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Differential effects of chronic 17β-estradiol treatment on rat behaviours relevant to depression

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Abstract

Sex differences are a prominent feature of the pathophysiology of psychiatric disorders, such as major depressive disorder which affects women at a higher incidence than men. Research suggests that the most potent endogenous estrogen, 17β-estradiol, may have therapeutic potential in treating depression. However, preclinical studies have produced mixed results, likely due to various methodological factors such as treatment duration. This study aimed to investigate the effects of ovariectomy and chronic 17β-estradiol treatment via a subcutaneous silastic implant on behaviours relevant to depression in adult female Sprague-Dawley rats. Rats were assessed in the forced swim test, saccharin preference test, novel object recognition memory test and for possible confounding behaviours, including locomotion and anxiety (open field test) and motivation and anxiety (novelty supressed feeding test). Treatment effects were verified using body and uterus weight, as well as serum concentrations of 17β-estradiol, progesterone and testosterone. Compared to ovariectomised rats, chronic 17β-estradiol treatment enhanced saccharin preference and novel object recognition performance. There were no group differences in passive or active coping behaviour when assayed using the forced swim test. Taken together, these results support an antidepressant-like action of estrogens but highlight that the beneficial effects of chronic 17β-estradiol treatment may be related to specific depression-related symptoms, particularly anhedonia and memory.

Keywords

Female rats; ovariectomy; forced swim test; novel object recognition; sucrose preference; locomotor; novelty supressed feeding; estrogen

1. Introduction

Major depressive disorder (MDD) is classified as an affective disorder typified by persistent sadness, a loss of interest in previously enjoyable activities and cognitive deficits including impairments in attention, learning and memory [1]. It is widely accepted that MDD is more prevalent in women than in men [2, 3]. Whilst this difference may be related to psychosocial and cultural factors, it is likely that neurobiological sex differences play an important role. In women, risk of developing a depressive illness appears to increase during key periods of the reproductive lifecycle when levels of estrogens and progesterone are changing: during puberty, postnatal period, perimenopause and postpartum [4, 5]. Strong links between fluctuations in mood and sex hormones have been reported [6, 7]. Additionally, the effects that estrogens have on cognition, particularly learning and memory, are well-established [8]. Popular theory posits that 17β-estradiol, the most potent of the endogenous estrogens, may be neuroprotective, and that the absence of this protection as a result of low estradiol levels may increase vulnerability to psychiatric disturbance [7, 9]. The notion that estradiol is protective against depression is supported by studies wherein perimenopausal women with a depressive disorder had significantly more remissions after treatment with transdermal 17β-estradiol [10].

Preclinical investigations targeting the potential antidepressant role of estrogens in depression have produced mixed results, likely due to various methodological factors. The forced swim test (FST) is the most widely used rodent assay of depressive-like behaviour, employed predominantly as a screening tool for antidepressants [11, 12]. The literature generally supports the notion of a protective role of estrogens in the FST. For example, some studies, but not all [13], show that intact female rats display a reduction in depressive-like behaviour, i.e. reduced immobility, during the proestrus (high estradiol) phase compared to other phases of the estrous cycle or to male rats [14-

[16]. By contrast, in ovariectomised (OVX) rats, where removal of ovaries results in depletion of endogenous ovarian hormone production, studies report increased immobility [17-19]; however this is dependent on the duration post-OVX [17]. The OVX-induced effects in FST were able to be reversed both by acute 17β-estradiol injection [17] and by daily 17β-estradiol injections for 7 days [19] or 26 days [20].

The sucrose (or saccharin) preference test (SPT) is a well-established behavioural measure of anhedonia, the loss of ability to experience pleasure [11, 21]. Few studies have investigated the role of estrogens in the SPT; however, one reported that acute treatment with estradiol valerate increased sucrose preference in middle-aged, stressed OVX rats [22]. However, the effect of OVX and subsequent chronic estradiol treatment in rodents has, to the best of our knowledge, yet to be investigated in the SPT.

Cognitive deficits in subjects with MDD may persist even after remission of affective symptoms [23]. In preclinical studies, increased endogenous levels of estradiol or exogenously administered estradiol have been shown to enhance performance on memory tasks that rely on the hippocampus [8, 24-27]. In the present study, we used the novel object recognition test (NORT) which measures recognition memory and is dependent on hippocampal circuitry and the frontal cortex [28]. The NORT is advantageous relative to other rodent memory tests because it does not require external motivation, reward or punishment and little training is required [29]. NORT performance can vary across the estrous cycle in rats; significantly more time was spent exploring a novel object during the proestrus phase compared to metestrus (low estradiol) [30-32]. OVX rats were found to have reduced recognition memory in the NORT when compared to intact controls [33], although the period of time post-surgery influences this [30]. The beneficial effects of acute 17β-estradiol treatment in OVX rats in the NORT has been demonstrated consistently [27, 32, 34, 35], however little is known about the effects of chronic 17β-estradiol treatment on adult OVX rats [30]. One study examined chronic 17β-estradiol treatment in adult OVX rats and found that there was no improvement in NORT performance compared to chance performance [36], while another found that chronic 17β-estradiol treatment reversed a drug-induced reduction in NORT performance in OVX rats [37].

Overall, there is evidence estrogens can improve depressive-like symptoms in rodents. Mixed results are likely due to various factors such as dose and type of estrogen administered, timing of estrogen administration, interaction with other hormones and

the specific task being tested [8, 17, 38]. Treatment duration is of particular importance as it not only alters experimental outcomes, but also likely reflects estradiol's action via genomic versus non-genomic mechanisms and the associated different estrogen receptors involved [9, 39]. Further, a chronic treatment regimen, as will be used in the present study, more closely mirrors the clinical setting of estradiol being used as a therapeutic agent. Few studies have investigated the effect of chronic treatment with 17β-estradiol on depressive-like symptoms and none have investigated chronic 17β-estradiol treatment to OVX rats in SPT. Further, we attempt to address some of the methodological limitations of previous studies by confirming serum hormone levels and by using a subcutaneous estradiol-filled implant rather than stressful daily injections. We aimed to investigate depressive-like behaviours by measuring five behaviours in rats, by the same experimenters in the same laboratory meaning there were consistent conditions. This is important because various environmental conditions, such as housing, are known to affect behaviour [40]. Thus, in the present study, we used our established chronic 17β-estradiol treatment model in rats [41-43] and measured three widely-used, validated behaviours relevant to depression: SPT, FST, NORT. We also measured activity in the open field test [44] and the novelty suppressed feeding test [45] in order to evaluate whether locomotion, anxiety and motivation are implicated in the effects of chronic 17β-estradiol treatment on depressive-like behaviour.

2. Methods

2.1. Animals

Two cohorts of adult (11 - 12 weeks of age) female Sprague-Dawley rats (Animal Resources Centre, WA, Australia) weighing 230-295 g at the time of surgery were used in this study (n = 27 and n = 30 respectively). Rats were housed in open-top cages, 2-3 per cage, in a temperature-controlled room (20-22°C) on a 12-hour light cycle (lights on 0600). Food and water were available *ad libitum*. Rats were acclimatised and handled regularly for one week prior to beginning experimentation.

All procedures were approved by the Florey Institute's animal ethics committee and carried out in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (1990) set out by the National Health and Medical Research Council of Australia, as well as the institutional guidelines for ethical animal care.

2.2. Ovariectomy and chronic 17β-estradiol treatment

Rats in both cohorts were randomly assigned to become sham-operated/intact (INT), ovariectomised and vehicle-treated (OVX), or OVX and 17β-estradiol-treated (EST; n = 9/10 per group). Surgical procedures were performed as reported previously [41]. In brief, rats were anaesthetised with an isoflurane/oxygen gas mixture and injected subcutaneously with the analgesic, anti-inflammatory agent, carprofen (5 mg/kg; Rimadyl®; Heriot AgVet, VIC, Australia). A small midline incision was made through the skin above the lower back, followed by an incision through the abdominal wall. The ovaries were bilaterally located and removed and the incisions closed. Intact rats were sham-operated; they received all procedures except that the ovaries were not removed. During surgery, rats received a subcutaneous silastic implant that was prepared as described previously [46, 47]. Briefly, implants were either empty (INT and OVX groups) or filled with 100% crystalline 17β-estradiol (EST group; 5 mm implants containing ~25 mg per implant; Sigma-Aldrich; MO, USA). These 17β-estradiol implants were based on our previous dose-finding studies and were aimed at producing pharmacologically active doses akin to levels reached during the proestrus phase [41, 47].

2.3. Experimental protocol

Cohort 1: During the surgical procedure, rats had blood samples of ~0.8 ml collected from the lateral tail vein ('baseline' measurement). Two weeks following surgery, rats were lightly anaesthetised and up to 0.8 ml blood was again collected from the lateral tail vein. One week later, rat behaviour was measured in the open field test (OFT) and two weeks later, the NORT. One week later (~6 weeks post-surgery), rats were euthanized and during this procedure, up to 8 ml of cardiac blood was collected. *Cohort 2:* Nine days following surgery, rats began the 6-day protocol for the SPT.

Three days following completion of the SPT, the novelty supressed feeding test (NSFT) was conducted after an overnight food deprivation. Three days later, the rats began the 2-day FST protocol. Upon completion of experiments, rats were euthanized. Testing was conducted by cage. In cohort 2, there were 10 cages of rats; each cage contained 3 rats, 1 from each group (1 INT, 1 OVX, 1 EST).

All behavioural testing was conducted between 0930 and 1630. For OFT, NORT and NSFT, a cuboid grey plexiglass open field arena (dimensions: 60 cm^3, length × width × height) was used, with indirect lighting producing approximately 40 lux inside the arena. A video camera above the arena recorded all movements. Rats were acclimatised to the room for 15-20 min prior to arena exposure for each of the tests. Before each trial the arena was wiped out with 10% ethanol and any objects used were sprayed with 10% ethanol solution, submerged in water and dried with towels to remove any latent odours/olfactory cues. Recorded data were de-identified and analysed independently by two experimenters using the automated behaviour analysis package *TopScan* (CleverSys Inc., VA, USA) for measures of locomotion, exploratory behaviours and object interest.

2.4. Open field test (OFT)

Rats were placed in the central zone of the arena and allowed to move freely for 6 min while all movements were recorded. The centre of the rat's body was used for data analysis. Behaviours of interest included distance travelled (m) and mean ambulatory velocity (m/min) in the total arena or the centre zone (middle 50%) and duration (s) spent in the centre zone.

2.5. Novel object recognition test (NORT)

The NORT relies on the rats' innate predisposition for investigating novelty when compared to a familiar stimulus [30, 48]. *Habituation:* rats were first placed in the empty arena together with their cage-mates and allowed to explore the arena freely for 3 min, before being returned to their home cage. *Familiarisation:* rats were individually returned to the arena, now containing two identical objects in adjacent corners. There were two sets of objects which were randomly presented: a large plastic cell culture flask (500 ml) filled with sand, or a plastic sports drink bottle (800 ml) containing

coloured liquid. Objects were oriented with their centre-point 12 cm from the two nearest walls. Object sets were counterbalanced for treatment, novel object and orientation. Rats were placed in the arena facing the wall directly opposite to both objects and allowed to move freely for 3 min. Rats were then returned to their cage. *Test:* following a 4-hour delay rats were individually returned to the arena, which now contained one familiar and one novel object and allowed to move freely for 3 min. The primary behaviour measure was novel object preference (novel object interest/TOTAL object interest) where interest was measured in both bouts (number of times of nose to object) and duration (s), when the rat's nose was within 2 cm of the object. Latency (s) to first explore the novel object, total distance travelled (m) and mean ambulatory velocity (m/min) were also measured.

2.6. Saccharin preference test (SPT)

Given that OVX and estrogen treatments have shown effects on metabolism and weight gain [41], the artificial sweetener, saccharin, was used rather than sucrose, as saccharin is non-caloric [49]. On day 1 of the SPT, rats were habituated to two bottles of normal drinking water, to adapt to the two-bottle apparatus [48]. On days 2-4, rats were familiarised to one bottle of normal water and one bottle of 0.1% saccharin (*o*-Benzoic sulfimide; Sigma-Aldrich) in water; the left and right placement of the bottles were randomised and then reversed on day 4. On day 5, all water/saccharin was withdrawn overnight (starting at 1630 or 1830). On day 6, rats were removed from their group housing conditions and individually placed in clean cages and given 15 min to habituate. They then received two drinking bottles: normal water and 0.1% saccharin water for 2 hours (starting at 0930 or 1130, with left/right positions randomly assigned and reversed 1 hr into testing). At the end of the testing period, all rats were returned to home cages with regular food and water conditions. Bottles were weighed at the start and end of each day. Preference on day 6 was calculated as a ratio of total consumption: (saccharin solution consumption/TOTAL consumption: saccharin + water), therefore a score above 0.5 indicates a preference for saccharin.

2.7. Novelty suppressed feeding test (NSFT)

Food pellets were withdrawn from cages at 1630 (overnight); water remained available *ad libitum* [50]. The following morning, rats were exposed to hyponeophagic challenge to assess anxiety-like behaviour and motivation to feed in a novel

environment. At the beginning of the test, rats were placed in a corner of the open field arena with eight food pellets neatly stacked at its centre. Rats could move freely for up to 8 min. The starting corner was rotated and counterbalanced for treatment. The primary behaviour of interest was the rat's latency (s) to feed - the time taken for the rat to begin eating from the pile of food pellets. Bouts (number of times rat's nose is within 2 cm of food) and latency to first bout were also measured.

2.8. Forced swim test (FST)

The FST was conducted as previously described [51, 52]. Briefly, clear plexiglass cylindrical tanks (45 cm height × 20 cm diameter) were filled to a depth of 30 cm (to ensure the rat's tail does not touch the bottom of the cylinder) with tap water (23 ± 1°C). On day 1, each rat was placed in the water and allowed to swim freely for 15 min (learning trial). The next day, rats were again placed in the tanks for the 5-min test session. Recorded data were de-identified and analysed independently by two experimenters using the automated *DepressionScan* (Forced swim version 2.0, CleverSys Inc.) analysis software. Software settings were optimised for our location, scored in real-time and experimenter watched to verify the software ratings. Results were expressed as both duration (s) and number of bouts performing each behaviour: either climbing or swimming (active coping behaviours) or immobility (passive coping behaviour).

2.9. Blood and tissue collection

While under anaesthesia, blood samples were collected from all rats in cohort 1: ~0.8 ml from the lateral tail vein (twice) and up to 8 ml from the heart. Following blood collection, whole blood was allowed to clot at room temperature, for serum isolation. Clotted blood samples were centrifuged (4°C, 2000 rpm, 10 min), sera aliquots were stored at -80°C until analysis. Serum was analysed for sex steroid levels (17β-estradiol (E2), progesterone (P4) and testosterone (T)) at the ANZAC Research Centre (University of Sydney, NSW, Australia) by liquid chromatography–tandem mass spectrometry [53]. Limit of quantification for each steroid was 17β-estradiol 5 pg/ml, progesterone 0.1 ng/ml, testosterone 25 pg/ml. A total of 24 rats had samples analysed at various time points (INT n = 9, OVX n = 6, EST n = 9); n = 5/group were sampled at baseline. One OVX rat from cohort 1 was excluded from data analysis due to detectable levels of 17β-estradiol (11 pg/ml at 2 weeks post-surgery; 12 pg/ml at 6

weeks post-surgery). At least three days after completion of experiments, all rats were euthanized using a lethal dose of pentobarbitone and then decapitated. The uteri and the hormone/vehicle implants were removed and weighed. Uterus weight was used as an index of ovariectomy and hormone treatment effects.

2.10. Statistical analysis

Data were analysed using SYSTAT 13 and differences were significant at $p \leq 0.05$. All data are expressed as mean ± S.E.M. Physiological measures (body and uterus weight, steroid levels) and behavioural data (OFT, NSFT) were analysed using one-way analysis of variance (ANOVA) between groups (INT, OVX, EST), with Bonferroni post-hoc correction applied where appropriate. For serum steroid data analysis, samples with a reading below the limit of quantification were deemed 'not detectable' and treated as missing data [53]. For NORT data analysis, repeated-measures ANOVA was used [group (INT, OVX, EST) x phase (familiarisation, test)]. For NSFT, there were 10 rats that did not feed during the maximum time (INT $n = 2$, OVX $n = 4$, EST $n = 4$); data for these rats was set at the maximum time (480 s) for latency to feed. For SPT, repeated-measures ANOVA was used [group (INT, OVX, EST) x time (0-60 min, 60-120 min)]. For FST data (test session day 2), one-way ANOVA was used to compare groups; the three behaviours were separately analysed: climbing, swimming, immobility.

3. Results

3.1. Body and uterus weight

A repeated-measures ANOVA comparing surgery and final body weights across the three groups revealed a significant main effect of group (cohort 1: $F_{(2,23)}=7.3$, $p=0.004$; cohort 2: $F_{(2,27)}=4.4$, $p=0.02$), time (cohort 1: $F_{(1,23)}=285.2$, $p<0.001$; cohort 2: $F_{(1,27)}=212.6$, $p<0.001$) and a group x time interaction (cohort 1: $F_{(2,23)}=45.5$, $p<0.001$; cohort 2: $F_{(2,27)}=25.7$, $p<0.001$). The pre-surgery body weight between groups in either cohort 1 or 2 showed no significant differences (Table 1). However, one-way ANOVA revealed significant differences in final body weight (cohort 1: $F_{(2,23)}=16.7$, $p<0.001$; cohort 2: $F_{(2,27)}=8.6$, $p=0.001$). Post-hoc analysis, using Bonferroni correction, revealed that in both cohorts the final body weight of OVX rats

was significantly greater than INT (cohort 1: p=0.002; cohort 2: p=0.007) and EST (cohort 1: p<0.001; cohort 2: p=0.002) rats. Similarly, significant differences were found in uterus weight between groups when expressed as an absolute weight (cohort 1: $F_{(2,23)}$=39.0, p<0.001; cohort 2, $F_{(2,27)}$=71.3, p<0.001) or as a percentage of body weight (cohort 1: $F_{(2,23)}$=44.4, p<0.001; cohort 2, $F_{(2,27)}$=77.3, p<0.001). As expected, post-hoc analysis revealed significantly greater uterus weight in INT and EST rats compared to OVX rats (all p<0.001 for absolute weight and as a percentage of body weight; Table 1). Body and uterus weights were not significantly different between INT and EST rats, highlighting that these physiological effects of ovariectomy were reversed by treatment with 17β-estradiol to levels similar to those of intact rats.

3.2. Circulating sex steroid levels

Circulating serum levels of 17β-estradiol, progesterone or testosterone in rats prior to surgical procedure (i.e. baseline; Table 2), were not significantly different between the groups. When combining the three groups of rats from cohort 1, the average levels of each steroid at baseline were: 17β-estradiol = 33.3 ± 8.7 pg/ml, progesterone = 8.5 ± 1.6 ng/ml and testosterone = 89.5 ± 24.9 pg/ml. At 2 and 6 weeks post-surgery, circulating levels of 17β-estradiol were undetectable in OVX rats (Table 2), reflecting successful removal of the ovaries. The EST group had the highest levels of 17β-estradiol, indicating effective chronic treatment with 17β-estradiol-filled implants. Significantly, EST rats had higher levels than INT rats (2 weeks: $F_{(1,11)}$=7.2, p=0.022; 6 weeks: $F_{(1,11)}$=14.6, p=0.003). Progesterone levels did not significantly differ between groups (p=0.096), however levels were highest in INT rats compared to OVX and EST rats (Table 2). Testosterone levels were undetectable in OVX or EST rats at 2 and 6 weeks post-surgery (Table 2).

3.3. Open field test

There was no significant difference between the groups for each of the locomotor parameters measured (Table 3). Thus, spontaneous locomotor activity (total arena) and anxiety-like behaviour (centre zone) were not different between INT, OVX and EST rats.

3.4. Novel object recognition test

During the familiarisation phase, both objects were identical and the side orientation for the subsequent novel object were counter-balanced. There was an unexpected significant group difference in preference for the side where the novel object will be introduced during the test phase (main effect of group for duration: $F_{(2,23)}$=4.3, p=0.03 and trend for bouts: $F_{(2,23)}$=2.9, p=0.08; data not shown). Bonferroni-corrected post-hoc comparisons revealed that OVX rats had an increased preference compared to EST rats (duration: p=0.02; bouts p=0.09), thus repeated-measures ANOVA was used for NORT data analysis.

When analysing novel object preference (duration; bouts) during the familiarisation and test phases (Figure 1), repeated-measures ANOVA revealed no main effect of group, but there was a significant group x phase interaction (duration: $F_{(2,23)}$=5.2, p=0.014; bouts: $F_{(2,23)}$=4.2, p=0.03). Further analysis comparing OVX rats with INT or EST rats revealed that the OVX rats had significantly reduced duration novel object preference than INT rats (group x phase interaction: $F_{(1,15)}$=7.1, p=0.02) and EST rats (group x phase interaction: $F_{(1,15)}$=8.6, p=0.01; Figure 1). For bouts, a similar pattern emerged; there was significantly reduced novel object preference (bouts) in OVX rats compared with INT rats (group x phase interaction: $F_{(1,15)}$=7.0, p=0.02) and EST rats (group x phase interaction: $F_{(1,15)}$=6.0, p=0.03; Figure 1).

When analysing total object interest (duration; bouts) during the familiarisation and test phases, repeated-measures ANOVA revealed there were no significant group differences nor group x phase interaction, suggesting that all rats showed a similar interest in overall exploration of the objects (data not shown). There was the expected significant effect of phase (duration: $F_{(1,23)}$=4.5, p=0.05; bouts: $F_{(1,23)}$=7.5, p=0.01), where overall exploration of objects was reduced during the test phase compared to the familiarisation phase. In terms of latency to explore the novel object, there was a significant main effect of phase ($F_{(1,23)}$=17.5, p<0.001) reflecting the overall increased latency to approach objects in the test phase. There was also a significant group x phase interaction ($F_{(2,23)}$=3.4, p=0.05), which was further explored by comparing INT and EST rats to OVX rats. OVX rats showed increased latency (s) compared to INT rats (group x phase interaction: $F_{(1,15)}$=6.2, p=0.03; INT: 10.5 ± 5.1 familiarisation vs. 17.5 ± 5.8 test; OVX: 8.7 ± 3.1 familiarisation vs 67.5 ± 22.6 test), further supporting a novel object recognition deficit in OVX rats. There were no group differences when comparing OVX rats with EST rats. Finally, there were no significant differences

between groups during either phase for distance travelled in the arena nor average velocity, consistent with behaviours observed in the open field test (data not shown).

3.5. Saccharin preference test

The day after all fluids were withdrawn, rats were tested over a 120-min period (Figure 2). When assessing saccharin preference across the two time points (0-60 min and 60-120 min), repeated-measures ANOVA revealed a significant main effect of group ($F_{(2,26)}$=4.9, p=0.02), but no main effect or interaction with time suggesting the group difference was similar over the two-hour period. Further analysis comparing OVX rats to INT and EST rats revealed that EST rats had a significantly greater preference for saccharin compared to OVX rats ($F_{(1,17)}$=7.9, p=0.01; Figure 2). There was no difference between OVX and INT rats. To account for the increased body weight of OVX rats, these ANOVAs were also conducted with body weight as a covariate. There was no significant group difference in total fluid consumption (Figure 2) or total fluid consumption/body weight (data not shown), demonstrating that the group difference in saccharin preference was not associated with increased drinking.

3.6. Novelty suppressed feeding test

There was no significant difference between the groups for the primary measure, latency (s) to feed (INT = 306 ± 41, OVX = 337 ± 44, EST = 369 ± 37). Further, the average number of bouts (INT = 7 ± 0.8, OVX = 7 ± 0.6, EST = 7 ± 0.9) and latency (s) to first bout (INT = 35 ± 6, OVX = 53 ± 17, EST = 67 ± 14) were also not significantly different between groups. Thus, there was no group difference in anxiety-like behaviour and motivation to feed in a novel environment.

3.7. Forced swim test

During the test session (day 2), one-way ANOVA revealed there were no significant group differences for either immobility, climbing or swimming in terms of duration (Figure 3) and bouts (data not shown).

4. Discussion

Using our established rat model of estrogen treatment, we investigated five behaviours relevant to symptoms of depression. The present study found that chronic

17β-estradiol treatment in OVX rats reduced anhedonia-like behaviour in the SPT and improved memory in the NORT. Chronic 17β-estradiol treatment had no significant effect on passive or active coping behaviours in the FST. It has previously been reported that group differences in locomotion, anxiety and motivation may confound the results of tests of depressive-like behaviour [54] and of cognition [29]. As a result, the OFT [44] and NSFT [45] were employed to assess the potential impact of these factors. These results showed no significant differences in measures of locomotor activity, motivation and anxiety-like behaviour; therefore it is unlikely that these factors confounded the results obtained from the SPT, FST and NORT.

As the ovaries are the primary source of sex hormones, the low circulating levels of 17β-estradiol, progesterone and testosterone in OVX rats confirmed successful ovariectomy, in addition to the low uterus weight and increased body weight gain that are typically reported after ovariectomy [41, 46, 47]. In OVX rats, levels of 17β-estradiol and testosterone were reduced to non-detectable levels; progesterone levels were reduced, however this did not reach significance, likely due to the small sample size. The hormonal status of the EST rats was confirmed by the high serum 17β-estradiol levels produced by the implants at 2 and 6 weeks post-surgery. Furthermore, the comparable body and uterus weight between EST and INT rats implies that 17β-estradiol treatment successfully reversed the physiological effects of OVX. This use of chronic treatment is advantageous over acute administration as it mimics clinical circumstances where treatments are administered long-term; however, the relevance to endogenous functioning of estradiol may be limited because this continual release differs from the cyclical levels normally produced. It should be noted that INT rats were naturally cycling and their steroid levels likely varied from test to test. Thus, EST rats, which are ovariectomised rats treated with 17β-estradiol, should be compared with OVX rats, which have non-detectable levels of 17β-estradiol, rather than INT rats.

The SPT is an animal behavioural assay of anhedonia which is sensitive to the actions of antidepressants. In this task, the absence of a preference for more palatable sweetened water over normal drinking water in rodents is suggested to reflect a defective reward system and thus an anhedonic-like state [21, 54]. Our results showed that chronic 17β-estradiol treatment improved anhedonia-like behaviour in OVX rats. Notably, 17β-estradiol treatment increased saccharin preference above the level of

intact rats. However, OVX rats did not show a deficit compared to intact rats, suggesting that other hormones may also regulate saccharin preference, such as testosterone and progesterone which were also significantly reduced. Given that estradiol modulates glucose homeostasis that regulates caloric intake [55], a benefit of this study is the use of the non-caloric artificial sweetener, saccharin. The present findings align with previous work showing that chronic estrogen treatment in OVX rats increased sucrose preference after chronic mild stress when administered in combination with fluoxetine [56]. Similar findings were seen in studies of male rodents [57] and of acute estradiol treatment in OVX rats [22].

Given the antidepressant-like action of chronic 17β-estradiol treatment evident in the SPT, the lack of effect observed in the FST was somewhat surprising. Our results contrast with previous literature that described a reduction in depressive-like behaviours in the FST in animals treated with estradiol [17-20, 58]. The reason for this remains unclear; however, methodological differences such as estradiol treatment regimens, rat strain or time after ovariectomy (1 week vs 3 weeks), may have contributed to the discrepancy. It is important to note that most previous studies have not measured the circulating levels of estradiol associated with their treatment, thus the comparisons that can be made between studies is limited. Future studies should measure hormone levels and also analyse whether there is a relationship between hormone levels and behaviour. Our use of a chronic treatment regimen meant that during the learning trial of the FST, the presence of these hormone treatments (OVX, EST) may have had an impact. However during the 15 minute learning trial we did not find any significant group differences (data not shown). In addition, the present study design used multiple testing in the same rat. While this is a strength of the study, it should be noted that the FST was the last behavioural test measured and it may have been impacted by previous tests. To summarise, the FST measures active and passive coping behaviours, with the latter resembling symptoms of despair or helplessness seen in people with MDD [11]; the present results suggest that chronic 17β-estradiol treatment will not affect these symptoms of depression.

The effect of 17β-estradiol on memory was of interest, given the previous evidence of estrogen's cognitive-enhancing abilities [27, 59] and the cognitive deficits associated with MDD [23]. Using the NORT, the results showed that when comparing the familiarisation and test phases, OVX rats had a reduced preference for the novel

object compared to intact rats, which chronic 17β-estradiol treatment corrected. This was evident in both the duration spent and bouts of investigation of the novel object, as well as the latency to approach the novel object. However, these results should be considered with caution given the unusual finding of a preference during the familiarisation phase. Nevertheless, these results align with previous data demonstrating acute [25, 27, 30, 32, 34, 35] and chronic 17β-estradiol treatment enhances NORT performance in OVX rodents [37, 60]. Although the memory-enhancing effects of chronic 17β-estradiol treatment in OVX animals has been demonstrated using other memory tasks, such as the Morris water maze or radial arm maze [61, 62], these tasks involve stress or rewarding/punishing stimuli that may influence motivational - rather than mnemonic - aspects of task performance. This advantage of NORT is particularly pertinent for assessing the effects of ovarian hormones specifically, as estrogens interact with the corticosteroids released in response to stress, which may act to confound the results [30, 63, 64]. Thus, the present NORT findings demonstrate a learning and memory enhancing effect of chronic 17β-estradiol treatment in OVX rats that is unlikely to be a product of confounding influences such as stress.

Despite the clinical data suggestive of an antidepressant action of estrogens [5, 10], few preclinical studies have assessed the impact of chronic estrogen treatment on depressive-like behaviours. The present results indicate that chronic 17β-estradiol treatment has some antidepressant effects. The efficacy of our chronic treatment, as opposed to an acute regimen, suggests some involvement of genomic mechanisms initiated by nuclear estrogen receptors [9, 39]. The differential effects of chronic 17β-estradiol treatment observed in the three behaviours measured may provide neurobiological insight into the underlying mechanisms. Estrogen's effects on neurotransmitter systems is well-established and has been linked to a number of CNS disorders [9, 65]. The effect of estrogens in the FST has been linked to the serotonergic and noradrenergic systems [66], while estrogen's effects in the SPT may rely more on the dopaminergic system [67, 68]. In contrast, estrogen's effects on NORT appear to be via direct activation of estrogen receptors in the hippocampus which can promote hippocampal synaptic plasticity [30]. Further, the FST can be considered a measure of affective components of depression whereas NORT measures cognitive components. However, the precise mechanisms of depression-associated deficits and how estrogens impact on these remains unclear and requires further investigation. Finally, while we

have focussed on 17β-estradiol, other hormones, in particular testosterone and progesterone, have been implicated in depressive-like behaviours in rodents [69-71] and warrant further investigation.

In conclusion, this study showed that chronic 17β-estradiol treatment improved anhedonia and recognition memory. Ovariectomy and 17β-estradiol treatment had little impact on helplessness and stress-coping behaviour as assessed in the FST. These data add to the literature supporting an antidepressant effect of estrogens but highlight that the effects of chronic 17β-estradiol are on selective depressive-like symptoms under specific methodological conditions. While this study focussed on the relevance of these behaviours to depression, they are also relevant to other CNS disorders; negative and cognitive symptoms of schizophrenia [9], or memory deficits in Alzheimer's disease. These results may also bear relevance to sex differences [2, 3] and links to ovarian hormone function [6, 7] associated with psychiatric disorders and supports the use of hormone treatment for both the affective and cognitive symptoms of depression.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

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Figure legends

Figure 1: Novel object preference in duration (top panel) and bouts (bottom panel). OVX rats had significantly reduced novel object preference (duration and bouts) than INT or EST rats. Novel object preference is the ratio of novel object interest/total interest. INT: intact, sham-operated rats; OVX: vehicle-treated ovariectomised rats; EST: 17β-estradiol-treated OVX rats (*n* = 8-9/group; mean ± S.E.M.).

Figure 2: Saccharin preference (top panel) and total fluid consumed (bottom panel) at 60 and 120 min. EST rats had a significantly greater preference for saccharin compared to OVX rats (top panel). Saccharin preference = saccharin solution consumption/total consumption; a score of 0.5 indicates no preference. INT: intact, sham-operated rats; OVX: vehicle-treated ovariectomised rats; EST: 17β-estradiol-treated OVX rats (*n* = 10/group; mean ± S.E.M.).

Figure 3: Duration (s) spent immobile, climbing and swimming during the 5 min forced swim test. INT: intact, sham-operated rats; OVX: vehicle-treated ovariectomised rats; EST: 17β-estradiol-treated OVX rats (*n* = 10/group; mean ± S.E.M.).

Tables

	n	Surgery BW (g)	Final BW (g)	UW (g)	%UW/BW
Cohort 1: OFT, NORT					
INT	9	263 ± 6	297 ± 8*	0.61 ± 0.07*	0.20 ± 0.02*
OVX	8	261 ± 6	344 ± 10	0.11 ± 0.007	0.03 ± 0.002
EST	9	256 ± 5	278 ± 7*	0.65 ± 0.04*	0.24 ± 0.02*
Cohort 2: SPT, NSFT, FST					
INT	10	263 ± 7	294 ± 10*	0.61 ± 0.04*	0.20 ± 0.01*
OVX	10	262 ± 6	346 ± 13	0.11 ± 0.004	0.03 ± 0.001
EST	10	253 ± 6	287 ± 9*	0.62 ± 0.04*	0.21 ± 0.01*

Table 1: Mean ± S.E.M. body weight (BW; g) and uterus weight (UW; g) of female rats of cohort 1 (used in the open field test (OFT) and novel object recognition test (NORT)) and cohort 2 (used in the saccharin preference test (SPT), novelty supressed feeding test (NSFT) and forced swim test (FST)). INT: intact, sham-operated rats; OVX: placebo-treated ovariectomised rats; EST: 17β-estradiol-treated OVX rats. * $p<0.01$ compared with OVX rats from the same cohort.

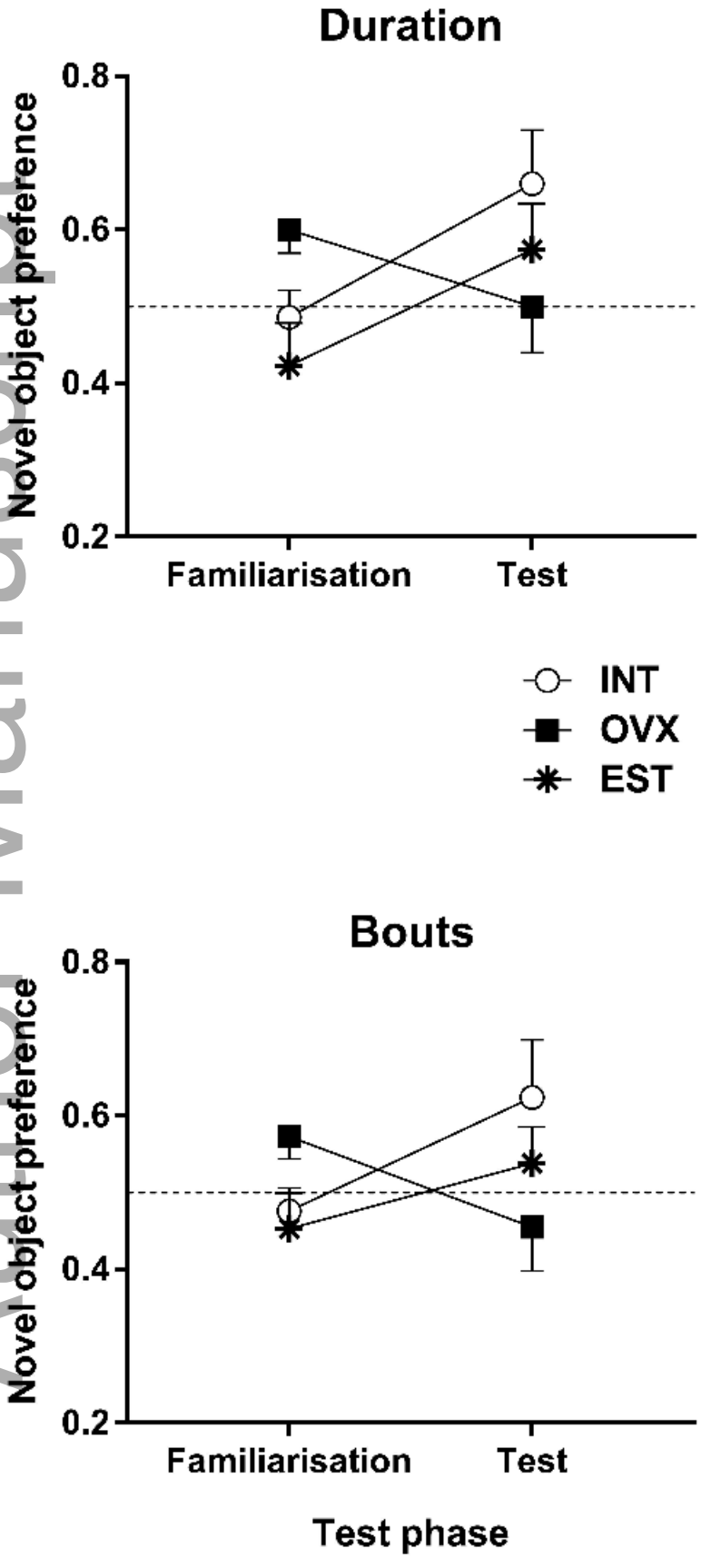
	17β-Estradiol (pg/ml)	Progesterone (ng/ml)	Testosterone (pg/ml)

Baseline – time of surgery			
INT	30.9 ± 16.0	8.8 ± 3.8	102.2 ± 66.4
OVX	48.9 ± 22.8	8.9 ± 2.3	87.9 ± 18.2
EST	22.9 ± 7.1	7.6 ± 2.9	74.6 ± 40.9
2 weeks post-surgery			
INT	32.1 ± 7.5	6.2 ± 1.9	173.1 ± 84.5
OVX	<5 (n.d.)	2.8 ± 1.4	<25 (n.d.)
EST	65.3 ± 8.5*	1.5 ± 0.3	<25 (n.d.)
6 weeks post-surgery			
INT	14.9 ± 3.1	7.3 ± 1.8	141.8 ± 53.6
OVX	<5 (n.d.)	3.1 ± 2.2	<25 (n.d.)
EST	42.9 ± 4.7*	3.4 ± 0.8	<25 (n.d.)

Table 2: Mean ± S.E.M. circulating sex steroid levels analysed from serum collected from female rats of cohort 1 at three time points: immediately before surgery (Baseline), 2 weeks post-surgery and at the end of experimentation (6 weeks post-surgery). Note that at baseline, all rats were still intact when blood was collected. Limit of quantification for each steroid: 17β-estradiol 5 pg/ml, progesterone 0.1 ng/ml, testosterone 25 pg/ml. INT: intact, sham-operated rats (n=5-7); OVX: placebo-treated ovariectomised rats (n=3-5); EST: 17β-estradiol-treated OVX rats (n=5-9); n.d.: not detected. * p<0.05 compared with INT rats at the same time point.

	Total distance (m)	Total velocity (m/min)	Centre distance (m)	Centre velocity (m/min)	Centre duration (s)
INT	28.2 ± 3.8	4.7 ± 0.6	2.7 ± 0.6	7.5 ± 1.0	20.9 ± 5.5
OVX	32.7 ± 5.5	5.5 ± 0.9	3.4 ± 0.6	10.2 ± 1.5	23.2 ± 4.8
EST	28.5 ± 3.7	4.8 ± 0.6	2.3 ± 0.6	8.6 ± 1.8	20.7 ± 4.6

Table 3: Mean ± S.E.M. open field test measures from female rats of cohort 1 (*n*=8-9/group), including: distance travelled (m) in 6 min and average ambulatory velocity (m/min) in the total arena and centre zone, and duration (s) spent in the centre zone. INT: intact, sham-operated rats; OVX: placebo-treated ovariectomised rats; EST: 17β-estradiol-treated OVX rats. There was no difference between groups in either measure.



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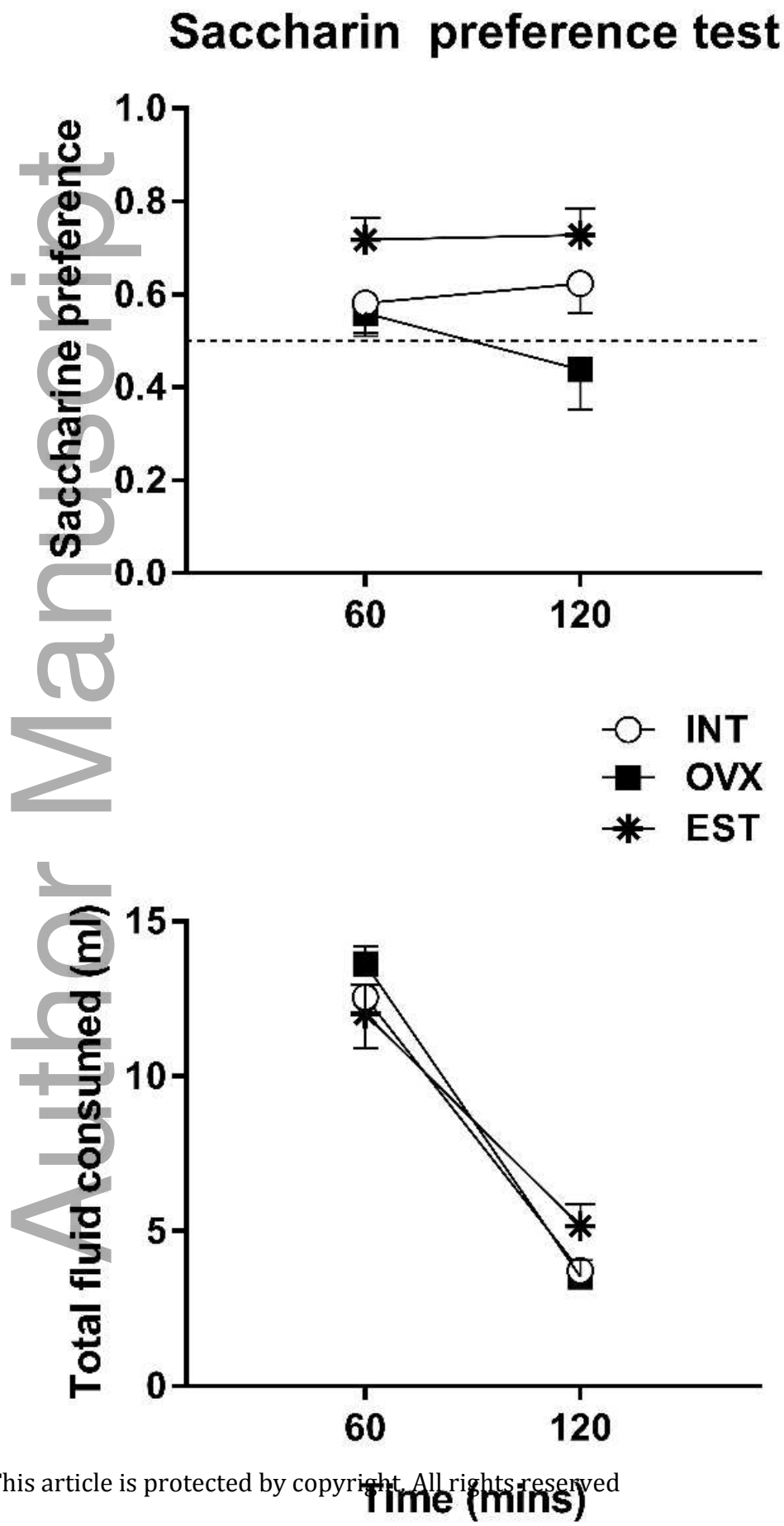

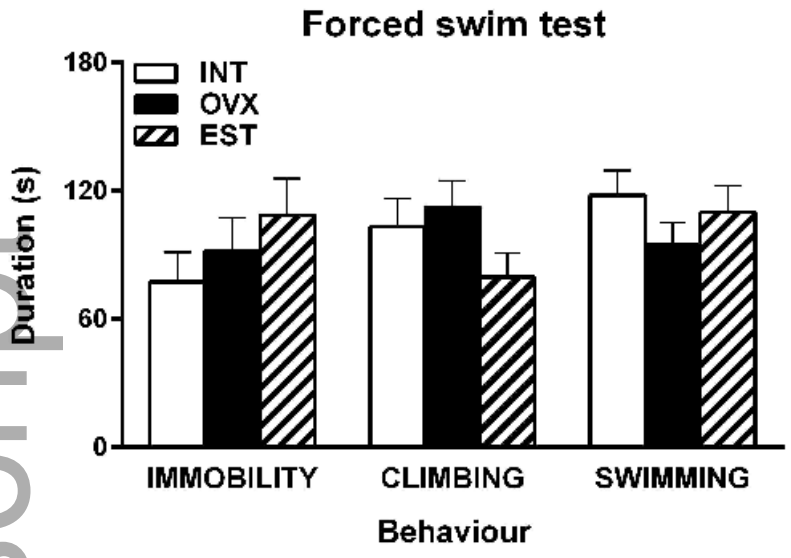

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