



Suppression of ovarian hormones in adolescent rats has no effect on anxiety-like behaviour or *c-fos* activation in the amygdala

Amy R. Hodgson¹ | Claire Richmond¹ | Javier Tello² | Gillian R. Brown¹ 

¹School of Psychology & Neuroscience, University of St Andrews, St Andrews, UK

²School of Medicine, University of St Andrews, St Andrews, UK

Correspondence

Gillian R. Brown, School of Psychology & Neuroscience, University of St Andrews, South Street, St Andrews, Fife, KY16 9JP, UK.
Email: grb4@st-andrews.ac.uk

Present address

Amy R. Hodgson, Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, UK
Claire Richmond, Centre for Discovery Brain Sciences, University of Edinburgh, Edinburgh, UK

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Abstract

In humans, sex differences in mood disorders emerge during adolescence, with prevalence rates being consistently higher in females than males. It has been hypothesised that exposure to endogenous ovarian hormones during adolescence enhances the susceptibility of females to mood disorders from this stage of life onwards. However, experimental evidence in favour of this hypothesis is lacking. In the present study, we examined the long-term effects of suppressing adolescent gonadal hormone levels in a group of female Lister-hooded rats via administration of a gonadotrophin-releasing hormone antagonist (Antide; administered on postnatal day [PND] 28 and 42) compared to control females and males ($n = 14$ per group). We predicted that, in adulthood, Antide-treated female rats would exhibit more male-like behaviour than control females in novel environments (elevated-plus maze, open field and light-dark box), in response to novel objects and novel social partners, and in an acoustic startle task. Progesterone and luteinising hormone assays (which were conducted on blood samples collected on PND 55/56 and 69/70) confirmed that the hypothalamic-pituitary-gonadal axis was temporarily suppressed by Antide treatment. In addition, Antide-treated females were found to exhibit a modest pubertal delay, as measured by vaginal opening, which was comparable in length to the pubertal delay that has been induced by adolescent exposure to alcohol or stress in previous studies of female rats. However, Antide-treated females did not substantially differ from control females on any of the behavioural tests, despite the evidence for predicted sex differences in some measures. Following the acoustic startle response task, all subjects were culled and perfused, and *c-Fos* staining was conducted in the medial and basolateral amygdala, with the results showing no significant differences in cell counts between the groups. These findings suggest that ovarian hormone exposure during adolescence does not have long-term effects on anxiety-related responses in female rats.

KEYWORDS

Antide, LH, progesterone, puberty, sex difference

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1 | INTRODUCTION

In humans, the prevalence of mood disorders is consistently reported to be higher in women than in men,¹⁻³ and this sex difference in prevalence emerges during adolescence.⁴⁻⁶ These observations have led to hypothesis that the fluctuations in ovarian hormone levels that accompany puberty increase the susceptibility to mood disorders amongst adolescent girls.⁷ This hypothesis is supported by evidence that pubertal status, rather than age, predicts the incidence of mood disorders^{8,9} and evidence that supplementation with synthetic ovarian hormones during adolescence (via hormonal contraceptive use) is associated with a raised incidence of depression and altered physiological stress responses.¹⁰⁻¹² However, because these human studies are correlational, caution is required when inferring a direct link between endogenous ovarian hormone exposure during adolescence and long-term mental health and stress reactivity.¹³

Studies on laboratory rodents provide the opportunity to experimentally investigate the immediate and long-term effects of adolescent gonadal hormone alterations on behavioural and brain development. In rodents, adolescence is generally defined as the period that encompasses pubertal sexual maturation¹⁴ and is characterised in female rodents by vaginal opening and onset of ovarian cycling and in male rodents by rising testicular hormone levels and sperm production.^{15,16} Sex differences in behaviour emerge during adolescence in rodents; for example, adolescent male rats (*Rattus norvegicus*) exhibit a higher preference for novel objects compared to adolescent females¹⁷ and locomote less than aged-matched females in novel environments.^{18,19} The hypothalamic-pituitary-adrenal (HPA) axis also undergoes significant changes during adolescence in rodents and humans,²⁰ which means that any factors affecting the developing HPA axis could have long-term effects on response to stressors.²¹

Previous research on adolescent rodents has shown that removing gonadal hormones during this stage of life has significant, long-term effects on behavioural development.^{22,23} The majority of these studies have been conducted on male rodents and have compared the behaviour of males that were castrated before pubertal onset with males that were castrated at the end of adolescence (ie, only the post-pubertally castrated males experienced normal gonadal hormone exposure during adolescence). Post-pubertally castrated male rodents are reported to exhibit higher levels of aggression and sexual proficiency, as well as spend less time in the aversive areas of novel environments, in adulthood than pre-pubertally castrated male rats,²⁴ Syrian hamsters (*Mesocricetus auratus*)²⁵⁻²⁷ and mice (*Mus musculus*).²⁸ These findings indicate that exposure to gonadal hormones during adolescence influences behavioural development in male rodents, consistent with the hypothesis that adolescence is a 'sensitive period' of development.²⁹

Fewer studies have investigated the immediate and long-term behavioural effects of manipulating adolescent gonadal hormone levels in female rodents; these studies have reported that exposure to gonadal hormones during adolescence increases maternal behaviour in adulthood (in mice³⁰) and has organisational effects on

subtle aspects of movement during social and sexual interactions (in rats³¹⁻³³). However, little is known about the effects of manipulating adolescent gonadal hormones on anxiety-like behaviour or fear responses. A recent study reported that pre-pubertally ovariectomised Siberian hamsters (*Phodopus sungorus*) spend more time than controls in the light area of a light-dark box,³⁴ whereas another study reported that pre-pubertally ovariectomised female rats spend less time in the centre of a novel open field in adulthood than sham-operated females,³⁵ which is consistent with data from adult rodents showing that low oestradiol levels are associated with enhanced anxiety-like behaviour (eg, in rats³⁶). However, the later study³⁵ did not include a separate control group of females ovariectomised after puberty, and so any apparent long-term behavioural differences could reflect the activational effects of adult hormones; such effects are not necessarily consistent with the organisational effects of the same hormone at earlier life stages.²⁴

Gonadal hormone exposure during adolescence could impact upon the development of brain regions that are involved in emotional processing, such as the amygdala, ventral striatum and prefrontal cortex, which undergo reorganisation in adolescent rodents and humans.³⁷⁻⁴¹ Gonadal hormone receptors are located within these brain regions,^{41,42} and these regions exhibit sex differences in structure and function.^{43,44} For example, the basolateral amygdala, which is highly sensitive to stress,⁴⁵ shows substantial sex differences associated with emotion-related behaviours corresponding to greater excitatory synaptic input in female rats.^{46,47} In addition, the posterior medial amygdala is sensitive to ovarian sex hormones,⁴⁸ showing robust post-pubertal differences in soma morphology and size.^{49,50} In humans, the developmental trajectories of the amygdala and prefrontal cortex have been reported to correlate with pubertal status.⁵¹⁻⁵⁴ Given that gonadal hormones are known to influence a range of neurodevelopmental processes,⁵⁵ adolescent hormone exposure could thus potentially have long-term, 'organisational' effects on later affective behaviour by influencing the development of these brain areas.

The present study aimed to examine the effects of suppressing ovarian hormones during adolescence on later anxiety-like behaviour, including responses to novelty, and amygdala function in female rats. Previous rodent studies have used ovariectomies, an approach that involves invasive surgery and results in the permanent loss of gonadal function, alongside significant elevation in circulating gonadotrophin levels as a result of the permanent disruption of negative-feedback loops. An alternative method for temporarily reducing gonadal hormone levels involves treatment with gonadotropin-releasing hormone (GnRH) peptide antagonists, which can be delivered via subcutaneous injection. GnRH peptide antagonists competitively bind to GnRH receptors in the pituitary, without activating these receptors (in contrast to GnRH agonists, which produce an initial phase of hyperstimulation), and reversibly suppress gonadotrophin and gonadal hormone production.⁵⁶ These antagonists have a long half-life and, once metabolised, the hypothalamic-pituitary-gonadal (HPG) axis is reactivated. Therefore, using a GnRH antagonist to suppress gonadal hormone levels has several advantages

compared to ovariectomy, including that negative-feedback is maintained in the HPG axis and that the treatment avoids the use of stressful surgical procedures and is reversible. This experimental design, involving juvenile treatment with a GnRH antagonist, delays the hormonal changes that normally accompany puberty, allowing for hormone-independent developmental changes to be dissociated from hormone-dependent processes.

We examined the effects of suppressing ovarian hormones during adolescence using a GnRH peptide antagonist (Antide), and the goal was to delay pubertal gonadal hormone exposure to a similar extent as seen with adolescent exposure to alcohol or stress, both of which can interfere with HPG functioning during this period of life.^{20,57} We predicted that, in adulthood, Antide-treated female rats would exhibit more male-like behaviour than control females in novel environments (elevated-plus maze, open field and light-dark box), in response to novel objects and novel social partners, and in an acoustic startle task. Previous studies have reported behavioural sex differences in these tasks⁵⁸; for example, on average, female rats usually spend more time than males in the exposed sections of the elevated-plus maze and light-dark box,¹⁸⁻²⁰ and male rats exhibit stronger acoustic startle responses than females.^{59,60} We also postulated that *c-fos* activation in the amygdala following the acoustic startle task would be greater in Antide-treated females than in control females.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

Ethical guidelines, as set out in the Principles of Laboratory Animal Care (NIH, Publication No. 85-23, revised 1985) and the UK Home Office Animals (Scientific Procedures) Act 1986, were adhered to throughout the study (Home Office Project Licence PC33CDA1C; Personal Licences IF9BFD0E9 and I09B3A36E).

2.2 | Subjects and housing

The subjects comprised 42 Lister-hooded rats (28 females and 14 males) that were bred in-house (stock animals were obtained from Charles River, Margate, UK). The subjects were derived from five litters, and an additional six males from these litters were used as social stimuli in one of the behavioural tests. Breeding females were individually housed in plastic and mesh cages (52 × 40 × 26 cm, length × depth × height) with access to water and pellet food (DBM Food Hygiene Supplies Ltd, Scotland) available ad lib. Pups were weaned at postnatal day (PND) 23 and housed in single-sex sibling groups until PND 27, then re-housed as same-sex pairs (cage dimensions same as above) with access to water and food available ad lib. All animals were housed in a holding room under a 12:12 hour light/

dark photocycle (lights on 7.00 AM) at 20 ± 1°C and 55 ± 5% relative humidity.

2.3 | Experimental design

Three sets of subjects were produced: (i) females (n = 14) that were treated on PND 28 and 42 with a gonadotrophin-releasing hormone (GnRH) peptide antagonist, Antide (Bachem, Germany), via s.c. injection at a dose of 6 mg kg⁻¹ in 1:1 propylene glycol: saline solution, as in our previous research⁶¹ (injection volumes [μL] were 2× animal weight [g]); (ii) females (n = 14) that received vehicle (propylene glycol: saline solution) injections on PND 28 and 42; and (iii) males (n = 14) that received vehicle injections on PND 28 and 42 (as a result of practical constraints, four males received the first injection on PND 29 rather than PND 28). Each bolus of Antide was predicted to suppress circulating gonadal hormone levels for between 2 and 3 weeks, as in previous studies,^{61,62} and so the regime of two injections 14 days apart was designed to suppress gonadal hormone levels from the late juvenile phase through to the end of late adolescence (age ranges based on previous studies^{18,63}). No more than five animals from each litter were assigned to a single experimental group, and, in the majority of cases, no more than four females from a single litter were used as subjects in the experiment (ie, two Antide-treated females and two control females), with all subjects paired with a littermate in the same experimental group.

All subjects were weighed once per week from PND 28 onwards. Vaginal opening (VO) was assessed in female subjects daily by visual inspection from PND 28, with VO recoded as the first day when the vagina was either partially or fully opened, and males received similar handling each day. Previous studies have shown that vaginal opening typically occurs around PND 32-34 in rats.^{64,65} Anogenital distance (AGD) was measured for all subjects on PND 35 and 49 using electronic calipers, with care being taken not to touch the genital area with the caliper tips, given that artificial genital contact can accelerate sexual maturation in adolescent rats.⁶⁶

Behavioural testing was undertaken during adolescence (PND 40-44) and adulthood (PND 79-103). All subjects underwent all of the behavioural tests in the order: (i) adolescent social behaviour (PND 40-44); (ii) response to novel environments (PND 79-85); (iii) response to novel objects and social partners (PND 89-92); and (iv) the acoustic startle response (ASR) task (PND 98-103, followed by perfusion 90 minutes after the ASR task). The same order of testing was maintained for all subjects, as in previous studies in our laboratory,^{18,19} because some tests had to be conducted at specific subject ages (adolescent social behaviour) and the ASR test had to precede perfusion for all subjects. Although this design does not allow the estimation of any carry-over effects that might result from the order of behavioural testing, between-group comparisons remain valid. All behaviour tests took place in a single testing room with the relevant piece of apparatus set-up directly below the ceiling-mounted camera, and a black curtain separated the apparatus from the rest of the testing room. Individual subjects were transported to the testing

room in a carrying box and returned to the home-cage immediately after completing the test.

Information on ovarian cyclicity was not collected for the female subjects in this experiment for three reasons. First, our research question did not involve investigating cycle-dependent effects, and it has been suggested previously that experimental designs do not need to take ovarian stage into account unless the study is specifically investigating cycle-dependent effects,^{67,68} particularly because the behaviour of unstaged females is not more variable than that of males.⁶⁹ Second, we did not require cyclicity data to confirm that Antide treatment had been successful because hormonal and vaginal opening data were considered to be sufficient. Third, cyclicity monitoring would have required swab sampling to be undertaken alongside the behavioural testing, which could have induced stress in both of the female groups (but not in the control male group) and thereby impacted upon the behaviour of subjects and influenced between-group comparisons.

Blood samples were taken from tail veins on PND 55/56 and 69/70, under gas anaesthesia (isoflurane), to confirm that treatment with Antide successfully suppressed the HPG axis, and serum was stored at -70°C prior to assaying of progesterone and luteinising hormone (LH) levels. All subjects were culled via perfusion following the final behavioural test (ie, 90 minutes after the acoustic startle response task) on PND 98-103, and the brain tissue was sectioned, mounted and frozen at -20°C prior to c-Fos immunohistochemistry and cell counting.

2.4 | Behavioural testing

2.4.1 | Adolescent social behaviour

On PND 40, 41, 43 and 44, subjects were paired with a same-aged, same-sex partner from the same experimental group (ie, not the cage-mate [insufficient non-experimental animals of the same age were available to conduct the social behaviour tests with fully naïve animals]). Social interaction sessions took place in a perspex arena ($49 \times 44 \times 47$ cm, length \times width \times height). The outer walls of the arena were black, and light illuminance in the arena was maintained at approximately 40 lux. A ceiling-mounted camera relayed video to a computer, and the arena floor was divided visually into four equal quadrants. A black curtain occluded views of the testing room and the experimenter. Subjects were transported to the testing room in an enclosed box, and the arena was cleaned with disinfectant solution after each test. On PND 35, 37 and 39, each pair of cage-mates was given a 5-minute habituation session in the arena, then returned to the home-cage. The social behaviour tests were subsequently conducted on 4 days (PND 40, 41, 43 and 44) to provide sufficient behavioural data. The two subjects were placed into the arena for a 10-minute test, then returned to the respective home-cages. The apparatus was cleaned with liquid disinfectant after each test. As a result of limited numbers of potential partners, each subject was paired with two different animals across the four sessions (ie, one

partner on PND 40 and 43, and another partner on PND 41 and 44), rather than four different animals. For each test, the (i) *total frequency of social investigations* (ie, sniffing the partners face, body or anogenital region) and (ii) *social play* (ie, nape-attack, pinning, boxing, chasing and evasion) was calculated (definitions in accordance with previous research^{70,71}).

2.4.2 | Response to novel environments in adulthood

Elevated-plus maze (EPM)

All subjects were tested once on the EPM on either PND 79, 80 or 81. The EPM was a wooden, grey-painted maze that consisted of four arms (51 cm \times 11 cm, length \times width) extending from a central area (11 cm \times 11 cm). Two of the opposing arms were enclosed by walls (40 cm height; 'closed' arms) and the other two opposing arms lacked walls ('open' arms). The maze was raised 56 cm off the floor on a metal frame, and the light illuminance was ~ 30 lux on the closed arms and ~ 65 lux on the open arms. At the start of a test, the subject was placed into the central area, facing an open arm. Each test lasted 5 minutes, and the apparatus was cleaned with liquid disinfectant after each test. The following behavioural measures were calculated: (i) *total number of entries into the open arms*, (ii) *total number of entries into the closed arms*, (iii) *percentage of time spent on the open arms*, and (iv) *percentage of time spent on the closed arms*.

Open field (OF)

All subjects were tested once in the OF on PND 82. The OF consisted of an area of vinyl floor (120 cm \times 120 cm, length \times width) enclosed on all four sides by a wooden, grey-painted wall (50 cm height). The floor of the arena was marked into nine areas (8 outer and 1 central area) by lines that were located 20 cm in from the walls, and the light illuminance at floor level was approximately 50 lux. At the start of the test, a subject was placed into the outer areas and left in the area for 5 minutes. The apparatus was cleaned with liquid disinfectant after each test. The following behavioural measures were calculated: (i) *total number of transitions between areas* (both outer areas and centre) and (ii) *total percentage of time spent in the centre vs the outer areas*.

Light-dark box (LDB)

All subjects were tested once in the LDB on either PND 83, 84 or 85. The LDB apparatus consisted of a Perspex arena that was separated into two sections using an opaque plastic divider with an opening at floor level (11-cm diameter archway). The 'light' section (70 \times 44 \times 47 cm, length \times width \times height) had white walls and was illuminated to approximately 90 lux, and a smaller 'dark' section (49 \times 44 \times 47 cm, length \times width \times height) had black walls and an opaque lid. At the start, the subject was placed into the dark section, and the test lasted for 5 minutes. The apparatus was cleaned with liquid disinfectant after each test. The following behavioural measures were calculated: (i) *latency to enter the light section*; (ii) *total*

number of transitions between the dark and the light sections; and (iii) percentage of time spent in the light section.

2.4.3 | Response to novel objects and social partners in adulthood

Novel object (NO) test

All subjects underwent the NO test on PND 89. The test was conducted in a painted, wooden arena measuring 67 × 67 × 45 cm (length × width × height). The arena floor was divided visually into four equal quadrants, and the light illuminance was approximately 20 lux at floor level. Three novel objects were used that were similar in size but differed in texture (glass or plastic) and colour (blue, pink or clear). During the first stage of the test, two objects were placed into the arena, and one in each of two adjacent quadrants. The subject was then placed in the empty half of the arena, facing away from the novel objects, and allowed to explore for 3 minutes. The subject was then removed and placed into a carrying box for 2 minutes, during which time the objects were removed and both the arena and the objects were cleaned with liquid disinfectant. One of the original objects was then placed back into the arena in its original position, and the third object was placed into the position previously occupied by the removed object. The subject was returned to the arena for a 3-minute period, then returned to the home-cage and the apparatus and objects cleaned. The objects were counterbalanced across subjects. In the NO test, time spent exploring the novel objects can be used as a measure of anxiety-like behaviour, whereas the relative time spent exploring the novel and familiar object can either reflect novelty preference or short-term memory.⁷² The following specific behavioural measures were calculated: (i) *total amount of time in each quadrant containing an object* and (ii) *a novel object preference score* (defined as [(time with novel object - time with familiar object)/(time with novel object + time with familiar object)] × 100, as in previous research^{17,61}).

Social novelty (SN) test

All subjects were tested once in the SN on either PND 90, 91 or 92. The SN apparatus consisted of a perspex arena (119 × 44 × 47 cm, length × width × height; approximately 15 lux) that contained two clear perspex boxes (24 × 21 × 46 cm, length × width × height) in opposite corners of the arena. One of the boxes contained a male stimulus animal and the other contained a novel object (a blue, yellow or blue-and-yellow plastic object, with objects counterbalanced across subjects). Stimulus animals received two habituation sessions in the apparatus and were used for no more than two consecutive tests. In one-half of the sessions, the animal was in the box closest to the testing room door and, in the remaining sessions, the animal was in the other box. The arena was divided visually into three areas, two of which included one of the boxes (49 × 44 × 47 cm, length × width × height) plus a smaller central area (21 × 44 × 47 cm, length × width × height). The subject was placed into the central area and allowed to explore for 5 minutes. The apparatus was cleaned with liquid disinfectant after each test. The following behavioural

measures were calculated: (i) *total amount of time spent in the area containing the social partner or the novel object* and (ii) *total number of entries into each area*.

2.4.4 | Acoustic startle response (ASR) task

All subjects were tested once in the ASR task between PND 98 and 103. The task was conducted in a perspex box (49 × 44 × 47 cm, length × width × height) that was illuminated at approximately 90 lux at floor level and had an electronic speaker attached to the top of one of the walls. The speaker was connected to a laptop computer and AUDACITY (<https://www.audacityteam.org>) was used to play a series of 1-second bursts of white noise at a volume of approximately 90 decibels. The subject was placed into the arena and, after 45 seconds, the first startle noise was played via the speaker. The noise was then repeated every subsequent 30 seconds, resulting in a total of nine acoustic startle stimuli across a 5-minute test. The apparatus was cleaned with liquid disinfectant after each test. As in previous studies,⁷³ immobility, or 'freezing' behaviour, was coded from the videos, and the following behavioural measures were calculated: (i) *total number of times that the startle noise resulted in the animal becoming immediately immobile* (ie, number of 'freezes', range 0-9) and (ii) *summed duration of time (seconds) spent immobile following the startle noises* (ie, duration of 'freezing'). Because we did not have access to an electronic startle monitor, we were unable to evaluate additional measures, such as startle amplitude; however, 'freezing' behaviour has previously been shown to correlate with startle amplitude in female rats.⁷⁴ At the end of the ASR task, the subject was returned to the home-cage for 90 minutes before being culled for brain tissue collection.

2.5 | Hormone assays

2.5.1 | Progesterone assay

Serum progesterone levels were measured using a commercially available progesterone enzyme-linked immunosorbent assay kit (ADI-900-011; Assay Designs, Enzo Life Sciences, Exeter, UK). Serum samples were diluted with assay buffer (sera from females were diluted 1:100, sera from males were diluted 1:25) and 100 µL of each diluted sample was assayed in duplicate. The assay has a detection limit of 8.57 pg mL⁻¹, an intra-assay coefficient of variation of 5.4% and an inter-assay coefficient of variation of 8.3%.

2.5.2 | LH assay

Serum LH levels were measured using established in-house enzyme-linked immunosorbent assays as described previously.⁷⁵ Serum samples were assayed neat or diluted (1:10) when necessary and 20 µL of each sample was assayed in duplicate. The 3,3',5,5'-tetramethylbenzidine

substrate (34021; Thermo Scientific, Waltham, MA, USA) was developed for 30 minutes at room temperature and stopped by the addition of 2 mol L⁻¹ H₂SO₄. The absorbance in each well was then read at 450 nm. The assay had a detection limit of 0.05 ng mL⁻¹, an intra-assay coefficient of variation of 2.2% and an inter-assay coefficient of variation of < 10%.

2.6 | Immunohistochemistry and cell counting

At the end of the behavioural testing (ie, 90 minutes after the ASR task), rats were transcardially perfused with 4% paraformaldehyde in 0.1 mol L⁻¹ phosphate buffer after anaesthesia with Euthatal® (Dopharma Research B.V., Raamsdonksveer, The Netherlands) (1.0 mL kg⁻¹, pentobarbital sodium administered i.p. 200 mg mL⁻¹). The brain tissue was removed, post-fixed and stored overnight in 20% sucrose solution at 4°C. The tissue was then set in egg yolk and fixed via exposure to 40% formaldehyde for 72 hours. Prior to sectioning, the left-hand side of the egg yolk was marked using a needle puncture to allow the hemispheres to be identified. The tissue was cut into 40 µm coronal sections using a freezing microtome (SM 2010R; Leica Microsystems, Wetzlar, Germany), then stored in ethylene glycol in sucrose solution at -20°C. After being removed from the freezer, the sections were rinsed in phosphate-buffered saline (PBS) and every fourth section (between 1.30 mm to 4.52 mm posterior to Bregma) was mounted onto SuperFrost Plus slides (Thermo Scientific). Slides were then dried at 37°C for 30 minutes and stored at -20°C. One set of sections for each subject then underwent c-Fos immunohistochemistry labelling using the Vectastain ABC kit with Rabbit IgG (PK-6101) in combination with the DAB peroxidase substrate kit (SK-4100) (Vector Laboratories, Burlingame, CA, USA). Briefly, the sections underwent heat-induced epitope retrieval by immersion in 10 mmol L⁻¹ sodium citrate (pH 6) at > 90°C for 10 minutes. Sections were then incubated in PBS for 5 minutes before being covered with BLOX-ALL reagent (SP-6000; Vector Laboratories) for 10 minutes. Sections were washed in PBS for 5 minutes before incubation with blocking serum for 20 minutes (100 µL per section). Blocking serum was then removed and a rabbit anti-c-Fos antibody (dilution 1:1000; sc-52; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied (100 µL per section), and sections were incubated overnight at 4°C in a humidified slide tray. The next day, sections were washed three times in PBS and incubated with biotinylated secondary antibody (anti-rb IgG) for 45 minutes (100 µL per section), then immersed in ABC reagent for 45 minutes and then washed again times. The sections were then incubated in peroxidase substrate solution for 9 minutes, rinsed in tap water and mounted using Fluoromount-G (00-4958-02; Invitrogen, Carlsbad, CA, USA).

Slides were imaged using a Axio Scan Z1 scanner (Carl Zeiss, Oberkochen, Germany) and quantification of c-Fos labelling was undertaken in four nuclei of the medial amygdala and three nuclei of the basolateral amygdala (right hemisphere only; n = 9 per group). At the anterior level of the medial amygdala, this included the dorsal (MeAD) and ventral (MeAV) nuclei and, at the posterior level, this included the dorsal (MePD) and ventral (MePV) nuclei. At the level of the basolateral amygdala, this included the anterior (BLA), posterior (BLP) and

ventral (BLV) nuclei. Using QUPATH, version 0.1.2,⁷⁶ regions of interest were outlined with reference to the rat brain atlas,⁷⁷ and c-Fos positive cells were quantified using the 'positive cell detection' function. The threshold for cell detection was set between 0.30 and 0.52, adjusted to account for differing levels of background staining. Sections from 1.30 mm to 2.80 mm posterior to Bregma were analysed for the MeAD; for the MeAV, from 2.12 mm to 2.30 mm posterior to Bregma; for the MePD, from 2.80 mm to 3.60 mm posterior to Bregma; for the MePV, from 2.56 mm to 3.30 mm posterior to Bregma; for the BLA, from 1.60 mm to 3.30 mm posterior to Bregma; for the BLP, from 2.30 mm to 4.52 mm posterior to Bregma; and, for the BLV, from 2.30 mm to 3.60 mm posterior to Bregma. Between four and eight sections were analysed per animal, and values were averaged over the available sections for each region. The experimenter was blind to the animal ID and treatment groups during the cell counting.

2.7 | Statistical analysis

Analyses were conducted using SPSS, version 23 (IBM Corp., Armonk, NY, USA) and R, version 3.5.3 (R Foundation for Statistical Computing, Vienna, Austria). Body weight and AGD data were analysed using repeated-measures ANOVAs (for one control female, AGD data were not included as a result of a missing value) and VO data were analysed using an independent samples *t* test. Hormone data were analysed using non-parametric statistics (Kruskal-Wallis test and Dunn's post-hoc test with Bonferroni correction) as a result of a lack of normality in the data. One Antide-treated female did not appear to exhibit suppression of the HPG axis (ie, both the progesterone and LH serum levels fell above of the 95% percentile on PND 55/56), which strongly suggests that the Antide treatment was not successful for this subject, and the data for this female were therefore excluded from all analyses (whether this subject was included or not had minimal effects on the alpha values throughout). Adolescent social interaction data were analysed using a repeated-measures ANCOVA, with PND as the repeated measure and the identity of the play partner as a covariate. The EPM and SN data were analysed using repeated-measures ANOVA with Tukey's post-hoc test (in the EPM analyses, one Antide-treated female was excluded because the animal left the apparatus during the test) and the remaining behavioural data were analysed using one-way ANOVAs. Cell count data were analysed using two repeated-measures ANOVAs. All data are presented as the mean ± SD, except hormone data, which are presented as medians and interquartile ranges.

3 | RESULTS

3.1 | Body weight

Antide-treated and control females did not differ in body weight ($F_{1,25} = 0.39, P = 0.540$) (Figure 1) and the interaction between group and age was also not significant ($F_{9,225} = 1.65, P = 0.103$). The main effect of age was significant ($F_{9,225} = 1843.89, P < 0.001$).

3.2 | VO and AGD

Antide-treated females exhibited VO at a significantly older age (PND 36.7 ± 3.04 days) compared to control females (PND 33.9 ± 1.94 days) ($t_{26} = 2.84$, $P = 0.009$). As expected, AGD was smaller at PND 35 than at PND 49 ($F_{1,24} = 780.92$, $P < 0.001$); however, AGD did not differ significantly between Antide-treated and control females ($F_{1,24} = 0.16$, $P = 0.679$) and the interaction between group and age was not significant ($F_{1,24} = 0.52$, $P = 0.476$; PND 35: Antide-treated = 14.8 ± 1.4 mm; control = 15.1 ± 1.0 mm; PND 49: Antide-treated = 21.3 ± 1.0 mm; control = 21.4 ± 1.4 mm).

3.3 | Hormone levels

Serum progesterone levels differed significantly between groups at PND 55/56 ($P < 0.001$) (Table 1), with Antide-treated females having lower average progesterone levels than control females and higher average progesterone levels than control males (all pairwise comparisons, $P < 0.05$). At PND 69/70, serum progesterone levels differed between groups ($P < 0.001$) as a result of control males having lower average progesterone levels than both control females ($P < 0.001$) and Antide-treated females ($P < 0.001$), with no difference between the two groups of females ($P = 1.000$).

Serum LH levels differed between groups at PND 55/56 ($P = 0.001$) (Table 1) as a result of Antide-treated females having lower average LH levels than both control males ($P = 0.001$) and control females ($P = 0.017$), whereas control males had similar LH levels to control females ($P = 1.000$). At PND 69/70, serum LH levels differed between groups ($P = .0007$) as a result of control males having higher average LH levels than Antide-treated females ($P = 0.015$) and control females ($P = 0.028$), with no difference between the two female groups ($P = 1.000$).

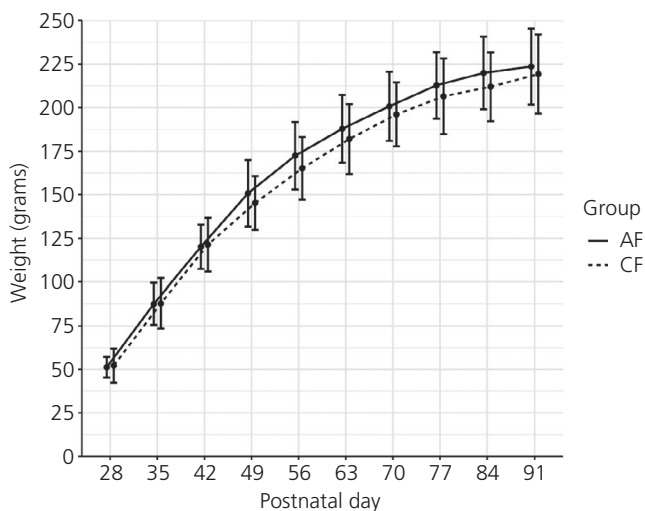


FIGURE 1 Body weight of Antide-treated females (AF, solid line, $n = 13$) and control females (CF, dotted line, $n = 14$) (mean \pm SD). PND, postnatal day

3.4 | Adolescent social behaviour

The total number of social interactions did not differ between groups ($F_{2,15} = 1.07$, $P = 0.367$) and did not differ across postnatal days ($F_{3,45} = 1.92$, $P = 0.140$) (Figure 2). Although Antide-treated females exhibited a reduction in social interactions on PND 43 (ie, 1 day after the second Antide injection), the interaction between group and day was not significant ($F_{6,45} = 1.36$, $P = 0.262$).

3.5 | Response to novel environments in adulthood

3.5.1 | EPM

The total number of entries onto open arms and closed arms of the EPM (ie, sum of open and closed arm entries) differed significantly between groups ($F_{2,37} = 9.67$, $P < 0.001$) (Figure 3A) as a result of control males making fewer transitions, on average, than both control females ($P = 0.003$) and Antide-treated females ($P = 0.001$). The main effect of arm type was not significant ($F_{1,37} = 1.15$, $P = 0.291$), neither was the interaction between group and arm type ($F_{2,37} = 0.64$, $P = 0.531$).

The percentage of time spent in either the open or closed arms (ie, time in open arms plus time in closed arms) differed between groups ($F_{2,37} = 3.35$, $P = 0.046$), which can be explained by control males spending more time, on average, in the alternative, central area of the EPM ($31.1 \pm 10.0\%$) than control females ($23.8 \pm 6.4\%$; $P = 0.036$), but not Antide-treated females ($27.4 \pm 4.71\%$; $P = 0.428$), with no difference between female groups ($P = 0.444$). The percentage of time spent in open arms vs closed arms did not differ ($F_{1,37} = 3.26$, $P = 0.079$) and the interaction between group and time spent in the open vs closed arms was also non-significant ($F_{2,37} = 1.30$, $P = 0.285$).

3.5.2 | OF

The total number of transitions between sections of the OF differed between groups ($F_{2,38} = 8.64$, $P < 0.001$) (Figure 3B), with, on average, control males making fewer transitions than Antide-treated females ($P = 0.002$) and control females ($P = 0.004$), with no difference between female groups ($P = 0.952$). The percentage of time spent in the centre of the OF did not differ between groups ($F_{2,38} = 0.94$, $P = 0.398$; control males = $19.0 \pm 11.2\%$; Antide-treated females = $21.7 \pm 6.1\%$; control females = $23.7 \pm 8.8\%$).

3.5.3 | LDB

The total number of transitions between sections of the LDB differed between groups ($F_{2,38} = 3.77$, $P = 0.032$) (Figure 4A), with control males, on average, exhibiting fewer transitions than control females ($P = 0.041$), but not Antide-treated females ($P = 0.091$), and with no difference between female groups ($P = 0.944$). Latency to enter the light section of the LDB differed between groups

		Control males	Antide-treated females	Control females
Progesterone (ng mL ⁻¹)	PND 55/56	1.00 (0.52) ^{*,#}	5.38 (2.59) [#]	9.13 (4.93)
	PND 69/70	0.57 (0.44) ^{*,##}	8.07 (4.79)	10.29 (12.69)
LH (ng mL ⁻¹)	PND 55/56	0.77 (0.40) ^{**}	0.35 (0.27) [#]	0.77 (0.62)
	PND 69/70	0.83 (0.26) ^{*,#}	0.39 (0.47)	0.47 (0.32)

* $P < 0.05$,

** $P < 0.001$ vs Antide-treated females;

$P < 0.05$,

$P < 0.001$ vs control females, (Kruskal-Wallis with Dunn's post-hoc).

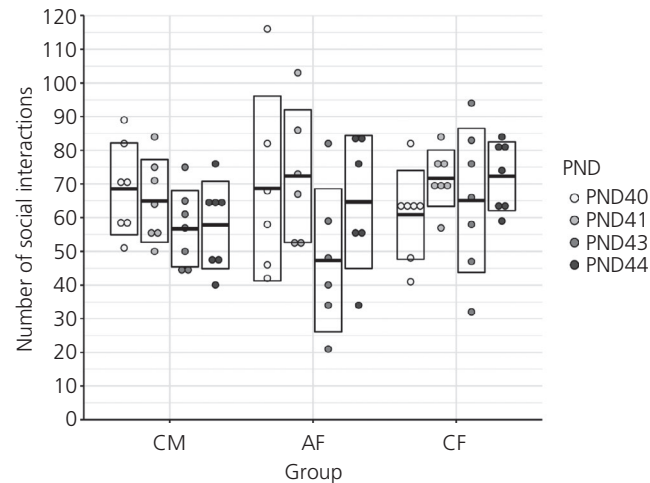


FIGURE 2 Total number of social interactions with a novel partner exhibited by control males (CM, $n = 14$), Antide-treated females (AF, $n = 13$) and control females (CF, $n = 14$) during 10-minute tests on postnatal day (PND) 40, 41, 43 and 44 (mean \pm SD)

($F_{2,39} = 3.40$, $P = 0.044$) (Figure 4B) as a result of control males having a longer latency (14.4 ± 13.5 seconds) than control females (5.4 ± 6.2 seconds; $P = 0.040$), but not Antide-treated females (8.0 ± 6.2 seconds; $P = 0.195$), with no difference between the female groups ($P = 0.750$). The percentage of time spent in the light section did not differ between groups ($F_{2,38} = 2.66$, $P = 0.083$; control males = $37.3 \pm 8.3\%$; Antide-treated females = $44.3 \pm 7.6\%$; control females = $43.0 \pm 9.4\%$).

3.6 | Response to novel objects and social partners in adulthood

3.6.1 | NO test

Total amount of time spent in the same section of the arena as the novel object did not differ between groups ($F_{2,38} = 3.18$, $P = 0.053$; control males = 90.0 ± 21.9 seconds; Antide-treated females = 78.7 ± 16.5 seconds; control females = 73.4 ± 14.2 seconds). The novel object preference score revealed a main effect of group ($F_{2,38} = 3.50$, $P = 0.040$), with control males having a

higher average preference score (23.3 ± 21.1) than control females (2.7 ± 22.0 ; $P = 0.030$), but not Antide-treated females (11.9 ± 18.2 ; $P = 0.333$), with no difference between the two female groups ($P = 0.486$).

3.6.2 | SN test

The percentage of time spent in the same section of the arena as the novel object or the social partner (ie, sum of the percentage of time spent in these two sections) differed between groups ($F_{2,38} = 4.04$, $P = 0.026$) as a result of control males spending slightly more time in the third, central section of the arena ($16.8 \pm 5.4\%$) than Antide-treated females ($12.4 \pm 4.1\%$, $P = 0.026$), but not more than control females ($13.6 \pm 2.7\%$, $P = 0.114$; Antide-females vs control females, $P = 0.763$). Across all groups, subjects spent more time in the section of the arena with the social partner than with the novel object ($F_{1,38} = 235.18$, $P < 0.001$) (Figure 5). The interaction between group and section of the arena was non-significant ($F_{2,38} = 1.42$, $P = 0.254$).

3.7 | ASR

The total number of immobilisation responses differed between groups ($F_{2,38} = 4.34$, $P = 0.020$) (Figure 6A), with control males, on average, exhibiting a higher number of immobilisations than control females ($P = 0.022$), but not Antide-treated females ($P = 0.081$), with no difference between the female groups ($P = 0.868$). The time spent immobile following a startle noise also differed significantly between groups ($F_{2,38} = 7.34$, $P = .002$) (Figure 6B), with control males, on average, spending longer immobile than both control females ($P = 0.004$) and Antide-treated females ($P = 0.009$), with no difference between the two female groups ($P = 0.960$).

3.8 | Cell counts

Cell counting, which was carried out on the tissue sections that were collected 90 minutes after the ASR task, revealed that the

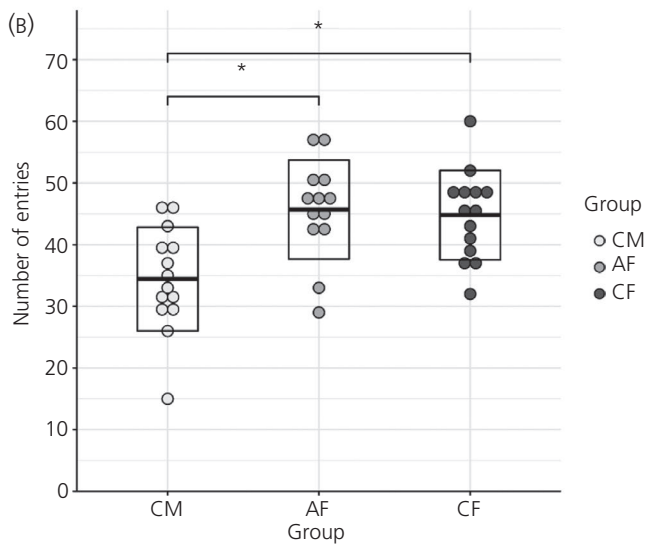
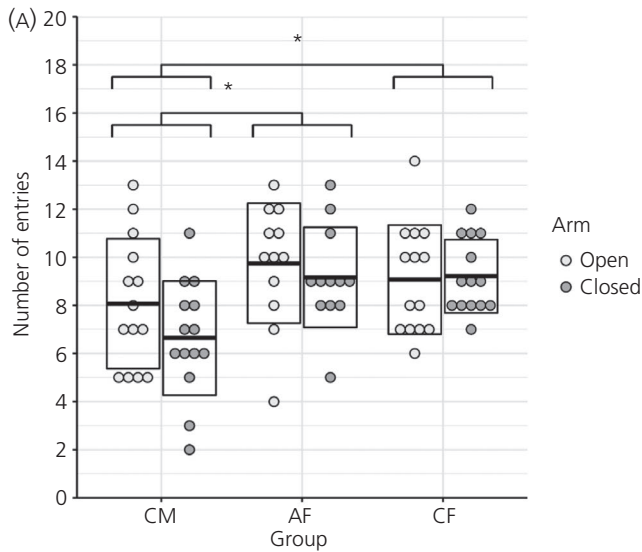


FIGURE 3 A, Number of entries onto the open arms (light grey points) and closed arms (dark grey points) of the elevated-plus maze (EPM) and (B) locomotion in the open field (OF) (total number of entries into sections), by control males (CM, $n = 14$), Antide-treated females (AF, $n = 12$ for EPM, $n = 13$ for OF) and control females (CF, $n = 14$) (mean \pm SD). * $P < 0.05$ (EPM = repeated-measures ANOVA; OF = one-way ANOVA with Tukey's post-hoc test)

number of *c-Fos* positive cells in the medial amygdala (MeA) nuclei did not differ between groups ($F_{2,17} = 2.29$, $P = 0.132$) (Table 2) or between MeA regions ($F_{3,51} = 2.50$, $P = 0.070$), and the interaction between group and region was not significant ($F_{6,51} = 1.45$, $P = 0.214$) (see Supporting information, Figure S1). Cell counts in the basolateral amygdala (BL) nuclei also did not differ between groups ($F_{2,24} = 0.51$, $P = 0.607$); however, cell counts did differ between BL regions ($F_{2,48} = 21.35$, $P < 0.001$) as a result of higher average cell counts in the BLA than the BLP ($P < 0.001$) and the BLV ($P < 0.001$; BLP vs BLV, $P = 0.719$). The interaction between group and BLA region was not significant ($F_{4,48} = 0.77$, $P = 0.552$).

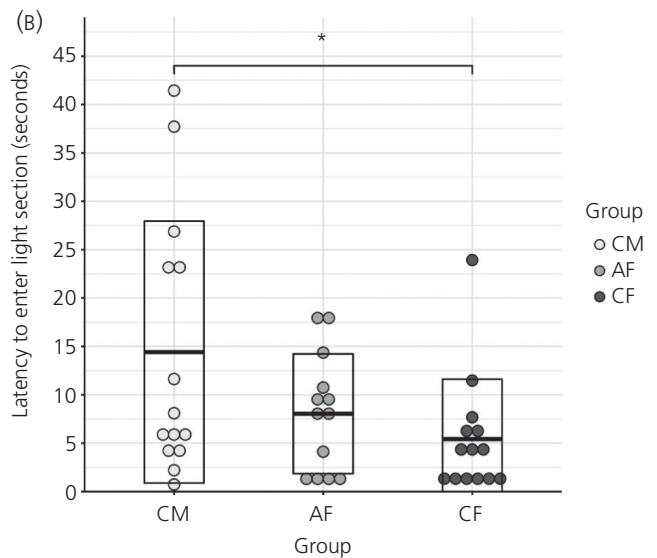
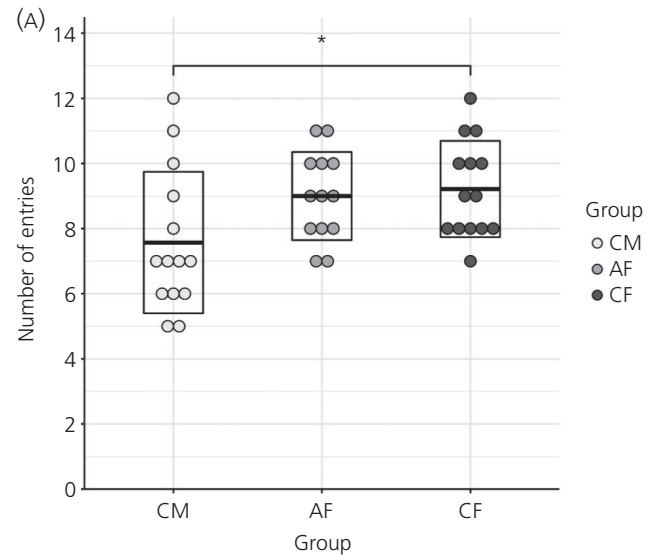


FIGURE 4 A, Locomotion in the light-dark box (LDB) (total number of entries into sections) and (B) latency to enter the light section of the LDB (seconds), by control males (CM, $n = 14$), Antide-treated females (AF, $n = 13$) and control females (CF, $n = 14$) (mean \pm SD). * $P < 0.05$ (one-way ANOVA with Tukey's post-hoc test)

4 | DISCUSSION

The results of the present study indicate that treatment of adolescent female rats with the GnRH antagonist, Antide, successfully suppressed the HPG axis and delayed pubertal development, as measured by reproductive hormone levels and the timing of VO, and Antide-treated and control females exhibited comparable levels of social behaviour during adolescence. However, contrary to our predictions, Antide-treated females did not differ from control females in behavioural tests involving exploration of novel environments, novel objects or novel social partners, or immobilisation responses to negative acoustic stimuli, in adulthood. In some behavioural tests, the expected sex differences in performance were observed; for

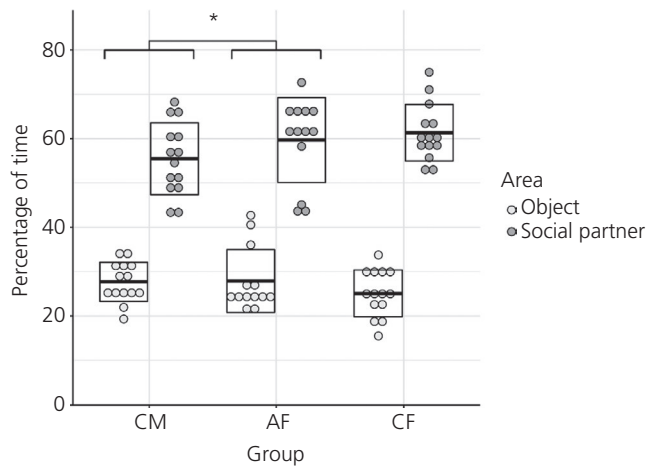


FIGURE 5 Percentage of time spent in the section of the arena with the novel object (light grey points) and social partner (dark grey points) in the social novelty (SN) test, by control males (CM, $n = 14$), Antide-treated females (AF, $n = 13$) and control females (CF, $n = 14$) (mean \pm SD). * $P < 0.05$ (repeated-measures ANOVA with Tukey's post-hoc test)

example, on average, control males exhibited less locomotion in the OF, as well as a higher preference for novel objects in the NOR task, than females, with Antide-treated females not differing from control females on these tasks. In addition, no group differences in c-Fos cell counts were found in nuclei of the medial and basolateral amygdala. Overall, these data suggest that a moderate delay in exposure to gonadal hormones during adolescence does not significantly impact upon anxiety-related behavioural and brain development in female rats, which contrasts with previous studies suggesting that adolescent exposure to testicular hormones has subtle, long-term effects on behaviour in male rodents.²⁴⁻²⁸

One of the benefits of using GnRH antagonists, rather than ovariectomies, to manipulate gonadal hormone levels is that, once the antagonist has been metabolised, the HPG axis is reactivated. The dose of Antide used in the present study was identical to the dose used in a previous study in our laboratory,⁶¹ and this previous study confirmed that administration of Antide on PND 28 resulted in a significant suppression of progesterone levels for at least 2 weeks.⁶¹ In the present study, Antide was administered on PND 28 and again on PND 42, and the hormone analyses of blood samples taken 2 weeks later (PND 55/56) showed that LH and progesterone levels in Antide-treated females were significantly lower than those of control females, confirming effective gonadotrophin and gonadal hormone suppression. The second blood sample, which was taken 4 weeks after the second Antide injection (PND 69/70), revealed that progesterone and LH levels did not differ between Antide-treated and control females at this point, indicating that the reproductive axis was reactivated. Collectively, these data confirm that the HPG axis can be temporarily suppressed in female rats by treatment with the GnRH antagonist, Antide.

With regard to pubertal development, Antide-treated females exhibited a modest delay (around 2-3 days) in the timing of VO relative to control females. This finding is consistent with previous evidence

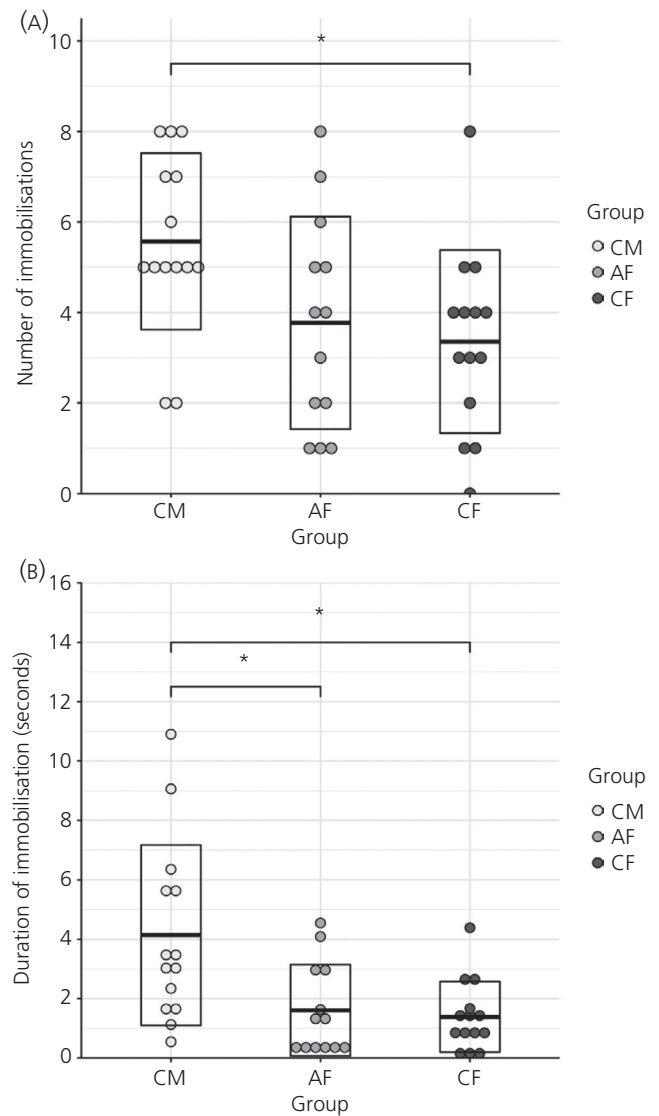


FIGURE 6 A, Number of times that subjects were immobile following the startle noise and (B) total duration of time spent immobile following startle noises by control males (CM, $n = 14$), Antide-treated females (AF, $n = 13$) and control females (CF, $n = 14$) (mean \pm SD). * $P < 0.05$ (one-way ANOVA with Tukey's post-hoc test)

indicating that suppression of the HPG axis in adolescent female rats moderately delays pubertal development,⁷⁸ whereas treatment with oestrogenic compounds can induce early puberty.^{79,80} A comparable delay in VO (2-3 days) has been found in previous studies that exposed female rats to either alcohol or stress during the adolescent period.^{81,82} The delay in VO in Antide-treated female rats did not appear to result from a more general developmental delay, given that average body weight did not differ between the two groups of females. If anything, Antide-treated females gained slightly more weight than control females, which is consistent with evidence that pre-pubertal ovariectomy leads to weight gain in female rats.^{33,35} Average AGD did not differ between the two female groups and thus AGD does not appear to be influenced by ovarian hormone exposure during this stage of life. This finding contrasts with studies of male

TABLE 2 c-Fos⁺ cell counts in the medial and basolateral amygdaloid nuclei in control males (n = 14), Antide-treated females (n = 13) and control females (n = 14) (per mm²; data are presented as the mean ± SD)

		Control males	Antide-treated females	Control females
Medial amygdala (per mm ²)	MeAD	46.95 ± 15.56	54.56 ± 16.13	60.26 ± 34.69
	MeAV	48.40 ± 31.72	38.41 ± 47.73	80.80 ± 59.85
	MePD	24.28 ± 11.71	69.59 ± 12.43	65.82 ± 41.23
	MePV	49.76 ± 22.46	89.28 ± 28.44	93.96 ± 108.85
Basolateral amygdala (per mm ²)	BLA ^{*#}	38.14 ± 22.81	38.30 ± 18.44	27.43 ± 15.97
	BLP	15.24 ± 11.37	11.74 ± 9.42	14.66 ± 12.09
	BLV	20.41 ± 16.36	17.79 ± 31.67	11.49 ± 11.11

Abbreviations: MeAD, anterodorsal medial; MeAV, anteroventral medial; MePD, posterodorsal medial; MePV, posteroventral medial; BLA, Basolateral anterior; BLP, Basolateral posterior; BLV, Basolateral ventral.

*P < 0.001 vs BLP,

#P < 0.001 vs BLV (repeated-measures ANOVA).

rodents where AGD can show some plasticity during adolescence and adulthood in response to circulating androgen levels.^{28,83,84}

During adolescence, Antide-treated and control females exhibited similar levels of social interactions with novel partners, which suggests that suppressing the HPG axis did not have immediate activational effects on social behaviour. This finding is consistent with a previous study showing that the total amount of adolescent social play was not influenced by pre-pubertal ovariectomy in female rats.³³ By contrast, a recent study reported increased frequencies of play with a familiar partner following pre-pubertal gonadectomy in female Siberian hamsters.⁸⁵ In addition, subtle aspects of posture during social interactions have been shown to be influenced by early gonadal hormone exposure in rats,^{31,33} and so we cannot exclude the possibility that Antide treatment might have had more fine-grained effects on social behaviour that were not evident in the present study. In adulthood, Antide-treated and control females did not differ in their response to novel male partners in the SN task, although more subtle effects might again have been revealed using other measures. One such measure of interest would be the frequency and type of ultrasonic vocalisations (USVs), given that female rats are known to exhibit high levels of USVs in the presence of novel social partners.⁸⁶

Several of the behavioural tasks involved placing subjects into a novel environment and measuring total locomotion and time spent in the potentially aversive areas of the apparatus (ie, open arms of the EPM, centre of the OF and light area of the LDB). In all of these tasks, although control males exhibited lower average levels of locomotion than either of the female groups, the two groups of females did not differ, which suggest that suppression of the HPG axis in females does not have long-term effects on exploratory behaviour. By contrast, pre-pubertal gonadectomy has been reported to increase exploratory behaviour in novel environments when tested during adolescence (in male rats²⁴ and female Siberian hamsters³⁴), which leaves open the possibility that peri-pubertal gonadal hormones have short-term effects on locomotor exploration. In the present study, control male and control female subjects did not differ in the amount of time spent in the exposed sections of each apparatus,

which could potentially be explained by the handling that subjects received during adolescent testing. Adolescent handling has been shown to reduce later anxiety-like behaviour,⁸⁷ whereas adolescent social interactions per se do not alter behavioural responses on these tasks.⁸⁸ The lack of sex differences in control subjects leaves open the possibility that the effects of suppressing the HPG axis might only be revealed following specific adolescent experiences.

In the NO task, control males exhibited a higher preference than control females for the novel object, which is consistent with the sex difference that we have previously reported in adolescent rats.¹⁷ In this task, Antide-treated females had an average preference score that was intermediate between that of control males and control females. Similarly, in the ASR task, the number of immobilisations shown by Antide-treated females was intermediate between the scores for control males and control females. By contrast, the total time spent immobile did not follow the same pattern and instead the standard sex difference was replicated, with males on average spending more time immobile than both groups of females.⁶⁰ Although these data suggest that Antide-treated females might differ slightly from control females on some measures of responsiveness to novelty and fear-inducing stimuli, this interpretation of the results was not supported by the underlying brain activity because no differences in c-Fos labelling were evident between groups in the basolateral or medial amygdaloid nuclei. This lack of between-group differences in c-Fos measures was unexpected, given the sensitivity of the BLA to stressors, including aversive acoustic noises.⁸⁹ A potential explanation is that measures of immobilisation in the ASR task do not strongly reflect aversive responses, and future studies could benefit from employing startle amplitude measures instead. Overall, we conclude that suppressing the HPG axis during adolescence did not result in marked behavioural effects or differences in c-fos activity in the basolateral or medial amygdaloid nuclei.

In summary, although previous research has suggested that adolescent exposure to ovarian hormones has long-term effects on subtle aspects of social behaviour,^{30–32} the results of the present study do not provide strong evidence for similar effects on anxiety-like

behaviour. One of the limitations of previous experimental studies that have used ovariectomy techniques is the possibility that the surgical procedure itself acts as a stressor and thus introduces a potential confounding factor.⁹⁰ The use of injectable GnRH antagonists to suppress the HPG axis provides a useful, alternative approach. Given that a range of factors can influence circulating gonadal hormone levels, including alcohol and stress exposure,^{91,92} the long-term effects of manipulating the HPG axis during adolescence deserve continued investigation.

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DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Gillian R. Brown  <https://orcid.org/0000-0002-0675-0780>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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