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# Ovariectomy Drives Asynchronous Changes in Serotonin Receptor 2A and Transporter Availability in Rats

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## Abstract

Ovarian hormones have potent effects on key features of the serotonergic neurotransmission. This includes effects that may counterbalance each other, e.g. postsynaptic receptor changes and change in serotonin transporter (SERT) levels, which regulates synaptic serotonin. Such mechanisms may be implicated in the heightened risk for depressive episodes seen in perimenopause or postpartum. However, to what extent transition to hypogonadism in it-self drives such serotonin related risk mechanism remains elusive. Here we evaluate if ovarian hormone withdrawal affects postsynaptic serotonin receptor 2A ($5\text{-HT}_{2A}$) and presynaptic SERT availability differently across the early withdrawal phase in ovariectomized (OVXed) relative to sham operated rats. Cortical (prefrontal cortex (PFC)) and subcortical (striatum) $5\text{-HT}_{2A}$ receptor and SERT binding were quantified with autoradiography at 8 and 23 days after ovariectomy (OVX). We observed that 8 and 23 days after withdrawal the level of $5\text{-HT}_{2A}$ receptor binding was decreased relative to sham, while SERT binding was unaltered, however, with a weak trend to decrease at day 23. The dataset available is small and the results should be viewed as preliminary. If replicated, these data highlight a potential phase-specific and predominantly early challenge of serotonergic neurotransmission when ovarian hormone levels decline abruptly, which may translate to the risk for psychopathology, e.g. depressive episodes postpartum or during menopausal transition in humans.

**Keywords:** Ovarian hormones; Estradiol; $5\text{-HT}_{2A}$; 5-HTT; Mood disorder; SERT

## Introduction

Epidemiological studies support an increased risk for developing major depression during phases in women's lives, where ovarian hormones decline rapidly or fluctuate, e.g across the pre- to postpartum and menopausal transition [1,2]. Whether changes in ovarian hormones, estradiol and progesterone, drives pathophysiological mechanisms that may elicit depressive symptoms remains elusive. Nevertheless, one factor considered to play a critical role in depression is an imbalance in serotonergic neurotransmission. Interestingly, it has become evident that ovarian hormones have effects on key components of the serotonergic system [3]. In particular, estradiol is thought to affect serotonin signalling via nuclear or membrane-bound estrogen receptors (ER) expressed within the central nervous system (CNS),,including serotonergic neurons, by changing gene expression of the serotonin transporter (SERT) and/or serotonin receptors [3-5].

Current treatments for major depression, including selective serotonin reuptake inhibitors (SSRIs), are inadequate and show delayed onset of action. Intriguingly, estradiol replacement therapy both postpartum and during perimenopause has been reported to alleviate depressive symptoms with a fast onset of action [6]. However, an optimal timing of estradiol replacement therapy in this context appears to be critical for the efficacy. If initiated in early menopause, cognitive functions in terms of verbal memory is preserved, conversely, if initiated when menopause is fully established, no effect or possibly even detrimental effects are observed [6]. Accordingly, it is critical to address the early phase of ovarian hormone withdrawal and characterize the impact on key markers of serotonergic neurotransmission that may be sensitive to ovarian hormone deprivation.

Most animal studies addressing effects of ovarian hormones on the serotonin system are based on an experimental paradigm of short-term ovariectomy (OVX) and acute estradiol, and progesterone in some cases, replacement with ovariectomized (OVXed) control animals [4,7,8]. However, the effect of OVX, i.e. hypogonadism, *per se* on serotonin regulation, and the impact of total time of estradiol deprivation have yet to be elucidated. In rodents and non-human primates, estradiol administration potentially facilitates serotonin signalling by increasing tryptophan hydroxylase activity [9], the key enzyme in serotonin synthesis, decreasing degradation of serotonin by decreasing the availability of monoamine oxidase (MAO) [10], decreasing auto-inhibition via serotonin receptor 1A ($5\text{-HT}_{1A}$) availability [11,12], and by increasing postsynaptic serotonin receptor 2A ($5\text{-HT}_{2A}$) availability [4,13]. However, this may be counterbalanced by the estradiol-induced increase in serotonin SERT gene expression in the raphe [8] and binding levels [14] predominantly in cortical projection areas [7], which in turn would lower synaptic serotonin. Nevertheless, the timing and relative contributions from post- and presynaptic features of such serotonergic integration of ovarian hormone information are far from clear. We hypothesise, that ovarian hormone withdrawal transiently compromise serotonin signalling by decreasing $5\text{-HT}_{2A}$ receptor levels, which may be counterbalanced by decreases in SERT across the early withdrawal phase 8 to 23 days after OVX.

## Materials and Methods

### Rats

Twenty-two Sprague-Dawley female rats (210-290 g) were

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purchased from Charles River (Charles River Laboratories International, Germany). Rats were housed in standard cages in the animal facilities, acclimatized, and maintained under conditions of 12-hour light/dark cycle (light on at 7:00 AM-7:00 PM) with access to water and standard chow ad libitum. All animal procedures were carried out in accordance with the regulations provided by the Danish Animal Experimentation Inspectorate (J.No. 2012-15-2934-00156) and treated in concordance with the European Communities Council Directive of 24th November 1986 (86/609ECC).

### Experiment 1

Six OVXed and six sham-OVXed rats were pre-ordered and delivered to our facility from Charles River, Laboratories International, Germany 3 days after surgery. During surgery, rats were anaesthetized with ketamine (43 mg/kg) and xylazine (8.7 mg/kg) and received buprenorphine (0.05 mg/kg). These rats were decapitated 8 days after the surgery, which corresponds to the very early ovarian hormone withdrawal phase.

### Experiment 2

Five rats were bilaterally OVXed and five rats were sham-OVXed at our facility using procedures similar to those applied in experiment 1. Rats were anaesthetized with a hypnorm-midazolam solution containing 2 ml hypnorm (0.315 mg fentanyl citrate, 10 mg fluanisone, 0.5 mg methyl parahydroxybenzoate, 0.05 mg propyl parahydroxybenzoate), 2 ml midazolam (5 mg/ml) and 4 ml sterile water (3 ml/kg, supplemented 1.5 ml/kg after 25 min) and received the analgesia carprofen (1 ml/kg Rimadyl; Orion Pharma Animal Health, Denmark). After surgery, these rats received carprofen (1 ml/kg) and buprenorphine (1 ml/kg Temgesic®), and were decapitated 23 days after surgery.

Successful OVX was confirmed with uterine weights and luteinizing hormone (LH) measurements (data not shown). In experiment 2, two outliers were removed; one shamOVX rat due to outlier in LH level and one OVXed rat due to remaining ovarian tissue leaving 4 rats available for analyses in each group.

### *In vitro* autoradiography

Autoradiography was performed separately on different imaging plates for experiment 1 and 2. Rats were sacrificed and brains removed and stored at -80°C until further processing. Frozen brains were sectioned in 12µm serial sections through the prefrontal cortex (PFC) and striatum. For detection of SERT binding, sections were pre-incubated for 20 min in 50 mM Tris-HCl buffer with 120 mM NaCl and 5 mM KCl, pH 7.4 and subsequently incubated for 60 min with 4 nM [$^3$H]-escitalopram (Specific activity 79 Ci/mmol, kindly supplied by H. Lundbeck A/S, Copenhagen, Denmark) diluted in the same buffer. Non-specific binding (NSB) was determined in the presence of 10µM paroxetine (kindly provided by GlaxoSmithKline, UK). After incubation, the sections were washed 3 × 2 min in ice-cold 50 mM Tris-HCL buffer at pH 7.4, and 1 × 10 sec in ice-cold distilled water ($dH_2O$).

For detection of 5-$HT_{2A}$ receptor binding sites, sections were pre-incubated with 50 mM Tris-HCL buffer (pH 7.4) for 15 min under constant gentle shaking. The 5-$HT_{2A}$ receptor binding was determined with [$^3$H]-MDL100907 (specific activity; 64 Ci/mmol, courtesy of Prof. Christer Halldin, Karolinska Institute, Stockholm, Sweden) and NSB in the presence of 10µM ketanserin tartrate (Sigma-Aldrich, USA).

All sections were fixed in paraformaldehyde vapor at 4°C overnight, and then transferred to an exicator box at room temperature for 3 hours before slides were exposed to a BAS-TR2040 Imaging Plate in a BAS-TR2040 TR-imaging plate cassette (Science Imaging Scandinavia AB, Nacka, Sweden) together with [$^3$H]-microscales (GE Healthcare, UK) for 7 days at 4°C. After exposure, imaging plates were scanned in a BAS-2500 scanner (Fujifilm Europe GmbH, Düsseldorf, Germany) at Resolution 50, Gradation (max) 16 bit and Dynamic Range Selector L5 S30000, after which autoradiograms were analysed with Quantity One 1-D Analysis Software (BioRad, USA). Image optical density was determined in the areas of interest in at least two neighbouring sections from each animal. The optical densities were converted to activity density in nCi/mg tissue equivalents (TE) using the [$^3$H]-microscales and converted to fmol/mg TE using the specific activity of the ligands. The specific receptor binding was determined by subtracting NSB from the total radioactivity in each animal, and the brain regional binding were analysed with Student's *t*-test. In the 8-day sham-OVXed group one 5-$HT_{2A}$ receptor measure was omitted due to technically suboptimal autoradiography. Data are reported as mean ± SEM; differences were considered statistically significant only if $P<0.05$.

## Results

5-$HT_{2A}$ receptor binding was significantly decreased with 15% 8 days after OVX relative to sham controls (P=0.02). This effect appeared to sustain at least at a borderline significant level up to 23 days after OVX (P=0.05) with a 13.5% decrease in PFC (Figure 1). In the same animals, SERT binding in PFC did not differ significantly at day 8 (P=0.65) or 23 (P=0.21) in the OVX group compared to sham (Figure 2A and 2B). In striatum, SERT was also unchanged both at day 8 (p=0.45) and at day 23 (P=0.09) however, if anything, showed a weak trend to decrease at day 23 (Figure 2C and 2D).

## Discussion

We demonstrate here that while 5-$HT_{2A}$ receptor availability in PFC is decreased after 8 days of ovarian hormone withdrawal, and with borderline significance until day 23, SERT availability remains unchanged in PFC and striatum 8 and 23 days after OVX.

Our study consists of two independent experiments, one for each time point. For each experiment, autoradiography was performed simultaneously, and control groups undergoing identical transport, housing and surgery procedures enable us to isolate changes in SERT and 5-$HT_{2A}$ arising from changes in ovarian hormone levels at each time point. Our data suggest asynchronous changes in post- and presynaptic features of serotonergic neurotransmission in the early phase of ovarian hormone deprivation. The combination of decreased 5-$HT_{2A}$ receptor capacity and sustained SERT levels, indicate that serotonergic neurotransmission is transiently challenged in prefrontal projection areas in the early phase of ovarian hormone deprivation. However, at a later time point (23 days) we cannot exclude from our experiments that SERT down-regulates since a weak trend was observed in striatum. We interpret with caution but note, that if replicated, this phenomenon would support a compensatory mechanism, which would tend to facilitate serotonergic signalling in the context of decreased receptor availability. This study is the first to evaluate 5-$HT_{2A}$ receptor binding responses to direct ovarian hormone withdrawal in the early phase (8 days after OVX). However, this is well in line with Cyr et al. [15] who showed both cortical and subcortical decreases in 5-$HT_{2A}$ receptor binding 2 weeks after OVX. Our data suggest that the effect attenuates 23 days post OVX. Studies in the opposite direction, i.e. estradiol replacement, support that estradiol drives brain 5-$HT_{2A}$ receptor expression; 5-$HT_{2A}$ receptor binding increases globally both in rats [3,4] and after hormonal replacement in postmenopausal women as seen with *in vivo* molecular imaging [16,17]. In addition to the serotonergic

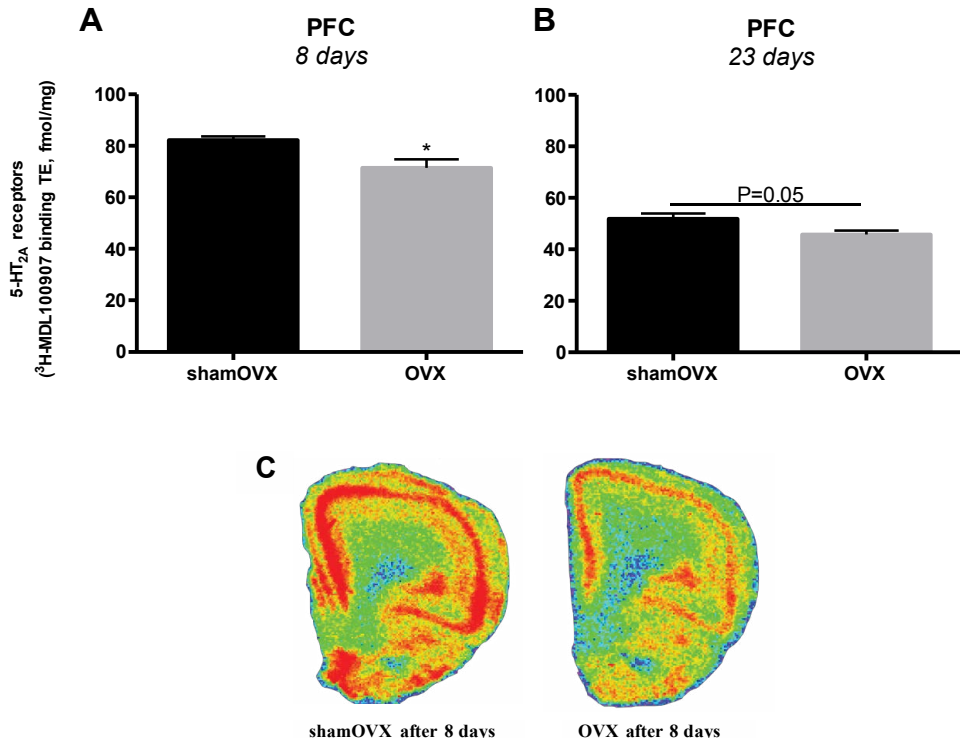


**Figure 1**. 5-$HT_{2A}$ receptor binding sites detected with [$^3$H]-MDL100907 in prefrontal cortex (PFC) were significantly decreased 15% 8 days after ovariectomy (OVX) (71.4 ± 3.4 fmol/mg tissue equivalents (TE)) relative to shamOVX (82.19 ± 1.5 fmol/mg TE) (Panel A). 23 days after OVX, [$^3$H]-MDL100907 binding was decreased 13.5% with borderline significance (45.7 ± 1.6 fmol/mg TE) relative to shamOVX (51.89 ± 2.0 fmol/mg TE) (Panel B). (C) Representative autoradiograms of 8 days shamOVX and OVX. The bars represent mean ± SEM (day 8: n=5/6 and day 23: n=4/4), *P<0.05 using Students *t*-test.

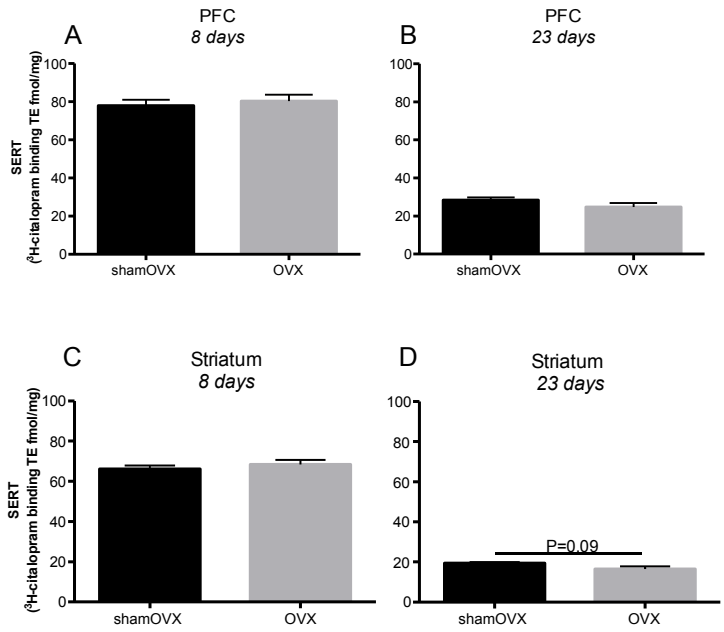


**Figure 2:** SERT binding sites detected with [$^3$H]-escitalopram in prefrontal cortex (PFC) were unchanged 8 days (Panel A) and 23 days (Panel B) after ovariectomy (OVX) (80.2 ± 3.5 fmol/mg tissue equivalents (TE) and 24.7 ± 2.2 fmol/mg TE respectively) compared to shamOVX groups (78.0 ± 3.1 fmol/mg TE and 28.4 ± 1.4 fmol/mg TE respectively). In striatum, SERT binding was unchanged 8 days after OVX (68.4 ± 2.2 fmol/mg TE) compared to shamOVX (66.1 ± 1.8 fmol/mg TE) (Panel C) and 23 days after OVX (16.5 ± 1.4 fmol/mg TE) relative to shamOVX (19.4 ± 0.5 fmol/mg TE) (Panel D). The bars represent mean ± SEM (day 8: n=6/6 and day 23: n=4/4), P=0.09 using Students *t*-test.

system, dopaminergic, GABAergic and glutamatergic neural circuits as well as hippocampal plasticity are also sensitive to ovarian hormones, showing complex and bidirectional effects depending on the dose, timing, and the duration of exposure (reviewed in ref. (18)). This further substantiates the hypothesis that ovarian hormone-dependent changes, which also implicate serotonergic neurotransmission in brain regions associated with depression, may transiently increase susceptibility to depression. Notably in support of this, chronic estradiol treatment initiated at the time of OVX reduced anxiety and depression-like behaviour as opposed to when administered after long-term ovarian hormone withdrawal (5 months after OVX) in female rats [19]. Also, this resonates well with why the timing of initiation of hormone replacement therapy in e.g. menopausal transition is critical, e.g. in order to preserve verbal memory [6].

In macaques, estradiol *replacemen*t with or without progesterone reduced SERT mRNA after 28 days [20]. However, also long-term (3 years) withdrawal of ovarian hormones was observed to reduce SERT mRNA expression [21], indicating that ovarian hormones may have bidirectional effects on SERT mRNA expression dependent on the timing and duration of ovarian hormone changes. The present study is in-line with the hypothesis that SERT binding may decrease in later phases of ovarian hormone withdrawal, relative to the earlier phases.

Interestingly, gene expression in blood from women in late pregnancy links a specific set of genes to ER function as predictors of later development of postpartum depression [2], which highlights possible adverse mechanisms coupled to placenta hormone exposure, including estradiol, that peaks in late pregnancy. Potentially, withdrawal subsequent to elevated ovarian hormone levels may be particularly adverse. Studies in rodents and non-human primates suggest that *exposure* to high estradiol increases SERT gene expression and protein levels [4,7,8]. Whereas, in response to short-term ovarian hormone *deprivation*, we here demonstrate that 5-$HT_{2A}$ receptor availability decreases without compensatory SERT changes. We thus speculate that the combination of estradiol exposure, which heightens SERT, and subsequent ovarian hormone withdrawal, would be particularly potent in compromising serotonergic neurotransmission.

Estradiol seems to be the main contributor to serotonergic effects observed after ovarian hormone withdrawal [3]; however, potential progesterone-withdrawal driven effects may also play a role. No study has investigated the effect of progesterone alone on 5-$HT_{2A}$. But for SERT function, it has been shown that both progesterone and estradiol affect SSRI-induced blocking of serotonin clearance from the synapse [22]. This phenomenon is possibly linked to estradiol and progesterone cancelling of antidepressant-like actions of SSRIs in rat behavioural models, which for estradiol appears to be ERα mediated [23]. Importantly, only estradiol affected serotonin clearance from the synapse in the absence of SSRI and this effect, mediated through the ERβ and GPR30, resulted in reduced serotonin clearance from the synapse [22,23], thereby increasing serotonergic tonus. Future studies are needed to determine the differential contributions from ovarian hormones in the effects on SERT and 5-$HT_{2A}$ of ovarian hormone withdrawal.

Previous studies evaluating effects of ovarian hormones on the serotonin system mainly use estradiol (with or without progesterone) replacement after short-term OVX with OVX as reference. The present study emphasises that OVX in itself most likely have consequences for serotonergic neurotransmission. This raises the question whether OVX is an appropriate control of the baseline condition in studies addressing serotonin related mechanisms of ovarian hormone fluctuations.

In conclusion, postsynaptic 5-$HT_{2A}$ receptor levels are decreased in the early phase of ovarian hormone withdrawal, while SERT levels are unaltered. At later time-points, a compensatory SERT down-regulation cannot be excluded. If replicated, our data highlight a phase-specific, transient and predominantly early challenge of serotonergic neurotransmission in response to ovarian hormone withdrawal, which represents a plausible mechanism by which risk for psychopathology, e.g. depressive episodes, may be heightened during menopausal transition or early postpartum. Thus, it provides a rationale for testing preventive strategies, e.g. in the immediate postpartum period or early in menopausal transition in humans.

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