-THP blood levels in women with PMS than in controls (430). This was thus suggested to uncover a blunted 5-HT activity in PMS women (430). This is also further evidence of a close relationship between neuroactive steroids, the 5-HT system and depressive symptoms. Other abnormalities of the 5-HT system, such as a blunted response to a fenfluramine challenge (144) or to tryptophan infusions (429), have been observed in PMS (56,336,502).

Depressed menopausal women also had lower platelet and blood 5-HT contents, which could be returned to control values by 17β-E and synthetic progesterone supplementation, which also alleviated the depressive symptoms (186). These data suggest that some women may be have altered sensitivity to hormonal modulations of the 5-HT system (501).

1.3 Steroids and depression

1.3.1 Steroids, postpartum and menopausal depression

Apart from the obvious fall in ovarian steroid plasmatic levels, there does not seem to be any correlation between estrogen or progesterone levels and postpartum depression since both depressed and non-depressed women have

similar plasmatic levels of these hormones (214,217,369,380). However, depressed women were reported to have lower serum levels of 3α,5α-THP as compared to non-depressed women during the postpartum period (369). Concerning menopause, depression was associated with low levels of DHEAS rather than with estrogen or progesterone (28). This could thus suggest a role for neuroactive steroids in the pathophysiology of female mood disorders.

1.3.2 Steroids and premenstrual syndrome

In the premenstrual syndrome (PMS), depressive symptoms occur during the late luteal phase of each menstrual cycle (209,397). Because of this cyclic recurrence, many have tried to find a link between levels of ovarian steroid and symptom severity. However, no distinct pattern has yet emerged. For instance, higher plasma concentrations of progesterone (P) (176,553), 3α,5α-THP (176) and 17β-estradiol (17β-E) (553) were observed in women suffering from PMS as compared to controls. This finding was partly contradicted by another study showing lower P and 3α,5α-THP levels but higher estradiol in women with PMS as compared to those who were symptom-free (355). An increase in negative mood symptom severity was also observed with increased plasma levels of pregnenolone (PREG) (553), pregnenolone sulfate (PS) (553), P (202,209) and 17β-E (209,469,553) as well as with decreased levels of 5α-pregnane-3,20-dione (5α-DHP) (553) and 3α,5α-THP (428,553). Finally, others reported no difference in plasma levels of either P (56,428,466,469,518), PREG, 3α,5α-THP

(466,469,518), 17β-E (56,448,518), dehydroepiandrosterone sulfate (DHEAS) or dihydrotestosterone (5α-DHT) (448) between PMS women and controls, and no correlation between hormone levels and severity of depressive symptoms (397,466).

Since no clear picture could be drawn from these studies, it has been proposed that ratios and variation rates were of greater importance than hormonal levels *per se*. Concerning the fluctuation of plasma 3α,5α-THP levels, the ratio between the luteal and follicular phases was three times less in women with PMS than in healthy women (355). The ratio of 3α,5α-THP to P during the late luteal phase of the menstrual cycle of PMS women was also lower than in controls (428). But, a third study showed the opposite; a greater plasma levels of both 3α,5α-THP and P as well as enhanced 3α,5α-THP to P ratio in PMS women compared to controls (176). Halbreich *et al.* reported faster rates of decrease in P levels, but not in those of estrogen, in PMS women as compared to controls as well as a correlation between higher rates of P decrease with increasing symptom severity (202). Together, these studies show that, as was the case for hormonal levels, there is no consensus as to ratios or variation rates of ovarian steroids and PMS symptoms.

It is clear from the lack of consistency that, although ovarian hormones are likely implicated in development or exacerbation of depressive symptoms, there is no simple correlation between hormonal levels or ratios and PMS. It is possible,

but unlikely, that such generalized inconsistency is merely due to protocol differences. Different sensitivities of the 5-HT system to hormonal variations have been suggested (502) and would represent a more convincing hypothesis. Considering the myriad of effects that multiple neuroactive steroids have in the CNS, through various mechanisms of action (as discussed in the following sections), this discrepancy could reflect individual imbalances, compensation mechanisms and/or vulnerabilities, all leading to depressive symptoms.

1.3.3 Steroid levels in depression and following antidepressant treatments

Altered levels of ovarian steroids were also observed during depressive episodes and were regularized by antidepressants. For instance, the CSF and plasma of depressed patients have been shown to contain lower levels of 3α,5β-THP (440,542) and 3α,5α-THP (440,507,508,542) than those of healthy subjects, and they could be elevated back to normal levels by successful antidepressant treatments (440,507,508,542). Interestingly, the increase in CSF levels of 3α,5α-THP was proportional to the mood improvement (542). Plasma levels of 3β,5α-THP were conversely found to be higher during depressive episodes than in healthy controls, and again antidepressant treatments reversed this effect (440,507). Some studies found no differences in PREG (440,542), P (440,508,542), 5α-DHP (440,508) or DHEA (440) levels, while others showed a decrease in PREG in the CSF of depressed inpatients (173) and lower levels of DHEAS in the plasma of depressed elderly women (39).

Likewise, modulation of steroid levels by antidepressants was observed in rats. Injection of the selective serotonin reuptake inhibitors (SSRIs) fluoxetine or paroxetine to male rats rapidly resulted in greater 3α,5α-THP cerebral content and concomitant decrease in 5α-DHP, without change in PREG, P or DHEA (541). Independent of extracellular 5-HT levels (187), this effect seemed to result from the interaction of SSRIs with the enzyme 3α-hydroxysteroid dehydrogenase (3α-HSD), favoring the conversion of 5α- and 5β-DHP into their respective 3α-reduced metabolites (3α,5α- and 3α,5β-THP) (180,187,541,542). Three different SSRIs (fluoxetine, paroxetine and sertraline) were shown to facilitate this conversion of 5α-DHP in 3α,5α-THP by the human 3α-HSD (180). These results were also reproduced in rat frontal cortical slices, with other classes of antidepressants such as amitriptyline (a tricyclic) and desipramine (a norepinephrine reuptake inhibitor), which both increased synthesis of 3α,5α-THP from 5α-DHP (236). This was apparently also due to direct interaction with 3α-HSD (236). Other 5-HT reuptake inhibitors (clomipramine and fluvoxamine) have also been shown to decrease P and 17β-E serum levels in female rats (435).

These data would suggest synergic interactions between neurosteroids and antidepressants to modulate the 5-HT system and/or induce beneficial effects on mood.

1.3.4 Antidepressant effects of steroids

In both clinical studies and animal models, different ovarian steroids have been shown to have antidepressant (or antidepressant-like in the case of animals) effects.

1.3.4.1 Clinical studies

Removal of hormonal fluctuations, either by pharmacologically induced anovulation (464,519) or by ovariectomy (84), has been proven efficient in alleviating premenstrual symptoms. However, for obvious reasons, this treatment is not suitable for the majority. Several clinical studies have found estradiol to be efficient in treating PMS (560), postpartum (9,10,179,218) and peri-menopausal (186,465,494) depressions. On the other hand, progesterone was reported to not be more efficient than placebo in treating PMS (151). Estrogen administration improved the response to antidepressants in depressed elderly women (467) as well as in both pre- and post-menopausal women who were initially not responding to treatment (257). Conversely, treatment of breast cancer with tamoxifen (an estrogen receptor antagonist) was associated with higher rates of depression than in cancer patients not treated with tamoxifen (85). These data clearly suggest antidepressant properties for estrogens.

DHEA was also shown to be beneficial in the treatment of dysthymia (68) and major depression (572-574). Patients treated with DHEA responded better

than those receiving placebo (68,572) and the response rates were similar to that obtained with other antidepressants (68). Interestingly, the plasma levels of DHEAS increased at least 3 times more than that of DHEA (68,573). Furthermore, the changes in mood ratings correlated with the increase in plasma DHEAS levels (68,573) rather than DHEA levels (68). This was attributed to the faster clearance of DHEA (68). In middle-aged and elderly patients, improvement of depressive symptoms seemed to correlate with increases in both DHEA and DHEAS levels (574).

1.3.4.2 Animal models

Several studies using animals have shown antidepressant-like effects for neuroactive steroids. The Porsolt forced swimming test is widely used for screening potential new antidepressants (133,414). In this experimental model, animals are forced to swim and after a certain period of time, they become immobile, a behavior which is then considered to indicate lowered mood or despair (414). Since most antidepressant treatments can effectively reduce the length of time they remain immobile, this test is useful for evaluating putative antidepressant properties of a given compound (133,414).

DHEAS was shown to reduce the mouse immobility time in this paradigm, suggesting an antidepressant-like effect (432,540). DHEA also reduced the immobility time in this paradigm but only in high-anxiety rats (415). In one study (432), PS had a similar effect in intact animals but in another, its antidepressant-

like effect was only apparent in ADX/CX mice (540). Together, these data might suggest a lower efficacy for DHEA and PS as compared to DHEAS. However, in the case of DHEA, it could also be due to species difference and/or use of lower doses.

Khisti and coworkers, using the same model, showed that 3α,5α-THP was also efficient in reducing the mouse immobility time (253,254). Interestingly, various 5-HT agents (SSRI, 5-HT releaser and respective 5-HT_{1A}, 5-$HT_{1B/1C}$ and 5-$HT_{2A/1C}$ receptor agonists) all potentiated this antidepressant-like effect of 3α,5α-THP at doses insufficient to affect the immobility time on their own (253). Conversely, decreasing 3α,5α-THP cerebral levels, by blocking P metabolism using finasteride, increased immobility time in proestrus rats, which otherwise swam more than diestrus females or males (see next paragraph for the rat's estrous cycle) (160). Administration of P to ovariectomized (OVX) rats also reversibly decreased immobility time (315), but whether this effect was mediated by elevated levels of 3α,5α-THP remains to be confirmed.

For clarity purposes, a brief description of the rat's estrous cycle and its hormonal fluctuations is included here. The cycle lasts about 4 to 5 days and consists of four phases: estrus, metestrus, diestrus and proestrus (154). Ovulation occurs at the end of the proestrus (154). Plasmatic levels of 17β-estradiol are low at estrus and gradually increase during metestrus and diestrus, to peak at mid-proestrus, and fall abruptly before ovulation (154). Progesterone is low during

most of the cycle, rises drastically at mid-proestrus and, like E, drops before ovulation (154).

Castration of male and female rats significantly increased their immobility time (161,382), while estradiol supplementation decreased it, indicating an antidepressant-like effect (133,161,382,425). This reduction could be blocked by both the 5-HT_{1A} receptor antagonist WAY100635 and the selective estrogen antagonist RU58668 (133), thus supporting a close relationship between the 5-HT system and neuroactive steroids. Using the tail suspension test, it was shown that the antidepressant-like effects of both estradiol and P were only apparent in OVX animals, which had longer immobility time as compared to sham-operated mice (38).

Most studies assessing the immobility time in the Porsolt forced swimming test as a function of the estrous cycle found it to be longer during diestrus than proestrus-estrus (104,160,161,316,317). Interestingly, this difference could be prevented by treatment with clomipramine (316) and exacerbated by stressors (317). This could suggest that the females' mood is sensitive to stressors especially during diestrus (317) and that this more labile mood can be stabilized by antidepressants. Using the same paradigm, Galea *et al.* designed a model of postpartum depression, which showed that 3 weeks of P administration, followed by 3 days of withdrawal, significantly increased the immobility time of female rats (163). They also showed that if estrogen was administered during these 3 days of withdrawal, the immobility time was not

different from control females (163). In general, these data suggest a detrimental effect of hormonal withdrawal and fluctuation as well as a beneficial effect of steroid supplementation on mood.

These studies, using the Porsolt forced swimming test, support a gender difference as well as an effect of estrous cycle in the susceptibility to develop depressive symptoms. Furthermore, both clinical studies and animal models indicate potential antidepressant effects for various steroids, including DHEA, DHEAS, PS, 3α,5α-THP and 17β-E. They also suggest a close interaction between neurosteroids and the 5-HT system as well as an important role in the neurobiology of depression.

1.4 Metabolism and Synthesis

Being lipophilic, most steroids can easily cross the blood-brain barrier (452). Moreover, several of them can also be synthesized by the brain and were thus named neurosteroids (452). In the CNS, the expression of steroidogenic enzymes is region- and cell type-specific, and is developmentally regulated (101). Only enzymes involved in the synthesis and metabolism of sex steroids will be discussed here (for partial metabolic pathway see figure 1).

1.4.1 Cytochrome P450 side-chain cleavage

The first step in steroid synthesis is the translocation of cholesterol from the cytoplasm into mitochondria, by mitochondrial peripheral-type benzodiazepine receptors (PBR) (389). Inside mitochondria, cholesterol is converted into PREG by the rate-limiting enzyme cytochrome P450 cholesterol side-chain cleavage (P450scc) (101,544). P450scc is present in the human (562) and the rat (162,256,258,280,337,509,539) brain, in which it is widely distributed (256,258,280,457,509) and has a constant expression across ages (258). In humans, but not in rats (258,337), a gender difference was observed as women have higher P450scc mRNA levels than men in the frontal lobe and temporal cortex (562). This enzyme is expressed by oligodendrocytes, astrocytes (242,243,255,337,457,562) and neurons (162,256,457,535,539,562), which were all shown to convert cholesterol into PREG (228,242,243,256,562). As expected from its function, P450scc is concentrated in mitochondria (242,243,337,539).

1.4.2 3β-hydroxysteroid dehydrogenase

P is formed from PREG by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) (457,538). This enzyme is also widely distributed in the rat brain (162,185,258,457) but its mRNA levels seem to decrease with age (258,538). Neurons (125,162,185,457,535,538,598) as well as glial cells (457,598) express

3β-HSD and were shown to synthesize P from PREG (29,242,243,538,598) as well as androstenedione from DHEA (598).

1.4.3 21-hydroxylase

P can be converted into 21-hydroxyprogesterone (deoxycorticosterone, DOC) by the enzyme 21-hydroxylase (P450c21)(101,509). This enzyme's mRNA is found in the rodent cerebellum, hypothalamus and brainstem (293,509,593) as well as in the human hippocampus (45). Enzymatic activity was detected in the rat brainstem (232) and cultured cerebellar astrocytes (293), which actively metabolized P into DOC (232,293). There is no sex difference in the amount of rat or human P450c21 mRNA (45,232) but it seemed to increase from childhood to adulthood (45).

1.4.4 5α-reductase and 3α-hydroxysteroid dehydrogenase

In the rat brain, P is mainly metabolized into 5α-DHP and then, to a lesser extent, into 3α,5α-HTP (88,126). The 5α-reductase-catalyzed conversion of P, DOC and testosterone (T) into 5α-DHP, 5α-pregnan-21-ol-3,20-dione (5α-DHDOC) and 5α-DHT, respectively, is irreversible (88,286,452). Oxidation of 5α-DHP into 3α,5α-THP by 3α-hydroxysteroid dehydrogenase (3α-HSD) is reversible but, although both reactions occur in the brain, the oxidative direction

predominates (88,90,261). 3α-HSD also converts 5α-DHDOC into 5α-pregnan-3α,21-diol,20-one (3α,5α-THDOC) (450).

The 5α-reductase (89,266,403) and 3α-HSD (96,403) enzymatic system appears to be widely distributed in the rat brain (96,266,403). Furthermore, there is evidence of functional cerebral conversion of T into 5α-DHT (89,266,285) and then into 5α-androstan-3α,17β-diol (3α-diol) (89). The rat olfactory bulb, striatum, hippocampus and frontal cortex were also shown to metabolize P into 5α-DHP and 3α,5α-THP to varying extent; the greatest enzymatic activity being observed in the olfactory bulb (95,236). Cultured astrocytes can also synthesize 3α,5α-THDOC from P (293). These data indicate a functional 5α-reductase-3α-HSD enzymatic system with region-dependent activity.

Although one study reported that 5α-reductase was mainly expressed by astrocytes (403), others showed that neurons have greater 5α-reductase activity than glial cells, independent of age (89,333-335). On the other hand, astrocytes (type 1) have a greater 3α-HSD activity than the other types of brain cells (334,335). In rats (403) and humans (506), no sex differences were observed in brain 5α-reductase (403) or 3α-HSD mRNA, protein (403) or activity. 5α-reductase seems to be located in cellular membranes (90).

1.4.5 Cytochrome P450 aromatase

Androgens are converted to estrogens by the cytochrome P450 aromatase (P450aro) (90,101,535). Evidence shows that P450aro is active in the adult rat brain (549). P450aro expression (456,598) and enzymatic activity (370,598,600) were found mostly in neurons (370,456,598) but also, to a lesser extent, in neonatal astrocytes (370,598,600). Although the extensive brain distribution of the enzyme is similar between sexes (456,509), female rats seem to have lesser P450aro mRNA (552) and activity (444,445) than males. No such gender differences were observed in the human brain (506). P450aro is located in the endoplasmic reticulum (101,456).

1.4.6 Cytochrome P450 17α-hydroxylase

The enzyme cytochrome P450 17α-hydroxylase (P450c17) is mostly responsible for converting PREG into DHEA and, to a lesser extent, P into androstenedione (101). There is no consensus regarding the expression of the P405c17 in the adult brain as some found that it was only transiently expressed during development (101) while others found its mRNA expressed during adulthood (258,509). Astrocytes and neurons isolated from neonatal rat cerebral cortex and hypothalamus, express functional P450c17 and can produce DHEA from PREG, with astrocytes the being most active (598,599). P450c17 is located in the smooth endoplasmic reticulum (101).

1.4.7 17β-hydroxysteroid dehydrogenase

Five types of 17β-hydroxysteroid dehydrogenases (17β-HSD) exist and they are responsible for the following reversible conversions: testosterone into androstenedione, androstenediol into DHEA and estrone into 17β-estradiol (101,269,499). All of these reactions were shown to occur in the human brain (499). In several regions of the rat brain (402), 17β-HSD is expressed and active only in astrocytes (402,598). No sex differences were found in rats (402) or humans (499,506) concerning cerebral 17β-HSD expression or activity.

1.4.8 Hydroxysteroid sulfotransferase and Steroid sulfohydrolase

PREG and DHEA can be sulfated into PS and DHEAS by the enzyme hydroxysteroid sulfotransferase (HST), while the steroid sulfatase (STS) catalyses the reverse reaction (101). STS expression and enzymatic activity were found in several brain regions of the rat (12,101,102) despite apparent decline during development (426). Active HST was found in hippocampal and cerebellar neurons (256).

It is clear that the mammalian brain has the enzymes required to functionally synthesize and metabolize a wide variety of sex steroids. Furthermore, evidence shows that these enzymes can be co-localized in a cell.

For instance, astrocytes from neonatal rat cerebral cortex and hypothalamus can metabolize DHEA into T and then into E, demonstrating the enzymatic activity of 3β-HSD, 17β-HSD and P450aro (599). Glial cultures can also synthesize PREG, P, 5β-DHP and 3α,5α-THP from cholesterol (242,243).

These data confirm that the brain efficiently synthesizes and metabolizes various neurosteroids, including P, 17β-E, T and DHEA, their precursors and metabolites. They also indicate that the brain does not need to rely on peripheral synthesis for its hormonal supplies. Furthermore, it would definitely hint at neurosteroids playing a role in the cerebral functioning beyond the sole control of the hypothalamic-pituitary-gonadal axis.

1.5 Mechanisms of action of steroids

Ovarian steroids exert a myriad of effects on the central nervous system through multiple mechanisms of action. In this section, only those that have been shown to occur in the brain will be described.

1.5.1 Classical genomic mechanism of action

The action of steroids on the genome is well characterized. First, they enter the cell and bind their specific receptor, a ligand-activated transcription factor, located in the cytoplasm or nucleus, which then undergoes complex

conformational changes (31,46,277,292). Chaperone proteins, responsible for maintaining the receptor in a steroid binding conformation, then dissociate from the receptor (30,31). This leads to dimerization of the receptor, which can then bind DNA on a specific sequence (the hormone response element) located in the promoter region of the target gene (30,31). Finally, with the interaction of other sequence-specific transcription factors, there is an up- or down-regulation of gene expression (30,31) leading to a long-term cellular response. While only one receptor is known for P (PR), two types of active nuclear receptors have so far been recognized for E: ERα and ERβ. It might be important to mention that in addition to P, both 5α- and 5β-DHP can bind PR and have genomic effects (453).

1.5.1.1 Ovarian steroid receptors in the DRN

ER and PR are present in the mammalian DRN. In the OVX monkey, both PR and ERβ (40,192,194), but not ERα (192,194), were detected in the DRN. Most 5-HT neurons were shown to express PR (40) and the mRNA for ERβ was detected in both 5-HT non-5-HT neurons (194). Hormonal replacement (E, P or E + P) does not affect the mRNA or protein expression of ERβ (192,194).

In the rat brain, the ERα is highly expressed throughout the brain, while the β isoform is more restricted to limbic regions (270) and its distribution is gender-independent (270). The rat DRN expresses very low levels of ERα (366,481) but higher levels of ERβ (366,475) and PR (16). ERα and PR are

undetectable on 5-HT cells (16,294) and are rather found on adjacent neurons (16), which are immunoreactive for excitatory amino acids (17). On the other hand, about 40% of 5-HT neurons express ERβ mRNA (294). ERβ are mostly nuclear but are also found in the cytoplasm of a few neurons (294). The distribution patterns for ERα and PR are similar between genders and so is the number of cells expressing ERα (16). However, 30% more female DRN cells expresse PR than male's (16). E treatment decreases ERα expression and enhanced PR immunoreactivity in both sexes (16). It does not increase the number of cells expressing ERβ but intensifies its mRNA signal (294).

In the mouse DRN, ERα and PR are expressed by 5-HT neurons as well as by non-5-HT neurons (14), which are immunoreactive for excitatory amino acids (17). No sex difference was observed in the number of cells expressing PR but this number is increased following E treatment, in both sexes (14). Interestingly, this is true for wildtype (WT) as well as for ERαKO mice (14). ERβ are found in the DRN of male WT (375) and ERαKO (375,476,477) mice, and are confined to the nucleus (375). Gonadectomy or E replacement both failed to modify the number of cells expressing ERβ receptors in either genotype (375). ERβ are more abundant than ERα in the DRN of OVX mice and are not restricted to the nucleus (349).

In the DRN of OVX guinea pigs, immunoreactivity for ERβ is restricted to the nucleus and ERα is barely detectable (556). ERβ mRNA, but not ERα or PR,

is expressed by their 5-HT neurons (297). Finally, in the OVX cat, ER are present in the nucleus, cytoplasm of cell body and dendrites of neurons in the periaqueductal gray (545).

To summarize, in the DRN of monkeys, rats, mice and guinea pigs, the expression of ERβ seems greater than ERα, which has a very low expression. PR is also expressed in the DRN of all these species. This strongly suggests that the expression of ovarian steroid receptors was well preserved during evolution. What appears to differ, however, is whether or not they are expressed by 5-HT neurons. Concerning ERβ, the information is only available for monkeys and rats, both of which express this receptor on 5-HT neurons. It could presumably be expected for other species. ERα, on the other hand, was only detected in mice 5-HT neurons. PR was found in 5-HT neurons of monkeys and mice, but not rats. Nevertheless, the cellular machinery necessary for steroid genomic effects to take place is present in the DRN. Furthermore, even if not directly occurring in 5-HT neurons, such effects in adjacent cells could lead to their subsequent modulation.

1.5.2 Non-genomic mechanisms of action

Some effects of steroids are too rapid (seconds to minutes) to be mediated by a genomic mechanism of action (46,137,367,368,529). Moreover, these effects are insensitive to transcription and translation inhibitors, thus further supporting independent processes (46,137,367). There is considerable evidence

indicating that steroids can initiate cellular responses at the neuronal cell membrane level through a variety of mechanisms of action.

1.5.2.1 Steroid membrane receptors

An increasing amount of data suggests the existence of progesterone and estrogen membrane receptors (mPR and mER) in the brain (292). An early study, using synaptic plasma membranes prepared from rat brains, showed specific binding sites for E, P and T (533). Bovine serum albumin (BSA) conjugated steroids, such as E-BSA and P-BSA, which cannot diffuse freely through the plasma membrane, have also been very useful to study putative membrane steroid binding sites and effects (246). Such studies have shown plasma membrane binding sites for both E and P in the rat brain (246,390,460,530), as well as a variety of membrane-mediated responses (47,81,121,157-159,340,363,460). For instance, 17β-E can enhance the excitability of rodent hippocampal neurons (81,148,149,183,363,529,575,576) by inhibiting Ca^{2+}-dependent K^+ channels (81), via reduced Ca^{2+} influx through voltage-gated Ca^{2+} channels (81). Furthermore, this effect appears to be initiated at the cell membrane level (81,149) by a specific ER (575). The steroid-binding membrane receptors can be classified into three types: classical "nuclear" ER/PR located at the neuronal membrane level (46,98), new specific membrane receptors which are different from the nuclear ER/PR (265,420,530,531) and other steroid-binding proteins, such as enzymes, neurotransmitter receptor, etc. (46).

a) Membrane receptors identical to nuclear receptors

There is indication that some mERs might be structurally similar, if not identical, to nuclear ERs (292,390) and be the products of a single gene (431). This would explain why cells expressing membrane steroid receptors also have the nuclear counterpart (559). Of the classical ERs, only ERα (and not ERβ) has so far been detected in the plasma membrane of astrocytes (460) as well as hippocampal (98,346,558) and cultured midbrain neurons (46). Activation of this membrane receptor in astrocytes, for instance, negatively regulates the glutamate-aspartate transporter, which results in the inhibition of L-glutamate uptake (460). It has been postulated that tissue- and cell-specific accessory proteins might also link this receptor to various intracellular signaling cascades (see section 1.5.2.2) (46).

b) New specific membrane receptors

Specific mER, which are different from nuclear ER, have also been reported. For instance, a new mER (ER-X) has been shown to be present in the neocortex of neonatal WT and ERαKO mice but to be minimally expressed in adults (531). This receptor has a different molecular weight (62-63 kDa) from ERα (67 kDa) and ERβ (60 kDa) and a greater affinity for 17α-E (531). Binding of 17β-E or 17α-E to this receptor results in activation of the mitogen-activated

protein kinase (MAPK) cascade (531) (see section 1.5.2.2). There is also report of yet another novel transmembrane ER, which is coupled to a G protein ($G\alpha_q$) and can activate an intracellular cascade, which finally results in phosphorylation and inhibition of inward rectifying K+ channels (420). It is unclear whether these two novel mER are a variant of the same gene. However, they seem pharmacologically different since 17α-E seemed unable to activate the second (420).

Recently, a 40-kDa PR was cloned from sea trout ovaries (597). This receptor appears to be a membrane receptor, coupled to an inhibitory G protein, and its mRNA was detected in the sea trout brain (597). A membrane protein of similar molecular weight (40-50 kDa), and with high affinity for P, had previously been shown in rat synaptosomal membrane preparations (530). Finally, another mPR, a 25-kDa protein called 25-Dx, is also expressed in the rat CNS (265,268), as well as in different brain regions of WT and PR knockout (PRKO) mice, where it seems to be localized in the membrane of neuronal cell bodies (265).

c) Other steroid-binding membrane proteins

Steroids may act on a wide variety of proteins located in the cell membrane such as ion channels, enzymes, transporters, receptors for molecules other than steroids, etc (559). For instance, in the rat brain, 17β-E and P were shown to bind and modulate the enzymatic activity of the membrane-bound

mitochondrial ATP synthase/ATPase (592) and glyceraldehyde-3-phosphate dehydrogenase (238). A dual binding site for P and E, which is coupled to G proteins, was also reported in the plasma membrane fraction of OVX female rat medial preoptic area-anterior hypothalamus (MPOA-AH) (76). Interestingly, the G protein coupling to this receptor and subsequent conformational changes appear to determine its relative affinity for the two steroids (76).

1.5.2.2 G proteins and intracellular second messenger systems

a) G proteins

As mentioned above, there is also accumulating evidence indicating that estrogen activates G-proteins and intracellular second messenger systems to rapidly alter synaptic transmission (247,271) and neuronal responses (248,340,363). 17β-E potentiates kainate-induced currents (183,184,363,364,576) and depolarizes (575,576) hippocampal neurons without implication of either ERα or ERβ (183,363,576), but rather by involving a G protein-coupled mechanism (184,363,364). PREG and PS also inhibit hippocampal Ca^{2+} channel currents via a pertussis toxin (PTX)-sensitive G-protein, through a mechanism initiated at an extracellular binding site (141). Similarly, in neostriatal neurons, 17β-E activates a G-protein-coupled membrane receptor, which triggers a second messenger cascade and finally inhibits L-type Ca^{2+} channels (340). Selective coupling of mERα to $G\alpha_i$, but not $G\alpha_q$ or $G\alpha_s$ protein was shown in endothelial

cells (580). Based on the above-mentioned results, a similar coupling could be expected in neuronal cells but needs to be confirmed.

b) MAPK cascade

17β-E can activate different proteins of the MAPK cascade, including B-Raf and ERK, by inducing their phosphorylation, probably via a tyrosine kinase (50,51,482-484). This was observed in rat hippocampal (49,51,309) and thalamic (50) neurons, where it led to the phosphorylation of the *N*-methyl-D-aspartate (NMDA) receptor (49-51), enhanced this receptor function and long-term potentiation (LTP) (49,51), as well as conferred neuroprotection (49,50). It can also lead to other effects such as secretion of nonamyloidogenic amyloid β precursor protein (APP) (309). Interestingly, it seems that 17β-E can activate the MAPK cascade even in neurons devoid of functional ER (309).

c) PKA and PKC cascades

Estrogen treatments of OVX rats increase PKC enzymatic activity in the preoptic area (POA), which activates adenylyl cyclase (AC) and leads to elevation of cAMP levels (20). Kelly *et al.* proposed a model in which 17β-E non-genomically activates PKC in hypothalamic neurons (247,248). Activated PKC, on one hand, uncouples μ-opioid and $GABA_B$ receptors from G protein-activated inwardly rectifying K^+ channels (GIRK) (247,248). On the other hand, it activates

PKA, through an AC-mediated increase in cAMP, and PKA also prevents the coupling of μ-opioid receptors to GIRK (247,248). They also observed that 17β-E potentiates the β_1-adrenoceptor mediated inhibition of Ca^{2+}-dependent K^+ channels (248). This led to the suggestion that 17β-E potentiates the response of $G\alpha_s$-coupled receptors (e.g. β_1-adrenoceptors) (248,364), while inhibiting that of $G\alpha_{i/o}$-coupled receptors (e.g. μ-opioid and $GABA_B$ receptors), thus leading, in both cases, to neuronal excitation (248). All of these estrogenic effects seem independent of the classical genomic mechanism of action (271,561,595) and appear to be mediated by a specific receptor (271) on the cell membrane (248,595).

1.5.2.3 Membrane-initiated genomic effects

Accumulating data has also shown that, in the brain, estrogen has transcriptional effects, initiated at the membrane level (468,532), which involve various transduction pathways and result in modulating gene expression (468,484,532), including those devoid of estrogen response element (ERE) in their promoter regions (484,532). For instance, 17β-E has been shown to rapidly and non-genomically increase intracellular cAMP concentrations in neural cells (561). This increase in cAMP activates PKA (271,561) and is followed by a rapid phosphorylation of the cAMP response element-binding protein (CREB) (561,595). In OVX rats, both 17β-E and P can non-genomically induce receptor-mediated increase in phosphorylated CREB (pCREB) in neurons of different

brain regions (181,595). pCREB is the activated form of CREB, which can influence gene expression via cAMP response element (CRE) containing gene promoters (561). Interestingly, it has been suggested that upregulation of CREB is associated with antidepressant-like effects whereas its downregulation could be implicated in the pathophysiology of depression (124). Activation of the ERK/MAPK pathway by 17β-E can also, in addition to the above-mentioned effects, lead to modulation of gene transcription (287) since activated ERK can interact with nuclear transcription factors (532). An ER different from ERα and ERβ has been suggested to trigger this enzymatic cascade (483).

1.5.2.4 Neurotransmitter receptors

In addition to these steroid-binding protein-mediated effects and the activation of intracellular cascades, steroids can also modulate the function of neurotransmitter receptors through various mechanisms of action. Nicotinic acetylcholine, glycine, dopamine and oxytocin receptors are all examples of receptors, which can be affected by steroids (452). Of greater interest for the purpose of this work, the modulation by neuroactive steroids of 5-HT, GABAergic, glutamatergic, noradrenergic and sigma receptors will be discussed in the following sections.

Taken together, these examples underscore the multiplicity of steroid mechanisms of action, which can occur concomitantly and interact together (364)

to produce a variety of effects as well as a combination of rapid and long lasting cellular responses (463). Rapid non-genomic effects can precede and/or complement later genomic responses by acting on the same or different effector protein (463). Modulation of ion channels or neurotransmitter receptor responses, for instance, can rapidly alter neuronal response and synaptic transmission. The time frame concerning the triggering of intracellular second messenger cascades can range from rapid to slow, depending on the resulting cellular responses. Also, whether it is membrane-initiated or occurs via nuclear steroid receptors, modulation of gene transcription and/or translation, while slower, may lead to longer lasting effects. Furthermore, these data undeniably demonstrate that neurosteroids can have important impacts on the modulation of cerebral functions.

1.6 Ovarian steroids and the 5-HT system

Female rats seem to have a greater cerebral 5-HT synthesis (42,79,80,206,543) than males, especially in the hippocampus (42,206). They also have higher 5-HIAA/5-HT ratio than males in various regions of the brain (79), including the DRN (122). This ratio is usually considered as an index of 5-HT turnover or metabolism (41). Increased synthesis and turnover rate, together, may suggest a greater potential for rapid 5-HT fluctuation in females. Indeed, the HIAA/5-HT ratio can be reduced by exposure of females to a stressor such as the elevated plus maze, whereas that of males is stable (122). This could thus suggest a more sensitive 5-HT system for females. These gender differences combined with those observed in humans (section 1.3.6), along with the putative role of

ovarian steroids in female affective disorders and the implication of the 5-HT system in the neurobiology of depression, all point to a modulation of this system by ovarian hormones. In order to characterize it, numerous studies have been performed on the different steps involved in the 5-HT neurotransmission.

1.6.1 Modulation of tryptophan hydroxylase

Tryptophan hydroxylase (TPH) is the rate-limiting enzyme in the synthesis of 5-HT and it converts tryptophan into 5-hydroxytryptophan, which is the precursor to 5-HT (44). In the DRN of ovariectomized (OVX) monkeys (42,43,401) and guinea pigs (297), but not of rats (15), estrogen (E) has been shown to increase TPH expression, with no significant additive effect of progesterone (P) (43,401). One of the functional consequences of this could be an E-induced elevation of the 5-HT content in the DRN.

1.6.2 Modulation of monoamine oxidase A

Most available data suggest that ovarian hormones negatively modulate the expression and/or activity of monoamine oxidase A (MAO-A), the enzyme degrading 5-HT and norepinephrine (NE) (4,387). For instance, E, P or E + P lowered the MAO-A mRNA levels in the DRN and hypothalamus of OVX monkeys (193). Both short- and long-term treatments of OVX rats with E or P alone, but not in combination, also reduced the hypothalamic MAO-A activity (225,383). In a human neuroblastoma cell line of neural origin (SK-ER3), E

significantly decreased the activity of MAO-A (299,300). Since MAO-A reduces 5-HT levels, such negative modulation of its expression and/or activity could be expected to increase the amount of 5-HT available for 5-HT neurotransmission.

1.6.3 Modulation of the 5-HT transporter

The 5-HT transporter (SERT) is responsible for removing extracellular 5-HT from the synaptic cleft, thus terminating synaptic transmission (4). In the DRN of OVX monkeys, 4-week E treatments have been shown to decrease SERT mRNA expression, with little additive effect of P (43,400). In a recent study, the same group showed that, in the DRN, the density of SERT binding sites was not affected by either E, P or E + P (296). However, in certain DRN projection areas (hypothalamus and basal ganglia), these treatments (E, P or E + P) increased SERT binding sites and functional 5-HT uptake (296). The reduction in SERT mRNA levels in the DRN and the increase in SERT density in projection areas may represent greater trafficking of SERT to terminal fields as an adaptive response to higher levels of 5-HT in the synaptic cleft (296). Indeed, as mentioned above (section 1.6.1), E increased TPH expression and 5-HT levels in different brain regions. Moreover, it has been shown that extracellular 5-HT, by activating the transporter, prevents SERT phosphorylation and subsequent internalization (427). Conversely, long-term blockade of SERT, by SSRIs, results in its downregulation (411).

In vitro, 17β-E has been shown to inhibit 5-HT uptake within minutes of incubation (92,343). A 7-day treatment with E, starting 2 weeks after ovariectomy, was found to decrease SERT mRNA levels in the rat midbrain (594), as well as SERT binding sites in the hippocampus (338). This would be consistent with a blockade-induced down-regulation, as observed with long-term SSRI treatments. The discrepancy between the results obtained in rats and in monkeys concerning SERT binding sites density in projection areas could be due to species, brain regions or timeframe differences. However, since a reduction in mRNA levels in the DRN were observed in both species, brain region or timeframe specificity seem more likely.

Studies investigating shorter timeframes have shown that, a single injection of E, just after ovariectomy, increased SERT mRNA levels in the rat DRN (329,330,515) and SERT binding sites in various projection areas (329,515). Similarly, castration of male rats reduced the levels of SERT mRNA in the DRN and this effect could be attenuated by administration of T and even more so by 17β-E, but not 5α-DHT (143). These data suggests that this increase is mediated through aromatization of T into 17β-E (143) and further support an upregulating effect of short-term 17β-E administration on SERT expression. Interestingly, female rats seem to have less SERT binding sites than males, at least in the hippocampus and dentate gyrus (338).

Taken together, these results regarding the effects of E on SERT expression and/or activity suggest species and brain region specificity. Furthermore, they appear sensitive to the timeframe of administration and long-term effects are likely to result, at least in part, from indirect adaptive changes. Finally, these data could suggest that ovarian steroids promote 5-HT neurotransmission rather than reduce it.

1.6.4 Modulation of 5-HT_{1A} receptors

1.6.4.1 5-HT_{1A} receptors

As previously mentioned (section 1.3.3) 5-HT_{1A} receptors are of particular importance. The richest brain region in 5-HT_{1A} receptors is the DRN (91,276,348), where they are mainly located on the soma and dendrites of 5-HT neurons (276,437,497,550) and regulate the firing activity of these neurons (116,497). These 5-HT_{1A} autoreceptors are directly coupled to K^+ channels through $G_{i/o}$ proteins (8,66,229,230,276,442). Postsynaptically, they are present in high densities in the DRN and limbic regions (276,348,479), such as the lateral septum, entorhinal cortex and hippocampus, which is especially rich in 5-HT_{1A} receptors (91,178,348,437,479). Activation of 5-HT_{1A} receptors, pre- or post-synaptically, triggers the opening of K^+ channels, which hyperpolarizes the neuronal membrane and decreases the neuronal firing rate (4,7,8,83,108,230,497,498). In the case of 5-HT_{1A} autoreceptors, this functionally

results in less 5-HT release and neurotransmission in the cell body and projection areas (83,224,472,473).

Several lines of evidence suggest different properties for pre- and post-synaptic 5-HT_{1A} receptors (60,66,67,116,198,438). For instance, an important distinction is seen in the adaptive desensitization of the somatodendritic autoreceptors, which is not present in postsynaptic hippocampal and cortical 5-HT_{1A} receptors, following their sustained activation either by 5-HT_{1A} agonists or increased 5-HT availability (59-63,93,94,116,198,220,241,276,480). There is also data suggesting a region specific and time-dependent modulation of particular types of G protein in response to administration of the SSRI fluoxetine (289). This could offer a basis for differential functional desensitization of 5-HT_{1A} receptors according to brain areas.

1.6.4.2 Modulation of 5-HT_{1A} autoreceptors

Bethea and coworkers have shown that E or E + P decreased 5-HT_{1A} receptor mRNA levels in the DRN of OVX monkeys; E reduced the number of cells expressing 5-HT_{1A} mRNA, while P reduced the quantity of mRNA per cell (399). Interestingly, this decrease was associated with a reduction of 5-HT_{1A} binding sites and G protein activation in the DRN of OVX macaques (295), indicating functional autoreceptor downregulation.

In OVX rats, a 3-week treatment with high levels of E and P also significantly reduced 5-HT_{1A} autoreceptor mRNA levels in the DRN (54). On the other hand, a 2-week administration of E alone led to either a trend towards 5-HT_{1A} receptor downregulation in the midbrain (594) or no effect in the DRN (275). This could suggest that longer treatments or addition of P would be needed to lower the 5-HT_{1A} autoreceptor expression in rats. However, electrophysiological experiments in the DRN of OVX rats showed a desensitization of the 5-HT_{1A} autoreceptor following 48h of E administration (272,273). Moreover, ovariectomy seemed to increase the expression and functional response of this autoreceptor (70). Interestingly, 7 days of E supplementation reversed this effect (70). Together, these results support a functional desensitization and/or downregulation effect of E on 5-HT_{1A} autoreceptors. Considering the autoinhibitory role of this receptor on the firing activity of 5-HT neurons, this estrogenic effect would facilitate 5-HT neurotransmission.

1.6.4.3 Modulation of postsynaptic 5-HT_{1A} receptors

a) mRNA levels

In OVX rats, acute E administration reduced 5-HT_{1A} receptor mRNA levels in various brain regions (e.g. medial amygdala, piriform, perirhinal, cingulate and motor cortices) (385,386), while longer E treatments did not result in any expression change in these or other cerebral regions (hypothalamus,

dentate gyrus, hippocampus, prefrontal and retrosplenial cortices) (275,384,594). One study, however, did show a reduction of 5-HT_{1A} mRNA levels in the dentate gyrus and dorsal hippocampus (CA2 region) following 3 weeks of E administration, as well as an increase in mRNA levels in the CA1 region of the hippocampus when P was added to this treatment (54). In spayed monkeys, two weeks of E or E + P administration does not modify the hypothalamic 5-HT_{1A} receptor mRNA levels (195). Therefore, in general, short-term, but not long-term, administration of E and P appears to modulate the mRNA expression of postsynaptic 5-HT_{1A} receptors.

b) Binding sites

Four-week treatments with E or E + P, but not P alone, reduced hypothalamic 5-HT_{1A} binding sites in OVX monkeys (295). Acute administrations of E or E + P do not seem to affect 5-HT_{1A} receptor binding sites in any of the rat brain regions investigated (hippocampus, hypothalamus, preoptic area, medial amygdala, piriform and perirhinal cortices) (150,233,385). Four days of E also seemed insufficient to alter the number of hippocampal 5-HT_{1A} binding sites (100). However, a 2-week E treatment reduced this number in the amygdala, perirhinal and motor cortices (384), but not piriform, retrosplenial, prefrontal or cingulate cortex (275,384). Concerning the hippocampus, one study found no effect (275) while another one showed a reduction (384) in 5-HT_{1A} binding sites after 2 weeks of E. This discrepancy could be due to the use of different 5-HT_{1A}

receptor ligands ([^{3}H]8-OH-DPAT and [^{3}H]MPPF (275) as opposed to [^{3}H]WAY100635 (384)).

These studies clearly indicate that the E-induced dowregulation of 5-HT_{1A} receptor binding sites is region-specific. Furthermore, the timeframe difference between the rapid effect on mRNA levels and the later reduction in binding sites, strongly suggest a genomic mechanism of action. E probably inhibits the 5-HT_{1A} receptor gene expression at a transcriptional level, which then translates into fewer 5-HT_{1A} receptors being expressed at the cell membrane. The relatively slow (days) turnover of 5-HT_{1A} receptors was suggested to account for this long time frame difference between the fast decrease in mRNA levels (hours) and later reduction (more than 4 days) in binding sites (384).

c) Functional studies

Two days of E replacement, in OVX rats, decreased the neuroendocrine response to 8-OH-DPAT, as well as hypothalamic levels of G_z, G_{i1} and G_{i3} (but not G_{i2}) proteins, suggesting that the E-induced desensitization of 5-HT_{1A} receptors could be due to a lower number of G proteins (422). This hypothesis was only partly supported by other studies, which found blunted neuroendocrine (423) and behavioral (233,234,320,455) responses to 8-OH-DPAT but no change in G_z protein levels (423) or in coupling of the receptor to G proteins (233) following longer E treatments (4 and 14 days). Another group also showed that a single E injection, but not a 14-day treatment, decreased the 5-HT_{1A} receptor-

mediated activation of G proteins in the hippocampus, cortex and amygdala of OVX rats (350). However, this was not supported by other studies showing an enhancement of the 5-HT_{1A}-mediated response to 5-HT in the hippocampus of OVX rats after a few (3-6) days of E replacement (32,99,100). Although these data indicate a functional modulation of 5-HT_{1A} receptors by E, the exact nature of this effect remains unclear. Again, timeframe of administration, cerebral region specificity as well as the type of G proteins, which are coupled to the receptor, might account for these apparent discrepancies.

Mize and coworkers have shown that activation of ER *in vitro*, in membranes prepared from the hippocampus and frontal cortex, resulted in rapid reduction of 5-HT_{1A} receptor function without altering the affinity of the G protein Gα subunit for GTP, as assessed by agonist-independent [^{35}S]-GTP-γ-S binding (352). They also showed that 17β-E, through increased PKA and PKC activities, non-genomicaly induced the phosphorylation of 5-HT_{1A} receptors and their consequential uncoupling (351).

It thus appears from these studies that the downregulation of 5-HT_{1A} receptors by E is paralleled by a reduced functional response and that genomic and non-genomic mechanisms of action are involved. In contrast to its beneficial presynaptic effect, this reduction of postsynaptic 5-HT_{1A} receptors expression and function by E would suggest lesser 5-HT neurotransmission. It is also possible that the reduction in postsynaptic sites arises as a homeostatic consequence of the

enhanced 5-HT neurotransmission. The net result on neurotransmission would nevertheless depend on the balance of these two effects.

1.6.5 Modulation of other 5-HT receptors

1.6.5.1 5-$HT_{1B/1D}$ receptors

5-$HT_{1B/1D}$ receptors are important in the mediation of 5-HT neurotransmission since they exert autoinhibitory control on the release of 5-HT in the synaptic cleft (63). Interestingly, these receptors also desensitize within two to three weeks of treatment with SSRIs, thus leading to greater 5-HT release and 5-HT neurotransmission (63). Unfortunately, little data is available on the effect of ovarian steroids on these receptors. One study showed region-selective E- and P-induced elevation of 5-HT_{1B} receptor binding sites in the ventromedial hypothalamic nucleus, but not in other regions of the hypothalamus, preoptic area or hippocampus in OVX rats (150). Such upregulation of 5-$HT_{1B/1D}$ receptors would expectedly reduce 5-HT release and neurotransmission in this area. Nevertheless, more studies would be needed to clearly establish the hormonal modulation of 5-$HT_{1B/1D}$ receptor expression and function.

1.6.5.2 5-HT_{2A} receptors

5-HT_{2A} receptors are widely distributed in the rat brain with varying expression intensity, the highest being in the hippocampus (106). In the DRN,

they are not present on cell bodies but only on very few dendrites, which express low levels of 5-HT_{2A} receptors (106). Their activation is mainly inhibitory for 5-HT neurons as it reduces their neuronal firing activity (169,314) as well as the extracellular 5-HT levels in the DRN (169) and projecting area (314). However, this effect does not appear to be direct (169), which is consistent with the very low expression of 5-HT_{2A} receptors by 5-HT neurons. Rather, DRN 5-HT_{2A} receptors might be part of a local feedback loop and activate GABAergic interneurons, thus leading to 5-HT neuronal inhibition (290).

In the OVX monkey hypothalamus, a four-week administration of E decreased 5-HT_{2C}, but not 5-HT_{2A}, receptor mRNA expression (195). On the other hand, in OVX rats, a single dose of E was shown to increase 5-HT_{2A} receptor mRNA levels as well as receptor density in the DRN and various projection areas (e.g. hippocampus, hypothalamus, nucleus accumbens, caudate-putamen, olfactory tubercle and cerebral cortex) (142,386,511-513,515). This effect could be blocked by the ER antagonist tamoxifen, which had otherwise no effect (515). Furthermore, the E surge occurring during proestrus was sufficient to increase forebrain 5-HT_{2A} binding sites as compared to diestrus females or males (514). This rapid upregulation in the DRN, striatum and frontal cortex could also be observed after longer (two weeks) treatments (109).

The elevation of 5-HT_{2A} receptor binding site being paralleled by increased mRNA levels, a genomic mechanism of action might be involved in this estrogenic effect. However, it might be noteworthy that throughout the brain, 5-

HT_{2A} receptors were found in the cytoplasm of neurons (dendrites and cell body) rather than in the plasma membrane, suggesting that they are normally internalized (106). Therefore, the E-mediated increase in binding sites may also represent a translocation of receptors from the cytoplasm to the cell membrane. As stated above, 5-HT_{2A} receptors inhibit the activity of 5-HT neurons. Therefore, greater expression in the DRN could be expected to reduce 5-HT neurotransmission. Conversely, a similar upregulation in postsynaptic regions would probably increase it. For instance, activation of 5-HT_{2A} receptors in the medial prefrontal cortex (mPFC) increased 5-HT release, probably by stimulating the release of glutamate, which can then activate AMPA/kainate receptors located on 5-HT terminals (314). These postsynaptic effects might then be enough to compensate for the inhibitory presynaptic effect, especially since there seems to be higher densities of 5-HT_{2A} receptors postsynaptically. Again, the balance between these two opposite influences would determine the net effect on 5-HT neurotransmission.

Castration of male rats decreased 5-HT_{2A} receptor mRNA levels in the DRN and a single injection of T or 17β-E, but not 5α-DHT, reversed this effect (143,511). The same was observed for 5-HT_{2A} receptor binding sites in different brain regions (piriform, frontal and cingulate cortex, olfactory tubercle and nucleus accumbens) (143,511). Again, this suggests that the effect of T is mediated through its aromatization into 17β-E (143), thus supporting an upregulating effect of E on 5-HT_{2A} receptors, irrespective of gender. In juvenile

rats, castration also induced a T-reversible reduction in 5-HT_{2A} mRNA levels in the hypothalamus but not in the hippocampus, thalamus, cortex, amygdala or DRN (591), which could suggest an influence of age.

Indeed, a PET study on humans has shown that age reduces 5-HT_2 receptor binding potential (342). Furthermore, PET studies supported what was observed in animals; several weeks of E and P administration increased 5-HT_{2A} binding potential in various cerebral cortical areas of postmenopausal women (361,362). Also, one PET study suggested reduced cortical 5-HT_2 binding potential in depressed patients as compared to healthy controls (583). It would be interesting to see if E treatment could prevent the age- and depression-related reduction in 5-$HT_{2(A)}$ receptor expression and whether it would be associated with mood improvement.

1.6.5.3 5-HT_3 receptors

5-HT_3 receptors are excitatory ligand-gated cation (mainly Na^+ and K^+) channels (569,579). 17β-E and P, but not 3α,5α-THP, act as non-competitive antagonists of 5-HT_3 receptors, possibly through allosteric interaction at the extracellular receptor-membrane interface (451,569,579). 17βE appears to be more potent than P in inhibiting 5-HT_3 receptor-mediated influx currents (27).

The putative effect of ovarian hormones on the 5-HT neurotransmission through their modulation of 5-HT_3 receptors is not clear. Since 17β-E and P are antagonists at 5-HT_3 receptors, one would probably expect them to reduce this neurotransmission. However, there is evidence that it could actually be the opposite. First, no 5-HT_3 antagonist has so far been shown to have any effect on the firing activity of DRN 5-HT neurons *in vivo* or *in vitro* (5), suggesting that they are not normally tonically activated (196). On the other hand, the 5-HT_3 agonist 2-Me-5-HT inhibits the firing activity of DRN 5-HT neurons when applied locally *in vivo* and *in vitro,* probably through indirect activation of 5-HT_{1A} autoreceptors (5,196). In the DRN, 5-HT_3 receptors appear to be extrasynaptically located (23) and their activation stimulates 5-HT release in the somatodendritic area (23). This greater extracellular 5-HT would likely activate 5-HT_{1A} receptors, which inhibit the firing activity of 5-HT neurons (23). This would explain the reduction in firing activity observed with 2-Me-5-HT. Systemic or local administration of 2-Me-5-HT in projection areas was also inhibitory on the firing activity of cortical and hippocampal neurons, thus suggesting the implication of inhibitory GABAergic interneurons (196). This is supported by the *in vitro* observation that superfusion with 2-Me-5-HT stimulated 5-HT release from frontal cortical, hippocampal and hypothalamic slices (58). Together these results suggest that even if excitatory, the 5-HT_3 receptors' influence is mostly inhibitory. Their blockade by ovarian hormones would therefore likely result in a positive net influence on 5-HT neurotransmission.

Taken together these data point to a beneficial effect for estrogen on 5-HT neurotransmission. It possibly increases the 5-HT pool by increasing the expression and/or activity of the synthesis enzyme TPH, while decreasing that of the degrading enzyme MAO-A. Of critical importance, E also appears to modulate functional desensitization and/or downregulation of the inhibitory 5-HT_{1A} autoreceptors. Moreover, E and P, as 5-HT_3 antagonists, might also prevent a potential 5-HT_3-mediated inhibition of 5-HT neurotransmission. The estrogenic modulation of SERT is less straightforward and may include species and cerebral region specificities. Nevertheless, a beneficial effect of ovarian steroids could still be speculated. The hormonal influence on 5-HT transmission through modulation of postsynaptic 5-HT_{1A} and 5-HT_{2A} receptors is, however, not as clearly beneficial. Concerning postsynaptic 5-HT_{1A} receptors, E and P seem to induce a rapid region-specific downregulation of mRNA levels, which is later followed by a decrease in binding sites and is also translated in reduced functional response. Different mechanisms of action, including genomic and non-genomic, appear to be involved. The E-induced greater expression of 5-HT_{2A} receptors in the DRN could reduce 5-HT neurotransmission, while in projecting areas it could have the opposite effect. In the end, the net result on 5-HT neurotransmission would depend on the balance of all these effects as well as on the cerebral region and the species investigated. Nevertheless, there is undisputable evidence of an ovarian hormonal modulation of several enzymes and receptors participating in 5-HT neurotransmission.

1.7- Effect of steroids on different 5-HT afferent systems

There are extensive projections to the DRN from various regions of the brain (407) and different afferent systems modulate the activity of 5-HT neurons. The best characterized heterologous modulatory systems of 5-HT neurons, if not the most important, are probably the GABAergic tonic inhibition and the glutamatergic and noradrenergic excitation.

1.7.1 GABA

1.7.1.1 GABA receptors

$GABA_B$ receptors are metabotropic receptors, which are coupled, via a G-protein, to inward rectifying K^+ channels (3,229,230), while $GABA_A$ receptors are chloride ion channel complexes (302). Activation of either type of receptors leads to hyperpolarization of the neuron and reduces its firing activity (230,302). The $GABA_A$ receptor complex has a pentameric structure (331), which can be composed of various subunits: α1-α6, β1-β3, γ1-γ3, δ, ε and π (274,331). The specific combination of subunits determines the pharmacological properties of the receptor (274) and varies greatly across cerebral regions (331).

1.7.1.2 GABAergic tonic inhibition of DRN 5-HT neurons

In the DRN, there is reciprocal inhibition between GABAergic and 5-HT neurons (22). Of interest here, is the well-characterized GABAergic tonic inhibition of DRN 5-HT neurons (3,524). Numerous GABAergic terminals make direct synaptic contact with 5-HT neuronal dendrites and cell bodies (167,212,554). These afferents originate from various brain regions (174), including local GABAergic interneurons (174,290,548,555). Both $GABA_A$ and $GABA_B$ receptors could mediate this inhibition, as they are both expressed by 5-HT neurons (2,167,471).

Local infusions of GABA or a $GABA_A$ receptor agonist (muscimol) in the DRN reduce the firing activity of 5-HT neurons (164,165,174,547) as well as the release of 5-HT locally (371,524,526) and in projection areas (e.g. nucleus accumbens, striatum, olfactory tubercle, substantia nigra and medial prefrontal cortex) (371,462,526). Conversely, application of $GABA_A$ receptor antagonists (bicuculline, picrotoxin or GABAzine) in the rat DRN enhances all of these parameters (174,245,523,524,526), indicating that 5-HT neurons are under tonic $GABA_A$ receptor-mediated inhibition. Activation of DRN $GABA_B$ receptors also inhibits 5-HT release (in both the DRN and nucleus accumbens) but to a lesser extent than that of $GABA_A$ receptors (526). Furthermore, the $GABA_B$ receptor antagonist phacolofen had no effect on 5-HT release, arguing against tonic activation of this receptor (2). Finally, blockade of $GABA_A$ receptors (with bicuculline or picrotoxin), but not of $GABA_B$ receptors (with pertussis toxin), can

efficiently prevent the inhibitory effect of locally applied GABA on 5-HT neurons (229). These data indicate that the GABAergic tonic inhibitory modulation of DRN 5-HT neurons is mainly mediated by $GABA_A$ receptors (229).

1.7.1.3 Effects of neuroactive steroids on $GABA_A$ receptors

a) Neuroactive steroid modulation of $GABA_A$ receptors

At physiological (nM) concentrations, the following neuroactive steroids are positive allosteric modulators of $GABA_A$ receptors: 3α,5α-THP, 3α,5β-THP, 3α,5α-THDOC, 3α,5β-THDOC, androsterone and ganaxolone (a synthetic analog of 3α,5α-THP) (77,82,171,213,215,305,319,359,360,405,418,419,436,536,571). The structural requirement for this modulation appears to be reduction of the A-ring in the 5α or 5β conformation, along with a hydroxyl group at position C3 in the α configuration (171,172,215,359). 3α,5α-THP, 3α,5α-THDOC and ganaxolone behave like barbiturates in displacing the binding of *t*-butylbicyclophosphorothionate (TBPS) from the chloride channels and in potentiating GABA-induced chloride currents as well as the binding and potency of muscimol and benzodiazepines to $GABA_A$ receptors (82,172,215,305,359,405,536). However, they seem to act via a different binding site from that of barbiturates or benzodiazepines (302,405,418,536). Furthermore, at high concentrations (mM), 3α,5α-THP, 3α,5β-THP and 3α,5α-THDOC were shown to act as proper $GABA_A$ receptor agonists in the absence of GABA

(33,259,418,571). Consistent with these effects, 3α,5α-THP and ganaxolone have been shown to have anxiolytic (55,74) and anticonvulsant properties (111,259,433).

P, PREG, 5α- and 5β-DHP, DOC, 5α- and 5β-DHDOC, 3β,5α- and 3β,5β-THP, 17β-E and androstenedione, on the other hand, lack a 3α-hydroxy group within the A-ring and do not modulate $GABA_A$ receptors (77,155,172,215,306,405,419,453). However, as mentioned in section 1.4, they can be converted to 3α,5α- and 3α,5β-THP, and 3α,5α- and 3α,5β-THDOC, respectively, and thus modulate $GABA_A$ receptors.

Sulfated steroids such as PS, 3α,5β-THP sulfate, 3α,5α-THP sulfate, 3β,5α-THP sulfate, 3β,5β-THP sulfate and DHEAS are $GABA_A$ receptor antagonists (11,119,127,303,306,307,332,344,391,474). DHEA can also, although less potently than DHEAS, inhibit the response to GABA (119) and binding of benzodiazepine, muscimol and TBPS (249). Because positive and negative modulators act on distinct sites on $GABA_A$ receptors, these receptors can be simultaneously potentiated and inhibited (391). It is thus clear that a slight shift in the equilibrium of the steroid metabolic pathway could result in an important difference in GABAergic modulation of a neuronal system, which could in turn significantly affect its activity.

b) Steroid-induced plasticity of $GABA_A$ receptors

Hormonal exposure and/or fluctuation can induce functional plasticity of $GABA_A$ receptors. For instance, chronic treatment of cultured neurons with 3α,5α-THP or 3α,5β-THP was shown to time- and dose-dependently reduce the efficacy of $GABA_A$-mediated responses to GABA, neuroactive steroids, barbiturates and benzodiazepines, probably due to heterologous uncoupling between the GABA site, the modulatory sites and the chloride channel (155,588-590). Furthermore, during pregnancy or P administration, there is a region-dependent alteration in $GABA_A$ binding potential (78,568), affinity (304) and/or response (103), which is probably due to higher levels of 3α,5α-THP and 3α,5α-THDOC (304). The effect of estradiol exposure on these parameters appears to depend on the endogenous hormonal levels since it increased [^{3}H]muscimol binding in various brain regions (hypothalamus, cortex, olfactory bulb and striatum) in OVX rats (279,404) but reduced it in intact females (210). Hormonal withdrawal, such as occurring following parturition or ovariectomy, is also associated with region-dependent changes in affinity (244,304), stimulated chloride currents (103) and receptor density (244,304).

As mentioned above, the $GABA_A$ receptor's pharmacological properties are greatly influenced by its subunit composition (33), suggesting that these hormonally induced changes in $GABA_A$ response could be brought about through alteration of subunit expression. Indeed, there is a large body of evidence indicating that P exposure (or pregnancy) modulates the $GABA_A$ receptor subunit

expression in a region- and neuron-specific manner (103,139,140,145,147,319,565), probably via metabolism into 3α,5α-THP (103,147). Accordingly, 3α,5α-THP has also been shown to induce such changes (139,147,565,587). Furthermore, these changes were associated with consistent modifications of $GABA_A$ receptor's pharmacological properties (103,145,147). There are less studies assessing the effect of E on $GABA_A$ receptor plasticity but it also appears similarly region- and subunit-specific (221,565). Interestingly, fluctuations of E levels across the estrous cycle were shown to be sufficient to induce plasticity of γ subunits (97).

Abrupt fall in P and/or 3α,5α-THP cerebral levels is also associated with region-specific dynamic changes in $GABA_A$ receptor subunits (146,147,319) and pharmological properties (145,147). The same was shown for the synthetic steroid ganaxolone (319). Smith and co-workers did a series of studies showing that initial exposure to P, as well as withdrawal from it, lead to upregulation of the α4 subunit of the $GABA_A$ receptors in the hippocampus (188,486-488). This was associated with important loss of sensitivity to benzodiazepines (BDZ) (107,188,227,356,486,487) and 3α,5α-THP (486-488), and increased response to barbiturates (107,486). Furthermore, they showed that it was, in fact, exposure to 3α,5α-THP that induced this $GABA_A$ receptor plasticity and cross-tolerance to both BDZ and 3α,5α-THP (107,188,486,488). However, since 3α,5β-THP had the same effect (189), it could also be involved, as it is also a metabolite of P. These receptor changes also had measurable consequences on rat behavior, as

shown by increased anxiety, loss of BDZ anxiolytic properties (166,188,189,356,487,488) and greater seizure susceptibility (357,433). These data indicate that this neuroactive steroid can induce transient $GABA_A$ receptor plasticity after both short-term and termination of chronic exposure, and that it is behavior relevant (188). In the hippocampus, there was no sex difference in this 3α,5α-THP-induced $GABA_A$ receptor plasticity (189) but in the amygdala it was observed in females only (190).

c) Subunits required for neuroactive steroid modulation

As already mentioned, the differential assembly of various subunits gives rise to multiple $GABA_A$ receptors having different sensitivity to neurosteroid modulation (33). $GABA_A$ receptors containing the δ subunit appear to be the most sensitive to positive modulation by THDOC, especially when in combination with the α1 subunit (52,571). Cerebral regions expressing $GABA_A$ receptors containing these two subunits might thus be especially sensitive to neurosteroid modulation (52). Furthermore, in δ subunit knockout (δ KO) mice, $GABA_A$ receptor-mediated currents are less responsive to THDOC (551) or the synthetic steroid alphaxolone (496). Alphaxolone and ganaxolone also have greatly reduced anxiolytic effects in δ KO mice (345). Interestingly, in these mice, there was also greater sensitivity to PS, thus reducing the total amplitude of $GABA_A$ receptor-mediated current (413). Only one study showed that δ subunit greatly reduces sensitivity to neurosteroids (THDOC, 3α,5α-THP and PS) (596).

Concerning other subunits, $GABA_A$ receptors containing α1 or α3 seem more responsive to modulation by 3α,5α-THP than the α6-containing ones, while those expressing α2, α4 or α5 are about 10 times less sensitive than those expressing α1 (33). Different γ subunits influence the responsiveness to 3α,5α-THP (γ2 conferring greatest sensitivity, followed by γ3 and then γ1), while β1-3 subunits do not appear to affect it (33).

Recent studies have shown that phosphorylation also determines the $GABA_A$ receptor's response to neurosteroids (213). Interestingly, stimulating PKC activity restored normal sensitivity to THDOC in cerebellar granule neurons from δ KO mice (551). However, phosphorylation can lead to both greater and lesser sensitivity depending on the brain region, type of neurons, receptor composition and which amino acid residues are phosphorylated (213). For instance, in neurons of the cortex (213) and supraoptic nucleus (260), PKC-mediated phosphorylation reduces sensitivity of $GABA_A$ receptors to 3α,5α-THP (213,260). Conversely, in hypothalamic magnocellular neurons, inhibition of G protein and PKC prevents 3α,5α-THP-mediated potentiation of the $GABA_A$ receptor response (138). In CA1 hippocampal pyramidal cells and dentate granule neurons, PKA- or PKC-mediated phosphorylation also enhances sensitivity to neurosteroids, while its inhibition prevents it (213).

In the rat DRN, the most importantly expressed subunits are α1, β2, γ2, γ3 and δ, followed by, in order, α2 and β1, α3, and finally by α5 and β3 (412). The

expression of α4 and γ1 subunits is minimal if even present (412). An older study revealed a different pattern of expression: moderate expression of α1, α3, β2,3 and γ2, low expression of α2, while α5 and δ were not detected (156). Despite contradictory results concerning the expression of the δ subunit, both these patterns of expression would suggest the DRN to be sensitive to neurosteroid modulation. Furthermore, most of the rat DRN 5-HT neurons express $GABA_A$ receptor α3 subunits on their dendrites and cell bodies (167), while only a minority expresses the α1 subunit (167). The latter is rather expressed by GABAergic neurons, along with α3 subunits (167). These observations represent other potential mechanisms of action for neurosteroid modulation of 5-HT neurons.

It is clear from these findings that $GABA_A$ receptors mediate the GABAergic inhibition of 5-HT neurons and that they can, themselves, be modulated by several neurosteroids. Steroid-induced alterations of this GABAergic inhibition of 5-HT neurons would therefore be likely and might modulate their firing activity. Since many steroids can modulate the $GABA_A$ receptor response and since this modulation can be brought about by various mechanisms of action it would be difficult to speculate on the net impact this modulation would have on the activity of 5-HT neurons. However, it can be extrapolated from these studies that physiological hormonal fluctuations, such as occurring during the estrous cycle or pregnancy, would be sufficient to modulate 5-HT neuronal activity through altered GABAergic inhibition.

1.7.2 Glutamate

1.7.2.1 Glutamatergic excitatory modulation of DRN 5-HT neurons

The DRN receives glutamatergic afferents from various brain regions (4,240,281,282). This input on 5-HT neurons positively modulates their firing activity (4,240). Early studies showed that iontophoretic applications of glutamate in the DRN elevate the firing activity of 5-HT neurons (25,547). Later, studies using microdialysis showed that this increase in firing activity is mediated by local AMPA/kainate and NMDA receptors and that it also results in increased extracellular 5-HT in the DRN and projection area (nucleus accumbens) (524,525,527,528). Antagonism of these receptors had little or no effect of its own (200,524,525,528), thus suggesting that they mediate only a weak tonic excitation of 5-HT neurons (524,525,528). On the other hand, NMDA and AMPA/kainate receptors have been shown to mediate non-tonic stimulation of 5-HT neuronal activity and release (87,522).

1.7.2.2 Effects of neuroactive steroids on glutamatergic receptors

a) Sulfated steroids

Various sulfated steroids modulate the activity of glutamatergic receptors. For instance, PS rapidly potentiates the NMDA receptor response

(1,71,86,231,393,578) by increasing the frequency and duration of channel openings (71). It is physiologically relevant since, for instance, this effect protects mice against NMDA antagonist-induced learning deficits and motor impairment (321). NMDA receptors are composed of an obligatory NR1 subunit and modulatory NR2 subunits, which influence the receptor's pharmacological properties and its modulation by neurosteroids (292). The NR2 receptor subunit is particularly important in determining the effect of PS on the NMDA response; a potentiation is observed with receptors expressing the NR2A or NR2B subunit (86,308), whereas an inhibition was shown for the NR2C- or NR2D-containing receptors (308).

Other sulfated steroids, such as DHEAS, have been shown to potentiate the NMDA receptor response but to a lesser extent than PS (71,392). PS also seems to dose-dependently and non-competitively inhibit the AMPA/kainate receptor (71,578,581). Conversely, some sulfated steroids such as 3β,5β-THP sulfate (231,393,581), 3α,5β-THP sulfate (1,231,308,392,563,581) and 3α,5α-THP sulfate (1) inhibit NMDA receptors. This inhibition was shown to be protective against glutamate- and NMDA-induced cell death as well as against NMDA-induced seizure (563). These antagonistic sulfated steroids and PS seem to act at different sites on the NMDA receptor, since no competitive interaction was observed between them (393).

b) 17β-estradiol and Progesterone

The effect of 17β-E on NMDA and AMPA receptor expression is region specific (110,112). For instance, in OVX rats, a two-week administration of 17β-E increased the NMDA receptor density in the hippocampal CA1 area and the dentate gyrus, decreased it in the frontal cortex and the dorsal striatum, and caused no change in the CA2/3 hippocampal regions, the nucleus accumbens or the hypothalamus (72,110). The hippocampal increase in NMDA receptors was already visible after two days of 17β-E administration, (170,564,577) and was associated with enhanced dendritic spine and synapse densities as well as greater functional NMDA receptor-mediated synaptic input (577). P had little or no additional effect (170,182,564). Interestingly, a functional estrogen responsive element (ERE) was found in the regulatory region of the gene coding for the NR2D subunit (557), thus offering a mechanism by which 17β-E can modulate the expression of NMDA receptors (557) and/or influence its pharmacological properties. Concerning estrogenic effects on AMPA receptor expression, 17β-E was shown to decrease AMPA binding sites in the frontal cortex, striatum and nucleus accumbens of OVX rats (110) without affecting them in the hippocampus (110,564,577).

The rat DRN expresses high levels of NMDA receptors (374,461) as well as mRNA for different subunits of non-NMDA glutamate receptor (GluR1-4) (459). A hormonal modulation of their expression, which could in turn affect the

activity of 5-HT neurons, is possible. However, there is no data available yet concerning the effect of ovarian steroids on glutamatergic receptors in the DRN.

Smith and colleagues, using cerebellar Purkinje neurons from OVX rats, have shown that local (490) and systemic (491) administrations of 17β-E, but not of 17α-E (490), rapidly and dose-dependently potentiate the excitatory response to iontophoretic applications of glutamate (490,491), quisqualate and NMDA (485). P (489,492), 5α-DHP and 3α,5α-THP (493) had the opposite effect and reduced the excitatory response to local applications of glutamate (489,492). 3β,5α-THP was without effect in this paradigm (493). Neither the ER antagonist tamoxifen nor a protein synthesis inhibitor (492) prevented these effects, thus suggesting a non-genomic mechanism of action (491,492). Furthermore, these 17β-E- and P-induced effects were additive, indicating that they are independent (492). Interestingly, in freely cycling females, 17β-E potentiated the excitatory response to glutamate during proestrus but not diestrus, i.e. when there are relatively high levels of 17β-E as compared to P (490).

As mentioned above, the glutamatergic excitation of 5-HT neurons does not seem tonic. Therefore, even if steroids induced a similar enhancement of the response of glutamatergic receptors expressed on 5-HT neurons, only a phasic effect on the firing activity would be expected. However, PS and DHEAS were shown to increase glutamate release from cultured hippocampal neurons (341).

Therefore, if these steroids similarly triggered glutamate release in the DRN, this might enhance the basal firing activity of 5-HT neurons.

1.7.3 Norepinephrine

1.7.3.1 Tonic noradrenergic excitatory input on DRN 5-HT neurons

The noradrenergic (NE) and 5-HT systems exert a complex reciprocal modulation of each other (647, 875, 929, 1385). Of particular relevance for this thesis, is the excitation of DRN 5-HT neurons by NE (534). Anatomically, the DRN is extensively innervated by NE terminals, which make direct synaptic contact with 5-HT neurons (26,406). These NE terminals seem to originate mainly from the locus coeruleus (LC) (417). α_2 autoreceptors, located on the cell body (446) and nerve terminals of NE neurons, exert a negative control on the firing activity of these neurons (875) and on NE release in the synaptic cleft (69,197). Activation of α_2-adrenoceptors inhibits 5-HT neuronal firing activity (521) and 5-HT release in the DRN and projection areas (69,416,417), whereas antagonism of these receptors elevates all of these parameters (168,330). Chemical lesion of the NE system using 6-OHDA leads to a 70-80% reduction in DRN NE content (454), as well as a transiently lower firing activity of 5-HT neurons firing activity, which then recovers within a week (521). *In vitro* DRN 5-HT neurons, devoid of afferents, also have a lower spontaneous activity and can

be stimulated by NE (546). Together these data support a non-obligatory NE excitation of DRN 5-HT neurons.

This tonic NE positive modulation appears to be mediated by α_1-adrenergic receptors located on the 5-HT neurons (6,69,318). Indeed, systemic or local application of α_1 adrenoceptor antagonists in the DRN decreases the 5-HT neuronal firing rate (24,25,318), as well as 5-HT release in the DRN (69,447) and projection areas (e.g. hippocampus, striatum and prefrontal cortex) (416,447), an effect which could be reversed by NE or an α_1-adrenergic agonist (24,25,318). Application of NE or an α_1-adrenergic agonist in the DRN increases 5-HT release in the prefrontal cortex (416) but has little effect on the extracellular 5-HT in the DRN (69,416). This could suggest that this α_1-adrenergic receptor-mediated tonic activation is maximal (69,264,416,417,521) or that this excitatory effect is counter-balanced by the inhibitory effect of NE on terminal α_2-adrenoceptor (4,417). However, inhibition of α_2-adrenoceptors results in an increase in firing frequency of DNR 5-HT neurons (197), thus arguing against a saturated tonic activation.

1.7.3.2 Effects of ovarian steroids on noradrenergic neurotransmission

There is some evidence of a steroid modulation of the NE system. For instance, 17β-E was shown to increase the mRNA levels of two enzymes implicated in NE synthesis (tyrosine hydroxylase and dopamine β-hydroxylase) in

the LC of OVX rats and monkeys (396,470). This effect was associated with greater release of NE in the monkey hypothalamus (396). A 2-day 17β-E treatment of OVX rats was shown to uncouple the hypothalamic terminal α_2 autoreceptor from its effector G protein, which reduces its function and also leads to greater NE release (135,136). Furthermore, 17β-E appears to selectively and competitively inhibit NE uptake in rat hypothalamic and cortical synaptosomes (175), suggesting that, *in vivo,* it might inhibit NE reuptake and thus increase its extracellular levels. Finally, two days of 17β-E administration increased α_1-adrenergic expression and functional activation in the hypothalamus and preoptic area of OVX rats (134,135). Taken together, these data suggest facilitation of the NE neurotransmission by 17β-E. It is unclear whether this is the case in the DRN but, if so, it would likely result in enhanced NE tonic activation of 5-HT neurons.

The data presented here demonstrate that steroids can modulate the function of different neurotransmitter systems through different mechanisms of action, ranging from activation of ionotropic receptors to modulation of gene expression. The systems presented here are all afferents of DRN 5-HT neurons. Therefore, in addition to direct modulation of the 5-HT system, steroids probably also impact on it indirectly through actions on these systems. The GABAergic inhibition has been suggested to be the most important tonic influence on DRN 5-HT neurons (523,524,528) and might thus play a particular role.

1.8 Steroids and sigma receptors

1.8.1 Sigma receptors

The existence of sigma (σ) receptors was first reported in 1976 (313) and so far, there are two generally accepted subtypes of σ receptors: σ_1 and σ_2 (421). In 1996, the sigma$_1$ (σ_1) receptor was cloned (211). To this day, however, the endogenous ligand(s) remains to be identified (117). The amino acid sequence and the deduced structure show homology with that of the product of the yeast gene encoding a sterol C_8-C_7 isomerase (ERG2), which has high affinity for several σ ligands (211,353). Also, σ_1 receptors share affinity for different compounds with a mammalian sterol C_8-C_7 isomerase (the emopamil binding protein or EBF), which is, however, structurally unrelated to the yeast counterpart (353). Since this enzyme is necessary for cholesterol synthesis and the brain relies on *de novo* synthesis for its cholesterol supplies, it was suggested that σ_1 receptors might be a brain-specific sterol C_8-C_7 isomerase (353).

Sigma receptors are widely distributed in the mammalian brain (13,191,328,409). They have a relatively high level of expression in the brainstem (327,409) and are moderately expressed in the DRN of guinea pigs (191,328). Their distribution being very similar between rats and guinea pigs (191), σ receptors may also be expressed in the rat DRN. σ binding sites appear to be limited to neurons (191,409), where they are found in both the cell body

and dendrites (13,191). More specifically, they appear to be located in the microsomal fraction (i.e. endoplasmic reticulum, mitochondrial and plasma membranes) (13,327,358) and concentrated in postsynaptic membranes (13).

1.8.2 Steroids modulation of sigma receptors

Several steroids (P, PS, T, DHEAS) have high affinity for σ receptors (324,326,327,510,582) and have been shown to dose-dependently and competitively inhibit the binding of the σ radioligand [^{3}H]-(+)-SKF-10,047 in mouse and rat brains (324,327,510). P has the highest affinity and is the most potent in inhibiting [^{3}H]-(+)-SKF-10,047 binding (324,510). It has thus been suggested to be a potential endogenous ligand for σ receptors (510,582). Interestingly, [^{3}H]-(+)-SKF-10,047 binding was also reduced during pregnancy, a period during which P levels are higher (324).

In different behavioral models, including the conditioned fear stress (373), the Porsolt forced swimming test (432,540) and a model of learning impairment, in which σ ligands have antiamnesic effects (322,323,325,408), DHEAS and PS have been shown to be functional agonists of σ receptors, and P to be a potent antagonist. In a cocaine-induced conditioned place preference paradigm, DHEA acted as a σ agonist, as it facilitated acquisition of this preference, while P antagonized it (443). It was also confirmed by electrophysiological studies. For instance, different σ ligands (DTG, JO-1784 and (+)-pentazocine), as well as

DHEA, potentiate the excitatory response of hippocampal CA3 pyramidal neurons to NMDA (35,36,118). While P, similarly to other σ antagonists, does not modify this neuronal activity *per se*, it prevents its potentiation by σ ligands and DHEA (35,36,118). A similar reduction in the σ receptor mediated potentiation of the NMDA response is also observed during late pregnancy, when plasma P levels are high (36). Conversely, during periods associated with an abrupt fall in P, such as the postpartum period and following ovariectomy, this effect was not only reversed but the potentiation of the NMDA response by DHEA, DTG and (+)-pentazocine is greater than in control females (35,36,118). This suggests a tonic inhibition of σ receptors by progesterone (35,118).

There is data indicating that activated σ_1 receptors translocate from the endoplasmic reticulum to the plasma membrane, where they can trigger intracellular signaling cascades involving G proteins, PLC and PKC, and thus modulate neuronal excitability (35,354,358,365,376,441) and/or neurotransmitter release (120,365,376,377). This increase in PLC activity is region-dependent (441) and occurs in rat hippocampal, striatal and cortical neurons, as well as in neurons from the guinea pig brainstem (354,358,441). It might explain why others, using guinea pig cerebellar tissue or rat pituitary, could not reproduce these results (226,298). The σ receptor-mediated G-protein activation was shown to modulate intracellular Ca^{2+} concentrations (216) and also to result in inhibition of two types of K^+ channels in frog pituitary cells, thus increasing their electrical activity (495). Interestingly, DHEA, DHEAS and PS dose-dependently

stimulated [^{35}S]GTPγS binding in synaptic membranes obtained from the mouse prefrontal cortex (537). This effect could be prevented by pertussis toxin (PTX) and blocked by both NE-100 (a selective σ_1 antagonist) and P, which could also block the (+)-pentazocine-induced stimulation of [^{35}S]GTPγS binding (537). PS was also shown to modulate inhibitory postsynaptic currents (IPSCs) by activating G protein-coupled σ_1 receptors in embryonic rat hippocampal neurons (365). These data strongly suggest that steroids can activate G proteins by binding σ receptors (216,537).

Activation of σ_1 receptors has been shown to modulate the electrical activity of different types of neurons (347,358,365), including DRN 5-HT neurons (37). Of particular interest, two different σ_1 agonists ((+)-pentazocine and 4-IBP) were shown to increase DRN 5-HT neuronal activity when administered for 2, 7 and 21 days (37). Thus, σ receptors may represent an additional way by which neurosteroids can affect the 5-HT system.

1.9 Overview

In summary, evidence indicates that ovarian steroids and the 5-HT system are implicated in the neurobiology of depression. The data presented here also demonstrate that most sex steroids can be synthesized and metabolized by the brain, and that they can alter cerebral function via numerous mechanisms of action, both genomic and non-genomic. More specifically, they have been shown

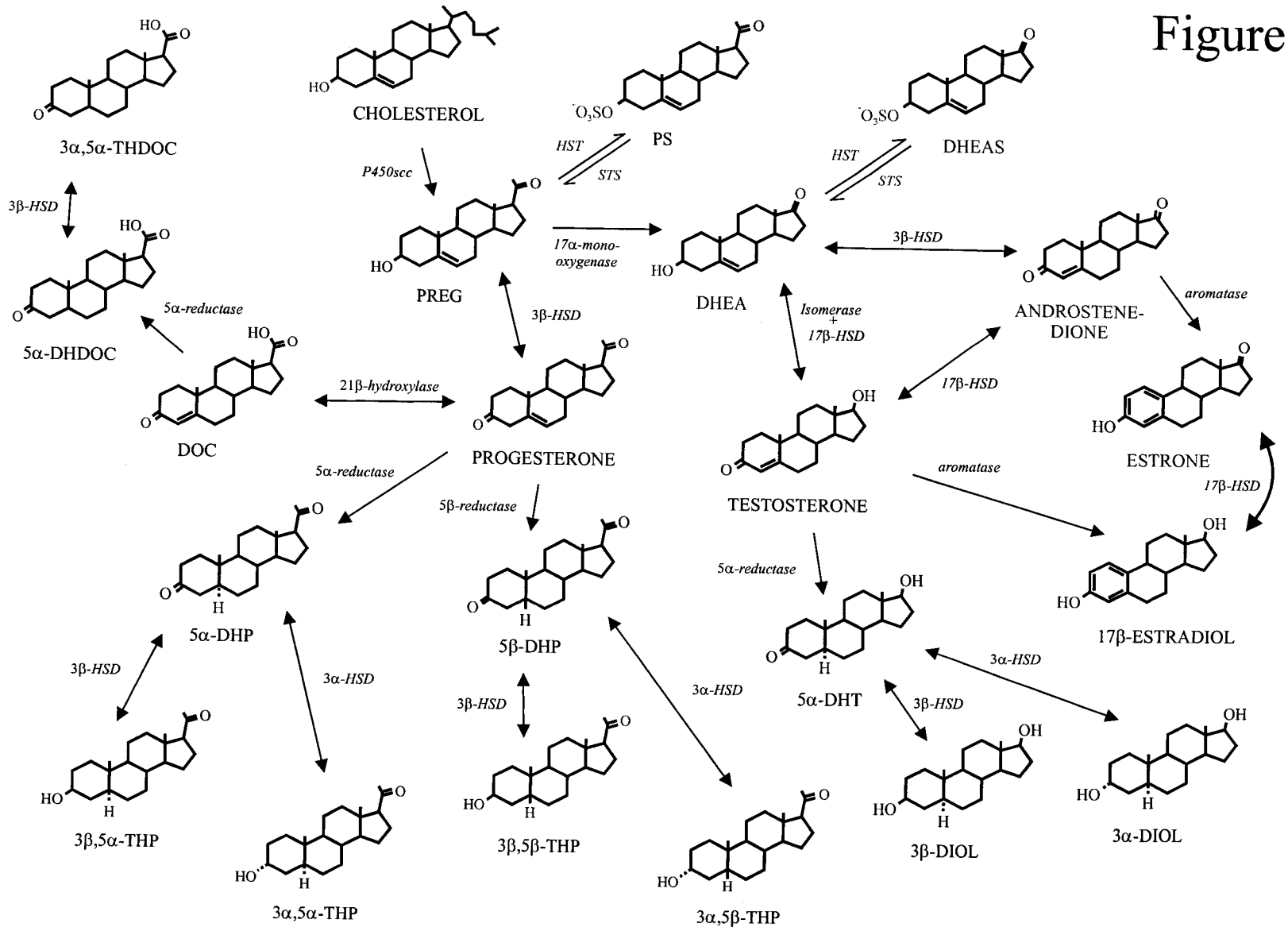
to affect different proteins involved in 5-HT neurotransmission. Furthermore, they have the ability to modify the function of other receptor or neurotransmitter systems, which are afferents or modulators of DRN 5-HT neurons. Taken together, these data strongly suggest that physiological hormonal fluctuations could affect 5-HT neurotransmission. Women with a more vulnerable or sensitive 5-HT system might thus be at greater risk of developing mood disorders.

1.10 Objectives

Important gender differences exist regarding affective disorders and accumulating evidence indicates that ovarian steroids and the 5-HT system are implicated in the neurobiology of depression. It is also clear that neuroactive steroids can alter cerebral function and there are data strongly suggesting that physiological hormonal fluctuations could affect 5-HT neurotransmission. The activity of 5-HT neurons located in the dorsal raphe nucleus (DRN) is a decisive factor in 5-HT neurotransmission. However, a bridge between the clinical and the molecular studies regarding the effects of sex steroids on the 5-HT system is still needed. Therefore, the goal of this thesis project was first to evaluate the effect of gender, gonadectomy and pregnancy on the spontaneous firing activity of DRN 5-HT neurons and to examine different potential mechanisms of action underlying these differences. The second objective was to directly assess the modulation by various neuroactive steroids of the 5-HT neuronal firing activity and compare this modulation between males and females. Finally, potential therapeutic applications in the treatment of depression were investigated for some of these steroids.

Figure 1: Steroids' partial metabolic pathways. Two-way arrows depict reversible reactions catalyzed by the same enzyme. Double arrows represent reactions, which are catalyzed by their respective enzyme.

Figure 1


3α,5α-THDOC
CHOLESTEROL
PS
DHEAS
HST
STS
P450scc
3β-HSD
17α-mono-oxygenase
PREG
DHEA
ANDROSTENE-DIONE
aromatase
5α-DHDOC
5α-reductase
DOC
21β-hydroxylase
PROGESTERONE
Isomerase + 17β-HSD
TESTOSTERONE
17β-HSD
ESTRONE
17β-ESTRADIOL
5α-DHP
5β-reductase
5β-DHP
5α-DHT
3α-HSD
3β-HSD
3β,5α-THP
3α,5α-THP
3β,5β-THP
3α,5β-THP
3β-DIOL
3α-DIOL

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Foreword to Chapter 2

Gender and gonadal status modulation of dorsal raphe nucleus serotonergic neurons. Part I: Effects of gender and pregnancy

As mentioned in the introduction, there are important gender differences concerning mood disorders, with women being more vulnerable, and there is accumulating evidence of a role for ovarian hormones in female affective disorders. Furthermore, a large body of evidence indicates a functional interrelationship between depression, neuroactive steroids and the serotonergic (5-HT) system, which has itself long been implicated in the neurobiology of affective disorders. The activity of 5-HT neurons located in the dorsal raphe nucleus (DRN) is crucial for 5-HT neurotransmission. The goal of this study was thus to assess whether ovarian hormones could modulate the activity of DRN 5-HT neurons. The effects of gender and different hormonal status on this activity were evaluated by measuring the spontaneous firing activity of DRN 5-HT neurons in male, freely cylcing female, ovariectomzed female and pregnant female rats.

Chapter 2

GENDER AND GONADAL STATUSMODULATION OF DORSAL RAPHE NUCLEUS SEROTONERGIC NEURONS. PART I: EFFECTS OF GENDER AND PREGNANCY

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SUMMARY

Gender differences in susceptibility to affective disorders are well documented. The ovarian steroids, estrogen (E) and progesterone (P), may modulate the function of the serotonergic (5-HT) system, implicated in the etiology and treatment of affective disorders. We tested the hypothesis that ovarian steroid modulation of 5-HT function could result in a modification of the 5-HT neuronal firing activity. Extracellular unitary recordings of dorsal raphe nucleus 5-HT neurons were obtained in male rats and in female rats during natural E and P fluctuations. The average firing activity of 5-HT neurons was significantly higher in males (41%) than in freely cycling (CF) and in ovariectomized (OVX) females. During pregnancy, it increased gradually and by up to 136% on gestational day 17, then declined before parturition. In the postpartum period (PP), the firing rate decreased markedly compared to P17 but remained 63% higher than in CF. During pregnancy, the firing rate variations were closely correlated with P plasmatic levels. Finally no modification of the basal firing activity of locus coeruleus noradrenergic neurons was found in any group tested. Our results thus reveal a gender and pregnancy-dependent modulation of 5-HT firing rate that would impact 5-HT-mediated neurotransmission.

INTRODUCTION

Susceptibility to affective disorders differs between genders; women are twice as likely to suffer from major depression than men (Weissman and Olfson, 1995). In addition, women can experience dramatic swings in affective states during and following pregnancy. During pregnancy, women typically report feelings of elation and tranquillity and are at lower risk for developing affective disorders (Pugh et al., 1963), whereas following pregnancy, psychiatric diseases are likely to occur in predisposed individuals (Davidson and Robertson, 1985). Ovarian hormones have thus been recognized as having influences on mood and affective states.

Serotonin (5-HT) is known to regulate mood and the 5-HT system is implicated in the etiology (Mann et al., 1996; Lesch, 1998) as well as the treatment of affective disorders. An enhancement in 5-HT neurotransmission is presumed to underlie the therapeutic effect of antidepressant medications (Blier and de Montigny, 1994; Owens, 1996). In view of the postulated role of ovarian hormones on mood, several indices of modulation of the 5-HT function by estrogen (E) and progesterone (P) have been sought for and demonstrated in many species. Most of these studies had focused on 5-HT levels and metabolism (Cone et al., 1981; Desan et al., 1988; Morissette et al., 1990) as well as on receptor binding in postsynaptic target areas (Biegon and McEwen, 1982; Frankfurt et al., 1994; Sumner and Fink, 1995). However, until recently, the locus (i.e. terminal field versus cell body region) at which E and P could regulate central 5-HT

activity was unknown. Data from primates (Bethea, 1994) and rodents (Alves et al., 1998a) have now established that the dorsal raphe nucleus (DRN), the largest nucleus providing 5-HT innervations to the forebrain, is a site at which E and P receptors are expressed. Thus, ovarian steroids can exert a direct receptor-mediated effect on the serotonergic function.

Lately, E and P modulation of the expression of genes directly regulating serotonergic neurotransmission has been reported. The mRNA levels for the tryptophan hydroxylase (Pecins-Thompson et al., 1996), the serotonin transporter (McQueen et al., 1997; Pecins-Thompson et al., 1998), and the 5-HT_{1A} somato-dendritic autoreceptor (Pecins-Thompson and Bethea, 1997) were all affected by hormonal treatments. However, these studies did not provide direct, conclusive evidence that the 5-HT neurotransmission was affected. The release of 5-HT in terminal fields is highly dependent on the neuronal discharges in the cell body region (Wilkinson et al., 1991), which is strictly correlated with behavioral state (Trulson and Jacobs, 1979). We have therefore tested the hypothesis that ovarian hormones can directly modulate the 5-HT neuronal discharge, independently of the behavioral state. We monitored *in vivo*, the baseline spontaneous unit activity of 5-HT neurons in the DRN of anesthetized male and female rats during periods of natural ovarian hormone fluctuations. Indeed, 5-HT discharge rate was found to differ between genders and, in females, to vary dramatically during pregnancy and the postpartum period. To demonstrate the specificity of this modulation we also examined spontaneous activity in the noradrenergic (NE) neurons of the locus coeruleus (LC).

MATERIALS AND METHODS

Animals

Sprague-Dawley rats were purchased from Charles River (St. Constant, Québec) and kept under standard laboratory conditions. Experimental groups were: intact freely cycling females (CF, 225-250 g); ovariectomized females (OVX, 225-250 g) used 15 to 21 days after surgery; pregnant females (250-350 g) used at 11 days (P11), 14 days (P14), 17 days (P17), and 21 days (P21) of gestational age (delivery was on day 22); intact females (250-275 g) used 1 to 7 days postpartum (PP); and males (275-325 g). We considered freely cycling females as our control female group (CF). Since high levels of progesterone can be maintained during lactation as a result of the suckling stimulus (Smith, 1981), mothers were separated from their pups after delivery to ensure that progesterone levels in PP were back to CF levels. Ethical approval was given by the McGill University Animal Ethical Care Committee and all their rules and regulations were followed. The suffering of animals as well as the number used were kept at minimum.

Extracellular single unit recordings

Rats were anesthetized with chloral hydrate (400 mg/kg i.p.) and mounted in a stereotaxic frame. Body temperature was maintained at 37±0.5 ºC. A 2 mm-diameter section of bone was removed from the skull at the appropriate location and a glass micropipette, tip diameter 1-3 μm, filled with a 1 M NaCl solution

was lowered to the appropriate depth. When the identity of a neuron was ascertained, output voltage was passed through a window discriminator then recorded simultaneously on a chart recorder and an on-line computer using in-house data acquisition software. Data were displayed as a 10 s bin integrated firing frequency histogram. For each neuron, the mean firing rate, in spikes per 10 s, was computed by averaging 5 or 6 consecutive bins.

Dorsal raphe recordings.

Unit activity was recorded along descents covering the nucleus from 300 μm anterior to lambda to 1500 μm anterior to lamba or until no 5-HT neurons were encountered. 6 tracks were done on the midline and 2 to 4 tracks 200 μm lateral to midline. Spontaneously active 5-HT neurons were encountered starting at the ventral border of the Sylvius aqueduct easily recognized by a sudden voltage drop, and could be seen for up to 1mm below this landmark. 5-HT and non-5-HT neurons were identified according to the criteria of Aghajanian et al. (1978). Only 5-HT neurons were recorded. The occurrence of non-5-HT neurons was simply noted.

Locus coeruleus recordings.

Unit activity was recorded along 5 descents, separated by 200 μm, centered on 1 mm posterior to lambda and 1 mm lateral to midline. Spontaneously active NE neurons were encountered right below the ventral border of the 4rth ventricle, and were identified according to the criteria of Cedarbaum and

Aghajanian (1976). No spontaneously active units other than NE were encountered. Recordings were interrupted 500 μm below the 4th ventricle so as not to sample spontaneously active units of the sub-coeruleus nucleus.

E and P plasmatic dosage

At the end of single unit recordings, peripheral blood was drawn by cardiac tapping. After centrifugation, plasma was stored at -70 ºC for later analysis. 17β-estradiol and progesterone concentrations were determined by a highly specific competitive immunoassay using the ACS:180 Automated Chemiluminescence System (Chiron Diagnostics, MA).

Statistical analysis

Statistical analysis was performed with "SigmaStat for Windows Version 2.0" software (Jandel Corporation). Average values are given as mean ± SEM. In some cases, the coefficient of variation (CV=standard deviation/mean) was calculated to characterize the spread of a distribution. One-way ANOVA, with alpha = 0.05, followed by a post-hoc analysis using Tukey's method of comparison versus control (controls being CF) was used for evaluating statistical significance. Results (F) of statistical analysis are expressed in terms of number of groups compared (p) and degrees of freedom between groups compared (df). Significance was considered for $P<0.05$. A correlation analysis was used to assess whether the firing activity of the 5-HT neurons in pregnant females followed the levels of plasma progesterone. This analysis was performed using

the software "GraphPad Prism version 3.0", with alpha = 0.05. Results of the correlation are expressed with r: correlation coefficient and P. Significance was considered for $P<0.05$.

RESULTS

Spontaneous DRN neuronal activity

We compared the spontaneous firing activity of DRN 5-HT neurons of male rats, female rats during pregnancy and following ovariectomy to that of freely cycling females. To quantify the 5-HT firing rate, electrode descents (Fig. 1A) covering the DRN were performed and the mean firing rate for each unit was evaluated in spikes per 10 s (spk/10s). In anesthetized male rats, DRN 5-HT neurons discharge in a slow (2-30 spk/10s), clock-like manner (Aghajanian et al., 1968; Aghajanian and Vandermaelen, 1982). The same rhythmic pattern was observed in females although a difference in firing rates was clearly apparent (Fig. 1A). The mean number of 5-HT neurons per descent, however, did not significantly differ between groups. Neither did the mean number of non-5-HT neurons encountered per rat, which indicates that variations in mean firing rate from one group to the other were not accompanied by a significant change in the number of spontaneously active 5-HT or non-5-HT neurons. In addition, the within-group variability of firing rates was equally low for all groups (Table 1).

Group comparisons revealed that the mean firing rates was significantly different between experimental populations using CF as the control. Indeed, a significant interaction was observed between CF, M and OVX [$F(2,3) = 27.1$, $P<0.001$]. This interaction was related to a higher mean basal firing rate for M (Tukey's test, $q = 9.3$, $P<0.05$). The mean firing rate in males was significantly higher (41%) than in CF while CF and OVX were not statistically different

(Tukey's test, q = 0.2, n.s.) (Fig. 1B). During pregnancy, the mean firing rate increased steadily with gestational age to culminate at P17, before declining abruptly at P21 (Fig. 2B). At its peak value, at P17, the firing rate was 136% higher than in CF. At day 19 of pregnancy, median firing rate (13.0 spk/10s, n=47; data not shown) had already substantially declined relative to P17 and was only 44% higher than in CF. The mean firing rates at P11, P14, P17, and P21 were all significantly higher than in CF [$F(5,6) = 68.9$, $P<0.001$, Tukey's test, $q = 7.9$, $P<0.05$, $q = 16.8$, $P<0.05$, $q = 24.0$, $P<0.05$, $q = 5.9$, $P<0.05$, respectively]. Following parturition, the mean firing rate in PP remained significantly (63%) higher than in CF [Tukey's test, $q = 10.8$, $P<0.05$] (Fig 2B). Surprisingly, following parturition, the declining trend seen between P17 and P21 seemed to be reversed (16.4 spk/10s for PP *vs* 13.7 spk/10s for P21).

Spontaneous LC neuronal activity

Estrogen is known to possess CNS activating and proconvulsant properties (reviewed in Smith, 1994). To exclude the possibility of a generalized modulation of neuronal activity produced during natural hormonal variations, we also assessed the spontaneous firing activity of NE neurons in the LC. A particular reason for selecting the LC was that, by the same token, a potential influence on DRN 5-HT firing activity could be investigated. Pharmacological evidence suggests that the 5-HT neuronal discharge is dependent on a tonic excitatory NE input (Baraban and Aghajanian, 1980). Also, anatomical studies have demonstrated that the DRN receives one of the heaviest NE innervations in the

brain (Levitt and Moore, 1979) and that the LC does, indeed, project to the DRN (Jones and Yang, 1985).

Electrode descents were therefore performed in the LC of CF, males, OVX and P17 (Fig. 3A), in a manner analogous to that in the DRN. The distribution of unit firing rates is shown in the bar graph of Fig. 3B, while Table 2 lists quantified parameters. No significant difference was found between the mean firing rates or between the mean number of spontaneously active NE units per descent of any of the groups [$F(3,4) = 1.3$, n.s.].

Relation of 5-HT firing activity to circulating E and P

During the estrous cycle, plasma levels of E (as 17β-estradiol) and P peak at about the same time, on the afternoon of proestrus (Freeman, 1994). However, during pregnancy, the E and P maxima are shifted by several days (reviewed in Rosenblatt et al., 1988). To determine if the modulation of 5-HT neuronal firing activity during pregnancy and the postpartum period followed circulating levels of one or the other steroid hormone, plasma levels of E and P were assessed in the same animals used for electrophysiological recordings. Figures 4 and 5 show, for each group, mean plasma levels of E (Fig. 4) and P (Fig. 5). In keeping with Rosenblatt et al. (1988), during pregnancy, we found low circulating concentrations of E, and a steady increase in P that peaks at P17 and then declines markedly before parturition. We have not been able to detect the sharp rise in E occurring immediately prior to parturition, although there was a trend for an

increase on P21. Also shown in these figures (4 and 5) are the mean firing rates of 5-HT neurons, for each group, superimposed on the mean plasma levels of E (Fig. 4) and P (Fig. 5). There was a striking correlation between circulating levels of P and the 5-HT neuronal discharge, but only during pregnancy ($r=0.94$, $P<0.05$; Fig. 5B). No other relation between firing rates and levels of E or P could be detected. Notably, hormonal levels were similar in OVX and males but firing rate was significantly higher in males (Fig. 1B); likewise, hormonal levels were similar in CF and PP but firing rate was significantly higher in PP (Fig. 2B).

DISCUSSION

Our findings demonstrate a clear modulation by gender and hormonal levels of the *in vivo* spontaneous firing activity of DRN 5-HT neurons. To our knowledge, this is the first report of a tonic variation in 5-HT neuronal discharge, which is not dependent on behavioral state. We have shown that the basal firing rate of 5-HT neurons is higher in males than in females. This difference does not seem to be solely tied to the circulating levels of E or P since the hormonal levels of OVX are similar to males while their 5-HT neurons firing activity is comparable to CF. We have also shown that in females, pregnancy and the postpartum period bring about dramatic changes in the basal discharge rate of 5-HT neurons.

Gender differences in the 5-HT system have long been demonstrated in rodents and humans (McEwen et al., 1998, Nishizawa et al., 1999). A study, using positron emission tomography (PET), showed that women have a smaller rate of serotonin synthesis, which is moreover, reduced about four times more than it is in men, following an acute tryptophan depletion (Nishizawa et al., 1999). In female rats, 5-HT levels, as well as, 5-HT function and receptor concentrations, were also shown to vary during periods of ovarian hormone fluctuations such as the estrous cycle, pregnancy and postpartum period (Biegon et al., 1980, Uphouse et al., 1986, Maswood et al., 1999). More specifically, in females, numerous studies were performed at different levels of the regulation of the 5-HT neurotransmission. Both E and P treatments lead to a decrease in DRN vesicular

monoamine transporter ($VMAT_2$) mRNA levels (Rehavi et al., 1998). Moreover, E treatments have been shown to decrease the serotonin reuptake transporter (SERT) mRNA levels in monkeys and increase it in rats (McQueen et al., 1997, Pecins-Thompson et al., 1998). It is clear that ovarian steroids can modulate the expression of different genes of the 5-HT system. However, modulations of mRNA levels are not necessarily reflected by a modification in protein expression or by physiological or behavioral changes. Our present results show that ovarian hormones can indeed functionally modulate this system by affecting the firing activity of 5-HT neurons.

The greater susceptibility of women to affective disorders is well documented (Weissman and Olfson, 1995). Moreover, some affective pathologies like the premenstrual syndrome, postpartum blues, postpartum depression, and postmenoposal depression are obviously selective to women. The significantly lower basal firing rates observed in CF relative to males would constitute the most parsimonious explanation for this clinical observation. It has been agreed upon, for some time, that the 5-HT system is the main common target of the different types of antidepressant treatments, and that they all result in augmentation of 5-HT neurotransmission (Blier and de Montigny, 1994; Owens, 1996). Impaired 5-HT function has been implicated in the etiology of affective disorders (Lesch et al., 1996) and a blunted 5-HT brain response in untreated depressed patients has been reported (Mann et al., 1996). If the difference in the basal activity of 5-HT neurons that we observed between males and females is sustained across

behavioral states, a reduced activity of the 5-HT system in females could imply a greater vulnerability to impairment when this system is challenged.

The most interesting and unexpected result of this study is, perhaps, our finding that, during pregnancy, a graded augmentation and diminution in 5-HT firing rate occurs, which mirrors that of circulating P concentrations. The striking parallelism between 5-HT discharge and P levels during pregnancy strongly suggests that P is implicated in the modulation of 5-HT neurons activity. However, during the postpartum period, even following the decrease of P levels, the firing activity of 5-HT neurons remained significantly higher than that of CF. This could be due to a sustained positive modulation of the in firing activity by different factors, which might take longer to return to baseline. Indeed, P metabolites, which increase with P levels during pregnancy, are also implicated in the enhanced firing activity of 5-HT neurons in pregnant rats. During pregnancy, they accumulate in the brain and their cerebral levels probably do not fall as abruptly at parturition as the plasma levels. On the other hand, other neurosteroids may take over following parturition and contribute to maintain a relatively high 5-HT neurons firing activity. For instance, it is possible that during pregnancy, when P levels are high, E has little effect on the firing activity of 5-HT neurons. As E levels rise when P levels drop, just before parturition, the effect of E on the firing activity of 5-HT neurons might become apparent. It may also serve to prevent a too large decrease in the 5-HT neuronal firing rate following parturition. Nevertheless, this sustained increase of the firing activity

of 5-HT neurons suggests that P is not the only neurosteroid involved in the modulation of 5-HT neurons.

The increased firing activity of 5-HT neurons in males seems to be a gender-dependent trait rather than due to lower levels of ovarian hormones as identical levels of E and P in OVX and males resulted in lower discharge rates in OVX, which were similar to that of CF. Following ovariectomy, when P levels are low, a possible compensation by other neurosteroids may prevent a decrease of the firing activity of 5-HT neurons. Similarly, males, having constantly low levels of P, may have a different neurosteroid equilibrium, which could maintain their 5-HT neuronal firing activity higher than that of females. For instance, it would be interesting to assess if testosterone, levels of which are high in males, has a positive modulatory effect on the firing rate of 5-HT neurons.

There is strong evidence supporting the hypothesis of a combined dysfunction of the noradrenergic (NE) and 5-HT systems in the pathophysiology of depression. For instance, antidepressant medications like desipramine or reboxetine, acting selectively on the NE system, have proved to be efficient in the treatment of depression. Moreover, clinical studies have shown that increasing both types of neurotransmission was clinically more efficient then treatments aiming at only one (reviewed in Mongeau et al., 1997). This is not inconsistent with the reciprocal modulatory influence that the NE and 5-HT systems exert on each other. In the DRN, 5-HT neurons are innervated by NE terminals, which make direct synaptic contacts with them and exert a tonic excitatory input on

these cells (Baraban and Aghajanian, 1980, 1981). 5-HT neurons also project back to the LC and tonically inhibit the firing activity of NE neurons (Szabo and Blier, 2001).

However, our data suggest that the differences in 5-HT neurons basal firing rate, observed between genders and brought about during pregnancy and the postpartum period, are not due to, nor do they result in, a modification of the activity of LC NE neurons. Indeed, the firing rate of LC neurons was similar in all our experimental groups. Obviously, one cannot totally rule out a possible involvement of the NE system in the modifications observed in the present study. However, even modifications such as the desensitisation of α_1-adrenoceptors observed following application of P to rat hypothalamic slices would rather tend to decrease the firing activity of 5-HT neurons (Petitti and Etgen, 1992). This phenomenon in the DRN, if present, would strengthen our suggestion that the observed modifications of the activity of 5-HT neurons are not due to a difference in the NE input. These results could also suggest that the greater vulnerability of women to affective disorders is not due to a difference in the NE function. Furthermore, our observation of the absence of any change in the firing activity of LC NE neurons could indicate a certain specificity of action for ovarian hormones on the dorsal raphe nucleus. This is in keeping with the failure to detect the presence of E and P receptors in the LC following ovarian hormone treatments (Schutzer and Bethea, 1997), and strengthens the contention that the NE system

may not contribute significantly to affective states related to changes in ovarian hormones (Schutzer and Bethea, 1997).

The presence of E and P nuclear receptors was reported in the DRN of primates (Bethea, 1994) and rodents (Alves et al., 1998a). The cellular substrate for the variation in 5-HT neuronal activity that we describe is therefore present in the DRN. Interestingly, the DRN of female rats was shown to contain 30% more PRs than males, while the amount of ERs seemed to be constant between sexes (Alves et al., 1998a). This could suggest a greater sensitivity of the female 5-HT system to P modulation. Interestingly, in rat DRN, the P receptors are not expressed by 5-HT neurons but by neighboring excitatory amino acid neurons (Alves et al., 1998a, Alves et al., 1998b). It could thus suggest a transsynaptic mechanism of action for the modulatory effects of P on 5-HT neurons.

Our results, however, may contrast with other studies. For instance, P has been shown to decrease the extracellular levels of 5-HT and its turnover rate in different regions of the hypothalamus and the midbrain central gray, following E priming (Gereau et al., 1993; Farmer et al., 1996; Maswood et al., 1999). Although the release of 5-HT in the projection areas is closely related to the firing activity of the 5-HT neurons, other mechanisms, such as nerve terminal autoreceptors, can contribute to the fine-tuning of the 5-HT release. Interestingly, one of these studies suggested that 5-HT terminal autoreceptors were implicated in the P-induced reduction in extracellular 5-HT (Maswood et al., 1999). Finally,

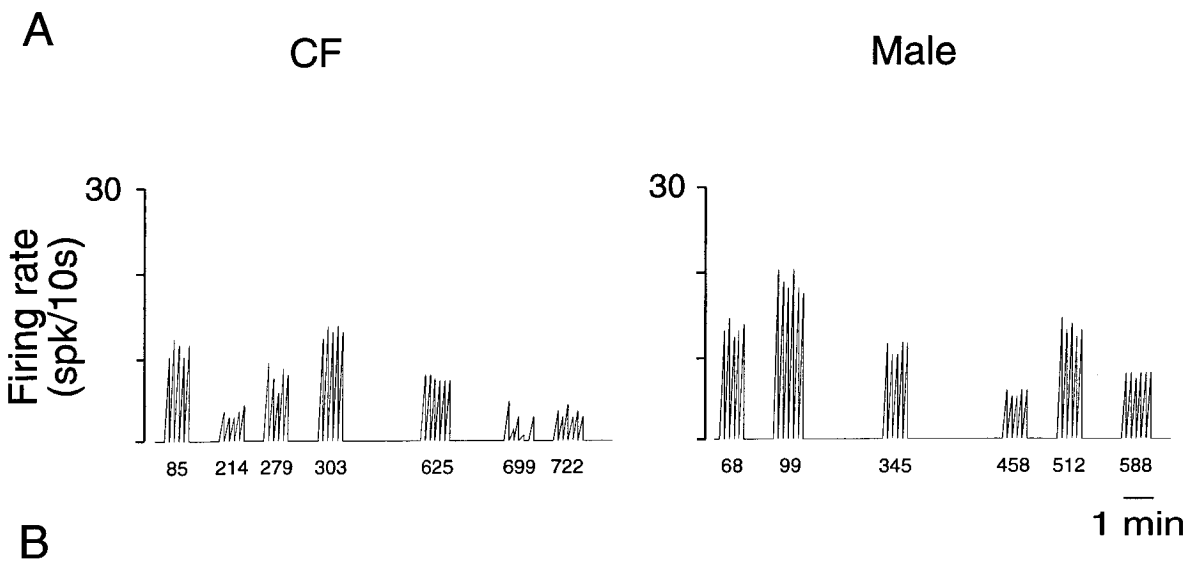
the possibility that the fetus or the placenta might play a role in modulating the firing activity of 5-HT neurons during pregnancy cannot be ruled out.

In conclusion, we demonstrated that the DRN 5-HT system significantly differs between genders and undergoes profound changes during pregnancy and the postpartum period. Furthermore, whether a neuroactive or genomic mechanism is implicated, it would be interesting to know what afferent or receptor modulating the firing activity of 5-HT neurons is also affected. Elucidating this question was the purpose of the second part of this study (see companion paper). Whatever the exact mechanism, the dramatic increase in basal discharge of 5-HT neurons during pregnancy is bound to bear an important outcome on various aspects of 5-HT-mediated neurotransmission. In particular, it could explain feelings of well-being and elation commonly reported by women during pregnancy.

FIGURE LEGENDS

Figure 1. Effect of gender on the spontaneous firing activity of DRN 5-HT neurons. (A) Integrated firing rate histograms of 5-HT neurons recorded in one electrode descent along the midline of the DRN in CF (*left*) and male (*right*). For each unit encountered, the 10s bin integrated firing rate was displayed for 5 or 6 bins. In this and the following figures, numbers under each unit indicate distance in μm below the Sylvius aqueduct. (B) Mean firing rate of DRN 5-HT neurons expressed in spike per 10s (spk/10s, mean ± SEM) for each experimental group (CF, OVX and males). In this and the following figures, the number of neurons recorded is indicated in a box at the bottom of each bar. Significance compared to CF is indicated by an asterisk ($P<0.001$).

Figure 1


A
CF
Male
30
30
Firing rate (spk/10s)
85 214 279 303 625 699 722
68 99 345 458 512 588
1 min

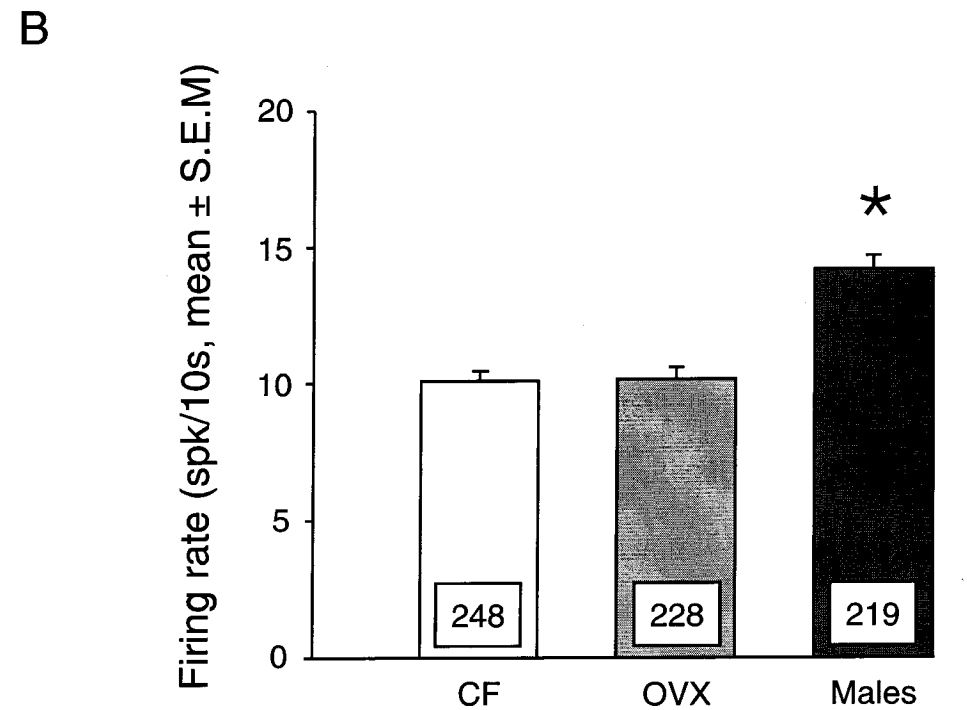
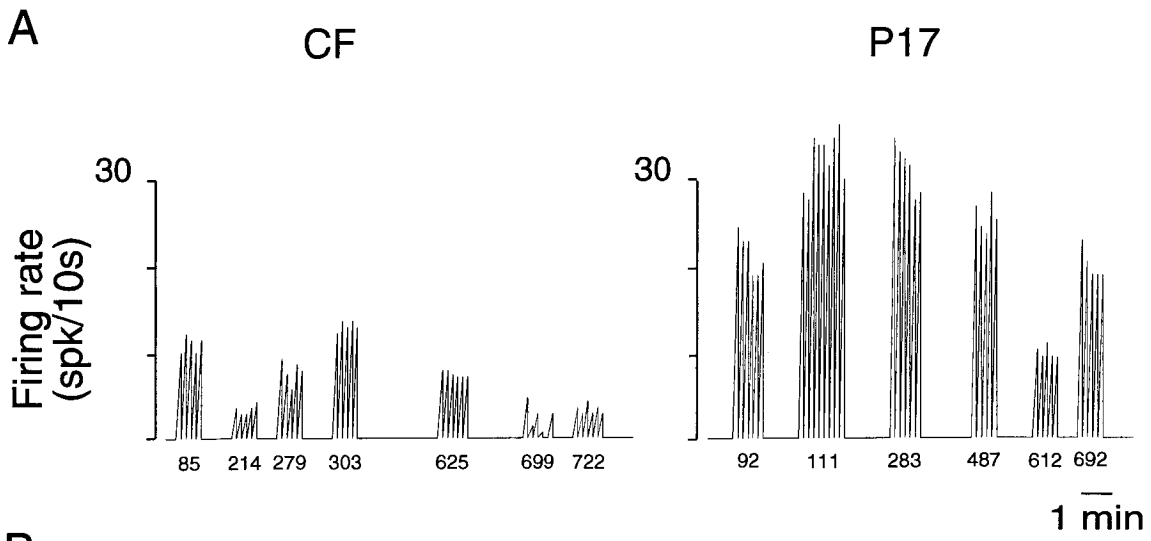

B
Firing rate (spk/10s, mean ± S.E.M)
20
15
10
5
0
*
248
228
219
CF
OVX
Males

Figure 2. Effect of pregnancy on the spontaneous firing activity of DRN 5-HT neurons. (A) Integrated firing rate histograms of 5-HT neurons recorded in one electrode descent along the midline of the DRN in CF (*left*) and P17 (*right*). (B) Mean firing rate of DRN 5-HT neurons expressed in spike per 10s (spk/10s, mean ± SEM) for each experimental group (CF, P11, P14, P17, P21 and PP). Significance compared to CF is indicated by an asterisk ($P<0.001$).

Figure 2


A
CF
P17
30
30
Firing rate (spk/10s)
85 214 279 303 625 699 722
92 111 283 487 612 692
1 min

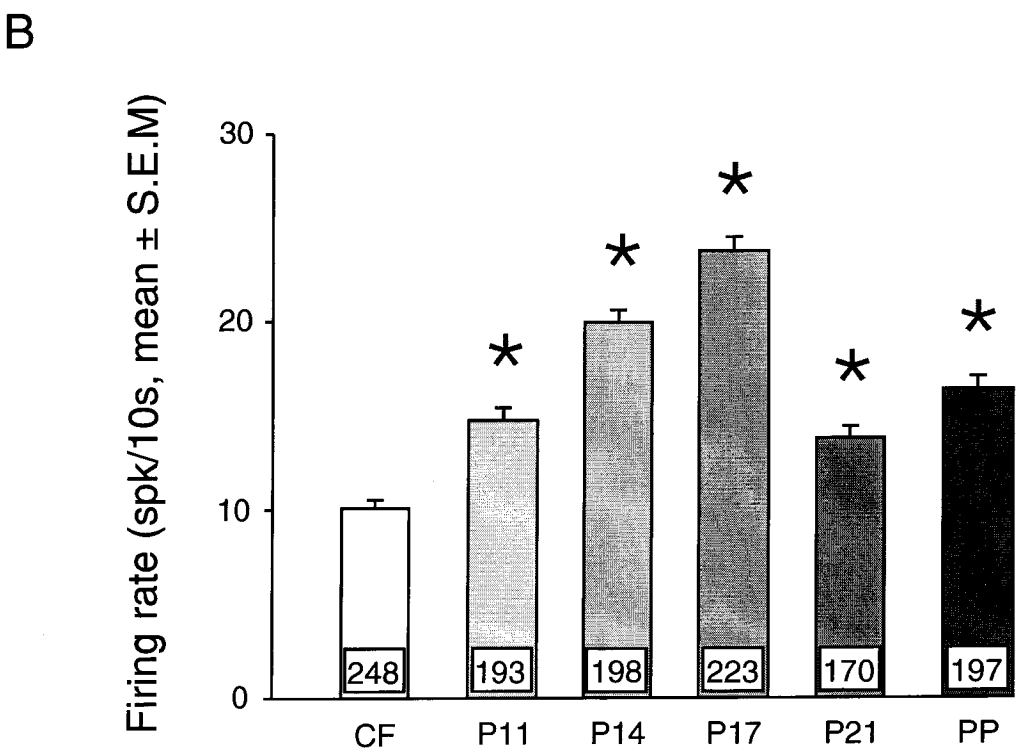
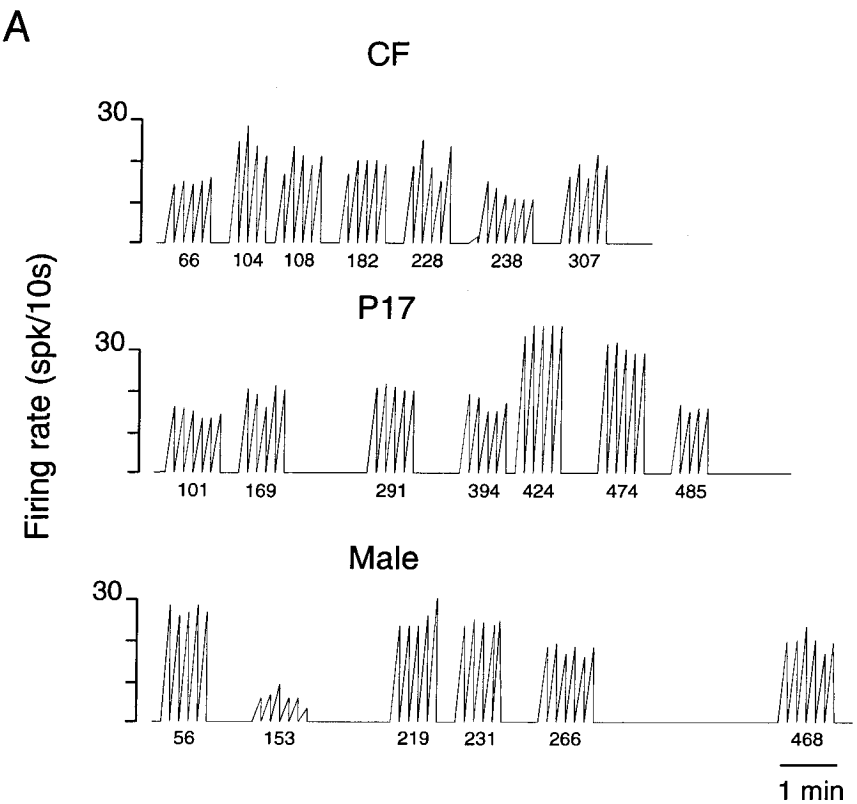

B
Firing rate (spk/10s, mean ± S.E.M)
30
20
10
0
248
193
198
223
170
197
CF
P11
P14
P17
P21
PP

Figure 3. Spontaneous NE firing activity. (A) Integrated firing histogram of typical electrode descents in the LC of CF (*left*) and male (right). Numbers under each unit indicate distance in μm below the 4th ventricle. (B) Mean firing rate of LC NE neurons expressed in spike per 10s (spk/10s, mean ± SEM) for each experimental group (CF, males, OVX and P17).

Figure 3


A
CF
30
66 104 108 182 228 238 307
P17
30
101 169 291 394 424 474 485
Male
30
56 153 219 231 266 468
1 min
Firing rate (spk/10s)

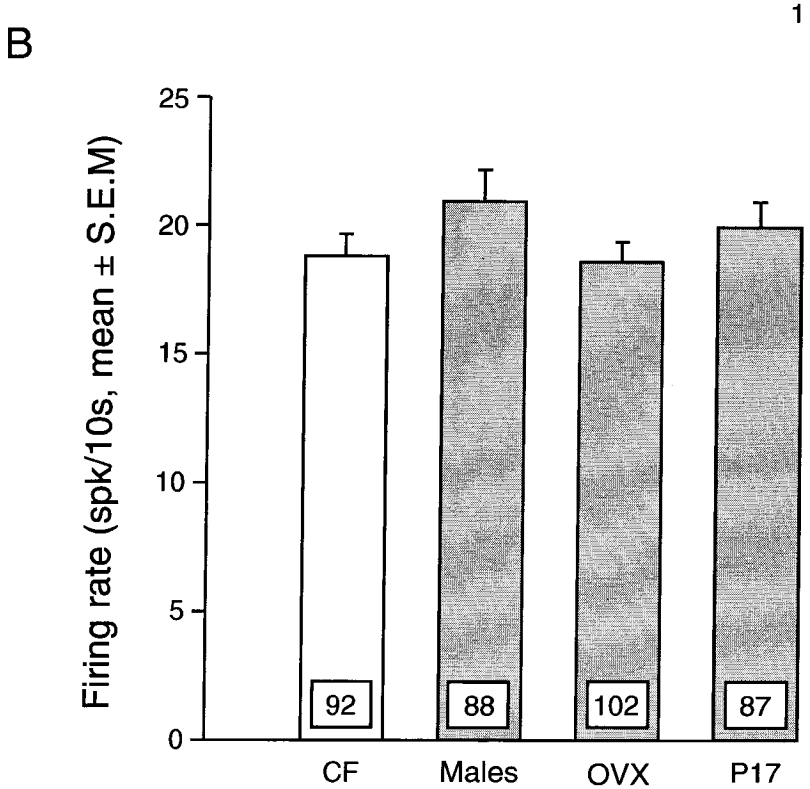

B
Firing rate (spk/10s, mean ± S.E.M)
25
20
15
10
5
0
92
88
102
87
CF
Males
OVX
P17

Figure 4. Plasma concentrations of E in relation to 5-HT neurons firing rate. Columns indicate the spontaneous firing rate of 5-HT neurons (spk/10s, mean ± SEM), scale on the left of each panel, values from Table 1. Scatter and line plots indicate the levels of E in pg/ml (mean ± SEM), scale on the right of each panel. From 6 to 14 animals were used per group.

Figure 4

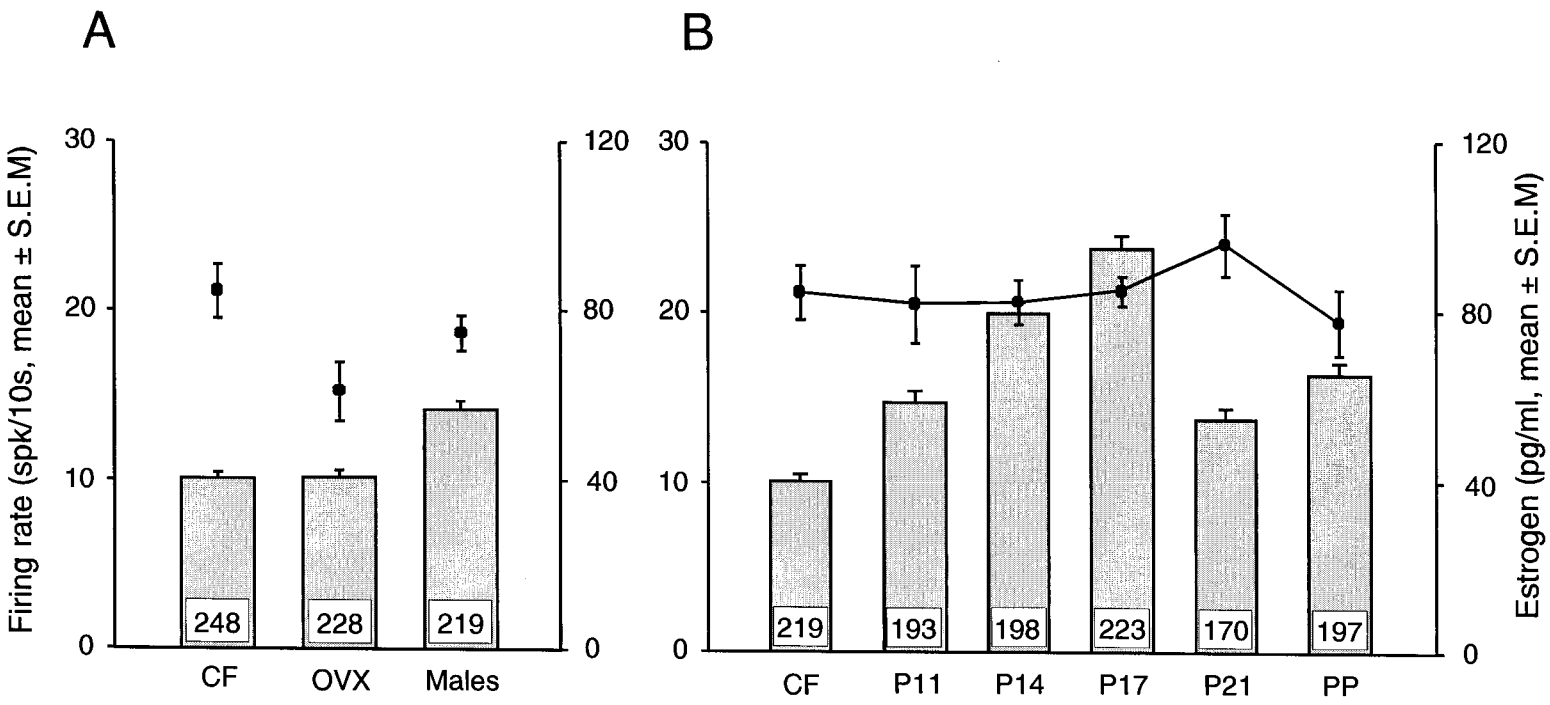

A
B
Firing rate (spk/10s, mean ± S.E.M)
Estrogen (pg/ml, mean ± S.E.M)
30
20
10
0
120
80
40
0
248
228
219
CF
OVX
Males
219
193
198
223
170
197
CF
P11
P14
P17
P21
PP

Figure 5. Plasma concentrations of P in relation to 5-HT neurons firing rate. Columns indicate the spontaneous firing rate of 5-HT neurons (spk/10s, mean ± SEM), scale on the left of each panel, values from Table 1. Scatter and line plots indicate the levels of P in ng/ml (mean ± SEM), scale on the right of each panel. From 6 to 14 animals were used per group. During pregnancy, the rise and fall of 5-HT neuronal discharge closely followed P levels (*right*).

Figure 5

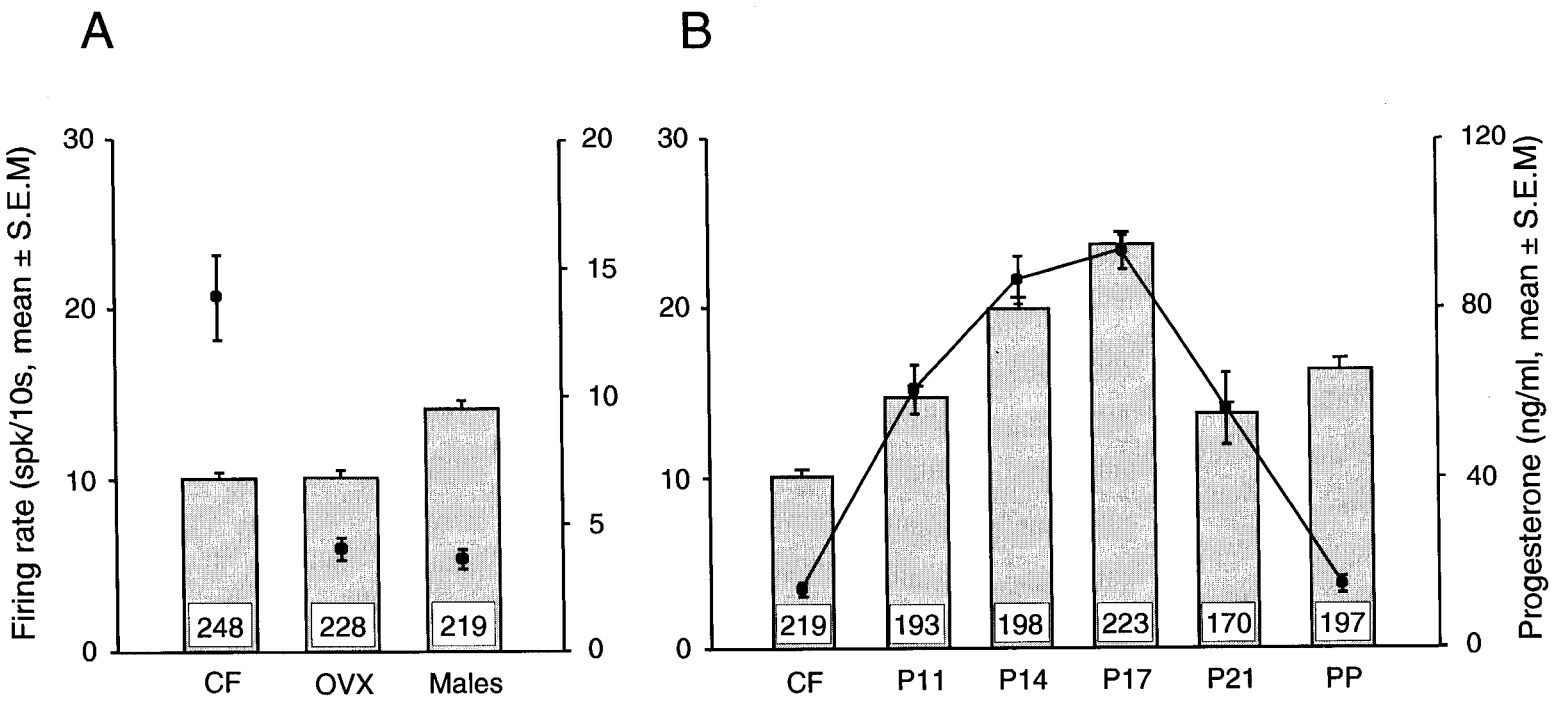

A
B
Firing rate (spk/10s, mean ± S.E.M)
Progesterone (ng/ml, mean ± S.E.M)
30
20
10
0
20
15
10
5
0
120
80
40
0
248
228
219
CF
OVX
Males
219
193
198
223
170
197
CF
P11
P14
P17
P21
PP

TABLE 1

group	total nb of 5-HT neurons	mean firing rate (spk/10s)	coefficient of variation	mean nb of 5-HT neurons/descent	mean nb of non-5-HT neurons/rat
M	219	14.2	0.08	4.0±0.4	3.0±0.1
CF	248	10.1	0.2	3.5±0.5	4.5±0.9
OVX	228	10.2	0.13	5.2±1.1	3.2±0.8
P11	193	14.7	0.06	3.6±0.2	3.8±0.9
P14	198	19.9	0.15	3.1±0.4	3.4±0.8
P17	223	23.8	0.19	2.0±0.4	4.5±1.5
P21	170	13.7	0.20	3.1±0.5	4.4±0.9
PP	197	16.4	0.23	4.4±0.9	4.0±0.8

Table 1. Parameters of spontaneous 5-HT neuronal discharge in the DRN. From 6 to 9 animals were used per group. To quantify the variability of the firing rate within a group, the distribution of mean firing rates within each group was characterized with the coefficient of variation (CV=mean/SD); a low CV reflects an homogeneous distribution of firing rates from rat to rat within the same group. The mean nb of 5-HT neurons/descent (mean ± SEM) and the mean nb of non-5-HT neurons encountered per rat (mean ± SEM) are a measure of the number of spontaneously active 5-HT and non-5-HT neurons within each group.

TABLE 2

group	total Nb of NE neurons	mean firing rate (spk/10s)	mean Nb of NE neurons/descent
Male	88	20.9	4.0±0.8
CF	92	18.8	4.7±1.5
OVX	102	18.6	4.4±0.1
P17	87	20	4.1±0.5

Table 2. Parameters of spontaneous NE neuronal discharge in the LC. From 3 to 4 animals were used per group. The mean nb of NE neurons/descent (mean ± SEM) is a measure of the number of spontaneously active NE neurons within each group.

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Foreword to Chapter 3

Gender and gonadal status modulation of dorsal raphe nucleus serotonergic neurons. Part II: Regulatory mechanisms

In the first part of this study, the spontaneous firing activity of DRN 5-HT neurons has been shown to be significantly higher in male rats than in freely cycling female rats. Also, during pregnancy, the 5-HT neuronal activity gradually increased, peaked at the 17^{th} day of pregnancy and then declined before parturition, thus paralleling circulating levels of progesterone. Ovariectomy, on the other hand, did not significantly modify the firing rate of 5-HT neurons.

In order to understand the basis of these gender differences and hormonal modifications, the second part of the study focussed on the role of different mechanisms regulating the 5-HT neuronal firing activity (as detailed in the introduction). The function of 5-HT_{1A} receptors and the GABAergic tonic inhibition of 5-HT neurons was pharmacologically assessed in males, ovariectomized females and pregnant females and compared to that of freely cycling females.

Chapter 3

GENDER AND GONADAL STATUSMODULATION OF DORSAL RAPHE NUCLEUS SEROTONERGIC NEURONS. PART II: REGULATORY MECHANISMS

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SUMMARY

In the companion paper, we showed that the spontaneous firing activity of DRN 5-HT neurons is significantly higher in male (M) than in freely cycling female (CF) rats. Moreover, during pregnancy, it increased in parallel to circulating levels of progesterone, peaking at day 17 of pregnancy (P17). In this second part, we assessed the role of three regulatory mechanisms potentially involved in these modifications of the 5-HT neurons firing activity. During pregnancy, the ED_{50} for the response to LSD was decreased by about 70%, indicating a partial desensitization of $5\text{-}HT_{1A}$ autoreceptors, which is consistent with the 5-HT neurons higher firing activity. The GABAergic tonic inhibition of 5-HT neurons was assessed using the responses to GABA, bicuculline and isoniazid. Together, they indicate a lower GABAergic tonic inhibition in males and P17 as compared to CF, which is in agreement with their greater 5-HT neurons firing rate. Finally, the efficacy of the long feedback loop, involving postsynaptic $5\text{-}HT_{1A}$ receptors, did not seem affected by gender, ovariectomy or pregnancy since the response to systemic 8-OH-DPAT was similar. These results constitute strong evidence of mechanisms by which gender and hormonal fluctuations can modulate the 5-HT neurons function and influence vulnerability to mood disorders.

INTRODUCTION

Several studies strongly support a functional modulation of the 5-HT system by ovarian steroids (reviewed in Bethea *et al.*, 1999). In order to further characterize this modulation, we used *in vivo* electrophysiology to study the effect of gender, ovariectomy and pregnancy on dorsal raphe nucleus (DRN) 5-HT neurons spontaneous activity. In the first part of this paper we have shown that the spontaneous firing activity of DRN 5-HT neurons is significantly higher in male (M) than in freely cycling female (CF) rats (companion paper). Ovariectomy (OVX), on the other hand, did not significantly modify the 5-HT neuron basal firing rate (companion paper). Interestingly, during pregnancy, the spontaneous firing rate increased gradually to peak at the 17^{th} day of pregnancy (P17) and then declined before parturition, thus following circulating levels of progesterone, but not of estrogen (see companion paper).

In this second part of the study, as an attempt to explain how these differences in basal firing rate are brought about, we investigated different mechanisms regulating the firing activity of 5-HT neurons. Activation of pre- or post-synaptic 5-HT_{1A} receptors triggers the opening of K+ channels. This induces a hyperpolarization of the neuron and decreases its firing activity (reviewed in de Montigny & Blier, 1992). Several lines of evidence suggest different properties for the pre- and the post-synaptic 5-HT_{1A} receptors (reviewed in de Montigny & Blier, 1992). Furthermore, several observations led to the hypothesis that 5-HT neurons are regulated by a small loop (the autoregulation via somatodendritic

autoreceptors) as well as by a long negative feedback loop involving postsynaptic 5-HT_{1A} receptors (Blier *et al.*, 1987; Blier & de Montigny, 1987; de Montigny & Blier, 1992). Briefly, the long feedback loop hypothesis implies the activation of post-synaptic 5-HT_{1A} receptors, which location still remains to be determined. It results in the activation of cortical neurons (presumably glutamatergic), which possibly activate inhibitory GABAergic interneurons, which, in turn, inhibit DRN 5-HT neurons (Blier *et al.*, 1987; Blier & de Montigny, 1987; Hajós *et al.*, 1998; Hajós *et al.*, 1999). Even if there is only one gene for post-synaptic and somatodendritic 5-HT_{1A} receptors, their pharmacological profile is different and this effect can be identified using two different 5-HT_{1A} agonists. At low doses, the systemic administration of the 5-HT_{1A} agonist R(+)-8-hydroxydipropylaminotetralin hydrobromide (8-OH-DPAT), exerts its inhibitory effect on DRN 5-HT neurons partly through the long negative feedback loop, via the activation of postsynaptic 5-HT_{1A} receptors, whereas diethylamide lysergic acid (LSD) causes the inhibition of 5-HT neurons through a direct activation of 5-HT_{1A} autoreceptors (Blier *et al.*, 1987; Blier & de Montigny, 1987; Ceci *et al.*, 1994; Hajós *et al.*, 1998; Hajós *et al.*, 1999).

The inhibitory effect of GABA on DRN 5-HT neurons is also well known. In this nucleus, GABAergic terminals are present in large numbers and have been shown to make direct synaptic contact with 5-HT neurons (Harandi *et al.*, 1987; Wang *et al.*, 1992). A local infusion of GABA or of muscimol (a $GABA_A$ agonist) into the DRN leads to a reduction of 5-HT neurons firing rate and of extracellular levels of 5-HT in the DRN (Gallager, 1978; Gallager &

Aghajanian, 1976; Nishikawa & Scatton, 1985; Tao *et al.*, 1996; Tao & Auerbach, 2000; Vandermaelen *et al.*, 1986). Conversely, infusions of bicuculline or picrotoxin ($GABA_A$ antagonists) in the DRN of anesthetized and freely moving rats, increase extracellular 5-HT in this nucleus and in the striatum (Kalén *et al.*, 1989; Tao *et al.*, 1996; Tao & Auerbach, 2000). This supports the existence of a putative GABAergic tonic inhibitory modulation of DRN 5-HT neurons. Furthermore, in rats, the bicuculline-induced increase of DRN 5-HT neurons firing activity was observed during waking periods as well as during slow-wave and REM sleep periods, suggesting that in this species, the tonic GABAergic inhibition of DRN 5-HT neurons is independent of the vigilance state (Gervasoni *et al.*, 2000).

Therefore, the effectiveness of these three regulatory mechanisms (the 5-HT_{1A} autoreceptors, the long feedback loop and the GABAergic tonic inhibition) of 5-HT neurons activity was assessed in different experimental groups of rats (CF, M, OVX and P17) and compared to that of CF.

EXPERIMENTAL PROCEDURE

Animals

All rats used were Sprague Dawleys weighing between 250g and 350g and kept under standard laboratory conditions (12:12 light-dark cycle with access to food and water *ad libitum*). The different rat populations used for the experiments consisted of the following: freely cycling females (CF); males (M); ovariectomized females operated at their 10^{th} week of life (OVX); females at the 17th day of pregnancy (P17); and postpartum females (PP) separated from their pups immediately, or a few hours after delivery, and used 1 to 7 days following parturition. Ethical committee approval was obtained from the McGill University Animal Ethical Care Committee and all their rules and regulations were followed. The suffering of animals as well as the number used were kept at minimum.

Electrophysiological Experiments

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

a) Extracellular recording

Extracellular unitary recording of serotonergic neurons were obtained with single-barelled glass micropipettes filled with a 1M NaCl solution and of final

impedance ranging between 2 and 6 MΩ. A 4 mm-diameter hole was drilled in the skull of each rat at the appropriate location (about 1 mm anterior of lambda and centered with respect to the midline). DRN 5-HT neurons unit activity was recorded by lowering the micropipette along descents covering the nucleus from 300μm to about 1500μm anterior of lambda. Spontaneously active DRN 5-HT neurons were identified by their slow and regular rythmical firing (Aghajanian *et al.*, 1978).

For each rat population, the basal firing rate of DRN 5-HT neurons was calculated by averaging the firing rate of each neuron measured in 1 to 5 complete descents in the DRN of 3 to 6 rats.

b) Microiontophoresis

In other experiments, the same approach was used for microiontophoresis, but with five-barrel micropipettes (ASI Instruments, Warren, MI), tip diameter 5-7 μm. The central barrel, used for unit recording, was filled with a 2M Na-acetate solution. Two of the side barrels were used for drug ejection and were filled with γ-aminobutyric acid (GABA) (2.5 mM in 200 mM NaCl) and bicuculline methiodide (BicuM) (3 mM in 200 mM NaCl). BicuM was used for iontophoresis because of the ionic charge it carries, whereas the blood-brain barrier permeable bicuculline was used for systemic administration (see below). The third side barrel was filled with 0.5 M Na-acetate and used for automatic current balancing. A retaining current of -1 nA was applied to each drug barrel

except during periods of active drug ejection. GABA was applied with currents of 2-8 nA for a constant duration of 100s. The number of spikes suppressed per nA, which consists in dividing the total number of spikes suppressed during drug application by the ejection current, was used as an index of receptor responsiveness. BicuM was applied with currents of 3-6 nA for a constant duration of 100 s. The number of spikes generated per nA was used as an index of endogenous activation of $GABA_A$ receptors. The spikes suppressed per nA, and spikes generated per nA were calculated by homemade data acquisition software. From one to four neurons were tested in each animal.

5-HT_{1A} Receptor Activity Evaluation

For each population (CF, M, OVX, and P17), a dose-response curve of the inhibitory effect of each of two 5-HT_{1A} receptor agonists, LSD and 8-OH-DPAT, was constructed. The LSD and the 8-OH-DPAT doses used ranged from 1 μg/kg to 40 μg/kg and from 0.5 μg/kg to 4.0 μg/kg, respectively. Each agonist was injected intraveneously via the tail vein after the basal firing rate of the neuron had been stable and recorded for at least 1 min. The inhibition was measured as a percentage of the initial firing rate. Following the stabilization of the inhibited firing rate, 100 μg/kg of N-[2-(4-[2-methoxyphenyl]-1-piperazinyl)ethyl)]-N-2-pyridinylcyclohexanecarboxamide (WAY 100635), a 5-HT_{1A} antagonist, was injected in the same manner in order to bring the neuron back to its initial firing activity and ascertain that the decrease in the firing rate was solely due to the effect of the 5-HT_{1A} agonist.

Bicucculine i.v. dose-response curve

For CF, M and P17, a dose-response curve of the effects of the $GABA_A$ receptor antagonist (+)-bicuculline was constructed with doses ranging from 50 μg/kg to 400 μg/kg. (+)-bicuculline, dissolved in 10 % DMSO, was injected intraveneously (i.v.) via the tail vein after the basal firing rate of the neuron had been recorded for about 1 min. The increase in firing rate was measured in terms of percentage of the initial firing rate. For the dose-response curve, doses inducing convulsion and/or death of the rats were not used. The dose producing these effects was determined to be 450 mg/kg in males and 500mg/kg in females. Only doses inducing a stable response of the neuron were used.

Effect of partial GABA depletion

The glutamic acid decarboxylase (GAD) is the enzyme responsible for the synthesis of GABA from glutamate. In order to evaluate the effect of a partial depletion in GABA on the tonic GABAergic inhibition of 5-HT neurons activity, a 500 mg/kg dose of isoniazid (a selective GAD inhibitor) was injected intraperitoneally (i.p.), to CF, M, and P17, 45 minutes before descending the electrode in the rat DRN and measuring the spontaneous firing rate of 5-HT neurons.

Statistics

Average values are given as the mean ± S.E.M. In some cases, the coefficient of variation (CV=standard deviation/mean) was calculated to characterize the spread of a distribution. Statistical analysis were performed using the software SigmaStat Version 2.0 (Jandel Corporation). One-way ANOVA, with alpha = 0.05, followed by a post-hoc analysis using Tukey's method of comparison versus control (controls being CF) was used for evaluating statistical significance. In the case of the experiment with isoniazid, the post-hoc analysis using Tukey's method allowed multiple pairwise comparisons. Results (F) of statistical analysis are expressed in terms of number of groups compared (p)and degrees of freedom between groups compared (df). The ED_{50} for each of the dose-response curves was calculated by a non-linear regression analysis, using the software Prism 3.0 (GraphPad). Statistical differences between the entire dose-response curve for the effect of each of the three drugs (8-OH-DPAT, LSD and (+)-bicuculline) was assessed using an F-test analysis of variance, with CF as the comparative control group. In each case, the F value and the total degrees of freedom (df) are indicated. In all cases, significance was considered for $P<0.05$.

Drugs

8-OH-DPAT, WAY 100635 (WAY), BicuM, (+)-bicuculline were purchased from RBI (Sigma-Aldrich, Ontario, Canada), GABA and isoniazid were purchased from Sigma (Sigma-Aldrich, Ontario, Canada) and LSD was obtained from U.S.P.C Inc (Rockville, MD).

RESULTS

Assessment of the long feedback loop activity

A dose-response curve was obtained for the effect of 8-OH-DPAT, a selective 5-HT_{1A} receptor agonist, in CF, M, OVX and P17. The intravenous administration of 8-OH-DPAT induced a dose-dependant and reversible inhibition of 5-HT neurons (Fig. 1A). No statistically significant difference was found between the 8-OH-DPAT dose-response curves of the different groups as compared to CF (M: [$F(9,2) = 0.9$, n.s.]; OVX: [$F(12,2) = 2.3$, n.s.] and P17: [$F(9,2) = 0.9$, n.s.] (Fig. 1B). The doses of 8-OH-DPAT causing a 50% inhibition of the neuronal firing rate (ED_{50}) in the various groups are the following: CF: 2.51 µg/kg, M: 1.71 µg/kg, OVX: 1.91 µg/kg, and P17: 2.27µg/kg.

Assessment of the 5-HT_{1A} autoreceptor activity

To assess the responsiveness of the 5-HT_{1A} autoreceptor, a dose-response curve for the effects of LSD, a somatodendritic 5-HT_{1A} agonist, was obtained in CF, M, OVX and P17. Intravenous injections of LSD caused a dose-dependant and reversible inhibition of the firing activity of 5-HT neurons (Fig. 2A). The ED_{50} values for LSD were the following: CF: 6.94 µg/kg; M: 7.61 µg/kg; OVX: 10.10 µg/kg; and P17: 11.83 µg/kg. An analysis of variance using an F-test indicated that the entire LSD dose-response curve was statistically different in M [$F(10,2) = 4.6$, $P < 0.01$] and P17 [$F(12,2) = 6.9$, $P < 0.01$] but not OVX [F(12,2)

= 3.0, n.s.] when compared to that of CF (Fig. 2B). The dose-response curve was shifted to the right in both OVX and P17 as compared to CF, although reaching statistical significance only in P17. These results suggest a partial functional desensitization of the 5-HT_{1A} autoreceptor during late pregnancy. Although the ED_{50} values are relatively similar in M and CF, the dose-response curves were statistically different.

Assessment of the GABAergic tonic inhibition

Many progesterone metabolites are $GABA_A$ receptor modulators. Since we observed a direct correlation between 5-HT neuronal activity and circulating levels of P from P11 to P21, the responsiveness of the DRN 5-HT neurons to GABA, as well as their GABAergic tonic inhibition, had to be explored.

In a first set of experiments, the response of DRN 5-HT neurons to microiontophoretic applications of GABA was examined in CF, M, OVX, P17 and PP. GABA applications (2-8 nA, 100 s) resulted in a suppression of the firing activity (Fig. 3A), which was totally reversed by the concurrent application of the $GABA_A$ receptor antagonist BicuM (3-6 nA), in all the neurons tested (illustrated for P17, Fig. 3A, middle). The onset and offset of GABA action was fast. The number of spikes suppressed per nA was used as an index of $GABA_A$ receptor sensitivity. Responses to GABA were homogenous in CF (CV = 0.29, n =18), M (CV = 0.28, n =16) and OVX (CV = 0.29, n =16, Fig. 3B, left). However, the mean number of spikes suppressed/nA was significantly larger in M (64.3 ± 4.5) than in CF (49.2 ± 3.3) [$F(2,3) = 4.2$, $P < 0.05$, Tukey's test, $q = 3.8$, $P < 0.05$].

The difference observed between OVX (61.6 ± 4.2) and CF did not reach statistical significance (Tukey's test, q = 3.1, n.s.).

Responses to GABA were more variable in P17 (CV = 0.47, n =21) and in PP (CV = 0.58, n = 21) than in CF (Fig 3B, right). The mean number of spikes suppressed/nA was lower in P17 (39.4 ± 4.0) and PP (26.5 ± 3.5) than in CF (49.2 ± 3.3) but the difference reached significance only in PP [$F(2,3) = 9.7$, $P < 0.001$, Tukey's test, $q = 3.8$, $P < 0.05$].

In order to compare the GABAergic tonic inhibition of DRN 5-HT neurons, three sets of experiments were carried out. First, BicuM was microiontophoretically applied onto 5-HT neurons of CF and P17. In CF, BicuM ejections (3 nA, 100 s) caused a highly variable increase in firing activity while, in P17, BicuM (4 nA, 100 s) resulted in an non-significant increase of only a few percent (Fig. 4A). The number of spikes generated per nA was taken as an index of the extent of endogenous inhibition of basal firing rate due to the activation of $GABA_A$ receptors (Fig. 4B). The mean number of spikes generated per nA was drastically reduced in P17 (1.9 ± 0.6, n = 6) [$F(1,2) = 51.3$, $P < 0.001$, Tukey's test, $q = 10.1$, $P < 0.05$] compared to CF (13.1 ± 1.3, n = 8). These results indicate that, in P17, $GABA_A$ receptors are activated to a much lesser extent by endogenous GABA, than they are in CF.

Also, for CF, M and P17 rats, an i.v. dose-response curve was obtained for the effect of the blood-brain barrier-permeable $GABA_A$ receptor antagonist

(+)bicuculline (Bicu). Only the excitatory responses of 5-HT neurons were used in the curve (Fig. 5A). An analysis of variance using an F-test indicated that the Bicu dose-response curve was statistically different in M [$F(5, 2) = 16.8$, $P < 0.001$] but not P17 [$F(6, 2) = 1.6$, n.s.] as compared to CF (figure 5B).

Isoniazid is a selective inhibitor of the glutamic acid decarboxylase (GAD), the enzyme converting glutamic acid into GABA. The effect, on the spontaneous firing rate of 5-HT neurons, of a 500 mg/kg i.p. injection of isoniazid, which has been shown to induce depletion of about 50% of the GABA content of the DRN, was investigated (Bagdy *et al.*, 2000). It was used to assess whether such a treatment would have an effect on the GABAergic tonic inhibition and, most importantly, whether it would affect the 5-HT neurons firing activity. It was also used as another mean to assess possible differences in the GABAergic tonic inhibition of the 5-HT neuronal firing rate between experimental groups. In CF, M and P17 rats, the basal firing rate of DRN 5-HT neurons was calculated by averaging the firing rate of each neuron encountered a few descents in the DRN. This was done in control animals and in rats treated with isoniazid 45 minutes prior to the experiment (Fig. 6). In CF, the isoniazid treatment induced a significant increase in 5-HT neuron spontaenous firing rate: 1.55 ± 0.09 Hz vs 0.98 ± 0.08 Hz [$F(5,6) = 11.7$, $P < 0.001$, Tukey's test, $q = 5.8$, $P < 0.05$]. In M and P17, isoniazid did not induce any significant change (M: 1.34 ± 0.65 Hz vs 1.38 ± 0.10 Hz, [Tukey's test, $q = 0.4$, n.s.]; P17: 1.88 ± 1.03 Hz vs 1.99 ± 0.14 Hz, [Tukey's test, $q = 1.1$, n.s.].

DISCUSSION

The dose-response curves of the effects induced by the systemic administrations of 8-OH-DPAT were not statistically different in M, OVX and P17 as compared to CF (Fig.1). However, our results suggest a gender difference in the 5-HT_{1A} autoreceptor-mediated response, as shown by the different dose-response curves for the effect of LSD (Fig. 2B). LSD is a non-selective agonist of 5-HT receptors and act on several other subtypes including the 5-HT_{2A} and an antagonist of the 5-HT_7.(Krebs-Thomson & Geyer, 1996, Chapin & Andrade, 2001) However, there is no indication that post-synaptic 5-HT_{2A} or 5-HT_7 receptors could be involved in a decrease of the firing activity of DRN 5-HT neurons and this effect of LSD is therefore assumed to be linked to its affinity for the 5-HT_{1A} autoreceptor (Blier & de Montigny, 1987; Blier & de Montigny, 1990, Krebs-Thomson & Geyer, 1996).

Strong evidence indicates that LSD and 8-OH-DPAT partly exert their effect through different mechanisms. For instance, in rats, long-term treatments with gepirone (a 5-HT_{1A} agonist) dampened the response of DRN 5-HT neurons to microiontophoresis application of 5-HT, LSD, 8-OH-DPAT, and to the systemic administration of LSD but not to that of 8-OH-DPAT (Blier & de Montigny, 1987; Blier & de Montigny, 1990). Second, short-term lithium treatments were shown to reduce the ED_{50} for i.v. 8-OH-DPAT, while leaving unaltered the response of 5-HT neurons to i.v. LSD or to local applications of LSD and 8-OH-DPAT (Blier *et al.*, 1987). Furthermore, ablation of the medial

prefrontal cortex (mPCF), which project to the DRN, was shown to dramatically increase the ED_{50} for systemic, but not local, 8-OH-DPAT administration (Ceci *et al.*, 1994; Hajós *et al.*, 1998; Hajós *et al.*, 1999). Also, systemic and local administration of 8-OH-DPAT in the mPCF inhibited 5-HT neurons, which was reversed by WAY-100635 (Casanovas & Artigas, 1999; Celada *et al.*, 2001; Hajós *et al.*, 1999). Finally, the electrical stimulation of mPCF results in an inhibition of the majority of DRN 5-HT neurons, which can be reversed or dampened by WAY-100635 and picrotoxin (Celada *et al.*, 2001; Fletcher *et al.*, 1996; Hajós *et al.*, 1998).

It has thus been hypothesized that, contrary to LSD, which produces its effect through the direct activation of 5-HT_{1A} somatodendritic autoreceptors, the systemic administration of low doses of 8-OH-DPAT exerts its effect on DRN 5-HT neurons, partly through a longer feedback loop (Blier *et al.*, 1987; Blier & de Montigny, 1987; de Montigny & Blier, 1992; Martin-Ruiz & Ugedo, 2001). According to this hypothesis, the systemic administration of 8-OH-DPAT would activate, not only 5-HT_{1A} autoreceptors, but also post-synaptic 5-HT_{1A} receptors located on inhibitory afferents to mPCF pyramidal glutamatergic neurons, thus resulting in their excitation (Celada *et al.*, 2001; Hajós *et al.*, 1999). The projection of mPCF glutamatergic neurons to GABAergic interneurons in the DRN would then lead to the inhibition of 5-HT neurons (Celada *et al.*, 2001; Hajós *et al.*, 1998; Hajós *et al.*, 1999). Moreover, some of the cortical neurons appear to stimulate a small portion of DRN 5-HT neurons, thus, causing 5-HT

release and consequently inhibiting surrounding 5-HT neurons through activation of 5-HT_{1A} autoreceptors (Celada *et al.*, 2001).

Our results are thus suggesting that the long feedback loop might not be affected by gender, ovariectomy, nor by pregnancy and might not be sensitive to ovarian steroids or their fluctuation. They also indicate that the long regulatory feedback loop does not appear to be implicated in the difference observed in the DRN 5-HT neurons basal firing activity of the four experimental groups (F, M, OVX and P17) (see companion paper).

Indeed, the dose-response curves for the effects of LSD are significantly different between genders. The main difference appears to be in the slope of the curves, which is steeper in M (Fig. 2B). In order to interpret this in terms of physiological implications, further experiments will be needed. Nevertheless, one could speculate either that there is a gender difference in the autoreceptor expression, conformation and/or functional mechanism. For instance, there could be greater cooperativity in the binding of 5-HT molecules to the male 5-HT_{1A} autoreceptor, thus resulting in a greater activation of the autoreceptor in the presence of high concentrations of 5-HT, as compared to the female one. This could account for the steeper slope observed in the dose-response curve for the effect of LSD. It is also possible that the male autoreceptor is tonically less activated by endogenous 5-HT than that of CF. A slightly greater tonic activation of 5-HT_{1A} autoreceptor in CF, however insufficient to desensitize it, would be in agreement with the similar ED_{50} between genders. In such a situation, doses of

LSD, lower than its ED_{50}, added to the slightly lower tonic activation in M, could lead to a lesser inhibition of the 5-HT neurons, as observed in the lower part of the LSD dose-response curve. Doses higher than the ED_{50} might be large enough for the slight difference in tonic activation to become unapparent and thus lead to a similar inhibition of the 5-HT neurons, as is observed at the higher doses of the curve. Moreover, a slightly less tonically activated 5-HT_{1A} autoreceptor in M would be in agreement with, and could partly explain, the higher basal firing rate of 5-HT neurons observed in M as compared to CF (see companion paper).

In P17 rats, the LSD dose-response curve has the same overall shape and is shifted to the right as compared to that obtained in CF, indicating that pregnancy leads to a partial desensitization of the autoreceptor without otherwise altering its functional properties (Fig 2B). In OVX rats, the same phenomenon is observed without, however, reaching statistical significance (Fig 2B). It could be hypothesized that P reduces the sensitivity of the 5-HT_{1A} autoreceptors, whether directly or through its metabolites. This would be consistent with both the P17 LSD dose-response curve, which is shifted to the right relative to CF, and the gradually increasing neuronal firing rate observed during pregnancy paralleling the increasing P levels (companion paper). It could be brought about through P neuroactive metabolites. A direct genomic mechanism of action seems unlikely since P receptors (PR) have not been found in the rat 5-HT neurons but have rather been shown to be present on neighbouring excitatory amino acid neurons in the DRN (Alves et al., 1998a, Alves et al., 1998b). A genomic action in those cells could result in a modulation of the firing activity of 5-HT neurons or of the

release of 5-HT in the DRN and remains a possibility, which could be investigated using PR antagonists. Also, a putative difference in the excitatory amino acids modulation of the 5-HT neuronal function between the experimental groups used in the present study would be another interesting avenue to explore. A reduction of the expression of the autoreceptor gene could also be implicated as it has been shown that ovarian steroid treatments reduce 5-HT_{1A} receptor mRNA in the DRN of OVX rats and monkeys (Birzniece *et al.*, 2001; Pecins-Thompson & Bethea, 1999). Different mechanisms of action could by hypothesized to explain this eventuality. For instance, an enhanced 5-HT release in the DRN could induce a desensitization / downregulation of the autoreceptor.

In the DRN, GABAergic terminals are present in large numbers and have been shown to make synaptic contact with the cell bodies and dendrites of 5-HT neurons (Harandi *et al.*, 1987; Wang *et al.*, 1992). Several lines of evidence suggest that there is a tonic GABAergic inhibitory modulation of DRN 5-HT neurons, which is mostly mediated by $GABA_A$ receptors (Gervasoni *et al.*, 2000; Innis & Aghajanian, 1987; Kalén *et al.*, 1989; Tao *et al.*, 1996; Tao & Auerbach, 2000). Furthermore, gender differences in $GABA_A$ receptor binding and function have been demonstrated and have been shown to be region specific (Bujas *et al.*, 1997; Jüptner & Hiemke, 1990; Wilson, 1992). Also, since many progesterone metabolites are $GABA_A$ receptor modulators, a putative gender- or steroid-mediated modulation of the GABAergic tonic inhibition of 5-HT neurons had to be explored (Majewska, 1992; McCauley *et al.*, 1995).

M and P17, having a higher basal firing frequency than CF, were expected to have a lower GABAergic tonic inhibition. This would translate into a smaller excitatory effect on the 5-HT neuron for a same dose of bicuculline (either systemically or locally administered). Indeed, the effect of iontophoretically applied bicuculline onto DRN 5-HT neurons was found to lead to a significantly smaller increase in firing rate in P17 as compared to CF, indicating a lower GABAergic tone than in CF (Fig 4). A similar trend, although not reaching significance, was observed following i.v. administration of bicuculline (Fig 5B). This indicates that 5-HT neurons of P17 rats are receiving a lower GABAergic tonic inhibition than the ones of CF.

Chronic neurosteroid treatments have been shown to induce important functional modifications of the $GABA_A$ receptor complex, which result in a net reduction of the efficacy of GABA-mediated Cl^- influx as well as the pentobarbital, benzodiazepine and neurosteroid-induced potentiation of these Cl^- currents (Le Foll *et al.*, 1997; Majewska *et al.*, 1989; Yu *et al.*, 1996a; Yu *et al.*, 1996b; Yu & Ticku, 1995; Zhu & Vicini, 1997). Furthermore, the neurosteroid potentiation of muscimol binding to synaptosomal $GABA_A$ receptors is reduced following a 5α-pregnane-3α-ol-20-one treatment and during late pregnancy (Majewska *et al.*, 1989). It could, thus, suggest that these modifications of the $GABA_A$ receptor function, induced by chronic neurosteroid treatments, are present during pregnancy, when levels of progesterone metabolites are high. This

would result in a lesser $GABA_A$ receptor responsiveness and would explain the lower GABAergic tonic inhibition of 5-HT neurons observed in the present study.

M rats present a significantly higher excitatory response to the i.v. administration of bicuculline than CF (Fig 5B). Although, at first glance, this data might suggest that M have a higher tonic inhibition than CF, the results obtained with isoniazid seem to argue against such conclusion (Fig 6). Following the administration of isoniazid, only in CF was there a significant increase in firing activity of the 5-HT neurons (Fig.6). A 500mg/kg dose of this inhibitor of GABA synthesis results in an approximate 50% depletion of the GABA content of the DRN (Bagdy *et al.*, 2000). Since the study with isoniazid was performed in male rats, no information is available on the percentage of GABA depletion induced by this drug in CF or P17. Nevertheless, our results suggest that in CF, it induces a GABA depletion important enough to reduce the GABAergic tonic inhibition on 5-HT neurons so that it results in an increase in firing activity (Fig 6). This could suggest that in males, the GABAergic inhibition is smaller than in the females. In CF, such a strong tonic inhibition would also explain the relatively low excitatory response observed with i.v. bicuculline. A 400 μg/kg (i.v.) dose of bicuculline, would be enough to suppress the male GABAergic inhibition of DRN 5-HT neurons, but possibly not sufficient to do so in CF. CF would have a greater number of tonically activated $GABA_A$ receptors than M. Therefore, reducing the level of GABA in the synaptic cleft (e.g. with isoniazid) would result in a greater excitatory response as is, in fact, observed in CF. Accordingly, a dose of

bicuculline important enough to block the majority of $GABA_A$ receptors in M would not do so in CF, explaining the relatively lower excitatory response.

When compared to CF, the mean number of spikes suppressed per nA (ss/nA) of iontophoretically applied GABA was significantly greater in M, indicating an apparent higher sensitivity the inhibitory effect of GABA. Again, this data is consistent with male 5-HT neurons being under lower GABAergic tonic inhibition than CF and subsequent GABA application having more pronounced inhibitory effect.

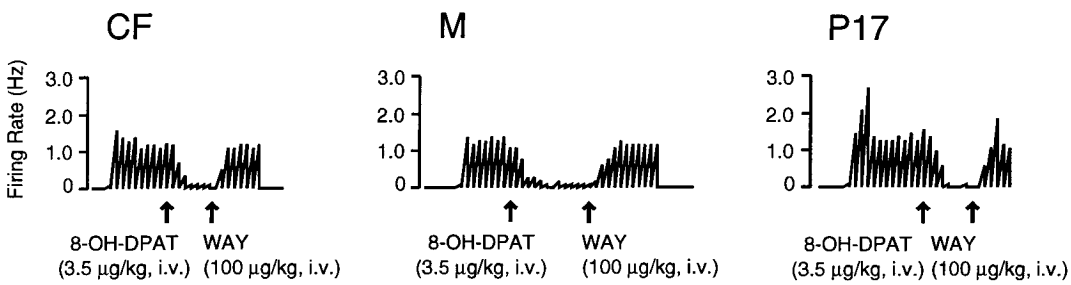
In summary, the present investigation of three different regulatory mechanisms suggest that the $5\text{-}HT_{1A}$ somatodendritic autoreceptor is partly desensitized in P17 as compared to CF, which is consistent with their increase in 5-HT neurons firing rate. Moreover, 5-HT neurons of both M and P17 appear to be under lower GABAergic tonic inhibition, which is also consistent with their higher basal firing rate as compared to CF. On the other hand, the long feedback loop does not seem to be affected by gender or by gonadal status and might not represent an important contribution to explain the difference in spontaneous firing rate observed between experimental groups.

FIGURE LEGENDS

Figure 1: Responsiveness of the 5-HT_{1A} receptor and effect on the 5-HT neurons firing activity. (A) Integrated firing rate histograms of DRN 5-HT neurons, showing the inhibitory effect of a single dose of 8-OH-DPAT (3.5 μg/kg, i.v.) and its reversal by the 5-HT_{1A} antagonist WAY 100635 (100 μg/kg, i.v.), in CF, M, P17. (B) Relationship between the degree of inhibition of the DRN 5-HT neurons firing activity and the dose of 8-OH-DPAT administered intravenously in CF (r^2 = 0.80), M (r^2 = 0.83), OVX (r^2 = 0.91), and P17 (r^2 = 0.95) on a logarithmic scale. The initial response to the first dose of 8-OH-DPAT of a single 5-HT neuron in each rat was used to construct the curve.

Figure 1

A


CF
M
P17
Firing Rate (Hz)
3.0
2.0
1.0
0
8-OH-DPAT (3.5 µg/kg, i.v.)
WAY (100 µg/kg, i.v.)

B

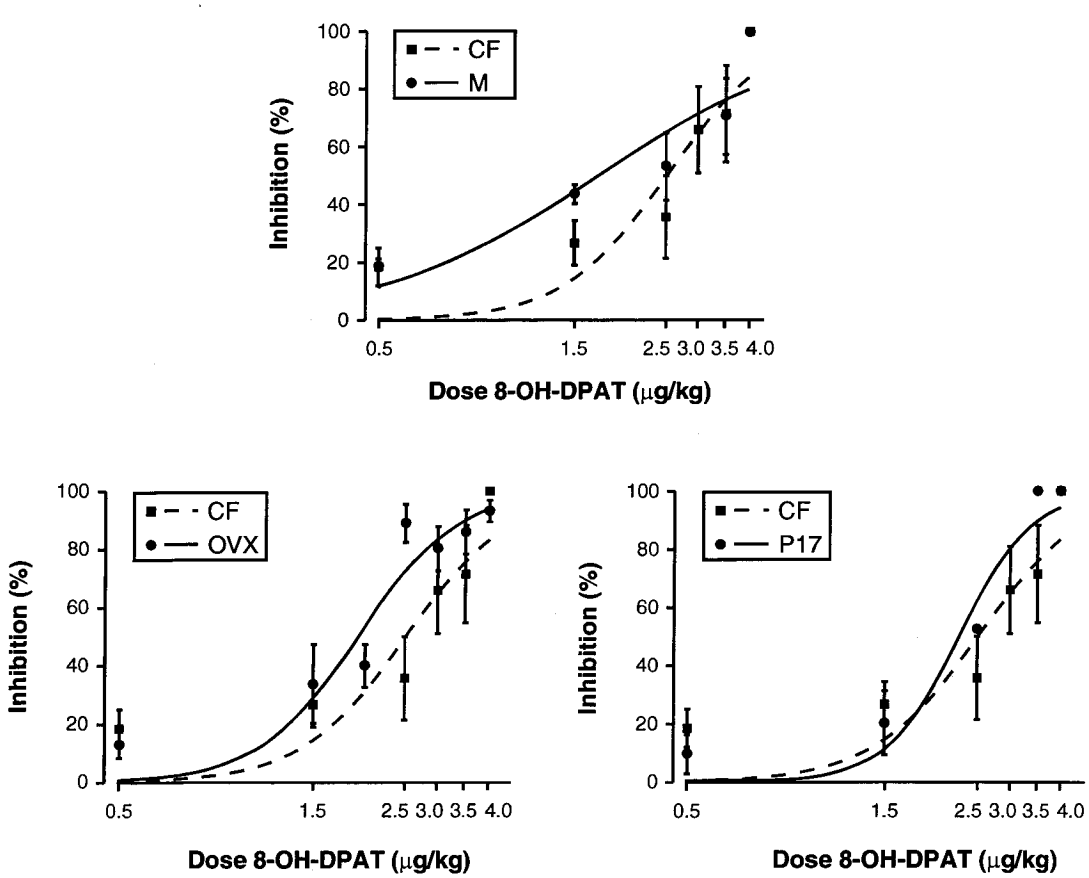
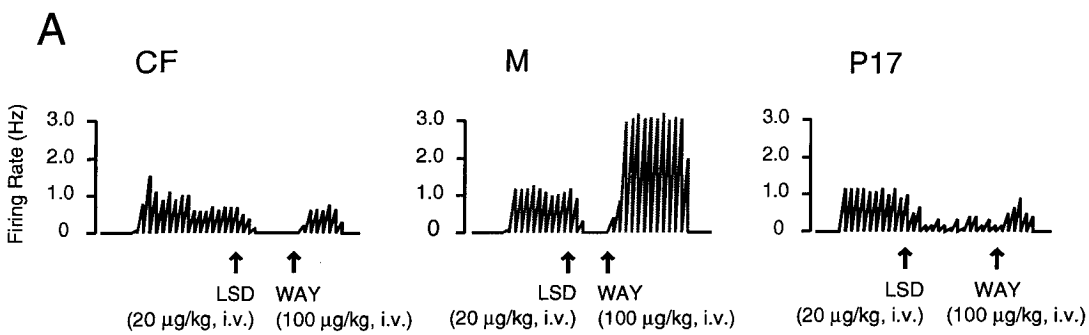

CF
M
OVX
P17
Inhibition (%)
100
80
60
40
20
0
0.5
1.5
2.5 3.0 3.5 4.0
Dose 8-OH-DPAT (µg/kg)

Figure 2: Responsiveness of the 5-HT_{1A} autoreceptor and effect on the 5-HT neurons firing activity. (A) Integrated firing rate histograms of DRN 5-HT neurons, showing the inhibitory effect of a single dose of LSD (20 μg/kg, i.v.) and its reversal by the 5-HT_{1A} antagonist WAY 100635 (100 μg/kg, i.v.), in CF, M, and P17. (B) Relationship between the degree of inhibition of the DRN 5-HT neurons firing activity and the dose of LSD administered intravenously in CF, M, OVX, and P17 on a logarithmic scale. The initial response to the first dose of LSD of a single 5-HT neuron in each rat, was used to construct the curve.

Figure 2


A
CF
M
P17
Firing Rate (Hz)
3.0
2.0
1.0
0
LSD
(20 µg/kg, i.v.)
WAY
(100 µg/kg, i.v.)

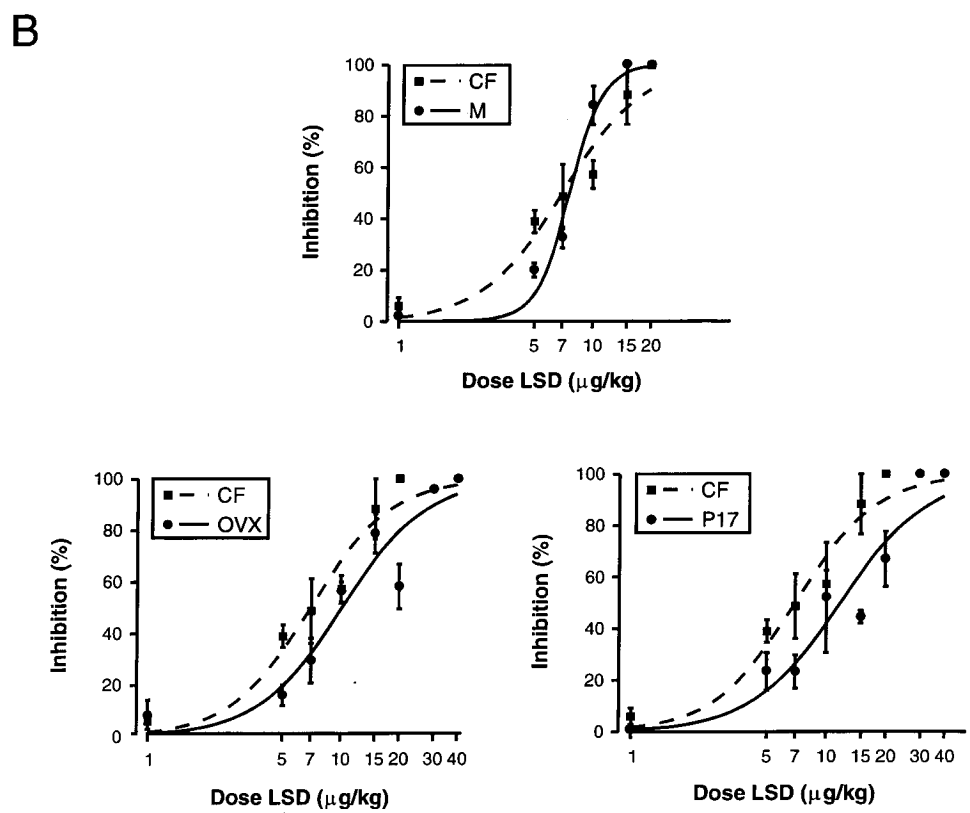

B
Inhibition (%)
CF
M
OVX
P17
Dose LSD (µg/kg)
1
5
7
10
15
20
30
40

Figure 3: Responsiveness of the $GABA_A$ receptor to GABA in 5-HT neurons. (A) Integrated firing rate histograms displaying the response to the microiontophoretic application of GABA (100 s) in CF, P17, and PP. In CF, GABA (2 nA) resulted in 55.4 ss/nA (*left*). In P17, GABA (3 nA) resulted in 41.4 ss/nA; the $GABA_A$ receptor antagonist BicuM (3 nA, 130s) ejected simultaneously with GABA, totally reversed firing inhibition caused by GABA, indicating that $GABA_A$ receptors are mediating responses to GABA (*middle*). In PP (7 days after parturition), GABA (2 nA) caused an almost imperceptible decrease in firing rate; GABA (8 nA) resulted in 7.5 ss/nA. (B) ss/nA corresponding to single applications of GABA in each group (columns of open symbols); the group mean is indicated by the horizontal bar.

Figure 3

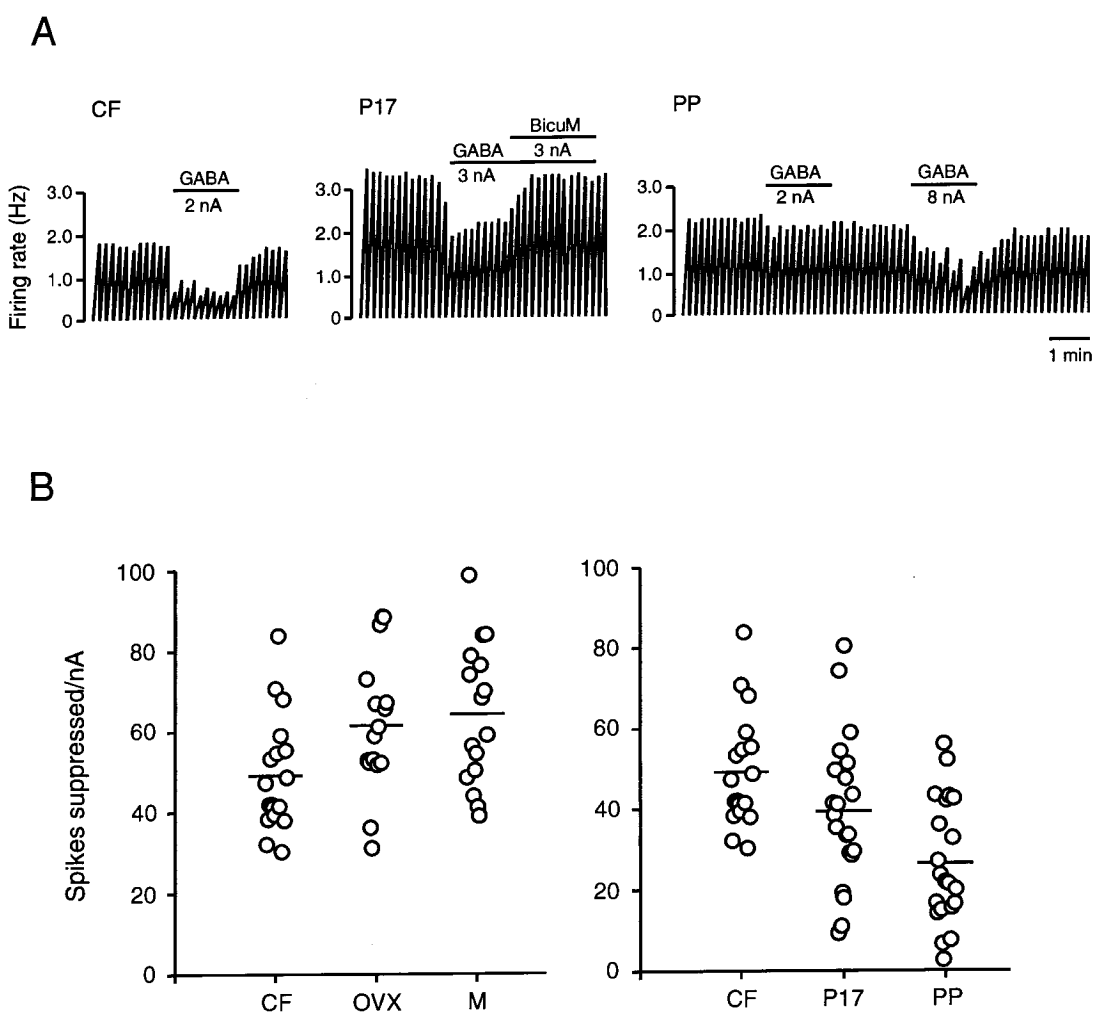
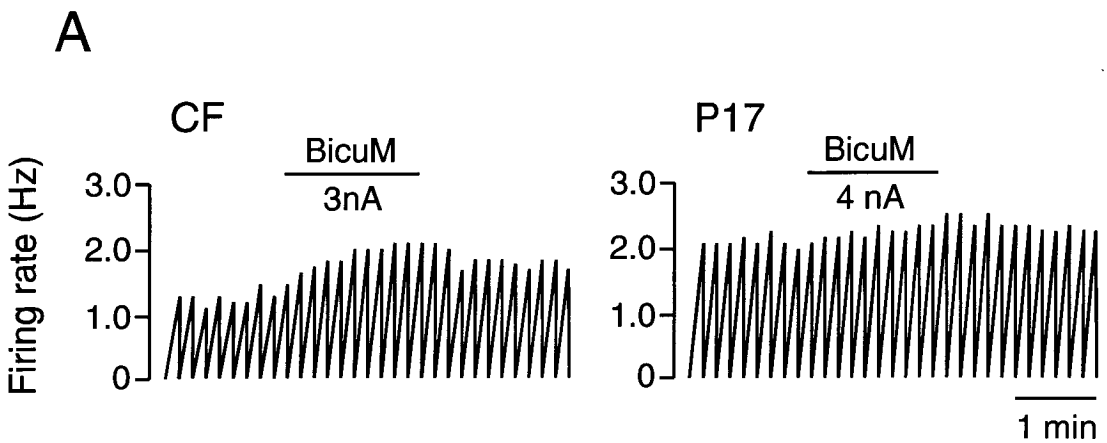

A
CF
P17
PP
BicuM
GABA
3 nA
GABA
3 nA
GABA
2 nA
GABA
2 nA
GABA
8 nA
Firing rate (Hz)
3.0
2.0
1.0
0
1 min
B
Spikes suppressed/nA
100
80
60
40
20
0
CF
OVX
M
CF
P17
PP

Figure 4: Responsivess of the $GABA_A$ receptor to local BicuM 5-HT neurons of CF and P17. (A) Integrated firing rate histograms showing the response of 5-HT neurons to the microiontophoretic application of BicuM (100s). In CF, BicuM (3 nA) resulted in 17.4 sg/nA (*left*). In P17, BicuM (4 nA) resulted in 2.7 sg/nA. (B) sg/nA corresponding to single applications of BicuM (columns of open symbols); the group mean is indicated by the horizontal bar.

Figure 4


A
CF
P17
BicuM
3nA
BicuM
4 nA
Firing rate (Hz)
3.0
2.0
1.0
0
1 min

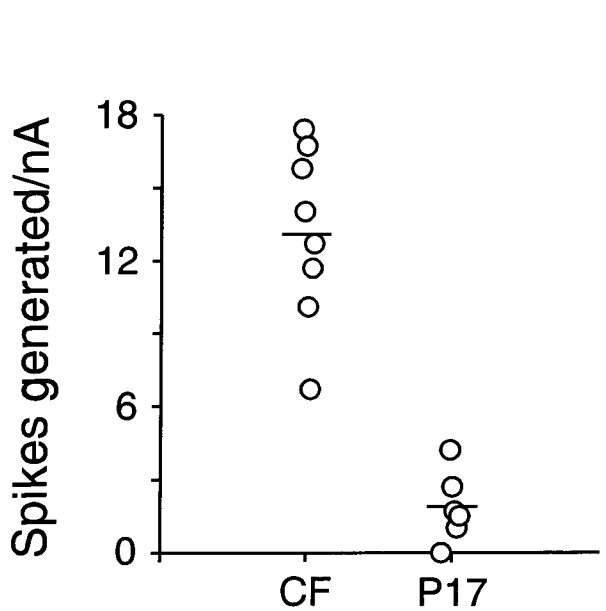
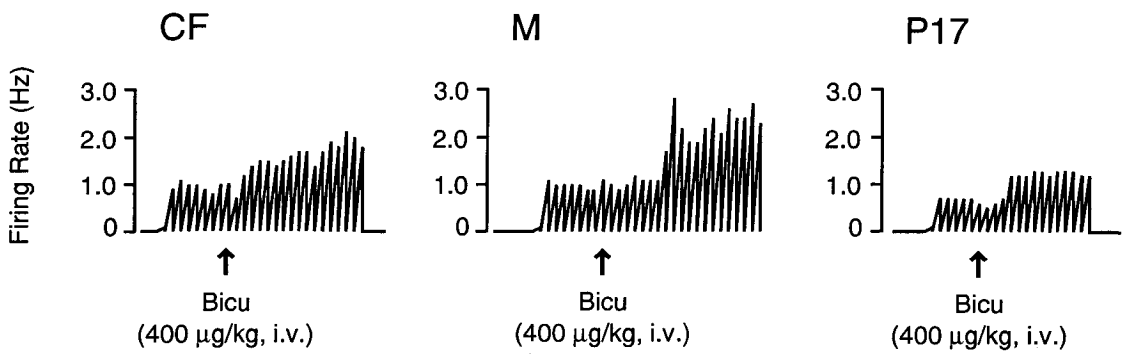

B
Spikes generated/nA
18
12
6
0
CF
P17

Figure 5: Responsivess of the $GABA_A$ receptor to intravenous Bicu and effect on the 5-HT neurons firing activity. (A) Integrated firing rate histograms of DRN 5-HT neurons, showing the excitatory effect of a single dose of bicuculline (400 μg/kg, i.v.), in CF, M, and P17. (B) Relationship between the degree of enhancement of the DRN 5-HT neurons firing activity and the dose of bicuculline administered intravenously in CF, M, and P17 on a logarithmic scale. The initial response to the first dose of Bicu of a single 5-HT neuron in each rat was used to construct the curve.

Figure 5

A


CF
M
P17
Firing Rate (Hz)
3.0
2.0
1.0
0
Bicu
(400 µg/kg, i.v.)

B

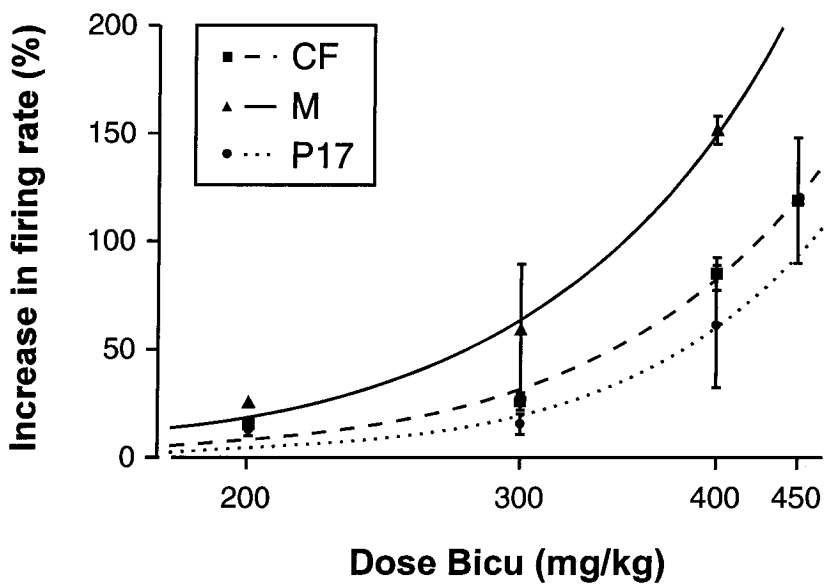
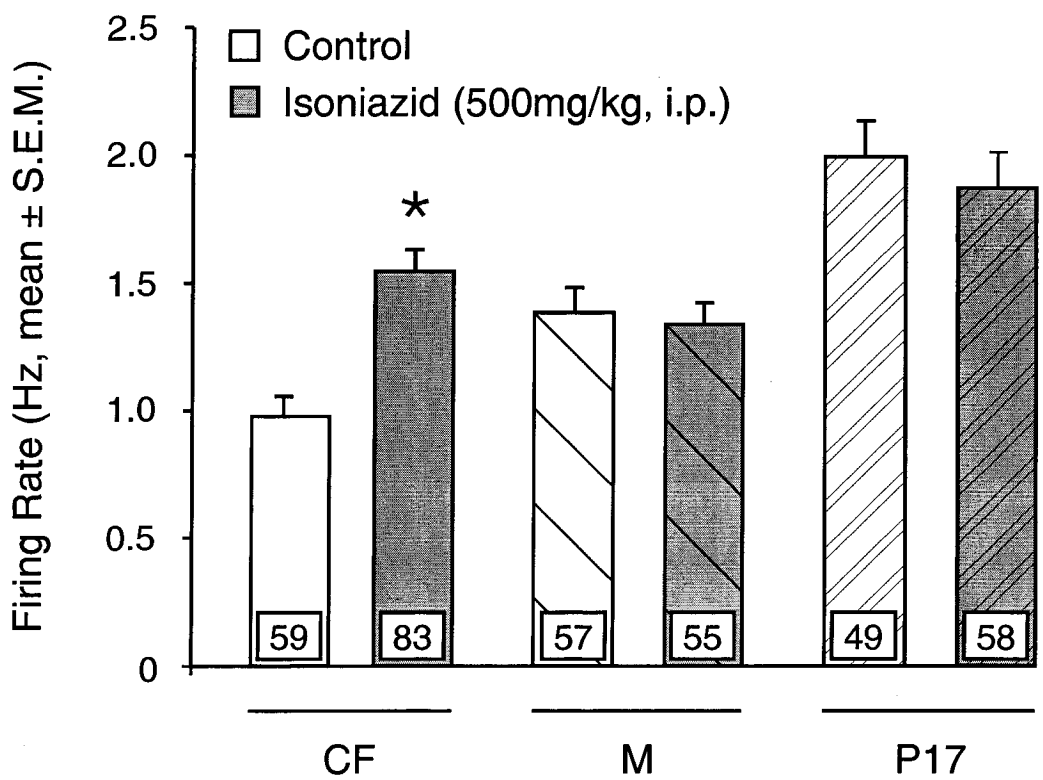

Increase in firing rate (%)
200
150
100
50
0
CF
M
P17
200
300
400
450
Dose Bicu (mg/kg)

Figure 6: Mean firing rate of DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) in CF, M, OVX, and P17 following, or not, an injection of isoniazid (500 mg/kg, i.p.). The number of neurons recorded is indicated in each box. Significance compared to the respective control group is indicated by a star ($p<$ 0.01).

Figure 6


Control
Isoniazid (500mg/kg, i.p.)
Firing Rate (Hz, mean ± S.E.M.)
2.5
2.0
1.5
1.0
0.5
0
*
59
83
57
55
49
58
CF
M
P17

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Foreword to Chapter 4

Modulation of the firing activity of female dorsal raphe nucleus serotonergic neurons by neuroactive steroids

Ovarian steroids have been shown to modulate the expression of several genes of the 5-HT system (see introduction). Furthermore, the previous manuscripts of this thesis have shown that the spontaneous firing are of DRN 5-HT neurons is significantly higher in males and in pregnant females than in freely cycling females. Interestingly, during pregnancy, the 5-HT neuronal firing activity rate follows circulating levels of progesterone. These data strongly suggested a modulation of 5-HT neuronal activity by ovarian steroids, especially progesterone. The aim of this study was thus to assess a possible modulation of 5-HT neuron firing activity by progesterone and its metabolites by preventing the synthesis and metabolism of progesterone as well as directly administering these steroids in the cerebrospinal fluid of female rats.

Chapter 4

MODULATION OF THE FIRING ACTIVITY OF FEMALE DORSAL RAPHE NUCLEUS SEROTONERGIC NEURONS BY NEUROACTIVE STEROIDS

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ABSTRACT

Important gender differences in mood disorders result in a greater susceptibility for women. Accumulating evidence suggest a reciprocal modulation between the 5-HT system and neuroactive steroids. Previous data from our laboratory have shown that during pregnancy, the firing activity of 5-HT neurons increases in parallel with progesterone (P) levels. This study was undertaken to evaluate the putative modulation of the 5-HT neuronal firing activity by different neurosteroids. Female rats received intracerebroventricularly (i.c.v.), for 7 days, a dose of 50 μg/kg/day of one of the following steroids: P, pregnenolone (PREG), 5β-pregnane-3,20-dione (5β-DHP), 5β-pregnane-3α-ol,20-one (3α,5β-THP), 5β-pregnane-3β-ol,20-one (3β,5β-THP), 5α-pregnane-3,20-dione (5α-DHP), 5α-pregnane-3α-ol,20-one (allopregnanolone, 3α,5α-THP), 5α-pregnane-3β-ol,20-one (3β,5α-THP) and dehydroepiandrosterone (DHEA). 5β-DHP and DHEA were also administered for 14 and 21 days (50 μg/kg/day, i.c.v.) as well as concomitantly with the selective σ_1 antagonist NE-100. In vivo extracellular unitary recording of 5-HT neurons performed in the dorsal raphe nucleus of these rats revealed that DHEA, 5β-DHP and 3α,5α-THP significantly increased the firing activity of the 5-HT neurons. Interestingly, 5β-DHP and DHEA showed different time-frames for their effects with 5β-DHP having its greatest effect after 7 days to return to control values after 21 days, whereas DHEA demonstrated a sustained effect over the 21-day period. NE-100 prevented the effect of DHEA but not of 5β-DHP, thus indicating that it σ_1

receptors mediate the effect of DHEA but not that of 5β-DHP. In conclusion, our results offer a cellular basis for potential antidepressant effects of neurosteroids, which may prove important particularly for women affective disorders.

Abbreviations: 3α,5α-THP, 5α-pregnane-3α-ol,20-one (allopregnanolone); 3α,5β-THP, 5β-pregnane-3α-ol,20-one; 3β,5α-THP, 5α-pregnane-3β-ol,20-one; 3β,5β-THP, 5β-pregnane-3β-ol,20-one; 5α-DHP, 5α-pregnane-3,20-dione; 5β-DHP, 5β-pregnane-3,20-dione; 5-HT (serotonin), 5-hydroxytryptamine; DHEA, dehydroepiandrosterone; DRN, dorsal raphe nucleus; FC, freely cycling females; GABA, γ-aminobutyric acid; NE-100, N,N-dipropyl-2-(4-methoxy-3-(2-phenylethoxy)phenyl)-ethylamine; OVX, ovariectomized females; P, progesterone; PREG, pregnenolone; SSRI, serotonin reuptake inhibitor.

INTRODUCTION

There are important gender differences in susceptibility for mood disorders. Women experience depressive episodes earlier in life, more frequently, more recurrently and more persistently than men (Weissman & Klerman, 1977; Weissman & Klerman, 1985; Maes *et al.*, 1986; Parry, 1989; Hamilton, 1993; Kessler *et al.*, 1994; Weissman *et al.*, 1996; Kornstein, 1997; Joffe & Cohen, 1998). The mechanisms underlying these gender differences are not understood. It has been suggested that the frequent hormonal variations experienced by women might contribute to their vulnerability to depression (Endicott, 1993; Pajer, 1995; Kornstein, 1997; Joffe & Cohen, 1998). Although the etiology of major depression remains unclear, the enhancement of serotonergic (5-HT) neurotransmission observed with antidepressant treatments suggests the implication of the 5-HT system in the biology of depression (Racagni & Brunello, 1999; Blier & de Montigny, 1999).

Ovarian steroids are known to affect brain areas not directly related to reproductive functions, such as the dorsal raphe nuclei (DRN) (McEwen *et al.*, 1998). This nucleus being a region rich in 5-HT neuron cell bodies, ovarian steroids may have a functional impact on the 5-HT system. Numerous studies, performed in females, indicate that ovarian steroids modulate the expression of several genes of the 5-HT system (e.g. tryptophan hydroxylase, vesicular monoamine transporter, serotonin reuptake transporter and different 5-HT receptors) (see review by Bethea *et al.*, 1999).

Moreover, there is accumulating evidence suggesting that depressive states may be associated with a decrease in 3α,5α-THP (allopregnanolone) levels. For instance, in a mouse model of depression, long-term social isolation decreases the cortical levels of 3α,5α-THP and 5α-DHP (Dong *et al.*, 2001). Also, the levels of 3α,5α-THP and 3α,5β-THP were shown to be significantly lower in the cerebrospinal fluid (CSF) and plasma of depressed patients as compared to controls (Romeo *et al.*, 1998; Uzunova *et al.*, 1998). Conversely, selective serotonin reuptake inhibitors (SSRIs) have been shown to increase the cerebral content of some neuroactive steroids both in rats and humans (Uzunov *et al.*, 1996; Griffin & Mellon, 1999; Serra *et al.*, 2001). Furthermore, successful antidepressant treatments not only regularize the levels of neuroactive steroids, but the extent of the increase in CSF contents of 3α,5α-THP and 3α,5β-THP is also proportional to the mood improvement (Romeo *et al.*, 1998; Uzunova *et al.*, 1998).

Using an *in vivo* electrophysiological paradigm of extracellular recordings, we have previously shown that the spontaneous firing activity of DRN 5-HT neurons is significantly higher in male (M) than in female (F) rats, while ovariectomy (OVX) did not significantly modify the female 5-HT neuronal basal firing rate. Interestingly, during pregnancy, the spontaneous firing rate increased gradually to peak at the 17^{th} day of pregnancy (P17) and then declined before

parturition, thus following circulating levels of progesterone (but not of estrogen) (Klink *et al.*, 2002).

Furthermore, we showed that, during pregnancy, 5-HT_{1A} autoreceptors were partly desensitized and that 5-HT neurons were probably under a much lower GABAergic tonic inhibition as compared to that of freely cycling females (FC) (Robichaud *et al.*, 2002). Both of these functional modifications are consistent with the enhanced firing activity of 5-HT neurons observed during pregnancy. They also provide possible mechanisms by which hormonal fluctuations can modulate the 5-HT neurons function and influence women vulnerability to mood disorders.

Taken together, the literature and our previous data suggest a reciprocal modulation between the 5-HT system and neuroactive steroids. Therefore, the goal of the present studies was to assess a possible modulation of 5-HT neuron firing activity by progesterone (P) and its metabolites (see Fig.1).

MATERIALS AND METHODS

Animals

Sprague Dawleys (Charles-River, St-Constant, Québec, Canada) weighing between 250g and 325g were kept under standard laboratory conditions (12:12 light-dark cycle with access to food and water *ad libitum*). Freely cycling females (FC), and ovariectomized females (OVX) were used for the experiments. Ethical committee approval was obtained from the McGill University Animal Ethical Care Committee and all their rules and regulations were followed. The suffering of animals as well as the number used were kept at minimum.

Treatment with inhibitors of progesterone synthesis and metabolism

Ovariectomized females (OVX) operated at their 8^{th} week of life were used for treatments with Trilostane (OVX-T) and Finasteride (OVX-F). Trilostane treatments began at the 9^{th} week of life. Trilostane suspended in sesame oil was administered by daily subcutaneous (s.c.) injections of 25 mg/kg for 14 days. Finasteride treatments began at the 10^{th} week of life. Finasteride suspended in 1.5 % methyl cellulose was administered for 5 days by daily gavage of 20 mg/kg. These doses of Finasteride and Trilostane have previously been shown to efficiently the 5α-reductase and 3β-hydroxysteroid dehydrogenase enzymatic activities, respectively (Potts *et al.*, 1978, Young *et al.*, 1994, Phan *et al.*, 1999, Micevych *et al.*, 2003). Experiments were carried out on the day following the last administration of either drug.

Treatments with steroids

All steroids were dissolved in 3% (v/v) ethanol/distilled water and administered continuously and intracerebroventricularly (i.c.v.) by mean of a subcutaneous osmotic minipump connected to a canulae (ALZA, Palo Alto, CA, USA), which was implanted in the left lateral ventricle of the rat brain. The surgery was performed as described by the manufacturer (ALZA, Palo Alto, CA, USA) and under chloral hydrate anesthesia. Each steroid was administered with a dose of 50μg/kg/day. NE-100, a sigma 1 (σ_1) receptor antagonist, was dissolved in distilled water and administered subcutaneously (s.c.) by an osmotic minipump (ALZA, Palo Alto, CA, USA) for a daily dose of 10 mg/kg.

FC received one of the following treatments: 7, 14 or 21 days with either 5β-pregnane-3,20-dione (5β-DHP) or dehydroepiandrosterone (DHEA); 7 days with either progesterone (P), pregnenolone (PREG), 5β-pregnane-3α-ol,20-one (3α,5β-THP), 5β-pregnane-3β-ol,20-one (3β,5β-THP), 5α-pregnane-3,20-dione (5α-DHP), 5α-pregnane-3α-ol,20-one (3α,5α-THP) or 5α-pregnane-3β-ol,20-one (3β,5α-THP); 7 days with either 5β-DHP or DHEA concomitantly with NE-100. A first control for the surgical procedure was obtained with i.c.v. administration of saline for 7 days. A second series of controls received 3% ethanol, i.c.v., for 7 days. Experiments were carried out the day following the last day of administration and after removal of the canulae. Following experiments, every

brain was removed, frozen at –80°C and sliced using a microtome to confirm the position of both the canulae and the recording electrode.

Electrophysiological Experiements

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

Extracellular unitary recording of serotonergic neurons were conducted with single-barelled glass micropipettes pulled in a conventional manner, filled with a 1M NaCl solution and of final impedance ranging between 2 and 6 MΩ. A 4 mm-diameter hole was drilled in the skull of each rat at the appropriate location (about 1 mm anterior of lambda and centered with respect to the midline). DRN 5-HT neurons unit activity was recorded by lowering the micropipette along descents covering the nucleus from 300μm to about 1500μm anterior of lambda. Spontaneously active DRN 5-HT neurons were identified according to the criteria of Aghajanian: a slow and regular rythmical firing rate and positive action potentials of long duration (Aghajanian *et al.*, 1978).

For each group of rats, 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 seconds, each 5-HT neuron encountered in complete descents in the DRN of 3 to 6 rats.

Statistics

Statistical analysis were performed with the software SigmaStat for Windows Version 4.0 (Jandel Corporation). Average values are given as the mean ± S.E.M. One-way ANOVA, with alpha = 0.05, followed by a post-hoc analysis using Tukey's method of comparison versus control was used for evaluating statistical significance. Results (F) of statistical analysis are expressed in terms of degrees of freedom between groups compared (df) and number of groups compared (p). Significance was considered for $P<0.05$.

Drugs

Trilostane was obtained from Sanofi Research Division (Malvern, PA), NE-100 was kindly provided by Taisho Pharmaceutical Co. Ltd. (Tokyo, Japan) and Finasteride was prepared from 5mg commercial pills of Proscar (Merck Frosst). Steroids used were: progesterone, pregnenolone, 5β-pregnane-3,20-dione, 5β-pregnane-3α-ol,20-one, 5β-pregnane-3β-ol,20-one, 5α-pregnane-3,20-dione, 5α-pregnane-3α-ol,20-one, 5α-pregnane-3β-ol,20-one (Steraloids), and DHEA (Sigma Aldrich).

RESULTS

Two types of controls were separately carried out in order to assess the potential effect of the surgical procedure and of the vehicle. Since the surgical control (saline, i.c.v.) were very similar to FC (1.01 ± 0.07 Hz, n = 70, and 1.02 ± 0.07 Hz, n = 72 (data not shown), respectively, the vehicle control was used for comparison with the subsequent treatments. P, administered i.c.v. for 7 days to FC did not significantly modify the firing activity of 5-HT neurons (1.21 ± 0.07 Hz, n = 95 vs 1.18 ± 0.10 Hz, n = 52, [F(1,2) = 0.09, n.s.]) (Fig.2).

Since in a previous series of experiments, ovariectomy did not decrease the basal firing activity of 5-HT neurons (Klink *et al.*, 2002), we decided to investigate if the cerebral *de novo* synthesis of P was sufficient to maintain the basal 5-HT neuron activity. Thus, to prevent local P synthesis, OVX rats were treated with trilostane (an inhibitor of the 3β-hydroxysteroid dehydrogenase, the enzyme responsible for converting PREG into P, see Fig.1). As reported before, the firing activity of 5-HT neurons was not significantly modified following OVX (as compared to FC, [F(1,2) = 1.19 ± 0.12 Hz, n = 43 vs 1.02 ± 0.07 Hz, n = 72, n.s.], Fig. 3A). Following the treatment with trilostane (25 mg/kg/day for 14 days), the firing activity of 5-HT neurons was increased by less than 10%, which was not statistically significant as compared to OVX (1.29 ± 0.11 Hz, n = 44 and 1.19 ± 0.12 Hz, n = 43, respectively, [F(1,2) = 0.37, n.s.], Fig. 3B).

As another way to assess the capacity of the cerebral *de novo* synthesis of P to influence the basal firing rate DRN 5-HT neurons, OVX were treated with finasteride (20 mg/kg/day for 5 days), a selective blocker of the 5α-reductase (the enzyme metabolizing P into 5α-DHP, see Fig.1). This was done to prevent the catabolism of P and, therefore, to increase its cerebral levels. Increasing P levels with finasteride did not significantly increase in 5-HT neuron firing activity as compared to OVX (1.34 ± 0.08 Hz, n = 42 and 1.19 ± 0.12 Hz, n = 43, respectively, [F(1,2) = 1.00, n.s.], Fig. 3B).

Since P does not seem to affect the firing activity of 5-HT neurons, the effects of its precursor and of some of its metabolites were investigated. As was the case with P, a 7-day treatment with PREG led to a non-significant increase in 5-HT neuronal firing rate (1.52 ± 0.17 Hz, n = 55 vs 1.19 ± 0.13 Hz, n = 41, [F(1,2) = 2.07, n.s.], Fig.4).

In parallel to P, DHEA is also synthesized from pregnenolone (Fig.1). The DHEA treatments led to an increase in firing activity, which persisted over time (Fig.5). After 7 days, the mean firing frequency of 5-HT neurons was increased from 0.96 ± 0.08 Hz, n = 52, to 1.48 ± 0.11 Hz, n = 78 ([F(5,6) = 7.92, P<0.001, Tukey's test, q = 4.8, P<0.01]), after 14 days, it was increased from 1.10 ± 0.09 Hz, n = 54 to 1.66 ± 0.11 Hz, n = 68 ([F(5,6) = 7.92, P<0.001, Tukey's test, q = 5.1, P<0.01]), and after 21 days it reached 1.82 ± 0.18 Hz, n = 40 as compared to 1.21 ± 0.11 Hz, n = 54 ([F(5,6) = 7.92, P<0.001, Tukey's test, q = 4.9, P<0.01).

Since the increase in 5-HT neurons' firing activity, measured following P treatments, was not present as expected from the results obtained during pregnancy (Klink *et al.*, 2002), the possible implication of different P metabolites was investigated. First, females were treated for 7, 14 and 21 days with 5β-DHP. The 7-day treatment led to a significant increase in 5-HT neurons basal firing rate (1.64 ± 0.11 Hz, n = 45, [$F(5,6) = 7.01$, $P < 0.001$, Tukey's test, $q = 6.0$, $P<0.01$], Fig. 6). This was followed by a gradual decrease towards control values (Fig. 6); after 14 days of administration, the increase in firing rate was still statistically significant as compared to its control (1.57 ± 0.09 Hz, n = 78, vs 1.10 ± 0.08 Hz, n = 63, [$F(5,6) = 7.01$, $P < 0.001$, Tukey's test, $q = 5.2$, $P<0.01$]) but not after 21 days of treatment (1.16 ± 0.13 Hz, n = 55, vs 1.22 ± 0.11 Hz, n = 46, [$F(5,6) = 7.01$, $P < 0.001$, Tukey's test, $q = 0.6$, n.s.).

The enzymes converting 5β-DHP into its metabolites 3α,5β-THP and 3β,5β-THP are present in the brain (Guennoun *et al.*, 1995; Celotti *et al.*, 1992). Therefore, FC were administered for 7 days with these metabolites but neither treatment led to a statistically significant increase in the firing rate of 5-HT neurons as compared to controls (3α,5β-THP: 1.47 ± 0.14 Hz, n = 50 vs 1.23 ± 0.13 Hz, n = 47, [$F(1,2) = 1.52$, n.s.] and 3β,5β-THP: 1.51 ± 0.12 Hz, n = 68 vs 1.23 ± 0.13 Hz, n = 47, [$F(1,2) = 2.39$, n.s.], Fig. 7).

The effect of the other P metabolite stereoisomers was also investigated. FC received a 7-day administration of 5α-DHP, 3α,5α-THP and 3β,5α-THP (Fig.

8). Neither 5α-DHP nor its metabolite 3β,5α-THP significantly modified the firing activity of 5-HT neurons (1.12 ± 0.09 Hz, n = 63, [F(1,2) = 0.33, n.s.] and 1.35 ± 0.11 Hz, n = 75, [F(1,2) = 0.80, n.s.], respectively as compared to 1.20 ± 0.10 Hz, n = 42 for the controls). However, 3α,5α-THP induced a pronounced increase in their firing rate (1.97 ± 0.13 Hz, n = 58 compared to 1.20 ± 0.10 Hz, n = 42, [F(1,2) = 18.23, P< 0.001).

DHEA has been shown to have agonistic properties at sigma (σ) receptors in a model using the NMDA response in the hippocampus (Bergeron *et al.*, 1996, Debonnel *et al.*, 1996). For this reason, FC were treated simultaneously with DHEA and the selective σ_1 antagonist NE-100 to investigate if the higher firing rate induced by DHEA was mediated by σ_1 receptors. NE-100, at a dose shown to block the effect of other σ ligands (Bermack & Debonnel, 2001), prevented the increase in firing rate induced by DHEA (controls: 0.96 ± 0.08 Hz, n = 52; DHEA: 1.48 ± 0.11 Hz, n = 78 [F(2,3) = 6.22, P< 0.005, Tukey's test, q = 5.0, P< 0.05]; DHEA + NE-100: 1.22 ± 0.10 Hz, n = 70, [F(2,3) = 6.22, P< 0.005, Tukey's test, q = 2.5, n.s.], Fig. 9) indicating that this effect was mediated by σ_1 receptors. Because the effect of 5β-DHP had a different time-frame, as compared to that of DHEA, the implication of σ_1 receptors was also investigated for this steroid. However, NE-100 did not prevent the 5β-DHP-induced increase in firing rate (controls: 1.06 ± 0.07 Hz, n = 86; 5β-DHP: 1.64 ± 0.11 Hz, n = 45 [F(2,3) =

14.95, P< 0.001, Tukey's test, q = 6.4, P< 0.05]; 5β-DHP + NE-100: 1.67 ± 0.14 Hz, n = 40, [F(2,3) = 6.23, P< 0.001, Tukey's test, q = 6.2, P< 0.05], Fig. 10).

DISCUSSION

The main findings of this study are 1) the increase in the firing activity of female 5-HT neurons following the chronic administration of some neuroactive steroids, i.e. 5β-DHP, 3α,5α-THP and DHEA, and 2) the effect of DHEA but not of 5β-DHP is mediated, at least in part, by σ_1 receptors. Furthermore, the larger effects of some metabolites compared with those of P, and the fact that P is rapidly metabolized in the brain, suggest that the metabolites may play an important role in the modulation of the 5-HT neuronal activity.

In a previous study, we have shown that during pregnancy the spontaneous firing rate of 5-HT neurons is more than doubled (Klink *et al.*, 2002). Moreover, the firing activity of 5-HT neurons changes in parallel with the plasma levels of P (Klink *et al.*, 2002). Our hypothesis was therefore that, progesterone could be responsible for increasing the 5-HT neuronal activity in females. However, in OVX rats, the plasma levels of P are much lower than in FC while the firing rate of DRN 5-HT neurons is unchanged when compared to FC (Klink *et al.*, 2002). This was suggesting that the *de novo* synthesis of P, which is known to occur in the brain (Guennoun *et al.*, 1995), might be sufficient to maintain a normal level of the 5-HT neuron firing activity following an ovariectomy.

In rats treated with P for 7 days, the absence of significant modification of the firing activity indicates that P is probably not directly involved in the increase

of 5-HT neuronal firing activity observed during pregnancy. Based on the literature, cerebral concentrations were extrapolated to be about four times higher than that reached by P during pregnancy (Corpéchot *et al.*, 1993) and are thus in the high physiological range. However, P was administered only for 7 days and an effect of P following a longer treatment cannot be ruled out.

Surprisingly, blocking cerebral P synthesis in OVX rats, with a Trilostane treatment, did not decrease the firing activity of the 5-HT neurons and there was even a trend towards an increase. Using different *in vivo* paradigms, similar or lower doses of Trilostane were shown to efficiently inhibit the enzyme 3β-hydroxysteroid dehydrogenase (Potts *et al.*, 1978, Young *et al.*, 1994, Phan *et al.*, 1999, Micevych *et al.*, 2003). A possible explanation was thus that the blockade of P synthesis could lead to a shift of the biosynthesis equilibrium towards a greater synthesis of DHEA (see Fig. 1). Rats were therefore treated with the precursor PREG as well as with DHEA. Administration of DHEA, but not of PREG, resulted in an enhanced firing activity of the DRN 5-HT neurons, which is in keeping with our hypothesis. Moreover, this DHEA-induced increase in 5-HT neuronal firing rate might explain the antidepressant effect observed with DHEA in humans (Wolkowitz *et al.*, 1997).

Another way to assess the possible effect of the local P *de novo* synthesis was to block its catabolism, therefore increasing P cerebral levels in OVX rats. Finasteride, a selective 5α-reductase inhibitor, was used for this purpose. It has

previously been shown that the systemic administration of similar doses of Finasteride efficiently blocks the enzymatic activity of cerebral 5α-reductase (Phan *et al.*, 1999). However, in our hands, this treatment did not significantly change the 5-HT neurons basal firing rate. This constitutes another indication that, contrarily to our previous hypothesis, P by itself is not involved directly in the control of the firing activity of DRN 5-HT neurons. This is also in keeping with the demonstration that following ovariectomy, despite local P synthesis, the cerebral content of P was decreased by more than 70% (Corpechot *et al.*, 1993) but that there was no change in the firing activity of 5-HT neurons. Therefore, it appears that neurosteroids, other than P, are involved in the modification of the firing activity of DRN 5-HT neurons observed during pregnancy.

P can be metabolized into 5α-DHP and 5β-DHP by the 5α- and 5β-reductase, respectively, and then further reduced into 3α,5α-THP and 3α,5β-THP by the enzyme 3α-hydroxysteroid oxidoreductase (3α-HSOD) (Kawahara *et al.*, 1975; Karavolas & Hodges, 1991; Celotti *et al.*, 1992; Compagnone & Mellon, 2000). These three enzymes (5α- and 5β-reductases, and 3α-HSOD) are present, and active, in the mammalian brain (Kawahara *et al.*, 1975; 1981; Celotti *et al.*, 1992). It appears that the principal metabolic pathway for cerebral P is its reduction into 5α-DHP and 3α,5α-THP (Karavolas & Hodges, 1991; Korneyev *et al.*, 1993) (see Fig. 1). Interestingly, 3α,5α-THP was the most potent steroid of the present study. Since this steroid is probably the principal metabolite of P, its

levels may be elevated enough during pregnancy to substantially contribute to the increase in the firing activity of 5-HT neurons observed in pregnant rats.

A rather unexpected finding of this study was the different time-frame for the increase in firing activity of the 5-HT neurons caused by 5β-DHP and DHEA. The 5β-DHP-induced increase in firing activity was maximal after a 7-day treatment, followed by a gradual decline to finally lose statistical significance after 21 days of administration. On the other hand, the enhancement of the firing rate caused by DHEA was sustained over the 21-day period of time. This would support the hypothesis that more than one receptor is implicated in the effects seen with the different steroids tested in this study. Indeed, the DHEA-induced enhancement of the firing activity of 5-HT neurons seems to be mediated, at least in part, by σ_1 receptors, as shown by the fact that co-administration of the σ_1 receptor antagonist NE-100 prevented this effect. Interestingly, other sigma ligands have been shown to increase the firing rate of 5-HT neurons in similar period of times and this effect could be blocked by NE-100 (Bermack and Debonnel, 2201). Furthermore, the present results could suggest a physiological basis to the antidepressant-like effects observed for some neuroactive steroids in an animal model of depression, which effects were also shown to be mediated by σ_1 receptors (Reddy *et al.*, 1998; Urani et al., 2001). On the other hand, NE-100 did not prevent the effect of 5β-DHP, thus indicating that other receptor(s) must mediate it. Also, the time-frame for the 5β-DHP-induced increase in firing activity could suggest a functional desensitization of the receptor mediating the

effect of 5β-DHP on 5-HT neurons, whereas in the case of DHEA, there is no such indication, which is also in agreement with what is found with other sigma ligands (Bermack & Debonnel, 2201).

The mechanism(s) by which P metabolites increase the firing activity of 5-HT neurons is still unclear. However, based on our previous studies with pregnant rats, a partial desensitization of 5-HT_{1A} autoreceptors appears as a likely component (Robichaud *et al.*, 2002). If neurosteroids, which levels rise dramatically during pregnancy, are indeed responsible for the pregnancy-induced desensitization of 5-HT_{1A} autoreceptors, the intracerebral administration of neuroactive steroids could also lead to such desensitization. Since 5-HT_{1A} autoreceptors are inhibitory, their partial desensitization would easily explain the enhanced firing activity of 5-HT neurons reported in the present study.

On the other hand, recent studies have shown that DHEA promotes neurogenesis in the hippocampal dentate gyrus and protects it from glucocorticoids' detrimental effects (Karishma & Herbert, 2002). Interestingly, Santarelli and colleagues showed that inhibition of hippocampal neurogenesis prevents the behavioral effects of antidepressants in different animal models of depression (Santarelli et al., 2003). Together, these data offer another mechanism of action for the antidepressant effect of DHEA (Kaminska et al., 2000). Further studies assessing the effect of other steroids on neurogenesis would be needed to

assess whether this mechanism of action is specific to DHEA or if it might extend to other neurosteroids.

The effect of neuroactive steroids on 5-HT neurons activity could also be mediated through their interaction with $GABA_A$ receptors. It is well-known that 3α-reduced steroids are $GABA_A$ receptor modulators and can even act as proper agonists (Harrison *et al.*, 1987; Morrow *et al.*, 1989; Puia *et al.*, 1990; McCauley *et al.*, 1995). P, 5α- and 5β-DHP, devoid of such property, are however rapidly converted into 3α,5α- and 3α,5β-THP, which can act on $GABA_A$ receptors (Bitran *et al.*, 1993; Lancel *et al.*, 1996). In rats, GABAergic neurons exert a tonic inhibition of DRN 5-HT neurons which seems to be mediated mostly by $GABA_A$ receptors (Innis & Aghajanian, 1987; Smith & Gallager, 1987; Gervasoni *et al.*, 2000). Interestingly, during pregnancy, the GABAergic tonic inhibition of the 5-HT neurons was dramatically reduced when compared to that of FC (Robichaud *et al.*, 2002). Also, accumulating evidence suggest that sustained high levels of neuroactive steroids reduce $GABA_A$ receptor responsiveness and the efficacy of modulators to potentiate the chloride influx (Concas *et al.*, 1998; Friedman *et al.*, 1993; Yu & Ticku, 1995a; Yu & Ticku, 1995b; Yu *et al.*, 1996; Gulinello *et al.*, 2001). It is thus possible that sustained neurosteroids administration could cause both a desensitization of $GABA_A$ receptors and/or a diminution of the tonic GABAergic inhibition on 5-HT neurons and thus increase the firing activity of these neurons. Interestingly, 3α,5α-THP has the most potent agonistic properties on $GABA_A$ receptors and induced the greatest increase in 5-

HT neurons firing activity, whereas P and 5α-DHP were devoid of significant effect, which is in keeping with this hypothesis.

The CSF and plasma of depressed patients have been shown to contain lower levels of 3α,5β-THP and 3α,5α-THP, than those of healthy volunteers, which could be brought back up to normal levels by successful antidepressant treatments (Romeo *et al.*, 1998; Uzunova *et al.*, 1998; Ströhle *et al.*, 1999). No differences in P levels were observed (Romeo *et al.*, 1998; Uzunova *et al.*, 1998). Lower serum levels of 3α,5α-THP were observed in both women suffering from premenstrual syndrome (PMS), during the luteal phase of their menstrual cycle, and in women with postpartum blues as compared to corresponding controls (Rapkin *et al.*, 1997; Nappi *et al.*, 2001). It was suggested that fluoxetine and other SSRIs interact with the enzyme 3α-HSD, responsible for the reversible conversion of 5α- and 5β-DHP into their respective 3α-reduced metabolites (3α,5α- and 3α,5β-THP). This interaction would favor the reduction reaction and would reduce the rate of the oxidative reaction (i.e. conversion of 3α,5α- and 3α,5β-THP into 5α- and 5β-DHP), thus leading to enhanced levels of 3α,5α- and 3α,5β-THP (Uzunov *et al.*, 1996; Uzunova *et al.*, 1998; Griffin & Mellon, 1999).

Together, these studies could suggest an association between depressive states with a decrease in 3α,5α-THP levels, and mood improvement with an enhancement of the steroid levels. In agreement with this hypothesis, our results show that 3α,5α-THP significantly increases the 5-HT neuron firing activity in

females. Since all antidepressant treatments increase the efficacy of the 5-HT neurotransmission, these results could suggest a potential antidepressant effect for some neuroactive steroids.

Even if a clear link has not yet been firmly established, recent reports have suggested that hormonal replacement therapy (HRT) could induce undesirable side effects (see review by Armitage *et al.*, 2003), which has led many patients to stop their HRT treatments. Natural hormones might, therefore, not be the best candidates as long-term adjuvants for antidepressant treatments, which would have to be administered presumably for several years to patients suffering from refractory depression. However, synthetic compounds having a similar pharmacological profile and the same effects on 5-HT neurons, which could be administered systemically, become interesting candidates. Therefore, the modulation by neurosteroids of the firing activity of 5-HT neurons, reported here, may prove important for the treatment of female mood disorders.

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FIGURE LEGENDS

Figure 1: Partial schematic representation of the progesterone metabolic pathway.

Figure 1

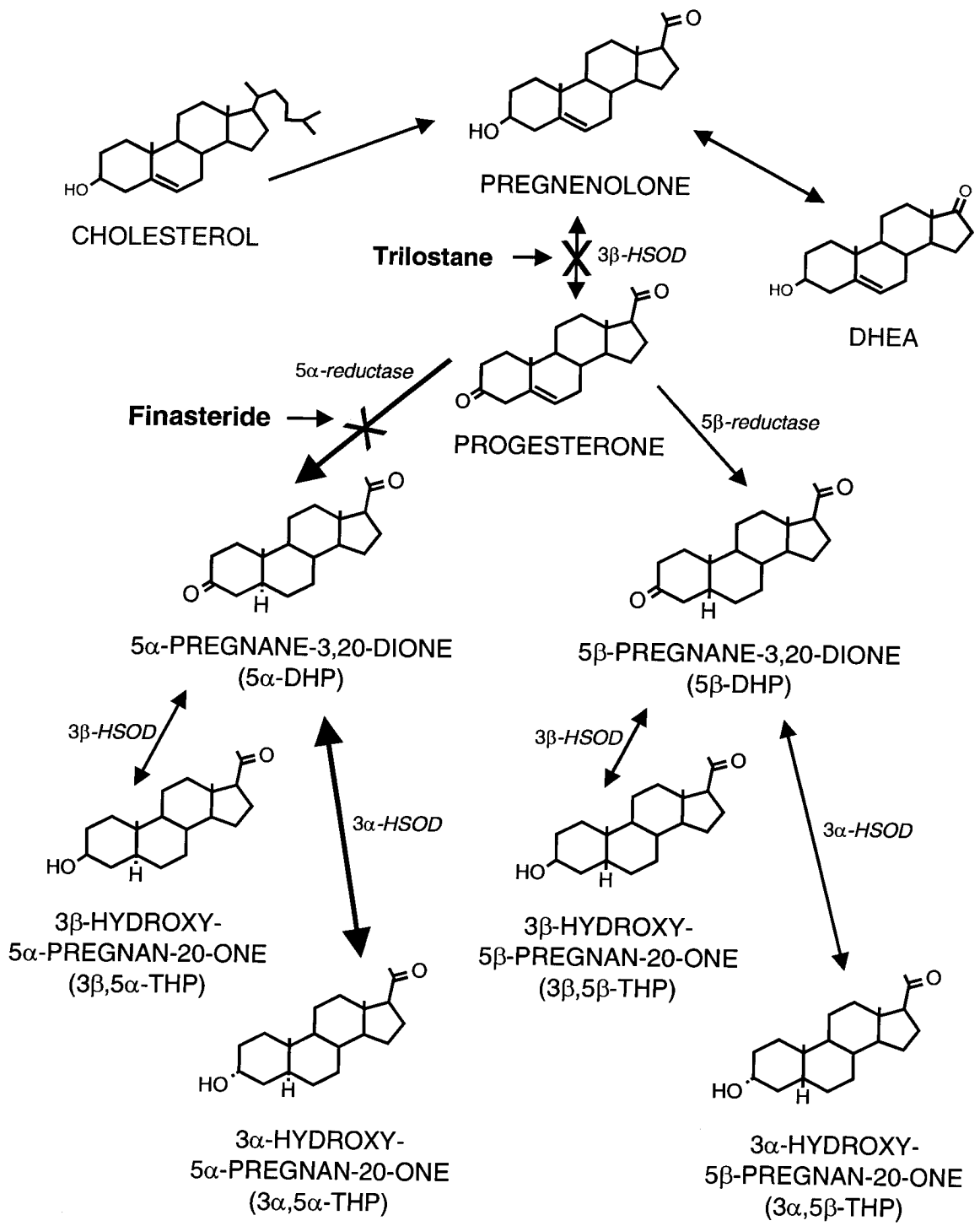
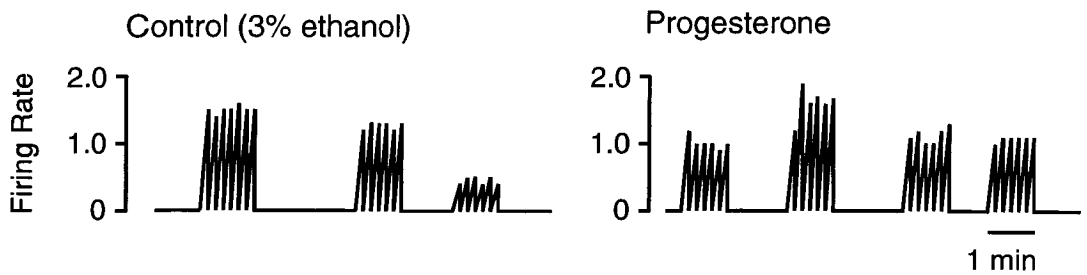

CHOLESTEROL
PREGNENOLONE
DHEA
Trilostane
3β-HSOD
PROGESTERONE
5α-reductase
Finasteride
5β-reductase
5α-PREGNANE-3,20-DIONE
(5α-DHP)
5β-PREGNANE-3,20-DIONE
(5β-DHP)
3β-HSOD
3α-HSOD
3β-HYDROXY-
5α-PREGNAN-20-ONE
(3β,5α-THP)
3β-HYDROXY-
5β-PREGNAN-20-ONE
(3β,5β-THP)
3α-HYDROXY-
5α-PREGNAN-20-ONE
(3α,5α-THP)
3α-HYDROXY-
5β-PREGNAN-20-ONE
(3α,5β-THP)

Figure 2: (A) Integrated firing rate histograms of 5-HT neurons, showing their spontaneous firing activity, recorded in one electrode descent in the DRN of females control (3% ethanol, i.c.v., 7 days) and following a treatement with P (50 μg/kg/day, i.c.v., 7 days). (B) Spontaneous firing rate of female DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) in controls and following a 7-day treatment with P. In this and the following figures, the number of neurons recorded is indicated in each box.

Figure 2

A)


Control (3% ethanol)
Progesterone
Firing Rate
2.0
1.0
0
2.0
1.0
0
1 min

B)

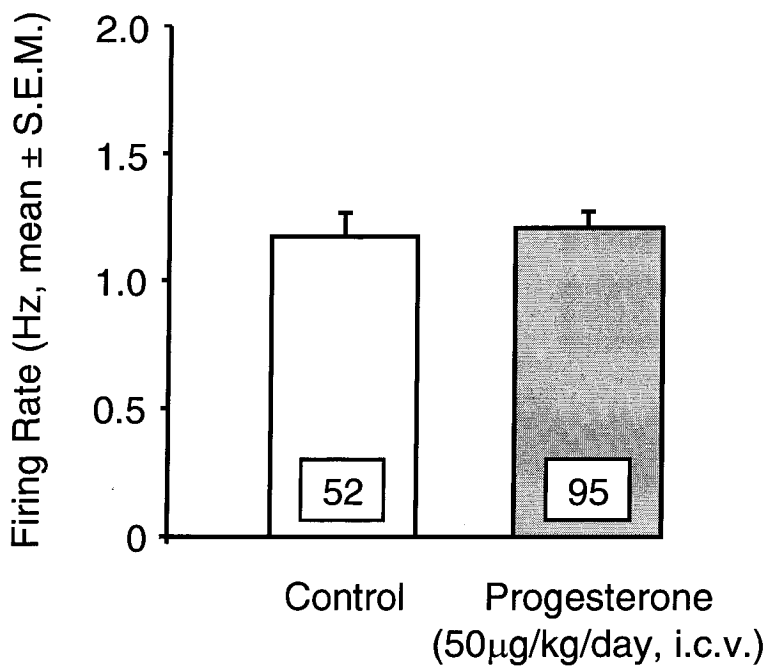

Firing Rate (Hz, mean ± S.E.M.)
2.0
1.5
1.0
0.5
0
52
95
Control
Progesterone
(50μg/kg/day, i.c.v.)

Figure 3: Spontaneous firing rate of DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) in FC, OVX (A) and in OVX, OVX treated with trilostane for 14 days (OVX + Trilostane, 20mg/kg/day, s.c.) and OVX treated with finasteride for 5 days (OVX + Finasteride, 25mg/kg/day, p.o.) (B).

Figure 3

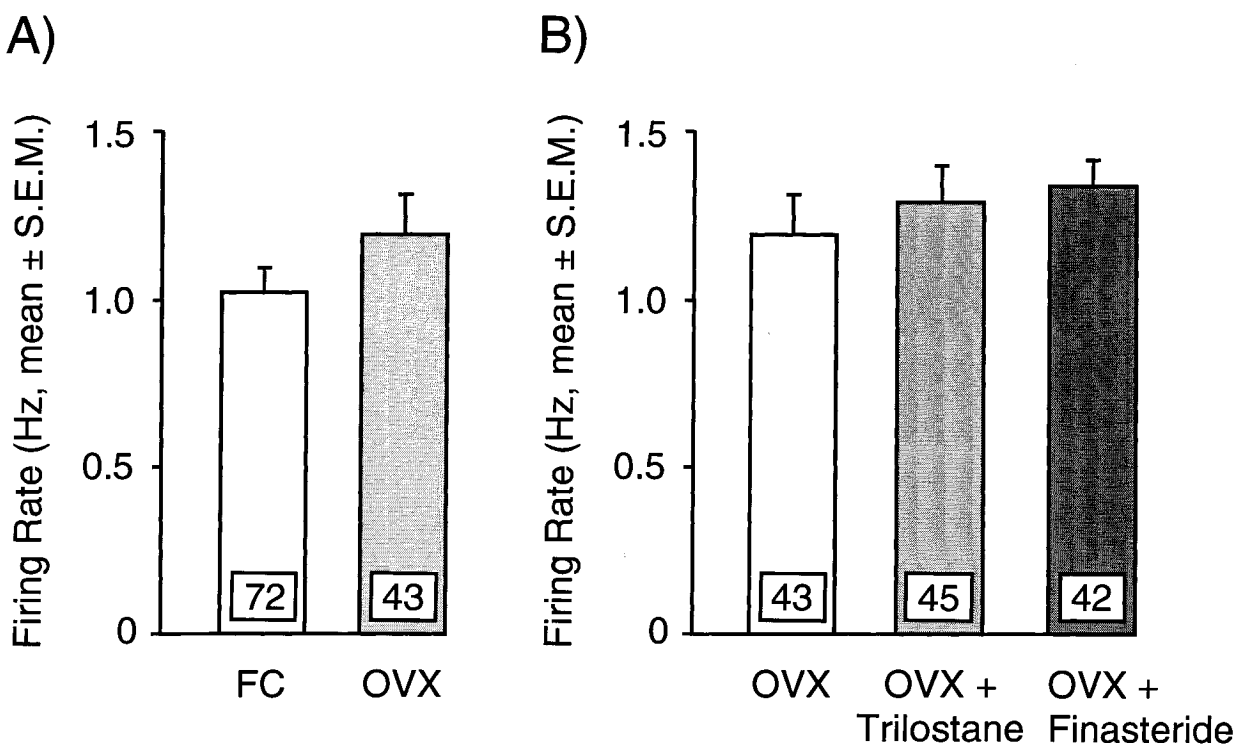

A)
B)
Firing Rate (Hz, mean ± S.E.M.)
1.5
1.0
0.5
0
72
43
FC
OVX
43
45
42
OVX
OVX + Trilostane
OVX + Finasteride

Figure 4: Spontaneous firing rate of female DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) in controls (3% ethanol, 7days, i.c.v.) and following a treatment with pregnenolone (50 μg/kg/day, i.c.v., 7days).

Figure 4

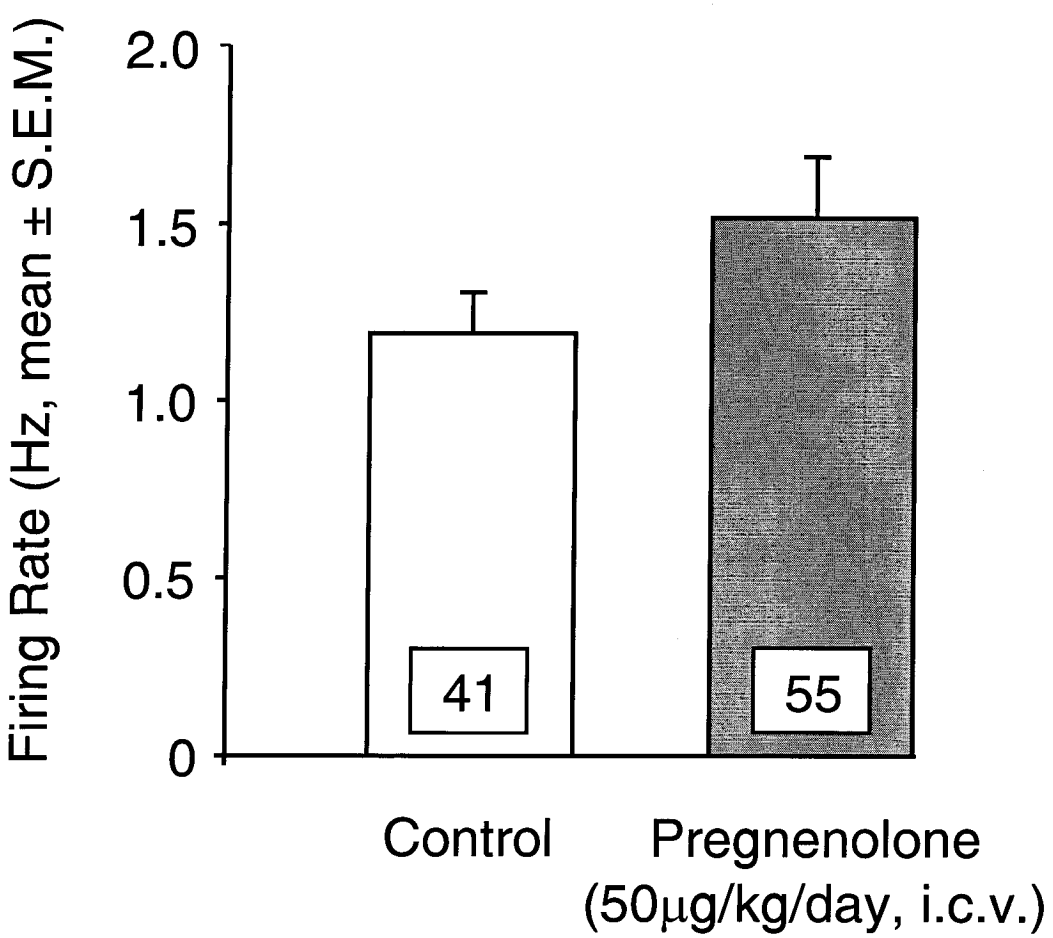

Firing Rate (Hz, mean ± S.E.M.)
2.0
1.5
1.0
0.5
0
41
55
Control
Pregnenolone
(50μg/kg/day, i.c.v.)

Figure 5: Spontaneous firing rate of female DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) following different treatment durations with DHEA (50 µg/kg/day, i.c.v.) or 3% ethanol (i.c.v.) for the controls. The stars indicate $P < 0.01$ as compared to the respective control.

Figure 5

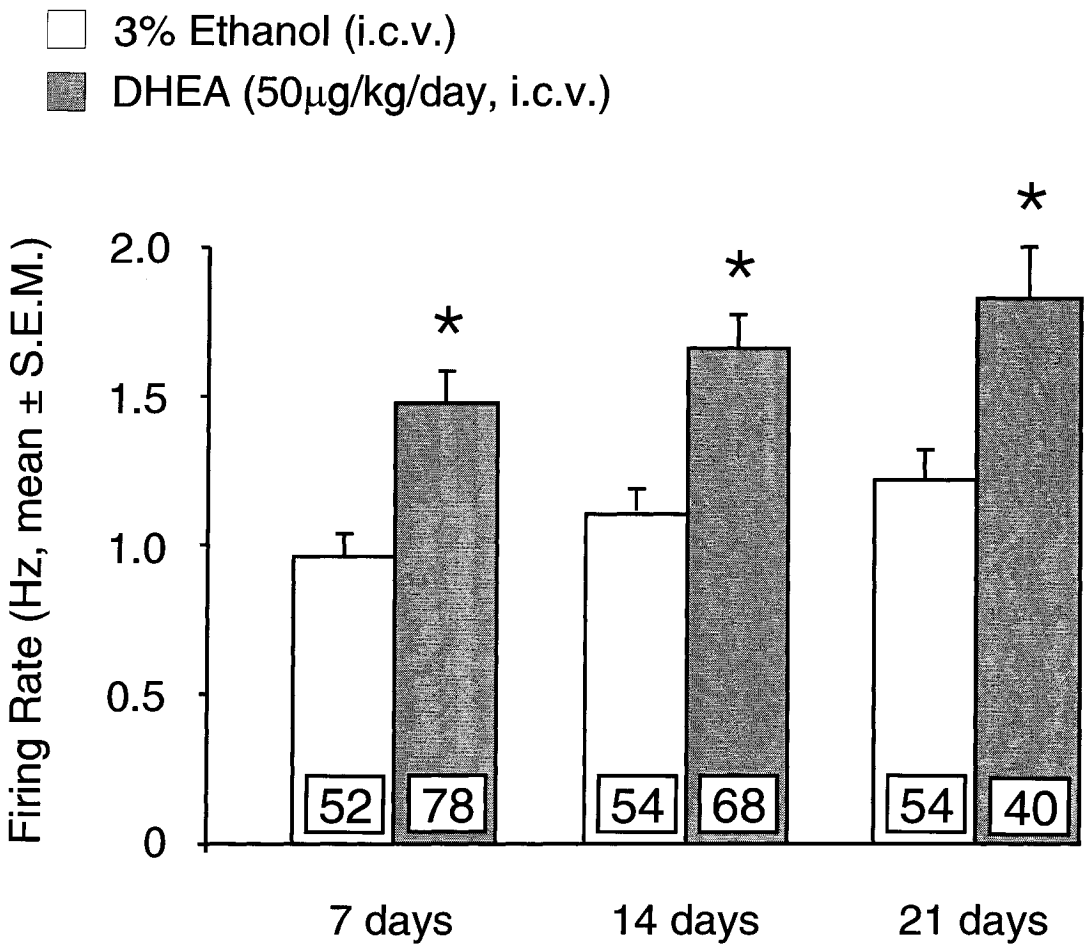

3% Ethanol (i.c.v.)
DHEA (50μg/kg/day, i.c.v.)
Firing Rate (Hz, mean ± S.E.M.)
2.0
1.5
1.0
0.5
0
*
*
*
52
78
54
68
54
40
7 days
14 days
21 days

Figure 6: Spontaneous firing rate of female DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) following different treatment durations with 5β-DHP (50 μg/kg/day, i.c.v.) or 3% ethanol (i.c.v.) for the controls. The stars indicate $P < 0.01$ as compared to the respective control.

Figure 6

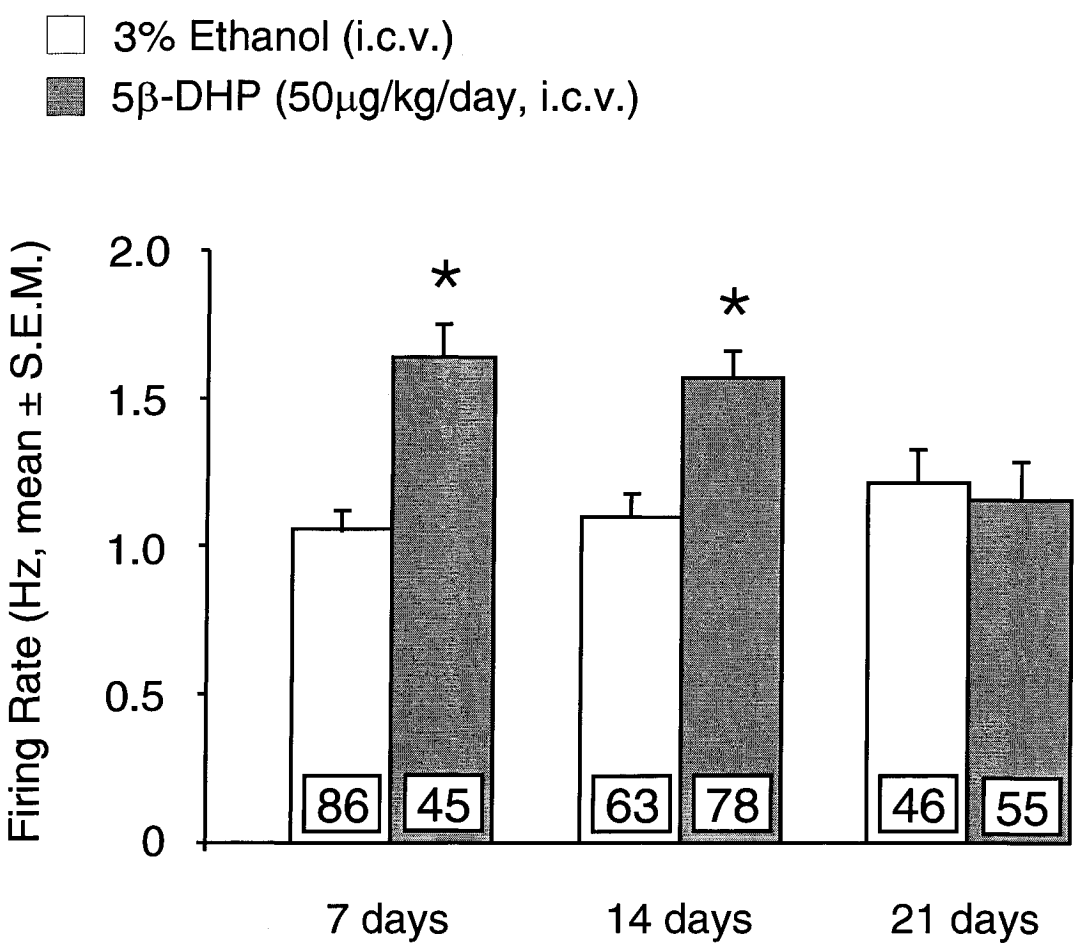

3% Ethanol (i.c.v.)
5β-DHP (50μg/kg/day, i.c.v.)
Firing Rate (Hz, mean ± S.E.M.)
2.0
1.5
1.0
0.5
0
*
*
86
45
63
78
46
55
7 days
14 days
21 days

Figure 7: Spontaneous firing rate of female DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) in controls (3% ethanol, i.c.v.) and following a 7-day treatment with either 3α,5β-THP (50 μg/kg/day, i.c.v.) or 3β,5β-THP (50 μg/kg/day, i.c.v.).

Figure 7

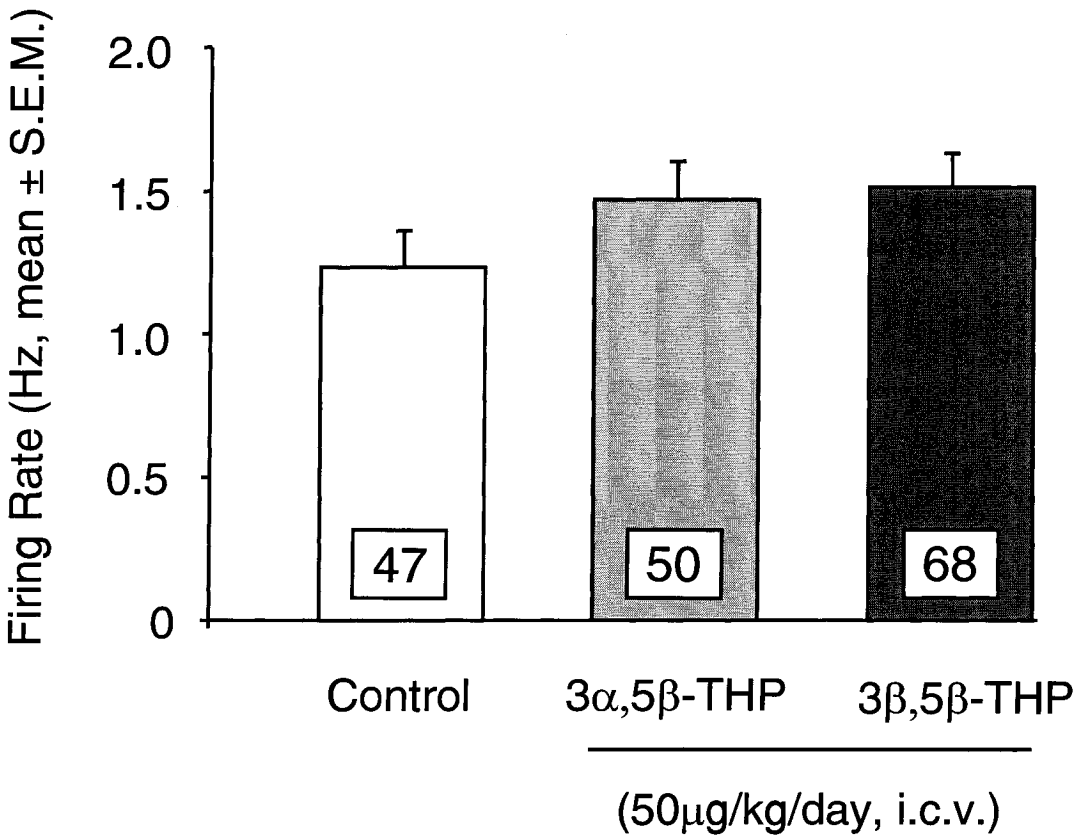

Firing Rate (Hz, mean ± S.E.M.)
2.0
1.5
1.0
0.5
0
47
50
68
Control
3α,5β-THP
3β,5β-THP
(50μg/kg/day, i.c.v.)

Figure 8: Spontaneous firing rate of female DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) in controls (3% ethanol) and following a 7-day treatment with either 5α-DHP, 3α,5α-THP or 3β,5α-THP (50 μg/kg/day, i.c.v., in each case). The star indicates $P < 0.001$.

Figure 8

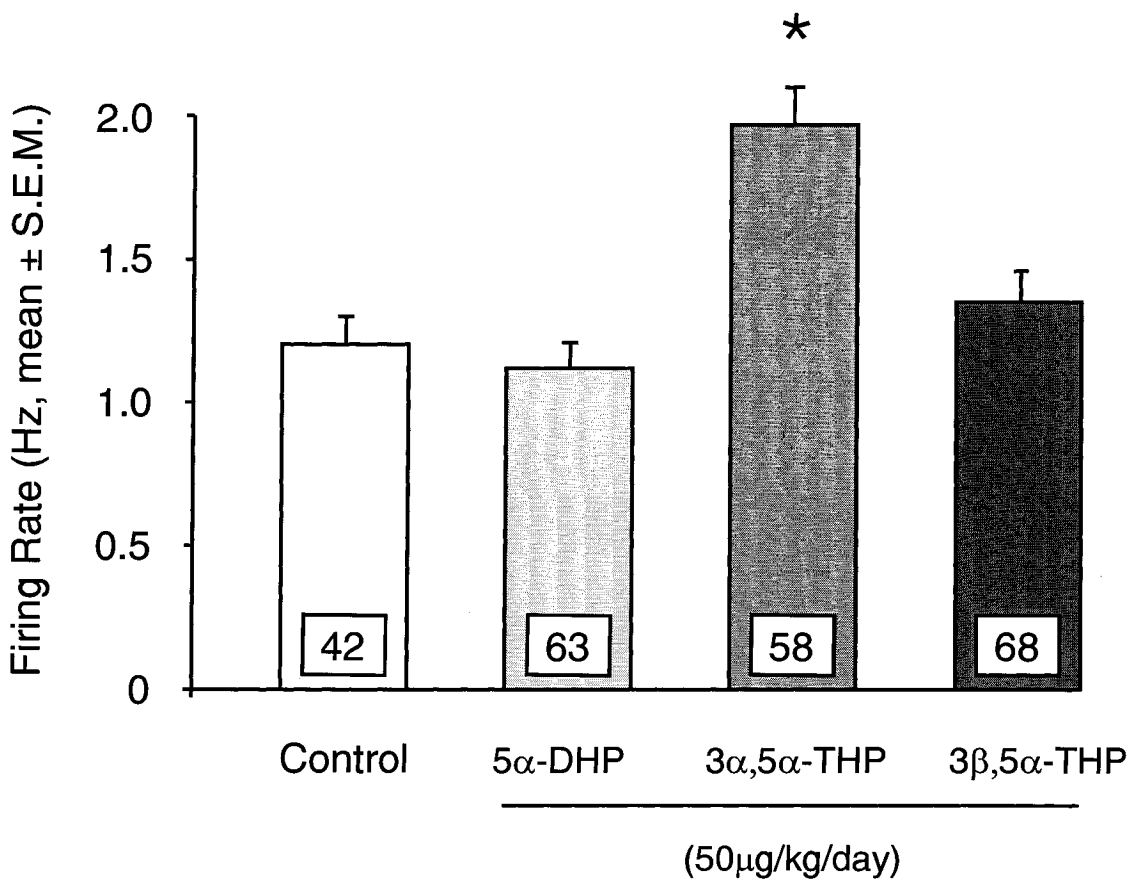

*
Firing Rate (Hz, mean ± S.E.M.)
2.0
1.5
1.0
0.5
0
42
63
58
68
Control
5α-DHP
3α,5α-THP
3β,5α-THP
(50μg/kg/day)

Figure 9: Spontaneous firing rate of female DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) in controls (3% ethanol) and following a 7-day treatment with either DHEA (50 μg/kg/day, i.c.v.) or both DHEA (50 μg/kg/day, i.c.v.) and NE-100 (10 mg/kg/day, s.c.). The star indicates $P < 0.05$.

Figure 9

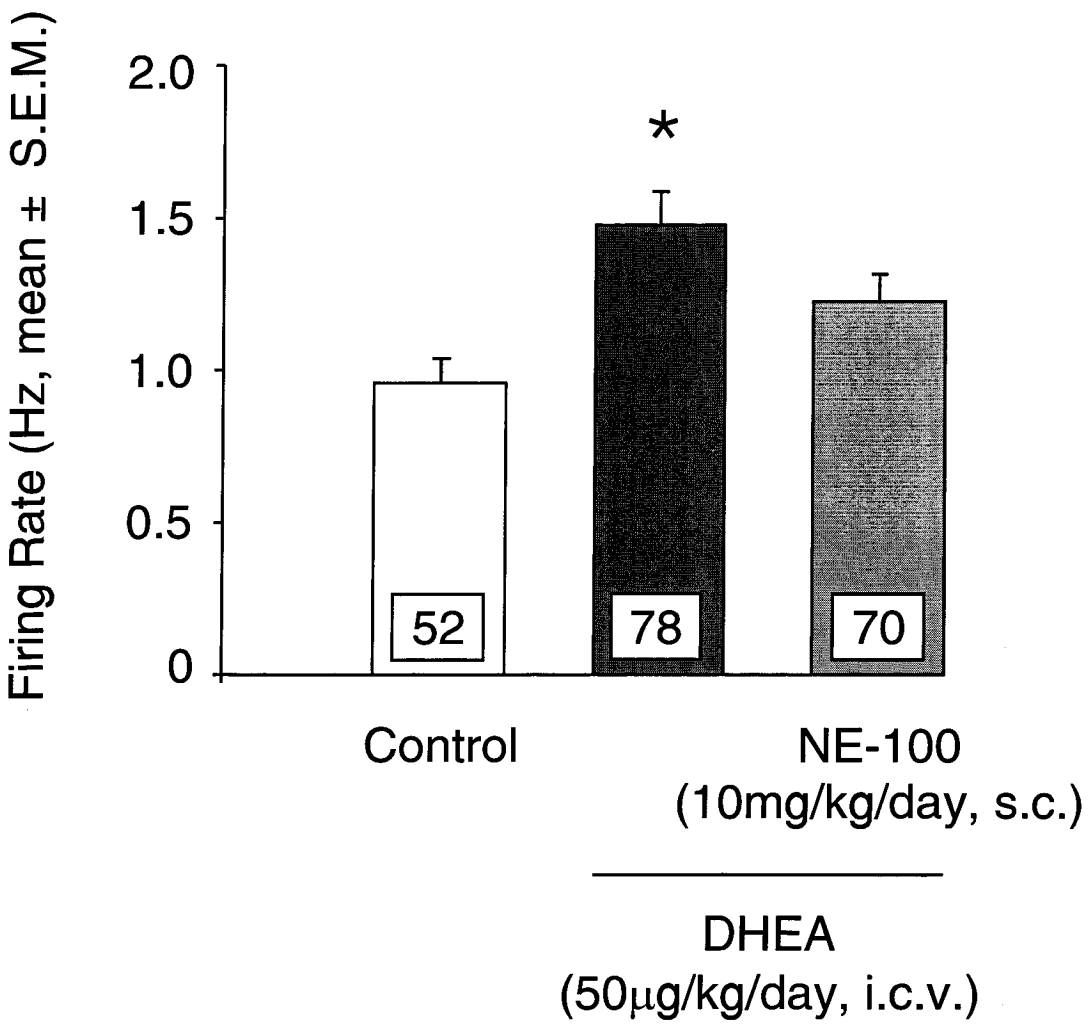

Firing Rate (Hz, mean ± S.E.M.)
2.0
1.5
1.0
0.5
0
*
52
78
70
Control
NE-100
(10mg/kg/day, s.c.)
DHEA
(50μg/kg/day, i.c.v.)

Figure 10: Spontaneous firing rate of female DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) in controls (3% ethanol) and following a 7-day treatment with either 5β-DHP (50 μg/kg/day, i.c.v.) or both 5β-DHP (50 μg/kg/day, i.c.v.) and NE-100 (10 mg/kg/day, s.c.). The stars indicate $P < 0.05$.

Figure 10

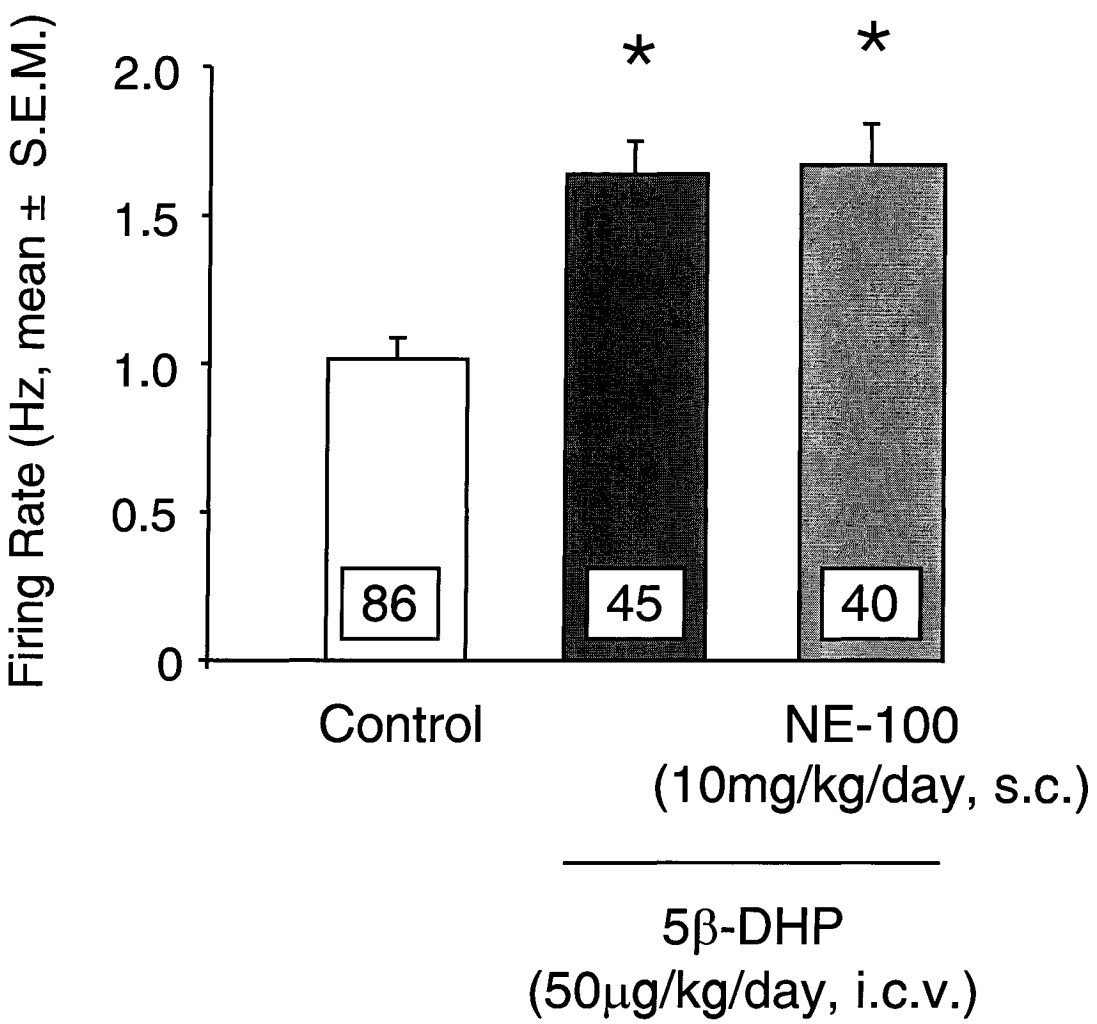

Firing Rate (Hz, mean ± S.E.M.)
2.0
1.5
1.0
0.5
0
*
*
86
45
40
Control
NE-100
(10mg/kg/day, s.c.)
5β-DHP
(50μg/kg/day, i.c.v.)

Table 1

Steroid	Effect on the firing activity of 5-HT neurons
Progesterone	--
Pregnenolone	--
DHEA	↑
5β-DHP	↑
3α,5β-THP	--
3β,5β-THP	--
5α-DHP	--
3α,5α-THP	↑
3β,5β-THP	--

Table1: Summary of the effect of each steroid after a 7-day treatment (50μg/kg/day, i.c.v.) on the firing activity of female DRN 5-HT neurons. Only statistically significant increases are identified by upward arrows.

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Foreword to Chapter 5

Gender differences in the neuroactive steroid modulation of the firing activity of dorsal raphe nucleus serotonergic neurons

The first study of this thesis has shown that female rats have a slower 5-HT neuronal firing activity than males. This observation may have implications in the greater vulnerability of women to develop depression. In the last manuscript, some neuroactive steroids were shown to modulate the firing activity of 5-HT neurons in female rats. As a difference in 5-HT neuronal spontaneous firing rate has been observed between sexes, a possible gender-dependent modulation of this activity by neuroactive steroids could also be possible. Therefore, the goal of this study was to further characterize the steroid modulation of 5-HT neurons. Male rats were thus treated with the steroids, which had been found to be effective in females (5β-DHP, 3α,5α-THP and DHEA). Also, the effects of estrogen and androgens were also assessed and compared between sexes.

Chapter 5

GENDER DIFFERENCES IN THE NEUROACTIVE STEROID MODULATION OF THE FIRING ACTIVITY OF DORSAL RAPHE NUCLEUS SEROTONERGIC NEURONS

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ABSTRACT

Women are twice as likely to suffer from mood disorders than men. Moreover, a growing body of evidence suggests a reciprocal modulation between sex steroids and the serotonin (5-HT) system. A previous study from our laboratory has shown that the progesterone metabolites 5β-pregnane-3,20-dione (5β-DHP) and 5α-pregnan-3α-ol,20-one (3α,5α-THP), as well as dehydroepiandrosterone (DHEA), increase the firing activity of dorsal raphe nucleus (DRN) 5-HT neurons in female rats. The present study was undertaken to assess the effects of these steroids in male rats as well as the effects of testosterone and 17β-estradiol (17β-E) in both sexes, and finally to evaluate gender differences in the modulation of the 5-HT neuronal firing activity by these different neuroactive steroids. Male rats were treated i.c.v., for 7 days, with a dose of 50 μg/kg/day of one of the following steroids: progesterone, 5β-DHP, 3α,5α-THP, DHEA, testosterone, 17β-hydroxy-5α-androstan-3-one (5α-DHT) and 17β-E. Some rats also received a 3-day administration of testosterone (50 μg/kg/day, i.c.v.). Females were treated in the same fashion with testosterone and 17β-E. Extracellular unitary recordings of 5-HT neurons, obtained *in vivo* in the DRN of these rats, revealed that testosterone and 17β-E increased the firing activity of 5-HT neurons in both males and females. In males, the effect of testosterone could already be seen after 3 days of treatment. Neither castration nor any treatment with other steroids significantly modified the firing rate of male 5-HT neurons. The results of the present study, taken together with previous

findings, indicate both similarities and differences between sexes in the modulation of 5-HT neurons by some steroids. This could prove important in understanding gender differences in mood disorders.

INTRODUCTION

Gender differences in mood disorders have long been documented. For instance, it is well established that major depression affects women twice more often than men (Robins *et al.* 1984; Maes *et al.* 1986; Regier *et al.* 1988; Kessler *et al.* 1993; Kessler *et al.* 1994; Breslau *et al.* 1995; Kessler & Walters 1998; Blazer *et al.* 2001; Angst *et al.* 2002; Bijl *et al.* 2002) and that this ratio appears at around 12-14 years of age (Breslau *et al.* 1995; Silberg *et al.* 1999). Since puberty is the time when ovarian hormones start to fluctuate in women, these hormones have been hypothesized to play a role in the differences between sexes in affective disorders (Pajer 1995; Joffe & Cohen 1998).

There is also increasing evidence suggesting a relationship between neuroactive steroids and depression, independent of sex. For instance, CSF and plasma levels of 5β-pregnan-3α-ol,20-one (3α,5β-THP) (Romeo *et al.* 1998; Uzunova *et al.* 1998) and 5α-pregnan-3α-ol,20-one (3α,5α-THP, allopregnanolone) (Romeo *et al.* 1998; Uzunova *et al.* 1998; Ströhle *et al.* 1999; Ströhle *et al.* 2000) are lower in depressed patients than in healthy volunteers, and can be elevated back to normal levels following successful antidepressant treatments (Romeo *et al.* 1998; Uzunova *et al.* 1998; Ströhle *et al.* 1999; Ströhle *et al.* 2000). Interestingly, the increase in CSF levels of 3α,5α-THP was proportional to the mood improvement (Uzunova *et al.* 1998). Injection of fluoxetine or paroxetine to male rats also resulted in a rapid increase in the brain

content of 3α,5α-THP and a concomitant decrease in 5α-DHP, without any change in pregnenolone (PREG), progesterone or dehydroepiandrosterone (DHEA) (Uzunov *et al.* 1996). In a mouse model of depression, long-term social isolation was shown to decrease the cortical levels of 3α,5α-THP and 5α-pregnane-3,20-dione (5α-DHP), but not progesterone or PREG, by reducing the 5α-reductase's mRNA and protein expression (Dong *et al.* 2001) (for partial steroid metabolism, see Fig. 8). Finally, clinical studies have shown antidepressant properties for DHEA (Wolkowitz *et al.* 1995; Wolkowitz *et al.* 1997; Wolkowitz *et al.* 1999; Bloch *et al.* 1999).

The serotonergic (5-HT) system has also long been implicated in the neurobiology of mood disorders since, amongst other evidence, most antidepressant treatments result, through various mechanisms of action, in an enhanced 5-HT neurotransmission (Blier & de Montigny 1994; Owens 1996). Interestingly, numerous studies indicate that ovarian steroids modulate the gene expression and functional activity of different components of the 5-HT system (reviewed in Bethea *et al.* 1999). We have previously shown, using *in vivo* electrophysiology, that this modulation could be observed with the electrical activity of dorsal raphe nucleus (DRN) 5-HT neurons. Indeed, during pregnancy, the firing rate of 5-HT neurons gradually increases, peaks at the 17th day of pregnancy and then declines before parturition, thus directly reflecting plasma levels of progesterone (Klink *et al.* 2002). Furthermore, we have shown that a 7-day administration of 5β-pregnane-3,20-dione (5β-DHP), 3α,5α-THP or DHEA

increases the firing activity of female 5-HT neurons (Robichaud & Debonnel 2004). Together, these data strongly suggest a reciprocal modulation between the 5-HT system and neuroactive steroids.

Furthermore, several studies indicate gender differences in the 5-HT system (Rosecrans 1970; Carlsson *et al.* 1985; Kennett *et al.* 1986; Carlsson & Carlsson 1988; Haleem *et al.* 1990; Maswood *et al.* 1995; Ellenbogen *et al.* 1996; Nishizawa *et al.* 1999; Zhang *et al.* 1999; Li *et al.* 2000; Okazawa *et al.* 2000). Interestingly, we have shown that males have a faster spontaneous 5-HT neuronal firing activity than females (Klink *et al.* 2002). There is also evidence that testosterone can modulate the expression of different 5-HT receptors in various regions of the brain (Sandrini *et al.* 1989; Mendelson & McEwen 1990; Sumner & Fink 1998; Flügge *et al.* 1998) as well as the function of the 5-HT system (Sundblad & Eriksson 1997; Cologer-Clifford *et al.* 1999; Thiblin *et al.* 1999).

The present study was thus undertaken to further characterize gender differences in 5-HT neuronal activity and in its modulation by neuroactive steroids since better understanding of these aspects could be important in elucidating the putative role of neurosteroids in mood disorders, the related sex differences and possibly new therapeutic approaches. Male rats were therefore treated with the steroids which had been shown to be the most potent on the female 5-HT neurons, i.e. 5β-DHP, 3α,5α-THP and DHEA (Robichaud & Debonnel 2004) as well as with progesterone. Moreover, males having higher

levels of testosterone than females, it was hypothesized that this hormone might also play a role in the gender difference observed in the spontaneous firing activity of 5-HT neurons (Klink *et al.* 2002). For this reason, the effects of testosterone on the firing activity of 5-HT neurons were also investigated in both male and female rats. Finally, since the putative effect of testosterone might be mediated through its conversion into estrogen, the effects of this hormone were also assessed in our paradigm.

MATERIALS AND METHODS

Animals

All rats used were Sprague Dawleys (Charles-River, St-Constant, Québec, Canada) weighing between 250g and 325g and kept under standard laboratory conditions (12:12 light-dark cycle with access to food and water *ad lib.*). Freely cycling females, males and castrated males (CX) were used for the experiments. CX were castrated at their 10^{th} week of life and used no sooner than one week after surgery. Ethical committee approval was obtained from the McGill University Animal Ethical Care Committee and all their rules and regulations were followed.

Treatments with steroids

All steroids were dissolved in 3% (v/v) ethanol/distilled water and administered continuously and i.c.v. by mean of a s.c. osmotic minipump connected to a canulae (ALZA, Palo Alto, CA, USA), which was implanted in the left lateral ventricle of the rat brain. The surgery was performed as described by the manufacturer (ALZA, Palo Alto, CA, USA) and under chloral hydrate anesthesia. Each steroid was administered at a dose of 50μg/kg/day.

Females were treated for 7 days with 3% ethanol (for the controls), 17β-estradiol (17β-E) or testosterone. Males received one of the following steroids for 7 days: progesterone, 5β-pregnane-3,20-dione (5β-DHP), 5α-pregnan-3α-ol,20-

one (3α,5α-THP), dehydroepiandrosterone (DHEA), 17β-E, testosterone or 17β-hydroxy-5α-androstan-3-one (5α-DHT). Some males were also treated with testosterone for 3 days. Experiments were carried out the day following the last day of administration and after removal of the canulae. Following experiments, each brain was removed, frozen at –80°C and sliced using a microtome to confirm the position of the canulae and of the recording site.

Electrophysiological Experiments

All rats were anesthetized 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 approximately 37°C throughout the experiment by a thermistor-controlled heating pad.

Extracellular unitary recording of serotonergic neurons were obtained with single-barelled glass micropipettes pulled in a conventional manner, filled with a 1M NaCl solution and of final impedance ranging between 2 and 6 MΩ. A 4 mm-diameter hole was drilled in the skull of each rat at the appropriate location (about 1 mm anterior of lambda and centered with respect to the midline). The unitary activity of DRN 5-HT neurons was recorded by lowering the micropipette along descents covering the nucleus from 300 μm to about 1500 μm anterior of lambda. Spontaneously active DRN 5-HT neurons were identified according to the criteria of Aghajanian: a slow and regular rythmical firing rate and a shape of action

potential with a large initial positive spike of 1-2 msec duration and a postspike hyperpolarization (Aghajanian *et al.* 1978; Aghajanian & Vandermaelen 1982).

For each group of rats, 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 seconds, each 5-HT neuron encountered in complete descents in the DRN of at least 5 rats.

Statistics

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

Drugs

The steroids used were: progesterone, 5β-pregnane-3,20-dione, 5α-pregnan-3α-ol,20-one, 17β-estradiol, testosterone, 17β-hydroxy-5α-androstan-3-one (purchased from Steraloids, New Port, RI, USA) and dehydroepiandrosterone (purchased from Sigma Aldrich, Oakville, Ontario, Canada).

RESULTS

In male rats, seven-day treatments with P, 5β-DHP, 5α3α-THP and DHEA did not induce any statistically significant change in the firing activity of 5-HT neurons as compared to controls (1.56 ± 0.14 Hz, n = 54, $F(1,2) = 1.49$, n.s.; 1.32 ± 0.08 Hz, n = 112, $F(1,2) = 0.01$, n.s.; 1.37 ± 0.10 Hz, n = 71, $F(1,2) = 0.04$, n.s.; 1.51 ± 0.09 Hz, n = 69, $F(1,2) = 1.47$, n.s. and 1.34 ± 0.11 Hz, n =51, respectively, Fig. 1).

The effect of testosterone was also investigated. First, males were castrated (CX) to assess if lowering testosterone levels would lead to a reduction in 5-HT neuronal firing rate. However, this surgery did not induce the expected reduction (1.63 ± 0.16 Hz, n=46 vs 1.37 ± 0.09, n = 62, $F(1,2) = 2.27$, n.s., Fig. 2).

Treatment of males with testosterone led to an increase in their 5-HT neuronal firing rate (Fig. 3). A 3-day administration elevated the firing activity by more than 50% (2.23 ± 0.22 Hz, n = 105 compared to 1.41 ± 0.11 Hz, n = 53, $F(1,2) = 6.39$, $P<0.05$, Fig. 3A). The 7-day treatment also led to a similar statistically significant enhancement as compared to the respective controls (1.96 ± 0.14 Hz, n = 121 vs 1.36 ± 0.11 Hz, n = 57; $F(1,2) = 10.96$, $P<0.001$, Fig. 3B). Testosterone was also administered to females for 7 days and increased the firing activity of their 5-HT neurons by 28% as compared to the controls' (1.53 ± 0.12 Hz, n = 71 vs 1.20 ± 0.10 Hz, n = 45; $F(1,2) = 3.96$, $P<0.05$, Fig. 4).

Interestingly, in males, but not in females, it seems that the T-induced enhancement of the mean firing rate was due to the dramatic response of only a few 5-HT neurons relatively to the whole neuronal population (Fig. 5). Indeed, the distribution of the firing rate of individual neurons shows that a small number of neurons are responsive to testosterone and acquire a very fast firing rate (Fig. 5). It is not excluded that the same phenomenon is also present in females. However, if it is the case, it is less spectacular since no recorded neurons had a firing frequency comparable to that observed in males.

In order to investigate if the effect of testosterone was mediated through its conversion into estrogen (see Fig. 8), females and males received a 7-day treatment with 17β-E, which induced, in both cases, an enhancement of the 5-HT neuronal firing activity (Fig. 6A, B). One-way ANOVAs indicated that this increase was statistically significant for both females (1.68 ± 0.14 Hz, n = 67 vs 0.99 ± 0.09 Hz, n = 68; $F(1,2) = 19.13$, $P<0.001$, Fig. 6A) and males (1.57 ± 0.11 Hz, n = 71 vs 1.22 ± 0.10 Hz, n = 51; $F(1,2) = 5.07$, $P<0.05$, Fig. 6B) as compared to their respective controls.

Males were also treated with 5α-DHT, which is another metabolite of testosterone. Unlike testosterone and 17β-E, 5α-DHT did not significantly modify the firing activity of males' 5-HT neurons (1.58 ± 0.15 Hz, n = 68 as compared to 1.39 ± 0.14 Hz, n = 45 in controls, $F(1,2) = 0.80$, n.s., Fig. 7).

DISCUSSION

The goal of this study was to assess potential gender differences in the modulation of the firing activity of 5-HT neurons by neuroactive steroids. Both similarities and differences were observed between males' and females' 5-HT neurons regarding their response to sustained steroid administrations. Testosterone and 17β-estradiol (17β-E) increased the firing activity of DRN 5-HT neurons of both males and females (Figs. 3, 4 and 6). Moreover, in males, similarly to what was previously found in females (Klink *et al.* 2002; Robichaud & Debonnel 2004), neither progesterone nor gonadectomy significantly modified the firing rate of 5-HT neurons (Figs. 1 and 2). However, unlike what was observed in females (Robichaud & Debonnel 2004), 5β-DHP, 5α,3α-THP and DHEA, did not significantly change the firing activity of 5-HT neurons in males (Fig. 1).

Testosterone

Some of these ovarian steroids, which were ineffective on the 5-HT neuronal firing activity in males, have potent effects on those of female rats (Robichaud & Debonnel 2004). It was, therefore, plausible that male 5-HT neurons might be more responsive to other neuroactive steroids, such as androgens. This is why the effect of testosterone on the firing activity of 5-HT neurons was investigated.

First, males were castrated to see if a decrease of endogenous testosterone would decrease the firing rate of their 5-HT neurons. The rationale behind this expectation was the faster firing rate observed in males as compared to females (Klink *et al.* 2002), which might have been due to the higher levels of testosterone. However, no such reduction in firing activity was observed following castration (Fig. 2). Similarly to what was observed with females (Klink *et al.* 2002), removal of the gonads did not significantly affect the firing rate of 5-HT neurons (Fig. 2). However, this is not completely surprising since the brain is known to produce neuroactive steroids and, thus, the reduction of steroid levels in the brain is less drastic than in the plasma (Aghajanian 1985; Guennoun *et al.* 1995). On the other hand, males were castrated once they were adults. Had they been castrated earlier, an effect might have been observed, as castration at the 3rd week of life has been shown to increase the 5-HT_{1A} mRNA levels in different brain regions, including the DRN (Zhang *et al.* 1999). In turn, this would reduce the firing activity of 5-HT neurons if indeed translated in greater expression of 5-HT_{1A} receptors on the soma.

In both males and females, 7 days of testosterone administration increased the firing activity of 5-HT neurons (Fig. 3 and 4). In males, this increase could already be seen after a 3-day treatment (Fig. 3). What is of particular interest is the fact that in males, apparently only a subpopulation of neurons seemed sensitive to testosterone (Fig. 5). The DRN is formed of heterologous neuronal populations and there is data showing that neurons, which are normally presumed to be 5-HT based on their firing characteristics may actually not be (Kirby *et al.*

2003). It is thus possible that the hyper-responsive neurons recorded in the current experiments be of another nature than 5-HT. However, other studies have shown subpopulations of DRN 5-HT neurons responding differently to a given pharmacological administration (Martin-Ruiz & Ugedo 2001; Lucas & Debonnel 2002) or to an electrical stimulation of the medial prefrontal cortex (mPFC) (Celada *et al.* 2001). In all these cases, it was suggested that postsynaptic regions were implicated, as part of a longer feedback loop (Martin-Ruiz & Ugedo 2001; Celada *et al.* 2001; Lucas & Debonnel 2002). The location of the receptor mediating the effect might contribute to this phenomenon (Martin-Ruiz & Ugedo 2001; Lucas & Debonnel 2002). Furthermore, direct synaptic contact on 5-HT cells by neurons projecting back to the DRN or, on the other hand, involvement of interneurons, might also give rise to differential responses (Celada *et al.* 2001). In the present study, not only is it unclear whether the observed effect is mediated by androgen receptors or by a nongenomic mechanism, the location of the effector receptor is also unknown since steroids were administered in the cerebrospinal fluid. It is thus possible that the T-induced increase in firing activity could be mediated via afferents to 5-HT neurons. Moreover, the neurons, which were responsive to T, presented a very high firing frequency (Fig. 5). Although this was surprising, it has recently been reported that some DRN neurons, which were immunolabelled as 5-HT neurons, had a firing frequency of up to almost 8 Hz in basal conditions (Allers & Sharp 2003). In females, the increase in 5-HT neuronal firing activity following the administration of testosterone was less dramatic but more generalized (Fig. 5). Therefore, even though the mean firing

rate was increased in both genders, a sex difference was nevertheless observed in the response of DRN neurons to testosterone.

In the brain, testosterone is mainly metabolized into 17β-E and 5α-DHT (Stoffel-Wagner 2001). In order to investigate if the T-induced enhancement of the firing activity could also be mediated by either one of these two metabolites, males were treated with 17β-E and 5α-DHT (Figs. 7B and 8). 17β-E but not 5α-DHT increased the firing activity of 5-HT neurons, thus suggesting a role for aromatization of testosterone into 17β-E rather than an androgenic effect. The lack of effect of 5α-DHT is in keeping with the report of McQueen *et al.* (1999), who investigated the effects of castration and hormonal (T, estrogen and 5α-DHT) replacements on the expression of the serotonin transporter (SERT) in different brain regions of male rats. They showed, for instance, an increase in SERT mRNA levels following testosterone or estrogen but not 5α-DHT administrations. If this increase in SERT mRNA expression leads to a greater SERT protein density, it would result in less 5-HT being present in the vicinity of the soma of 5-HT neurons. This would reduce the activation of the 5-HT_{1A} autoreceptor and lead to an increase in the neuronal firing activity. Such an effect might therefore represent one of the mechanisms of action through which 17β-E and testosterone increase the firing activity of 5-HT neurons. These results also support the hypothesis that the effect of testosterone might be, at least in part, mediated by its aromatization into estrogen. However, since both estrogen and

androgen receptors are expressed by DRN neurons (Simerly *et al.* 1990), a direct effect of testosterone is also possible.

Estrogen

The effect of estrogen on the 5-HT system has been extensively studied. For instance, E has been shown to alter the gene expression and binding sites for SERT in the DRN and other brain regions of ovariectomized (OVX) monkeys and rats (Mendelson *et al.* 1993; McQueen *et al.* 1997; Pecins-Thompson *et al.* 1998; McQueen *et al.* 1999; Sumner *et al.* 2000; Zhou *et al.* 2002; Bethea *et al.* 2002). Of particular interest is the effect of E on the 5-HT_{1A} autoreceptor, which is responsible for regulating the firing activity of 5-HT neurons. Bethea and colleagues have shown that E decreases 5-HT_{1A} receptor mRNA (Pecins-Thompson & Bethea 1999), 5-HT_{1A} binding sites as well as the G protein activation in the DRN of OVX macaques (Lu & Bethea 2002), indicating a functional downregulation of the autoreceptor. In rats, however, data are not as consistent. For instance, a 2-week treatment with E led to a trend towards a decrease in 5-HT_{1A} receptor mRNA levels in the midbrain (Zhou *et al.* 2002) but had no effect on 5-HT_{1A} mRNA levels or binding sites in the DRN of OVX rats (Landry & Di Paolo 2003). A 3-week treatment with high levels of E and P, however, caused a significant reduction in DRN 5-HT_{1A} autoreceptor mRNA levels (Birzniece *et al.* 2001). Most interestingly, however, 2 days of E administration led to a reduced 5-HT_{1A} autoreceptor response in a paradigm identical to ours (Lakoski 1989), which could easily explain the higher firing activity of 5-HT neurons.

Other systems and receptors might also be implicated. For instance, E was found to reduce $GABA_A$ receptor binding sites in various brain regions of OVX females (O'Connor *et al.* 1988) and to have a region-dependent effect on glutamate receptors in females (Cyr *et al.* 2000). It is thus possible that 17β-estradiol decreases the GABAergic tonic inhibition and increases the glutamatergic input on 5-HT neurons, thus enhancing the firing activity of 5-HT neurons. Indeed such a reduced GABAergic tonic inhibition leading to faster 5-HT neuronal firing has already been shown during pregnancy (Robichaud *et al.* 2002).

Ovarian Steroids

Since 5α3α-THP is a potent $GABA_A$ receptor modulator, we have previously proposed that 5α3α-THP modulated the firing activity of 5-HT neurons via $GABA_A$ receptors (Robichaud & Debonnel 2004). It is thus surprising that 5α3α-THP had no effect on the firing activity of male 5-HT neurons. Gender differences in the functional responses of $GABA_A$ receptors have, however, been reported (Jüptner & Hiemke 1990; Wilson & Biscardi 1992; Wilson 1992; Bujas *et al.* 1997) and might explain this unexpected result. For instance, we have shown earlier that male 5-HT neurons were more sensitive than female ones to the $GABA_A$ antagonist bicuculline as seen by their greater excitatory response following its administration (Robichaud *et al.* 2002). Interestingly, it has also been shown that, even though the basal levels of GABA-

activated chloride influx were not different between genders in the amygdala and the hypothalamus, the potentiation by high doses of THDOC (a steroid with similar $GABA_A$ positive modulatory properties to 5α3α-THP) was greater in males than females (Wilson & Biscardi 1997). This indicates gender- and region-specific differences in the response of $GABA_A$ receptors to neuroactive steroids, which might explain the present observations. The subunit composition of $GABA_A$ receptor varies between brain regions and influences greatly the functional response of the receptor (see review 543). It is thus possible that a difference in $GABA_A$ receptor subunit assembly exists between sexes and leads to a greater sensitivity of females' 5-HT neurons to 5α3α-THP modulation as compared to that of males.

In conclusion, the main results of this study are the increase in 5-HT neuronal firing activity induced by testosterone and 17β-estradiol in both male and female rats. Taken together with previous findings, these data indicate not only similarities but also sex differences in the modulation of 5-HT neurons by some steroids, which could prove important in the understanding of gender differences in mood disorders.

ACKNOWLEDGEMENTS

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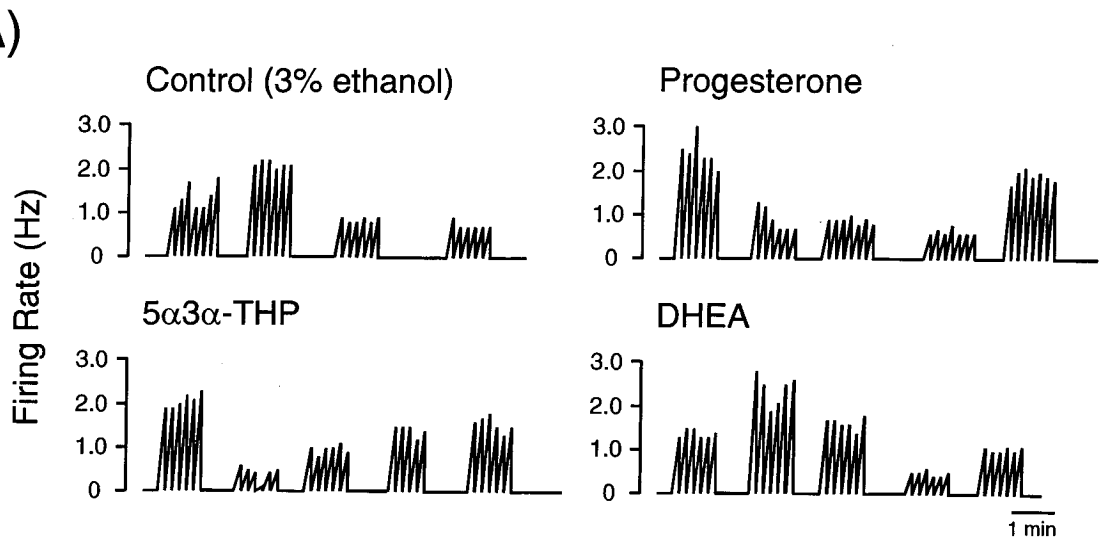
FIGURE LEGENDS

Figure 1: (A) Integrated firing rate histograms of 5-HT neurons, showing their spontaneous firing activity, recorded in one electrode descent in the DRN of a male control (3% ethanol, i.c.v., 7 days) and following a treatment with P, 5α,3α-THP and DHEA (50μg/kg/day, i.c.v., 7 days, each). (B) Spontaneous firing rate of male DRN 5-HT neurons expressed in Hz (mean ± SEM) in controls and following 7-day treatments with P, 5β-DHP, 5α,3α-THP and DHEA. In this and the following figures, the number of neurons recorded is indicated in each box.

Figure 1


A)
Control (3% ethanol)
Progesterone
5α3α-THP
DHEA
Firing Rate (Hz)
3.0
2.0
1.0
0
1 min

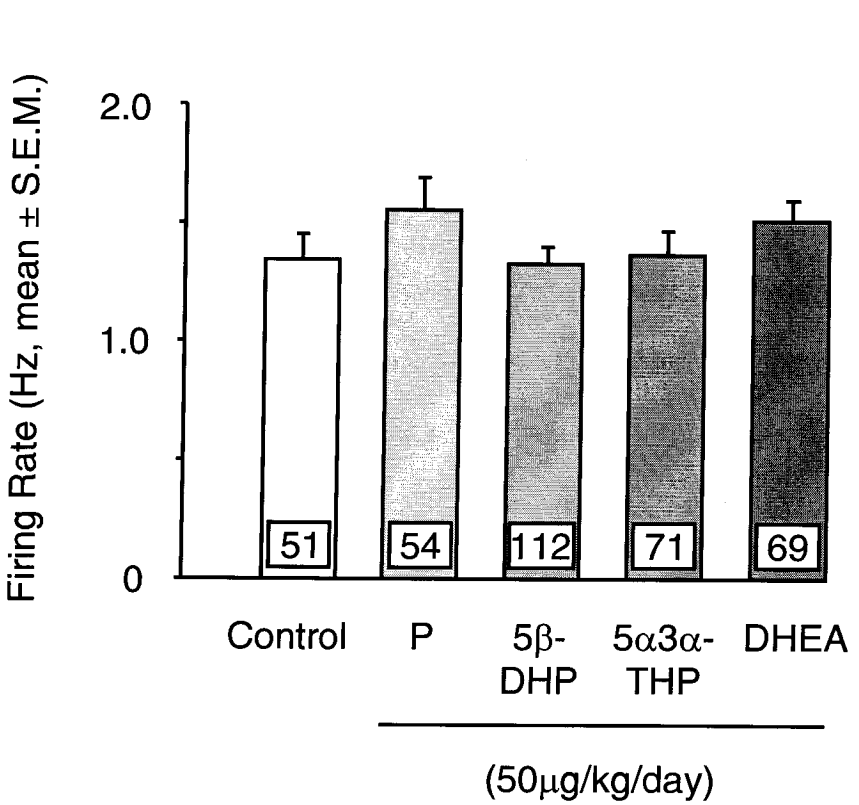

B)
Firing Rate (Hz, mean ± S.E.M.)
2.0
1.0
0
51
54
112
71
69
Control
P
5β-DHP
5α3α-THP
DHEA
(50μg/kg/day)

Figure 2: Spontaneous firing rate of DRN 5-HT neurons expressed in Hz (mean ± SEM) in controls and castrated males (CX).

Figure 2

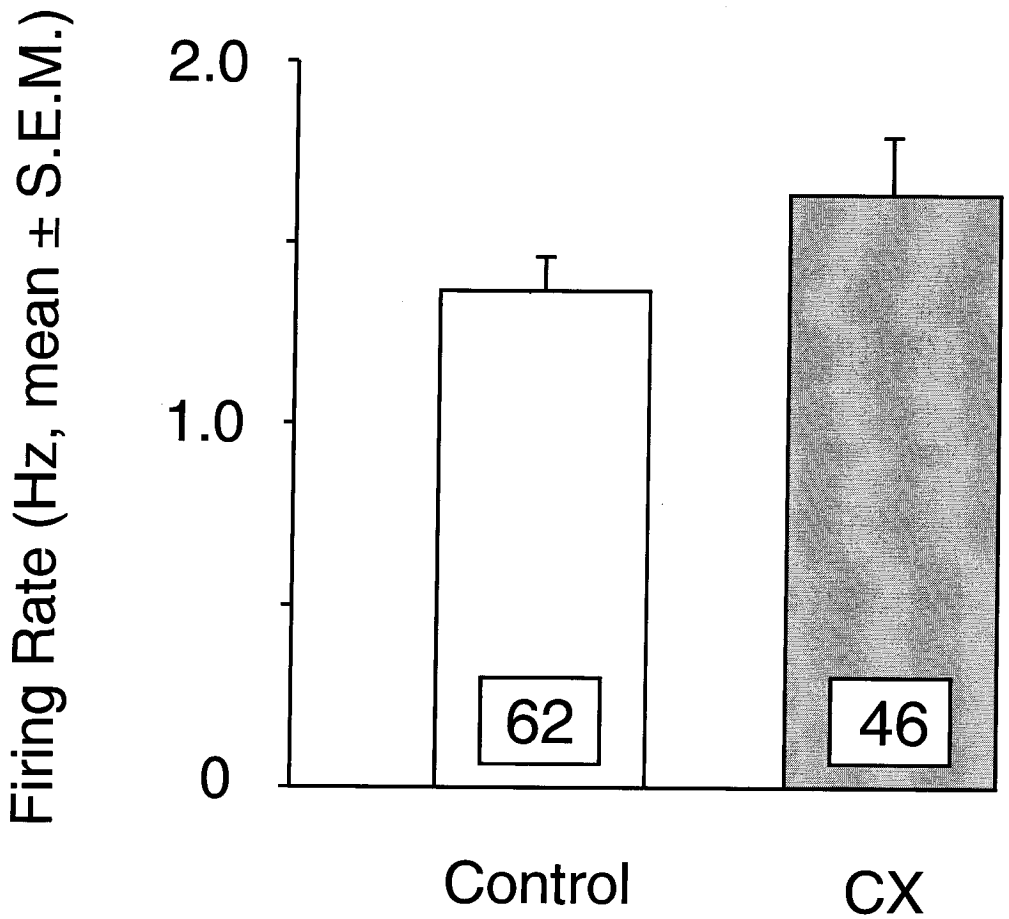


Figure 3: Spontaneous firing rate of male DRN 5-HT neurons expressed in Hz (mean ± SEM) following A) a 3-day treatment with testosterone (50 μg/kg/day, i.c.v.) and B) a 7-day treatment with testosterone (50 μg/kg/day, i.c.v.), and their respective controls (3% ethanol, i.c.v., for 3 or 7 days). The star indicates $P < 0.05$.

Figure 3

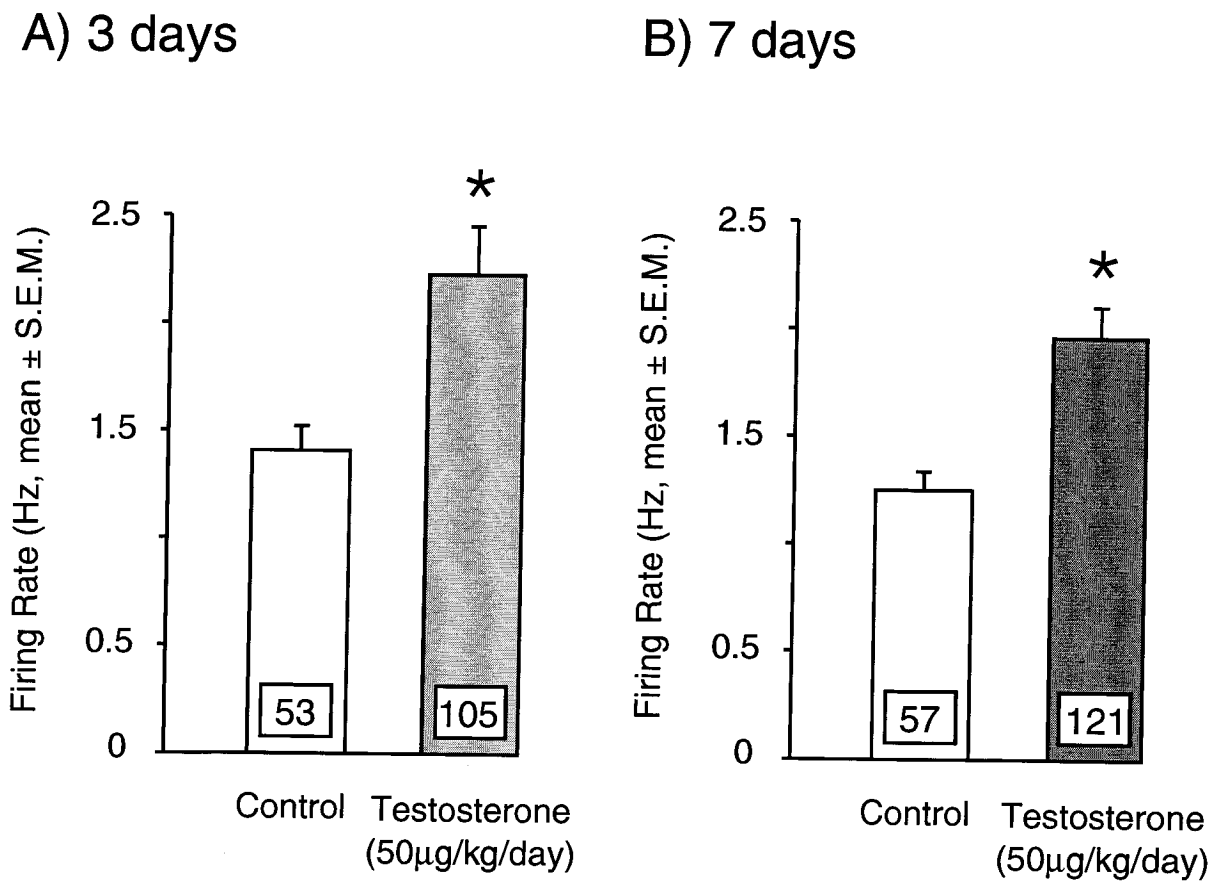

A) 3 days
B) 7 days
Firing Rate (Hz, mean ± S.E.M.)
2.5
1.5
0.5
0
*
53
105
57
121
Control
Testosterone
(50µg/kg/day)

Figure 4: Spontaneous firing rate of female DRN 5-HT neurons expressed in Hz (mean ± SEM) in controls (3% ethanol, i.c.v., 7days) and following a treatment with testosterone (50 μg/kg/day, i.c.v., 7 days). The star indicates $P < 0.05$.

Figure 4

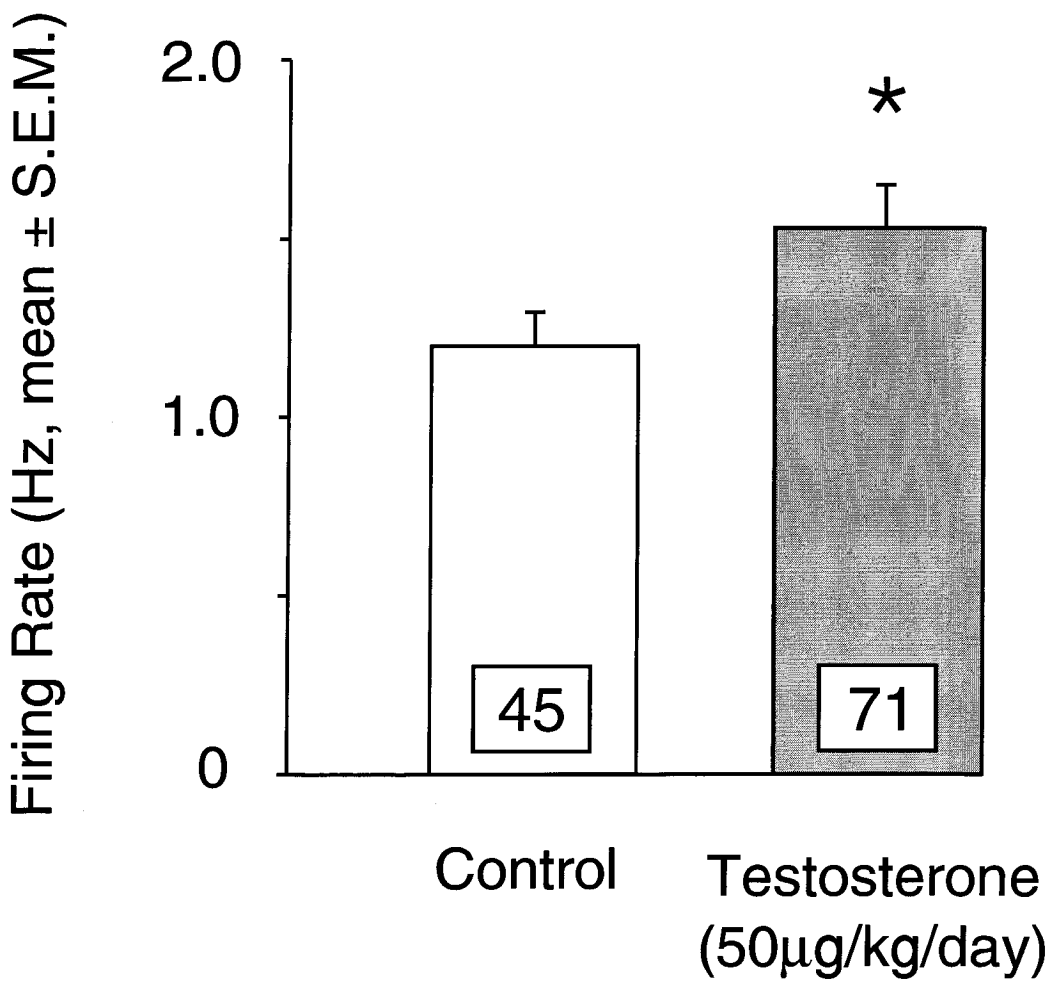

Firing Rate (Hz, mean ± S.E.M.)
2.0
1.0
0
*
45
71
Control
Testosterone
(50µg/kg/day)

Figure 5: Distribution of the spontaneous firing rate of DRN 5-HT neurons expressed in Hz, in males treated with testosterone for 3 or 7 days (50 μg/kg/day, i.c.v.), their respective controls (3% ethanol, i.c.v., for 3 or 7 days) and in female controls (3% ethanol, i.c.v., 7days) and following a 7-day treatment with testosterone (50 μg/kg/day, i.c.v.).

Figure 5

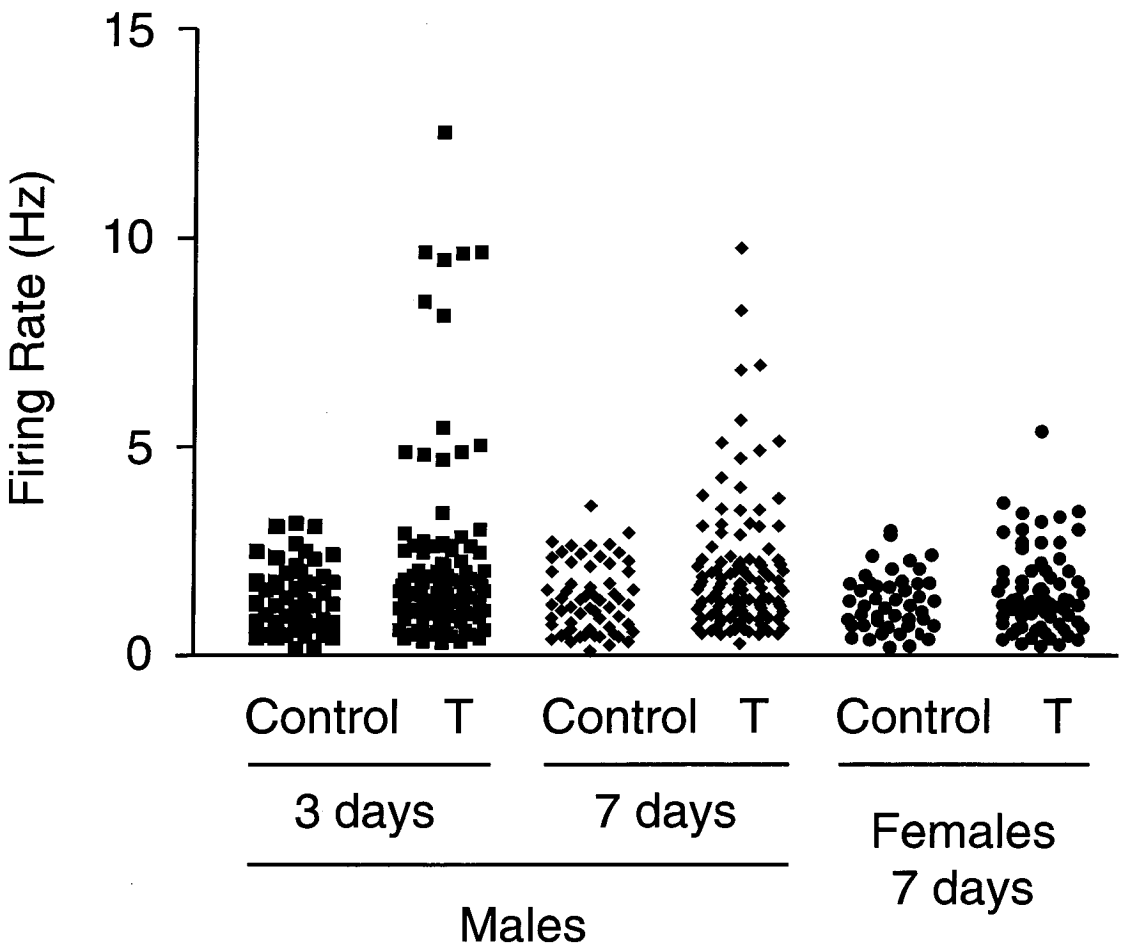
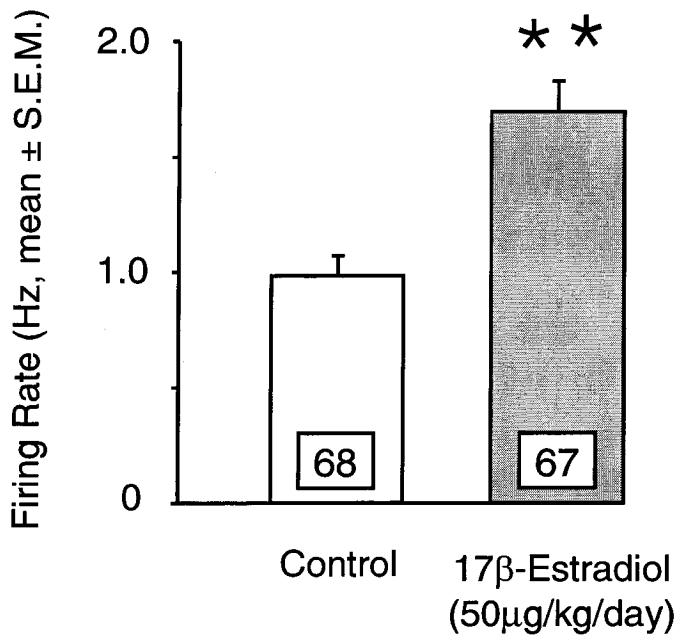

15
10
5
0
Firing Rate (Hz)
Control
T
Control
T
Control
T
3 days
7 days
Females
7 days
Males

Figure 6: Spontaneous firing rate of DRN 5-HT neurons expressed in Hz (mean ± SEM) in A) females and B) males following a treatment with 17β-estradiol (50 μg/kg/day, i.c.v., 7 days) and their respective controls (3% ethanol, i.c.v., 7days). The single star indicates $P < 0.05$ and the double stars, $P < 0.001$.

Figure 6

A) Females



B) Males

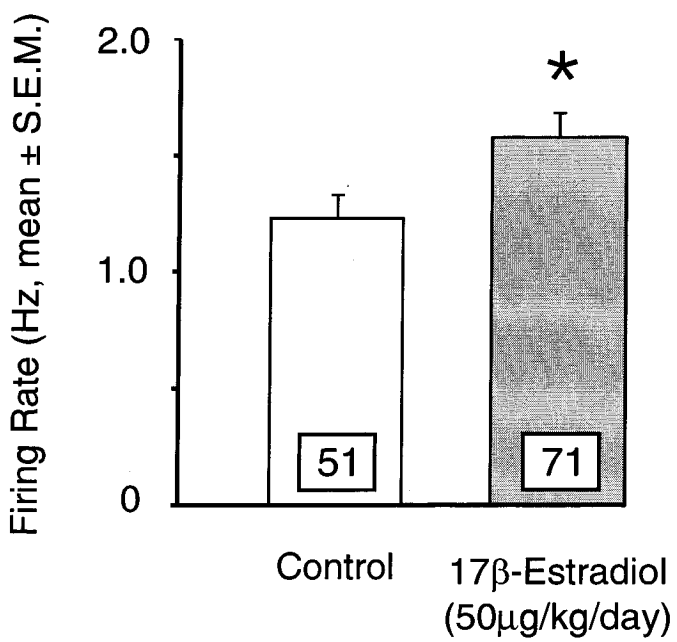


Figure 7: Spontaneous firing rate of male DRN 5-HT neurons expressed in Hz (mean ± SEM) in controls (3% ethanol, i.c.v., 7days) and following a treatment with 5α-DHT (50 μg/kg/day, i.c.v., 7 days).

Figure 7

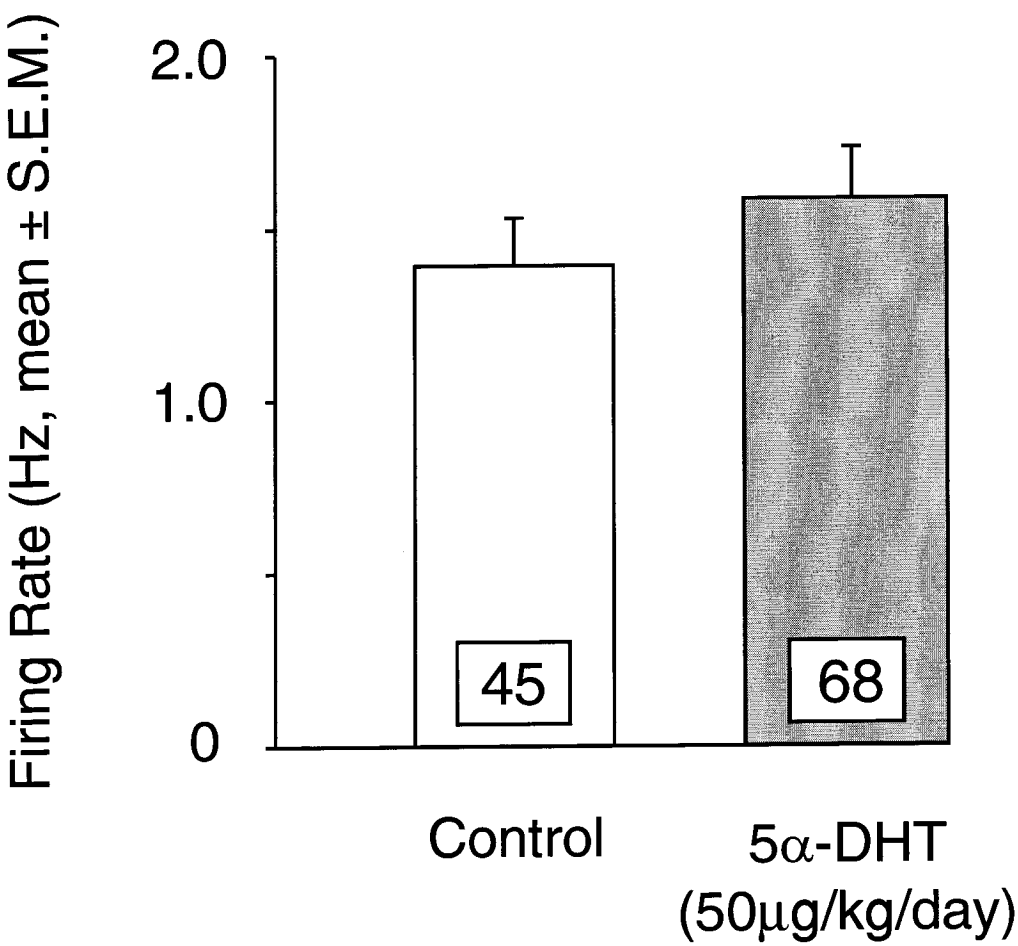
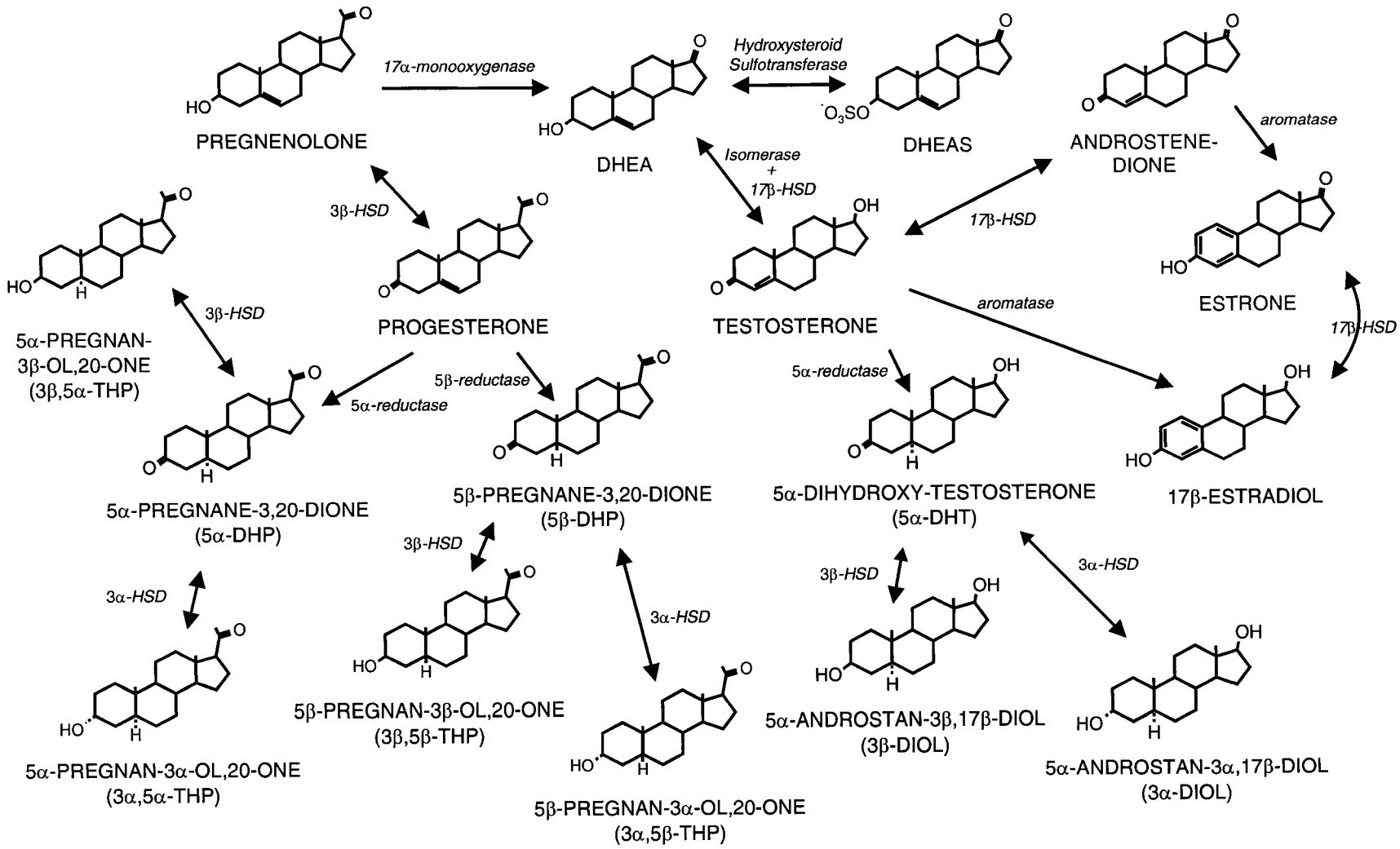


Figure 8: Partial schematic representation of the sex steroids' metabolic pathway.

Figure 8


PREGNENOLONE
17α-monooxygenase
DHEA
Hydroxysteroid Sulfotransferase
DHEAS
ANDROSTENE-DIONE
aromatase
ESTRONE
17β-HSD
3β-HSD
PROGESTERONE
Isomerase + 17β-HSD
TESTOSTERONE
17β-HSD
5α-PREGNAN-3β-OL,20-ONE (3β,5α-THP)
3β-HSD
5α-reductase
5β-reductase
5α-PREGNANE-3,20-DIONE (5α-DHP)
5β-PREGNANE-3,20-DIONE (5β-DHP)
aromatase
5α-reductase
5α-DIHYDROXY-TESTOSTERONE (5α-DHT)
17β-ESTRADIOL
3α-HSD
3β-HSD
3α-HSD
3β-HSD
3α-HSD
5α-PREGNAN-3α-OL,20-ONE (3α,5α-THP)
5β-PREGNAN-3β-OL,20-ONE (3β,5β-THP)
5β-PREGNAN-3α-OL,20-ONE (3α,5β-THP)
5α-ANDROSTAN-3β,17β-DIOL (3β-DIOL)
5α-ANDROSTAN-3α,17β-DIOL (3α-DIOL)

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Foreword to Chapter 6

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

Amongst the neuroactive steroids tested in female rats, the most potent one in modulating the firing activity of 5-HT neurons was found to be 3α-hydroxy-5α-pregnane-20-one (3α,5α-THP, allopregnanolone). Interestingly, there is accumulating evidence suggesting antidepressant properties for this steroid (see introduction). The 3α,5α-THP-induced enhancement of the 5-HT neuronal activity could offer a biological basis for its antidepressant effects. This study was undertaken to assess the potential of this steroid in antidepressant treatments. First, the time frame of this modulation was better characterized. Second, the effects of ganaxolone, a synthetic analog of 3α,5α-THP devoid of hormonal properties, were similarly assessed. Since many women are reluctant to hormonal therapy, ganaxolone might be an interesting substitute. Finally, the potential for these two steroids to reduce the antidepressants' delay in therapeutic onset of action was investigated.

Chapter 6

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-ol,20-one (3α,5α-THP, allopregnanolone), and the serotonergic (5-HT) system. Furthermore, both 5-HT and neurosteroids seem to play an important role in the pathophysiology of major depression. We have previously shown that a 7-day 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 analog of 3α,5α-THP. Female rats received 3α,5α-THP or ganaxolone for 3 and 7 days (50 μg/kg/day, i.c.v.). Others received 3α,5α-THP concomitantly with the antiprogestin RU486 (50 μg/kg/day, i.c.v., 7 days, each), which was also administered alone. Acute experiments were done by means of a single injection of 3α,5α-THP (1 μg/kg, i.c.v). Finally, both 3α,5α-THP and ganaxolone were administered along with the selective serotonin reuptake inhibitor (SSRI) citalopram. *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 days of treatment. A 7-day treatment with RU486 had the same effect. Furthermore, an increase could be seen as soon as after 30-60 minutes 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-day 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.

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; Kessler *et al.* 1994; Kessler & 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 (Hamilton 1993; Parry 1989), menstrual cycles (Endicott 1993; Endicott & Halbreich 1988; Hamilton 1993; Parry 1989; Weissman & Klerman 1985; Yonkers & White 1992), the postpartum period (Hamilton 1993; Kornstein 1997; Parry 1989; Weissman & Klerman 1985) and menopause (Burt & Stein 2002; Kornstein 1997; Weissman & Klerman 1985). Ovarian hormones have thus been hypothesized to play an important role in women mood disorders (Endicott 1993; Eriksson 1999; Joffe & Cohen 1998; Pajer 1995; Parry 1989). Serotonin (5-HT) has also long been implicated in the pathophysiology of depression (Coppen 1967; Lapin & Oxenkrug 1969). The most compelling evidence probably is the enhancement of 5-HT neurotransmission seen following all antidepressant treatments (Blier & de Montigny 1999; Racagni & Brunello 1999).

Interestingly, ovarian steroids have clearly been shown to 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 seem altered during depressive episodes and regularized by antidepressant treatments (Romeo *et al.* 1998; Ströhle *et al.* 1999;

Ströhle *et al.* 2000; Uzunova *et al.* 1998). This could suggest, in addition to their respective implication in depression, a functional interrelationship between ovarian steroids and the 5-HT system.

More specifically, accumulating evidence suggest a link between lowered levels of 5α-pregnane-3α-ol,20-one (3α,5α-THP, allopregnanolone) and depressive states. Indeed, depressed patients have lower CSF and plasma levels of 3α,5α-THP (Romeo *et al.* 1998; Ströhle *et al.* 1999; Uzunova *et al.* 1998) than healthy subjects (Ströhle *et al.* 1999; Uzunova *et al.* 1998). Furthermore, in addition to being regularized by successful antidepressant treatments (Romeo *et al.* 1998; Ströhle *et al.* 1999; Uzunova *et al.* 1998), the mood improvement was correlated with the increase of 3α,5α-THP levels in the CSF (Uzunova *et al.* 1998).

Studies in rodents also support these observations. In a model of depression, long-term social isolation decreased cerebral levels of 3α,5α-THP in mice (Dong *et al.* 2001) and rats (Serra *et al.* 2000). In rats, bilateral olfactory bulbectomy (OBX), a well recognized model of depression, also results in an important reduction of 3α,5α-THP levels in specific cerebral regions, which are relevant to depression such as the amygdala, the frontal cortex and the hippocampus (Uzunova *et al.* 2003). Chronic treatments (3 weeks) with various antidepressants (desipramine, fluoxetine, sertraline and venlafaxine) completely reversed this reduction in the cerebral cortex of OBX rats (Uzunova *et al.* 2004).

Furthermore, fluoxetine and venlafaxine also increased 3α,5α-THP levels in the cerebral cortex of sham-operated rats (Uzunova *et al.* 2004). This had previously been shown with the selective serotonin reuptake inhibitors (SSRIs) fluoxetine or paroxetine, which rapidly increased the rat cerebral content of 3α,5α-THP (Uzunov *et al.* 1996). 3α,5α-THP was also reported to have antidepressant-like effects in the Porsolt forced swimming test (Khisti *et al.* 2000; Khisti & Chopde 2000). Conversely, inhibition of its formation, using finasteride (a selective inhibitor of 5α-reductase (Azzolina *et al.* 1997), which is the enzyme metabolizing progesterone into 5α-pregnane-3,20-dione), leads to depressive-like symptoms in the same paradigm as well as to anxious behavior in the open field task (Frye & Walf 2002).

We have previously shown, using *in vivo* electrophysiological extracellular recordings, that a 7-day treatment with 3α,5α-THP drastically increased the spontaneous firing activity of 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 analog of 3α,5α-THP (Monaghan *et al.* 1997), were also assessed. Finally, potential therapeutic avenues were also investigated for these two steroids.

METHODS

Animals

Freely cycling female Sprague Dawley rats (Charles-River, St-Constant, Québec, Canada), weighing between 250g and 325g 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 and all their rules and regulations were followed.

Treatments with steroids

All steroids were dissolved in 3% (v/v) ethanol/distilled water and administered intracerebroventricularly (i.c.v.) by mean of a canulae (ALZA, Palo Alto, CA, USA), which was implanted in the left lateral ventricle of the rat brain. For acute administrations, the canulae was attached to a 5 µl Hamilton syringe, while for chronic treatments it was connected to a subcutaneous osmotic minipump (ALZA, Palo Alto, CA, USA), which continuously delivered the steroids. Surgeries were performed as described by the manufacturer (ALZA, Palo Alto, CA, USA) and under chloral hydrate anesthesia. Steroid doses used during acute and chronic administrations were 1 µg/kg and 50µg/kg/day, respectively.

Females received an acute injection, a 3- or a 7-day treatment with 5α-pregnane-3α-ol,20-one (3α,5α-THP, allopregnanolone). This steroid was also administered concomitantly with the progesterone receptor antagonist RU486 for 7 days. Some females received only RU486 for 7 days. Ganaxolone was administered for 3 or 7 days. Controls were treated with the vehicle (3% ethanol) for the appropriate period of time. Finally, 3α,5α-THP, ganaxolone and the vehicle were co-administered with citalopram (a selective serotonin reuptake inhibitor or SSRI, 10mg/kg/day) for 3 days. Citalopram was dissolved in distilled water and administered by mean of a subcutaneous osmotic minipump (ALZA, Palo Alto, CA, USA).

Electrophysiological Experiments

All rats were anesthetized 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 approximately 37°C throughout the experiment by a thermistor-controlled heating pad.

Extracellular unitary recording of serotonergic neurons were obtained with single-barelled glass micropipettes pulled in a conventional manner, filled with a 1M 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 about 1 mm anterior of lambda and centered with respect to the midline. The unitary activity of DRN 5-HT

neurons was recorded by lowering the micropipette along descents covering the nucleus from 300 μm to about 1500 μm anterior of lambda. Spontaneously active DRN 5-HT neurons were identified according to the criteria of Aghajanian: a slow and regular rythmical firing rate and a shape of action potential with a large initial positive spike of 1-2 msec duration and a postspike hyperpolarization (Aghajanian *et al.* 1978; Aghajanian & 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 seconds, each 5-HT neuron encountered in complete descents in the DRN of at least 5 rats. Regarding acute experiments, however, at least 10 rats were used for each experimental group.

Statistics

Statistical analyses were performed with the software SigmaStat for Windows Version 2.0 (Jandel Corporation). 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 versus control were used for evaluating statistical significance. Results (F) of statistical analysis are expressed in terms of degrees of freedom between groups and number of groups compared. Significance was considered for $P<0.05$.

Drugs

Steroids used were: 5α-pregnane-3α-ol,20-one, RU486 (purchased from Steraloids) and 3α-hydroxy-3β-methyl-5α-pregnan-20-one (Ganaxolone, a generous gift from Dr. Purdy, Department of Psychiatry, University of California, San Diego, CA, USA). Citalopram was kindly provided by Lundbeck (Copenhagen, Denmark).

RESULTS

As previously shown (Robichaud & Debonnel 2004), a 7-day treatment with 3α,5α-THP increased the firing activity of 5-HT neurons in female rats (1.88 ± 0.19 Hz, n = 77 compared to 1.26 ± 0.09 Hz, n = 63, [$F(2,3) = 4.40$, $P<0.05$, Tukey's test, $q = 3.4$, $P<0.05$], Fig. 1B). In order to rule out the implication of progesterone receptor (PR) in mediating this effect, some rats were concomitantly treated with the PR antagonist RU486. As expected, an elevated firing activity was still present after this combined treatment as compared to controls (2.04 ± 0.26 Hz, n = 56 compared to 1.26 ± 0.09 Hz, n = 63, [$F(2,3) = 4.40$, $P<0.05$, Tukey's test, $q = 3.9$, $P<0.05$], Fig. 1B). However, RU486 had an unexpected effect on its own and enhanced the firing activity of 5-HT neurons (1.75 ± 0.18 Hz, n = 79 compared to 1.16 ± 0.09 Hz, n = 64, [$F(1,2) = 7.85$, $P<0.01$, Tukey's test, $q = 4.0$, $P<0.05$], Fig. 2).

This 3α,5α-THP-induced elevation in 5-HT neuronal firing rate could also be observed following only 3 days of administration (1.92 ± 0.23 Hz, n = 51 vs 0.98 ± 0.07 Hz, n = 58, [$F(1,2) = 16.59$, $P<0.001$, Tukey's test, $q = 5.8$, $P<0.05$], Fig. 3). Furthermore, acute experiments showed an increase in firing activity as soon as 30 to 60 minutes following a single injection of 3α,5α-THP (1 μg/k, i.c.v., Fig. 4). Before, and up to 30 minutes following the injection, no difference in 5-HT neuronal firing rate was observed between treated rats and controls (1.35 ± 0.13 Hz, n = 42 vs 1.23 ± 0.13 Hz, n = 25, [$F(1,2) = 0.38$, n.s.] and 1.49 ± 0.12 Hz, n = 64 vs 1.20 ± 0.11 Hz, n = 50, [$F(1,2) = 3.21$, n.s.], respectively, Fig. 4).

However, in the following timeframes (i.e. 30 to 59 and 60 to 90 minutes post-injection), this difference became statistically significant (1.80 ± 0.13 Hz, n = 79 vs 1.24 ± 0.10 Hz, n = 66, [$F(1,2) = 11.49$, $P<0.001$, Tukey's test, $q = 4.8$, $P<0.05$] and 1.75 ± 0.11 Hz, n = 77 vs 1.28 ± 0.11 Hz, n = 54, [$F(1,2) = 8.26$, $P<0.005$, Tukey's test, $q = 4.1$, $P<0.05$], respectively, Fig. 4).

It is well established that short-term administration of SSRIs decreases the firing activity of 5-HT neurons in male rats (Chaput *et al.* 1986; Romero *et al.* 1996). This was also true in the current experiments with females, as a 3-day administration of citalopram reduced the firing activity of 5-HT neurons (0.58 ± 0.06 Hz, n = 54 compared to 1.17 ± 0.10 Hz, n = 51, [$F(2,3) = 13.25$, $P<0.001$, Tukey's test, $q = 6.3$, $P<0.05$], Fig. 5). It was thus 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 really differ from that of controls (1.16 ± 0.12 Hz, n = 50 compared to 1.17 ± 0.10 Hz, n = 51, [$F(2,3) = 13.25$, $P<0.001$, Tukey's test, $q = 0.13$, n.s.], Fig. 5).

The effect of ganaxolone was then investigated. Both a 3- and a 7-day administration of ganaxolone increased the firing activity of 5-HT neurons as compared to the appropriate controls; 1.55 ± 0.11 Hz, n = 72 vs 1.15 ± 0.09 Hz, n = 52, [$F(2,3) = 6.99$, $P<0.01$, Tukey's test, $q = 3.7$, $P<0.05$] and 1.52 ± 0.11 Hz, n = 56 vs 1.12 ± 0.09 Hz, n = 43, [$F(1,2) = 7.54$, $P<0.01$, Tukey's test, $q = 3.9$, $P<0.05$], respectively (Fig. 6). Ganaxolone was also able to partially prevent the

citalopram-induced decrease in firing activity of 5-HT neurons; the firing rate was significantly lower in citalopram-treated rats as compared to controls (0.51 ± 0.06 Hz, n = 48 vs 1.08 ± 0.09 Hz, n = 51, [$F(2,3) = 8.86$, $P<0.001$, Tukey's test, $q = 5.7$, $P<0.05$]) but this difference no longer reached statistical significance when ganaxolone was co-administered (0.94 ± 0.12 Hz, n = 57 vs 1.08 ± 0.09 Hz, n = 51 [$F(2,3) = 8.86$, $P<0.001$, Tukey's test, $q = 1.5$, n.s.], Fig. 7).

DISCUSSION

The first finding of this study is the enhanced firing activity of 5-HT neurons following a treatment with either 3α,5α-THP or ganaxolone. A greater firing activity of 5-HT neurons, which was previously shown in female rats after 7 days of 3α,5α-THP administration, was confirmed by the present experiments. Furthermore, this increase was already present after 3 days of treatment and as soon as 30-60 minutes following an acute administration. This suggests a very rapid onset of action of 3α,5α-THP, which appears to be sustained during at least 7 days of treatment. Similarly, both a 3- and a 7-day administration of the synthetic analog ganaxolone also enhanced the firing activity of 5-HT neurons.

The mechanism(s) by which 3α,5α-THP and ganaxolone increase the 5-HT neuronal firing activity remains unclear. Nevertheless, the fact that 3α,5α-THP induced a very rapid (within a few minutes) enhancement of the firing activity of 5-HT neurons suggests that a genomic mechanism of action, such as mediated via PR, is less likely. We have previously shown that during pregnancy the spontaneous firing rate of 5-HT neurons increases in parallel with plasmatic P levels, to finally reach an enhancement greater than a 100% in late pregnancy as compared to control females (Klink *et al.* 2002). In rats, the principal metabolic pathway for cerebral P seems to be its sequential reduction into 5α-DHP and 3α,5α-THP (Karavolas & Hodges 1991; Korneyev *et al.* 1993). Furthermore, 3α,5α-THP has often been shown to be responsible for various effects observed with P on neuronal activity (Costa *et al.* 1995; Gulinello *et al.* 2001; Smith *et al.*

1998a; Smith *et al.* 1998b). It is therefore possible that 3α,5α-THP mediates the modulation of 5-HT neuronal activity observed during pregnancy (Klink *et al.* 2002). This is also supported by the lack of effect of P itself on this firing activity (Robichaud & Debonnel 2004). During pregnancy, we showed that 5-HT_{1A} autoreceptors were partially desensitized (Robichaud *et al.* 2002). If this desensitization was brought about by 3α,5α-THP, which levels rise dramatically during pregnancy, intracerebral administration of this steroid would also be expected to reduce the function of 5-HT_{1A} autoreceptors. This could explain, at least in part, the enhanced 5-HT neuronal firing activity reported by the present study.

The modulation of 5-HT neuronal activity by these neuroactive steroids could also result from their interaction with $GABA_A$ receptors since both of them are potent positive allosteric modulators (Carter *et al.* 1997). 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 & Aghajanian 1987). Interestingly, the GABAergic tonic inhibition of 5-HT neurons was dramatically reduced during pregnancy as compared to virgin 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, might have a comparable effect. In addition, it could explain the increase in firing activity of 5-HT neurons induced

by ganaxolone. Accumulating evidence suggests that high levels of neuroactive steroids reduce $GABA_A$ receptor responsiveness (Concas *et al.* 1998; Friedman *et al.* 1993; Gulinello *et al.* 2001; Yu *et al.* 1996; Yu & Ticku 1995a; Yu & Ticku 1995b)(Yu & Ticku 1995a). This could thus be a mechanism of action whereby 3α,5α-THP and ganaxolone could reduce $GABA_A$ receptor function and the tonic GABAergic inhibition on 5-HT neurons, and thus enhance the firing rate of these neurons.

One of the objectives of this study was to rule out the implication of PR in the effects observed with 3α,5α-THP. Therefore, the PR antagonist RU486 was used. Not only was it not able to prevent the increase in 5-HT neuronal firing activity induced by 3α,5α-THP but RU486 also enhanced it by itself. Although puzzling, these results are interesting. RU486 is not selective to PR and can also bind glucocorticoid receptors (GR) (Nordeen *et al.* 1995). Antagonistic effects on GR are unlikely to underlie this increase in 5-HT neuronal firing activity for two reasons. First, corticosterone was shown to impede the function of $5\text{-}HT_{1A}$ autoreceptors via GR (Fairchild *et al.* 2003; Laaris *et al.* 1995; Laaris *et al.* 1999) and second, the GR antagonist RU38486 alone had no effect on the function of $5\text{-}HT_{1A}$ autoreceptors (Laaris *et al.* 1995). However, there is evidence of RU486 acting as a full agonist at PR and GR in certain conditions such as stimulation of the cAMP cascade (Beck *et al.* 1993), activation of protein kinase A (Nordeen *et al.* 1995), or when more coactivators than corepressors are present in the cell (Liu *et al.* 2002). Agonistic properties of RU486 at PR are not likely to lead to an

increase in 5-HT neuronal firing activity since P itself was ineffective in this regard. On the other hand, since activation of GR reduced the 5-HT_{1A} autoreceptor function, if the conditions for RU486 to act as an agonist at GR were present in the present experiments in 5-HT neurons, it might explain the increase in their firing activity. Needless to say that further experiments would be needed to clarify the mechanism of action underlying these observations.

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 SSRIs cause an initial decrease in the firing activity of 5-HT neurons (Chaput *et al.* 1986; Romero *et al.* 1996). The main mechanism of action whereby this occurs is well characterized (see review by Blier and de Montigny, 1999). Briefly, SSRIs, by blocking reuptake, increase the amount of extracellular 5-HT, which activates somatodendritic 5-HT_{1A} autoreceptors. This leads, initially to an inhibition of the firing activity of 5-HT neurons and, later, to gradual desensitisation of these receptors. Therefore, 5-HT neurons eventually recover their initial action potential firing frequency, and the SSRI-induced increase in synaptic 5-HT concentration can finally enhance 5-HT neurotransmission. This desensitisation process takes about two to three weeks, which is consistent with the delayed therapeutic onset of action of antidepressants. This underscores the importance of developing adjuvant treatments, which could reduce this delay. The reduction of the firing activity of 5-HT neurons following short-term

administration of SSRIs has been repeatedly shown in male rats (Chaput *et al.* 1986; Romero *et al.* 1996). However, to the authors' knowledge, this is the first report confirming that it is also true concerning females. Furthermore, the present report indicates that a 3-day treatment with either 3α,5α-THP or ganaxolone, 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. Thus, 3α,5α-THP or ganaxolone could be good candidate as adjuvants to SSRI for the treatment of depressed women.

Both 3α,5α-THP and ganaxolone are potent positive allosteric modulators of $GABA_A$ receptors (Carter *et al.* 1997). Accordingly, both of them inhibit [^{35}S]TBPS binding and enhance the muscimol and benzodiazepine binding to rat brain membranes (Carter *et al.* 1997), and they both potentiate GABA-induced chloride currents (Carter *et al.* 1997; Mascia *et al.* 2002). Furthermore their efficacy and potency are similar, with ganaxolone being just a little less potent than 3α,5α-THP (Carter *et al.* 1997). However, an important distinction between them is found in the fact that ganxolone 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 & Rogawski 2000a; Reddy & Rogawski 2000b) and to have antiepileptic activity in humans (Laxer *et al.* 2000). 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. Furthermore, high doses of ganaxolone (twice its ED_{50}) did not induce tolerance after 7 days of administration (Reddy & Rogawski 2000a). Also, based on experiments assessing changes in $GABA_A$ 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). This lack of tolerance development and little withdrawal effects further supports ganaxolone as a good therapeutic candidate.

There are data showing an inverse correlation between CSF and plasma levels of 3α,5α-THP and the intensity of major depression in humans (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 behavior (Dong *et al.* 2001; Frye & Walf 2002; Uzunova *et al.* 2003; Uzunova *et al.* 2004) while administration of this steroid leads to antidepressant-like effects (Khisti *et al.* 2000; Khisti & Chopde 2000). Unfortunately, no study has yet assessed the antidepressant-like effect of ganaxolone. However, different pieces of evidence could suggest similar

properties as compared to 3α,5α-THP. For instance, the antidepressant-like effect of 3α,5α-THP in the Porsolt forced swimming test could be potentiated by the $GABA_A$ agonist muscimol and prevented by the antagonist bicuculline, indicating that this effect is mediated, at least in part, by $GABA_A$ receptors (Khisti *et al.* 2000; Khisti & Chopde 2000). Ganaxolone, having similar pharmacological characteristics at $GABA_A$ receptors as 3α,5α-THP (Carter *et al.* 1997), may potentially lead to similar behavioral effects. Furthermore, ganaxolone has anticonvulsant activity (Carter *et al.* 1997; Reddy & Rogawski 2000a; Reddy & Rogawski 2000b) similar to that of 3α,5α-THP (Finn & Gee 1994; Kokate *et al.* 1994) and could be predicted from their respective *in vitro* pharmacological properties at $GABA_A$ receptors (Monaghan *et al.* 1997). Finally, 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 that ganaxolone might have such beneficial properties. Furthermore, these data suggest that these steroids could be interesting adjuvant to reduce the delay before therapeutic onset, seen with SSRIs. Interestingly, in clinical trials, ganaxolone was shown to have an interesting pharmacokinetic profile and to be safe and well tolerated, up to relatively high doses, in both men and women (Monaghan *et al.* 1997). Since naturally occurring neuroactive steroids are not suitable for chronic treatments (due to their very short half-life)(Gasior *et al.* 2000), if ganaxolone had antidepressant properties, it could be especially promising.

The present data clearly demonstrate the ability of 3α,5α-THP and ganaxolone to positively modulate the firing activity of DRN 5-HT neurons in female rats within a short period of time. Moreover, these results suggest that these neuroactive steroids could reduce the delay of therapeutic onset of citalopram and possibly of other SSRIs. Considering the pharmacological profile of ganaxalone, this neuroactive steroid might represent an interesting adjuvant in the treatment of mood disorders in female.

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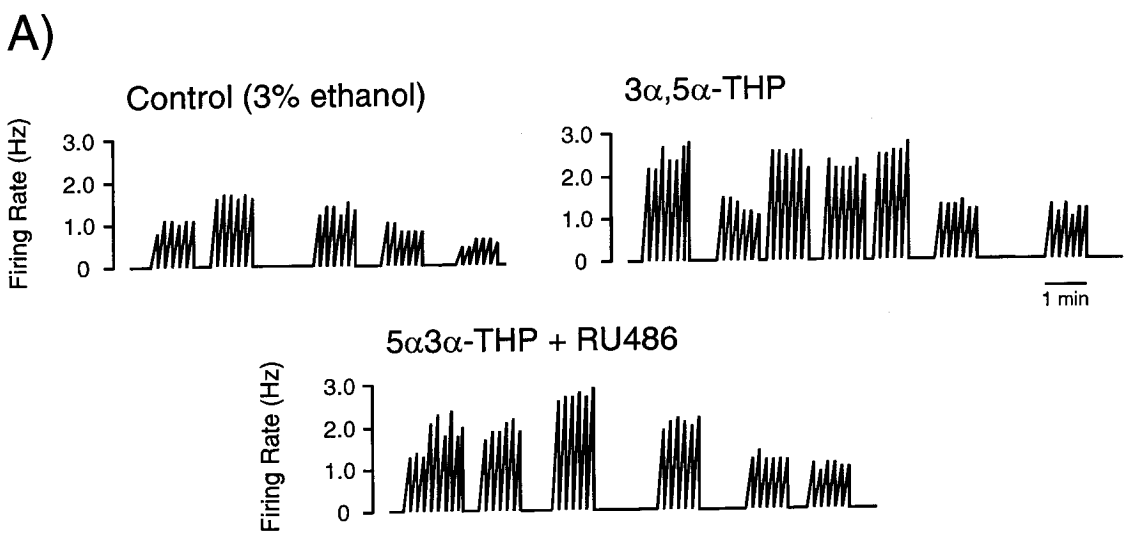
FIGURE LEGENDS

Figure 1: (A) Integrated firing rate histograms of 5-HT neurons, showing their spontaneous firing activity, recorded in one electrode descent in the DRN of female controls (3% ethanol, i.c.v., 7 days) and following a treatment with 3α,5α-THP alone or concomitantly with RU486 (50μg/kg/day, i.c.v., 7 days, each). (B) Spontaneous firing rate of female DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) in controls and following a 7-day treatment with 3α,5α-THP alone or with RU486 (50μg/kg/day, i.c.v., each). In this and the following figures, the number of neurons recorded is indicated in each box. Stars indicate $P < 0.05$.

Figure 1


A)
Control (3% ethanol)
3α,5α-THP
Firing Rate (Hz)
3.0
2.0
1.0
0
1 min
5α3α-THP + RU486

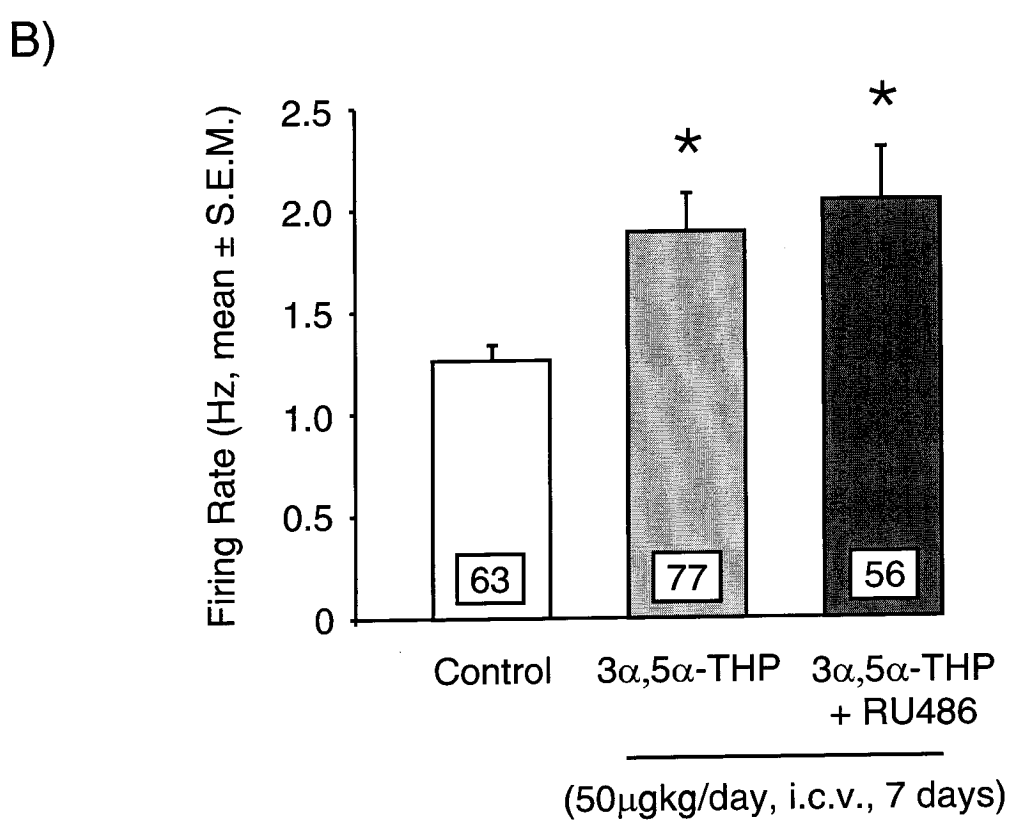

B)
Firing Rate (Hz, mean ± S.E.M.)
2.5
2.0
1.5
1.0
0.5
0
*
*
63
77
56
Control
3α,5α-THP
3α,5α-THP
+ RU486
(50μgkg/day, i.c.v., 7 days)

Figure 2: Spontaneous firing rate of DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) in controls and female treated with RU486 (50 μg/kg/day, i.c.v., 7 days). The star indicates $P < 0.01$.

Figure 2

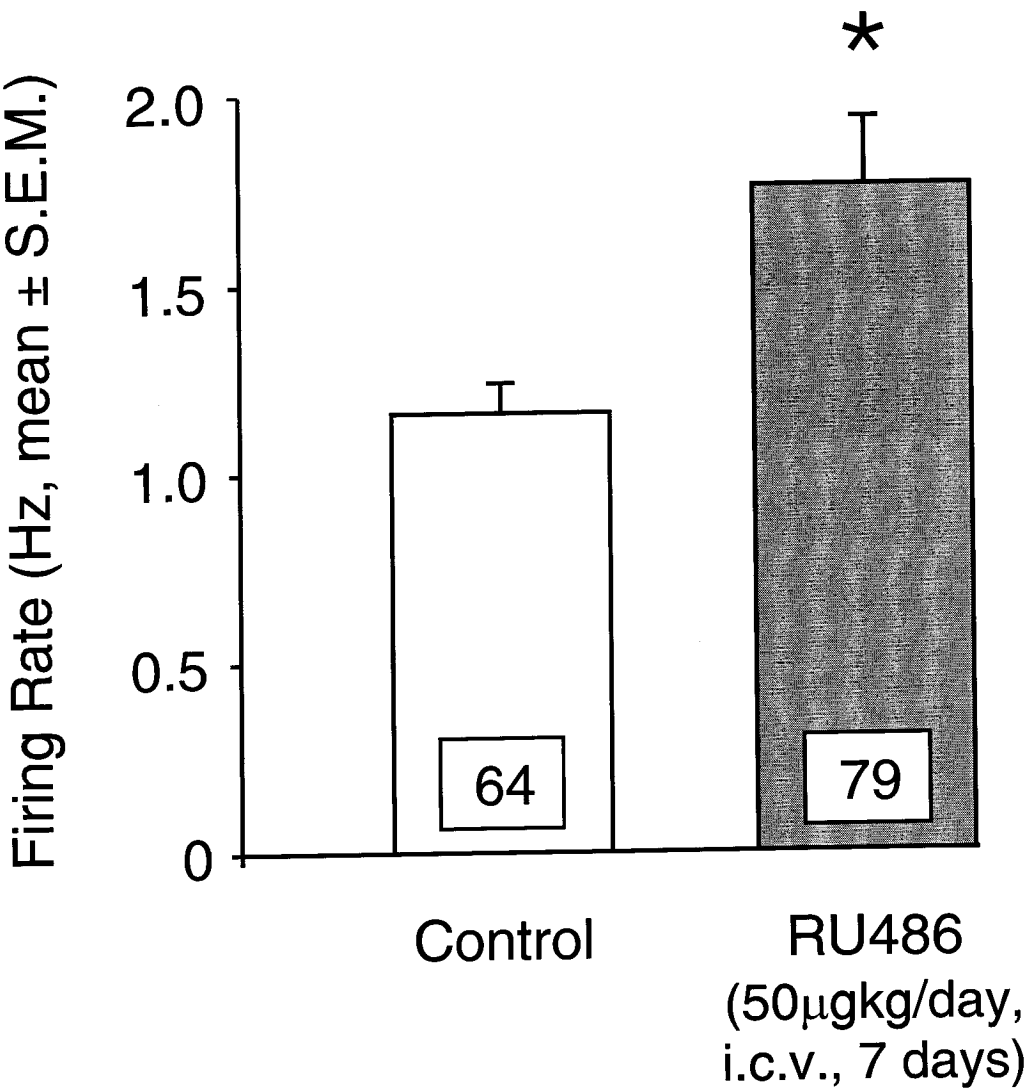

*
Firing Rate (Hz, mean ± S.E.M.)
2.0
1.5
1.0
0.5
0
64
79
Control
RU486
(50μgkg/day,
i.c.v., 7 days)

Figure 3: Spontaneous firing rate of DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) following a 3-day treatment with 3α,5α-THP (50 μg/kg/day, i.c.v.) or the vehicle (3% ethanol, i.c.v.). The star indicates $P < 0.001$.

Figure 3

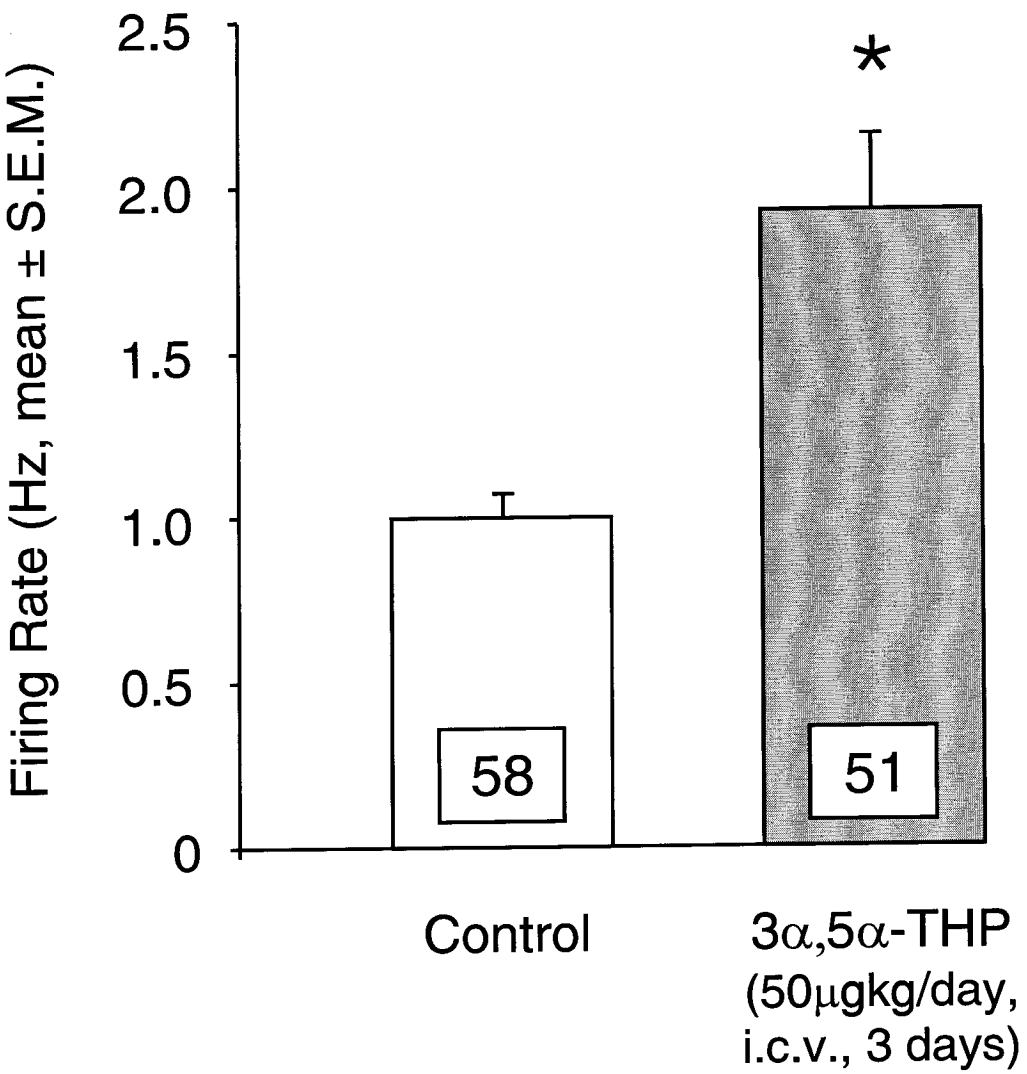

Firing Rate (Hz, mean ± S.E.M.)
2.5
2.0
1.5
1.0
0.5
0
*
58
51
Control
3α,5α-THP
(50μgkg/day,
i.c.v., 3 days)

Figure 4: Spontaneous firing rate of DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) before as well as at various time period (00-29, 30-59 and 60-90 minutes) following a single injection of 3% ethanol (controls) or 3α,5α-THP (1 μg/kg, i.c.v.). Stars indicate $P < 0.05$.

Figure 4

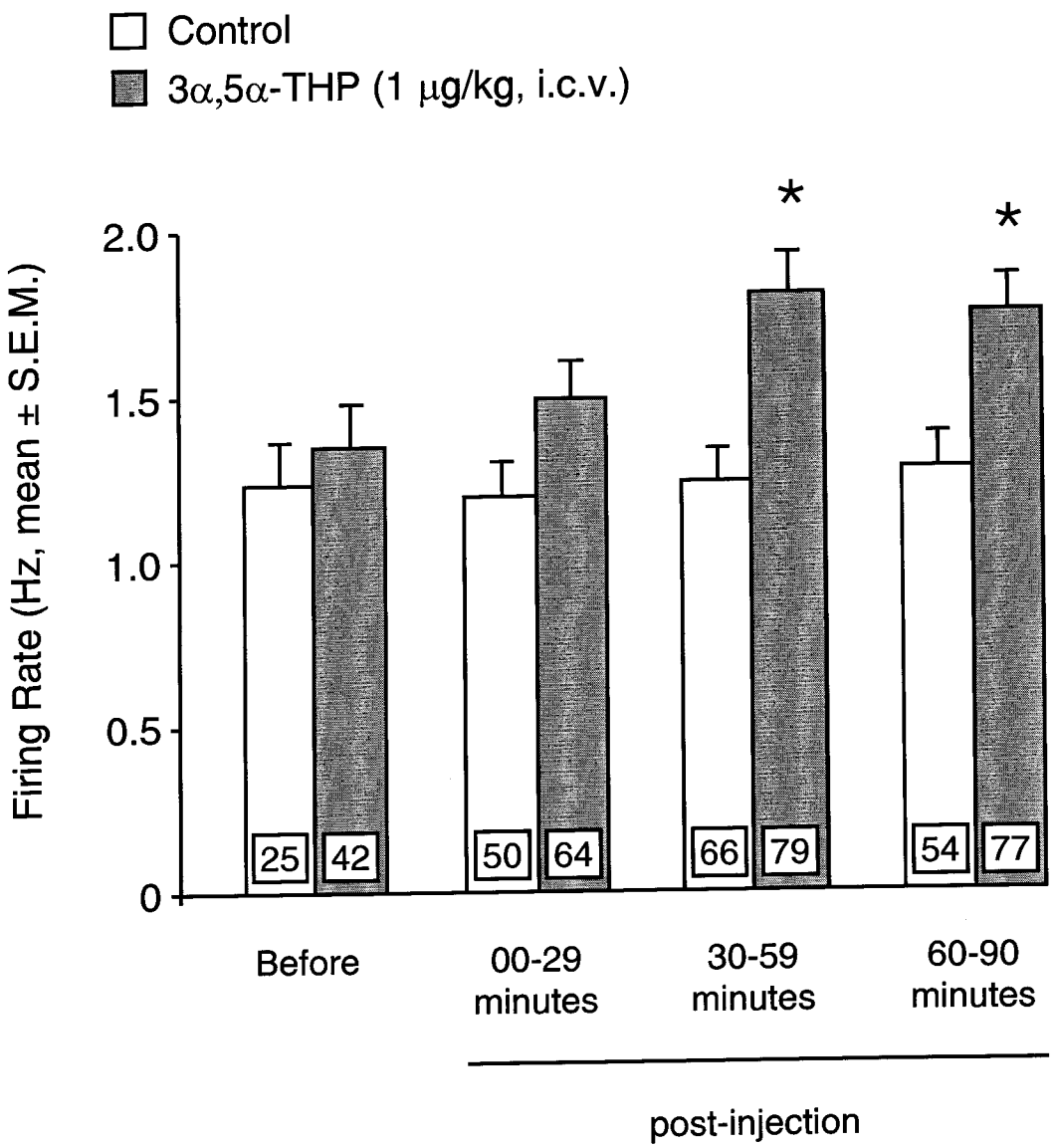

Control
3α,5α-THP (1 μg/kg, i.c.v.)
Firing Rate (Hz, mean ± S.E.M.)
2.0
1.5
1.0
0.5
0
*
*
25
42
50
64
66
79
54
77
Before
00-29 minutes
30-59 minutes
60-90 minutes
post-injection

Figure 5: Spontaneous firing rate of DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) following a 3-day co-treatment with citalopram (10 mg/kg/day, s.c.) and either the vehicle (3% ethanol, i.c.v.) or 3α,5α-THP (50 μg/kg/day, i.c.v.). The star indicates $P < 0.05$.

Figure 5

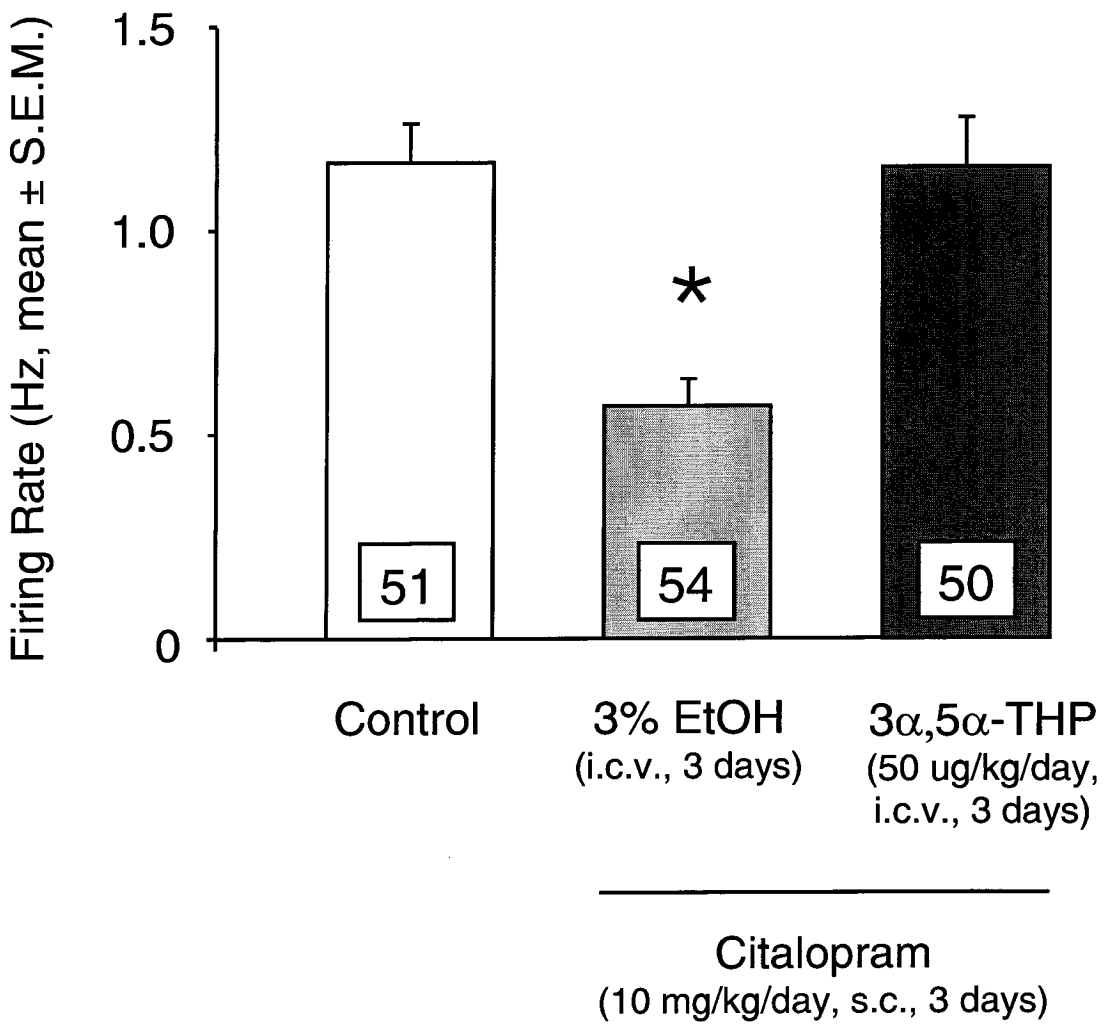
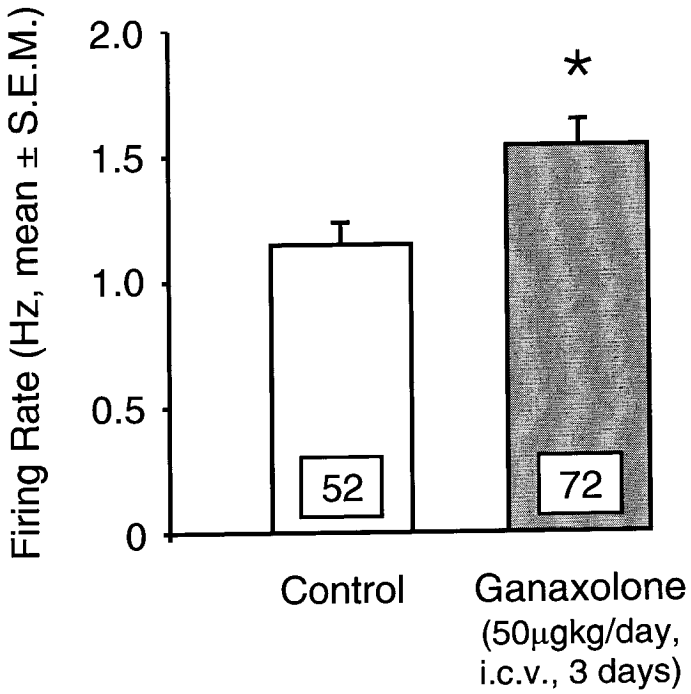

Firing Rate (Hz, mean ± S.E.M.)
1.5
1.0
0.5
0
*
51
54
50
Control
3% EtOH
(i.c.v., 3 days)
3α,5α-THP
(50 ug/kg/day,
i.c.v., 3 days)
Citalopram
(10 mg/kg/day, s.c., 3 days)

Figure 6: Spontaneous firing rate of DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) following A) a 3-day and B) a 7-day treatment with ganaxolone (50 μg/kg/day, i.c.v.), and their respective controls (3% ethanol, i.c.v. for 3 or 7 days). The stars indicate $P < 0.001$.

Figure 6

A) 3 days



B) 7 days

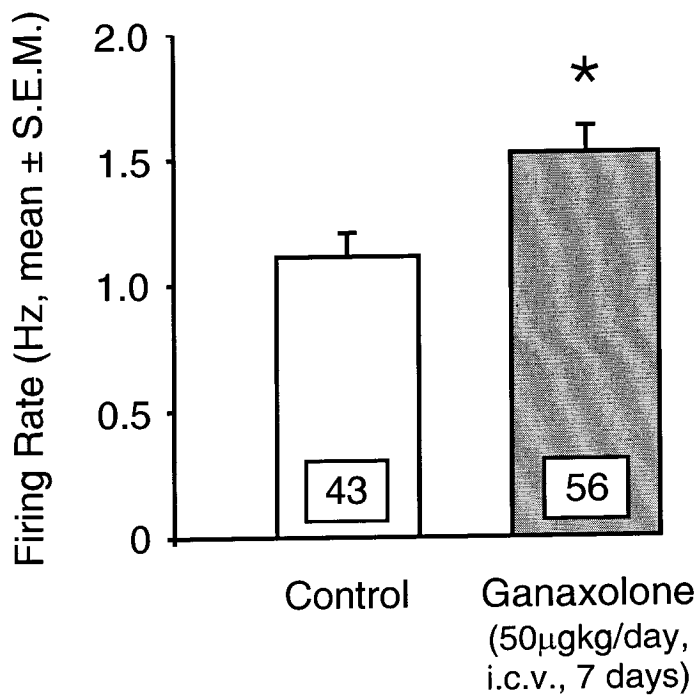
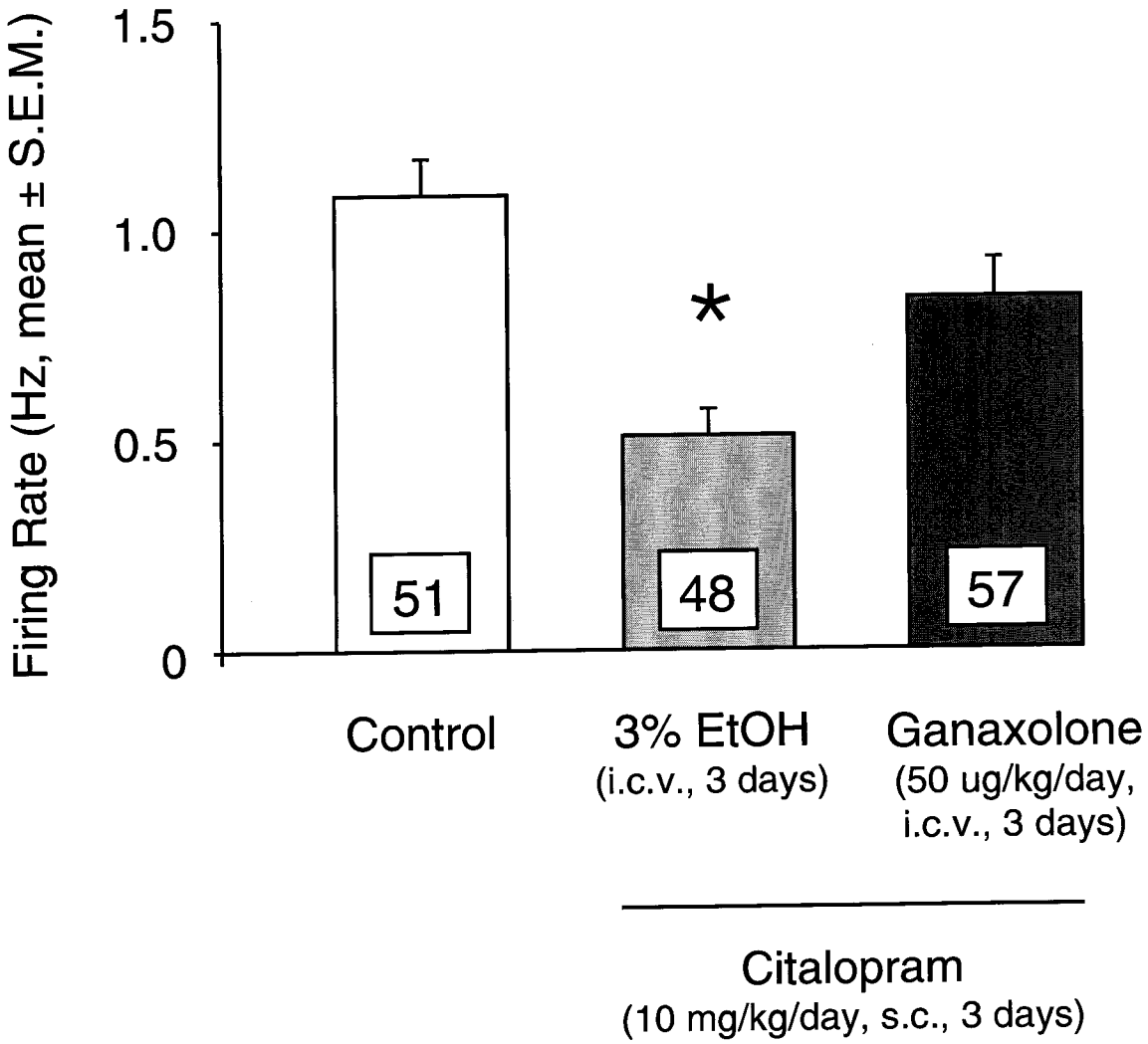


Figure 7: Spontaneous firing activity of DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) following a 3-day co-treatment with citalopram (10 mg/kg/day, s.c.) and either the vehicle (3% ethanol, i.c.v.) or ganaxolone (50 μg/kg/day, i.c.v.). The star indicates $P < 0.05$.

Figure 7


Firing Rate (Hz, mean ± S.E.M.)
1.5
1.0
0.5
0
51
48
57
*
Control
3% EtOH
(i.c.v., 3 days)
Ganaxolone
(50 ug/kg/day,
i.c.v., 3 days)
Citalopram
(10 mg/kg/day, s.c., 3 days)

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Foreword to Chapter 7

DHEAS modulation the firing activity of dorsal raphe nucleus serotonergic neurons

The last study indicated a rapid increase in 5-HT neuronal firing activity following administration of 3α,5α-THP and ganaxolone to females. Furthermore, both these steroids were able to prevent the reduction in firing frequency induced by the SSRI citalopram. If also true in humans, these data would suggest that these steroids could be interesting candidates as adjuvants to SSRIs in the treatment of depression. However, one of the earlier studies had shown that the effect of 3α,5α-THP was limited to females.

Evidence suggests antidepressant properties for dehydroepiandrosterone sulfate (DHEAS, see introduction). In hope of finding a steroid, which has antidepressant potential and which could be effective regardless of gender, the aim of this study was to assess the effects of DHEAS on the spontaneous 5-HT neuronal firing activity and its ability to prevent the citalopram-induced reduction of this activity in both males and females.

Chapter 7

DHEAS Modulation of the Firing Activity of Dorsal Raphe Nucleus Serotonergic Neurons

Malika Robichaud and Guy Debonnel

Short Communication, to be submitted to: Journal of Endocrinology

ABSTRACT

Accumulating evidence suggest antidepressant properties for DHEA and DHEAS in both animal models and clinical trials. We have already shown that DHEA increased the firing activity of dorsal raphe nucleus (DRN) serotonergic (5-HT) neurons in female but not male rats. This study was thus undertaken to assess the effect of DHEAS on the 5-HT neuronal firing activity in both males and females. *In vivo* extracellular unitary recording of 5-HT neurons performed in the DRN of these rats revealed that the intracerebroventricular (i.c.v.) administration of DHEAS for either 3 or 7 days increased the firing activity of 5-HT neurons in both males and females. Furthermore, when co-administered with citalopram (s.c.) for 3 days, DHEAS partially prevented the SSRI-induced reduction in firing frequency, irrespectively of gender. The present findings are in accordance with the putative antidepressant properties of DHEAS and also offer a physiological basis for these effects. Finally, these results could suggest a potential therapeutic application for DHEAS as an adjuvant to SSRIs in the treatment of depression.

INTRODUCTION

There is evidence suggesting that dehydroepiandrosterone and its sulfated moiety (DHEA and DHEAS) have antidepressant properties. For instance, DHEA was shown to be beneficial in the treatment of dysthymia (Bloch *et al.* 1999) and major depression (Bloch *et al.* 1999; Wolkowitz *et al.* 1999; Wolkowitz *et al.* 1995; Wolkowitz *et al.* 1997). The response rate of patients treated with DHEA was greater than with placebo (Bloch *et al.* 1999; Wolkowitz *et al.* 1999). Interestingly, during these treatments, the plasma levels of DHEAS increased at least 3 times more than that of DHEA (Bloch *et al.* 1999; Wolkowitz *et al.* 1995). Furthermore, the changes in mood ratings were correlated with the increase in plasma levels of DHEAS (Bloch *et al.* 1999) rather than DHEA (Bloch *et al.* 1999). In older patients, improvement of depressive symptoms seemed associated with increases of both DHEA and DHEAS levels (Wolkowitz *et al.* 1995; Wolkowitz *et al.* 1997).

In the Porsolt forced swimming test, used to predict antidepressant effects, DHEAS was shown to reduce the immobility time, indicating antidepressant-like effects for this steroid (Reddy *et al.* 1998; Urani *et al.* 2001). DHEA also reduced the immobility time in this paradigm but only in high-anxiety rats (Prasad *et al.* 1997), which might suggest a lower efficacy for DHEA as compared to that of its sulfated counterpart. Taken together these data suggest antidepressant properties for DHEA(S) and that DHEAS might in fact be responsible for these effects.

The serotonin (5-HT) system has also long been implicated in the neurobiology of depression (Coppen 1967; Lapin & Oxenkrug 1969). The most compelling evidence relies on the enhancement of 5-HT neurotransmission observed with antidepressant treatments (Blier & de Montigny 1999). Supporting the putative antidepressant properties of DHEA, we previously showed that this neurosteroid increased the firing activity of 5-HT neurons from the dorsal raphe nucleus (DRN) in female rats (Robichaud & Debonnel 2004). However, this was not true in males (Robichaud & Debonnel, submitted). Based on the above-mentioned literature, it deemed important to test DHEAS in our paradigm. The rationale was that if DHEAS was indeed the active molecule in that respect, it might have a more pronounced effect than DHEA in males and might be potent enough to modulate the firing activity of their 5-HT neurons. The goal of this study was thus to assess the effect of DHEAS on the spontaneous firing activity of DRN 5-HT neurons in both males and females, and to investigate a potential therapeutic application.

METHODS

Animals

All rats used were Sprague Dawleys (Charles-River, St-Constant, Québec, Canada), weighed between 225 g and 325 g and were kept under standard laboratory conditions (12:12 light-dark cycle with access to food and water *ad libitum*). Freely cycling females and males were used for the experiments. Ethical committee approval was obtained from the McGill University Animal Ethical Care Committee and all their rules and regulations were followed.

Treatments

DHEAS was dissolved in 3% (v/v) ethanol/distilled water. A dose of 50 μg/kg/day was administered continuously and intracerebroventricularly (i.c.v.) by means of a subcutaneous osmotic minipump connected to a canulae (ALZA, Palo Alto, CA, USA). The canulae was implanted in the left lateral ventricle of the rat brain and the surgery was performed as described by the manufacturer (ALZA, Palo Alto, CA, USA), under chloral hydrate anesthesia. Both females and males received a 3- or a 7-day treatment of DHEAS. Controls received the vehicle (3% ethanol) for the same period of time. DHEAS and the vehicle were also co-administered with citalopram (a selective serotonin reuptake inhibitor or SSRI, 10mg/kg/day, s.c.) for 3 days. Citalopram, dissolved in distilled water, was administered by means of a subcutaneous osmotic minipump (ALZA, Palo Alto, CA, USA), for a daily dose of 10 mg/kg.

Electrophysiological Experiments

All rats were anesthetized 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 approximately 37° C throughout the experiment by a thermistor-controlled heating pad. Extracellular unitary recordings of serotonergic neurons were obtained with single-barelled glass micropipettes pulled in a conventional manner, filled with a 1M NaCl solution and of final impedance ranging between 2 and 6 MΩ. A 4 mm-diameter hole was drilled in the skull of each rat at about 1 mm anterior of lambda and centered with respect to the midline. The unitary activity of DRN 5-HT neurons was recorded by lowering the micropipette along descents covering the nucleus from 300 μm to about 1500 μm anterior of lambda. Spontaneously active DRN 5-HT neurons were identified according to the criteria of Aghajanian: a slow and regular rythmical firing rate and a shape of action potential with a large initial positive spike of 1-2 msec duration and a postspike hyperpolarization (Aghajanian *et al.* 1978; Aghajanian & Vandermaelen 1982). 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 seconds, each 5-HT neuron encountered in complete descents in the DRN of at least 5 rats.

Statistics

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

Drugs

Dehydroepiandrosterone sulfate (DHEAS) was purchased from Steraloids and Citalopram was kindly provided by Lundbeck Canada (Montréal, Québec, Canada).

RESULTS

A 7-day treatment with DHEAS increased the firing activity of 5-HT neurons in both females (1.59 ± 0.12 Hz, n = 92 compared to 1.26 ± 0.09 Hz, n = 75, [$F(1,2) = 4.66$, $P<0.05$, Tukey's test, $q = 3.1$, $P<0.05$], Fig. 1) and males (2.02 ± 0.18 Hz, n = 74 compared to 1.35 ± 0.11 Hz, n = 62, [$F(1,2) = 8.73$, $P<0.005$, Tukey's test, $q = 4.2$, $P<0.05$], Fig. 2). This increase could also be observed as early as after 3 days of treatment in both females (1.78 ± 0.17 Hz, n = 48 compared to 1.38 ± 0.11 Hz, n = 61, [$F(1,2) = 4.29$, $P<0.05$, Tukey's test, $q = 2.9$, $P<0.05$], Fig. 1) and males (1.69 ± 0.10 Hz, n = 82 compared to 1.25 ± 0.11 Hz, n = 69, [$F(1,2) = 8.88$, $P<0.005$, Tukey's test, $q = 4.2$, $P<0.05$], Fig. 2).

It is well known that the short-term administration of SSRIs decreases the firing activity of 5-HT neurons (Chaput *et al.* 1986; Romero *et al.* 1996). This was also observed in the present experiments, in both sexes, as a 3-day administration of citalopram significantly reduced the firing activity of 5-HT neurons in females (0.53 ± 0.06 Hz, n = 52 compared to 1.14 ± 0.10 Hz, n = 42, [$F(2,3) = 9.33$, $P<0.001$, Tukey's test, $q = 6.1$, $P<0.05$], Fig. 3A) and males (0.63 ± 0.07 Hz, n = 61 compared to 1.25 ± 0.11 Hz, n = 69, [$F(2,3) = 11.97$, $P<0.001$, Tukey's test, $q = 6.8$, $P<0.05$], Fig. 3B). It was then investigated whether the DHEAS-induced increase in firing rate could compensate for the suppressant effect of citalopram. In both sexes, co-treatment with DHEAS with citalopram only partially prevented the reduction in firing activity (females: 0.82 ± 0.10 Hz, n = 69 compared to 1.14 ± 0.10 Hz, n = 42, [$F(2,3) = 9.33$, $P<0.001$, Tukey's test, $q = 3.4$, n.s.], Fig. 3A; males: 0.87 ± 0.08 Hz, n = 69 compared to 1.25 ± 0.11 Hz, n = 69, [$F(2,3) = 11.97$, $P<0.001$, Tukey's test, $q = 4.4$, n.s., Fig. 3B).

DISCUSSION

The main finding of this study is the increase in 5-HT neuronal firing activity following short (3 days) and longer (7 days) treatments with DHEAS in male and female rats. This supports the proposed antidepressant properties of DHEAS (Bloch *et al.* 1999; Reddy *et al.* 1998; Urani *et al.* 2001; Wolkowitz *et al.* 1995; Wolkowitz *et al.* 1997) and offers a physiological mechanism through which they may take place. Interestingly, DHEAS seemed equally effective in both sexes as opposed to DHEA, which increased the firing activity of 5-HT neurons only in females (Robichaud & Debonnel 2004, submitted). This is in accordance with our initial hypothesis that DHEAS might be the active molecule in this respect. In males, there might not be enough of DHEAS formed from DHEA to modulate the firing activity of 5-HT neurons but when DHEAS itself is administered, such changes can occur.

The mechanism of action underlying this effect of DHEAS is unclear. However, based on its pharmacological properties on various receptors, several hypotheses can be proposed. For instance, DHEAS is an allosteric antagonist of $GABA_A$ receptors (Demirgören *et al.* 1991; El-Etr *et al.* 1998; Hansen *et al.* 1999; Majewska *et al.* 1990; Mehta & Ticku 2001; Shen *et al.* 1999; Sousa & Ticku 1997) much more potent than DHEA in this respect (Demirgören *et al.* 1991; Imamura & Prasad 1998; Mehta & Ticku 2001). The rat DRN 5-HT neurons are under a tonic GABAergic inhibition, which is mostly mediated by $GABA_A$ receptors (Gervasoni *et al.* 2000; Innis & Aghajanian 1987). DHEAS, by

negatively modulating $GABA_A$ receptors, might thus reduce this tonic inhibition of 5-HT neurons and increase their firing activity. The lesser antagonistic potency of DHEA as compared to DHEAS may be a reason for the lack of noticeable effect of DHEA on 5-HT neuronal activity in males. In females, 5-HT neurons appear to be under greater GABAergic tonic inhibition (Robichaud *et al.* 2002), which may explain why DHEA nevertheless increased their neuronal activity (Robichaud & Debonnel 2004).

DHEAS has also been shown to slightly potentiate the response of NMDA receptors (Bowlby 1993; Park-Chung *et al.* 1994), which are involved in the glutamatergic stimulation of 5-HT neuronal activity (Jolas & Aghajanian 1997; Tao & Auerbach 2000). A greater NMDA-mediated glutamatergic excitatory input on 5-HT neurons could therefore explain their faster firing rate. However, the glutamatergic input on 5-HT neurons does not appear to be tonic (Tao *et al.* 1997; Tao & Auerbach 2000). Therefore, it is less likely to be responsible for the DHEAS-induced increase in 5-HT neuronal activity.

The presently reported effect of DHEAS could also be mediated by sigma (σ) receptors. Although DHEAS binds σ receptors with only low affinity (Maurice *et al.* 1996), its effects have often been shown to be σ receptor-mediated (Maurice *et al.* 1997; Maurice *et al.* 1998; Meyer *et al.* 2002; Noda *et al.* 2000; Ueda *et al.* 2001). For instance, DHEAS was shown to be beneficial in models of learning impairment (Maurice *et al.* 1997), conditioned fear response (Noda *et al.* 2000) and amnesia (Maurice *et al.* 1998), through activation of σ receptors.

Interestingly, the antidepressant-like effect of DHEAS in mice appears to be mediated, at least in part, by σ_1 receptors since it was efficiently prevented by co-administration a selective σ_1 antagonist (Reddy *et al.* 1998; Urani *et al.* 2001). Studies from our lab have also shown that σ_1 receptors were implicated in the DHEA-induced increase in 5-HT neuronal activity (Robichaud & Debonnel 2004) and that other sigma ligands had similar effects in this paradigm (Bermack & Debonnel 2001). Together, these data could support a role for σ receptors in the excitatory effect of DHEAS on 5-HT neurons. Additionally, DHEAS can increase glutamate release from cultured hippocampal neurons via σ_1 receptors (Meyer *et al.* 2002). If this was the case in the DRN, it could increase the glutamatergic tone on 5-HT neurons and thus increase their firing activity as mentioned above.

Another interesting finding of this study was that co-administration of DHEAS with citalopram for 3 days could partially compensate for reduction in firing activity of 5-HT neurons caused by this SSRI alone. Chronic treatments with SSRIs are known to cause an initial decrease in the firing activity of 5-HT neurons (Chaput *et al.* 1986; Romero *et al.* 1996). The underlying mechanism of action has been reviewed elsewhere (Blier & de Montigny 1999). Briefly, 5-HT reuptake blockade enhances extracellular 5-HT, thus leading to greater activation of somatodendritic 5-HT_{1A} autoreceptors. This produces an initial inhibition of the 5-HT neuronal firing rate, which is followed by a gradual desensitisation of these autoreceptors. 5-HT neurons can thus eventually recover their normal firing

activity, and the SSRI-induced greater extracellular 5-HT concentration can finally be expressed as enhanced 5-HT neurotransmission. This desensitisation process has a timeframe consistent with the delayed therapeutic onset of action seen with antidepressants, which emphasizes the importance of reducing this timeframe.

In females, 3α,5α-THP and ganaxolone efficiently prevented this initial reduction of the 5-HT neuronal firing activity caused by citalopram {Robichaud & Debonnel, submitted}. However, 3α,5α-THP did not modulate the firing activity of 5-HT neurons in males {Robichaud & Debonnel, submitted}. Therefore, this effect of DHEAS, which is irrespective of gender, could be exploited in developing new therapeutic approaches for treating depression. For instance, this partial recovery of a normal firing activity following administration of both DHEAS and citalopram suggest that this combination might be helpful to accelerate the onset of therapeutic action of SSRIs in men, as well as women. DHEAS could thus be used as an adjuvant to already existing treatments. This is supported by the literature on clinical studies (Bloch *et al.* 1999; Wolkowitz *et al.* 1995; Wolkowitz *et al.* 1997) and animal model of depression (Reddy *et al.* 1998; Urani *et al.* 2001), which suggests antidepressant effects for this steroid.

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FIGURE LEGENDS

Figure 1: Spontaneous firing rate of DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) in females treated for 3 or 7 days with DHEAS (50 μg/kg/day, i.c.v.) and their respective controls (3% ethanol, i.c.v.). In this and the following figures, the number of neurons recorded is indicated in each box. The star indicates $P < 0.05$.

Figure 1

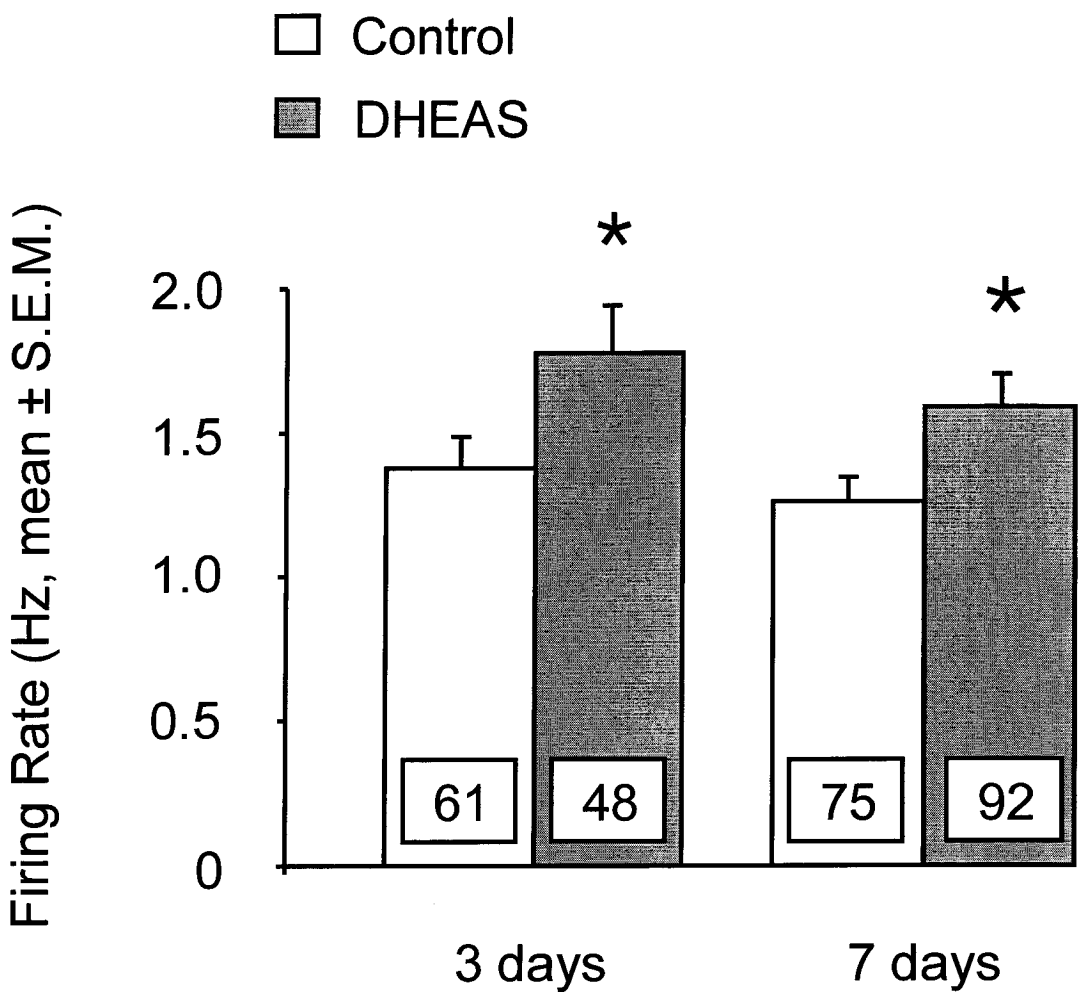

Control
DHEAS
Firing Rate (Hz, mean ± S.E.M.)
2.0
1.5
1.0
0.5
0
*
*
61
48
75
92
3 days
7 days

Figure 2: Spontaneous firing rate of DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) in males treated for 3 or 7 days with DHEAS (50 μg/kg/day, i.c.v.) and their respective controls (3% ethanol, i.c.v.). The star indicates $P < 0.005$.

Figure 2

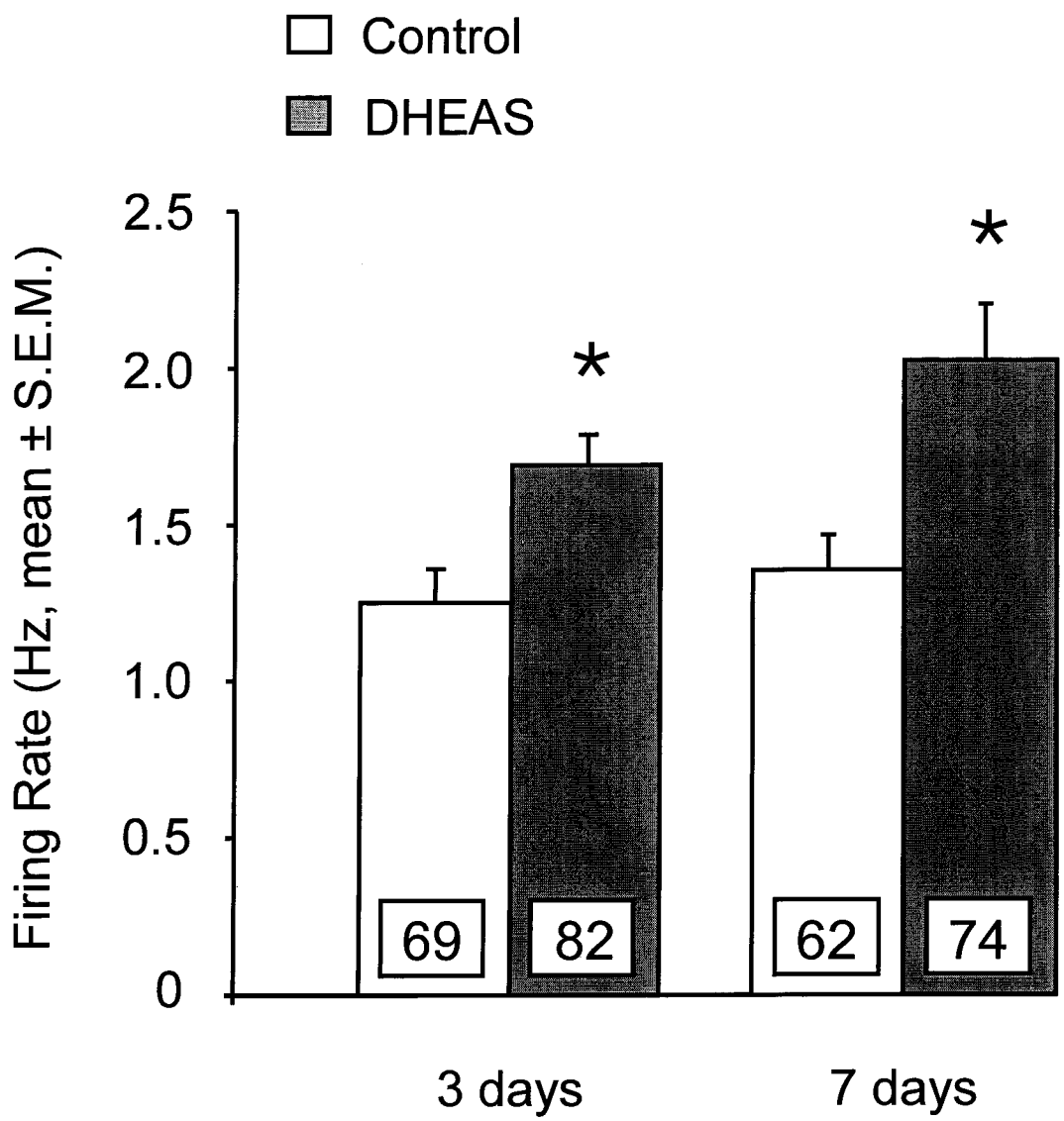
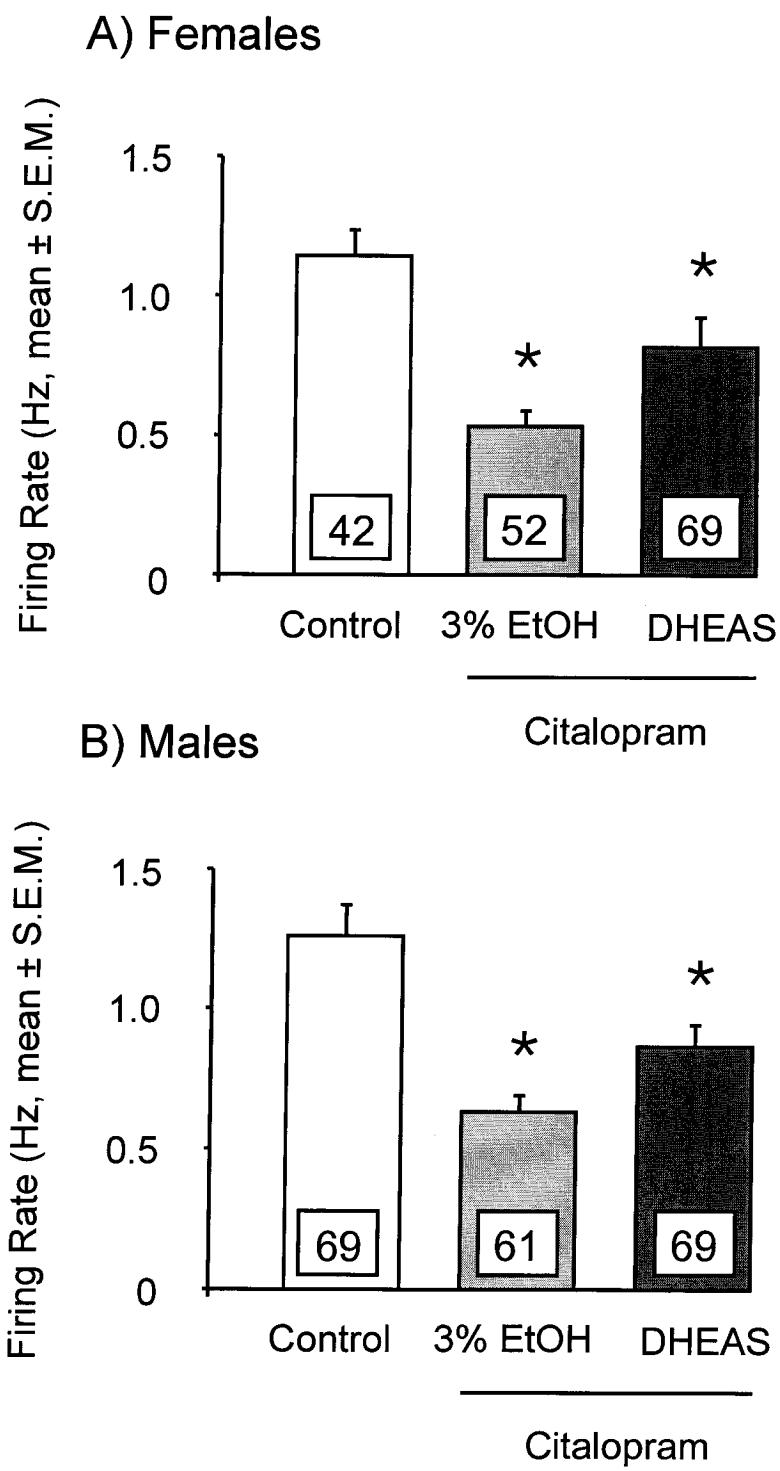

Control
DHEAS
Firing Rate (Hz, mean ± S.E.M.)
2.5
2.0
1.5
1.0
0.5
0
*
*
69
82
62
74
3 days
7 days

Figure 3: Spontaneous firing rate of DRN 5-HT neurons expressed in Hz (mean ± S.E.M.) following a 3-day co-treatment with citalopram (10 mg/kg/day, s.c.) and either the vehicle (3% ethanol, i.c.v.) or DHEAS (50 μg/kg/day, i.c.v.) in A) females and B) males. The star indicates $P < 0.05$.

Figure 3


A) Females
Firing Rate (Hz, mean ± S.E.M.)
1.5
1.0
0.5
0
42
52
69
*
*
Control
3% EtOH
DHEAS
Citalopram
B) Males
Firing Rate (Hz, mean ± S.E.M.)
1.5
1.0
0.5
0
69
61
69
*
*
Control
3% EtOH
DHEAS
Citalopram

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Chapter 8

CONCLUSION

Important gender differences exist regarding affective disorders, with women being more at risk than men (5,12,14,17,31-33,42,59,60). There is strong evidence of a role for ovarian hormones in female mood disorders (20,21,29,52,54). Furthermore, a large body of evidence indicates a functional interrelationship between depression, neuroactive steroids and the serotonergic (5-HT) system, which has itself long been implicated in the neurobiology of affective disorders (15,56). Since the activity of 5-HT neurons located in the dorsal raphe nucleus (DRN) is crucial for 5-HT neurotransmission, the first goal of this thesis project was to evaluate gender differences in the spontaneous firing activity of DRN 5-HT neurons and to examine different potential mechanisms of action underlying these differences. The second objective was to assess the modulation of the 5-HT neuronal firing activity by various neuroactive steroids and compare this modulation between males and females. Finally, a potential therapeutic application for some of these steroids was investigated.

As a whole, this thesis supports current molecular studies, which suggest that the 5-HT system is modulated by neuroactive steroids. Furthermore, the results presented in this thesis suggest a biological basis for the greater susceptibility of women to mood disorders. They also offer an explanation as to why some steroids have antidepressant properties.

In the first study, a greater 5-HT neuronal firing frequency in males and pregnant females as compared to freely cycling females was shown. Interestingly, during pregnancy, the 5-HT neuronal firing activity closely paralleled the variations in

progesterone (P) plasmatic levels. Both gradually increased, peaked at the 17th day of pregnancy (P17) and then dropped just before parturition. In the postpartum period, the firing rate decreased as compared to P17 but was still greater than in freely cycling females. These results demonstrated a clear modulation by gender and heightened hormonal levels of the *in vivo* spontaneous firing activity of DRN 5-HT neurons.

A distinction was made between gender and hormonal status because, even though males have lower ovarian hormonal levels, reducing that of females through ovariectomy did not modulate their firing activity. This suggests that the sex difference in firing activity is not solely due to hormonal levels but is rather a gender-dependent trait, which probably arises from more complex and developmentally regulated mechanisms. The clear correlation between the neuronal activity and P levels during pregnancy strongly suggested an implication of P and/or its metabolites in the modulation of 5-HT neurons. The involvement of P metabolites and/or other steroids was also suggested by the fact that following parturition, the firing activity ceased to correlate with P levels and did not return to baseline as quickly.

The second part of this study assessed the role of different regulatory mechanisms potentially involved in these gender differences and hormonal modifications of the 5-HT neuronal firing activity: 5-HT$_{1A}$ receptors and the tonic GABAergic inhibition of 5-HT neurons. The results indicated that during pregnancy, there was a partial desensitization of the 5-HT$_{1A}$ autoreceptor as well as a greatly reduced GABAergic tonic inhibition of 5-HT neurons. Both of these observations are in accordance with and could explain the

greater firing activity of 5-HT neurons observed during pregnancy. In males, 5-HT neurons also appeared to be under a lesser GABAergic tonic inhibition, which is also in agreement with their faster firing activity as compared to freely cycling females. A gender difference in the function of the 5-HT_{1A} autoreceptor was also observed but could not be simply expressed in terms of sensitivity. However, a lesser tonic activation of this receptor in males was hypothesized and could explain the pharmacological results as well as the faster firing frequency observed in males. These results constitute strong evidence of mechanisms by which gender and hormonal fluctuations could modulate the 5-HT neurons function and influence vulnerability to mood disorders.

The following study investigated the modulation of 5-HT neuronal activity by P and its metabolites in females in order to elucidate which neuroactive steroids participate in the enhanced neuronal activity observed during pregnancy. The hypothesis was that these steroids could thus alter 5-HT neurotransmission and be relevant to mood disorders. Interesting findings concerning the steroid modulation of the 5-HT system were obtained. First, 7 days of administration of 5β-DHP, 3α,5α-THP and DHEA clearly increased the firing activity of 5-HT neurons in females. Considering that P and its precursor PREG did not significantly modify the firing activity, and that P is rapidly metabolized in the brain, these results suggest that P metabolites, rather than P itself, play an important role in the modulation of the 5-HT neuronal activity observed during pregnancy.

Second, the effects of 5β-DHP and DHEA had a different timeframe. There was a net increase in firing activity following 7 days of treatment with 5β-DHP, which

gradually faded and returned to baseline values within 21 days. This could suggest that the receptor, which mediates the effect of 5β-DHP is desensitized within this time period. On the other hand, the effect of DHEA, which was already clearly present after 7 days, increased continuously during the 21-day period of investigation. Using the selective σ_1 antagonist NE-100, we showed that σ_1 receptors mediated, at least in part, the effect of DHEA but not that of 5β-DHP. Together these results indicate that more than one type of receptor is involved in the steroid modulation 5-HT neuronal activity. It is still unclear whether a given steroid acts via more than one type of receptor. However, given the variety of receptor-mediated effects of many steroids (see introduction), some of them could plausibly have a multiplicity of effectors. Furthermore, this could well differ between various steroids.

The steroid which induced the greatest increase in 5-HT neuronal firing activity was 3α,5α-THP. Amongst steroids, this is also the most potent positive modulator of the $GABA_A$ receptor. Moreover, during pregnancy, the levels of this steroid increase dramatically while the GABAergic tonic inhibition of 5-HT neurons, which is mainly $GABA_A$ receptor-mediated, is greatly reduced. Together, these data could suggest that 3α,5α-THP is important for the increase in firing activity observed during pregnancy and that it modifies the GABAergic tonic inhibition of 5-HT neurons through its action at $GABA_A$ receptors. They also further support the hypothesis of multiple types of receptors taking part in the steroid modulation of the 5-HT system.

Considering that antidepressant treatments increase 5-HT neurotransmission, these results, showing that 3α,5α-THP and DHEA enhance the firing activity of 5-HT neurons, offer a physiological basis for the antidepressant-like effects of 3α,5α-THP observed in animals (34,35) as well as the antidepressant property of DHEA in humans (16,80,81).

Since a difference in 5-HT neuronal spontaneous firing rate had been observed between sexes in the first study, a possible gender-dependent modulation of this activity by neuroactive steroids was then investigated. In the third study, males were thus treated with the steroids which had been found to be effective in females. Surprisingly, neither 5β-DHP, 3α,5α-THP nor DHEA had an effect on the firing activity of 5-HT neurons in males. For this reason and the observation that the basal firing rate differs between sexes, the role of androgens was then investigated. Of castration and treatments with T and 5α-DHT, only T enhanced the firing activity of 5-HT neurons in males. For the purpose of gender comparison, females received T and it also resulted in a faster firing frequency of their 5-HT neurons. Despite this similar net effect in both males and females, a gender difference was nevertheless observed. Indeed, in males, only a small proportion of neurons seemed responsive to T and they acquired a very fast firing activity, whereas in females, this effect seemed more generalized but less dramatic. This constitutes a further demonstration of intrinsic gender differences in the 5-HT neuronal modulation, which could potentially have dramatic physiological consequences regarding the neurobiology of depression. The results obtained in theses studies suggest that the female 5-HT system is more sensitive to neuroactive steroid modulation than that of males. This could

contribute to women's greater vulnerability to mood disorders, especially when considering the frequent hormonal variations that they experience throughout their life.

In the brain, T is mainly metabolized into 17β-E and 5α-DHT (67) and since 5α-DHT did not modulate the activity of 5-HT neurons in males, the effect of 17β-E was examined. It increased the firing activity of 5-HT neurons in both males and females, thus supporting the hypothesis that the effects of T could be mediated through its aromatization. These results are also in accordance with the antidepressant-like effects of 17β-E (see introduction). In terms of mechanisms of action, this steroid was shown to act on various proteins of the 5-HT system as well as on afferent systems.

Since, in females, the most potent steroid was 3α,5α-THP, the time-frame of its modulation of the 5-HT neuronal firing activity was further characterized. This study confirmed the previous findings and showed that this 3α,5α-THP-induced increase in firing frequency was already present after 3 days of administration and even appeared as early as 30 to 60 minutes following a single injection. This steroid thus seems to have a very rapid onset of action for sustained effects on the 5-HT neuronal activity. Not only did these results support the antidepressant-like properties of 3α,5α-THP but the time frame of its action also made it attractive as a potential adjuvant for treating depression. Naturally occurring hormones may, however, not be the best therapeutic candidates. It was thus important to assess whether ganaxolone, a synthetic analog to 3α,5α-THP, had similar effects on the firing activity of 5-HT neurons. Indeed, both a 3- and a 7-day treatment with ganaxolone enhanced the 5-HT neuronal activity.

There is a well-documented delay that precedes the therapeutic onset of action of antidepressants. In the case of SSRIs this delay is thought to be due to the initial reduction in firing activity of 5-HT neurons. Because of the rapid increase in 5-HT neuronal firing activity observed in our paradigm with 3α,5α-THP and ganaxolone, the potential of these steroids to prevent the reduction in firing frequency induced by the SSRI citalopram was then assessed. Interestingly, they blocked the citalopram-induced reduction in 5-HT neuronal firing activity. In humans, if this initial reduction is indeed responsible for the delay in therapeutic onset of action, these steroids could be interesting candidates as adjuvants to SSRIs in the treatment of depression. Ganaxolone might be particularly interesting in this regard since it is not metabolized into hormonally active compounds, does not seem to induce tolerance (58) and has been shown to be safe for humans (39,48).

These exciting results seemed, however, limited to females. Therefore, we sought another steroid, which could possibly prevent the SSRI-induced initial reduction of 5-HT neuronal firing activity in both males and females. Some studies addressing the antidepressant properties of DHEA have suggested that DHEAS might be the active form of this steroid. For this reason, the effect of DHEAS was assessed in this paradigm. In both males and females this steroid increased the firing activity of 5-HT neurons with a short onset of action and a lasting effect. However, in neither sex was it able to significantly prevent the citalopram-induced decrease in firing activity.

Possible mechanisms of action for the modulation of 5-HT neurons by steroids

As mentioned in the introduction, steroids could modulate the 5-HT systems through different mechanisms of action, which could involve various types of receptors and different neurotransmitter systems afferent of DRN 5-HT neurons (for a schematic representation of the main modulatory inputs on 5-HT neurons see figure 1).

The classical genomic mechanisms of action cannot be ruled out but is unlikely, at least in the case of 3α,5α-THP, because its action takes place within a very short time-frame. Aside from this, 3α,5α-THP does not bind the classical steroid receptors such as ER or PR and P, which binds PR was not effective in our paradigm. On the other hand 17β-E was effective and can bind ER, indicating that ER might be involved. The rat DRN expresses very low levels of ERα (50,64) but higher levels of ERβ (50,63) and, while ERα and PR are undetectable in 5-HT cells (4,41), about 40% of 5-HT neurons express ERβ mRNA (41). Therefore, if the mechanism for 17β-E's effect is genomic, it is more likely to occur via ERβ than ERα. Membrane steroid receptors and activation of intracellular signalling cascades could also constitute ways though which neuroactive steroids might influence 5-HT neuronal activity. No data is yet available to speculate on the details of these processes.

It is well known that 5-HT neurons receive noradrenergic (NE) and glutamatergic (1,30) excitatory input. An involvement of these two systems in the modulation of 5-HT neuronal activity by neuroactive steroids is thus possible (see introduction). However,

pregnancy did not modify the firing activity of locus coeruleus NE neurons, suggesting that the NE system was not the primary site of action of ovarian steroids. More studies would be needed to address these issue but they were beyond the scope of this thesis. Experiments were aimed at modulatory mechanisms, most likely involved in the steroid modulation of the 5-HT neuronal activity, such as 5-HT_{1A} and σ receptors and the tonic GABAergic inhibition of 5-HT neurons.

There is evidence of an ovarian hormonal modulation of several enzymes and receptors participating in 5-HT neurotransmission (see introduction). The data presented here point to a beneficial effect for estrogen in this regard. For example, evidence suggests that E reduces the function of 5-HT_{1A} autoreceptors in female rats (13,83). This was confirmed by electrophysiological experiments, similar to ours, showing a desensitization of the 5-HT_{1A} autoreceptor in the DRN of OVX rats following 48h of E administration (37,38). Considering the autoinhibitory role of this receptor on the firing activity of 5-HT neurons, this estrogenic effect might facilitate 5-HT neurotransmission. The results of this thesis support these findings. Indeed, during pregnancy a partial desensitization of this autoreceptor was observed along with an increase in basal firing activity. This increase in firing activity was also reproduced by administration of different ovarian steroids. These results suggest that the molecular effects on the 5-HT_{1A} autoreceptor reported by others have functional consequences on the firing activity of 5-HT neurons and possibly on the 5-HT neurotransmission efficacy.

Various steroids have high affinity for σ receptors (44-46,70,82) and activation of σ_1 receptors has been shown to enhance the firing activity of DRN 5-HT neurons in the same paradigm (10). Thus, σ receptors represented another potential way through which neurosteroids could modulate the 5-HT system. Indeed, the involvement of σ_1 receptors has been confirmed for the DHEA-induced enhancement of the firing activity of 5-HT neurons. These results thus suggest a physiological basis for the σ_1 receptors-mediated antidepressant-like effects obtained with some neuroactive steroids in rodents (57,72). They also suggest that σ receptors might be implicated in the antidepressant effect of DHEA observed in humans. P also has high affinity for σ receptors but seems to act as an antagonist (7,8). This could explain why P did not modulate the firing activity of 5-HT neurons. What remains intriguing is the fact that other σ ligands were shown to increase the firing activity of 5-HT neurons in males (11) while DHEA did not. P levels are higher in females than males, which would suggest that higher doses of DHEA might be necessary to compensate for P's antagonistic action at σ receptors and thus to have sigma-related effects in females. One would then expect that a given dose, which increases the firing activity of 5-HT neurons in females, would be enough to do so in males. However, σ ligands seem to have a bell-shaped dose-response curve, with high doses of agonists acting as antagonists (9,49). It is thus possible that the doses of DHEA used in these experiments were too high for males and that reducing the dose might be effective in modulating the firing activity of their 5-HT neurons.

DRN 5-HT neurons are under a GABAergic tonic inhibition, which is mainly mediated by $GABA_A$ receptors (27), whose function can be modulated by several

neuroactive steroids. The results of this thesis strongly suggest that the steroid-induced alterations of this GABAergic inhibition increase the 5-HT neuronal firing activity. Indeed, pregnancy was associated with reduced GABAergic tonic inhibition of 5-HT neurons and 3α,5α-THP, a potent modulator of $GABA_A$ receptors, was the most effective steroid in enhancing the firing frequency of 5-HT neurons. The putative $GABA_A$ receptor-mediated increase in 5-HT neuronal activity might also explain the report that $GABA_A$ receptors are implicated in the antidepressant-like effects of 3α,5α-THP (34,35).

DHEAS and, although less potently, DHEA, act as $GABA_A$ receptor antagonists (18,43,47,53). Both of them increased the firing activity of 5-HT neurons in females, while DHEAS was also effective in males. This would support the greater potency of DHEAS as compared to DHEA and could thus suggest the involvement of $GABA_A$ receptors in mediating this effect. It is however puzzling that antagonists have the same net effect as the potent agonists 3α,5α-THP and ganaxolone, within very similar time frames. In the case of DHEA and DHEAS, considering the high affinity of DHEA and DHEAS for σ receptors, it is possible that these receptors are implicated in this effect. Nevertheless, these results would suggest a combination of mechanisms of action.

Further experiments will be needed to fully characterize the mechanisms of action underlying the effects of steroids on the activity of 5-HT neurons. However, the results of this thesis strongly suggest that multiple types of receptors are likely implicated. Each steroid possibly acts through one or more receptors to increase the firing activity of 5-HT neurons. And, based on these results, it appears that steroids might not all act through the

same mechanism of action. The results of this thesis indicate that 5-HT_{1A} autoreceptors, and σ receptors contribute to this effect but there might also be others.

Clinical implications

Women are especially at risk of developing depression during period of ovarian steroid fluctuation and the data obtain herein show that the females' 5-HT neurons are more sensitive to ovarian steroid modulation. In addition, altered levels of ovarian steroids were observed during depressive episodes and normalized by successful antidepressant treatments (61,68,69,76). Interestingly, the increase in CSF levels of 3α,5α-THP was proportional to the mood improvement (75). Different types of antidepressants were shown to facilitate the synthesis of 3α,5α-THP from 5α-DHP (23,25,28,73) through direct interaction with the enzyme 3α-HSD (23,25,28,73). It has been suggested that the therapeutic action of antidepressants may involve increased levels of 3α,5α-THP (23,25,74). The results of this thesis showing that many steroids increase the firing activity of 5-HT neurons would support this hypothesis. If, indeed this is the case, these steroids could prove good adjuvants in the treatment of depression, especially with such a fast onset of action.

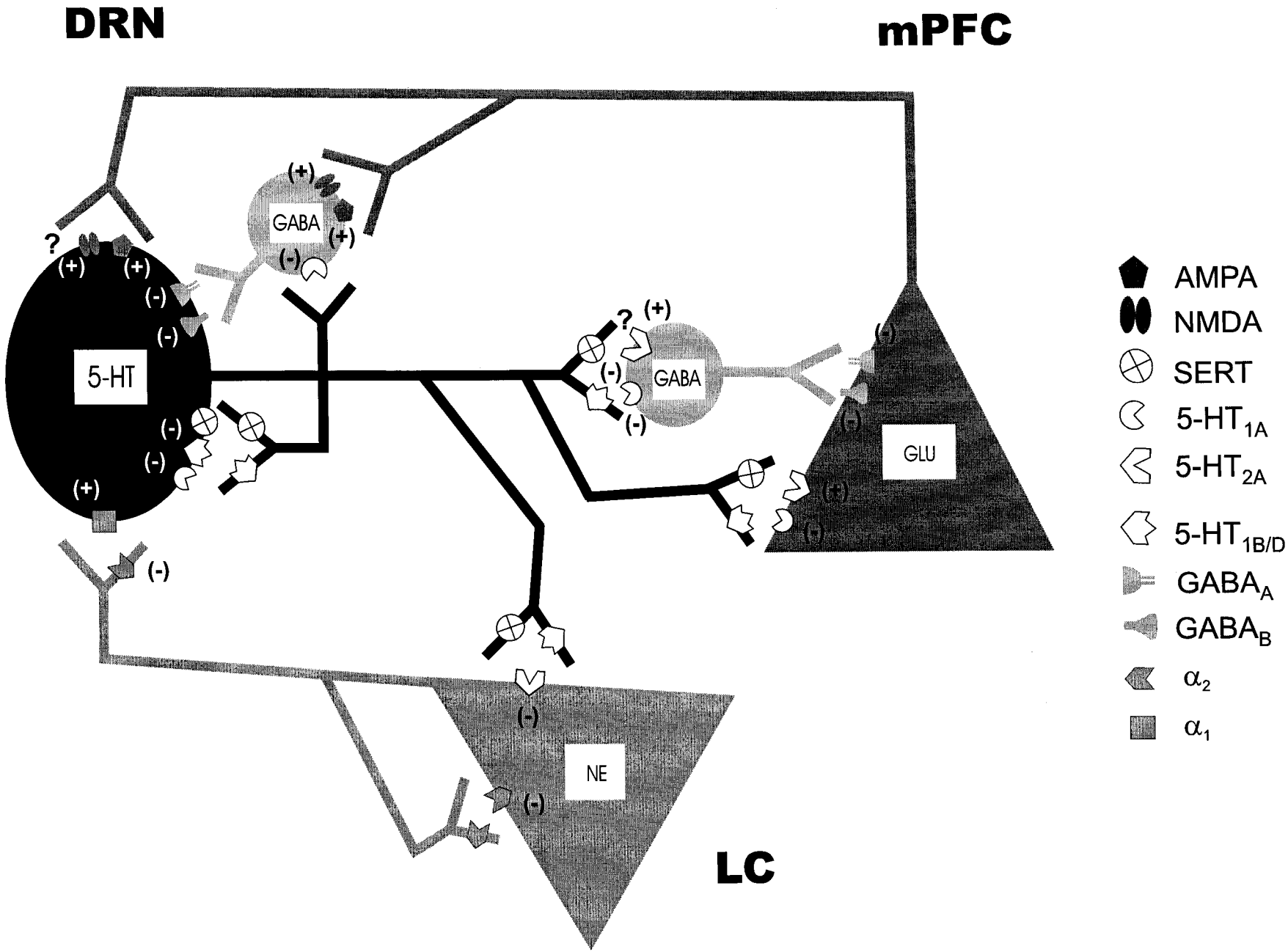
This is also in keeping with clinical studies and animal models showing antidepressant (or antidepressant-like in the case of animals) properties for different ovarian steroids including estradiol (2,3,22,24,26,36,62,65,77,78), DHEA(S) (16,57,71,79-81) and 3α,5α-THP (34,35), which are clearly supported by the steroid-

induced increase in 5-HT neuronal firing activity. Taken together with the literature, the present results support synergetic interactions between neurosteroids and antidepressants to modulate the 5-HT system and/or induce beneficial effects on mood.

There is evidence of anatomical and functional dissimilarities between men and women's 5-HT systems (6,19,40,51,55), which might explain gender differences related to depression and women's greater vulnerability to mood disorders. The slower firing activity of 5-HT neurons in females as compared to males, if also occurring in humans, would further support this. Furthermore, data suggest that some women may be have altered sensitivity to hormonal modulations of the 5-HT system (66). The female 5-HT system appears to be especially sensitive to hormonal modulation. In women with a certain predisposition, this might be enough to increase their vulnerability to developing mood disorders.

Finally, our results clearly show yet another gender difference in the function of the 5-HT system and in the ability of neurosteroids to modulate it. These findings, added to the previous data, further characterize not only similarities but also sex differences in the modulation of 5-HT neurons by some steroids, which could prove important in the understanding of gender differences in mood disorders. Furthermore, the present data offer a biological basis to the reported antidepressant properties of certain steroids and suggest that they could be used as adjuvants to antidepressants in the treatment of depression.

Figure 1: Schematic representation of excitatory and inhibitory inputs on DRN 5-HT neurons.


DRN
mPFC
LC
5-HT
GABA
GABA
GLU
NE
?
(+)
(-)
AMPA
NMDA
SERT
5-HT1A
5-HT2A
5-HT1B/D
GABAA
GABAB
α2
α1

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