available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

The effect of estrogen on dopamine and serotonin receptor and transporter levels in the brain: An autoradiography study

Carolina Chavez[a,b], *Marianne Hollaus*[a], *Elizabeth Scarr*[c,d], *Geoff Pavey*[c], *Andrea Gogos*[a,d], *Maarten van den Buuse*[a,b,*]

[a]*Behavioural Neuroscience Laboratory, Mental Health Research Institute, Melbourne, Australia*
[b]*Department of Pharmacology, University of Melbourne, Australia*
[c]*Rebecca L. Cooper Laboratory, Mental Health Research Institute, Melbourne, Australia*
[d]*Centre for Neuroscience, University of Melbourne, Australia*

ARTICLE INFO

Article history:
Accepted 16 December 2009
Available online 14 January 2010

Keywords:
Estrogen
Dopamine receptor
Serotonin receptor
DAT
SERT
Autoradiography

ABSTRACT

The aim of the present study was to elucidate the effect of estrogen on dopaminergic and serotonergic regulation of prepulse inhibition (PPI) by measuring its effects on the density of dopamine transporters (DAT), dopamine D_1 and D_2 receptors, serotonin transporters (SERT), serotonin-1A (5-HT_{1A}) and 5-HT_{2A} receptors using radioligand binding autoradiography. Three groups of female Sprague–Dawley rats were compared: sham-operated controls, untreated ovariectomized (OVX) rats and OVX rats with a 17β-estradiol implant (OVX+E). These groups were identical to our previous prepulse inhibition (PPI) studies, allowing comparison of the results. Results showed that in the nucleus accumbens, DAT levels were 44% lower in OVX rats than in intact controls. Estrogen treatment completely reversed the effect of OVX in this brain region to levels similar to those in intact controls. Dopamine D_2 receptor density was increased in OVX rats by 28% in the nucleus accumbens and 25% in the caudate nucleus compared to intact controls. Estrogen treatment reversed this increase and, in addition, reduced dopamine D_2 receptor levels by a further 25% and 20%, respectively, compared to intact control rats. There were no differences between the groups with respect to the densities of dopamine D_1 receptors, SERT, 5-HT_{1A} receptors or 5-HT_{2A} receptors. These results show effects of estrogen treatment on central indices of dopaminergic, but not serotonergic function. The observed changes do not provide a direct overlap with the effects of these estrogen treatment protocols on drug-induced disruptions of PPI, but it is possible that a combination of effects, i.e. on both DAT and dopamine D_2 receptor density, is involved. These data could also be relevant for our understanding of the potential protective effect of estrogen treatment in schizophrenia.

* *Corresponding author*. Behavioural Neuroscience Laboratory, Mental Health Research Institute of Victoria, 155 Oak Street, Parkville, Melbourne, Victoria 3052, Australia. Fax: +61 3 93875061.
E-mail address: m.vandenbuuse@mhri.edu.au (M. van den Buuse).

Abbreviations: PPI, prepulse inhibition; DAT, dopamine transporters; SERT, serotonin transporter; 5-HT, serotonin; OVX, ovariectomized; 8-OH-DPAT, 8-hydroxy-dipropylaminotetralin; ETE, estimated tissue equivalent; dpm/mg, disintegration units per min per mg; Sham, sham-operated; S.E.M., standard error of the mean; ANOVA, analysis of variance; NA, nucleus accumbens; CN, caudate nucleus; CC, cingulate cortex; DG, dentate gyrus; DRN, dorsal raphe nucleus; MRN, median raphe nucleus

0006-8993/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2009.12.093

1. Introduction

Recently, a clinical trial revealed that adjuvant transdermal estradiol treatment attenuated positive and general psychopathological symptoms, but not negative symptoms, in women with schizophrenia (Kulkarni et al., 2001,2008). This confirmed and extended earlier epidemiological, neurochemical and pre-clinical findings, that estrogen may play a role in schizophrenia (Seeman, 1997; Stevens, 2002). For example, it has been recognized for several years that there are gender differences in the age-of-onset, symptom severity and treatment response in schizophrenia (for references, see Castle et al., 1998; Häfner, 2003; Seeman, 1997). However, despite many reports on the effects of estrogen on brain neurotransmitter activity (for reviews, see Bethea et al., 1998; Di Paolo, 1994), the mechanism by which estrogen might be involved in schizophrenia, remains unclear.

We previously focused on prepulse inhibition (PPI) of acoustic startle to study the potential role of estrogen in schizophrenia. PPI is the reduction of a startle response to a loud, sudden noise pulse, if it is preceded by a low-intensity prepulse. PPI has been widely used as a model for sensory gating, i.e. the ability to filter sensory information and allow focused attention (Braff et al., 2001). PPI is reduced in patients with schizophrenia and other neurological/psychiatric illnesses (Braff and Geyer, 1990; Braff, 1993). One advantage of PPI is, that it can be studied across species with similar methodology, stimulus characteristics and behavioral responses (Geyer and Swerdlow, 1998). In rats and mice, PPI is disrupted by several dopaminergic and serotonergic drugs (Geyer et al., 2001). We initially studied the effect of the serotonin-1A (5-HT_{1A}) receptor agonist, 8-hydroxy-dipropylaminotetralin (8-OH-DPAT) and observed that in both species, estrogen treatment modulates its effects on PPI (Gogos and Van den Buuse, 2004; Gogos et al., 2006a). For example, in ovariectomized rats chronically treated with a high dose, but not a low dose of estradiol, the disruption of PPI induced by treatment with 8-OH-DPAT was prevented (Gogos and Van den Buuse, 2004). A subsequent proof-of-concept study in human volunteers showed the same effect and confirmed the translational relevance of these results (Gogos et al., 2006b). Thus, in healthy women treated with estradiol, the disruption of PPI caused by treatment with the partial 5-HT_{1A} receptor agonist, buspirone, was prevented (Gogos et al., 2006b), similar to the effect of 8-OH-DPAT in rats. Subsequent studies in rats extended these findings to suggest that the action of 8-OH-DPAT (and presumably buspirone) was mediated by modulating dopaminergic activity (Gogos et al., in press). In these experiments, chronic estrogen treatment similarly inhibited the effect of 8-OH-DPAT and the dopamine receptor agonist, apomorphine, on PPI (Gogos et al., in press). In other studies we had already observed that dopamine D_2 receptor antagonists, such as haloperidol, could block the action of 8-OH-DPAT on PPI (Van den Buuse and Gogos, 2007), suggesting that serotonergic pathways and 5-HT_{1A} receptors are functionally 'in series' with dopaminergic pathways and dopamine D_2 receptors to modulate PPI.

In the present study we use the chronic estradiol treatment rat model to further investigate mechanisms involved in the effect of estrogen on the brain, particularly transporter and receptor densities. Because of our previous results (see above), we focused on serotonergic and dopaminergic parameters. With respect to serotonin, we included 5-HT_{1A} receptor levels, as well as the other major serotonergic receptor in the brain, the 5-HT_{2A} receptor, and the serotonin transporter (SERT). With respect to dopamine, we included dopamine D_2 receptors, as well as the other major dopaminergic receptor, the dopamine D_1 receptor, and dopamine transporter (DAT). Brain regions chosen were those with the highest level of the respective transporters or receptors. The results show significant changes in dopamine D_2 receptor and DAT levels caused by chronic estrogen treatment. These results may help to explain our *in vivo* results using PPI and, potentially, elucidate the role of estrogen in schizophrenia.

2. Results

2.1. *Ovariectomy and estradiol treatment*

Body weight at the time of surgery was not different between the groups (Table 1). However, at the end of the chronic treatment period, final body weight and weight gain were significantly different between the groups ($F(2,42)=20.3$ and 103.8, respectively, both $P<0.001$). This difference was caused by higher final body weights and weight gain in the OVX group compared to the other groups, which did not differ from each other (Table 1). Uterus weight and uterus/body weight ratio were also significantly different between the groups ($F(2,42)=94.1$ and 66.1, respectively, both $P<0.001$). Both measures were significantly lower in OVX than in intact rats, confirming effective removal of the ovaries. Estrogen treatment increased

Table 1 – Body weight and uterus weight of the experimental groups used in the present study.

Group	Initial BW	Final BW	Weight gain	Final UW	UW/BW × 1000
Intact ($n=14$)	255.3±7.4	282.0±8.0	26.7±4.2	0.47±0.02	1.68±0.10
OVX ($n=16$)	247.6±5.1	340.4±6.5[a]	92.8±4.7[a]	0.14±0.01[a]	0.41±0.04[a]
OVX+E ($n=15$)	273.1±5.9	287.6±7.4[b]	14.5±3.6[b]	0.70±0.04[a,b]	2.47±0.20[a,b]

Groups: Intact=sham-operated; OVX=ovariectomized; OVX+E=ovariectomized plus estradiol implant.
Initial BW=body weight (g) at the time of surgery; Final BW=bodyweight (g) at dissection; Weight gain=Final BW−Initial BW; Final UW=uterus weight (g) at dissection; UW/BW×1000=final uterus weight divided by final body weight×1000.
[a] $P<0.05$ compared to intact rats.
[b] $P<0.05$ compared to OVX rats.

uterus weight and uterus/body weight ratio compared to OVX alone and these values were also slightly, but significantly higher than in intact rats (Table 1).

2.2. Dopamine transporter (DAT) binding

DAT levels were high in the nucleus accumbens and caudate nucleus, with lower levels present in the cingulate cortex (Fig. 1). In the nucleus accumbens, there was a significant difference between the three treatment groups ($F(2,16)=11.9$, $P<0.001$). Post-hoc analysis showed that DAT levels were significantly reduced in OVX rats compared to intact, sham-operated rats. In OVX+E rats, DAT density was significantly higher than in OVX rats, but not different from sham-operated controls (Fig. 1). There were no significant group differences in the caudate nucleus or cingulate cortex (Fig. 1).

2.3. Dopamine D_1 receptor binding

Similar to DAT, dopamine D_1 receptor levels were high in the nucleus accumbens and caudate nucleus, with lower levels present in the cingulate cortex (Fig. 1). ANOVA revealed a trend towards significant group differences in the caudate nucleus ($F(2,14)=3,6$, $P=0.057$). However, while D_1 receptor levels tended to be higher in intact rats compared to the other groups, there were no significant group differences in this brain region. Similarly, there were no differences between groups in the nucleus accumbens or cingulate cortex (Fig. 1).

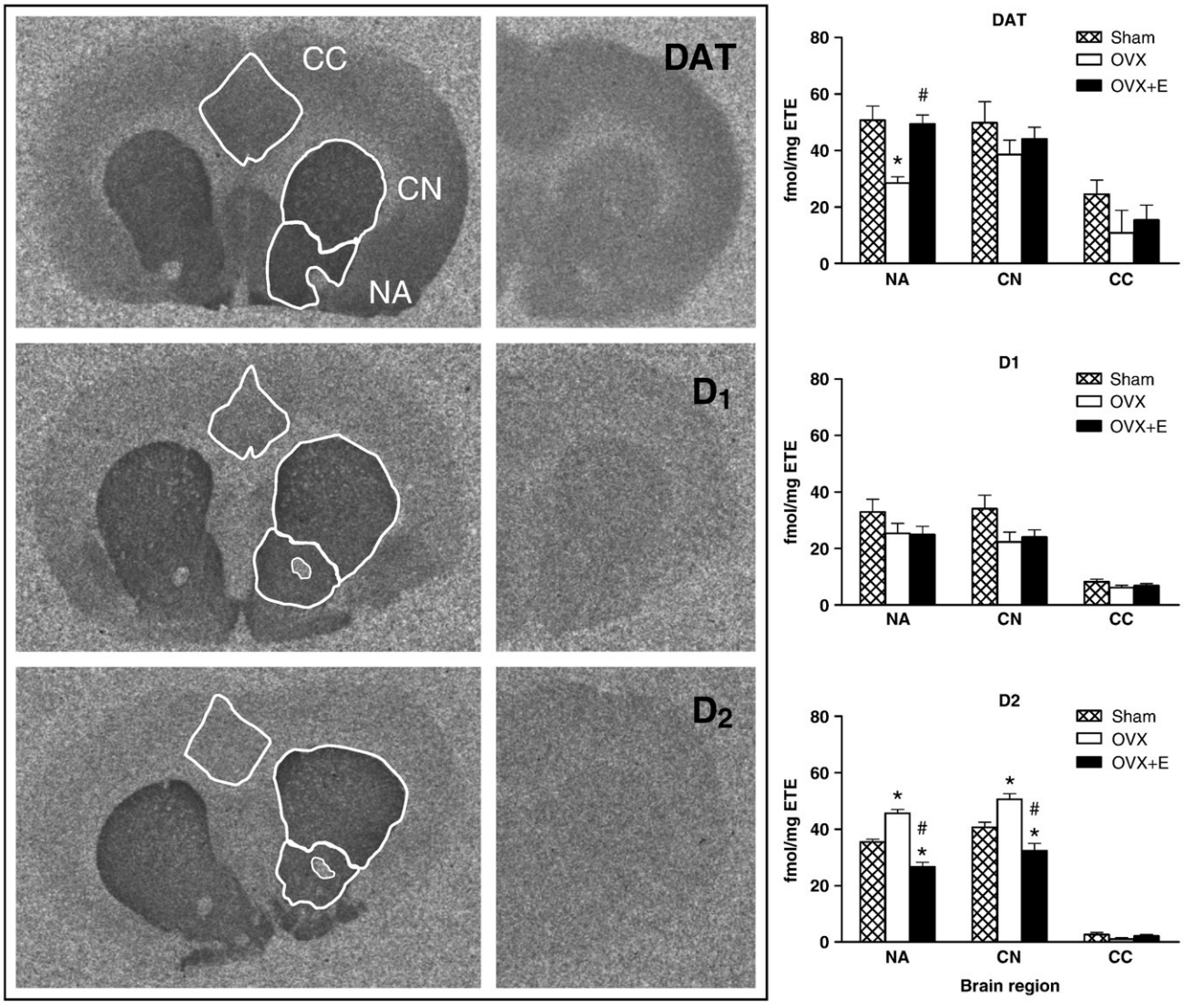

Fig. 1 – Density of dopamine transporter (DAT, top row), dopamine D_1 receptors (middle row) and dopamine D_2 receptors (bottom row) in intact, sham-operated rats (Sham), ovariectomized rats (OVX) and ovariectomized rats treated with estrogen (OVX+E). Left panels show autoradiograms of total binding and non-specific binding, respectively. Brain regions outlined are the nucleus accumbens (NA), caudate nucleus (CN) and the cingulate cortex (CC). DAT density was significantly reduced in the nucleus accumbens in OVX rats compared to Sham rats and this effect was reversed by estrogen treatment. There were no significant group differences in dopamine D_1 receptor binding. Dopamine D_2 receptor binding was significantly increased in the nucleus accumbens and caudate nucleus in OVX rats compared to Sham but decreased in OVX+E rats. *$P<0.05$ for difference with Sham rats; # $P<0.05$ for difference with OVX rats. There were 5–7 rats per group.

2.4. *Dopamine D_2 receptor binding*

Similar to DAT binding, dopamine D_2 receptor binding density was highest in the nucleus accumbens and caudate nucleus, with lower levels present in the cingulate cortex (Fig. 1). There was a significant difference between the three treatment groups in the nucleus accumbens ($F(2,15)=48.4$, $P<0.001$) and the caudate nucleus ($F(2,15)=18.1$, $P<0.001$). For both regions, post-hoc analysis showed that, compared to intact, sham-operated rats, dopamine D_2 receptor levels were significantly higher in OVX rats and significantly lower in OVX+E rats (Fig. 1). In addition, dopamine D_2 receptor levels were significantly lower in OVX+E rats compared to OVX rats. In contrast, there were no differences between groups in the cingulate cortex (Fig. 1).

2.5. *Serotonin transporter (SERT) binding*

SERT density was highest in the dorsal raphe nucleus, followed by the median raphe nucleus, the CA1 and CA3 region of the hippocampus, and the dentate gyrus (Fig. 2). At the hippocampus level, SERT density tended to be slightly higher in OVX rats than in intact and OVX+E rats. However, neither ANOVA nor pair-wise comparisons revealed any significant differences between the groups in dorsal raphe nucleus, median raphe nucleus, CA1, CA3 or dentate gyrus (Fig. 2).

2.6. *5-HT_{1A} receptor binding*

The density of 5-HT_{1A} receptors was highest in the dorsal raphe nucleus and dentate gyrus, followed by the CA1 and CA3 regions of the hippocampus, and the median raphe nucleus (Fig. 3). In the CA1 region of the hippocampus, 5-HT_{1A} receptor binding tended to be slightly lower in OVX+E rats than in intact and OVX rats. However, in neither the hippocampus nor the raphe were there any significant differences between the groups (Fig. 3).

2.7. *5-HT_{2A} receptor binding*

The density of 5-HT_{2A} receptors was highest in specific layers of the cingulate cortex with lower levels in the nucleus accumbens and caudate nucleus (Fig. 4). ANOVA showed a borderline significant group difference in the nucleus accumbens ($F(2,15)=4.0$, $P=0.040$). However, while both OVX rats and OVX+E rats tended to show higher 5-HT_{2A} receptor binding density in the nucleus accumbens (Fig. 4), pair-wise comparison showed no significant differences between individual groups in this region. No group differences were observed in other regions of the brain.

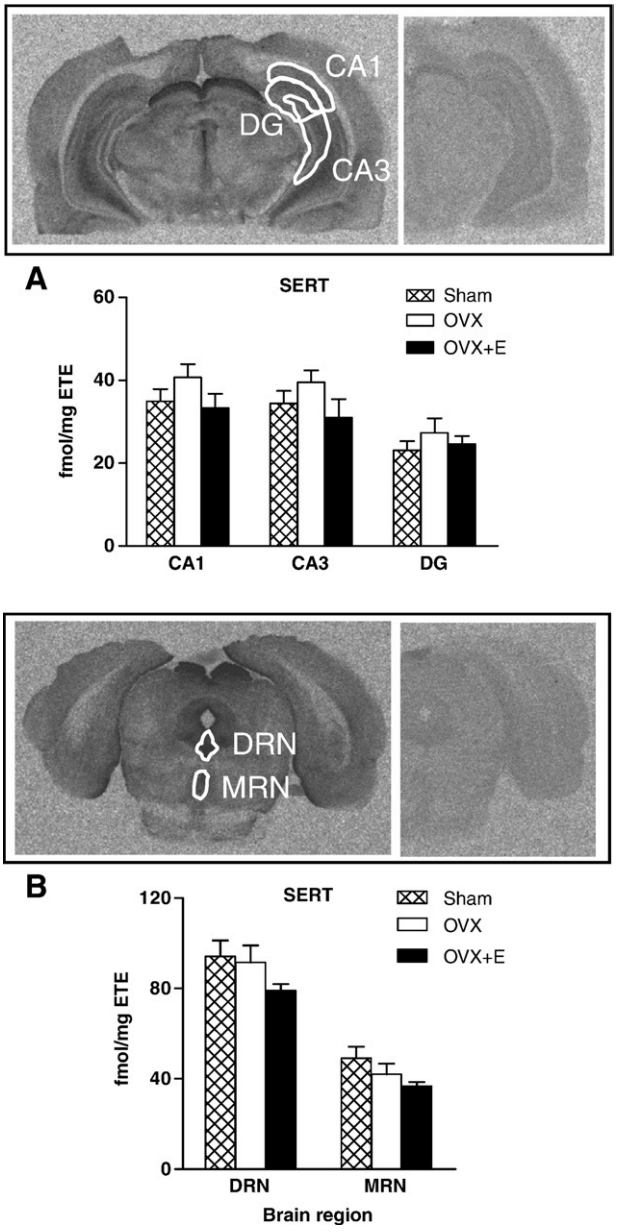

Fig. 2 – Density of serotonin transporters (SERT) in the hippocampus (A) and hindbrain (B) of intact, sham-operated rats (Sham), ovariectomized rats (OVX) and ovariectomized rats treated with estrogen (OVX+E). Brain regions outlined are the CA1, CA3 and dentate gyrus (DG) regions of the hippocampus (top two panels) and the dorsal raphe nucleus (DRN) and median raphe nucleus (MRN) (bottom two panels). Autoradiograms depict total binding and non-specific binding, respectively. There were no group differences in SERT density. N=5–7 rats per group.

3. Discussion

In this study, we compared dopamine and serotonin transporter and receptor levels between intact female rats, untreated OVX rats and OVX rats chronically treated with estrogen. Previously, we showed that estrogen treatment prevented the disruption of PPI induced by the 5-HT_{1A} receptor agonist, 8-OH-DPAT (Gogos and Van den Buuse, 2004), and by the dopamine receptor agonist, apomorphine (Gogos et al., in press). Therefore, this same estrogen replacement level was used here in an attempt to explain the mechanism by which PPI disruption was prevented. Estrogen treatment reversed OVX-induced changes in body weight and uterus weight, similar to our previous studies in rats (Gogos and Van den Buuse, 2004; Gogos et al., in press).

We found differential effects of the three hormonal states on binding densities (Table 2). The most important results

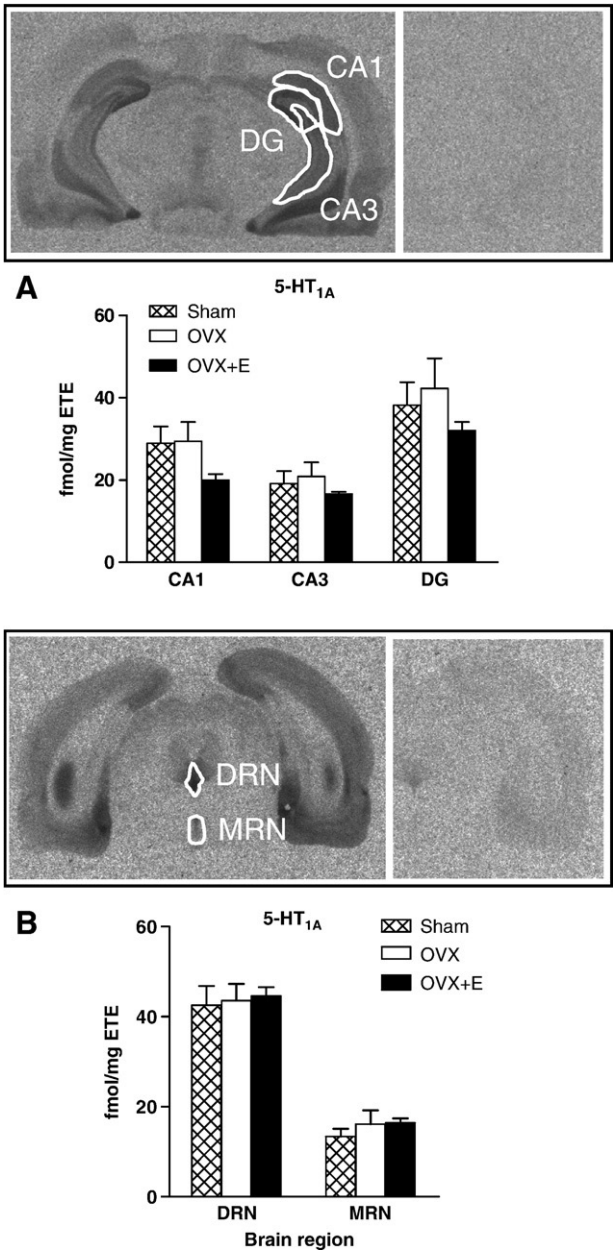

Fig. 3 – Density of serotonin-1A (5-HT_{1A}) receptors in the hippocampus (A) and hindbrain (B) of intact, sham-operated rats (Sham), ovariectomized rats (OVX) and ovariectomized rats treated with estrogen (OVX+E). Brain regions outlined are the CA1, CA3 and dentate gyrus (DG) regions of the hippocampus (top two panels) and the dorsal raphe nucleus (DRN) and median raphe nucleus (MRN) (bottom two panels). Autoradiograms depict total binding and non-specific binding, respectively. There were no group differences in 5-HT_{1A} receptor density. N=5–7 rats per group.

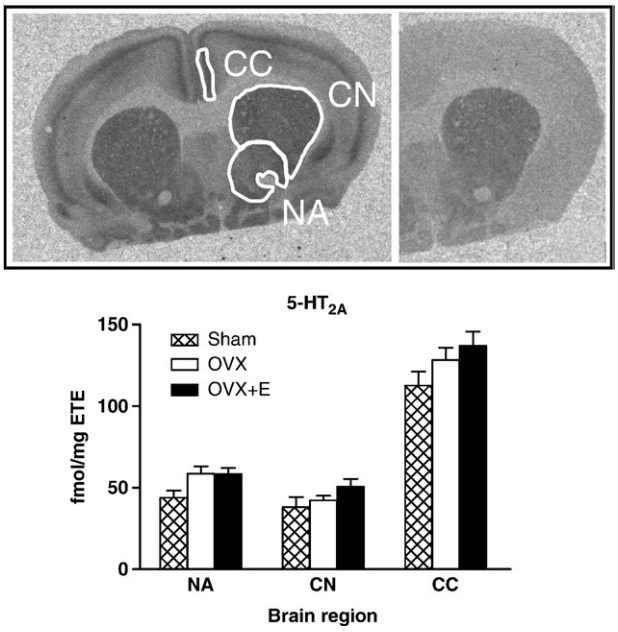

Fig. 4 – Density of serotonin-2A (5-HT_{2A}) receptors in intact, sham-operated rats (Sham), ovariectomized rats (OVX) and ovariectomized rats treated with estrogen (OVX+E). Autoradiograms show total binding and non-specific binding, respectively. Brain regions outlined are the nucleus accumbens (NA), caudate nucleus (CN) and the cingulate cortex (CC). There were no group differences in 5-HT_{2A} receptor density. N=5–7 rats per group.

were a significant reduction of DAT binding in the nucleus accumbens of OVX rats, which was reversed by estrogen treatment. In contrast, dopamine D_2 receptor density was higher in untreated OVX rats than in intact rats in the nucleus accumbens and caudate nucleus and this effect was reversed by estrogen treatment to a level lower than that seen in intact controls. No significant changes were observed in dopamine D_1 receptor levels, SERT levels or 5-HT_{1A} and 5-HT_{2A} receptor levels in any of the brain regions investigated. These results may help to explain some of the functional effects of estrogen treatment in our model on disruption of PPI by activation of 5-HT_{1A} or dopamine receptors.

Table 2 – Summary of the present results and comparison with previous studies on PPI regulation (Gogos and Van den Buuse, 2004; Gogos et al., in press).

Density	Intact	OVX	OVX+E
DAT (nucleus accumbens)	+++	+	+++
Dopamine D_1 receptors	+++	+++	+++
Dopamine D_2 receptors (nucleus accumbens; caudate nucleus)	++	+++	+
SERT	++	++	++
5-HT_{1A} receptors	++	++	++
5-HT_{2A} receptors	++	++	++
8-OH-DPAT effect	PPI disruption	PPI disruption	PPI disruption blocked
Apomorphine effect	PPI disruption	PPI disruption	PPI disruption blocked

Groups: Intact=sham-operated; OVX=ovariectomized; OVX+E=ovariectomized plus estradiol implant. +++, ++ and+indicate relatively high, moderate or low densities of binding, respectively.

The results suggest that the effect of estrogen treatment on 8-OH-DPAT-induced disruption of PPI (Gogos and Van den Buuse, 2004) cannot readily be explained by effects on 5-HT_{1A} receptor density as no changes were observed in [^{3}H]8-OH-DPAT binding in either the hippocampus or raphe nuclei. Although we chose to analyze brain regions with the highest density of 5-HT_{1A} receptors (Chalmers and Watson, 1991; Mengod et al., 2006), it can obviously not be excluded that changes in 5-HT_{1A} receptor density occurred in brain regions which were not included in the present study. Further studies will have to address this possibility. Furthermore, OVX and estrogen treatment may have elicited changes in 5-HT_{1A} receptor signaling rather than density. Previous studies using GTPγS binding have shown that estrogen may uncouple 5-HT_{1A} receptors from their G-proteins both in vivo (Mize and Alper, 2000) and in vitro (Mize et al., 2001) in the hippocampus, but not the frontal cortex (Mize et al., 2003). Such uncoupling would render 8-OH-DPAT less effective to disrupt PPI. Further studies, including more extensive dose–response experiments both in vivo and using GTPγS binding, are needed to address this possibility.

In addition to a lack of effects on 5-HT_{1A} receptor density, there were also no changes in the density of SERT or 5-HT_{2A} receptors. SERT affects the availability of serotonin in the synapse and thus could indirectly influence the effect of 8-OH-DPAT on PPI. Enhanced effects of 5-HT_{1A} receptor stimulation have been described previously in mice with reduced SERT levels (Fox et al., 2007) and estrogen-induced changes in SERT expression (Bertrand et al., 2005; Bethea et al., 2002) could thus have been responsible for the altered responses to 5-HT_{1A} receptor stimulation in our previous studies (Gogos and Van den Buuse, 2004). However, the lack of effect of either OVX or estrogen treatment at either level on SERT density, makes this unlikely. McQueen et al. (1997) also observed no significant changes in SERT binding in the cortex or hippocampus of OVX rats after estrogen treatment (McQueen et al., 1997). On the other hand, previous studies have observed changes in 5-HT_{2A} receptor density in the brain after chronic treatment with sex steroid hormones (Cyr et al., 2000; Fink et al., 1998). In addition, stimulation of the 5-HT_{2A} receptor leads to disruption of PPI (Sipes and Geyer, 1994). However, it is unclear how this is relevant to our previous work, where estrogen treatment blocked PPI disruption by 5-HT_{1A} receptor agonists (Gogos and Van den Buuse, 2004; Gogos et al., 2006b) in the absence of changes in 5-HT_{2A} receptor density.

The greatest changes in binding after OVX or estrogen treatment were observed for DAT and dopamine D_2 receptors, but not dopamine D_1 receptors. This suggests the possibility that estrogen-induced modulation of the effects of 8-OH-DPAT or other 5-HT_{1A} receptor agonists on PPI (Gogos and Van den Buuse, 2004; Gogos et al., 2006a,b) may be mediated by changes in dopaminergic activity. Several previous studies have investigated the effect of estrogen treatment on dopamine receptor density, however there is large variation in the dose, duration of treatment, experimental protocols and methods used (e.g. Bazzett and Becker, 1994; Di Paolo et al., 1988; Gordon and Fields, 1989; Guivarc'h et al., 1995; Zhou et al., 2002). Thus, while it is clear that estrogen interacts with dopaminergic function, the specificity, direction and extent of this interaction critically depend on methodological factors. This is why we used the same estrogen treatment model here as we did in earlier behavioral studies, allowing comparison of the two approaches (Table 2).

An involvement of dopamine D_2 receptors in the action of 8-OH-DPAT on PPI was already suggested by our previous observation that pretreatment with the dopamine D_2 receptor antagonist and antipsychotic, haloperidol, could block its effects (Gogos et al., in press; Van den Buuse and Gogos, 2007). More recent studies in female rats showed, that estrogen treatment could block the disruption of PPI caused by treatment with the dopamine receptor agonist, apomorphine, as well as 8-OH-DPAT (Gogos et al., in press). Apomorphine is an agonist at both dopamine D_1 and D_2 receptors but its effect on PPI in rats is predominantly mediated by dopamine D_2 receptors (Geyer et al., 2001; Swerdlow et al., 1991) although an involvement of dopamine D_1 receptors may become important at higher apomorphine doses (Hoffman and Donovan, 1994). Overall, it appears likely that the effect of 8-OH-DPAT on PPI is mediated by downstream modulation of dopaminergic activity, perhaps via 5-HT_{1A} receptor-mediated changes in dopamine release (Di Matteo et al., 2008) which subsequently activates dopamine D_2 receptors to disrupt PPI (Gogos et al., in press). Estrogen could then modulate the action of 8-OH-DPAT by an effect on dopamine release itself or on either post-synaptic dopamine receptor density or dopamine re-uptake mechanisms. The lack of effect of estrogen on dopamine D_1 receptor density in the present study is consistent with a more prominent role of dopamine D_2 receptors in the effect of apomorphine on PPI. Reduced dopamine D_2 receptor density as found in estrogen treated rats could explain reduced effects of both apomorphine or 8-OH-DPAT in these animals (Gogos and Van den Buuse, 2004; Gogos et al., in press; Van den Buuse and Gogos, 2007). However, it should be noted, that dopamine D_2 receptor density was significantly increased in OVX rats, despite these animals showing no change in their response to either 8-OH-DPAT or apomorphine in these previous studies Thus, the effects of estrogen treatment on dopamine D_2 receptor density still do not provide a straightforward explanation for changes in the action of apomorphine and 8-OH-DPAT on PPI in these animals.

Previous studies have examined the effect of estrogen treatment on DAT density and function and, again, there is substantial variability between studies (e.g. Attali et al., 1997; Bossé et al., 1997; Disshon et al., 1998; Zhou et al., 2002). For the purpose of the present study, it is important to compare the changes in DAT density with the behavioral results obtained in earlier studies (Table 2). It would appear that, similar to dopamine D_2 receptors, the changes in DAT do not correspond to the changes in drug effects in PPI. However, perhaps changes in dopamine D_2 receptor density and DAT density should be considered together. Reduced DAT levels in OVX rats may synergize with the enhanced dopamine D_2 receptor density to lead to a lack of a functional change in PPI regulation in these animals. In contrast, in OVX+E rats, there is no change in DAT density and the reduced dopamine D_2 receptor density in these animals is functionally unopposed and may contribute to the reduced effect of both 8-OH-DPAT and apomorphine in PPI. Clearly this is speculative at this point and further experiments are needed to confirm this.

In conclusion, in the present study we used a chronic estrogen administration model in which we previously

demonstrated functional protection against PPI disruption, at least at the level of 5-HT_{1A} receptor and dopamine D_2 receptor activation. The most important changes currently observed were for dopamine D_2 receptor and DAT density. While the direction of the observed neurochemical effects did not completely overlap with the previously observed behavioral changes, the data suggest that estrogen is more likely to affect dopaminergic function than serotonergic function in this model. These changes could be at least partly responsible for the effects of estrogen on PPI. One shortcoming of the present work is, that we only measured receptor and transporter binding. Further studies are therefore needed to elucidate the relationship between estrogen treatment and functional indices of central dopaminergic activity, including release and receptor signaling, and drug effects on PPI. These studies could help to explain the protective action of estrogen in schizophrenia.

4. Experimental procedures

4.1. *Animals and surgery*

A total of 45 female Sprague–Dawley rats were obtained from the Department of Pathology, University of Melbourne. The rats were housed in groups of two or three in standard rat cages with free access to standard pellet food and water, and maintained on a 12-h light/dark cycle (lights on at 6.30 a.m.), with a constant temperature of 22±2 °C. All surgical techniques, treatments and experimental protocols were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes set out by the National Health and Medical Research Council of Australia.

Animals were 12 weeks of age at the time of surgery (Gogos and Van den Buuse, 2004). Briefly, the animals were anesthetized by isoflurane inhalation and ovariectomized via a skin incision on the back (Gogos and Van den Buuse, 2004; Gogos et al., in press). After removal of the ovaries, the muscle layer was suture-closed and the skin incision closed with surgical staples. During the same procedure, the rats were subcutaneously implanted with silastic hormone implants (E100, see Gogos and Van den Buuse, 2004) at the nape of the neck. The implants were either empty or filled with 100% crystalline estradiol (17β-estradiol, salt; Sigma-Aldrich, St. Louis, MO, USA). In intact, sham-operated animals all steps were conducted apart from removal of the ovaries. Thus, there were three different groups of animals: sham-operated rats (Sham) receiving an empty implant; ovariectomized rats receiving an empty implant (OVX) and ovariectomized rats receiving an estradiol implant (OVX+E) (Table 1). We included these three groups because the implants release estrogen at a steady level and the comparison with the lack of that level in the OVX group is then the most appropriate. We also included intact female rats in our studies to allow comparison with the 'normal' condition. It is not clear what the circulating levels of estrogen in these animals is because they cycle and are unlikely to be all in the same stage of that cycle (Schank, 2001). Cyclical changes in estrogen have been shown to affect receptor levels (see for example Levesque et al., 1989 and Di Paolo et al., 1988). However, because of the random stage of the estrous cycle in the intact group, we did not expect consistent changes in receptor or transporter density. It would be reasonable to assume that, on average, estrogen levels in these animals are likely to be lower than those in the E100 group, as suggested also by the uterus weights (Table 1). At the same time, estrogen levels in the intact group are obviously higher than those seen in OVX rats.

4.2. *Tissue preparation*

The rats were killed by decapitation 4–5 weeks after surgery to coincide with the time interval used in previous behavioral studies (Gogos and Van den Buuse, 2004). The whole brains were removed, frozen over dry ice and stored at −80 °C. At this point, uterus weight was also determined to verify the effectiveness of the OVX procedure and estrogen treatment (Table 1).

Serial coronal 20 μm sections of the brain at relevant levels were cut on a cryostat (Leica CM18-50, Leica Microsystems Nussloch GmbH, Germany) and thaw-mounted onto gelatinised microscope slides. The sections were collected at the level of the nucleus accumbens (bregma 2.28 to 0.96 mm), hippocampus (bregma −5.16 to −5.64 mm) and raphe nuclei (bregma −7.2 to −7.92 mm) (Paxinos and Watson, 1986). The available sections were allocated randomly to different binding assays, such that each binding result had $n=5–7$.

4.3. *Dopamine transporter (DAT) binding*

DAT autoradiography experiments were performed as previously described (Choy and van den Buuse, 2008). Briefly, at room temperature, sections at the level of the nucleus accumbens were pre-incubated in buffer containing 50 mM NaH_2PO4 (Sigma), 70 mM NaCl (Ajax Chemicals, Auburn, Australia) and 0.025% BSA (Bovine Serum Albumin, Sigma) at pH 7.5 for 60 min and then incubated with the binding solutions for 60 min. For total binding the buffer contained 2 nM [^{3}H] GBR12935 (Perkin Elmer, Boston, USA) and 1 μM cis–flupenthixol (Sigma). Adjacent sections were incubated with this buffer in the presence of 10 μM GBR12909 (Sigma) for non-specific binding measurement.

4.4. *Dopamine D_1 receptor binding*

Dopamine D_1 receptor autoradiography was done as previously described (Choy et al., 2009). Briefly, at room temperature, sections at the level of the nucleus accumbens were pre-incubated in buffer containing 50 mM Tris–HCl (Sigma), 120 mM NaCl (Ajax), 5 mM KCl (Ajax), 2 mM $CaCl_2$ (Ajax) and 1 mM $MgCl_2$ (Ajax) at pH 7.4 for 30 min and then incubated with the binding solutions for 30 min. For total binding the buffer contained 1 nM [^{3}H] SCH23390 (Perkin Elmer) with additional 1 μM cis–flupenthixol (Sigma) for non-specific binding.

4.5. *Dopamine D_2 receptor binding*

Dopamine D_2 receptor autoradiography was done as previously described (Choy et al., 2009). The procedure was the same as for D_1 binding except that the sections were incubated with the binding solutions for 60 min. For total binding the

buffer contained 1 nM [^{3}H]YM09151 (Perkin Elmer), 0.1 μM pindolol (Sigma) and 0.5 μM DTG (1,3-di-o-tolylguanidine; Sigma). The non-specific binding buffer contained an additional 10 μM sulpiride (Sigma).

4.6. *Serotonin transporter (SERT) binding*

SERT autoradiography was done as previously described (Bertrand et al., 2005; Kusljic and van den Buuse, 2006). At room temperature, sections at the level of the raphe nuclei and hippocampus were pre-incubated in buffer containing 50 mM Tris–HCl (Sigma) and 120 mM NaCl (Ajax) at pH 7.4 for 15 min and then incubated with the binding solutions for 60 min. The total binding buffer contained 2 nM [^{3}H] citalopram (Perkin Elmer) whereas the non-specific binding buffer contained an additional 10 μM fluoxetine (Sigma).

4.7. *5-HT$_{1A}$ receptor binding*

5-HT$_{1A}$ receptor autoradiography was done as previously described (Gogos et al., 2006a) with slight modifications. At room temperature, sections at the level of the raphe nuclei and hippocampus were pre-incubated for 30 min in buffer containing 170 mM Tris–HCl (Sigma) at pH 7.6 and then incubated with the binding solutions for 60 min. For total binding the incubation buffer contained 1 nM [^{3}H]8-OH-DPAT (Amersham Biosciences, Buckinghamshire, UK). Adjacent sections were incubated in non-specific binding buffer containing an additional 1 μM unlabelled 5-HT (Sigma).

4.8. *5-HT$_{2A}$ receptor binding*

The 5-HT$_{2A}$ receptor autoradiography protocol was modified from previous studies (Dean and Hayes, 1996). Briefly, at room temperature, sections at the level of the hippocampus and nucleus accumbens were pre-incubated in buffer containing 170 mM Tris–HCl (Sigma) at pH 7.7 for 30 min and then incubated with the binding solutions for 60 min. For total binding the incubation buffer containing 10 nM [^{3}H]ketanserin (Perkin Elmer). Adjacent sections were incubated with non-specific binding buffer containing an additional 1 μM spiperone (Sigma).

4.9. *Binding protocol*

In all binding protocols, to ensure the correct concentration of the radioligands, the level of radioactivity was assessed by mixing 50 μl of the respective radioligand with 4 ml of scintillation mixture (Ready Protein$^+$, Liquid Scintillation Cocktail, Beckman Coulter, Fullerton, CA, USA) and using a scintillation counter (Packard 1500 Tri-Carb Liquid Scintillation Analyser). Final concentrations of the total and non-specific binding solutions were measured by scintillation count as well. In all cases, the concentration of the ligand was chosen as around 2–3 the reported Kd of these receptors in these conditions to ensure optimal specific binding (Dean et al., 1999; Pavey et al., 2002).

After incubation, sections were washed, air-dried and partially fixed in paraformaldehyde vapor overnight. After fixation, the slides were apposed to a BAS-TR2025 phosphor imaging plate (Imaging Plate, Fuji Imaging Plates, Berthold Australia) for 6 days except the slides of the SERT binding study which were apposed for 10 days. High and low [^{3}H] micro-scale autoradiography standards (Amersham Biosciences) were included to allow subsequent quantification (Pavey et al., 2002). Autoradiographic images were scanned from the BAS-TR2025 phosphor imaging plate and images were retrieved for analysis using MCID image analysis software (InterFocus, Cambridge, England). Relevant brain regions were outlined on the computer screen and the density of binding was compared to a standard curve obtained from the standard tritium micro-scales. The standard curve allowed for the conversion of photo-stimulated luminescence to disintegration units per min per mg (dpm/mg) estimated tissue equivalent (ETE) (Pavey et al., 2002). Specific binding density was calculated by subtracting the density from the non-specific samples from that of the total binding samples. The numbers were then converted from dpm/mg ETE to fmol/mg ETE.

4.10. *Data analysis*

All data were expressed as mean ± standard error of the mean (S.E.M.). Group differences were assessed using one-way analysis of variance (ANOVA) and subsequent pair-wise comparison with Bonferroni-corrected *t*-test. Differences between groups were considered significant if $P<0.05$.

REFERENCES

Attali, G., Weizman, A., Gil-Ad, I., Rehavi, M., 1997. Opposite modulatory effects of ovarian hormones on rat brain dopamine and serotonin transporters. Brain Res. 756, 153–159.

Bazzett, T.J., Becker, J.B., 1994. Sex differences in the rapid and acute effects of estrogen on striatal D2 dopamine receptor binding. Brain Res. 637, 163–172.

Bertrand, P.P., Paranavitane, U.T., Chavez, C., Gogos, A., Jones, M., van den Buuse, M., 2005. The effect of low estrogen state on serotonin transporter function in mouse hippocampus: a behavioral and electrochemical study. Brain Res. 1064, 10–20.

Bethea, C.L., Pecins-Thompson, M., Schutzer, W.E., Gundlah, C., Lu, Z.N., 1998. Ovarian steroids and serotonin neural function. Mol. Neurobiol. 18, 87–123.

Bethea, C.L., Mirkes, S.J., Su, A., Michelson, D., 2002. Effects of oral estrogen, raloxifene and arzoxifene on gene expression in serotonin neurons of macaques. Psychoneuroendocrinology 27, 431–445.

Bossé, R., Rivest, R., Di Paolo, T., 1997. Ovariectomy and estradiol treatment affect the dopamine transporter and its gene expression in the rat brain. Mol. Brain Res. 46, 343–346.

Braff, D.L., 1993. Information processing and attention dysfunctions in schizophrenia. Schizophr. Bull. 19, 233–259.

Braff, D.L., Geyer, M.A., 1990. Sensorimotor gating and schizophrenia. Human and animal model studies. Arch. Gen. Psychiatry 47, 181–188.

Braff, D.L., Geyer, M.A., Swerdlow, N.R., 2001. Human studies of prepulse inhibition of startle: normal subjects, patient groups, and pharmacological studies. Psychopharmacology 156, 234–258.

Castle, D., Sham, P., Murray, R., 1998. Differences in distribution of ages of onset in males and females with schizophrenia. Schizophr. Res. 33, 179–183.

Chalmers, D.T., Watson, S.J., 1991. Comparative anatomical distribution of 5-HT1A receptor mRNA and 5-HT1A binding in

rat brain: a combined in situ hydridisation/in vitro receptor autoradiography study. Brain Res. 561, 51–60.
Choy, K.H., van den Buuse, M., 2008. Attenuated disruption of prepulse inhibition by dopaminergic stimulation after maternal deprivation and adolescent corticosterone treatment in rats. Eur. Neuropsychopharmacol. 18, 1–13.
Choy, K.H.C., de Visser, Y., Van den Buuse, M., 2009. The effect of 'two hit' neonatal and young-adult stress on dopaminergic modulation of prepulse inhibition and dopamine receptor density. Br. J. Pharmacol. 156, 388–396.
Cyr, M., Landry, M., Di Paolo, T., 2000. Modulation by estrogen-receptor directed drugs of 5-hydroxytryptamine-2A receptors in rat brain. Neuropsychopharmacology 23, 69–78.
Dean, B., Hayes, W., 1996. Decreased frontal cortical serotonin2A receptors in schizophrenia. Schizophr. Res. 21, 133–139.
Dean, B., Pavey, G., Chai, S.Y., Mendelsohn, F.A.O., 1999. The localisation and quantification of molecular changes in the human brain using *in situ* radioligand binding and autoradiography. In: Dean, B., Kleinmann, J.E., Hyde, T.M. (Eds.), Using CNS Tissue in Psychiatric Research — A Practical Guide. Harwood Academic Publishers, Amsterdam, pp. 67–83.
Di Matteo, V., Di Giovanni, G., Pierucci, M., Esposito, E., 2008. Serotonin control of central dopamine function: focus on in vivo microdialysis studies. Prog. Brain Res. 172, 7–44.
Di Paolo, T., 1994. Modulation of brain dopamine transmission by sex steroids. Rev. Neurosci. 5, 27–42.
Di Paolo, T., Falardeau, P., Morissette, M., 1988. Striatal D-2 dopamine agonist binding sites fluctuate during the rat estrous cycle. Life Sci. 43, 665–672.
Disshon, K.A., Boja, J.W., Dluzen, D.E., 1998. Inhibition of striatal dopamine transporter activity by 17beta-estradiol. Eur. J. Pharmacol. 345, 207–211.
Fink, G., Sumner, B.E.H., McQueen, J.K., Wilson, H., Rosie, R., 1998. Sex steroid control of mood, mental state and memory. Clin. Exp. Pharmacol. Physiol. 25, 764–775.
Fox, M.A., Jensen, C.L., Gallagher, P.S., Murphy, D.L., 2007. Receptor mediation of exaggerated responses to serotonin-enhancing drugs in serotonin transporter (SERT)-deficient mice. Neuropharmacology 53, 643–656.
Geyer, M.A., Swerdlow, N.R., 1998. Measurement of startle response, prepulse inhibition, and habituation. In: Crawley, J.N., Skolnick, P. (Eds.), Current Protocols in Neuroscience. John Wiley & Sons, New York, pp. 8.7.1–8.7.15.
Geyer, M.A., Krebs-Thomson, K., Braff, D.L., Swerdlow, N.R., 2001. Pharmacological studies of prepulse inhibition models of sensorimotor gating deficits in schizophrenia: a decade in review. Psychopharmacology 156, 117–154.
Gogos, A., Van den Buuse, M., 2004. Estrogen and progesterone prevent disruption of prepulse inhibition by the serotonin-1A receptor agonist 8-OH-DPAT. J. Pharmacol. Exp. Ther. 309, 267–274.
Gogos, A., Martin, S., Jones, M.E., Van den Buuse, M., 2006a. Oestrogen modulation of the effect of 8-OH-DPAT on prepulse inhibition: effects of aromatase deficiency and castration in mice. Psychopharmacology 188, 100–110.
Gogos, A., Nathan, P.J., Guille, V., Croft, R.J., Van den Buuse, M., 2006b. Estrogen prevents 5-HT_{1A} receptor-induced disruptions of prepulse inhibition in healthy women Neuropsychopharmacology 31, 885–889.
Gogos, A., Kwek, P., Chavez, C.A., Van den Buuse, M., in press. Estrogen treatment blocks 8-OH-DPAT- and apomorphine-induced disruptions of prepulse inhibition: involvement of dopamine and serotonin receptors. J. Pharmacol. Exp. Ther. doi:10.1124/jpet.109.162123.
Gordon, J.H., Fields, J.Z., 1989. A permanent dopamine receptor up-regulation in the ovariectomized rat. Pharmacol. Biochem. Behav. 33, 123–125.
Guivarc'h, D., Vernier, P., Vincent, J.D., 1995. Sex steroid hormones change the differential distribution of the isoforms of the D_2 dopamine receptor messenger RNA in the rat brain. Neuroscience 69, 159–166.
Häfner, H., 2003. Gender differences in schizophrenia. Psychoneuroendocrinology 28, 17–54.
Hoffman, D.C., Donovan, H., 1994. D_1 and D_2 dopamine receptor antagonists reverse prepulse inhibition deficits in an animal model of schizophrenia. Psychopharmacology 115, 447–453.
Kulkarni, J., Riedel, A., de Castella, A.R., Fitzgerald, P.B., Rolfe, T.J., Taffe, J., Burger, H., 2001. Estrogen — a potential treatment for schizophrenia. Schizophr. Res. 48, 137–144.
Kulkarni, J., de Castella, A., Fitzgerald, P.B., Gurvich, C.T., Bailey, M., Bartholomeusz, C., Burger, H., 2008. Estrogen in severe mental illness: a potential new treatment approach. Arch. Gen. Psychiatry 65, 955–960.
Kusljic, S., van den Buuse, M., 2006. Differential involvement of 5-HT projections within the amygdala in prepulse inhibition but not in psychotomimetic drug-induced hyperlocomotion. Behav. Brain Res. 168, 74–82.
Levesque, D., Gagnon, S., Di Paolo, T., 1989. Striatal D1 dopamine receptor density fluctuates during the rat estrous cycle. Neurosci. Lett. 98, 345–350.
McQueen, J.K., Wilson, H., Fink, G., 1997. Estradiol-17 beta increases serotonin transporter (SERT) mRNA levels and the density of SERT-binding sites in female rat brain. Mol. Brain Res. 45, 13–23.
Mengod, G., Vilaró, M.T., Cortés, R., López-Giménez, J.F., Raurich, A., Palacios, J.M., 2006. Chemical neuroanatomy of 5-HT receptor subtypes in the mammalian brain. In: Roth, B.L. (Ed.), The Serotonin Receptors: From Molecular Pharmacology to Human Therapeutics. Humana Press, Totowa, NJ, pp. 319–364.
Mize, A.L., Alper, R.H., 2000. Acute and long-term effects of 17β-estradiol on $G_{i/o}$ coupled neurotransmitter receptor function in the female rat brain as assessed by agonist-stimulated [^{35}S]GTPγS binding. Brain Res. 859, 326–333.
Mize, A.L., Poisner, A.M., Alper, R.H., 2001. Estrogens act in rat hippocampus and frontal cortex to produce rapid, receptor-mediated decreases in serotonin 5-HT_{1A} receptor function. Neuroendocrinology 173, 166–174.
Mize, A.L., Young, L.J., Alper, R.H., 2003. Uncoupling of 5-HT1A receptors in the brain by estrogens: regional variations in antagonism by ICI 182, 780. Neuropharmacology 44, 584–591.
Pavey, G.M., Copolov, D.L., Dean, B., 2002. High-resolution phosphor imaging: validation for use with human brain tissue sections to determine the affinity and density of radioligand binding. J. Neurosci. Methods 116, 157–163.
Paxinos, G., Watson, C., 1986. The Rat Brain in Stereotaxic Co-ordinates2nd edition. Academic Press, New York. Vol.
Schank, J.C., 2001. Do Norway rats (*Rattus norvegicus*) synchronize their estrous cycles? Physiol. Behav. 72, 129–139.
Seeman, M.V., 1997. Psychopathology in women and men: focus on female hormones. Am. J. Psychiatry 154, 1641–1647.
Sipes, T.A., Geyer, M.A., 1994. Multiple serotonin receptor subtypes modulate prepulse inhibition of the startle response in rats. Neuropharmacology 33, 441–448.
Stevens, J.R., 2002. Schizophrenia: reproductive hormones and the brain. Am. J. Psychiatry 159, 713–719.
Swerdlow, N.R., Keith, V.A., Braff, D.L., Geyer, M.A., 1991. Effects of spiperone, raclopride, SCH 23390 and clozapine on apomorphine inhibition of sensorimotor gating of the startle response in the rat. J. Pharmacol. Exp. Ther. 256, 530–536.
Van den Buuse, M., Gogos, A., 2007. Differential effects of antipsychotic drugs on serotonin-1A receptor mediated disruption of prepulse inhibition. J. Pharmacol. Exp. Ther. 320, 1224–1236.
Zhou, W., Cunningham, K.A., Thomas, M.L., 2002. Estrogen regulation of gene expression in the brain: a possible mechanism altering the response to psychostimulants in female rats. Brain Res. Mol. Brain Res. 100, 75–83.