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Research report

Investigation of sex differences in behavioural, endocrine, and neural measures following repeated psychological stressor exposure

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Abstract

Animal models of repeated stressor exposure have generally been limited to physical stressors, despite the fact that the purpose of such models is to represent repeated stress in humans, which is usually psychological in nature. The present study was undertaken to investigate the behavioural, endocrine, and neural responses to a repeated psychological stressor exposure in male and female rats. Long-Evans rats were exposed to cat odour or a control condition for 1 h each day from Day 1 to Day 22. Every fourth day, defensive (e.g. hiding), and non-defensive (e.g. grooming) behaviour was quantified, during both the initial and the final 10 min of the hour. Defensive behaviours in cat odour-exposed animals remained vigorous during the initial 10 min of exposure across 22 exposure days. Non-defensive behaviours were suppressed during early exposures, but this suppression habituated across repeated exposures. Overall, the pattern of behavioural results indicated enhanced responses to novelty and to repeated cat odour exposure, in females, relative to males. Plasma corticosterone (CORT) levels were higher in females relative to males overall. However, males, but not females, exposed to cat odour had higher levels of CORT following exposure on Days 1 and 22, relative to controls. Finally, mRNA levels of glucocorticoid receptor, mineralocorticoid receptor, and brain-derived neurotrophic factor, all of which are modulated by CORT, were examined in hippocampus at the completion of stressor exposure, but none was affected by repeated stressor exposure. Results are discussed within the context of potential differences in effects of repeated psychological versus physical stressors.

Keywords: Stress; Corticosterone; BDNF; GR; Predator odour; Sex difference

1. Introduction

An organism's ability to deal with environmental stressors effectively (i.e. mount a set of responses that permit survival) is critical. The volume of research characterizing stress responding in rodents has increased dramatically, reflecting the mounting evidence supporting a link between stress responding and neural disease processes in humans [1].

One of the major systems activated during stressor exposure is the hypothalamic–pituitary–adrenal (HPA) axis, culminating with the release of glucocorticoids (corticosterone (CORT) in rats; cortisol in humans) from the adrenal glands, which exert widespread actions on physiology and function of peripheral and neural systems. In the short term, these hormones mobilize energy stores to allow for the expression of an adaptive fight-or-flight response [2]. These responses include context-specific behaviours, evoked at least in part by the binding of glucocorticoids to receptors in brain regions critical for stress responding. As the concentration of free circulating glucocorticoids increases, and as high-affinity mineralocorticoid receptors become saturated, glucocorticoids go on to initiate negative feedback on the HPA axis by binding to glucocorticoid receptors (GR) in the hippocampus [3,4].

Although short-term increases in glucocorticoids mediate adaptive responses, chronic and/or repeated exposure to stress or glucocorticoids leads to anatomical and functional changes of the hippocampus [5,6], including the retraction of dendritic spines [7,8], reduced inhibitory input to hippocampus [9], and suppressed neurogenesis [9,1,10]. The functional significance of these cellular changes is a matter of debate as some of

Abbreviations: ACTH, adrenocorticotrophic hormone; BDNF, brain-derived neurotrophic factor; CO, control odour; CORT, corticosterone; GR, glucocorticoid receptor; HB, hide-box; HO, head-out; MR, mineralocorticoid receptor; OA, odour area; PO, predator odour.

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them are reversible following chronic stress in rodents (e.g. dendritic retraction) [11]. Neurotrophins, such as brain-derived neurotrophic factor (BDNF), modulate the health and function of adult neurons [12]. Changes in protein and mRNA levels of BDNF [13–15], and its receptor, tyrosine receptor kinase B (trkB) [16,17], have been observed following stressor exposure, and are postulated to be part of the molecular mechanisms underlying hippocampal atrophy. Although there appears to be differential involvement of hippocampal BDNF changes in response to acute versus chronic stress [18], it remains unclear how that relates to other cellular changes and to endocrine and behavioural stress responses.

Animal studies of stress responding have demonstrated that males suffer from acute and chronic stress, while females are resistant. At the neural level, only male rats show alterations in hippocampal pyramidal neurons [19–21] following 21 days of repeated stress. At the behavioural level, sex differences in the effects of stress have largely been limited to learning paradigms. For example, chronic or repeated stressor exposure has deleterious effects in males on object recognition [22], spatial memory [23], and eye-blink conditioning [7]. Despite the lack of behavioural changes in learning paradigms in female rodents, various measures of HPA axis activation are higher in females, including CORT and ACTH levels following stress [24,25], as well as hippocampal glucocorticoid receptor (GR) levels [26]. Also, exogenous manipulation of sex steroids in females affects responses to repeated stress [27]. These findings demonstrate the importance of examining stress responses in both sexes.

The vast majority of stressor models (i.e. footshock, restraint, forced swimming) involve a physical component, making it difficult to examine behavioural effects of repeated psychological stressor exposure. Humans are more likely to be repeatedly exposed to psychological stressors, and diagnoses of clinical depression occur at the behavioural level. Thus, an ideal animal model would include repeated exposure to a psychological stressor and allow for the measurement of behaviour during stressor exposure. This is particularly important, given that dissociations between behavioural and physiological responses to stressors occur in rodents [22,28,29]. For example, a single footshock stressor, followed by weekly situational reminders, blunts the CORT response to the context after 3 weeks, but increases anxiety-like behaviour [30].

Predation threat represents an excellent model of repeated psychological stressor exposure because it induces robust and well-characterized changes in defensive and non-defensive behaviours in both males and females of many species of rodent [31–35], as well as changes in HPA axis function [28,36,29]. Predatory cues, such as cat odour, are sufficient to alter defensive behaviours, and these effects are not simply a function of the novelty or noxious nature of the odour [37]. Predatory odours selectively stimulate the neural circuitry involved in stress responding and defensive behaviour [38,39]. Here, we use this natural model of repeated stress to examine behavioural (defensive behaviour), endocrine (CORT levels) and neural changes (GR and BDNF mRNA levels) in both male and female rats. The first objective was to compare behavioural and endocrine responding across the repeated exposure period in males and females. The second objective was to examine how repeated stressor exposure would affect levels of the stress-related transcripts in hippocampus at the completion of the exposure.

2. Materials and methods

2.1. Animal housing

Twelve male and 12 female Long-Evans hooded rats (Charles River Canada, St. Constant, Quebec) were purchased at 60 days of age and acclimated to the colony room for 2 weeks prior to handling. Animals were housed in same-sex pairs upon arrival, but individually once habituation began. They were housed in polypropylene cages (47 cm × 24 cm × 20.5 cm) with wire lids, containing pine shavings (Hefler Forest Products Inc., Sackville, NS, Canada), and a black piece of PVC tubing (12 cm length, 9 cm diameter) for enrichment. Rat chow (Purina Lab Chow) and water were available *ad libitum*. A 12 h light:12 h dark reversed cycle (lights off at 09:30 h) was maintained in the colony room, which was kept at a temperature of 21 ± 1 °C. All experimental procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals. An effort was made to use the minimum number of animals required for statistical comparison and to minimize pain and suffering in experimental subjects.

2.2. Stressor exposure and assessment of physiological and behavioural measures

Fig. 1A illustrates the experimental timeline across days. Detailed descriptions of procedures are provided below.

2.2.1. Handling

Beginning 2 weeks after arrival (∼75 days of age) all rats were familiarized with being handled in the colony room once every day for 7 days prior to initiating experimental stress procedures. Handling familiarization involved picking up each rat and placing it onto the arm of the researcher for approximately 1 min, and then returning it to its home cage.

2.2.2. Pre-stressor blood sampling

In order to determine the physiological baseline for CORT levels, a blood sample was collected from each animal via the saphenous vein at 21:30 h (0.5 h after lights ON) during the evening prior to the first day of repeated stressor exposure (pre-baseline sample). During collection, each rat was transported from the colony room in its home cage to a lab space nearby, a space separate from experimental rooms. The experimenter restrained the animal using a surgical drape, leaving the hind leg exposed. The experimenter held the exposed leg between the thumb and forefinger, to apply pressure until the saphenous vein was visible through the skin and could be punctured with a sterile 23 g needle. Blood was collected and placed immediately on ice, while the puncture wound was clotted with gauze. The procedure took approximately 5 min for each animal.

2.2.3. Repeated stressor exposure

Stressor exposure took place in clear rectangular Plexiglas test arenas (80 cm × 39 cm × 30 cm), within which was integrated a hide box (HB; 27 cm × 39 cm × 30 cm) built from three black opaque walls (to increase darkness) and one clear wall to allow for observations (see Fig. 1B for a schematic representation). The HB contained an opening (7 cm × 7 cm) through which the animal could enter and exit. During experimental trials, the HB was placed at the end of the arena opposite to where the odour stimulus was placed. To ensure that rats were familiar with the testing apparatus prior to the beginning of the experiment, rats were placed individually into the test arena containing the HB (without any odour stimulus) for 15 min on 2 consecutive days prior to the beginning of stressor exposure and behavioural assessment.

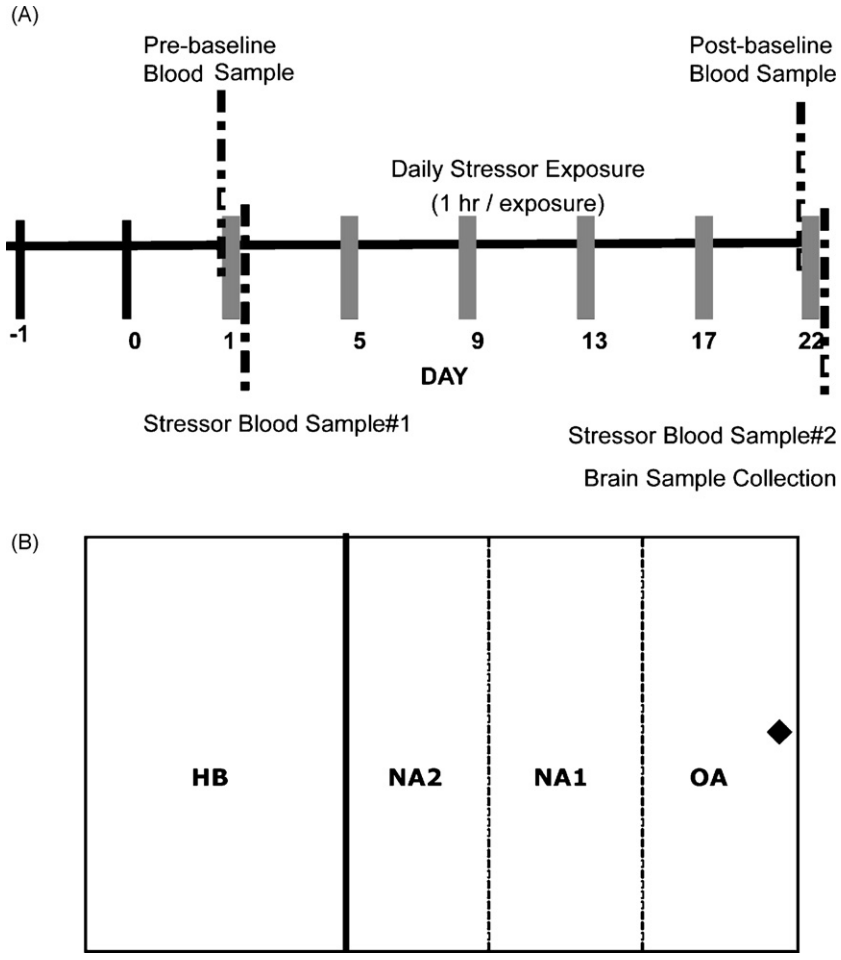

Fig. 1. (A) Prior to the beginning of the experiment (Days −1 and 0), rats were habituated to the test arenas. Beginning on Day 1, rats were individually exposed to the odour of a cat (PO) or a control condition for 60 min on each day from Day 1 to Day 22. Every fourth day, behaviour of the rats was recorded (these days are represented by thick gray lines). Blood samples were taken at select times during the experiment for assessment of corticosterone (CORT) levels. A pre-experiment baseline CORT sample was taken at 21:30 h (0.5 h after lights ON) on the night prior to Day 1 and two samples were taken during the experiment, within 5 min of the end of the stressor exposure (Days 1 and 22). A post-experiment baseline blood sample was taken at the end of the experiment in the same manner as the pre-experiment sample. (B) A top-down schematic representation of the test arena used to expose rats to PO or control. The arena consists of a hide box (HB) with three opaque walls (one side is clear) at the opposite end to an alligator clip (represented by the diamond), which is used to secure a piece of j-cloth containing the odour stimulus. For the purpose of scoring behaviour, the area outside of the HB was divided into three equal areas (odour area (OA), neutral area 1 (NA1), neutral area 2 (NA2)), allowing for the analysis of space use and assessment of crude locomotor activity (line-cross frequency).

On the day following the second habituation trial, subjects were randomly assigned to receive exposure to predator odour (PO; cat odour) or exposure to a control condition for 1 h per day for 22 days. Rats were exposed individually in the test arenas described above. The PO condition was administered by placing a strip of j-cloth (2.5 cm × 15 cm) containing cat hair and dander at one end of the test arena. To obtain the PO, a clean non-antibacterial j-cloth was taken each day to a cat breeding colony room within the Psychology Department at Dalhousie University. The cloth was rubbed vigorously on 2–4 reproductively active cats residing in the colony room and the cloth was then cut into equal-sized strips. The control condition involved presenting a clean strip of j-cloth in an identical manner. At the beginning of a stressor session rats were placed individually into the center of the testing arena, facing the j-cloth strip (odour stimulus). This ensured consistency in terms of each rat being in close proximity to the odour stimulus, an important procedural consideration when using cat odour, which is not very volatile [37]. All exposures took place during the dark phase of the light:dark cycle (between 09:30 and 18:00 h). The actual time of testing within this period for each subject was randomized across test days to prevent development of time-based conditioning. Control exposures took place in different testing rooms than stressor exposures throughout the experiment. The testing arena and hide-box were washed with unscented laboratory soap (Fisherbrand Versa Clean, Fisher Scientific), rinsed, and dried after each use.

2.2.4. Assessment of behaviour during repeated stressor exposure

Non-defensive and defensive behaviours were recorded on six occasions, corresponding to every fourth day of the repeated stressor exposure period (Days 1, 5, 9, 13, 17 and 22; see Fig. 1A). On these days, stressor/control exposures were video-recorded through the clear Plexiglas side of the testing apparatus using a camera mounted on a tripod. The Observer software package (Noldus, Wageningen, Netherlands) was used to score behaviours based on standardized operational definitions in accordance with previous investigations from this laboratory using a similar apparatus [40,35]. Please see Fig. 1B for a schematic representation of the arena for scoring purposes. The initial 10 min and the final 10 min of each session were scored separately for: duration of *rearing* (non-defensive behaviour defined as any motion made by the rat whereby both front paws left the floor of the apparatus), duration of *grooming* (non-defensive behaviour including head and body grooming; scored as bouts longer than 2 s), frequency of *head-out postures* from within the HB (defensive behaviour scored when the head protruded from the opening in the HB), and frequency of *odour source contacts* (defensive behaviour including sniffing with contact, biting, pawing). Furthermore, the duration of time spent in the HB, in the vicinity of the odour stimulus (in the odour area; OA) and within two other areas between the HB and OA (neutral areas 1 and 2; NA1 and NA2) was measured. The frequency of transitions among areas OA, NA1, NA2, and HB constituted a crude measure of spontaneous activity (line-cross frequency). Results of

Table 1
Statistical results for non-defensive and defensive behaviours measured during the initial 10 min of stressor exposure

Behavioural measure	Significant statistical effects	F- and p-values	Figure
Line-cross frequency	Day × Condition	$F_{4,80} = 2.459$, $p = 0.051$	Fig. 2A and B
Rearing duration	Condition	$F_{1,20} = 36.837$, $p < 0.001$	Fig. 2C and D
	Sex	$F_{1,20} = 5.525$, $p = 0.029$	
	Day × Sex	$F_{3.308,66.153} = 3.192$, $p = 0.025$	
Grooming duration	Condition	$F_{1,20} = 8.324$, $p = 0.009$	Fig. 2E and F
	Day	$F_{2.549,\ 50.980} = 9.340$, $p < 0.001$	
	Sex	$F_{1,20} = 15.229$, $p = 0.001$	
	Day × Sex	$F_{2.549,\ 50.980} = 3.112$, $p = 0.042$	
Time spent in the odour area	Condition	$F_{1,20} = 4.522$, $p = 0.046$	Fig. 3A–D
Time spent in the hide box	Condition	$F_{1,20} = 34.081$, $p < 0.001$	Fig. 3A–D
Odour source contact frequency	Condition	$F_{1,20} = 5.360$, $p = 0.031$	Fig. 4A and B
Head-out posture frequency	Condition	$F_{1,20} = 15.724$, $p = 0.001$	Fig. 4C and D
	Day	$F_{3.975,79.502} = 4.906$, $p = 0.001$	
	Day × Sex	$F_{3.975,79.502} = 2.600$, $p = 0.043$	
	Day × Sex × Condition	$F_{3.975,79.502} = 4.424$, $p = 0.003$	

behavioural analyses for the initial and final 10 min of the test hour are presented separately.

2.2.5. Statistical procedures used for analyzing behaviour

Results from each behavioural measure were analyzed using a mixed-design analysis of variance (ANOVA) with Sex and Condition (Control, PO) as between-subject factors and Day (1, 5, 9, 13, 17, 22) as the repeated measure. Simple effects analyses were conducted by Day and/or by Sex, wherever indicated by interaction effects.

To gain a sense of how space use within the test arena changes with repeated stress exposure, and to assess whether such patterns are influenced by sex, multivariate analysis of variance (MANOVA) was used to compare durations of time spent in OA, NA1, NA2, and HB among groups on each day separately. MANOVA combines these measures in such a way as to maximize group differences. In other words, the individual measures are each differentially weighted depending on the results (but weightings are consistent across animals) and together comprise a combined measure, labeled overall space use.

2.2.6. Assessment of physiological measures during repeated stressor exposure

Please see Fig. 1A for a schematic of the timeline for physiological measures. In order to determine stress-induced CORT levels, a blood sample was collected from each animal via the saphenous vein, as described for pre-stressor blood sampling, within 5 min of stressor exposure cessation (at 60 min) on Day 1 (D1 sample) of repeated stressor exposure. A second blood sample was taken to determine stress-induced CORT levels from trunk blood at the time of sacrifice on Day 22 (D22 sample) of repeated stressor exposure (within ∼5 min of stressor exposure cessation at 60 min). Trunk blood collection took place after decapitation following CO_2 anesthesia, at a site away from testing rooms. To determine if there was evidence for a shift in baseline levels of CORT with repeated stressor exposure, a second baseline sample was taken at 21:30 h (0.5 h after lights ON) prior to the last day (i.e. Day 22) of repeated stressor exposure (post-baseline sample).

All blood samples were collected into heparinized tubes (Becton Dickinson, NJ) and centrifuged at 6000 × g at 4 °C. Resulting plasma was retained and stored at −80 °C until assay. Levels of CORT were determined using the Correlate-EIA Corticosterone immunosorbent assay kit (Assay Designs, MI). Samples were diluted 1:50 prior to assay. Intra- and inter-assay variability were determined to be 6.6–8 and 7.8–13.1 pg/ml, respectively, and the level of detection of this assay is 27 pg/ml.

Body weight (g) was recorded early in the dark phase for each animal prior to testing on Days 1, 7, 14, and 21 of stressor exposure.

2.2.7. Collection of brain tissue and gene expression analysis

Following decapitation, brains were quickly removed and placed onto a piece of Plexiglas kept cold on dry ice. Tissue was microdissected from the hippocampus by making a saggital incision along the midline to separate the two hemispheres. The cortical structures were peeled away to reveal the hippocampus, which was then dissected and flash-frozen in liquid N_2. Samples were maintained at −80 °C until RNA extraction and subsequent quantitative PCR (qPCR).

Frozen rat hippocampi samples (both hemispheres) were homogenized using a Polytron homogenizer and total RNA was isolated using an RNeasy Mini Kit as per the supplier's (Qiagen Inc., Mississauga, ON) instructions. Total RNA (1 μg) was reverse transcribed, using Stratascript RT reverse transcriptase with random hexamers $pd(N)_6$, according to the supplier's (Stratagene, Cedar Creek, TX) instructions, with the exception that a final dNTP concentration of 1 mM was used. A 2 μl aliquot of the reverse transcription reaction was used as a template for quantitative PCR, using a Stratagene MX3000p thermocycler, in a total volume of 20 μl with Brilliant SYBR Green QPCR Master Mix (Stratagene). The following primer pairs were used for quantitative PCR analysis:

rGR qPCR-F1, 5′-GCTTCAGGATGTCATTACGGGG-3′
rGR qPCR-R1, 5′-GCTTCAAGGTTCATTCCAGCC-3′ (Accession #: NM_012576)
rCYPHA qPCR-F1, 5′-ATGGTCAACCCCACCGTGTTCTTC-3′
rCYPHA qPCR-R1 5′-ATCCTTTCTCCCCAGTGCTCAGAG-3′ (Accession #: NM_017101)
rBDNF F1: 5′-GGATGAGGACCAGAAGGTTCG-3′
rBDNF R1: 5′-GATACCGGGACTTTCTCCAGG-3′ (Accession #: M61175)
rMR F1: 5′-TCGGCGAAAGAACTGTCCTG-3′
rMR R1: 5′-TTGGTCGGAGCGATGTATGT-3′ (Accession #: NM_013131)

Thermal cycling conditions were identical for each primer pair and were as follows: a single cycle of 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 20 s, and elongation at 72 °C for 30 s. Melting curves were generated from 60 to 94 °C at the end of the PCR protocol to ensure the amplification of a single product. The PCR products were then separated on a 2.5% agarose gel and visualized by ethidium bromide staining to ensure the formation of a single product at the appropriate size. Product sizes were as follows: BDNF, 305 bp; MR, 183 bp; GR, 188 bp; CYPHA, 136 bp. Relative C_T values were obtained by the $\Delta\Delta C_T$ method [41], using a threshold of 10 standard deviations above background for C_T.

Table 2
Statistical results for non-defensive and defensive behaviours measured during the final 10 min of stressor exposure

Behavioural measure	Significant statistical effects	F- and p-values	Figure
Line-cross frequency	Day	$F_{5,90} = 2.955$, $p = 0.016$	Fig. 5A and B
	Day × Sex	$F_{5,90} = 2.454$, $p = 0.039$	
Grooming duration	Day	$F_{5,90} = 2.757$, $p = 0.023$	DNS
Time spent in the odour area	Condition	$F_{1,18} = 13.976$,p = 0.002	Fig. 6A–D
	Day	$F_{3.105,55.891} = 3.814$, $p = 0.014$	
	Day × Condition	$F_{3.105,55.891} = 4.483$, $p = 0.014$	
Time spent in the hide-box	Condition	$F_{1,18} = 17.862$, $p = 0.001$	Fig. 6A–D
	Day	$F_{3.304,\ 59.470} = 2.652$, $p = 0.052$	
	Day × Sex	$F_{3.304,59.470} = 2.663$, $p = 0.051$	
	Day × Condition	$F_{3.304,\ 59.470} = 4.463$, $p = 0.005$	
Odour source contact duration	Condition	$F_{1,18} = 22.352$, $p < 0.001$	DNS
Head-out posture duration	Condition	$F_{1,18} = 22.352$, $p < 0.001$	DNS

DNS, data not shown.

3. Results

A large number of results were generated from the planned analyses. In order to focus on the major findings, we have compiled the statistical results for main effects of Day, Condition, and Sex, and interactions among them, in Table 1 (initial 10 min of stressor exposure sessions) and Table 2 (final 10 min of stressor exposure sessions). A description of the subsequent simple effects analyses and post hoc comparisons that were performed are described in sections below.

3.1. Behavioural assessment—initial 10 min of stressor exposure

3.1.1. Non-defensive behaviours

Measures of non-defensive behaviour are depicted in Fig. 2. Overall, during the initial 10 min of the stressor sessions, rats exposed to PO showed reduced levels of non-defensive behaviour relative to levels displayed by control rats. General activity in the form of rearing was suppressed on each day of the repeated stressor exposure. There were subtle sex differences in the effect of PO on these activity measures but no profound differences between males and females were evident.

3.1.1.1. Line-cross frequency. Upon initial odour exposure (Day 1; $p = 0.020$), and again on Day 13 ($p = 0.024$), PO-exposed rats crossed significantly fewer lines than CO-exposed rats (see Fig. 2A and B; Table 1).

3.1.1.2. Rearing duration. PO-exposed rats consistently reared for a shorter period of time relative to control rats (∼5–10% difference) and females reared for longer relative to males on Day 9 ($p = 0.001$) (see Fig. 2C and D; Table 1). In order to investigate a difference in the pattern of response across time between males and females, we conducted simple effects analyses to compare rear duration on Day 1 versus Day 9 for each sex separately. Indeed, in females there was a significant increase in rear duration on Day 9 relative to Day 1 ($F_{1,10} = 8.588$, $p = 0.015$), and in males there was a significant decrease ($F_{1,10} = 5.752$, $p = 0.037$).

3.1.1.3. Grooming duration. PO-exposed rats spent less time grooming relative to control rats and this was a consistent reduction across all days of the stressor period. Males groomed for a longer duration than females on Days 9 ($p = 0.048$), 13 ($p = 0.001$), and 17 ($p < 0.001$; see Fig. 2E and F; Table 1).

3.1.2. Space use

3.1.2.1. Space use patterns. MANOVAs were used to compare the patterns of space use (see Fig. 3A–D; Table 1) among groups on each day separately. There were significant main effects of Sex and Condition (p's < 0.001 for each day), except a presumably anomalous lack of effect of Condition on Day 5.

3.1.2.2. Time spent in OA. Rats exposed to PO spent less total time in OA (the area containing the odour source), relative to control rats, an effect that was maintained across all the days of the stressor period (see Fig. 3A–D; Table 1).

3.1.2.3. Time spent in HB. As expected, PO-exposed rats consistently spent more time in the HB (20% increase) relative to control rats (see Fig. 3A–D; Table 1).

3.1.3. Defensive behaviours

In general, the expression of risk assessment defensive behaviours was increased in PO-exposed rats relative to control rats. There were some effects of Sex and Day on these measures (see Table 1).

3.1.3.1. Odour source contact frequency. PO-exposed rats made contact with the odour source less frequently relative to control rats (see Fig. 4A and B; Table 1).

3.1.3.2. Head-out posture frequency. While in the secure position of the HB, PO-exposed rats adopted a head-out posture more frequently (see Fig. 4C and D; Table 1) than control rats. Simple effects analyses were conducted for each sex separately to reveal further information about the three-way interaction for the frequency data. These analyses revealed a main effect of Condition ($p = 0.020$) for females. For males, a significant effect of Day

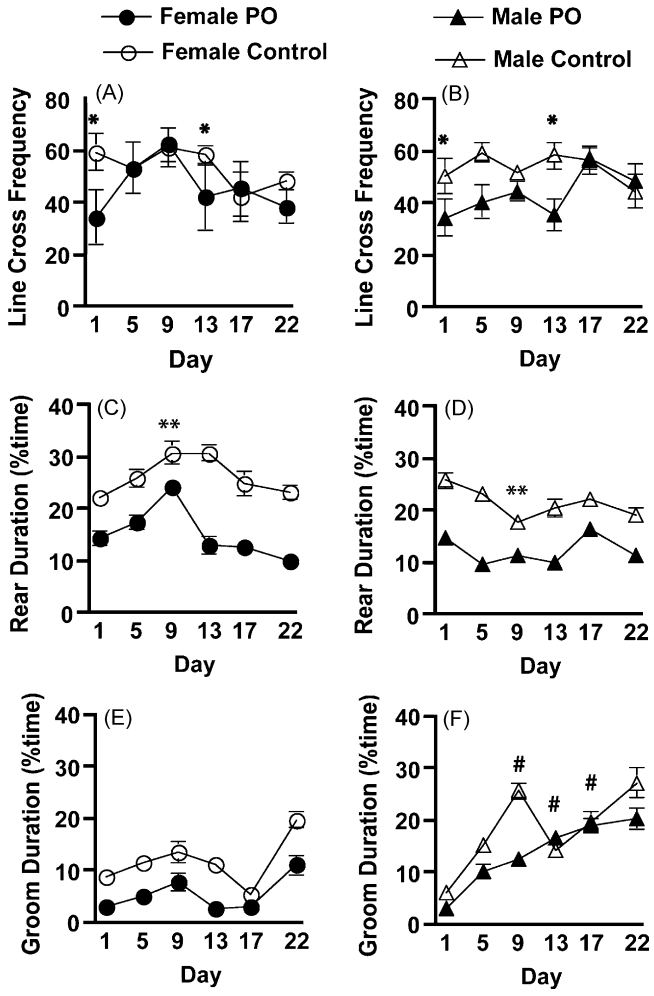

Fig. 2. Rats exposed to repeated predator odour (PO) displayed changes in a number of non-defensive behaviours relative to control rats. Males and females are graphed separately for ease of presentation but statistics on behavioural measures included Sex, Condition (PO vs. control) as between-subject factors and Day as the repeated measure. (A and B) Line-crosses were lower in PO animals, but mainly only on the first day of exposure. (*) Significantly different ($p = 0.020$) from Control on that day, collapsed across Sex. (C and D) In general, PO-exposed rats reared less than control-exposed rats. During the first 9 days, females increased their rearing while males decreased levels. (**) Day 9 is significantly different from Day 1 in females ($p = 0.015$) and males ($p = 0.037$). (E and F) PO exposure decreased grooming and males groomed more than females on select days. (#) Significantly different from females on the same day (collapsed across condition; $p < 0.05$).

($p < 0.001$), and a significant Day by Condition interaction effect ($p < 0.012$), were noted. PO-exposed males initially showed an elevated head-out frequency relative to controls ($p = 0.004$), but this dissociation was only maintained until Day 5 ($p = 0.010$).

3.2. Behavioural assessment—final 10 min of stressor exposure

3.2.1. Non-defensive behaviours

The effects of PO on a number of behaviours observed during the initial 10 min of the exposure were no longer present by the end of the stressor session. However, PO-exposed rats still spent more time hiding in the HB.

3.2.1.1. Line-cross frequency. Males were less active during the final 10 min as the stressor sessions went on ($p = 0.014$), an effect not observed in females (see Fig. 5A and B; Table 2).

3.2.1.2. Rearing and grooming. There were no significant differences in rearing duration (data not shown). Grooming behaviours changed significantly across repeated stressor exposures, but not in a clearly consistent fashion (data not shown).

3.2.2. Space use

3.2.2.1. Space use patterns. A MANOVA revealed significant main effects of Sex ($p = 0.043$) and Condition ($p < 0.001$) for the first Day (see Fig. 6A–D; Table 2). Interestingly, these effects were lost for Days 5 and 9. Significant effects of Condition reemerged for Day 13 ($p = 0.017$) and Day 17 ($p = 0.008$), but not Day 22.

3.2.2.2. Time spent in OA. During the final 10 min of exposure sessions, PO-exposed rats continued to spend less time in the OA relative to control rats on Days 1, 13, and 17 (p's < 0.05; see Fig. 6E and F; Table 2). Only control rats showed habituation of this defensive behaviour across time ($p = 0.01$).

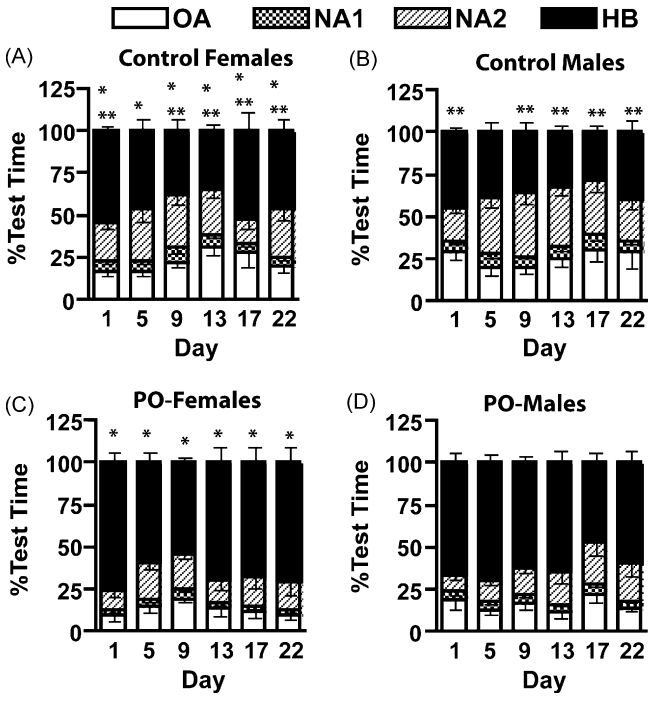

Fig. 3. Rats exposed to repeated predator odour (PO) displayed changes in patterns of space use relative to control rats. (A–D) The pattern of space use within the test arena was affected by both stressor exposure as well as sex on each of the days that behaviour was examined. Examination of the composite scores for time spent in the odour area (OA; area containing the odour stimulus), 2 neutral areas (NA1 and NA2; see Fig. 1B) and the hide box (HB) reveals that rats exposed to PO spent more time hiding in the HB and avoiding OA. Moreover, females spent more time in the HB relative to males. (*) Significantly different pattern relative to males on the same day, collapsed across Condition; (**) Significantly different pattern relative to PO animals on the same day, collapsed across Sex.

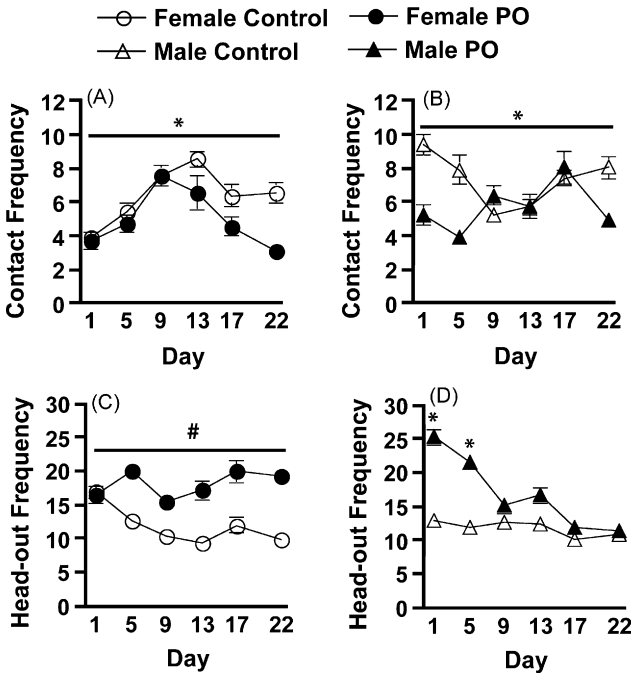

Fig. 4. Rats exposed to repeated predator odour (PO) displayed changes in a number of defensive risk-assessment behaviours relative to control rats. Males and females are graphed separately for ease of presentation but statistics on behavioural measures included Sex, Condition (PO vs. control) as between-subject factors and Day as the repeated measure. (A and B) Both male and female rats exposed to PO spent less time in contact with the odour stimulus relative to those exposed to a control condition. (*) Significant difference between PO and control, collapsed across Sex ($p < 0.05$). (C and D) While head-out frequency is consistently elevated in PO females relative to control females, the pattern of response in males with respect to this variable is more complex. In males, head-out frequency is only increased in PO-exposed animals on the Days 1 and 5 of the repeated stressor period. Thereafter, levels in PO males fall to those of control males. (#) Significant difference between PO-females and control females ($p = 0.02$). (*) Significantly different from control males on the same day ($p < 0.01$).

3.2.2.3. Time spent in HB. PO-exposed rats continued to spend more time in the HB relative to control rats, during the final 10 min of exposures. Simple effects analyses revealed that this measure changed across sessions in males only ($p = 0.006$ vs. 0.425 in females; see Fig. 6G and H; Table 2).

3.2.3. Defensive behaviours

During the final 10 min of an hour-long exposure to cat odour rats still responded with avoidance of the odour source, although for both PO and control rats, behaviour was more variable than it was during the initial 10 min of the exposure.

3.2.3.1. Odour source contact frequency. PO-exposed rats maintained decreased levels of odour source contact, relative to control rats, into the final 10 min segment of odour exposures, through the exposure period (data not shown).

3.2.3.2. Head-out posture frequency. There were no significant differences between PO- and control-exposed rats in head-out frequency while in the HB (data not shown).

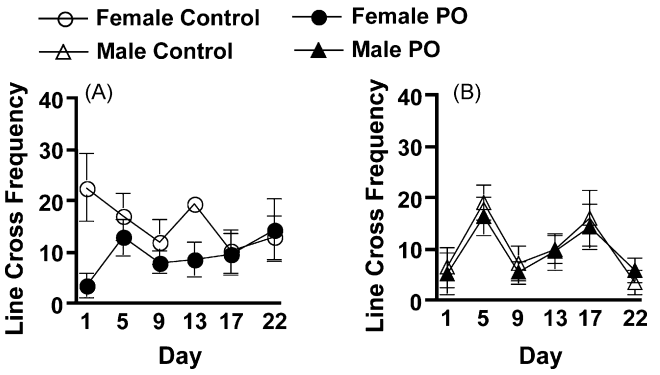

Fig. 5. Rats exposed to repeated predator odour (PO) displayed changes in non-defensive behaviours relative to control rats during the final 10 min of the stressor exposure session. Males and females are graphed separately for ease of presentation but statistics on behavioural measures included Sex, Condition (PO vs. control) as between-subject factors and Day as the repeated measure. (A and B) Line-cross frequency changed significantly across exposure days in males but not in females.

3.3. Physiological stress response measures

3.3.1. Body weight

Because of the well-characterized differences in growth rate of male and female rats, separate mixed-design ANOVAs, with Condition (PO, control) treated as the between-subject factor and Day (1, 7, 14, 22) treated as the repeated measure, were run for each sex separately (see Table 3 for means and S.E.M.'s). Body weight changed significantly across the test period in males, in both stressed and control rats (main effect of Day ($F_{3,30} = 79.9$, $p < 0.001$). Body weight significantly increased in females also across the 22-day session (main effect of Day; $F_{3,30} = 29.1$, $p < 0.001$). There were no significant effects of Condition for either sex.

3.3.2. Plasma CORT

3.3.2.1. Physiological baseline CORT levels. In order to determine if baseline levels changed across the stressor period a mixed-design ANOVA was performed with Sex and Condition (control vs. PO) as between-subject factors and time of baseline sampling (pre-baseline sample vs. post-baseline sample) as the repeated measure. Because there was no effect of Condition in males or females, data were collapsed for presentation and are presented as normalized to the pre-experiment average baseline levels of the respective group. Fig. 7A illustrates that there were no differences in the two baseline samples for males or females.

Table 3
There was no significant effect of repeated predator odour stressor on body weight in either males or females

Group	Day 1	Day 7	Day 14	Day 22
Cont-M	387.0 ± 8.0	425.0 ± 8.9	451.7 ± 6.5	465.0 ± 7.6
PO-M	370.0 ± 10.7	396.7 ± 11.5	416.7 ± 13.3	436.7 ± 14.8
Cont-F	285.0 ± 5.0	295.0 ± 4.3	303.3 ± 4.2	303.3 ± 5.6
PO-F	280.0 ± 7.8	288.3 ± 7.0	288.3 ± 7.5	298.3 ± 8.3

Means (±standard error of the mean) of body weight measured every 6 days across the 22 days of repeated stressor exposure.

Table 4
Females have higher levels of corticosterone (CORT) than males under basal and experimental conditions

Sex	D1-B	D21-B	D1-E	D22-E
Females	241.6 ± 40.0	154.6 ± 32.4	287.1 ± 32.4	370.0 ± 21.5
Males	80.0 ± 15.8	91.5 ± 19.4	100.1 ± 13.3	112.0 ± 12.2

Mean non-normalized plasma levels of CORT (±standard error of the mean) for baseline (B) and experimental (E; collapsed across PO and control conditions) samples are displayed for Day 1 (D1) and Day 22 (D22). Please see Section 2.2.6 for details. Statistical analyses revealed a main effect of sex; females had significantly higher levels than males across all time points.

3.3.2.2. Experimental CORT levels. Stress-induced levels of CORT were normalized for each animal to the mean physiological baseline determined for the group for D1 and D22. A 3-factor ANOVA with Sex, Condition (PO vs. control), and Day (D1 vs. D22) as between-subject factors was used to test for significant effects of stressor exposure on CORT levels. A significant main effect of Sex ($F_{1,40} = 12.1$, $p = 0.001$) was noted and in order to better show the sex difference (which exists for baseline and experimental CORT levels), Table 4 displays non-normalized CORT levels for males and females. A significant main effect of Condition ($F_{1,40} = 8.5$, $p = 0.006$) was also noted for normalized stress-induced CORT levels with PO-exposed rats having higher levels of CORT than controls. However, there was also a significant Sex by Condition interaction ($F_{1,40} = 12.14$, $p = 0.001$) and subsequent one-way ANOVAs for each sex separately revealed a main effect of Condition ($F_{1,20} = 14.4$, $p = 0.001$) for males only. Thus, PO exposure increased plasma CORT levels relative to control exposure in males, but not females (see Fig. 7B).

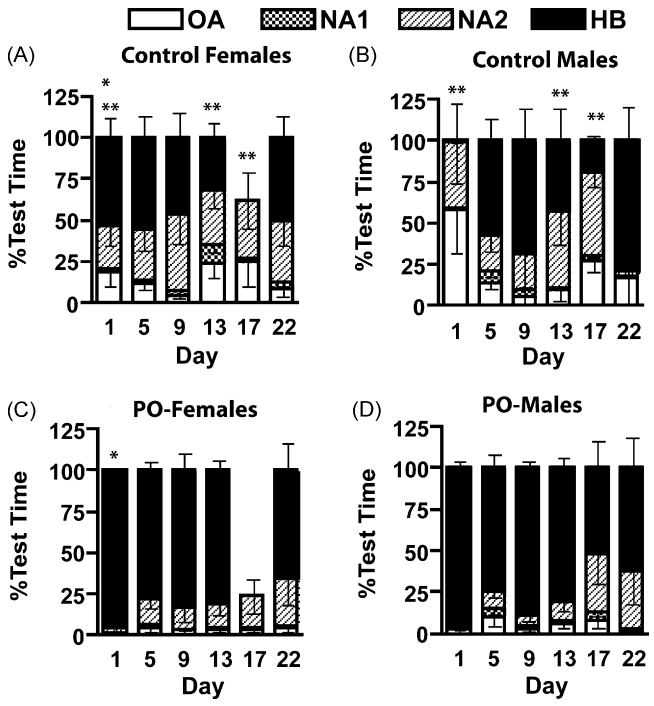

Fig. 6. Rats exposed to repeated predator odour (PO) displayed changes in patterns of space use during the last 10 min of the stressor session relative to control rats. (A–D) During the final 10 min of the stressor session, the pattern of space use within the test arena was affected by stressor exposure on specific days with sex differences only on Day 1. Examination of the composite scores for a number of areas (see Fig. 1B) reveals that rats exposed to predator odour (PO) are still spending a great deal of time hiding in the hide box (HB), some time in the neutral areas (NA1 and NA2) and avoiding the odour area (OA) relative to control rats. Similar to the initial 10-min analysis, females appear to use the hide-box more than males. (*) Significantly different pattern relative to males on the same day, collapsed across Condition; (**) significantly different pattern relative to PO animals on the same day, collapsed across Sex.

3.3.2.3. CORT and behaviour relationship. In order to examine the relationship between CORT levels and behaviour, HB duration measured during the final 10 min of the stressor session on D1 and D22 was correlated with CORT levels measured on D1 and at sacrifice on D22, separately for males and females using Pearson's correlation. Levels of CORT on D1 were not significantly related to hiding on D1 in males ($r(10) = 0.185$, $p > 0.05$) or females ($r(11) = 0.154$, $p > 0.05$). On D22, CORT levels were negatively related to the duration of time spent in the hide-box for males ($r(11) = -0.619$, $p = 0.04$) and for females ($r(10) = -0.682$, $p = 0.01$; see Fig. 7C).

3.3.3. Hippocampal gene expression

Fig. 7D–F depicts levels of hippocampal GR, MR, and BDNF mRNA. Separate two-factor ANOVAs with Sex and Condition (control, PO) were run for each transcript. Main effects of Sex were found for hippocampal expression of BDNF ($F_{1,19} = 12.338$, $p = 0.002$; see Fig. 7D) and GR ($F_{1,19} = 6.197$, $p = 0.022$; see Fig. 7F) mRNA, with females expressing higher levels relative to males. There was also a trend toward increased levels of MR transcription in females relative to males ($F_{1,19} = 3.824$, $p = 0.065$; see Fig. 7E). There was no effect of Condition or interaction between Sex and Condition for any transcript.

4. Discussion

Males and females displayed robust behavioural responses to the presence of cat odour throughout 3 weeks of daily exposure. However, there were sex differences in the temporal pattern of habituation of some defensive behaviours. Basal and stress-induced plasma levels of CORT were higher in females than in males but CORT levels were higher in PO-exposed males, but not females, after the first, and the last, stressor exposure relative to control-exposed males. Interestingly, higher levels of CORT at sacrifice were associated with lower levels of time spent hiding during the final 10 min of the final stressor session in both sexes. Hippocampal levels of GR, MR, and BDNF mRNA were not significantly altered by repeated stressor exposure, although levels of GR and BDNF were significantly greater in females relative to males.

During the first 10 min of the hour of cat odour exposure rats displayed decreased levels of non-defensive behaviours (rearing and grooming) and displayed defensive behaviours, including reduced contact with the odour source and increased risk assessment (head-out postures from within the hide box). They also altered their patterns of space use, increasing time spent hiding and avoiding the area containing the odour. Although these behavioural responses were robust in both sexes, there were sub-

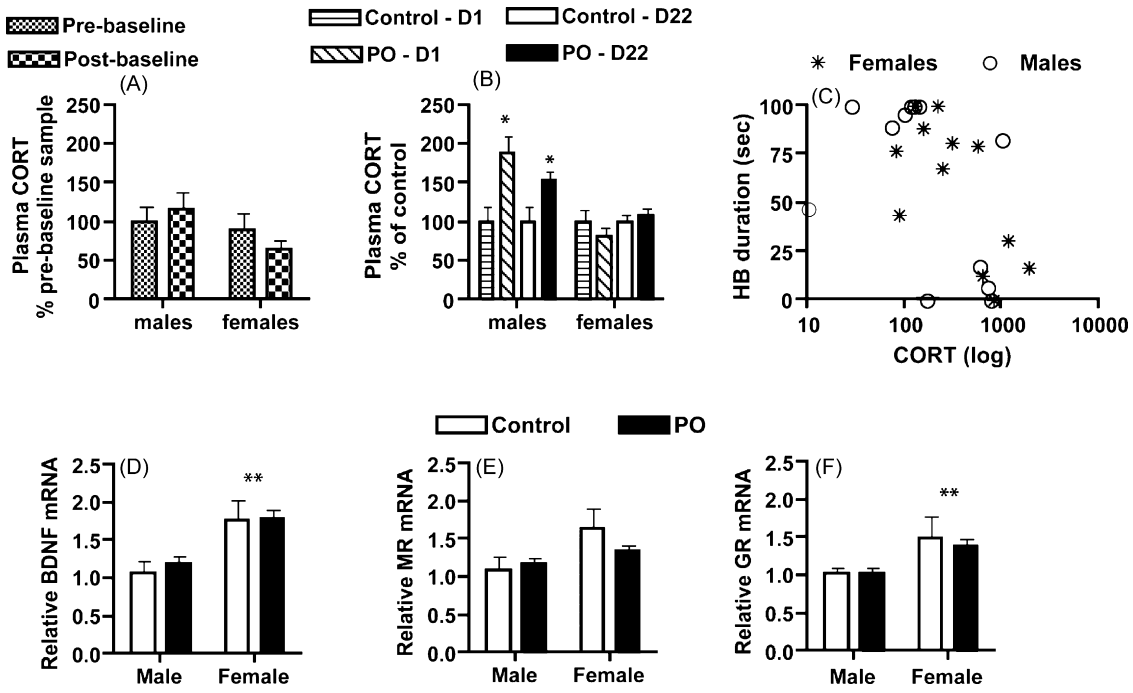

Fig. 7. (A) When sampled following 21 days of stressor exposure (i.e. during physiological nadir on Day 21; post-baseline), basal plasma levels of corticosterone (CORT) were similar to levels sampled during the physiological nadir the day prior to initiation of the stressor (pre-baseline). Data are normalized to pre-experiment baseline levels. (B) Exposure to predator odour (PO) increased plasma levels of CORT in males only, as measured on Day 1 (D1) and on Day 22 (D22). There was no significant effect of PO exposure on CORT levels in females on either day. (*) Significantly higher than control levels for the same day ($p < 0.05$). (C) Plasma levels of CORT sampled at sacrifice on D22 were negatively related to the amount of time spent in the hide box (HB) during the last 10 min of stressor exposure on D22 in males and females ($p = 0.04$ and 0.01, respectively). (D–F) Hippocampal levels of brain-derived neurotrophic factor (BDNF), mineralocorticoid receptor (MR), and glucocorticoid receptor (GR) were not affected by repeated exposure to predator odour (PO). Levels of GR and BDNF were significantly higher in females relative to males. (**) Significantly different from males, collapsed across condition, $p < 0.05$.

tle sex differences in the patterns of habituation across days of exposure that, overall, suggest enhanced responses to novelty and cat odour exposure in females relative to males. This suggests that females are more defensive in general and this is consistent with previous investigations noting that females exhibit higher levels of defensive behaviour, regardless of the testing condition [42,43]. In addition to rats, sex differences in behavioural responses to predator cues have been found previously in mice [44] and meadow voles [45]. The present study is the first to examine sex differences in behavioural responses to the psychological stress of predator cues over repeated exposures. One of the more interesting sex differences occurred with respect to habituation of risk assessment behaviour, assessed as the frequency of head protrusions from the hide-box. By Day 9 of repeated exposure, PO-exposed males were not displaying an increase in this defensive behaviour relative to control-exposed males. In contrast, PO-exposed females failed to show this defensive behaviour on Day 1 but displayed high levels on each subsequent day across the 22-day exposure period. The lack of head-out behaviour following an acute exposure to cat odour (i.e. Day 1 in the present study) is consistent with past work in Long-Evans rats [35]. Our results suggest that females show a slightly different pattern of defensive responding to acute versus repeated stressor exposure.

Results of prior studies with male rats have demonstrated habituation of some defensive responses, but not others [37,29,46]. Reductions in line-cross rates and rearing have typically shown fairly rapid habituation across exposures while defensive behaviours are less sensitive to habituation [32]. The most persistent responses to repeated predator odour cues appear to be odour avoidance measures, such as ‘sheltering’ [29] and reductions in odour source ‘approach-time’ [37]. For example, File et al. [29] demonstrated PO-induced sheltering across five 5-min exposures administered on consecutive days. Rats exposed to 20 days of daily 60 min presentations of a live cat separated from the rat by a wire screen mesh displayed a defensive response termed ‘crouching’ [28]. While crouching behaviour showed some evidence of habituation across the 60 min exposure periods, a vigorous crouching response was observed at the completion of each daily session and persisted across all 20 days [28].

Comparisons between prior studies and the present one are limited, as prior studies have typically employed shorter duration exposures (30 s to 20 min) administered over fewer repeated sessions (typically 5 days). The design and results of the Blanchard study (1998) are most similar to our own. Results from that study and the present results are consistent with the notion that defensive responses to cat odour (‘crouching’, spending more time in the HB, surveying the environment from a protected position) are persistent; that is, they are still evident at the end of an hour exposure. In contrast, non-defensive responses, such as rearing and grooming reductions were observed consistently during the first 10 min of each exposure, but these reductions were not present during the final 10 min.

Male rats exposed to PO showed significantly elevated CORT, relative to control-exposed males, at the completion of 60 min on the first and the last day of exposure, and the magnitude of the elevation was similar on the 2 days. These findings are consistent with reports showing an increase in CORT in response to an acute predation threat [36,29]. Few studies have examined the effect of repeated predatory stress on HPA axis output. Exposure to a live cat each day (60 min) for 20 days resulted in increased CORT in trunk blood, but the results are difficult to compare to the present study, as the animals were exposed to restraint stress on Day 19 [28]. A more recent investigation demonstrated that daily 1 h exposures to a cat initially increased plasma CORT in male Sprague–Dawley rats but did not result in increased CORT or ACTH on Day 7 or Day 14 [36]. This is not consistent with the present results in which we measured increased CORT levels in males on Day 22 following 1 h of cat odour exposure. The reason for the apparent discrepancy is unclear but could be related to differences in the stimulus (live cat vs. odour), the extent of the exposure period (14 day vs. 22 day), or the strain of rat.

In contrast to males, there was no discernable effect of repeated PO exposure on CORT levels in females. This finding is interesting, given the similar behavioural responses exhibited by males and females in response to the cat odour across the exposure period. However, studies demonstrating sex differences in CORT responses to stressor exposure have largely been performed using stressors containing a physical component, such as restraint stress (e.g. [24,25]) and thus, it is possible that females respond differentially to psychological versus physical stressors in terms of CORT release. However, because we only examined CORT levels at the end of stressor exposure (60 min after PO was introduced), effects in females may have been missed if the profile of CORT release is sexually dimorphic. One could argue against this, as CORT release in males was still elevated at 60 min, and that is consistent with past work using cat exposure [36]. Also, direct comparison of males and females following noise stress did not reveal any differences in the profile of CORT release (although females did show higher CORT release) [47]. Another possibility that should be considered is the fact that in the present study, naturally cycling females were used. Stage of estrus cycle impacts upon stress responding with higher levels of activation in stress-related regions in estrous and proestrous females relative to males and diestrous females [24]. We chose to use naturally cycling females because of past work demonstrating dramatic differences in changes in hippocampal BDNF following restraint stress in naturally cycling females and OVX females with hormones replaced to physiological levels [13].

Although females did not display a CORT response to PO, we observed significantly higher levels of CORT (both basal and stressor-induced) and levels of hippocampal GR mRNA in females, relative to males. This is consistent with findings that females display higher levels of HPA output than males, including plasma CORT secretion at rest [48,49]. Finally, females displayed higher levels of hippocampal BDNF mRNA relative to males, which is consistent with higher levels of BDNF protein observed in CA3 in intact females relative to males [13].

High overall CORT and/or BDNF levels in females may be causally related to enhanced defensive behaviour in general although future work will be necessary to make that determination directly. Indirect evidence for a relationship between CORT and behaviour was obtained in the present study. Higher plasma levels of CORT were correlated with lower levels of time hiding. This was the case for both males and females but was only observed at the end of the 22 days of stressor exposure. This relationship was not observed after acute exposure to PO (i.e. on Day 1), indicating that a relationship between CORT and defensive behaviour may develop with repeated exposures (in both sexes). Thus, it may be that behaviour is modulated over time to fit individual HPA profiles. Alternatively, behavioural responding to predation threat may feed back to modulate HPA axis output over repeated exposures. As there was no evidence for sensitization or habituation of the CORT response across repeated PO exposures in males, the former may be a more likely scenario.

The fact that we found no evidence for sensitization or habituation of the CORT response in males is somewhat surprising, given that chronic mild stress, social defeat and repeated restraint procedures can produce a sensitized CORT response [50]. However, we also found no evidence for changes in molecular markers of HPA function resulting from effects of repeated PO exposure, including mRNA levels of GR, MR or BDNF in hippocampus. It has been demonstrated previously that repeated restraint stress can produce a blunted HPA axis response, along with no significant changes in GR mRNA levels in hippocampus, relative to naïve restraint-stressed animals [51]. The role of BDNF in stress responding is still unclear, as BDNF mRNA in hippocampus can be increased by acute restraint stress [52], decreased following acute restraint stress, and decreased by repeated restraint, but less robustly [18]. Our results are difficult to compare with the aforementioned studies, however, because of the distinction between physical stressors, such as restraint and social defeat, and purely psychological ones, such as PO.

Consistent with a lack of effect of repeated PO exposure on various molecular markers of stress responding, we did not observe significant body weight changes in PO-exposed males or females relative to the control animals. These results are consistent with past findings showing that 20 days of repeated live cat exposure did not affect body weight, despite there being changes in adrenal and thymus weights [28]. In contrast, repeated exposure to a live cat reduced body weight on Day 7 with recovery by Day 14, with no evidence for HPA hyperactivity (ACTH and CORT) [36].

In conclusion, we have demonstrated that exposure to a repeated psychological stressor for 22 days induced defensive behavioural responding in male and female rats, with some evidence for sex differences defensive behaviour habituation. This stressor regimen did not alter hippocampal levels of GR, MR or BDNF mRNA in either sex. In the context of past work using physical stressors, these results suggest that habituation/sensitization processes in response to repeated stressor exposure might be influenced by the nature of the stressor. We also showed that males display a robust CORT response to acute PO and to PO at the end of 22 days of repeated exposure and that this response was not observed in females. This is consistent with the effects of psychological stress in humans, with men showing greater HPA axis reactivity than women [53].

In the present study, females had significantly higher levels of defensive behaviour, CORT (basal and in response to PO), and hippocampal GR and BDNF, relative to males. These findings are of particular interest when considered in conjunction with differences in both neural and endocrine responses to stress within the human and animal literature. Further characterization of the predator odour paradigm in relation to classical models of stress responding (i.e. physical stress, such as footshock or restraint) may lead to modeling the consequences of chronic psychological stress experience in humans more effectively. In contrast to other ecologically relevant models of repeated stress [54], using predatory threat is advantageous because it is relevant for both male and female rats. As such, it represents a useful model for studying the effects of sex on the stress response. Examining both sexes is critical to resolve the potential connections between repeated stress and affective disorders, which are more prevalent in women [55,56].

Acknowledgements

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