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Defensive aggregation to predatory threat in the laboratory rat: behavioural, neural, pharmacological and epigenetic correlates

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Statement of authentication

This thesis is submitted to the University of Sydney in fulfilment of the requirement for the Degree of Doctor of Philosophy.

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

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Co-author declaration

I the undersigned acknowledge the parts of the following statement pertaining to the publications presented in this thesis on which I am a co-author:

Michael T. Bowen was the primary author of the publications featured in Chapters 2 through 5 and Appendix 2 of this thesis. For these publications Mr. Bowen took the lead role in: the conception and design of the research; conducting the research; analysis and interpretation of the findings; and writing and critically appraising the manuscripts.

The publication in Appendix 1 contains two experiments. Mr. Bowen led experiment 2 for which he played the principal role in: the conception and design of the experiment; conducting the experiment; analysis and interpretation of the findings; and writing and critically appraising the parts of manuscript pertaining to experiment 2.

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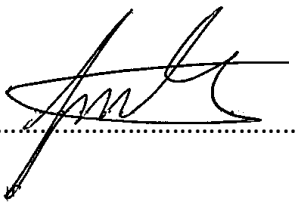
Signed……  ……Date:31/03/14

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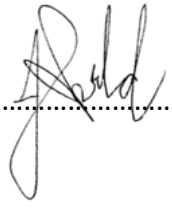
Signed……  ……Date:31/03/14

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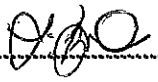
Signed……  ……Date:28/03/14

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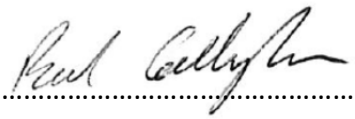
Signed  Date: 30/03/14

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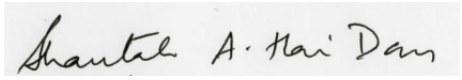
Signed  Date: 30/03/14

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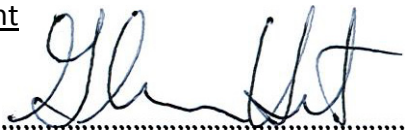
Signed  Date: 28/03/14

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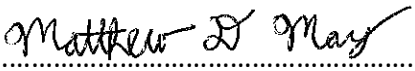
Signed  Date:27/03/14

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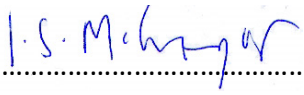
Signed  Date:27/03/14

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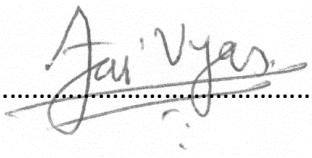
Signed  Date:30/03/14

Publications

The publications that form a major part of the contents of this thesis are:

1. **Bowen, M.T.**, Keats, K., Kendig, M.D., Cakic, V., Callaghan, P.D., McGregor, I.S., 2012. Aggregation in quads but not pairs of rats exposed to cat odor or bright light. Behavioural Processes 90, 331-336.
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2. **Bowen, M.T.**, Kevin, R.C., May, M., Staples, L.G., Hunt, G.E., McGregor, I.S., 2013. Defensive aggregation (huddling) in *Rattus norvegicus* toward predator odor: individual differences, social buffering effects and neural correlates. PLoS ONE 8, e68483.
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    - This paper is presented in Chapter 5.
5. May, M.D., **Bowen, M.T.**, McGregor, I.S., Timberlake, W., 2012. Rubbings deposited by cats elicit defensive behavior in rats. Physiology and Behaviour. 107, 711-718.
    - This paper is presented in Appendix 1.

6. **Bowen, M.T.**, Carson, D.S., Spiro, A., Arnold, J.C., McGregor, I.S., 2011. Adolescent oxytocin exposure causes persistent reductions in anxiety and alcohol consumption and enhances sociability in rats. PLoS ONE 6, e27237.

    - This paper is presented in Appendix 2.

7. Suraev, A., **Bowen, M.T.**, Ali, S.O., Hicks, C., Ramos, L., McGregor, I.S., 2014. Adolescent exposure to oxytocin, but not the selective oxytocin receptor agonist TGOT, increases social behavior and plasma oxytocin in adulthood. Hormones and Behaviour. 65, 488 – 496.

    - This paper is presented in Appendix 3

Additional publications

As part of my PhD studies, I also made a substantial contribution to the following publications which are complimentary to the present thesis but do not form a part of its content:

Book Chapters

1. McGregor, I.S., **Bowen, M.T.,** 2013. Oxytocin and addiction: recent preclinical advances and future clinical potential, in: Choleris, E., Pfaff, D.W., Kavaliers, M. (Eds.), *Oxytocin, Vasopressin and Related Peptides in the Regulation of Behavior.* Cambridge University Press, Cambridge UK, pp. 270 - 287.

Reviews

2. McGregor, I.S., **Bowen, M.T.**, 2012. Breaking the loop: Oxytocin as a potential treatment for drug addiction. Hormones and Behavior. 61, 331-339.

Original research

3. Kendig, M.D., **Bowen, M.T.**, Kemp, A.H., McGregor, I.S., 2011. Predatory threat induces huddling in adolescent rats and residual changes in early adulthood suggestive of increased resilience. Behavioural Brain Research. 225, 405-414.
4. Motbey, C.P., Hunt, G.E., **Bowen, M.T.**, Artiss, S., McGregor, I.S., 2011. Mephedrone (4-methylmethcathinone, 'meow'): acute behavioural effects and distribution of Fos expression in adolescent rats. Addiction Biology. 17, 409-422.
5. Motbey, C.P., Clemens, K.J., Apetz, N., Winstock, A.R., Ramsey, J., Li, K.M., Wyatt, N., Callaghan, P.D., **Bowen, M.T.**, Cornish, J.L., McGregor, I.S., 2013. High levels of intravenous mephedrone (4-methylmethcathinone) self-administration in rats:

Neural consequences and comparison with methamphetamine. Journal of Psychopharmacolgy. (Oxf.) 27, 823-836.

Abbreviations

AcbSH	Nucleus accumbens shell
ACTH	Adrenocorticotropic hormone
ANS	Autonomic nervous system
AOB	Accessory olfactory bulb
AOL	Anterior olfactory nucleus
AVP	Vasopressin
BC	somatosensory barrel cortex
BLA	Basolateral amygdala
CeA	Central amygdala
CPuM	Medial caudate putamen
CSF	Cerebrospinal fluid
DMPAG	Dorsomedial PAG
GAD	Generalised anxiety disorder
HAB	High anxiety behaviour
HPA	Hypothalamic-pituitary-adrenal
ICV	Intracerebroventricular
LAB	Low anxiety behaviour
LAL	Long attack latency
LHb	Lateral habenula
LPO	Lateral preoptic nucleus
LSV	Ventral part of the lateral septum
MeA	medial amygdala

MePV	Posteroventral medial amygdala
MOB	Main olfactory bulb
OT	Oxytocin
OTR	Oxytocin receptor
PAG	Periaqueductal grey
PCP	Phecyclidine
PFC	Prefrontal cortex
PKA	Protein kinase A
PMD	Dorsal premammillary nucleus of the hypothalamus
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus of the hypothalamus
SAL	Short attack latency
SON	Supraoptic nucleus of the hypothalamus
TMT	Trimethylthiazoline
V1AR	Vasopressin V1A receptor
V1BR	Vasopressin V1B receptor
V2R	Vasopressin V2 receptor
VNO	Vomeronasal organ

Abstract

Defensive aggregation, the tight clumping together of conspecifics in response to predatory threat, is a ubiquitous defensive response across many species, sometimes referred to as *huddling* (in mammals), *shoaling* (in fish) or *flocking* (in birds). Defensive aggregation affords a number of important survival advantages for prey species. These include improving predator detection, diluting the probability of predator attack, and enhancing defensive capabilities against a predator. While much research has been focused on defensive aggregation in the field there have been very few laboratory studies. The inherent lack of control and unpredictability of field studies has meant that a thorough exploration of the neurobiology and pharmacology of defensive aggregation, and the more subtle benefits it provides prey species, has not been possible.

The current thesis presents a novel laboratory approach to studying defensive aggregation, which is based on the innate defensive response elicited in laboratory rats when they detect the fur/skin odour of a natural predator (cat). More than a decade of research has shown that individual rats presented with cat fur show a characteristic defensive response that includes arrest of ongoing activity, inhibition of non-defensive behaviours, and retreat. When rats are exposed to cat odour in groups, however, cat odour causes an additional defensive response of defensive aggregation. This laboratory phenomenon provides an unprecedented opportunity to study defensive aggregation under controlled laboratory conditions.

Chapter 1 provides a comprehensive review of defensive aggregation in animal species, and the related phenomena of *social buffering* (the diminished stress response seen in animals encountering a stressor in the presence of conspecifics) and *coping style*

(the tendency for some animals to show passive behaviours, and others more active behaviours, in the presence of stressors).

Chapter 2 provides an initial study of defensive aggregation based in groups of rats presented with cat fur or with bright light. The importance of group size is demonstrated by showing that groups of four, but not two, rats exposed to either cat fur or bright light huddle closely together for long periods. This study thus provided important proof of concept in demonstrating that defensive aggregation can be readily elicited in laboratory rats by cat fur, affording a novel opportunity to explore the underlying neurobiology and pharmacology of defensive aggregation in mammals.

Chapter 3 comprises of two studies examining the behavioural and neural correlates of defensive aggregation, and of active and passive coping styles, in response to cat fur. In a cohort of outbred rats, a clear distinction could be seen whereby some rats showed active and others passive coping styles when exposed to cat fur in a groups of four animals. Active responders engaged in more approaches towards the predator odour stimulus and showed less defensive aggregation. Passive responders, on the other hand, spend a large amount of time engaged in huddling with two or more conspecifics. These coping styles were shown to be highly consistent across repeated exposures to cat fur and generalised to other behavioural tests, with an active coping strategy associated with lower levels of generalised anxiety-like and depression-like behaviour and less susceptibility to the negative impacts of chronic stress exposure. Expression of an active coping style also appeared to be partly socially mediated since the active coping style was not expressed by rats exposed to predatory threat alone.

Fos immunohistochemistry was used to examine the neural correlates of group *versus* individual exposure to predatory threat, and of the different coping styles. An active coping style was associated with: greater cat fur-induced activation of the accessory olfactory bulb, reflecting greater olfactory stimulation in rats with higher levels of approach to the fur stimulus; lowered activation of somatosensory cortex, reflecting reduced huddling with conspecifics; and reduced activation in the lateral septum, a key brain region involved in passivity in the face of stress. Exposure to predatory threat in a group of four rats, as opposed to exposure alone, resulted in clear differences in the behavioural and neural response to the same stressor. Group exposed rats showed less locomotor suppression, higher levels of grooming, higher levels of approach towards cat fur, and lowered c-Fos expression in the dorsomedial periaqueductal grey, medial caudate putamen and lateral habenula. These findings provide support for the hypothesis that an important and hitherto unreported function of defensive aggregation is to provide social buffering of the stress response.

Chapter 4 reports a series of experiments that explore whether the neuropeptides oxytocin (OT) and vasopressin (AVP) can influence defensive aggregation towards predatory threat in groups of four rats exposed to cat fur. OT and AVP were of interest as they play a highly conserved evolutionary role in regulating complex appetitive social behaviours across species. However, their involvement in regulating social behaviour under conditions of threat has not been examined. Peripheral administration of either OT or AVP was found to increase defensive aggregation in rats under predatory threat. However, these were linked to different mechanisms involving different receptors, specifically the V_{1A} and V_{1B} vasopressin receptors (referred to as $V_{1A}R$ and $V_{1B}R$ henceforth). The augmentation of the

social response to threat by OT was prevented by co-administration of the $V_{1A}R$ antagonist SR49059, consistent with the known affinity of OT for $V_{1A}Rs$ in addition to the OT receptor (OTR). When SR49059 was given alone, it reduced huddling in response to predatory threat, showing a role for tonic activation of $V_{1A}Rs$ in defensive aggregation. Interestingly, neither OT nor SR49059 affected the defensive responses of individual rats presented with cat fur. AVP also increased defensive aggregation in response to predatory threat. However, unlike OT, the increased huddling to predatory threat induced by AVP was not blocked by SR49059. Rather, the AVP augmentation of defensive aggregation was prevented by administration of the $V_{1B}R$ antagonist SSR149415. Unlike OT, AVP also increased huddling under baseline conditions when no predatory threat was present, and also increased the defensive response to predatory threat in rats individually exposed to cat fur, an effect that was also prevented by SSR149415.

These findings suggest that OT, via an action at $V_{1A}Rs$, specifically enhances the social responding to threat without affecting the more general anxiety response to cat fur. Conversely, AVP causes a more global enhancement of anxiety via its actions at the $V_{1B}R$ and this leads to huddling under baseline (non-threatening) conditions and magnifies the defensive response to predatory threat in individually exposed animals. It is proposed that drugs targeting the $V_{1A}R$ may have potential as novel treatments to enhance sociability under conditions of threat. This could be particularly beneficial for the treatment of psychiatric diseases such as schizophrenia and depression, which are characterised by social withdrawal by sufferers in the face of stress rather than the seeking out of social support.

Chapter 5 explored the endocrine and epigenetic correlates of active and passive coping styles. The major individual differences in coping styles towards predatory threat,

first reported in Chapter 3, were confirmed in another large cohort of rats. Again, coping style was stable over time with active responders engaged in far more contact with the cat fur than passive responders over repeated exposures, and passive responders spending much greater time engaged in defensive aggregation. Active responders were shown to have substantially lower plasma levels of corticosterone and progesterone than passive responders three days after the final stressor exposure. Plasma and testicular testosterone levels did not differ between active and passive responders at this time-point. At an epigenetic level, active responders had markedly less methylation of the AVP CGCG promoter region located at base 4970 in the posterodorsal region of the medial amygdala, but did not differ in the methylation status of the CCGG sequence located at base 2243. This is in agreement with prior research suggesting that AVP and progesterone act in an oppositional fashion within the medial amygdala to modulate stress-related behaviours. These findings suggest that epigenetic regulation of AVP gene expression may be involved in maintaining the differential activity in this system which underpins different coping strategies.

The model presented in this thesis will hopefully be a stimulus for future studies that further probe the neurobiology of social responses to threat and of active and passive coping styles. These findings may have implications for human wellbeing and psychiatric disease. It is suggested that defensive aggregation may be a fourth “pillar” of the rat defensive behaviour repertoire in addition to reduced ongoing activity, cessation of non-defensive activities, and avoidance of the threat. There are subtle benefits accruing from defensive aggregation other than the immediate survival advantages identified in the field literature. Indeed, it is possible that defensive aggregation has a major benefit of *social*

buffering that reduces stress responsivity and facilitates reengagement in important non-threat related behaviours. A hitherto unrecognised role of the neuropeptide OT acting at V_{1A}Rs was identified as a means of selectively promoting social responding to threat without increasing anxiety-like behaviour. Finally, we provide the first report of striking epigenetic differences in the medial amygdala AVP system between active and passive coping rats, providing a potential mechanism through which the proactive response style seen in some animals confronted with threat might be maintained. It is hoped that the work presented in this thesis has served as a foundation for the future investigation of the neurobiological mechanisms driving, and adaptive benefits underlying, the social response to threat.

Chapter 1: General introduction and literature review

1.1. Chapter overview

Imagine you were to find yourself with several close friends in a large field surrounded by 10 metre high electrified fences. Imagine there was also a fully grown Bengal Tiger sleeping at the other side of that field. Your initial response would likely be to cautiously assess the situation and probe for a means of escape. Do you think you would lead the way in those tasks or would someone else within your group take on that role? Once you realise there is simply no way out of the field, what would be your response? Let's say you have contacted the emergency services but due to your location it will be 30 min before they can come to your rescue, what would you do in those 30 min? Would you run off by yourself or stay with your friends? Let's say you escape physically unscathed, what sort of lasting psychological impact might the experience have on you? How do you think your experience of the situation would differ if you were to find yourself in the field alone?

For members of countless species – from the marine insect *Halobates robustus* to *Homo sapiens* – the overwhelming response in such a situation is to aggregate with conspecifics (Fig. 1) (Chen and Kolokolnikov, 2014; Foster and Treherne, 1981; Fryxell, 1995; Kirkwood and Robertson, 1999). From an evolutionary biology analysis, the survival advantage accruing to the individual from this response to a predator is believed to have been a fundamental factor driving the emergence of social behaviour across such a wide array of different species (Bertram, 1978; Shultz *et al.*, 2011). It is also apparent that not all conspecifics within a group exposed to threat will respond identically (Mloszewski, 1983; Zahavi, 1990; Zahavi and Zahavi, 1997). A clear distinction between active and passive responders emerges during threat exposure, with the active responders showing greater

active behavioural responses and greater resilience during and after the cessation of the traumatic event (Koolhaas *et al.*, 2010; Koolhaas *et al.*, 1999).

Figure 1. **Huddling is a defensive response to predatory threat that is extraordinarily well conserved across species.** Clockwise starting from top left: Elephants huddling together to protect a calf and a female giving birth from a nearby pack of hyenas (source: *Daily Mail,* Paolo Torchio, Barcroft Media); Zebra huddling in response to prowling lions (source: *Wordpress*); Meerkat huddling together to ward off a snake (source: *SA-Venues*); Elk huddling for protection from the two wolves in the distance (source: *UC Berkeley,* Dan Hartman); and Sardines shoaling in response to swooping gannets (source: *The Telegraph,* Jason Heller, Barcroft Media).

While much research has been focused on defensive aggregation in the field (Gilbert *et al.*, 2010), the inherent lack of control and unpredictability in field studies has prevented a thorough exploration of the neurobiology and pharmacology underlying this ubiquitous behaviour and the more subtle benefits that may arise from this important social response to threat. In the present thesis, a laboratory approach to studying defensive aggregation was explored which was based on the innate defensive response elicited in rodents when they are exposed to predator odours (Apfelbach *et al.*, 2005; Dielenberg and McGregor,

2001). An opportunity was evident to develop a novel laboratory model of the social response to threat and within-group differences in stress responsiveness.

The research conducted in this thesis has thus focused on the development of a novel rodent model of the social response to predatory threat. This model involves group exposure of laboratory rats to an ethologically valid stressor (predator odour), which permitted a fine-grained analysis of the phenomenon of defensive aggregation, and the closely related phenomena of *social buffering* and individual coping styles. *Social buffering* refers to the reduction in the magnitude of the response to a stressor when a conspecific is present during or immediately after stress exposure (Hennessy *et al.*, 2009). The use of a laboratory setting allowed a level of detailed analysis that would not be feasible in field studies. One important aim of this approach was to improve our understanding of the underlying neurobiology of social responses to threat, especially in mammalian species. Also of major interest was the role of oxytocin (OT) and arginine vasopressin (AVP) in regulating the social response to threat. These neuropeptides are rightly famous for their role in mediating social behaviour (Hoyle, 1999; Neumann, 2008). However, their possible role in regulating defensive aggregation in mammals had yet to be explored

The first chapter of the thesis provides an overview of the current literature on the defensive responses of individual animals to predatory threat in the field and laboratory and explores how such studies have contributed to our knowledge of defensive behaviour, pathological anxiety, and the neurobiology and pharmacology of individual defensive behaviours. This discussion also examines the theoretical, field and experimental studies conducted on the phenomenon of defensive aggregation, almost exclusively in non-rodent species.

Following this, the literature on *social buffering* is explored. Social buffering may be a benefit of defensive aggregation, but this likelihood has not been formally examined to date. Another key issue is individual differences in the tendency to socially aggregate in the face of predatory threat. Some animals are very proactive and investigatory in the face of threat, adopting a so-called "active coping style". Other animals are overwhelmingly passive in their coping style and seek out others to aggregate with. This phenomenon is discussed, as is the neural basis of such coping strategies. Discussion of the neuropeptides OT and AVP, which play a crucial role in many social and anxiety behaviours, follows, with particular emphasis placed on how these systems may have a critical role in the social response to threat, *social buffering* and the expression of individual coping styles.

The implications of these issues for human wellbeing are an important focus. Why do some people cope well with stress and others do not? Why are some people more motivated to seek out social support during challenging times? Many psychiatric disorders are associated with maladaptive stress coping strategies and a response to threat that involves withdrawal and isolation rather than seeking out social support. Understanding the neurobiology of social aggregation may aid in the development of new treatments that promote a prosocial response to stress and/or a more resilient coping strategy. The work presented in this thesis may serve as a foundation for the future investigation of the neurobiological mechanisms driving, and adaptive benefits underlying, the social response to threat and adaptive coping strategies.

1.2. Anti-predator defence

Survival of prey species is dependent upon effective detection, avoidance and defence against predators. When an animal is exposed to a live predator or to a cue that

predicts predatory threat (e.g. a predator odour), they show marked behavioural, physiological, and neural changes indicative of an anxiety-like state (Apfelbach *et al.*, 2005). These changes are discussed in detail below. This characteristic response of individual rats to predatory threat has been the subject of a large number of recent studies both in the laboratory and the field. There is interest in the utility of such approaches in modelling human anxiety disorders such as specific phobias, generalised anxiety disorder (GAD) and post-traumatic stress disorder (PTSD). However, few, if any, studies have examined the role of social factors in regulating the behavioural response to, and anxiety-like states arising from, exposure to predators or predator-associated cues.

1.2.1. Behavioural changes in response to predatory threat

Both field and laboratory studies examining the response of prey species to predatory threat have identified three major behavioural changes elicited by the threat. These are: (1) reduction of ongoing activity; (2) cessation of non-threat-related behaviours such as mating, foraging, feeding and grooming; and (3) movement to a safe location, if available (Apfelbach *et al.*, 2005). These behavioural adaptations all offer survival advantages through reducing the probability of being detected by predators, allowing greater resources to be dedicated to anti-predator behaviours, and providing cover from detection or attack.

Examples of anti-predator defensive behaviours are numerous. Wild rats are nocturnal; however, when they are preyed upon by a nocturnal predator such as a fox they will temporarily shift their active period to daylight until the predation risk is removed (Fenn and Macdonald, 1995). Prey will also reduce their foraging range in response to the presence of a live predator (Borowski, 1998). Voles drastically reduce their engagement in

non-defensive behaviours, such as foraging and mating, and shift their foraging area when predation risk is produced by introduction of a weasel (Jędrzejewski and Jędrzejewska, 1990; Jochym and Halle, 2012; Koivisto and Pusenius, 2003). Similar changes are observed in rats (Blanchard and Blanchard, 1989).

As noted above, defensive behaviours are not only elicited by a live predator, but also by cues that are reliably associated with increased predatory risk. For example, armadillos, who are nocturnal foragers, reduce foraging activity during moon-phase periods of brighter illumination to reduce risk of predation by pumas and jaguars (Harmsen *et al.*, 2011). Similar behaviour has been observed in desert rats and deermice during the full moon (Clarke, 1983; Daly *et al.*, 1992). Perhaps one of the most important signals of increased predatory risk comes from predator odours: such as the urine, faeces, fur or scent gland markings of a predator. Odours derived from predator fur or scent gland markings appear particularly potent in promoting long-lasting defensive responses and evidence suggests they are processed in a specialised way by prey species.

1.2.2. Kairomonal processing of predator odours

When laboratory rats are exposed to cat fur or skin odours by presentation of a cloth rubbed on a cat, a collar worn by a cat, or cat fur itself, the accessory olfactory system that processes pheromones is activated (McGregor *et al.*, 2004; Staples *et al.*, 2008a; Takahashi, 2014) and a striking repertoire of defensive responses is elicited (Apfelbach *et al.*, 2005; Dielenberg and McGregor, 2001). The accessory olfactory system consists of several structures. A specialized sensory organ, the vomeronasal organ (VNO), expresses G-protein coupled receptors that have evolved narrow sensitivity to chemical cues of species-specific importance. The VNO contains pheromone and kairomone receptors on its sensory neurons

which project to the accessory olfactory bulb (AOB). The AOB in turn projects to the posteroventral medial amygdala (MePV), bed nucleus of the stria terminalis (BNST) and hypothalamus, where highly stereotyped behavioural responses are organised (Chamero *et al.*, 2012; Ma, 2012). These responses are of critical importance to reproduction, aggression, territorial demarcation and predation. The involvement of the accessory olfactory system suggests that the cat odour may be processed by the rat as a *kairomone*: defined as a substance serving the function of pheromonal chemical communication within the predator species but intercepted by the prey species as a signal of predatory threat (McGregor *et al.*, 2004; Wyatt, 2003).

Cats will frequently rub up against an object in the environment, a person or a conspecific, with the "body-rubbing" action starting from their cheek and running along most of their flank (Houpt and Wolski, 1982; Verberne and Deboer, 1976). Cats deposit a residue from their cheek gland during this process which appears to act as a feline social cue for marking territory and conveying information about the proximity, identity, familiarity, sex and dominance of the marker (Bateson and Turner, 2000; Feldman, 1994; Wemmer and Scow, 1977). In a study conducted by our group (see Appendix 1)(May *et al.*, 2012), the residue left by house cats on objects they rubbed was collected on a cotton pad. When this pad was presented to rats in the laboratory they showed high levels of hiding, decreased exploration, and avoidance of the odour-containing stimulus. They also showed a conditioned fear response, whereby hiding remained elevated in the environment in which predator odour exposure occurred for several days afterwards, even in the absence of the odour stimulus. These findings are consistent with the kairomone theory of predator odours, suggesting that markings left by cats, presumably as a signal to other cats, are

intercepted by rats and are a potent stimulus in eliciting defensive behaviours. It is possible that these same residues are responsible for the profound defensive response elicited in rats by worn cat collars and by cat fur (May *et al.*, 2012). Overall, such stimuli are far more effective in repelling rats than predator urine or faeces (Apfelbach *et al.*, 2005).

When rats are exposed to cat odours they show a marked decrease in locomotor activity (Blanchard *et al.*, 2001b), much the same as prey species exposed to predatory threat in the wild. They also display a pronounced reduction in non-defensive behaviours such as foraging, mating and grooming (Dent *et al.*, 2013; McGregor *et al.*, 2004; Voznessenskaya *et al.*, 2003). When an option to hide is available, rodents will do so for extended periods and if hiding is not possible they will avoid the location containing the predator odour (Blanchard *et al.*, 2001b; Dielenberg and McGregor, 2001; Perrot-Sinal *et al.*, 2004). These effects of cat odour can extend well beyond the exposure period with rats and mice displaying a prominent conditioned-fear response to cues and/or contexts associated with the threat following single or repeated predator odour exposure (Blanchard *et al.*, 2001b; Dielenberg *et al.*, 2001a; Muñoz-Abellán *et al.*, 2008; Muñoz-Abellán *et al.*, 2011).

A recent study involved a task wherein mice must weigh up the benefit of gaining food *versus* the cost of exposure to a predatory cue. This showed that mice are highly sensitive to the cost-benefit trade-off in such scenarios, with their behaviour being determined by the salience of the predatory cue relative to the value of the reward (Dent *et al.*, 2013). Aspects of the cue used by rodents to determine the risk *vs* reward may include the age of the olfactory predatory cue, with aging predator cues associated with greater amounts of foraging behaviour but continuing risk-assessment of the threat stimulus (Bytheway *et al.*, 2013). Indeed, it is has been argued that one reason fur based predator

odours are far more effective than predator urine or scat may be because they suggest a predator is in much closer temporal and/or spatial proximity (Apfelbach *et al.*, 2005).

1.2.3. The neural substrates of predator odour detection and responsivity

Numerous studies have explored the neural substrates involved in predator odour detection and the subsequent behavioural response. These studies have employed a variety of different methods. These include: lesions of target regions; immunohistochemistry; and pharmacological and genetic manipulations.

The accessory olfactory system

Mammalian species generally have two olfactory subsystems – the *main* and *accessory* olfactory systems – and these may be differentially involved in the detection of different predator odours. The main olfactory system is primarily involved in the processing of the fox faeces-derived compound trimethylthiazoline (TMT), while the accessory olfactory system is the primary system involved in processing cat odour derived from fur or rubbings (Takahashi, 2014). However, the status of TMT as a true predator odour is highly debatable, with several studies suggesting it is an acrid compound that repels rodents due to its foul smell rather than because of any predatory connotations (Apfelbach *et al.*, 2005; Blanchard *et al.*, 2003b; McGregor *et al.*, 2002; Staples *et al.*, 2008b).

Cat fur/skin odours appear to be primarily detected by the VNO, which is a specialised organ located at the base of the naval cavity containing neurons with specialised receptors for pheromones and kairomones (Chamero *et al.*, 2012). Removal of the VNO in mice reduces their customary avoidance of a worn cat collar (Samuelsen and Meredith, 2009). Moreover, mice lacking TrpC2, which is a protein that provides the primary signal

transduction channel for VNO sensory neurons, display a non-functional VNO and have profound deficits in avoidance and risk assessment when exposed to odours of their predators, including cat and rat odours (Isogai *et al.*, 2011; Papes *et al.*, 2010).

VNO sensory neurons primarily project to the AOB which in rodents is located above the main olfactory bulb (MOB) in the most rostral part of the brain (Apfelbach *et al.*, 2005; Takahashi, 2014). Cat odour causes a pronounced increase in Fos expression in the posterior AOB (Staples *et al.*, 2008a) but only a modest increase in Fos expression in the MOB (McGregor *et al.*, 2004). The heavy involvement of the accessory olfactory system (VNO and AOB) in processing skin and fur derived predator odours is consistent with the hypothesis that these odours are being processed as a kairomone, rather than as a conventional odour.

The amygdala

The AOB projects to the medial amygdala (MeA) and BNST (Fig. 2), both of which are strongly activated in rats by cat odour (Dielenberg *et al.*, 2001b). The amygdala appears to play a critical role in threat detection, elicitation of defensive and fear-related behaviour, and modulation of fear learning and memory (Gross and Canteras, 2012). The MeA, particularly in its posteroventral division (MePV), and the basolateral amygdala (BLA), play roles in both the unconditioned and conditioned fear responses to predator odour. The MeA receives direct and indirect projections from the AOB and modulates hypothalamic-pituitary-adrenal (HPA) axis hormone secretion induced by predator odours (Takahashi, 2014). Lesions to either the MeA or BLA result in decreased freezing to, and avoidance of, cat odour (Takahashi *et al.*, 2007). Acute pharmacological inactivation of the BLA immediately following cat odour exposure disrupts predator odour memory consolidation and subsequent conditioned fear of the context in which the predator odour was

encountered (Takahashi *et al.*, 2007). Conversely, temporary inhibition of the MeA during the conditioned fear test, but not immediately after initial cat odour exposure, inhibits the conditioned fear response, suggesting interference with retrieval of the fear-related memories (Takahashi *et al.*, 2007). The central amygdala (CEA) does not appear to be involved in the predator odour response with CeA lesions affecting neither the unconditioned nor conditioned defensive responses to predatory threat (Martinez *et al.*, 2011).

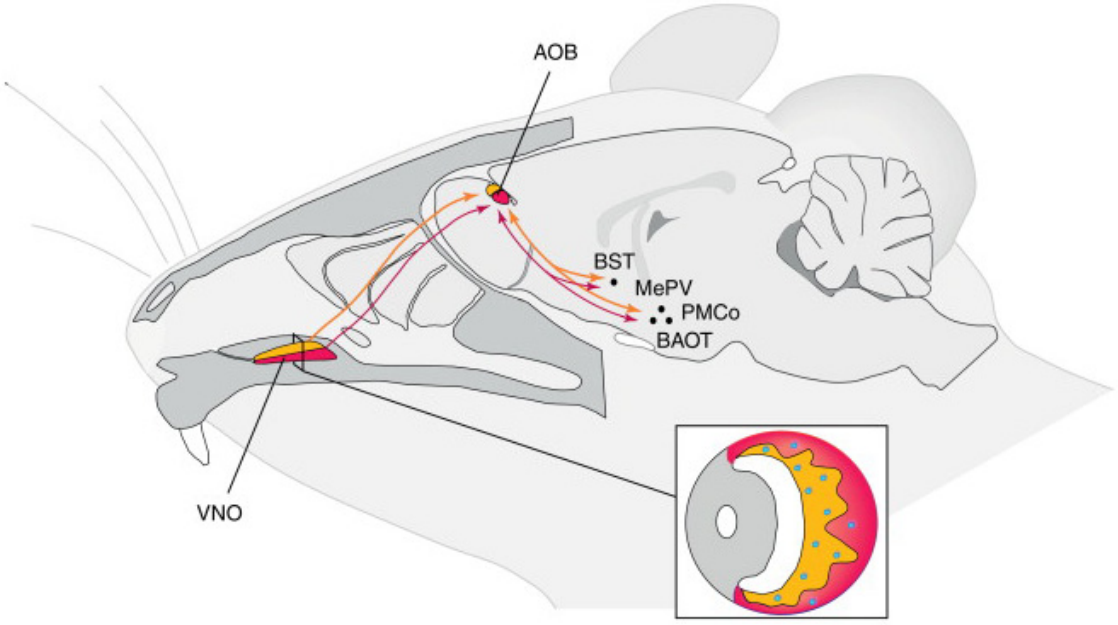

Figure 2. **Pathways involved in predator odour detection and the subsequent defensive behavioural response.** Cat odour is processed in the posterior part of the VNO which then projects to the posterior AOB, which is strongly stimulated by cat odour, which sends signals to limbic regions involved in organising and eliciting the defensive response to predatory threat. Pathways originating in the posterior VNO are shown in red and those originating in the anterior part of the VNO are shown in yellow. The red separation (posterior) of the VNO contains the vomeronasal V2 receptors that appear to be involved in sensory detection and processing of predator odours. Figure adapted from Chamero *et al* (2012).

The amygdala appears to play a critical role in regulating risk-taking behaviour in rats exposed to predatory threat. Lesion or inactivation (by muscimol infusion) of the amygdala increase the propensity to forage versus hide in response to predatory threat whereas disinhibition (by bicuculline methiodide infusion) of the amygdala promotes hiding over

foraging (Choi and Kim, 2010). Similarly, lesion of either the posterior basomedial or lateral amygdala nuclei increases risk assessment (crouch-sniff and stretch-attend postures) and decreases freezing in response to cat odour (Martinez *et al.*, 2011). Protein kinase A (PKA) activity in the amygdala may be critical in the response to predatory threat with Prkar1a heterozygous mice, possessing a haploinsufficiency for the main regulatory subunit (R1α) of PKA, showing indifference towards a predator odour (Keil *et al.*, 2013).

The hypothalamic-periaqueductal grey axis

Both a live cat and cat odour strongly activate the dorsal premammillary nucleus of the hypothalamus (PMD) in rodents (Canteras *et al.*, 1997; Dielenberg *et al.*, 2001b). The PMD lies adjacent to the ventral premammillary nucleus which is heavily involved in reproductive behaviour. It has been argued that interplay between these adjacent and interconnected hypothalamic nuclei may be involved in the pronounced inhibitory effect of predator odour exposure on reproductive behaviour (Apfelbach *et al.*, 2005). Lesions of the PMD eliminate cat odour-induced fear (Blanchard *et al.*, 2003a; Canteras *et al.*, 2008) while blockade of β-adrenoreceptors in the PMD reduces unconditioned and conditioned freezing induced by cat odour or cat odour-associated contexts (Do Monte *et al.*, 2008).

Cat odour also strongly activates the periaqueductal grey (PAG), which is densely innervated by projections from the PMD (Cezario *et al.*, 2008). The PAG consists of dorsolateral, dorsomedial, ventrolateral and lateral divisions. The PAG appears to play a role in regulating the motoric aspects of fear responses such as flight, freezing, and behavioural inhibition (see Bandler *et al.*, 2000 for a review). The hypothalamic-PAG axis may play a critical role in inhibiting foraging and other behaviours in response to predatory threat. For example, lesions of the dorsal PAG interfere with both the cardiovascular and behavioural

responses to cat odour exposure in rats (Dielenberg *et al.*, 2004), while electrical stimulation of the dorsal PAG elicits freezing and/or escape responses similar to those observed in rats in response to cat odour (Vianna *et al.*, 2003).

1.2.4. Autonomic and endocrine effects of predator odour exposure

Cat odour has considerable effects on the autonomic nervous system (ANS) and endocrine system. Activation of the ANS by threat plays an important role in facilitating the "fight-or-flight" response that is critical for survival in dangerous situations (Ulrich-Lai and Herman, 2009). Exposing rats to a worn cat collar induces a lasting increase in blood pressure that is accompanied by reduced exploration and heightened risk assessment and avoidance of the predator stimulus (Dielenberg *et al.*, 2001a). This ANS response to cat odour appears to be linked to the dorsal PAG as rats with lesions of this region display no increase in blood pressure in response to cat odour (Dielenberg *et al.*, 2004).

Exposure to cat odour causes acute activation of the HPA axis in rodents which is accompanied by subsequent release of adrenocorticotropic hormone (ACTH) and corticosterone (Cohen *et al.*, 2006; File *et al.*, 1993a; Muñoz-Abellán *et al.*, 2008; Muñoz-Abellán *et al.*, 2011). Heightened release of ACTH, but not corticosterone, is observed in rats for up to seven days after fur exposure when rats are returned to the context in which they were exposed to cat odour (Muñoz-Abellán *et al.*, 2008; Muñoz-Abellán *et al.*, 2011). This suggests that the conditioned response to predatory threat encompasses an endocrine component, as is the case with other stressors such as foot shock (Ottenweller *et al.*, 1992). Rats with fibre sparing lesions of the MeA show reduced ACTH and corticosterone responses to ferret odour, suggesting the MeA plays an important role in regulating the HPA axis response to predatory threat (Masini *et al.*, 2009).

1.2.5. Predator odour induced defensive behaviour as an animal model of anxiety disorders

The benefits of laboratory models of the response to predator threat extend beyond our improved understanding of the neurobiology of defensive behaviour. The response of laboratory rats to live predators or predator odours, especially cat odours, may model various aspects of human psychopathology, in particular various anxiety disorders. The first such analysis was conducted by File (File *et al.*, 1993b) who argued that the behavioural response to predator odours modelled innate phobic avoidance, equivalent to, for example, human fear of snakes.

Exposure of laboratory animals to predators or predatory threat is seen as a useful approach for producing analogous symptoms to those observed in humans suffering from PTSD (Berardi *et al.*, 2012; Siegmund and Wotjak, 2006; Zoladz *et al.*, 2012). The strong and enduring conditioned fear elicited by environments or cues associated with predatory threat are of particular interest in these models. Predatory threat produces exaggerated startle responses in rodents, heightened anxiety, cognitive impairments, amplified cardiovascular reactivity and inflated responsiveness to yohombine administration, all effects that are analogous to the symptoms and other phenomena observed in humans suffering from PTSD (Diamond and Zoladz, 2010; Zoladz *et al.*, 2008; Zoladz *et al.*, 2012).

It is argued that the direct response to cat odour more closely resembles symptoms of GAD and panic disorder (Blanchard *et al.*, 2001a; Blanchard *et al.*, 2011). Risk assessment behaviours (such as stimulus approach) in predator odour paradigms are thought to reflect aspects of GAD (Blanchard *et al.*, 1997; Staples, 2010). Consistent with this, the increased vigilance and risk assessment observed in rats exposed to cat odour are reduced by benzodiazepines which are effective in the treatment of GAD (Blanchard *et al.*, 1997;

McGregor *et al.*, 2004). Flight responses (such as escape attempts and fleeing followed by avoidance) and freezing induced by predator stimuli are argued to reflect a response profile more similar to panic disorder, and again, these responses are inhibited by drugs that are effective in the treatment of panic disorder (Blanchard *et al.*, 2011; Blanchard *et al.*, 1997). In particular, the response to an actual predator is thought to be a particularly suitable model of panic disorder as panic attacks are thought to be related more to perception of a proximal rather than distal threat (i.e. to be more of a fear rather than anxiety response) (Graeff and Del-Ben, 2008).

It is the case, then, that different aspects of the response to different predatory stimuli appear useful in modelling various anxiety disorders. The innate avoidance of predatory cues is perhaps most suited to modelling innate phobias whereas the strong conditioned fear response to those cues is reflective of aspects of PTSD. The heightened risk assessment induced by more distal predator cues (such as odours) are reflective of aspects of GAD whereas the strong flight or freezing response elicited by more proximal cues (such as a live predator) are closer in kind to the panic attacks observed in panic disorder.

1.2.6. The social response of laboratory rats to predatory threat

As noted above, the social aspects involved in anti-predator defensive responses have been largely overlooked in laboratory studies to date. Field research indicates that rats rarely experience threats such as predation in isolation (Macdonald *et al.*, 1999). As such, removing the ability of the rodent to interact with conspecifics during predatory threat may neglect important ethological aspects of the defensive response and lead to an artificial testing situation that does not allow proper expression of the innate defensive response repertoire. Appreciation of the social environment in which stress occurs also presents the

opportunity to model an important aspect of the human response to threat which has received little attention in laboratory animal models: the social response to threat.

One classic study that did examine these issues was that of Blanchard and Blanchard (1989). This study examined the behaviour of rats in a laboratory "visible burrow system" that mimicked the burrows inhabited by wild rats in the field. When a live cat was introduced on the surface of the burrow system, the authors reported a hasty retreat by foraging rats to their burrows and a subsequent increase in non-sexual, non-aggressive social contacts within the burrow. This early report of increased social contact in laboratory rats exposed to predatory threat suggests that aggregation with conspecifics may form a critical part of the rat defensive behaviour repertoire. The reason this social response to predatory threat has gone largely overlooked is perhaps in part due to the lack of a more accessible laboratory model of group exposure of rodents to predatory threat.

1.3. Defensive aggregation

Despite the dearth of laboratory rodent studies exploring the response of groups of rats to predatory threat, much can be learned from field and experimental studies in other species. The phenomenon described by Blanchard and Blanchard (1989) is akin to the phenomenon well-known to field biologists as *defensive aggregation* or, more broadly, as *huddling*. Huddling is an adaptive response defined as "an active and close aggregation of animals" (Gilbert *et al.*, 2010) and is observed across a wide variety of species, both social and solitary, in response to a variety of stressors. Most obviously, thermoregulatory huddling occurs in many mammals, with aggregation providing substantial energetic benefits during cold conditions (Gilbert *et al.*, 2010).

The key theoretical work on defensive aggregation (i.e. huddling in response to predatory threat) was presented in the 1970s and 1980s by William D. Hamilton and provides a core element in Richard Dawkin's classic work *The Selfish Gene* (Dawkins, 1976). Hamilton's article *Geometry for the Selfish Herd* (Hamilton, 1971) has perhaps been the most influential work in this area. In essence, Hamilton proposes that individuals within a population aggregate to reduce their own individual risk of predation and he argues, more broadly, that the survival advantages afforded by such an approach were a major driving force behind the development of social behaviour.

According to Hamilton's theory, the desire to aggregate should be strongest under predatory threat, which is indeed the case across a plethora of species. To present just a few examples, tight aggregation with conspecifics is observed in response to predatory threat in: Emperor Penguins (Kirkwood and Robertson, 1999); the marine insect *Halobates robustus* (Foster and Treherne, 1981); and Serengeti ungulates such as wildebeest, zebra, and Thomson's gazelle (Fryxell, 1995). This desire to aggregate is manifest in humans in the *need for affiliation* which is strongest in stressful and threatening situations (Gump and Kulik, 1997; Miller, 1966). This strong desire to seek out the social group when under threat is sometimes referred to as the *tend-and-befriend* response, which is argued to be an alternative to the *fight-or-flight* response (Taylor, 2006; Taylor *et al.*, 2000). In humans, this manifests in the increased need to be with others when presented with cues that signify potential harm such illness, danger, nightfall and disaster (for a review see Rofe, 1984).

Confirmatory work conducted subsequent to Hamilton's theoretical framework has characterised several phenomena that demonstrate the survival advantages provided by huddling in response to predator threat. In ethological terms, huddling can be seen as both

a *primary defense* - making an individual animal less susceptible to predator attack – and a *secondary defense* – minimising harm after detection by a predator has occurred. The first advantages of huddling operate pre-attack (in the *primary defense* phase) and are due to an *encounter effect* and *group vigilance effect*. The *encounter effect* states that the probability of detection by a predator does not increase in direct proportion with group size; thus a group of 50 prey is not 50 times more likely to be detected than an individual animal (Inman and Krebs, 1987). The *group vigilance effect* refers to the fact that as group size increases the probability of detecting a predator increases, the time taken to detect a predator decreases, and subsequently the amount of time individual animals can spend on non-vigilance related activities increases (Elgar, 1989; Hamilton, 1971; Lima, 1995; Roberts, 1996; Rogovin *et al.*, 2004).

Other benefits arising from defensive aggregation operate after detection by the predator (during the *secondary defense* phase) and involve the *dilution effect*, *confusion effect*, and *group defense effect*. The *dilution effect* refers to a phenomenon whereby for any one predator attack, the larger the group of prey the lesser the probability that any one particular animal will fall victim (Foster and Treherne, 1981; Hamilton, 1971; Inman and Krebs, 1987; Morgan and Godin, 1985). The *confusion effect* sees a predator make more mistakes when tracking an individual prey within a moving group compared to when tracking the individual on its own. As a result, the predator's success of catching an individual prey within a group is reduced (Landeau and Terborgh, 1986; Schradin, 2000). Finally, a *group defense effect* is present whereby if escape from attack is not possible, a group is better able to defend against the predator than an individual (Inman and Krebs, 1987).

While defensive aggregation has been explored primarily in response to predator threat, it is also of interest to see if other anxiogenic stimuli are able to induce huddling. Hamilton (1971) noted a number of anecdotal observations of animals huddling together in response not just to predator threat, but to a range of other potentially dangerous stimuli, such as lightning. It is therefore plausible that elicitation of the huddling response is not restricted to stimuli directly related to predators; however, this possibility has received little empirical assessment.

Laboratory studies on predatory threat have rarely addressed the social aspects of predator defence, while the field studies exploring defensive aggregation generally lack the experimental control required to penetrate the underlying neurobiology and nuances of the behavioural response. As such, the development of a laboratory model of defensive aggregation is highly desirable as it will facilitate a more comprehensive understanding of the pharmacological, neurobiological and physiological determinants of this extraordinarily well conserved behaviour. The laboratory rat is a species that is particularly well suited for such studies given its ability to thrive in a laboratory setting and its well described physiology, neurochemistry and behavioural repertoire.

1.4. Social Buffering

Some of the work discussed in the previous section (Elgar, 1989; Hamilton, 1971; Roberts, 1996; Rogovin *et al.*, 2004) indicates that the *group vigilance* effect of defensive aggregation serves not only to improve predator detection but also to allow conspecifics to re-engage in other important non-defensive behaviours (such as foraging, feeding, grooming and mating) much sooner than would otherwise be possible. In order for this to occur, the presence of those conspecifics should be accompanied by a concurrent reduction in the

magnitude of the stress response to predatory threat. This requirement suggests a largely overlooked benefit of defensive aggregation may be *social buffering* of the stress response.

The term "social buffering" describes how social interaction with conspecifics during or after exposure to a stressor may reduce the impact of that stressful situation (Hennessy *et al.*, 2009). Social buffering is observed in both humans and animals during the infant and adolescent stage of development when interactions with the mother during or after stressor exposure reduce the fear response and dampen HPA axis responsiveness (Albers *et al.*, 2008; Hostinar *et al.*, 2014; Nachmias *et al.*, 1996; Seltzer *et al.*, 2010; Shionoya *et al.*, 2007; Wiedenmayer *et al.*, 2003). Later in life the same social buffering effect can be elicited by contact with partners, or familiar or unfamiliar non-familial conspecifics of the same or opposite sex (Adams *et al.*, 2011; Ditzen *et al.*, 2007; Hennessy *et al.*, 2009; Hostetler and Ryabinin, 2014; Hostinar *et al.*, 2014; Kirschbaum *et al.*, 1995; Livia Terranova *et al.*, 1999).

1.4.1. The neurobiology of social buffering

There is still much to learn about the biological mechanisms governing social buffering. From research conducted thus far it appears that many neural pathways support social buffering. The neural substrates underlying any social phenomena are complex and we are only just beginning to gain an understanding of the mechanisms driving these various social behaviours. Most of the work on social buffering has focused on how the presence of a conspecific during or shortly after stressor exposure can inhibit the HPA axis response to stress (Hostinar *et al.*, 2014). For example, rats returned to a box in which they received electric shocks show markedly reduced c-Fos immunoreactivity in the paraventricular nucleus of the hypothalamus (PVN) in response to the stressful environment if a partner is present (Kiyokawa *et al.*, 2004). Social buffering induced reductions of stress

induced cortisol, in humans, and corticosterone, in rodents, have been documented in many studies in response to a wide array of stressors (see Hennessy *et al.*, 2009; Hostinar *et al.*, 2014 for reviews). For example, in one experiment 7- to 12-year-old girls were required to give a speech and perform mental arithmetic tasks in front of an audience (Seltzer *et al.*, 2010). This task resulted in an elevation of cortisol levels that was blunted if their mother was present during the performance. Some authors have hypothesised that social buffering may also involve dampening of stressor actions at the level of the prefrontal cortex (PFC), amygdala and hypothalamus (Hennessy *et al.*, 2009; Hostinar *et al.*, 2014).

In support of this, more direct evidence of social buffering effects upstream of the HPA axis has started to emerge. Juvenile rhesus monkeys exposed to a novel environment in the presence of their mother show less right dorsolateral PFC activation, more left dorsolateral PFC action and a reduced cortisol response relative to when their mother is absent (Rilling *et al.*, 2001). Furthermore, lesions of the prelimbic or anterior cingulate PFC block the social buffering effect of an adult partner in rodents (Herman *et al.*, 2005). Human neuroimaging studies also indicate a role for the PFC in social buffering with participants given social support during a social-stress task showing reduced activation of the right ventral PFC compared to participants without social support during the task (Eisenberger *et al.*, 2007). Another imaging study in humans associated greater psychosocial support and subsequent stress buffering during a public-speaking stress-task with reduced amygdala activation (Taylor *et al.*, 2008).

These studies indicate that the behavioural outcomes of social buffering appear to be accompanied by a buffering of the physiological and neural response to stress. However, to date there has been no direct examination of social buffering of responses to predatory

threat or to stressors that pose an innate and evolutionarily ancient threat to survival. If social buffering of these most primitive and ingrained defensive responses can occur it will indicate that social buffering plays an important role in regulating the response to even the most salient and well conserved threats and may provide clues as to the evolutionary origin of social buffering.

1.4.2. Potential benefits of promoting a social response to threat

What are the biological mechanisms driving an individual to seek out social contact or social support when faced with a stressful or threatening situation? The majority of social buffering studies have examined the benefits of social support, rather than the motivational mechanisms that make an individual seek out social support. Many of these studies were conducted either under conditions in which the stressed animal had no choice between social contact and isolation; or were designed in such a way that it was difficult to assess the social response of the stressed animal. For example, a common model of social buffering involves shocking rodents either alone or with a partner present in a small chamber. The small size of the chamber provides little choice but to interact with the other rodent and it is thus difficult to assess social motivation during and following stressor application (Kiyokawa *et al.*, 2004). A model that is able to explore the motivational aspect of social buffering would be of considerable utility in furthering our understanding of the phenomenon.

One of the major factors contributing to psychiatric distress and inhibiting recovery in persons suffering from psychological problems is social isolation and withdrawal in the face of threat or stress (Berkman, 2001; Cohen and Wills, 1985; Fletcher *et al.*, 2013; Lam and Wong, 1997; Matheson *et al.*, 2013). This is in contrast to healthy individuals who seek out social support during times of stress (Cohen and Wills, 1985; Fletcher *et al.*, 2013; Lam

and Wong, 1997). People suffering from PTSD, schizophrenia, autism and addictions often suffer from social withdrawal and isolation (Bell *et al.*, 2013; Chevallier *et al.*, 2012; Matheson *et al.*, 2013), which means a loss of the benefits of social buffering. For example, people suffering from bipolar disorder show a deficiency in psychosocial functioning and are less likely to seek social support when faced with stress (Fletcher *et al.*, 2013; Lam and Wong, 1997). It is widely accepted that deficits in social motivation, and a subsequent inability to utilise social support, play a central role in autism spectrum disorders (Chevallier *et al.*, 2012). As such, gaining a better understanding of the mechanisms motivating subjects to seek out conspecifics during and after stress will help us to understand how adaptive social responding to stressors and subsequent social buffering might be targeted in novel treatments of psychiatric disorders.

1.5. Individual differences in stress coping

Not every individual within a species responds to the same stressor in the same way. The term "coping style" is often used to refer to the idiosyncratic behavioural responses that are seen in individuals towards a given stressor. Coping strategies tend to be stable for a given individual across time and different types of stressors (Koolhaas *et al.*, 2010).

A general distinction is frequently made between *active* and *passive* coping styles, sometimes referred to as *proactive* and *reactive* coping styles. Active responders proactively confront threats, have a more aggressive phenotype, and show less immobility and HPA axis activity and reactivity compared to their passive counterparts. Conversely, passive responders tend to avoid threats, have a less aggressive phenotype, and respond actively only when it is absolutely necessary (Coppens *et al.*, 2010; Koolhaas *et al.*, 2010; Koolhaas *et al.*, 1999). This distinction between active and passive responders is observed not only in

rodents, but across a wide range of species from zebrafish to humans (Tudorache *et al.*, 2013; van Zeeland *et al.*, 2013).

1.5.1. The evolution of different coping styles

Active and passive coping styles may have emerged as adaptations to the changing environments that animals experience in the wild. Maintenance of diversity in coping styles may be due to different responder types being better suited to particular environmental conditions (Koolhaas *et al.*, 2010). Thus, shifts in the environmental conditions and availability of resources, and concurrent shifts in the survival and reproductive success of one responder type over another, may function to maintain the balance of responder types observed within populations.

An example of this comes from studies of common house mice. During the establishment of a colony, passive, non-aggressive mice appear to have superior survival. In contrast, active, aggressive mice appear have better survival chances in established colonies (Van Oortmerssen and Busser, 1989). It is argued that this is due to the more cautious approach of passive mice better suiting the risks associated with establishing a new colony, whereas the more aggressive approach of active responders makes them better able to capitalise on the resources available in the competitive environment of an established colony. In the passerine bird species *Parus major* (Dingemanse *et al.*, 2004a), males with a more active response style had greater rates of survival in years when food was plentiful. Conversely, in years when food was scarce, males with a more passive coping strategy had greater rates of survival. The enhanced ability of active responding males to secure and hold onto territory in resource-rich years improves their chances of survival. Conversely, their more overtly aggressive and territorial phenotype in resource-scarce years may result in

more net costs than benefits. Interestingly, the relationship between response style, availability of resources, and rate of survival was the opposite for female *Parus major*, further driving diversity in the offspring of these different responder types.

1.5.2. Laboratory models of active and passive coping styles

Many models and tests have been used in the laboratory to examine coping styles. One of the most commonly used experimental paradigms is the *resident-intruder test*. In this paradigm, a resident male mouse or rat inhabits a home cage for several days with a female before an intruder of the same sex and species is introduced. This elicits an aggressive territorial response in the resident male. Lower levels of offensive aggression in the resident, and higher levels of freezing are indicative of a more passive coping style (Ebner *et al.*, 2005). In this paradigm, and others, there is a close relationship between aggression and coping style, with a strong positive association between active coping and propensity to display offensive aggression (Koolhaas *et al.*, 2010; Veenema and Neumann, 2007). As such, offensive aggression is often used as a measure of coping style (Koolhaas *et al.*, 2010; Veenema and Neumann, 2007). Active responding in the resident intruder paradigm is associated with increased activation, as indicated by number of c-Fos positive neurons, to the presentation of the intruder in the amygdala, BNST, ventrolateral hypothalamus, nucleus accumbens shell (AcbSH), orbital PFC; and reduced activation in the lateral septum and dorsolateral PAG (Haller *et al.*, 2006; Koolhaas *et al.*, 2010; van der Vegt *et al.*, 2003; Veenema and Neumann, 2007).

The resident-intruder paradigm has proven very useful for studying differences in coping styles; however, there are two primary issues with this model. Firstly, the model's reliance on aggression as a proxy for coping style is less than ideal. Secondly, the success of

the competing strategies (active vs passive) in avoiding the negative outcome (physical harm and/or subordination) cannot be made equivalent for each animal as the outcome is largely dictated by the behaviour of the other rodent.

Another relevant behavioural assay is the *defensive burying test*. In this test individually housed rodents have an electric shock probe placed in their homecage and their response to a brief shock is assessed. Mice and rats with a passive coping style engage in freezing behaviour and avoidance of the probe, while an active coping style involves using the bedding from their cage to bury the probe. This model does not suffer from a lack of standardisation in the provoking stressor. However, this paradigm relies on electric shock, a stressor that lacks ethological validity, and also assesses coping strategy in socially isolated subjects. This latter point is particularly problematic as social factors may influence the expression of coping styles.

Field research shows that an important determinant of outward expressions of active coping may be the presence of other conspecifics (Zahavi, 1990; Zahavi and Zahavi, 1997). For example, male Arabian babblers of the highest social status engage in sentinel behaviour more frequently than other babblers. Sentinel behaviour involves going to the highest part of the canopy, where they are most exposed and are at greatest risk of being attacked, to keep watch for predators (Zahavi, 1990; Zahavi and Zahavi, 1997). Similar observations have been made with herds of African Buffalo, where the strongest males take up the most dangerous positions at the perimeter of the herd (Mloszewski, 1983). In the laboratory “visible burrow system” study discussed earlier in this chapter, it is the dominant male rats that are the first to return to the burrow surface following the introduction of the live predator (Blanchard and Blanchard, 1989). It has been argued that this overtly

dangerous active responding by males is one way an animal establishes and maintains their status within a group (Zahavi, 1990; Zahavi and Zahavi, 1997).

An interesting aspect of coping styles is that they can be selectively bred in subjects. Thus, active or passive response tendencies, or related behavioural traits such as anxiety and aggression, appear to have a heritable basis. Examples include the Low Anxiety Behaviour (LAB) rats and Short Attack Latency (SAL) mice who display an active coping style. Their more passive counterparts are the High Anxiety Behaviour (HAB) rats and Long Attack Latency Mice (LAL) (Koolhaas *et al.*, 2010; Veenema and Neumann, 2007). This selective breeding approach can be useful for exploring the genetic components associated with coping style. However, it is also important to explore intra-strain variations in coping style in outbred rats living in a group with natural variation in coping styles analogous to that seen in the field. This variation in coping style is critical for the survival of the species as it promotes survival under changing environmental circumstances, as discussed earlier (Dingemanse *et al.*, 2004b; Van Oortmerssen and Busser, 1989). As such, interaction in an environment containing conspecifics with a variety of coping styles is the norm.

The biological systems determining coping style show large variations across strains and species. Comparisons between two different strains that display active versus passive coping sometimes yields results that are inconsistent with those observed in studies examining within-strain variations in coping styles (Koolhaas *et al.*, 2010; Veenema and Neumann, 2007). For example, increased activity in the MeA-BNST-lateral septum AVP system is found to be associated with an active coping style when intra-strain variations in coping style are examined (Bester-Meredith *et al.*, 1999; Delville *et al.*, 1996; Ferris *et al.*, 1984; Irvin *et al.*, 1990; Koolhaas *et al.*, 1998; Wersinger *et al.*, 2002). However, when inter-

strain comparisons are made (for instance between the active coping SAL mice or LAB rats and the passive coping LAL mice and HAB rats), active coping is sometimes found to be associated with decreased activity in this AVP system (Beiderbeck *et al.*, 2007; Compaan *et al.*, 1993). Many of the systems associated with coping strategies show large variation between sexes, across the lifespan, between strains and between species, making it difficult to utilise inter-strain comparisons to study the involvement of these mechanisms in the expression of coping styles (Koolhaas *et al.*, 2010; Veenema and Neumann, 2007).

The ability to differentiate coping styles within a strain might be usefully done in an outbred strain of laboratory rat and in a paradigm involving standardised group exposure to a naturalistic stressor. In their natural environment, perhaps the most biologically relevant stressor for a rat is predatory threat. As discussed above, this is something that they rarely experience in isolation from other rats (Macdonald *et al.*, 1999). Therefore, group exposure to predatory threat could be a useful and ethologically valid method of identifying active and passive responders that appreciates the possible role social factors play in determining the expression of coping style. Furthermore, the use of a predator odour, rather than a live predator, would ensure the outcome (avoidance of predator attack) is equivalent for both active and passive responders. Aggression is not required as a proxy for coping style in such a model, as approach and avoidance of the threat stimulus, along with other social responses (e.g. defensive aggregation), could be used as, arguably, more direct measures of active and passive coping. Finally, the magnitude and consistency of the stress response elicited by predatory threat could facilitate identification of clear and consistent responder types within a single strain.

1.5.3. The relationship between coping style, social support and social buffering: implications for psychological disorders

Several studies have suggested that coping style may be an important determinant of whether a person seeks out social support when confronted with a stressor. In particular, it seems that persons with a more passive coping style can benefit more from the stress buffering effects of social support, and are more likely to seek such support out (Nolen-Hoeksema and Davis, 1999). Similar effects have been observed in other species with, for example, passive coping pigs gaining the greatest social buffering from the presence of another pig during a restraint-stress test (Reimert *et al.*, 2014).

A passive coping style in humans is associated with serious psychiatric disorders such as schizophrenia (Xu *et al.*, 2013), drug abuse (Scott *et al.*, 2013), bipolar disorder (Fletcher *et al.*, 2013), PTSD (Zhang *et al.*, 2014), depression (Muris, 2002), and GAD (LeDoux and Gorman, 2001). People suffering from a number of these disorders appear afflicted by the compounding effects of a passive coping style combined with a deficiency in seeking social support (as discussed in the previous section). As social support and subsequent social buffering appear to be an extremely important means of regulating and recovering from stress in passive responders (Nolen-Hoeksema and Davis, 1999; Reimert *et al.*, 2014; Taylor *et al.*, 2000), the combination of a passive response style and a deficient social response to threat could be particularly pathological and might play a significant role in the aetiology of psychiatric disease and be a major hindrance to recovery.

1.6. Oxytocin and vasopressin

OT and AVP are neuropeptides that consist of nine amino acids and differ in only two of the amino acid positions. OT and AVP are part of the arginine-vasotocin family of neuropeptides (Acher *et al.*, 1972). Neuropeptides of this family are highly evolutionarily

conserved in both structure and function across mammalian and non-mammalian species (Hoyle, 1999). The primary sites of synthesis of OT and AVP are in the magnocellular neurons of the PVN and supraoptic nucleus of the hypothalamus (SON). OT and AVP are released into the bloodstream via axonal projections to the neurohypophysis. Primary peripheral actions of OT include stimulation of uterine contractions and controlling the milk-letdown reflex during lactation (Freund-Mercier and Richard, 1984; Higuchi *et al.*, 1985), whereas AVP regulates water resorption in the kidney and controls the constriction of vascular smooth muscle cells (Goldsmith, 1987).

The main orthosteric binding site for OT is the OT receptor (OTR) whereas AVP has three primary receptors: the V_2 receptor (V_2R), which is the main AVP binding site in the periphery; and the V_{1A} receptor ($V_{1A}R$) and V_{1B} receptor ($V_{1B}R$) which are the main AVP binding sites in the central nervous system (Chini and Manning, 2007; Manning *et al.*, 2012). However, OT also appears to elicit some of its actions via binding at the $V_{1A}R$, for which it also has relatively high affinity. This is discussed in more detail later in this section.

Perhaps the most fascinating functions of OT and AVP are those involving its actions in the central nervous system. OT and AVP are released from the dendrites and perikarya of the magnocellular neurons within the hypothalamus (Ludwig and Leng, 2006) as well as from axonal and collateral projections from parvo- and magnoceullar neurons to regions in the limbic system such as the amygdala and lateral septum (Buijs *et al.*, 1983). AVP is also synthesized in extra-hypothalamic regions such as the BNST and the MeA (De Vries and Buijs, 1983). The AVP cells in these regions project to areas such as the lateral septum, parts of the hippocampus, PFC, olfactory tubercle, locus coeruleus and lateral habenula (LHb) (Caffe *et al.*, 1987; Dabrowska *et al.*, 2011; de Vries, 2008; van Leeuwen and Caffé, 1983).

There is a clear overlap between the OT and AVP systems of the brain and the structures involved in the defensive response to cat odour, social buffering, and coping strategies. Beyond this general structural overlap, there is mounting evidence that OT and/or AVP in these systems may be directly involved in the aforementioned phenomenon. Furthermore, there is reason to believe that OT and AVP systems may play a critical role in controlling the social response to threat. However, the overall number of studies in this area is relatively small.

1.6.1. The involvement of neuropeptides in defensive behaviours and social buffering

Anti-predator defence

Several studies have implicated a role for OT and AVP and their receptors in aspects of the defensive response to predator odours. Intracerebroventricular (ICV) infusion of an OTR antagonist blocks cat odour-induced activation of the MeA and increases contact with the cat odour stimulus (Samuelsen and Meredith, 2011). Central administration of either an OTR or $V_{1A}R$ antagonist inhibits cat odour induced increases in blood oxygen dependent signalling in the amygdala of rats (Reed *et al.*, 2013). This is consistent with a broader role for OT in regulating anxiety and stress responses via its central actions in areas such as the amygdala and via direct inhibition of the HPA axis stress response (Neumann and Landgraf, 2012).

Social buffering

OT has received considerable attention in research into social buffering. Individually housed hamsters, but not socially housed ones, that received a cutaneous wound followed by immobilisation stress show greater cortisol release and impaired wound healing

(Detillion *et al.*, 2004). Peripheral administration of OT to the socially isolated hamsters reduced the stress-induced cortisol release and aided wound healing. In contrast, administration of an OTR antagonist to the socially housed hamsters impaired wound healing. This study indicates that social contact may facilitate repair of physical wounds via OT-induced inhibition of the HPA axis. Similar effects of OT have been found in humans: for example, intranasal OT administration paired with social support dampened the HPA axis and subjective anxiety response to a psychosocial stressor in males (Heinrichs *et al.*, 2003).

A recent study in prairie voles provides further direct evidence for a role of OT in social buffering at the level of the HPA axis (Smith and Wang, 2014). Female prairie voles that recover with a partner following immobilisation stress show a less pronounced corticosterone response, reduced anxiety-like behaviours, and increased OT release from the PVN as assessed by microdialysis. Infusion of OT into the PVN of females recovering in isolation following stress elicited a similar effect to social buffering itself, while infusion of an OTR antagonist into the PVN blocked the social buffering effect evident in females recovering in the presence of a partner.

There appears to be a reciprocal relationship between OT and social contact whereby OT regulates the beneficial effects of social contact on stress and wellbeing, while social contact earlier in life appears to play a critical role in the development of the OT system. Mice reared communally have increased OTR binding in areas such as the lateral septum and BNST and reduced $V_{1A}R$ binding in the lateral septum (Bales and Perkeybile, 2012; Curley *et al.*, 2009a; Curley *et al.*, 2009b). Rhesus monkeys removed from their mothers shortly after parturition and reared in a nursery show reduced OT levels in cerebrospinal fluid (CSF) compared to monkeys reared by their mother (Winslow *et al.*,

2003). Similarly, women suffering from abuse as a child show lower levels of CSF OT (Heim *et al.*, 2009). The quality of care provided during early life also appears to be a critical factor influencing the development of the OT system with more involved maternal care of rodents (indicated by high licking and grooming behaviour by the dams) associated with increased OTR binding in areas such as the lateral septum, BNST, PVN and amygdala (Champagne and Meaney, 2007; Francis *et al.*, 2000; Francis *et al.*, 2002).

In recent studies (see Appendix 2 and 3) our group has found evidence that increased OT activity during early life, such as that observed in animals reared in a positive social environment, may play an important role in shaping social, anxiety and addictive behaviours later in life (Bowen *et al.*, 2011; Suraev *et al.*, 2014). Thus, peripheral administration of OT to rats during early adolescence was associated with increased OT system activity, greater sociability, lower anxiety, and less likelihood of developing excessive alcohol consumption in adulthood. Similar positive outcomes are observed in rodents who have a positive rearing environment (Baldini *et al.*, 2013), suggesting stimulating OT release during adolescence can lead to enduring positive effects and that OT stimulation by close attachment and contact during rearing may be a critical mechanism involved in the enduring positive effects of such a rearing environment. Furthermore, these studies suggest that OT administration during early life may prevent some of the negative outcomes of a poor up-bringing, perhaps acting as a sort of pharmacological proxy for a healthy rearing environment.

1.6.2. A possible role for neuropeptides in the social response to threat

The studies above suggest that the development of the OT system is partly dependent on social contact and that OT released during social contact has a direct

buffering effect via its inhibitive actions on the HPA axis. However, OT may play another role in social buffering by promoting the social contact that leads to buffering effects. Much work to date has focused on the role of OT and AVP in social bonding, social interaction, social preference and social memory, all of which are facilitated by OT (Donaldson and Young, 2008; Lukas *et al.*, 2011; Ramos *et al.*, 2013; Young and Wang, 2004).

An important point is that recent studies suggest that not all effects of OT on social behaviour are mediated by the OTR. OT has relatively high affinity for the $V_{1A}R$ as well as the OTR (Chini and Manning, 2007; Manning *et al.*, 2012). Some of the social effects of OT, especially those related to social interaction, may, in fact, be mediated by the $V_{1A}R$ rather than the OTR. Strikingly, exogenous OT ameliorates impaired sociability in OTR knockout mice and this effect is blocked by the selective $V_{1A}R$ antagonist SR49059 (Sala *et al.*, 2011). Moreover, the acute prosocial effects of peripheral OT and AVP in Long-Evans rats tested in the social interaction paradigm can be prevented by pre-treatment with SR49059 but not an OTR antagonist (Ramos *et al.*, 2013). Furthermore, the enduring increase in social interaction seen following adolescent OT administration was not observed with the selective OTR agonist TGOT, suggesting this effect may too involve stimulation of a receptor other than the OTR (Appendix 3 (Suraev *et al.*, 2014)).

These studies provide support for the hypothesis that OT helps to restore homeostasis following stress by motivating prosocial behaviours that lead to social buffering effects. However, most of these studies exploring OT's ability to increase social contact and interaction have been conducted in animals that are not facing the stressful or threatening situations that would most benefit from a social response. As such, a model that

investigates the action of OT under conditions of defensive aggregation might be very instructive.

Evidence that neuropeptides might modulate defensive aggregation comes from several studies exploring the role of these evolutionarily ancient neuropeptides in flocking and shoaling behaviour in birds and fish, respectively. Predator defence is a primary factor driving flocking and shoaling and thus these behaviours are viewed as forms of defensive aggregation (Caraco et al., 1980; Seppälä et al., 2008). Specifically, mesotocin (a non-mammalian analogue of OT) promotes flocking behaviour in estrildids (Goodson et al., 2009), while blockade of vasotocin V_{1A}-like receptors in the lateral septum reduces flocking behaviour in zebra finches (Kelly et al., 2011). In zebrafish, peripheral administration of isotocin (the teleost analogue of OT), OT, vasotocin or AVP increases shoaling (Braida et al., 2012). It is clearly of interest to determine whether OT and AVP also drive defensive aggregation in mammalian species.

1.6.3. The involvement of the amygdala-septum AVP system in active and passive coping

OT and AVP have also been closely linked to anxiety-like behaviours and coping styles, although the underlying mechanisms appear rather complex and region-dependent. AVP levels in the hypothalamus appear to be positively correlated with levels of anxiety (Neumann and Landgraf, 2012). However, AVP acting in other brain regions, such as the amygdala and septum, appears to promote an aggressive and active response to threat (Koolhaas *et al.*, 2010), although this may differ in some genetically selected strains of rodents (Veenema and Neumann, 2007).

Microinfusion of AVP into the cerebral ventricles (Winslow and Insel, 1993), or within the BNST or lateral septum, increases offensive aggression in both hamsters and rats

(Delville *et al.*, 1996; Ferris *et al.*, 1984; Irvin *et al.*, 1990; Koolhaas *et al.*, 1998). Denser AVP staining and greater receptor abundance is found in the lateral septum of more aggressive mouse strains (Bester-Meredith *et al.*, 1999) and deletion of the AVP $V_{1B}R$ gene essentially blocks all offensive aggression but does not alter defensive aggression in mice (Wersinger *et al.*, 2002). These studies suggest that the AVP system projecting from the MeA and BNST to the lateral septum plays a critical role in the expression of active and passive coping styles.

1.7. Conclusions

The literature discussed in this chapter suggests that anti-predator behaviour, defensive aggregation, social buffering and coping strategies may be interconnected. However, to date, these phenomena have mostly been studied in isolation, allowing only anecdotal or highly generalised inferences to be made about their possible relationships with each other. In this thesis, a laboratory model involving group exposure of rats to predatory threat is outlined. This model is initially used to establish whether defensive aggregation to threat forms part of the rodent defensive behaviour repertoire. The value of this model is in allowing such behaviours be assessed in a strictly controlled environment, and allowing the physiology, pharmacology and neurobiology of defensive aggregation to be studied objectively, at a level of detail not hitherto possible.

The ability to make within-group comparisons of response styles in conspecifics exposed to predatory threat would provide a novel, ethologically valid model for examining within-strain differences and their neurobiological underpinnings. It would also allow close examination of social factors that may influence coping strategies. Ultimately, a rodent model of defensive aggregation to predator threat could allow us to learn much about how

to promote adaptive social responding to stressors and a more resilient coping strategy and this could have benefits for human health.

1.8. Overview of experimental chapters in this thesis

The work presented in this thesis gathers evidence to assess a number of primary hypotheses derived from the literature review presented in this chapter:

1. Rats may aggregate in response to cat odour and other unconditioned stressors.

2. Defensive aggregation may allow social buffering of behavioural and neural responses to predatory threat.

3. There may be a clear and consistent distinction between active and passive coping styles in outbred rats exposed to predatory threat in groups.

4. The expression of an active coping style in response to predatory threat may depend upon the presence of conspecifics.

5. Passive coping styles may be associated with a greater social response to threat.

6. Active coping towards predatory threat may foster greater resilience during chronic stress and lower levels of anxiety and depression-like behaviour.

7. The neuropeptides OT and AVP are likely to be involved in the social response to threat and in the mediation of active and passive coping styles.

Chapter 2 (Bowen *et al.*, 2012) describes a novel rodent model of defensive aggregation based on the response of groups of rats to cat odour and bright light. The importance of group size is established by comparing the aggregation response of rats exposed to predator threat in groups of two versus groups of four.

Chapter 3 (Bowen *et al.*, 2013) uses Fos immunohistochemistry to characterise the behavioural and neural correlates of defensive aggregation, and of active and passive coping styles, in response to predatory threat. It demonstrates the expression of active and passive coping strategies within groups of rats exposed to predatory threat, the stability of these coping styles across time, and the extent to which they generalise to other assays of anxiety-like and depression-like behaviour. The second component of Chapter 3 is the assessment of possible social buffering effects of group versus individual exposure to predatory threat and the neural correlates of social buffering of predatory threat responses. It determines whether social factors influence the expression of active and passive coping styles toward predatory threat and identifies neural correlates of these different coping styles.

Chapter 4 (Bowen and McGregor, 2014) explores whether the neuropeptides OT and AVP can influence the social response to predatory threat. It establishes the involvement of the $V_{1A}R$ and $V_{1B}R$ in defensive aggregation and in mediating the effects of exogenously applied OT and AVP. The specificity of effects of OT and AVP on defensive aggregation are assessed relative to more general effects they may have on anxiety-like behaviour in response to cat odour.

Chapter 5 (Bowen *et al.*, 2014) examines neuroendocrine differences and epigenetic differences in the MeA AVP system between rats displaying an active versus passive coping strategy in response to predatory threat. It confirms our previous behavioural characterisation of the active and passive coping styles and provides important insights into how neurosteroids and epigenetic regulation of the AVP system might play an important role in the expression of active or passive coping toward threat.

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Chapter 2: Aggregation in quads but not pairs of rats exposed to cat odor or bright light

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Aggregation in quads but not pairs of rats exposed to cat odor or bright light

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ABSTRACT

In many prey species aggregation of individuals is a defensive strategy commonly employed in response to predators and predator-related cues. However, very little work has explored this adaptive response in laboratory rats. It is known that individual rats show characteristic defensive responses to predator odors, such as hiding, avoidance, inhibition of foraging, feeding and reproduction, and risk assessment directed toward the odor source. However, whether these species-typical responses in individuals are altered in the presence of other conspecifics is yet to be characterized. The present study therefore examined the defensive response of groups of two rats (dyads) or four rats (quads) to two unconditioned stressors: bright ambient light and cat odor (a 2 g ball of cat fur). The dyads and quads were formed from familiar cage mates and test sessions (20 min) occurred in a large open arena (1200 mm^2) to which the rats had been extensively habituated under dark conditions. The results showed that when quads of rats were exposed to either cat odor or bright light in this arena, they showed characteristic increases in close social proximity, termed "huddling". A tight grouping of 3 (triplet) or 4 (quad) rats was commonly seen in response to cat fur, while triplets were more commonly seen in response to bright light. Interestingly there was no evidence for increased social proximity in dyads exposed to either stressor, only in quads. However, cat odor caused other signs of fear (such as decreased locomotor activity and increased defecation) in both quads and dyads. It is concluded that huddling is a rodent defensive strategy in rats when anxiogenic stimuli are encountered by larger groups of rats.

1. Introduction

Rattus norvegicus is one of the most widely used laboratory animals and is essential to a large variety of modern scientific pursuits (Lindsey and Baker, 2006). Laboratory rats are often used to model human psychopathologies such as depression and anxiety, allowing improved understanding of the biological mechanisms that underlie such disorders and the efficacy of novel pharmacological interventions. To maximize the effectiveness of animal models it is important to have comprehensive knowledge of the laboratory rat's response to stressors.

One stressor applied to rats to mimic aspects of human anxiety is the presentation of predators or predator-related stimuli such as their odor. It has been argued that using naturalistic stimuli such as cat odor, as opposed to more traditional stressors such as foot shock or restraint, leads to more effective modeling of human anxiety disorders such as phobia, panic and post-traumatic stress disorder (Dielenberg et al., 2001b; Apfelbach et al., 2005; Blanchard and Blanchard, 1988, 1989b). An impressive body of work originating from the Blanchards (e.g. Blanchard and Blanchard, 1989a,b; Blanchard et al., 1989) and File (e.g. Zangrossi and File, 1992; File et al., 1993) provides considerable insight into the rat's defensive responses to predatory odors, with many characteristic neural, physiological and neurochemical correlates now identified (McGregor et al., 2004; Dielenberg et al., 2001a,b; Apfelbach et al., 2005). Furthermore, cat odor can produce strong and lasting cue and context conditioning (Blanchard et al., 2001; Dielenberg et al., 1999, 2001a) whereby previously neutral stimuli that have been associated with predatory stimuli come to elicit defensive behaviors in their own right. There may also be social transmission of fear with cage mates relaying information to each other about fearful contexts and stimuli (Bruchey et al., 2010; Knapska et al., 2010; Kim et al., 2010).

Somewhat surprisingly, very little research has been done on the response of groups of rats to predator threat. Given that rats are social animals and live in highly gregarious colonies, it is likely that wild rats will typically experience predatory threat in the company of other colony members (Macdonald et al., 1999). As such, it

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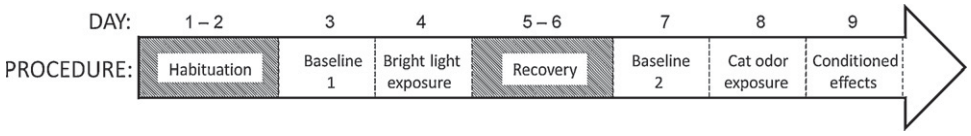

Fig. 1. Experimental timeline. Subjects in the dyad ($n = 16$) and quad ($n = 32$) condition underwent 3 days of habituation under red light conditions, the final one of which served as a baseline for the bright light exposure day. There were then 2 recovery days during which no testing took place. On the following day subjects were again tested under red light conditions to give a baseline for the following test the next day with cat odor present in the arena. On the final day subjects were returned to the arena under normal conditions to test for any conditioned effects of cat odor exposure.

is of interest to examine the response of groups of rats to predator-related stimuli. One relevant study by Blanchard and Blanchard (1989a) housed laboratory rats in a naturalistic visible burrow system and presented a cat in the open area above the burrow. Not unexpectedly, rats rapidly retreated to the safety of the burrow system when confronted with a cat. Interestingly, the authors noted that non-aggressive social contact was significantly increased during and after the introduction of the cat and this was accompanied by a significant increase in the number of observations in which the subjects were found in very close proximity to each other.

This increased social proximity, or huddling, in response to anxiogenic stimuli is the specific focus of this study. A recent study by our group demonstrated that this huddling effect can also occur in response to a predator-related cue (cat fur) rather than a predator per se, and also when there is no option to hide (Kendig et al., 2011).

Huddling is an adaptive response defined as "an active and close aggregation of animals" (Gilbert et al., 2010) and has been documented across a wide variety of species, both social and solitary. While huddling has clear thermoregulatory and energetic benefits (Alberts, 1978; Alberts and May, 1984; Withers and Jarvis, 1980; Schmidt et al., 1986; Yahav and Buffenstein, 1991), cold and food shortages do not appear to be the only determinants. In particular, the anti-predator advantages of huddling have garnered considerable attention as an adaptive response in many species. It has even been proposed that predator defense was one of the major evolutionary forces leading to gregariousness and group living (Hamilton, 1971). In ethological terms huddling can be seen as adaptive in terms of increased predator detection, and also maximization of individual survival after detection. In particular, it has been established that as the number of animals huddling together increases, the probability that any one prey will be attacked decreases, a phenomenon known as the *dilution effect* (Foster and Treherne, 1981; Hamilton, 1971; Inman and Krebs, 1987; Morgan and Godin, 1985).

While defensive huddling has been explored primarily in response to predator threat, it is also of interest to establish whether other anxiogenic stimuli also induce huddling. For example, Hamilton (1971) noted animals huddle together not only in response to predatory threat, but to a wide range of fear-inducing stimuli. However, to date there has been little work done to systematically explore Hamilton's assertion. Laboratory rats have a natural aversion to bright light which, like cat odor, induces a pronounced neural, physiological and behavioral response indicative of anxiety (Walker and Davis, 1997). While a cat odor stimulus is more obviously related to predator threat, it is also possible that rats' innate fear of bright light stems from adaptations related to predator avoidance given that rats are far more easily detected by aerial, feline and vulpine predators under bright light. It is of interest to note here that in one laboratory study, two unfamiliar rats engaged in *less* social interaction under bright light than under low light conditions, indicative of increased generalized anxiety (File and Seth, 2003). However, other literature suggests that aggregation usually takes place between familiar conspecifics (Gilbert et al., 2010) so such social avoidance under bright light may only occur between unfamiliar pairs of animals.

To further examine such issues, the present study characterized the group response of laboratory rats' to both cat fur and bright light to establish if the defensive huddling response is specific to predator threat. Furthermore, we examined the response to these anxiogenic stimuli in pairs of familiar rats (dyads) and also in groups of four familiar rats (quads) to see if group size has any effect on the huddling behavior as the *dilution effect* would predict.

2. Materials and methods

2.1. Subjects

The subjects were 48 male AAW rats (ARC, Perth, Australia) weighing an average of 663 g at the start of testing. Subjects were housed four per cage in a temperature controlled colony room ($22 \pm 2\,^{\circ}C$) on a reverse light–dark cycle. Subjects had ad libitum access to standard rat chow and water in the home cage. All subjects were handled extensively for several weeks before the start of testing.

2.2. Design

This was a $2 \times (7)$ mixed design study. Sixteen of the subjects were assigned to the dyad condition and 32 subjects were assigned to the quad condition. In the dyad condition rats were paired with another rat from the same home cage to form a familiar pair that underwent experimental procedures together. In the quad condition all four rats from a home cage underwent experimental procedures together.

2.3. Apparatus and materials

Testing took place during the dark cycle in an arena with wooden walls and floor. The walls and flooring were painted matt black. The area of the arena was 1200 mm^2, three of the walls were 900 mm high and the fourth wall was 610 mm high to facilitate access to the arena.

The predator odor stimulus was a 2 g ball of cat fur obtained from a dead cat carcass acquired from the company *Australian Feral Pest Management*. Feral cats are routinely shot in Australia as they pose a serious threat to native Australian wildlife. Deceased cats were stored at $-80\,^{\circ}C$. To prepare the predator stimulus, a 2 g ball of fur was shaved from the back and neck of the cat which was stored at $-20\,^{\circ}C$ when not in use. Prior to the first test session the fur was removed from the freezer and placed in a scientific oven (Binder; Crown Scientific, Australia) at $40\,^{\circ}C$ for 30 min. In between each test session the fur was returned to the oven for 5 min at $40\,^{\circ}C$ to ensure temperature was consistent across sessions. The heating process makes the temperature for the fur close to the body temperature of a live predator and appears to more reliably elicit fear responses in rodents (unpublished observations).

2.4. Procedure

For an overview of the experimental timeline see Fig. 1. The habituation period consisted of three days of testing, during which

the dyads and quads were placed in the arena for one 20 min session each day under red light. The final day of this habituation period was used as the baseline for the bright light test. On the fourth day the dyads and quads were placed in the arena for one 20 min session each under bright light conditions. To produce the bright light, four PAR70 150 W spotlights were placed above the arena pointing directly down onto the open field.

Following the bright light test day there were two recovery days during which no testing was conducted. On the seventh day, dyads and quads were placed in the arena for one 20 min session each under the baseline conditions described above. The data from this day was used as the baseline for the cat odor and conditioning test days as the data did not differ significantly from the initial habitation and baseline period on any measures (all p-values > 0.05). On the eighth day, all dyads and quads were placed in the arena for one 20 min session each in the presence of the predator odor stimulus. The predator odor stimulus was placed on the floor flush against the center of one of the walls. On the ninth day, all dyads and quads were placed in the arena for one 20 min session each under baseline conditions to test for conditioned effects.

2.5. Data collection and statistical analysis

All data except those otherwise indicated were collected using Trackmate Social v. 1.0 (Motion Mensura, Cook Hill, NSW, Australia). This video tracking software can be used to measure the social behavior of up to 4 rats simultaneously. For both dyads and quads the dependent variables of interest were time spent in a quad huddle, distance traveled and number of fecal boli. A complete huddle (see Fig. 2 for an illustration) involved all rats from a dyad or quad together so they were touching or were within less than one body length of each other. For quads, the time three rats spent

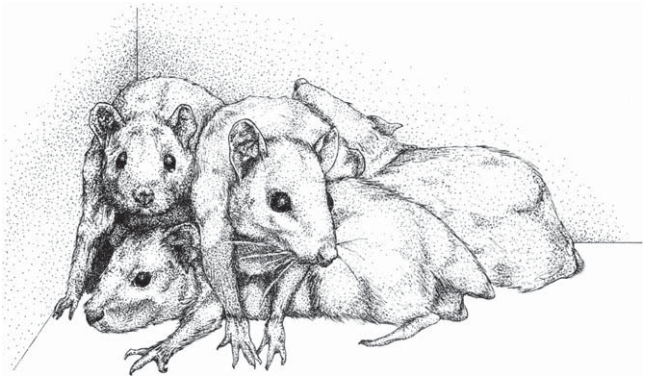

Fig. 2. Illustration of a "complete huddle" of four rats seen in response to cat fur.

in the huddle formation was another variable of interest – this is referred to as a triplet huddle. Traveled distance was the average mm traveled per rat in a 20 min session and was simply the total distance traveled by all rats in a dyad or quad in a session divided by two for dyads, or by four for quads. Defecation was recorded at the end of each session as the number of fecal boli left in the arena after each session divided by two for dyads or by four for quads.

Data were analyzed using SPSS version 18 (SPSS Inc., Chicago, IL). A 2 × (7) mixed design analysis was conducted with one repeated measures variable (stressor) with seven levels (baseline one, bright light, baseline two, cat odor and conditioning) and one between subjects variable (group size) with two levels (dyad or quad). Planned contrasts were used to assess any overall effect of bright light or cat fur on variables of interest, to determine if the stressors differed in their impact on the variables of interest, and to assess

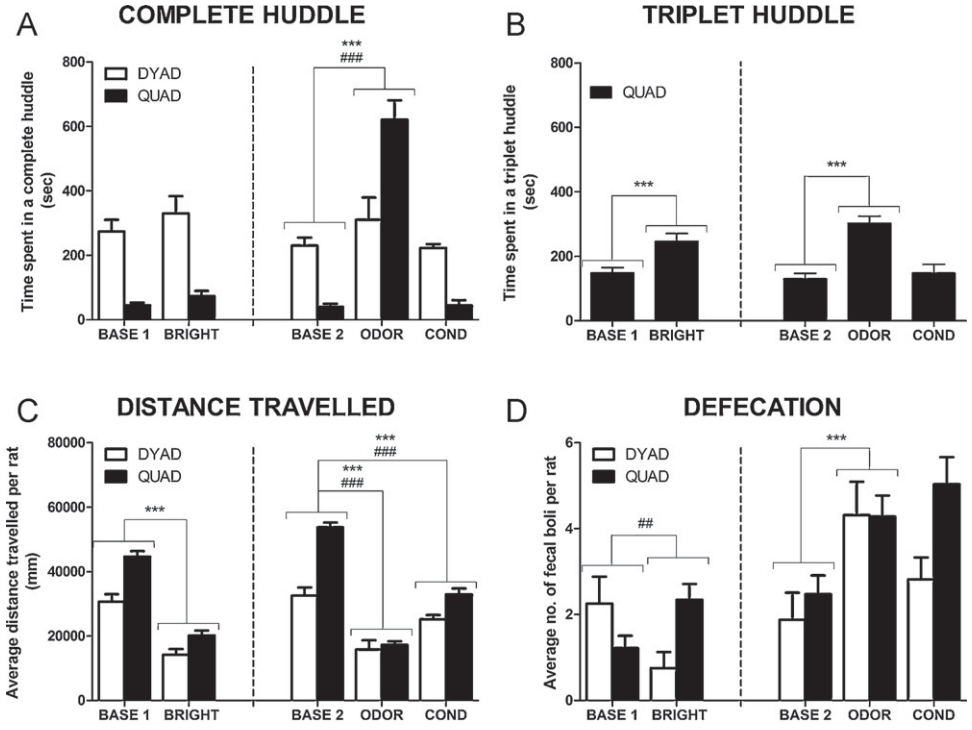

Fig. 3. Behavioral results. Only the primary main effect and interaction effect contrasts are indicated on the figure, please see the main text for other comparisons. Stippled line indicates the 2-day recovery period after bright light exposure. (A) Time spent in a complete huddle: there was no change for dyads across experimental conditions, but quads spent significantly more time in a complete huddle when cat fur (ODOR) was present. (B) The time quads spent in a triplet huddle was significantly increased by both bright light (BRIGHT) and cat fur. (C) Distance traveled was significantly decreased under bright light and cat fur with a more pronounced reduction for quads. There was also a significant conditioned effect (COND) of cat odor on distance traveled that was more pronounced for quads. (D) Number of fecal boli was increased for quads under bright light but decreased for dyads; number of fecal boli was increased for both dyads and quads when they were exposed to cat odor. $^{***}p \leq 0.001$ main effect of stressor; $^{###}p \leq 0.001$ interaction of stressor with group size; $^{##}p \leq 0.01$ interaction of stressor with group size.

whether there was evidence of any conditioning to cat fur. Planned interaction contrasts were used to assess whether group size had any impact on these comparisons. The Scheffé procedure was used to control the family wise error rate at 0.05 across all possible comparisons which gave a critical *t*-value of 3.19. Unadjusted *p*-values are reported. The Scheffé procedure was used because it controls the error rate across all possible comparisons (complex and pairwise) and, as the assumption of sphericity was met for all variables, it can be used to assess comparisons involving repeated measures variables (Skene and Kenward, 2010).

3. Results

Fig. 3a and b provides an overview of the results examining the huddling behavior of dyads and quads. Averaged across group size, there was no significant effect of bright light on time spent in a complete huddle, $p = 0.230$. However, bright light did result in a significant increase in the time quads spent huddled in a triplet (see Fig. 3b), $t(7) = 5.86$, $p < 0.001$. Quads also spent significantly more time huddled in a triplet when they were exposed to cat odor [$t(7) = 5.83$, $p < 0.001$] and the time spent huddling in a triplet did not differ for the two stressors, $p = 0.210$.

Averaged across group size, there was a significant increase in time spent in a complete huddle when cat odor was present, $t(14) = 7.32$, $p < 0.0001$. However, a significant interaction contrast revealed that while there was only a very minor, non-significant ($p = 0.282$), increase in time spent in a complete huddle for dyads, there was a dramatic increase for quads (refer to Video 1 in the electronic version for footage of a quads behavior in the presence and absence of cat odor), $t(14) = 5.55$, $p < 0.0001$. Furthermore, averaged across group size, the cat odor stressor was significantly more effective at inducing complete huddling than the bright light stressor, $t(14) = 4.11$, $p < 0.001$. However, the significant interaction contrast revealed that while the cat odor stimulus induced markedly more huddling than the bright light stimulus for quads, there was little difference in the efficacy of the stimuli in producing social proximity in dyads [$t(14) = 4.46$, $p < 0.001$] who showed no significant change in social proximity across the experiment, all *p*-values > 0.282. Finally, there was no evidence to suggest that the cat fur stimulus had a conditioned effect on time spent in a complete huddle, $p = 0.943$.

Fig. 3c provides an overview of the results examining distance traveled. Averaged across the testing conditions, the dyads traveled significantly less distance than the quads [$F(1, 14) = 62.96$, $p < 0.0001$]. Averaged across group size, distance traveled was significantly less under bright light conditions compared to baseline [$t(14) = 12.331$, $p < 0.0001$]. The interaction contrast revealed that the magnitude of this reduction in traveled distance did not differ significantly between dyads and quads after controlling the type 1 error rate (see 2.5 for more information on the statistical analysis), $t(14) < 3.19$, $p = 0.029$. However, averaged across conditions there was a reduction in distance traveled when cat odor was present [$t(14) = 11.13$, $p < 0.0001$] but this was qualified by a significant interaction contrast which indicated that while there was a reduction in traveled distance when cat odor was present for both dyads and quads, the magnitude of this reduction was significantly more pronounced for quads [$t(14) = 4.13$, $p = 0.001$]. Similarly, averaged across conditions there was a conditioned reduction in traveled activity as a result of cat odor exposure [$t(14) = 9.58$, $p < 0.0001$], however, the interaction contrast revealed that the conditioned effect was significantly more pronounced in the quads, $t(14) = 4.63$, $p < 0.001$. Distance traveled did not differ significantly when comparing the two stressors ($p = 0.783$) and there was no significant interaction with group size, $p = 0.348$.

Fig. 3d provides an overview of the results examining the average number of fecal boli per rat across the testing conditions. Averaged across group size, there was no significant effect of bright light on number of fecal boli ($p = 0.603$). However, this was qualified by a significant interaction with group size which revealed that the average number of fecal boli per rat increased in quads under bright light conditions but decreased in dyads, $t(14) = 3.72$, $p = 0.002$. Averaged across group size, the average number of fecal boli per rat was significantly higher in the presence of cat odor compared to baseline [$t(14) = 3.78$, $p = 0.002$]. The interaction contrast revealed that the magnitude of this increase in the number of fecal boli per rat did not differ significantly between dyads and quads, $p = 0.587$. Average number of fecal boli per rat was significantly higher with the cat odor stressor compared to the bright light stressor [$t(14) = 5.64$, $p < 0.0001$] and there was no significant interaction with group size, $p = 0.118$. Averaged across group size, there was no significant conditioned effect of cat odor on average number of fecal boli per rat after controlling the type 1 error rate [$t(14) < 3.19$, $p = 0.02$] and the interaction contrast revealed the magnitude of this conditioned effect did not differ significantly between dyads and quads, $p = 0.243$.

4. Discussion

The current study shows that huddling in groups of 4 is a characteristic defensive response in rats exposed to two naturalistic stressors (bright light and cat fur). These quads showed a dramatic increase in huddling to cat odor as well as a marked triplet huddling response to cat odor and bright light. In contrast, there was no increase in social proximity when pairs of rats were exposed to the same stressors under exactly the same environmental conditions. There was, however, clear evidence of an anxiety response to both stressors in both dyads and quads with the traveled distance of both groupings significantly reduced in the presence of both stressors. Similarly, the number of fecal boli increased under bright light for quads and increased for dyads and quads in the presence of cat odor.

The findings of the present study are consistent with those reported by Blanchard and Blanchard (1989a) who report that rats living in a visible burrow system cluster together in one of the burrows when a live cat is introduced into the open area of the arena. The present findings also confirm our recent observation that the huddling response occurs in adolescent rats exposed to predator odor and not a live cat *per se*, and when there is no option to hide (Kendig et al., 2011). Importantly, the present study substantially extends these findings by demonstrating that: the huddling response is not predator specific, with it also occurring in response to an anxiogenic bright light stimulus; and group size is an important determinant of huddling with a group of four, but not two, rats engaging in threat-induced huddling. Furthermore, as our previous study used adolescent rats and the current study used adult rats, it demonstrates that huddling is a defensive response present in both age groups.

This study also provides an interesting link between the findings of the zoological studies exploring predator-induced huddling/herding (e.g. Hamilton, 1971; Kim et al., 2010; Roberts, 1996; Rogovin et al., 2004; Morgan and Godin, 1985) and work examining the effects of cat odor and predatory threat on the laboratory rat (e.g. Zangrossi and File, 1992; Blanchard and Blanchard, 1989a; Blanchard et al., 2001; Dielenberg et al., 2001a,b). It has been hypothesized that one of the benefits of huddling after detection by the predator involves a *dilution effect*. The dilution effect refers to a phenomenon whereby for any one predator attack, the larger the group of prey the lesser the probability that any one particular

animal will fall victim (Foster and Treherne, 1981; Hamilton, 1971; Inman and Krebs, 1987; Morgan and Godin, 1985).

The failure to observe increased social proximity in dyads suggests that the dilution effect could be involved in the adaptive huddling response of rodents just as appears to be the case in other species (Foster and Treherne, 1981; Hamilton, 1971; Inman and Krebs, 1987; Morgan and Godin, 1985). The lack of huddling in the dyads may be due to minimal benefit being afforded by huddling when only two rats are present. The theoretical work of Hamilton (1971) and subsequent experimental work (e.g. Foster and Treherne, 1981; Morgan and Godin, 1985) have shown that in many species the general trend is the more animals in a huddle the greater the survival benefits. Ethical considerations obviously preclude direct assessment of the survival cost and benefit of huddling in laboratory studies such as the present one due to the possibility that numerous subjects will suffer physical harm and death.

It is also interesting that the bright light did not cause a reduction in social contact in the dyads as occurs when two unfamiliar rats are exposed to bright light (File and Seth, 2003). A possible explanation is that the unfamiliarity of conspecfics is critical in reducing social interaction given that huddling in many species occurs primarily with familiar conspecifics (Gilbert et al., 2010). It would clearly be of interest in future studies to explore the differential response of familiar versus unfamiliar dyads and quads to anxiogenic stimuli.

Some unexpected findings were obtained in the current study. For example, the reduction in traveled distance when cat odor was present was significantly more marked for quads. This may partly reflect the higher baseline activity in quads than in dyads. It was expected that the quads might have been more active in the presence of predatory threat due to reduced anxiety or "buffering" afforded by membership in a larger group. This relates to the so-called group vigilance effect which has been shown to allow individual animals within a larger group to focus less on the threat and engage in other important activities such as exploration (Roberts, 1996; Rogovin et al., 2004; Elgar, 1989; Hamilton, 1971). It is likely, however, that the more marked effect of cat fur in decreasing traveled activity in the quads reflects a floor effect in traveled activity in the dyads. Baseline distance traveled was higher in the quads providing a greater possible margin for reduction. It is also possible that the dyads showed a less marked reduction in traveled distance in response to cat odor due to a panic response with more time spent engaging in escape and panic related behaviors rather than huddling. In future studies it would be of interest to directly measure panic-related behaviors such as escape attempts.

There was a more pronounced conditioned effect of cat fur on distance traveled in the quads. This might also be explained by the lower baseline levels of activity in the dyads. Alternatively, it is possible that the context was more salient to the quads due to the increased number of conspecifics reacting to the environment, leading to a greater conditioned response. Indeed, recent work demonstrating fear conditioning by proxy supports this possibility (Bruchey et al., 2010; Knapska et al., 2010; Kim et al., 2010).

While an increase in triplet huddling was seen in the presence of both bright light and cat odor, an increase in complete huddle time was only seen in the presence of cat odor. There are a number of possible explanations for this. Firstly, it is likely that bright light is less anxiogenic and, as a result, it was more common for one rat to be out of the huddle exploring. Alternatively, the increased triplet huddling and lack of a change in complete huddling in response to bright light might be due to the nature of the stimulus. Whereas the source of the cat odor was localized to the small area of the arena containing the fur, the bright light stimulus was more diffuse which may have led to greater exploration by one rat at a time to try and identify any potential sources of threat. This hypothesis could be tested by putting small amounts of fur throughout the entire arena to mimic the diffuseness of the bright light anxiogenic stimulus and see if a similar triplet and complete huddling behavioral pattern is observed.

It is an exciting possibility that defensive huddling could be used as a novel behavioral index with which to test the effects of anxiolytics. If it is presumed that the greater the anxiety response, the greater the huddling, then an anxiolytic would be expected to reduce the amount of time spent in a huddle. A future study might therefore characterize the effects of anxiolytics such as benzodiazepines on the huddling response. It is also of considerable interest to study the neural basis of the huddling response and possible "social buffering" benefits arising from stressor exposure in the presence of conspecifics (Kikusui et al., 2006). Huddling animals are able to decrease their individual vigilance and devote more time to other non-vigilance related activities (Roberts, 1996) suggesting an overall anxiolytic effect of multiple conspecifics. Future studies might therefore characterize whether the acute and long-term adverse consequences of predatory stress (Apfelbach et al., 2005) are diminished somewhat by the presence of conspecifics during exposure.

The current study provides one of the first systematic documentations of predator threat-induced huddling in the laboratory rat. It detailed a profound huddling response in groups of four but not two rats exposed to both a direct predator threat and bright light stressor. Importantly, it paves the way for future research to examine the neural and behavioral advantages of being exposed to a powerful stressor such as cat odor in a group as opposed to alone. Furthermore, it presents the exciting possibility that new, more ecologically valid animal models of anxiety can be developed around the defensive huddling response to threat.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.beproc.2012.03.014.

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Chapter 3: Defensive aggregation (huddling) in *Rattus norvegicus* toward predator odor: Individual differences, social buffering effects and neural correlates

NOTE: The supplementary methods referred to in the publication presented in this chapter can be found in Appendix 4

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Defensive Aggregation (Huddling) in *Rattus Norvegicus* toward Predator Odor: Individual Differences, Social Buffering Effects and Neural Correlates

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Abstract

Aggregation is a defensive strategy employed by many prey species in response to predatory threat. Our group has characterized defensive aggregation (huddling) in *Rattus norvegicus* in response to a ball of cat fur. In this situation some rats huddle less, and approach the threatening cue more than others (active vs. passive responders). The present study explored whether active responding is a stable phenotype associated with behaviors outside direct predatory encounters. The neural substrates of active and passive responding under predatory threat were explored using c-Fos immunohistochemistry. Finally, we examined whether the presence of conspecifics during predatory threat biases behavior towards active responding. Active and passive responding styles were found to be stable in individual rats across consecutive group exposures to cat fur, and were predicted by anxiety-like behavior in an open-field emergence test. Active responders displayed less conditioned fear in an environment associated with predatory threat, and had higher post-exposure intake of a weak sucrose solution (a test of ''anhedonia''). Active responding was associated with: greater cat fur-induced activation of the accessory olfactory bulb, reflecting greater olfactory stimulation in rats actively approaching the fur; lowered activation of somatosensory cortex, reflecting reduced huddling with conspecifics; and reduced activation in the lateral septum. Social exposure to cat fur promoted active responding relative to individual exposure, and lowered c-Fos expression in the dorsomedial periaqueductal grey, medial caudate putamen and lateral habenula. We conclude that individual differences in anti-predator behavior appear stable traits with active responders having a more resilient phenotype. Social exposure to predatory threat has an acute buffering effect, subtly changing the neural and behavioral response towards threat and encouraging active responding. An association between active responding and lower c-Fos expression in the lateral septum is consistent with previous studies that highlight this region as an important neurobiological substrate of defensive aggregation.

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Introduction

Defensive aggregation is a ubiquitous response in prey species involving the tight grouping (e.g. huddling, flocking or shoaling) of animals in response to predatory threat. Defensive aggregation is observed across a wide array of mammalian and non-mammalian species [1], for example: Emperor Penguins [2]; the marine insect *Halobates robustus* [3]; and Serengeti ungulates such as wildebeest, zebra, and Thomson's gazelle [4]. Investigating the behavioral dynamics and neurobiological underpinnings of defensive aggregation is of increasing interest to researchers in both the laboratory and the field (e.g. [5–10]).

Laboratory models of defensive aggregation allow systematic study of the neural substrates, pharmacology and genetic determinants of this behavioral phenomenon. Our own research has focused on groups of laboratory rats confronted with predatory threat in the form of a ball of cat fur. Cat fur and skin odors cause characteristic behavioral and neural changes in individual rats that are indicative of a profound anxiety-like state (for a review see [11]). The response of groups of laboratory rats to predatory threat was first described by Blanchard & Blanchard [12] using a laboratory ''visible burrow system''. These authors reported hasty retreat and a subsequent increase in non-sexual, non-aggressive social contacts in the burrow system when rats encountered a live cat at the burrow surface. In more recent work [13,14], our group has described high levels of huddling when groups of four cage mates are exposed to cat fur in an open arena. This does not occur, however, when only two rats are present [13]. Such results are predicted by the *dilution effect*, which sees the survival benefit afforded by aggregation as increasing with the size of the group, through a decrease in the probability that any individual animal will be attacked [3,15].

Cat fur/skin odor appear to function as *kairomones*: defined as chemosensory signals that are emitted by one species and intercepted by another to the advantage of the recipient [16]. In

other words, the sensitivity of rodents to cat fur confers a survival advantage, and they are most likely intercepting signals that cats use in their own social communication. Studies using c-Fos immunohistochemistry show that cat odor activates pheromone processing circuitry in the rat brain localised within the accessory olfactory bulb (AOB) and its projection areas in the medial amygdala, bed nucleus of the stria terminalis, and ventromedial hypothalamus [17–19]. A medial hypothalamic circuit, with the dorsal premammillary nucleus (PMD) at its center, integrates this sensory input and organizes behavioral output via projections to the periaqueductal gray (PAG) and cuneiform nucleus. This system organises behavioral responses that may include escape attempts, immobility and inhibition of feeding, foraging and reproduction – all of which are characteristic responses to predatory threat [11,17,18,20–22].

Our informal observations of huddling in outbred laboratory rats exposed to predatory threat [13,14] suggests divergent response styles in the individuals within each group of four rats exposed to cat fur. Some rats (''active responders'') show relatively high levels of investigation of the cat fur, and relatively low levels of immobility and huddling with conspecifics. In contrast, many rats are ''passive responders'' and barely investigate the threatening stimulus, showing high levels of immobility and huddling. This is in accord with previous suggestions of active and passive responder styles in outbred rats exposed to other forms of stress [23]. Active responders proactively confront threats, have a more aggressive phenotype, and show less immobility and hypothalamic-pituitary-adrenal (HPA) axis activity and reactivity compared to their passive counterparts. Conversely, passive responders avoid threats, have a less aggressive phenotype, and respond actively only when it is absolutely necessary [24,25].

Active and passive coping styles may have emerged as adaptations to the changing environments that animals experience in the wild [26]. For example, Dingemanse and colleagues [26] studied a population of *Parus major* and found that in years when food was plentiful, active responding males had greater rates of survival. Conversely, in years when food was scarce, more passive responding males had greater rates of survival. The enhanced ability of active responding males to secure and hold onto territory in resource-rich years improves their chances of survival. Conversely, their more overtly aggressive and territorial phenotype in resource-scarce years may result in more net costs than benefits. Interestingly, the relationship between responder type, availability of resources, and rate of survival was the opposite for females, further driving diversity in offspring responder types [26].

In the present study, our initial aim was to examine whether active or passive responding to predatory threat was a stable behavioral trait in laboratory rats. As such, we individually marked rats and then scored their approach and huddling response to cat fur over consecutive exposures in groups. We also examined whether the passive versus active responder style was associated with behavioral differences in situations other than cat fur exposure, including the open field emergence test [27] and an environment previously associated with predatory threat [28]. We also examined whether active and passive responders differed in their consumption of a weak 1% sucrose solution, a marker of anhedonia, or depressive-like behavior, in rats [29]. Our highly developed knowledge of the patterns of neural activation elicited in rats individually exposed to cat odor [17–19,30] also allowed an opportunity to examine how active and passive responding may manifest itself at a neural level. In this regard we used c-Fos immunohistochemistry to determine neural activation to cat fur in rats exhibiting an active or passive coping style.

We also hypothesised that exposure to threat in a group may encourage a more active style of coping, by reducing the acute anxiety arising from the experience. The term ''social buffering'' describes how social interaction with conspecifics during or after exposure to a stressor may reduce the impact of that stressful situation [31]. A small number of studies with rodents suggest that the behavioral, physiological and neural responses to stressors may be reduced by the presence of conspecifics either during or after a stressor such as shock [31,32]. To examine possible social buffering effects, we compared behavior and brain activation in rats exposed to cat fur when alone, with those exposed in a group of four conspecifics. We predicted that group exposure might provide some buffering effects, resulting in a less pronounced neural and behavioral response to cat odor in the socially exposed rats.

In summary, the primary aims of the present study were twofold. The first aim was to formally examine the proposition that within a group of rats exposed to cat odor there are active and passive responders and that these characteristics are relatively stable over repeated exposure to threat, and associated with differential neural activation. The second aim was to characterize how group exposure to predatory threat differs from individual exposure, and whether social buffering might promote a more active style of responding.

Methods

All experimental procedures were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition, 2004) and were approved by University of Sydney Animal Ethics Committee (approval number L29/7-2010/3/5360).

2.1. Experiment 1

2.1.1. Aims. Experiment 1 examined the stability of active and passive response styles in groups of four rats repeatedly exposed to cat odor in daily sessions. A secondary aim was to see if responder style could be predicted from behavior in other situations, in which immediate predatory threat was not present. An overview of the tests conducted and their timeline is provided in Figure 1.

2.1.2. Subjects. The subjects were 48 male outbred Albino Wistar rats (Animal Resources Centre, Perth, Western Australia) weighing between 291 and 386 g and aged 8 weeks at the start of

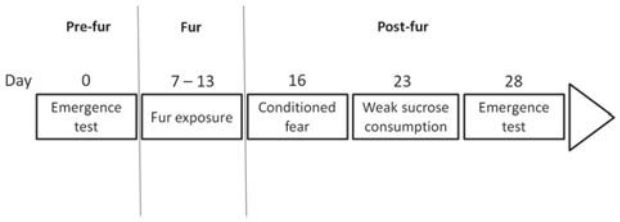

Figure 1. Timeline for experiment 1. An emergence test was initially conducted prior to cat fur exposure. Rats were exposed to cat fur in groups of four once per day for 7 days. Three days after the final fur exposure rats were returned to the test arena individually with a hide box present but no fur present. This allowed testing for conditioned fear to context. Ten days after the final fur exposure rats were tested in a 10 min session for their consumption of a 1% sucrose solution. Finally, 15 days after the final fur exposure the emergence test was once again conducted to test for chronic stress-induced changes in anxiety-like behavior.
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testing. They were housed in groups of 4 in a temperature controlled colony room (21±2°C) on a reverse light-dark cycle (lights on 21:00). Food and water were available *ad libitum* in the home cage and all behavioral testing took place during the dark cycle. Following their arrival at the laboratory, rats were thoroughly handled daily for 3 days before the start of testing. Subjects were randomly assigned to either a no cat odor control condition (n = 16, consisting of 4 quads) or a cat odor condition (n = 32, consisting of 8 quads).

2.1.3. Fur exposure. *Cat odor stimulus:* The predator stimuli used were 2 g balls of cat fur acquired from male cat carcasses kindly provided by *Australian Feral Pest Management*. Feral cats are routinely shot in Australia due to the threat they pose to native Australian wildlife. Deceased cats were stored at −20°C. To prepare each predator stimulus, a 2 g ball of fur was shaved from the back and neck of the cat. The fur sample was stored in an airtight jar at − 20°C when not in use. Prior to use, the cat fur was heated in a scientific oven (Binder; Crown Scientific, Australia) at 40°C for 30 min. In between testing sessions the ball was placed in the oven for 5 min at 40°C to ensure the temperature was consistent across sessions. The heating process makes the temperature of the fur close to the body temperature of a live predator.

Apparatus: Testing was conducted in two identical 120 cm×120 cm×90 cm (l x w x h) wooden framed arenas, located adjacent to each other. The removable arena base comprised of a 120 cm x 120 cm wire mesh sheet (0.1 cm thick wire rods, 1 cm apart) secured to a 7 cm high wooden frame. The interior walls and arena base were painted matt black. The testing room was lit with infrared light and overhead infrared-sensitive cameras fixed directly above each arena sent their outputs to two computers in an adjacent room that digitized and recorded the sessions.

Procedure: Rats were exposed to cat fur in groups consisting of 4 cage mates, hereafter referred to as a ''quad''. Each rat in a quad was marked with a non-toxic Sharpie permanent marker each day before testing to allow ready visual identification of individual animals within a group during subsequent video scoring of behavior. Marking involved holding the rat securely and gently making a unique mark on its back with the permanent marker. Subjects were marked with either a: cross running vertically from the upper to lower back and horizontally from shoulder to shoulder; a horizontal line running from shoulder to shoulder; a vertical line running from the upper to lower back; or a circle marked in the middle of the back.

A 2 g ball of fur was placed into each arena against the center of one wall. Quads were then placed into one of the arenas opposite the fur for 20 min. Control rat quads were handled identically but with no fur placed in the arena. This process was repeated once a day for 7 days. Each quad was always exposed in the same arena and the arenas were thoroughly cleaned with 30% v/v ethanol in between each session.

Over time, rats tend to habituate to cat fur such that they will show less defensive behavior over repeated exposures [33]. To guard against habituation to the fur, the fur was exchanged for that of another cat on the fourth day. A reinstatement of defensive responding to cat fur occurs when fur from a different cat is presented to habituated rats [14,34].

Classification of responder type: Subjects in the cat odor condition were designated as either a passive, neutral or active responder based on their number of contacts with the cat odor stimulus. A stimulus contact was defined as a rat placing its nose within 3 cm of the cat fur. Rats with 2 or less stimulus contacts in a 20 min session were classified as passive responders, animals with 3–4 stimulus contacts were classified as neutral responders, and rats with 5 or more stimulus contacts were classified as active responders. Criteria based assignment was used, as opposed to a simple median split, as it allowed greater equivalence of assignment thresholds between Experiment 1 and 2, and avoided some of the major issues presented by median split dichotomization [35], which might not provide adequate separation between active and passive responders. The overall classification as active, passive, or neutral was based on the average daily number of stimulus contacts over the seven exposure sessions.

Data collection and analysis of exposure sessions: Two of the dependent variables of interest were number of stimulus contacts and time spent in the half of the arena containing the cat fur. These behaviors were manually scored for each individual rat from the videos by an experimenter, using ODLog (Macropad Software). Data were analysed using mixed model ANOVA and planned contrasts were computed using the values of the dependent variables averaged across the 7 exposures to compare: (1) the cat odor exposed rats versus the control rats; and (2) the active responders versus the passive responders within the cat odor-exposed cohort.

The number of fecal boli present in the arena were counted after each session: obviously this was a group measure given that fecal boli could not be attributed to individual rats. The total number of fecal boli, averaged over the 7 sessions, were compared for control quads versus fur exposed quads using a planned contrast following a mixed model ANOVA.

Huddling behavior was scored automatically using MotMen Social Tracker 2.7 (as used in [13,14]) and was defined as three or four rats clumping together in a single tight group with each rat touching at least one other rat. The time that control quads spent huddling on each of the 7 exposure days was compared to fur-exposed quads using a planned contrast following mixed model ANOVA.

Consistency of responder type (active or passive) and huddling behavior across the 7 repeated exposures were assessed by computing Cronbach's alpha (Cronbach's α) and the Intraclass Correlation (ICC) using reliability analysis with a two-way random model examining absolute agreement and average measures. Cronbach's α is a measure of internal consistency, with a Cronbach's $\alpha > 0.9$ considered an indication of excellent internal consistency [36]. The ICC assesses the level of agreement between repeated ratings, with an ICC >0.8 indicating excellent agreement [37]. Essentially, these statistics indicate the consistency with which a rat was classified as the same responder type over the 7 days, and how consistent each quads huddling time was across the 7 repeated exposures (see [38]). Consistency of responder type indicates their classification as active or passive remains stable across the repeated exposures, and consistency of huddling behavior indicates the amount of time a quad spends huddling remains consistent across the repeated exposures.

2.1.4. Post exposure test 1: Conditioned fear. Three days after the final cat fur (or control) exposure, individual rats were returned to the same testing arena but with no fur present. A wooden hide box with a red Perspex lid (40×24×17 cm), identical to that used in the emergence test (see below), was placed against the center of one wall. The 5 min session started with an individual rat being placed in the hide box. The following dependent variables were automatically scored by Trackmate 5.5 video tracking software (Motion Mensura, Cook's Hill, NSW): (i) time in hide box, (ii) latency to emerge, and (iii) distance travelled. After each test the arena was cleaned with 30% v/v ethanol. Data were analysed using one-way ANOVA with four levels of the independent variable (control, passive, neutral, active) and

planned contrasts comparing: (1) control to active responders; (2) control to passive responders; and (3) active responders to passive responders.

2.1.5. Post exposure test 2: weak sucrose consumption (anhedonia). Testing took place 10 days after the final day of fur exposure. Individual rats were trained for 2 days before the start of any testing to consume sucrose in a small plastic cage (50×35×30 cm) with a sucrose dispenser (Sippy, Ferplast, Italy) placed in one of the short walls. Training sessions lasted 10 min with access to a highly preferred 10% sucrose solution. On the test day the rats were placed in the small plastic cage for 10 min with a 1% sucrose solution available from the dispenser. The scored behavior was the volume of sucrose solution consumed by each rat. Data were analysed using one-way ANOVA with four levels of the independent variable (control, passive, neutral, active) and contrasts comparing: (1) Active responders to passive responders; (2) Active responders to control; and (3) Passive responders to control.

2.1.6. Pre-exposure test and post exposure test 3: anxiety-like behavior. To assess individual differences in anxiety-like behavior before and after exposure to predatory threat, the emergence test was conducted 7 days before the first fur exposure and 15 days after the final fur exposure. This also allowed any increases in anxiety-like behavior as a result of the chronic stress exposure to be identified [39].

Testing occurred in a 120×120×60 cm wooden arena, with a black floor and white walls. A wooden hide box (40×24×17 cm), painted black with a red Perspex lid, was placed against the center of one wall. The arena was illuminated by two floodlights (with 240V, 150W globes), producing a bright open field, and a camera mounted above the arena provided video footage to a computer in another room, where behavior was scored automatically using Trackmate 5.5 (Motion Mensura, Cook's Hill, NSW). Rats were individually tested for 5 min after being placed into the hide box and after each test the arena was thoroughly cleaned with 30% v/v ethanol. The dependent variables were: (i) time in hide box, (ii) latency to emerge, and (iii) distance travelled. Note that these behaviours are usually highly correlated, with long emergence latency associated with greater time in hide box and less distance travelled. Nonetheless, we have analysed all of them here to show the consistency of any differences in anxiety-like behaviours across all of the major behaviors examined in the emergence test. Data were analysed using two-way ANOVA with a (2) (pre-exposure, post exposure) x 4 (control, passive, neutral, active) design and planned contrasts comparing: (1) active and passive responders; (2) pre-test and post-test for active responders; and (3) pre-test and post-test for passive responders.

2.2. Experiment 2

2.2.1. Aims. Experiment 2 aimed to assess the neural correlates of active and passive responder types and also to compare the behavioral and neural differences between rats exposed to predatory threat alone versus in a group.

2.2.2. Subjects. Subjects were 32 male Albino Wistar rats (Animal Resources Centre, Perth, WA) weighing between 460 and 597 g and aged 12 weeks at the start of the experiment. Rats were housed in groups of 4 as described for Experiment 1 (see above). All rats were thoroughly handled prior to testing, and all behavioral testing took place during the dark cycle.

2.2.3. Design and responder type classification. This was a 2×2 fully factorial design with 8 subjects in each of the four conditions. The independent variables were cat odor stimulus (present or absent) and exposure condition (exposed to fur either alone or in a quad).

In addition, subjects in the fur exposure conditions were classified as either active or passive using a modified version of the criteria used in Experiment 1 to account for the increased session length of 50 min. Accordingly, rats were classified as passive when they engaged in 3 or less stimulus contacts in 50 min, neutral when they engaged in 4–8 stimulus contacts and active when they engaged in 9 or more stimulus contacts. Pilot studies (unpublished findings) indicated that these slight changes to the criteria used in Experiment 1 were necessary to maintain a clear distinction between active and passive responders over the longer test session.

2.2.4. Apparatus and materials. The testing arena and fur samples were the same as described for Experiment 1.

2.2.5. Procedure. As in Experiment 1, all rats were uniquely marked on their backs with a Sharpie non-toxic permanent marker each day to allow identification of individual rats within a group. Prior to testing, all rats received 4 days of habituation to the test arena, but with no fur stimulus present. On the first day, half the rats were placed in one of the two arenas for 50 min alone and the other half were placed in one of the arenas in groups of 4. At the end of the habituation period rats were placed individually into small holding cages for 30 min. The following day this same procedure was repeated, but with rats that had been habituated individually on day one given group habituation and vice versa. On days three and four the procedure for days one and two were repeated. This extensive habituation procedure was used to minimise any c-Fos expression on test day due to extraneous environmental stimuli or novelty.

Testing took place over two days, with conditions counterbalanced to control for time of day effects. Subjects were placed into the arena either alone or in quads, with the cat odor stimulus either absent (no odor conditions) or flush against the center of the lower wall of the arena (cat odor conditions). Subjects were left in the arena for 50 min, after which they were removed and placed into a holding cage for 30 min. Following this they were removed from the holding cages and perfused (see below). In between test sessions the fur was heated as described earlier and the arenas were thoroughly cleaned with 30% v/v ethanol as well as a vacuum cleaner to remove any remnants of the fur, or the previous subjects.

2.2.6. Immunohistochemistry. The methods used for c-Fos immunohistochemistry were as described previously (see, for example, [18,19,34]). Briefly, rats were deeply anesthetized then perfused transcardially. Following perfusion, brains were extracted and prepared for slicing. Tissue was sliced at 40 μm then stained for c-Fos immunoreactivity. The number of c-Fos positive cells in the regions of interest were quantified (see Table 1 for the regions and counts for areas where significant results were obtained) and images of regions of interest were prepared. A detailed description of the methods used for: tissue collection, preparation, and staining; the counting of labelled cells; and the preparation of representative images can be found in the supporting information for this paper (File S1).

2.2.7. Data collection and analysis. Video files were given coded names unrelated to condition and were scored by an experienced blind observer using ODLog (Macropod Software). Time spent: immobile in the first 10 min of the session (rat is stationary with all four paws on the ground and no body movement); in the stimulus half of the arena; unsupported rearing (rat rears up on its hind legs without placing its forepaws on anything for support); supported rearing (rat rears up on its hind legs and places its forepaws on wall for support); and self-grooming (rat licks, scratches or face washes itself) were analysed with two-way ANOVA with the independent variables being cat odor stimulus (present or not present) and exposure (alone or group).

Table 1. Mean number (± SEM) of c-Fos-positive cells in brain regions of interest.

Region	Bregma	CONTROL ALONE	CONTROL QUAD	CAT ALONE	CAT QUAD
Sites where group vs individual exposure affected cat fur-induced c-Fos expression					
AOBmc	5.70	5.29 (1.97)	9.67 (3.71)	11.33 (3.04)	19.43 (1.57)[a,b,c]
CPuM	0.70	.12 (.12)	1.12 (.40)	6 (1.49)	2.57 (.20)[a,b,c]
LPO	−0.26	1.62 (.96)	.75 (.62)	13.75 (2.37)[a,b]	8.57 (2.11)[a,b,c]
LAmg	−3.14	.5 (.76)	.37 (.37)	5.25 (1.28)	2.37 (.80)[a,b,c]
LHb	−3.14	9.87 (2.29)	8.5 (2.88)	38 (5.49)[a,b]	26.5 (4.56)[a,b,c]
DMPAG	−6.04	2.12 (.51)	1.12 (.64)	8.75 (1.06)	3.25 (2.96)[a,b,c]
Sites where cat fur increased c-Fos expression irrespective of group vs individual exposure					
AOV	5.20	3.62 (.65)	3 (.90)	10.29 (1.81)[a,b]	9.62 (2.87)[a,b]
MPC	3.20	2.62 (.68)	2.37 (1.08)	16.57 (4.05)[a,b]	20.75 (4.42)[a,b]
IL	3.20	5.25 (.75)	5.25 (.65)	13.71 (2.57)	12 (2.56)
AOP	3.20	2 (.91)	1.62 (.80)	5.29 (1.49)	5.62 (1.62)
LSV	0.70	11.5 (2.38)	13.88 (3.46)	37.63 (5.51)[a,b]	36.63 (5.085)[a,b]
AcbSh	0.70	.87 (.48)	1.12 (.40)	6.87 (1.41)[a,b]	7.28 (1.44)[a,b]
BSTMA	−0.26	1.37 (.82)	1.87 (.74)	9.25 (1.79)[a,b]	9.25 (2.11)[a,b]
MPA	−0.26	4 (.82)	5.87 (1.76)	11 (3.36)	11.71 (3.39)
SON	−1.30	.25 (.25)	.62 (.62)	9.12 (2.38)[a,b]	5.25 (2.37)[a,b]
PVN	−1.80	2.87 (.93)	2.27 (1.26)	18.37 (3.70)[a,b]	16.87 (2.90)[a,b]
BC	−2.12	1.12 (.79)	.87 (.40)	6.12 (2.12)[a,b]	2.87 (.51)[a,b]
CeAmg	−2.80	.12 (.12)	.75 (.37)	3.75 (1.06)[a,b]	3.87 (1.20)[a,b]
BLAmg	−2.80	.5 (.19)	.25 (.25)	4 (.98)[a,b]	3.12 (.77)[a,b]
DMH	−2.80	6.25 (1.25)	9.5 (2.37)	25.25 (3.15)[a,b]	28.5 (3.25)[a,b]
MEPV	−3.14	2 (.78)	5.25 (1.57)	25.62 (4.08)[a,b]	23.62 (2.25)[a,b]
MEPD	−3.14	1.25 (.56)	1.37 (.65)	3.62 (.92)	3.12 (.81)
VMH	−3.14–.30	0 (0)	0 (0)	19.12 (2.52)[a,b]	22.62 (4.12)[a,b]
PMD	−4.16	.25 (.25)	1.37 (.56)	65.37 (8.31)[a,b]	67 (7.55)[a,b]
VLPAG	−8.72	1.5 (.80)	1.62 (.50)	22 (2.96)[a,b]	26.87 (3.54)[a,b]
CnF	−8.72	.75 (.41)	.25 (.25)	12.12 (1.24)[a,b]	11 (1.35)[a,b]
LC	−9.68	0 (0)	0 (0)	7.14 (1.96)[a,b]	8 (1.16)[a,b]

NOTE: In all of the above regions there was a significant difference between the cat odor exposure groups, on average, and the no cat odor exposure groups, on average. a = significantly different to no cat odor alone condition; b = significantly different to no cat odor quad condition; c = significantly different to cat odor alone condition.

AcbSH = Shell of the nucleus accumbens; AOBmc = mitral cell layer of the AOB; AOP = posterior part of the anterior olfactory nucleus; AOV = ventral part of the anterior olfactory nucleus; BC = somatosensory barrel cortex; BLAmg = Basolateral amygdala; BSTMA = medial division of the anterior part of the bed nucleus of the stria terminalis; CeAmg = Central nucleus of the amygdala; CnF = Cuneiform nucleus; CPuM = Medial caudate putamen; DMH = Dorsomedial nucleus of the hypothalamus; DMPAG = Dorsomedial PAG; IL = infralimbic cortex; LAmg = Lateral Amygdala; LC = locus ceruleus; LHb = lateral habenula; LPO = Lateral preoptic nucleus; LSV = ventrolateral septum; MePD = posterodorsal part of the medial amygdala; MePV = posteroventral part of the medial amygdala; MPA = medial preoptic area; MPC = medial prefrontal cortex; PMD = dorsal part of the premammillary nucleus; PVN = paraventricular nucleus of the hypothalamus; SON = supraoptic nucleus of the hypothalamus; VLPAG = ventrolateral PAG; VMH = ventromedial nucleus of the hypothalamus.

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Immobility was scored only for the first 10 min of the session as rats exposed to cat odor in a group begin to spend a substantial amount of time huddling after 10 min and it became difficult to score immobility due to obstruction by other rats.

As it was only possible to score the number of stimulus contacts when the stimulus was present, an independent samples t-test comparing rats exposed to cat odor alone to those exposed in quads was computed. Huddling, allogrooming (rat grooms another rat) and play behavior (rat pounces on, pins or chases another rat) are social behaviors and thus are only applicable to the quad exposed rats, so independent sample t-tests were computed to compare control quads to the quads exposed to cat odor. There is some debate as to whether or not adult rats engage in social play behavior, with some arguing what is classified as social play is just aggressive behavior. However, Schneider and Koch [40] argue that social play behavior occurs throughout the lifespan and simply becomes less frequent in adulthood. In the present study we have used their classification of pouncing, pinning and chasing as social play behavior.

In addition to the behaviors scored in Experiment 1, we examined the additional behaviors in Experiment 2 for several reasons. Firstly, it was of particular interest to examine immobility and grooming in Experiment 2 as the stress induced changes in these behaviors is known to be augmented by the presence of conspecifics [32,41,42]. Secondly, the measurement of additional behaviors was desirable so as to provide greater opportunity to identify possible links between patterns of brain activation associated with group exposure and behavior.

The number of c-Fos positive cells in regions of interest were analysed as previously described [18,43]. Problems with homogeneity of variance were dealt with by performing a log10+1 transformation and a square root +1 transformation and selecting the transformation that resulted in the highest p value in Levene's Test for Equality of Error Variances. ANOVAs with four levels of the independent variable (Alone no cat odor, alone cat odor, quad no cat odor and quad cat odor) were then used to compare between all groups, using the Student-Newman-Keuls procedure to control the type 1 error rate at 0.05 across all comparisons. The main effect of cat odor was compared using a planned contrast comparing the two cat odor conditions (alone and group, on average) to the two no cat odor conditions (alone and group, on average).

To compare the frequency of active and passive responders within the cat odor alone compared to cat odor group exposure conditions, a Chi-Square Test of Independence was conducted with the two variables being exposure configuration (alone or group) and responder type (active or passive). As no rats in the alone condition met the criteria for active responders, comparisons of behaviors and c-Fos immunoreactivity were made between active and passive responders within the cat odor group exposed condition using independent samples Student's *t*-tests.

Correlations were also calculated between huddling and the number of c-Fos positive cells in the regions of interest.

Results

3.1. Experiment 1

3.1.1. Responder type classification. Style of responding appeared to be highly consistent: rats classified as passive overall were classified as passive on 6.1 out of 7 exposure days (87%) on average, while rats classified as active were classified as active on 5.5 out of 7 exposure days (79%) on average. Reliability analysis indicated that responder type (active or passive) was highly consistent across the 7 exposure days, Cronbach's $\alpha = 0.928$, ICC = 0.927. Neutral responders displayed a far more variable response pattern, being classified, on average, as neutral on only 2.43 out of 7 exposure days (34.7%), passive on 2.86 out of 7 (40.8%) and active on 1.71 out of 7 (24.5%).

3.1.2. Fur exposure. Fur exposed quads huddled significantly more than control quads on all of the 7 exposure days (see Fig 2A) (all $p<0.001$). Reliability analysis revealed the magnitude of the huddling response across the 7 days was highly consistent, Cronbach's $\alpha = 0.978$, $ICC = 0.970$. Averaged over the 7 exposure days, fur exposed quads ($M = 24.8$, $SE = 1.1$) deposited, on average, 11 more fecal boli than control quads ($M = 13.9$, $SE = 2.6$), $F(1, 10) = 21.344$, $p = 0.001$, and spent significantly less time in the stimulus half of the arena compared to the control rats, $F(1, 44) = 363.09$, $p<0.001$.

Within the fur-exposed cohort, active responders had significantly more stimulus contacts (Fig 2B) and spent significantly more time in the stimulus half of the arena than passive responders (Fig 2C) averaged over the 7 exposure days [stimulus contacts: $F(1, 29) = 229.67$, $p<0.001$; stimulus half: $F(1, 44) = 82.14$, $p<0.001$]. Neutral responders did not differ significantly from passive responders in time spent in the stimulus half of the arena and fell between passive and active responders in number of stimulus contacts (statistics not shown).

3.1.3. Post exposure test 1: Conditioned fear. When returned to the arena in the absence of cat fur and with a hide box present, passive responders spent significantly more time hiding (Fig 2D); took significantly longer to emerge from the hide box and travelled significantly less distance than the no odor control rats [hiding: $F(1, 44) = 6.98$, $p = 0.011$; latency to emerge: $F(1,44) = 9.69$, $p = 0.003$; distance travelled: $F(1,44) = 33.13$, $p<0.001$].

Conversely, there was no significant difference between active responders and the no odor control rats in the time spent hiding ($p = 0.253$), latency to emerge ($p = 0.781$) or distance travelled ($p = 0.978$). Compared to passive responders, active responders spent significantly less time hiding, took significantly less time to emerge from the hide box and travelled significantly more distance [hiding: $F(1,44) = 12.04$, $p = 0.001$; latency to emerge: $F(1,44) = 9.10$, $p = 0.004$; distance travelled: $F(1,44) = 14.71$, $p<0.001$]. Neutral responders did not differ significantly from passive responders in time spent hiding, latency to emerge, or distance travelled (statistics not shown).

3.1.4. Post exposure test 2: Weak sucrose consumption (anhedonia). Results of the weak sucrose consumption test for anhedonia are shown in Figure 3A. Active responders consumed significantly more 1% sucrose than passive responders, $F(1,44) = 5.65$, $p = 0.022$. There was also a trend towards active responders consuming significantly more 1% sucrose than control rats, $F(1,44) = 3.39$, $p = 0.072$.

3.1.5. Pre-exposure test and post exposure test 3: Emergence test. The emergence test was conducted before and after fur exposure to test for differences in anxiety-like behavior between active and passive responders, and to examine whether exposure to a chronic stressor (7 days of fur exposure) altered this behavior. The results are shown in Figure 3B – D.

In the pre-exposure and post-exposure emergence test, active responders spent significantly less time hiding than passive responders (pre exposure: $p = 0.007$; post exposure: $p = 0.007$) and travelled greater distance (pre exposure: $p = 0.012$; post-exposure: $p = 0.001$). Within-group analysis showed no significant difference between pre-exposure and post-exposure hide times or activity for active responders (hide time: $p = 0.778$; $p = 0.534$) or passive responders (hide time: $p = 0.841$; activity: $p = 0.086$), although there was a trend towards passive responders being significantly less active during the post exposure test.

Conversely, there was no significant difference between active and passive responders in time taken to emerge from the hide box during the pre-exposure test ($p = 0.379$) whereas active responders took significantly less time to emerge than passive responders during the post-exposure test ($p = 0.004$). This change was due to an increased latency to emerge in passive responders: this cohort alone took significantly longer to emerge during the post-exposure test compared to the pre-exposure test ($p = 0.018$), while there was no change from pre- to post-exposure emergence latencies for active responders ($p = 0.967$). Neutral responders did not differ from passive responders on any of the pre- or post-exposure measures (statistics not shown).

3.2. Experiment 2

3.2.1. Behavioral responses to cat odor that did not differ as a function of group exposure. Rats exposed to cat fur (either individually or in a quad) spent significantly less time in the half of the arena that contained the fur stimulus, showed less unsupported rearing and had significantly more escape attempts (jumps) compared to rats that were not exposed to cat odor [time in stimulus half: $F(1,28) = 214.45$, $p<0.001$; unsupported rearing: $F(1,28) = 12.26$, $p<0.01$; jumps: $F(1, 28) = 28.27$, $p<0.001$]. None of these variables interacted significantly with configuration (alone and quad) ($p>0.2$).

3.2.2. Effects of cat odor exposure on huddling and social behaviors. Rats exposed to cat odor in a quad spent significantly more time huddling compared to the rats in quads

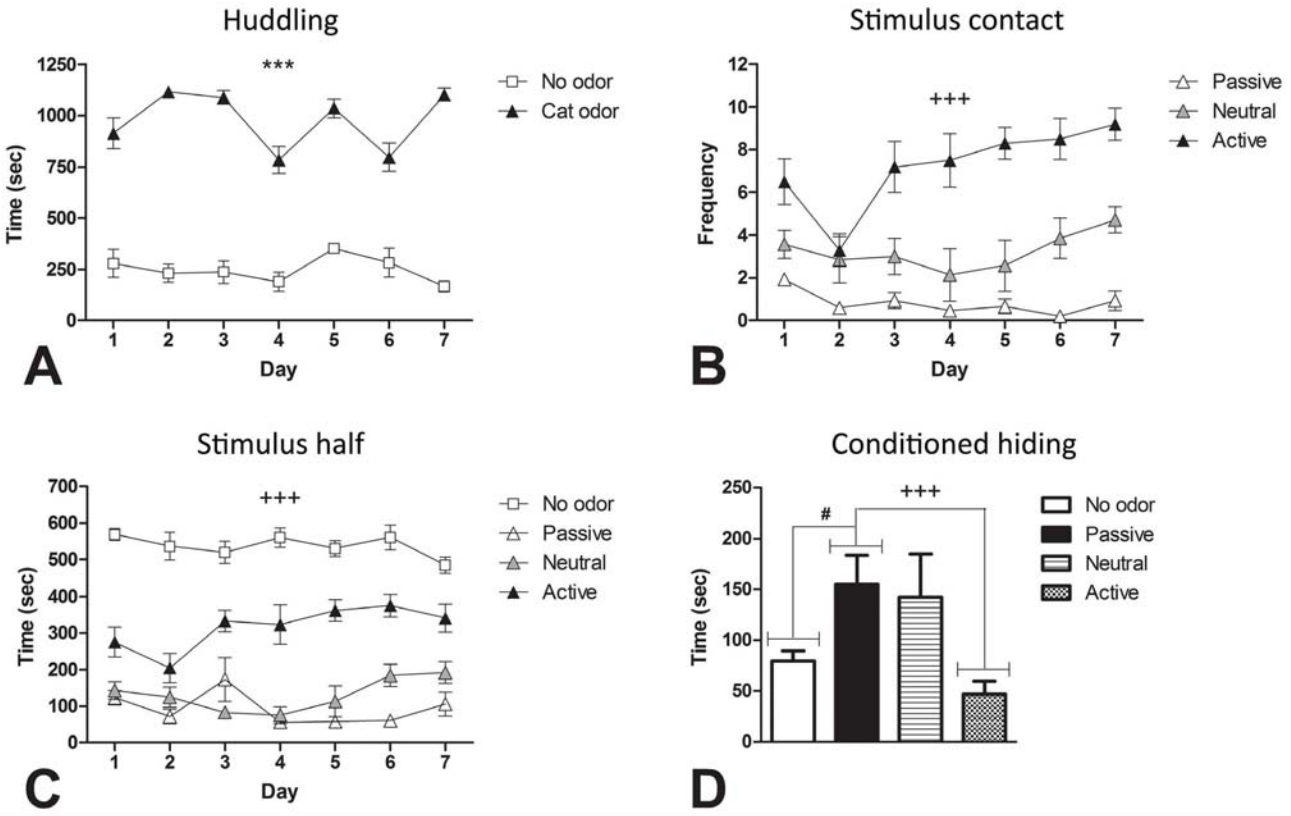

Figure 2. Differences between active and passive responders in the immediate and conditioned responses to cat odor. Averaged across the seven exposure days, cat odor exposed quads spent significantly more time huddling (A). Averaged across the seven exposure days, compared to passive responding rats, active responders had significantly more stimulus contacts (B) and spent significantly more time in the stimulus half of the arena (C). Active responders showed significantly less conditioned hiding when placed back in the arena 3 days after the final fur exposure with a hide box present and no fur present (D). Furthermore, relative to no odor controls, a significant conditioned hiding response was seen in passive responders, but no such response was seen in the active responders (D). *** $p<0.001$ for no odor vs cat odor; $^{+++}p<0.001$ for active vs passive responders; $^{\#}p<0.05$ for no odor vs passive responders.
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that were not exposed to cat odor (Fig 4A), $t(14) = 11.68$, $p<0.001$. File S2 is a representative video recording of two quads from Experiment 2 demonstrating the pronounced huddling response observed in groups of four rats exposed to cat odor. Exposure to cat odor completely abolished all allogrooming (Fig 4B) and play behavior (Fig 4C) in quads of rats [cat odor quads vs control quads: allogrooming, $t(14) = 3.92$, $p<0.001$; and playing, $t(14) = 3.48$, $p<0.01$].

3.2.3. Behavioral responses to cat odor modulated by social exposure. Rats exposed to cat fur spent significantly less time grooming and significantly more time immobile in the first 10 min of the session compared to rats that were not exposed to cat odor, [Grooming: $F(1,28) = 47.96$, $p<0.001$; Immobility: $F(1,28) = 99.00$, $p<0.001$]. However, the inhibition of grooming and increase in immobility in the presence of cat odor was significantly more pronounced for rats exposed alone than in a quad (Fig 5A and 5B), [Grooming: $F(1,28) = 4.67$, $p = 0.039$; Immobility: $F(1,28) = 16.28$, $p<0.001$]. Furthermore, rats exposed to cat odor in a quad had a significantly greater number of contacts with the cat odor stimulus compared to rats exposed to cat odor alone (Fig 5C), $t(14) = 2.23$, $p = 0.043$. File S3 is a representative video from Experiment 2 that illustrates the differential response of rats exposed to cat odor alone versus in a group.

3.2.2. c-Fos expression. c-Fos expression in each brain region of interest for the no odor conditions (alone and quad) and the cat odor conditions (alone and quad), are presented in Table 1. In all of the regions listed in Table 1 there was a significant difference between the odor exposure groups, on average, and the no odor exposure groups, on average.

Cat fur exposure increased c-Fos expression in a number of regions irrespective of exposure condition (individual or quad). These included the ventral part of the anterior olfactory nucleus (AOV), medial prefrontal cortex (MPC), infralimbic cortex (IL), posterior part of the anterior olfactory nucleus (AOP), LSV, Shell of the nucleus accumbens (AcbSH), medial division of the anterior part of the bed nucleus of the stria terminalis (BSTMA), medial preoptic area (MPA), supraoptic nucleus of the hypothalamus (SON), paraventricular nucleus of the hypothalamus (PVN), somatosensory barrel cortex (BC), central nucleus of the amygdala (CeAmg), basolateral amygdala (BLAmg), dorsomedial nucleus of the hypothalamus (DMH), Posteroventral part of the medial amygdala (MePV), posterodorsal part of the medial amygdala (MePD), ventromedial nucleus of the hypothalamus (VMH), PMD, ventrolateral PAG (VLPAG), Cuneiform nucleus (CnF), locus ceruleus (LC), anterior part of the hypothalamus (AH) and dorsolateral PAG (DLPAG).

Cat odor exposure caused an increase in the number of c-Fos positive cells in the mitral cell layer of the AOB (AOBmc), but this

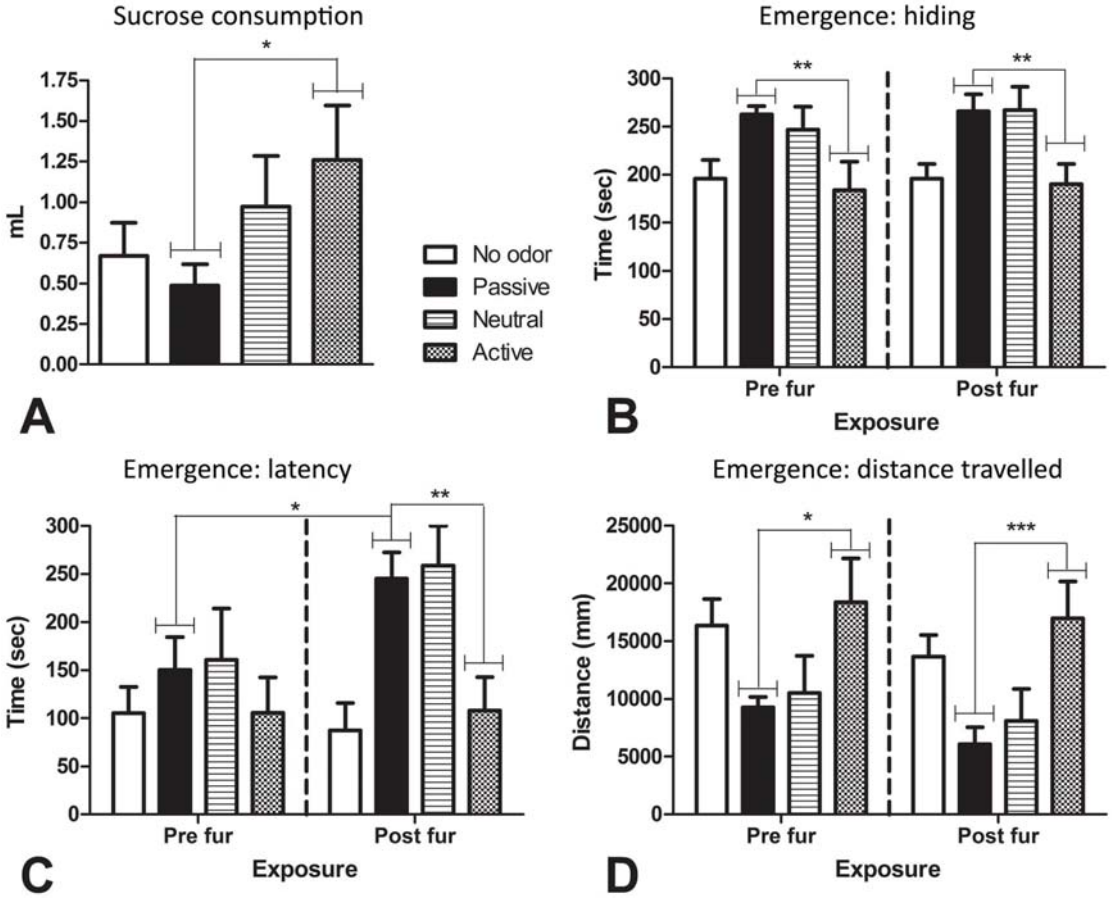

Figure 3. Tests of anhedonia-like and anxiety-like behavior. In the weak sucrose consumption test conducted after chronic fur exposure (A), active responders consumed significantly more of a 1% sucrose solution than passive responders. In the emergence tests, active responders spent significantly less time hiding in the pre- and post-exposure test (B). There was no significant difference in latency to emerge between active and passive responders in the pre-exposure test (C). However, there was a significant increase in latency to emerge from the pre- to post-exposure test for passive responders, but no change for active responders, with active responders having a significantly shorter latency to emerge during the post-exposure test (C). Active responders were significantly more active than passive responders during the pre- and post-exposure tests (D). * $p<0.05$; ** $p<0.01$; *** $p<0.001$.
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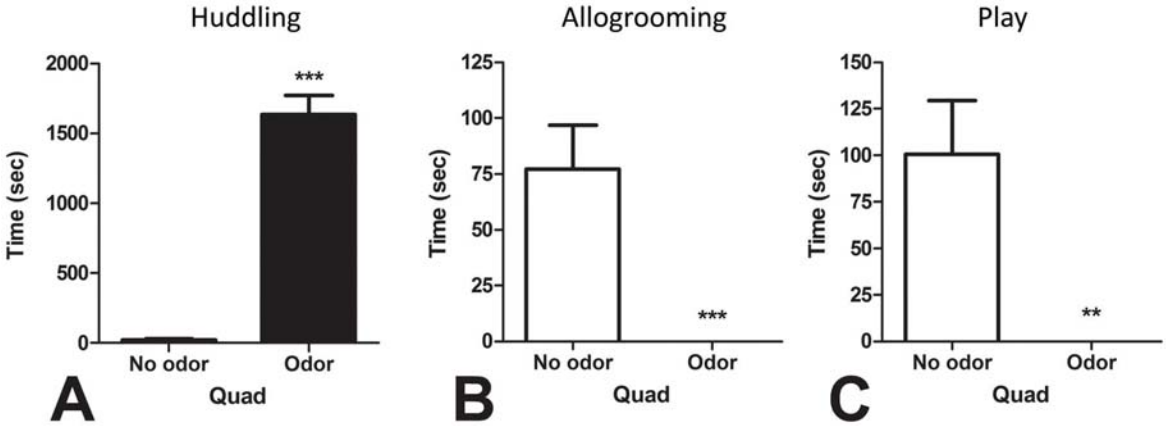

Figure 4. The effect of cat odor exposure on social behaviors during a 50 min exposure session. Compared to control quads, rats exposed to cat odor in quads spent more time huddling (A), and had allogrooming (B) and play behavior (C) completely abolished by the odor exposure. ** $p<0.01$; *** $p<0.001$.
doi:10.1371/journal.pone.0068483.g004

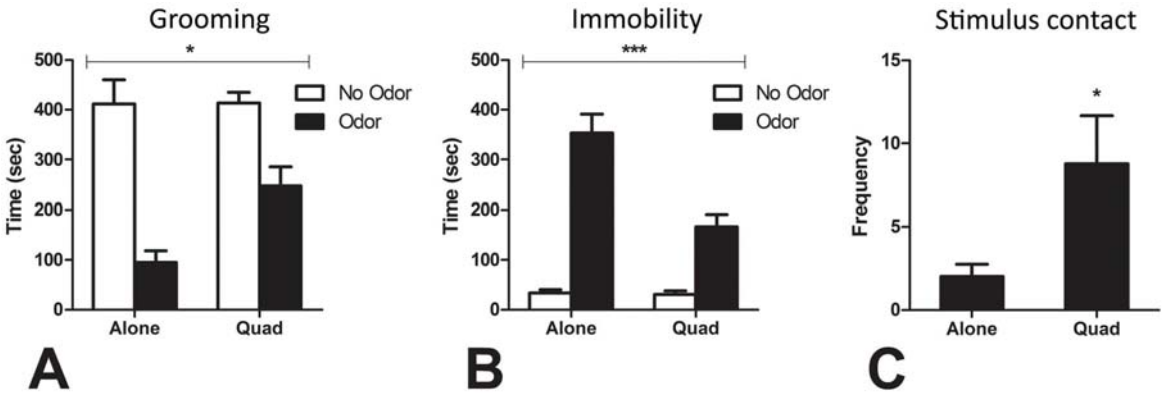

Figure 5. The effect of social exposure on the behavioral response to cat odor. Compared to rats exposed to cat odor when alone, rats exposed to cat odor in quads had less pronounced inhibition of grooming throughout the 50 min session (A), less pronounced induction of immobility in the first 10 min of the session (B), and made contact with the cat odor stimulus more times throughout the 50 min session. Note that the contacts (C) are only for the cat odor exposed rats as there was no stimulus present for the no odor rats to make contact with. Main effect of odor for grooming (A) and immobility (B) was significant [$p<0.001$], the asterisks refer to the interaction effects. * p for interaction effect <0.05 (A); p for odor alone vs. odor quad <0.05 (C); *** p for interaction effect <0.001 (B).
doi:10.1371/journal.pone.0068483.g005

effect was greater in rats exposed to cat fur in a group (see Fig 6). Conversely, the effect in the medial caudate putamen (CPuM), lateral preoptic nucleus (LPO), lateral amygdala (LAmg), dorsomedial PAG (DMPAG), and lateral habenula (LHb) was significantly lower in group-exposed rats (see Fig 6).

Finally, time rats exposed to cat odor in a quad spent in a huddle with three or four rats was significantly positively correlated with the number of c-Fos positive cells in the lateral part of the anterior olfactory nucleus (AOL) [$N=7$, $r=.770$, $p=0.043$], LSV [$N=8$, $r=0.713$, $p=0.047$] and CPuM, $N=7$ [$r=0.926$, $p<0.01$].

3.2.4. Differences between Active and Passive Responders. None of the rats exposed to cat odor alone met criteria for being an active responder, with one rat being classified as neutral and the rest classified as passive. Conversely, 4 rats from the quads exposed to cat odor met the criteria for being an active responder and the remaining 4 met the criteria for being a passive responder. The proportion of rats exposed to cat odor in a quad

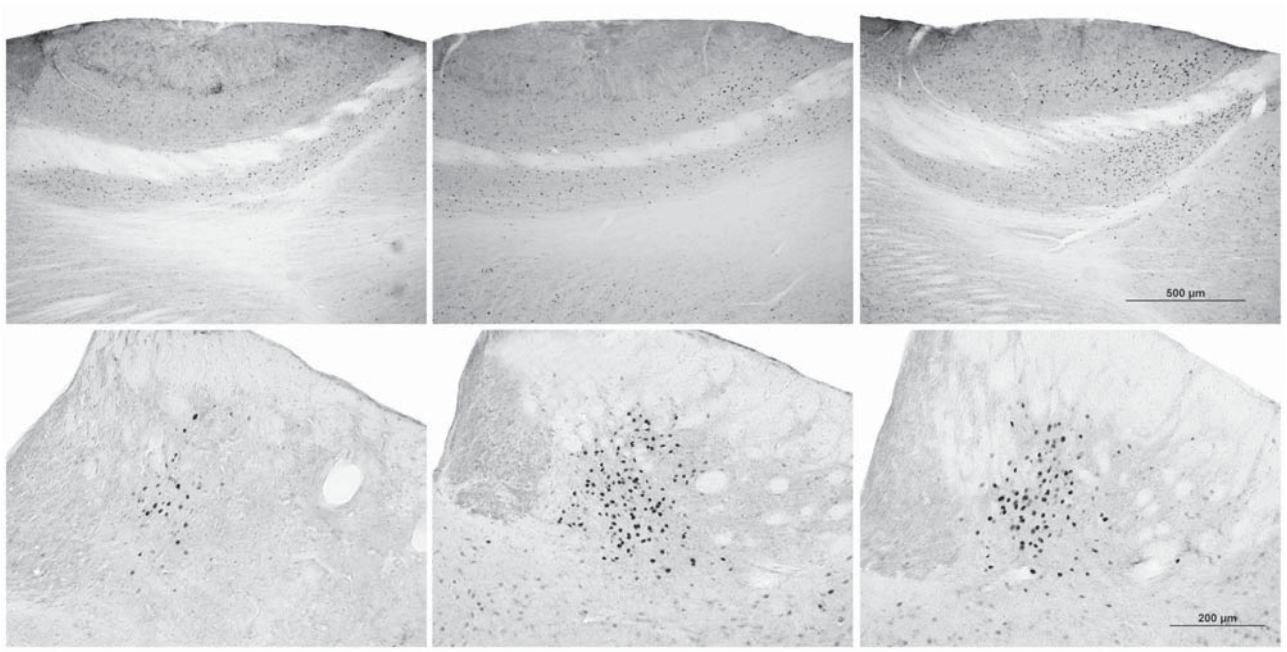

Figure 6. Representative images of brain slices from the mitral cell layer of the AOB (top) and lateral habenula (bottom) stained for c-Fos immunoreactivity. Mitral cell layer of the AOB: Rats not exposed cat odor (top left) had significantly less activation than rats exposed to cat odor (top middle and right). However, rats exposed to cat odor alone (top middle) had significantly less activation than rats exposed to cat odor in a group (top right). Lateral habenula: Rats not exposed to cat odor (bottom left) had significantly less activation than rats exposed to cat odor (bottom middle and right). However, rats exposed to cat odor alone (bottom middle) had significantly more activation than rats exposed to cat odor in a group (bottom right).
doi:10.1371/journal.pone.0068483.g006

that were classified as active (50%) was significantly greater than the proportion of rats exposed to cat odor alone that were classified as active (0%), $\chi^2 = 4.31$, $N = 31$, $p < 0.05$.

Compared to passive responders in quads, active responders (all from quads) spent significantly less time huddling and immobile, made contact with the cat odor stimulus significantly more times, and spent significantly more time in the half of the arena containing the cat odor stimulus (see Table 2 for the means for active and passive responders for these behaviors) [huddling: $t(6) = 3.40$, $p = 0.014$; immobility: $t(6) = 2.83$, $p = 0.03$; stimulus contacts: $t(6) = 5.13$, $p < 0.01$; time in stimulus half: $t(6) = 2.56$, $p = 0.043$]. There was a trend towards active responders spending more time grooming compared to passive responders, $t(6) = 2.23$, $p = 0.067$.

C-Fos expression in each brain region of interest for active responders and passive responders are reported in Table 2. Compared to passive responders, active responders had significantly more c-Fos positive cells in the mitral cell layer of the AOB [$t(5) = 2.71$, $p = 0.042$], and significantly fewer c-Fos positive cells in the AOL [$t(5) = 4.22$, $p < 0.01$], LSV (see Fig 7) [$t(6) = 4.21$, $p < 0.01$], nucleus accumbens shell [$t(6) = 3.31$, $p = 0.021$], and somatosensory barrel cortex [$t(6) = 3.35$, $p = 0.015$]. There was a strong trend towards significantly fewer c-Fos positive cells in the medial CPU of active responders compared to passive responders [$t(5) = 2.53$, $p = 0.052$].

Discussion

This study provides some novel insights into defensive aggregation in laboratory rats using our recently developed laboratory model. As we have recently reported [13,14], rats exposed to cat odor in a group showed a striking huddling response, and here we show that individual differences in this response and in stimulus approach are consistent over repeated exposures. The characteristic behavior of active and passive responders appears to reflect differing behavioral styles outside of situations involving acute predatory threat.

In Experiment 2, defensive aggregation was found to be associated with a social buffering effect whereby group exposure appears to promote more active styles of responding and greater performance of non-defensive behaviors such as grooming. This illustrates an important benefit of defensive aggregation that extends beyond the immediate survival advantages provided by a dilution effect. Finally, using c-Fos immunohistochemistry, we have identified brain regions associated with huddling, social buffering, and active responder styles.

4.1. The General Defensive Response to Cat Odor

In the present study, cat fur elicited a reliable anxiety-like response in rats exposed either alone or in a group. Fur-exposed rats avoided the half of the arena in which the fur stimulus was located, engaged in escape attempts, and spent more time immobile and less time grooming. These findings are consistent with previous demonstrations of cat odor effects in rats: including avoidance [44,45]; immobility [45]; escape attempts [19]; and reduced grooming [18,19,28,44].

Inconsistent effects of predator odor exposure on rearing have been observed, with some studies reporting increases [18,28], others decreases [44,46]; and others finding no effect [19] on rearing. Here we report a decrease in unsupported rearing in rats exposed to cat odor. Discrepancies may exist because lower levels of perceived threat may tend to increase rearing whereas higher levels of perceived threat inhibit rearing [47,48]. Most studies that report an increase in rearing in response to cat odor have used a hide box paradigm [18,28], whereas those that have reported a decrease in rearing have used either no hide box (the present study, [46]) or a hide wall that provides less concealment than a hide box [44], potentially elevating the level of perceived threat.

In addition to the changes in individual behaviors, rats exposed to cat odor in a group displayed an inhibition of social behaviors

Table 2. Behavioral and neural differences between active and passive responders.

Behavioral differences, Mean (± SEM)			
Behavior		**PASSIVE RESPONDERS**	**ACTIVE RESPONDERS**
Huddling (sec)		1930 (111)	1338 (133.7)*
Immobility (sec)		216.3 (28.43)	115.9 (21.21)*
Stimulus contacts (freq)		1.75 (0.48)	15.75 (2.69)**
Stimulus half (sec)		77.86 (34.69)	193.2 (28.68)*
Mean number (± SEM) of c-Fos-positive cells in brain regions of interest			
Region	**Bregma**	**PASSIVE RESPONDERS**	**ACTIVE RESPONDERS**
Sites where active responders had significantly higher c-Fos expression			
AOBmc	5.70	16 (2)	22 (1.22)*
Sites where active responders had significantly lower c-Fos expression			
AOL	5.70	9.33 (2.33)	.75 (.48)**
LSV	0.70	48.25 (3.79)	25 (4.02)**
AcbSH	0.70	10.67 (1.45)	4.75 (1.12)*
BC	−2.12	4 (.41)	1.75 (.48)*

*$p < 0.05$ versus passive responders; ** $p < 0.01$ versus passive responders.
AcbSH = Shell of the nucleus accumbens; AOBmc = mitral cell layer of the AOB; AOL = lateral part of the anterior olfactory nucleus; BC = somatosensory barrel cortex; LSV = ventrolateral septum This document contains a detailed description of the procedure used in Experiment 2 to extract, prepare, slice, stain and count the tissue.
doi:10.1371/journal.pone.0068483.t002

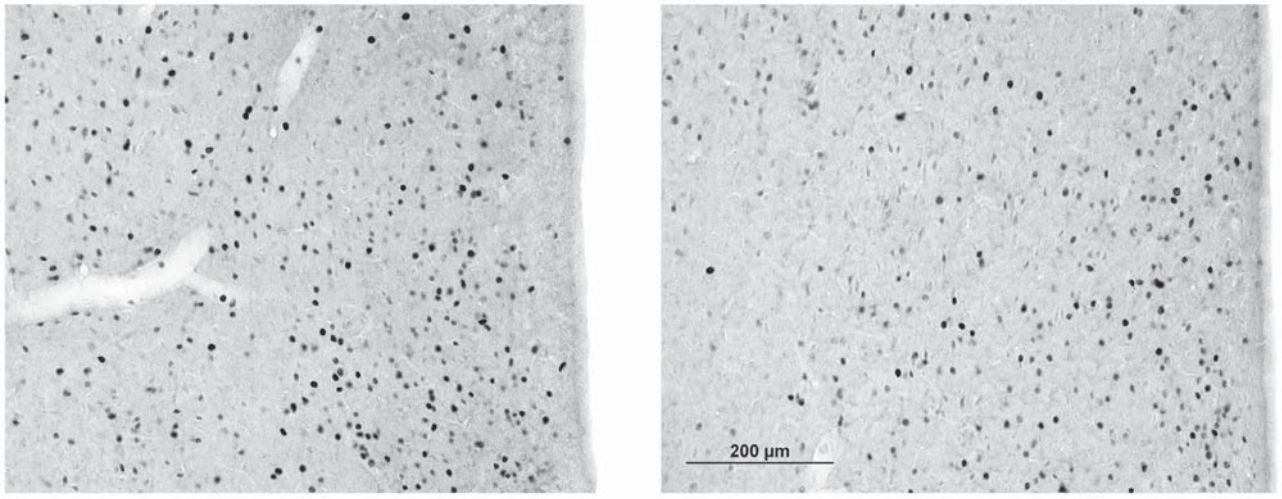

Figure 7. Representative images of brain slices from the lateral septum stained for c-Fos immunoreactivity. Passive responders (left) had nearly double the number of fos positive cells compared to active responders (right).
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such as allogrooming and play behavior. To the best of our knowledge, this is the first report of decreased allogrooming in rats in response to predator odor; and this agrees with Louvart and colleagues [49] who reported a long term decrease in allogrooming in female rats after a single intense footshock. This fits within a general pattern of inhibition of non-defensive behaviors, such as self-grooming and play, observed in response to the cat odor in the present study, as well as previous reports of decreased play behavior in juvenile rats in response to predator odor [50,51].

The overall distribution of cat odor-induced c-Fos immunoreactivity we observed here was consistent with previous studies [17–19] and highlights the role of accessory olfactory regions in the sensory processing of predator odor stimuli and the activation of characteristic downstream hypothalamic, limbic, midbrain and brainstem circuits. The LHb emerged in the current study as a site activated by cat odor, not previously reported in the literature, and very strongly activated in rats exposed to cat odor when alone (Table 1). Recent analyses suggest that the LHb may play a crucial role in suppressing locomotor activity when an aversive outcome is anticipated or when pain, stress or anxiety is experienced [52–54]. Thus the LHb may drive some of the avoidance behaviour, and inhibition of locomotor activity observed during predator threat in the present study, and others (e.g. [44,45]). This may be achieved through a gating influence on midbrain dopaminergic and serotoninergic systems as well as connections from the LHb to the DMPAG, which was also strongly activated in the present study, and again more strongly in rats exposed to cat fur when alone.

The PAG has an important role in controlling the behavioral and autonomic aspects of the defensive response [54] with lesions of the dorsal PAG interfering with the behavioral and cardiovascular response to cat odor [55] as well as being associated with both escape and freezing responses to aversive stimuli [56]. The greater activation of the DMPAG in rats exposed to cat odor when alone is consistent with their different profile of defensive behavior relative to rats exposed in groups, with greater passivity, less active approach and greater inhibition of non-defensive behaviors.

4.2. Neural Correlates of Defensive Aggregation

This study provides an examination of the possible neural basis of defensive aggregation and shows for the first time that the propensity to huddle and the magnitude of the huddling response is highly consistent across repeated cat odor exposures. Rats that huddled more, with a passive responder style, had greater c-Fos immunoreactivity in the AOL, LSV and CPuM. The lateral septum (LS) is an area of particular interest given that it plays a demonstrable role in defensive aggregation in other species. Goodson and colleagues [6,7] demonstrated that vasotocin V1a receptors and mesotocin in the LS potently promote flocking and flock size selection in Estrildids. It is also interesting that increasing vasopressin V1a receptor expression in the septum of Wistar rats, using viral vector mediated gene transfer, enhances their social discrimination and active social behavior [57].

The "chill of fear" [58,59] refers to the sensation of cold that comes over people when they are fearful [60] and may possibly be related to connections between the LPO and PAG. LPO neurons are activated by cold exposure and these neurons project to the PAG where they play a crucial role in eliciting non-shivering responses to cold [61,62]. In the present study, LPO neurons were activated by exposure to cat odor as were neurons in the PAG. It is therefore fascinating that both cold temperatures and exposure to predator odor (and, indeed, other unconditioned stressors such as bright light [13]) induce huddling, suggesting there may be some crossover in the neural substrates governing thermoregulatory huddling and defensive huddling. Notably, the LPO and PAG were more activated in rats exposed to cat odor when alone rather than in groups (Table 1).

4.3. Acute social Buffering

Rats exposed to cat odor in a group showed increased grooming, locomotor activity, and stimulus contacts than those exposed alone. This suggests an acute social buffering effect consistent with previous research showing the presence of conspecifics during or after a stressful event ameliorates stressor effects [32,42,63,64]. For example, rats tested for contextual fear conditioning to cat odor with a non-fearful unfamiliar partner hid less, engaged in more risk assessment and had greater overall activity [65]. Kiyokawa et al. [42] reported that the presence of a conspecific reduced conditioned freezing and stress induced hypothermia to footshock, and reduced the number of c-Fos positive cells in the PVN.

Our present findings suggest a social buffering effect in groups of familiar rats that are exposed to predatory threat together. Familiarity may be a very important factor as threat induced aggregation occurs primarily between familiar conspecifics in virtually all species that huddle [1]. The social buffering effect might be directly related to a group vigilance effect [66,67]. As a group of animals enjoys improved predator detection, individually, the animals in the group are able to devote less time to vigilance and more time to important non-threat related activities such as foraging and self-maintenance. The higher level of grooming present in socially exposed rats speaks to such an effect. This group vigilance phenomenon may operate through social buffering lessening stress, and thus freeing the animal up from vigilance-related tasks so it can continue on with other non-defence related tasks important for survival.

Several brain regions exhibited differences in c-Fos activation for rats exposed to the predator stimulus alone versus socially. Increased c-Fos expression in the AOB of socially exposed rats most likely reflects the sensory impact of the greater stimulus approach seen in socially exposed rats [18,19]. It may also conceivably reflect exposure to alarm pheromones released by the other rats present in the social group [68].

On the other hand, social exposure to threat was associated with lesser activation in the LPO, CPuM, LHb and DMPAG. All of these regions play an important and interconnected role in threat response [52–54]. As noted above, the LHb is an important region that connects limbic regions such as the LPO, and parts of the basal ganglia, such as the CPuM, to the DMPAG to influence the motoric aspects of the defensive response such as immobility, cessation of non-essential activities (e.g. grooming), and avoidance of aversive stimuli [52–54]. Given the present finding of reduced activation in all of these regions in socially exposed rats and their more active defensive responses, future studies might explore the specific role each region has via lesion and microinfusion studies.

4.4. Different Responder Types

This study confirmed our prediction from informal observations that not all members of a group respond to cat fur in the same way. Specifically, we identified a clear distinction between active and passive responding. Active rats engaged in more stimulus contacts, spent more time in the stimulus half of the arena and less time immobile and huddling compared to their passive conspecifics. Importantly, the active or passive phenotype of a rat remained highly consistent across repeated exposures to cat fur. It is worth noting that on day 2 of the fur exposure in Experiment 1 there was a striking drop in the number of stimulus contacts by active responders, who then returned to the day 1 levels of contact for days 3–7. Rats exposed to cat odor individually display very similar behavior on the first and second exposure to cat odor, however, the pattern of neural activation is found to differ on second exposure, and benzodiazepines only diminish fear when given on first exposure [69,70]. In a recent study from our group [14] there was also a decline in contacts on day 2 of group cat fur exposure, suggesting that this is a systematic effect that perhaps reflects increasing familiarity with the inescapable nature of the threat situation.

Beyond the immediate response to cat fur, active responders displayed lower anxiety and greater resilience in a number of behavioral tests. They displayed no conditioned fear to the arena in which they had been repeatedly exposed to the cat fur, whereas a pronounced conditioned fear was observed in the passive responders. Active responders also had lower levels of anxiety-like behavior in the emergence test both before and after fur exposure. Interestingly, a lasting increase in anxiety-like behavior was observed in passive responders on the emergence test following the chronic fur exposure, whereas no such increase was observed in active responders. This suggests greater resistance to the lasting anxiogenic effects of chronic stress in the active responders. Finally, active responders showed greater consumption than passive responders of a weak 1% sucrose solution post cat-odor, suggesting an absence of anhedonia in the active responders [29]. However, it is worth noting that the passive responders did not show a significant reduction in sucrose consumption relative to unstressed controls, so it remains possible that the differences in anhedonia between active and passive responders may be present irrespective of predatory stress. The behavioral correlates of active and passive responders reported here are in general agreement with other studies of active and passive coping styles in rats (for a review see [23]) and are also consistent with studies in humans which indicate passive coping strategies are associated with generalised anxiety disorder (GAD) and depression [71,72].

When c-Fos expression was compared in active and passive responders exposed to cat odor, the active responders showed greater sensory activation in the AOB, again most likely reflecting greater approach to the cat odor source. However, they showed lesser activation in the AOL, LSV, AcbSH, BC, and CPuM. The decreased activation in the LSV of passive responders was the most pronounced neural difference between active and passive responders in the present study and is of particular interest. Previous studies involving, for example, exposure of mice to an innately aversive ultrasonic stimulus have found a similar difference between active and passive responders in the LSV [24,73], with greater activation in the LSV in response to threat correlating with a more reactive (or passive) threat response [73].

The basal ganglia in general, and nucleus accumbens in particular, also play a role in determining whether an individual favours an active or passive coping strategy, possibly through changes in dopamine neurotransmission in this region [74]. For instance, active responding in *A. carolinensis* lizards is associated with increased dopamine levels, with increased activity of D1 receptors in the striatum as a result of the elevated dopamine thought to activate the basal ganglia to facilitate proactive responding [74]. A specific 'defensive' region has been identified in the nucleus accumbens with, for example, microinjections of the $GABA_A$ agonist muscimol or the AMPA antagonist DNQX into caudal parts of the medial AcbSH producing fear accompanied by motivated defensive responses such as escape [75].

A key observation in the present study was that experiencing threat in a group appears to influence the distribution of coping strategies, with group exposure shifting the overall response type from primarily passive to a more equal division between passive and active responding, more closely resembling the distinct phenotypes that are observed in the wild [23]. Active responding is in some ways then a socially-mediated phenomenon as Zahavi [76,77] suggests in his studies of the sentinel behavior of Arabian babblers. Sentinel behavior involves a babbler going to the highest part of the canopy, where they are most exposed to predators, to keep watch and alert other members of the species of a potential predator attack. Zahavi [76,77] argues that this type of behavior has a social function, acting to elevate and reinforce social status, leading to greater access to food and reproduction. Obviously, these social benefits are not present when animals are alone, and thus the rewards of active responding are minimized and the risks maximized.

It is interesting, therefore, to consider whether these different responder types reflect a consistent inherited trait or whether there is also phenotypic plasticity. On the one hand, the active responder type only emerged during cat fur exposure in a social

context, suggesting some plasticity. On the other hand, the generalization of the active responder type to other testing paradigms in which the rats were individually exposed, such as the conditioned fear test, the emergence test, and the weak sucrose consumption test, indicates trait consistency. It is therefore possible that in certain circumstances where threat is high (such as in the large open arenas in which cat fur exposure took place) the presence of conspecifics is required to allow expression of the active phenotype, possibly through social buffering and the aforementioned social motivators. Conversely, in assays that are less anxiogenic (such as the emergence test) the active phenotype is expressed in the absence of conspecifics.

4.5. Translational Implications

Exposure to predatory threat in laboratory animals has been seen by some authors as a possible approach for producing analogous symptoms to those of PTSD seen in humans [78,79]. The strong conditioned fear seen in environments associated with predatory threat may be of particular interest in these models. It is therefore fascinating that active responders, in addition to their reduced anxiety and anhedonia relative to passive responders, showed an apparent lack of a conditioned fear in the testing arena where cat odor was experienced, whereas passive responders were highly anxious in this context. This suggests there is the potential for modeling susceptibility to PTSD by closely examining the active and passive responders.

The direct response to cat odor may more closely resemble symptoms of GAD and panic disorder as opposed to PTSD [80,81]. Risk assessment behaviours (such as stimulus approach) in predator odor paradigms are thought to reflect aspects of generalized anxiety, while flight responses (such as escape attempts and fleeing followed by avoidance) suggest a response profile more similar to panic disorder [80]. The increased risk assessment and decreased freezing and stimulus avoidance in the socially exposed animals may reflect the profound benefit of social support in treating these disorders. Furthermore, the greater risk assessment and lesser freezing and stimulus avoidance in active responders than passive responders illustrates the utility of studying these distinct phenotypes to learn more about the behavioral, physiological, epigenetic and genetic factors that cause resilience or vulnerability to GAD and panic disorder.

Conclusions

The present study suggests that defensive aggregation not only offers an immediate advantage of dilution of predatory threat, but has a buffering effect where the behavioral response to the threat is more active, and the neural response subtly altered. We confirmed our hypothesis that different, stable, coping styles exist within a cohort of rats exposed to predator threat: active responders and passive responders. Interestingly, the active phenotype emerged only when rats were exposed in a group, demonstrating the importance of social context in determining the expression of this phenotype in certain circumstances. An active style was associated with lower levels of anxiety-like and depression-like behavior and a less pronounced impact of chronic stress on behavior, as well as reduced activation in key brain regions such as the LSV while under predatory threat.

Supporting Information

File S1 Detailed immunohistochemistry methods. This document contains a detailed description of the procedure used in Experiment 2 extract, prepare, slice, stain and count the tissue.
(DOCX)

File S2 Video of the huddling response to cat fur. This video is of the final 20 min of a 50 min session in Experiment 2 for a quad that was not exposed to cat odor (left) and a fur exposed quad (right). In the video on the right, you can see the cat fur sample placed flush against the center of the left hand wall. There is a pronounced huddling response in the fur exposed rats which is completely absent in the control rats. Note: this video has been sped up to 8x normal speed.
(MP4)

File S3 Video of the acute social buffering effect. This video is of the first minute of a 50 min exposure session in Experiment 2 for a rat individually exposed to cat fur (left) and four rats exposed to cat fur in a group (right). In both videos the cat fur sample can be seen placed flush against the center of the left hand wall. This video clearly illustrates the increased stimulus contacts, less pronounced fur induced immobility, and greater time spent in the stimulus half of the arena in the group exposed rats – indicative of an acute social buffering effect.
(MP4)

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Author Contributions

Conceived and designed the experiments: MTB RCK GEH ISM. Performed the experiments: MTB RCK MM LGS GEH. Analyzed the data: MTB. Contributed reagents/materials/analysis tools: LGS GEH ISM. Wrote the paper: MTB ISM.

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Chapter 4: Oxytocin and vasopressin modulate the social response to threat: a preclinical study

NOTE: The supplementary methods referred to in the publication presented in this chapter can be found in Appendix 5

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ARTICLE

Oxytocin and vasopressin modulate the social response to threat: a preclinical study

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Abstract

Individuals in many species increase their proximity to others in threatening situations (defensive aggregation), increasing their chance of survival and reducing the adverse psychological impact of stressors. However, the basic neurobiology of defensive aggregation is not well understood. Here we examined the role of the social neuropeptides oxytocin (OT) and vasopressin (AVP) in this response. Groups of rats were exposed to a ball of cat fur (an innate threat stimulus) in a large arena, causing prolonged periods of tight social grouping (huddling). The modulatory effects of OT and AVP on huddling were examined both alone and in conjunction with relevant antagonists. To determine specificity of treatment effects to social grouping, the effects of the same treatments were also assessed in individual rats exposed to cat fur and given the opportunity to hide. OT (0.5 mg/kg, i.p.) and AVP (0.01 mg/kg, i.p.) increased huddling in rats socially exposed to cat fur, whereas the selective V_{1A} AVP receptor antagonist SR49059 (3 mg/kg, i.p.) decreased huddling. The effects of OT were prevented by pre-treatment with SR49059 (3 mg/kg), while those of AVP were prevented by the V_{1B} receptor antagonist SSR149415 (30 mg/kg, i.p.). OT had no effect on huddling when groups of four rats were tested with no cat fur present whereas AVP increased huddling under these conditions. Neither OT, nor SR49059, affected hiding in individual rats exposed to cat fur. However, AVP increased hiding, an effect prevented by SSR149415 (30 mg/kg, i.p.). These results suggest that OT acts on V_{1A} receptors to promote a social response to threat without altering the more general defensive response. Conversely, AVP appears to increase generalised anxiety via V_{1B} receptors, which subsequently results in huddling. A hitherto unrecognised function of oxytocin is therefore to promote social affiliation during threatening situations.

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Introduction

One of the most important and well-conserved forms of social interaction in animals is *defensive aggregation*: the tight clustering of conspecifics seen during threat (Hamilton, 1971). Examples include flocking in birds, schooling in fish and huddling in mammals. Defensive aggregation is also observed in humans, typified by the *need for affiliation* in stressful or fearful situations (Miller, 1966; Gump and Kulik, 1997).

Defensive aggregation accrues survival advantages for the individual. An individual animal's probability of predation diminishes with increasing group size, a phenomenon known as the *dilution effect* (Hamilton, 1971; Foster and Treherne, 1981). Grouping with conspecifics can also diminish the lasting adverse psychological impact of stressors through *social buffering* (Kikusui et al., 2006; Siviy, 2008, 2010). Psychiatric disorders are often characterised by social withdrawal in the face of threat rather than adaptive social responding (Beels, 1981), and this can deny sufferers the important benefits of social buffering (Norman et al., 2005; Meyer-Lindenberg and Tost, 2012). As current medications do little to treat the social deficits present in such disorders (Sergi et al., 2007), greater understanding of the neurobiology of adaptive social responding may be potentially useful in developing novel treatments.

We have recently developed a laboratory model of the social response to threat with which to probe its neural and pharmacological substrates. In this model, groups of laboratory rats are placed in a large arena and exposed to a ball of cat fur. Cat fur and skin odours are innately anxiogenic to rodents, and produce hiding, risk assessment and inhibition of foraging, feeding and other non-defensive behaviours (for a review see Apfelbach et al., 2005). Our recent results show that cat fur and other unconditioned stressors (such as bright light) also induce defensive aggregation in rats, whereby they huddle together tightly for long periods (Kendig et al., 2011; Bowen et al., 2012, 2013).

A burgeoning literature focuses on the role of the oxytocin (OT) and vasopressin (AVP) systems in appetitive social situations (Neumann, 2008), such as pair-bonding

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(Young and Wang, 2004), maternal care (Blanchard et al., 2005; Slattery and Neumann, 2008; Bosch and Neumann, 2012), social interaction (Witt et al., 1992; Bowen et al., 2011; Ramos et al., 2013), social preference (Lukas et al., 2011) and social proximity in rat pups (Odya et al., 2002; Alberts, 2007; Kojima and Alberts, 2011). Other studies demonstrate an important role for AVP and vasotocin (the non-mammalian analogue of AVP) in sociability. Importantly, the distribution of vasotocin binding sites in the lateral septum predicts gregariousness of estrildid species (Goodson et al., 2009) while the distribution of V_{1A} receptors (V_{1A}Rs) in this same region influences the gregariousness of mammals (Young et al., 1999).

Evidence that neuropeptides modulate defensive aggregation in non-mammalian species comes from several important studies that explored the role of neuropeptides in flocking and shoaling behaviour in birds and fish, respectively. Whilst these studies examined these behaviours under non-threating conditions, predator defence is a primary factor driving flocking and shoaling and thus these behaviours are viewed as forms of defensive aggregation (Caraco et al., 1980; Seppälä et al., 2008). Specifically, mesotocin (a non-mammalian analogue of OT) promotes flocking behaviour in estrildids (Goodson et al., 2009), while blockade of vasotocin V_{1A}-like receptors in the lateral septum reduces flocking behaviour in zebra finches (Kelly et al., 2011). In zebrafish, peripheral administration of isotocin (the teleost analogue of OT), OT, vasotocin or AVP increases shoaling (Braida et al., 2012). It is clearly of interest to determine whether OT and AVP also drive defensive aggregation in mammalian species.

The pharmacological exploration of neuropeptide effects on behaviour is complicated by the significant crosstalk between OT and AVP (Thibonnier et al., 1994; Chini and Manning, 2007). Specifically, OT has a relatively high affinity for V_{1A}R and low affinity for V_{1B}R (Chini and Manning, 2007; Manning et al., 2012). Conversely, AVP has high binding affinity for both V_1Rs, with affinity being the highest for V_{1B}R (Chini and Manning, 2007). Furthermore, OT and V_{1A} receptors tend to be distributed in separate regions and discrete loci when expressed within the same region (Tribollet et al., 1988; Johnson et al., 1993), suggesting these peptides may act at different sites to cooperatively regulate complex behaviours (Sala et al., 2011). Indeed, some of the social effects of OT, especially those related to social interaction, may, in fact, be mediated by V_{1A}R rather than the oxytocin receptor (OTR). Thus, exogenous OT ameliorates impaired sociability in OTR knockout mice and this effect is blocked by the selective V_{1A}R antagonist SR49059 (Sala et al., 2011). Moreover, the acute pro-social effects of peripheral OT and AVP in Long-Evans rats tested in the social interaction paradigm can be prevented by pre-treatment with SR49059 but not an OTR antagonist (Ramos et al., 2013).

In the present study we examined the receptor-specific influences of OT and AVP on predator odour induced defensive aggregation in rats. In a series of experiments we studied the effects of SR49059 alone (Experiment 1); OT alone (Experiment 2); OT and SR49059 combined (Experiment 3); and, AVP alone or in combination with SR49059 or the selective V_{1B} receptor antagonist SSR149415 (Experiment 4). We also determined whether OT or AVP had any effect on huddling in the absence of the predatory threat stimulus (Experiment 5). Furthermore, as OT and AVP can have more general effects on anxiety-related behaviours in rodents (see Neumann and Landgraf, 2012 for a review) it was important to determine whether any effects of these nonapeptides on defensive aggregation might simply reflect increased or decreased generalised anxiety in individual rats. We therefore examined the effects of the same pharmacological manipulations on the defensive responses of individual rats exposed to cat odour when given the opportunity to hide (Experiments 6 and 7). This utilised our cat odour avoidance paradigm used previously in many published studies (Dielenberg and McGregor, 2001; Dielenberg et al., 2001, 2004; May et al., 2012).

Method

All experimental procedures were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition, 2004) and were approved by the University of Sydney Animal Ethics Committee (approval number L29/7-2010/3/5360). Further information on the subjects, their housing, habituation, as well as details on how the drug solutions were prepared can be found in the supplementary methods section (S1) published online with the electronic version of this manuscript.

Cat odour stimulus and testing arenas

Testing for all experiments took place during the dark cycle. The threat stimuli used were 2 g balls of cat fur (for more information see S1). Testing for Experiments 1–5 was conducted in 2 identical 1200 mm × 1200 mm × 900 mm (l × w × h) wooden framed arenas painted matte black, located adjacent to each other (Kendig et al., 2011; Bowen et al., 2012, 2013). The cat fur was placed flush again the centre of one of the walls either on a plastic platform (75 mm^2, Experiment 1) or underneath a wire mesh cylinder (80 mm diameter, 95 mm high, 2.5 mm^2 aperture). The cylinder was used for Experiments 2–4 as it eliminated unwanted moving of the fur by the rats that occurred occasionally with the platform. The platform (Experiment 1) or cylinder (Experiments 2–5) was also present in the arena during all sessions in which no fur was present in the arena.

Testing for Experiments 6 and 7 occurred in the cat odour avoidance apparatus: this incorporates a chamber measuring 60 × 25 × 35 cm with a red Perspex hide box

(23×14×22 cm) placed at one end (May et al., 2012). Cat fur was placed at the opposite end of the chamber to the hide box inside an open-top plastic container (55 mm high, 45 mm diameter) fastened to a clip on the wall. The cylinder was also present in the arena during habituation (no fur) sessions.

Behaviours of interest

Variables of interest in the social experiments (Experiments 1–5) were (1) huddling: the total time that three or four rats were clumped together in a single tight group with each rat touching at least one other rat; (2) contacts: the number of times an individual member of a quad came close to the platform on which the fur was placed (maximum distance of 3 cm); (3) the number of faecal boli left in the arena; and (4) the average distance travelled by a rat in a session.

Videos of sessions for Experiment 1–3 and 5 were scored for these measures by an observer blind to experimental conditions using the ODLog program (Macropod Software, www.macropod.com). In between conducting Experiments 1–3 and Experiment 4, we developed custom 'Trackmate Social: Kinect' tracking software that uses the Microsoft Xbox 'Kinect' camera to track the movement and proximity of up to four rats in real-time (for more information see S1). A random sample of five videos was hand-scored for huddling by a blind observer and the correlation with the automatic scoring by 'Trackmate Social: Kinect' was 0.952 ($p<0.001$). As such, the automated scoring was used for Experiment 4 to quantify huddling and the average distance travelled by a rat in a session.

The dependent variables of interest in the individual exposure experiments (Experiments 6 and 7) were; time spent hiding, time spent approaching the stimulus, number of contacts with the stimulus and distance travelled. Data were automatically scored by tracking software Trackmate Quad Version 5.5 (MotMen Ltd, Australia).

Experiment 1: the effects of SR49059 on defensive aggregation

The design of Experiment 1 can be seen in Fig. 1. Thirty-two adult male Albino Wistar rats (8 quads) were used in a 2×(2) study to test the effect of the $V_{1A}R$ antagonist SR49059 at 2 doses (1 and 3 mg/kg, i.p.) on defensive aggregation. Doses were as shown to be effective in previous studies involving various behavioural models (Serradeil-Le Gal et al., 1993; Tsukada et al., 2005; Manaenko et al., 2011; Ramos et al., 2013).

Experiments 2–5: the effects of OT and AVP on defensive aggregation: involvement of the $V_{1A}R$ and $V_{1B}R$

These studies utilised a within-subjects design to minimise the effect of inter-group variability in defensive aggregation and to reduce the total number of rats required (four rats are required for each data point). All studies used adult male Albino Wistar rats.

The experimental designs for Experiments 2 ($n=24$), 3 ($n=24$) and 4 ($n=64$) are presented in Fig. 1. The doses of drugs used were: 0.5 mg/kg OT; 3 mg/kg SR49059; 0.01 mg/kg AVP and 30 mg/kg SSR149415. The doses of OT and AVP were chosen on the basis of their efficacy at increasing social interaction in a recent study from our laboratory (Ramos et al., 2013). The dose of SR49059 (3 mg/kg) that was most effective in Experiment 1 was chosen, while a 30 mg/kg dose of SSR149415 was chosen given the efficacy of this dose in influencing social and anxiety-like behaviours in rodent models (Griebel et al., 2002). Relevant VEH injections were used for each experiment.

In Experiments 2 and 5, the OT, AVP or VEH were administered 5 min before rats were placed into the testing arena for a 60 min session. In Experiments 3 and 4, the first injection (VEH, SR49059 or SSR149415) was administered 5 min prior to the second injection (VEH, OT or AVP). Five minutes later the rats were placed in the test arena for 60 min. In Experiments 1–4, drug treatments were administered prior to a fur exposure session. In Experiment 5, treatments were administered to experimentally naïve rats prior to a session with no fur exposure to allow assessment of their baseline effects on huddling under non-threatening conditions.

Experiment 6: the effect of OT and SR49059 on the defensive response of individual rats

In Experiment 6 (Fig. 1), 18 adult male Albino Wistar rats were randomly assigned to one of three conditions: VEH ($n=6$); SR ($n=6$); or OT ($n=6$). The rats were injected with vehicle and initially placed in the cat odour avoidance testing apparatus for 20 min with no fur present (referred to as 'baseline' henceforth). On the following test day, rats received (according to group allocation) OT (0.5 mg/kg, i.p.), SR49059 (3 mg/kg), or vehicle. Half of the VEH group rats received saline while the other half received the vehicle for SR49059 (these groups were combined as there was no difference in their behaviour across any measures). Five minutes later the rats were placed in the hide box apparatus with fur present.

Experiment 7: the effect of AVP and SSR149415 on the defensive response of individual rats

Experiment seven (Fig. 1) involved twelve adult male Albino Wistar rats randomly assigned to one of three conditions: VEH+VEH; AVP+VEH; and SSR149415+AVP ($n=4$ per condition). These rats were tested in a baseline 20 min session followed by a 20 min fur exposure session as described for Experiment 6. On the test day, rats received (according to group allocation) either vehicle+AVP (0.01 mg/kg), SSR+AVP (30+0.01 mg/kg) or vehicle +vehicle.

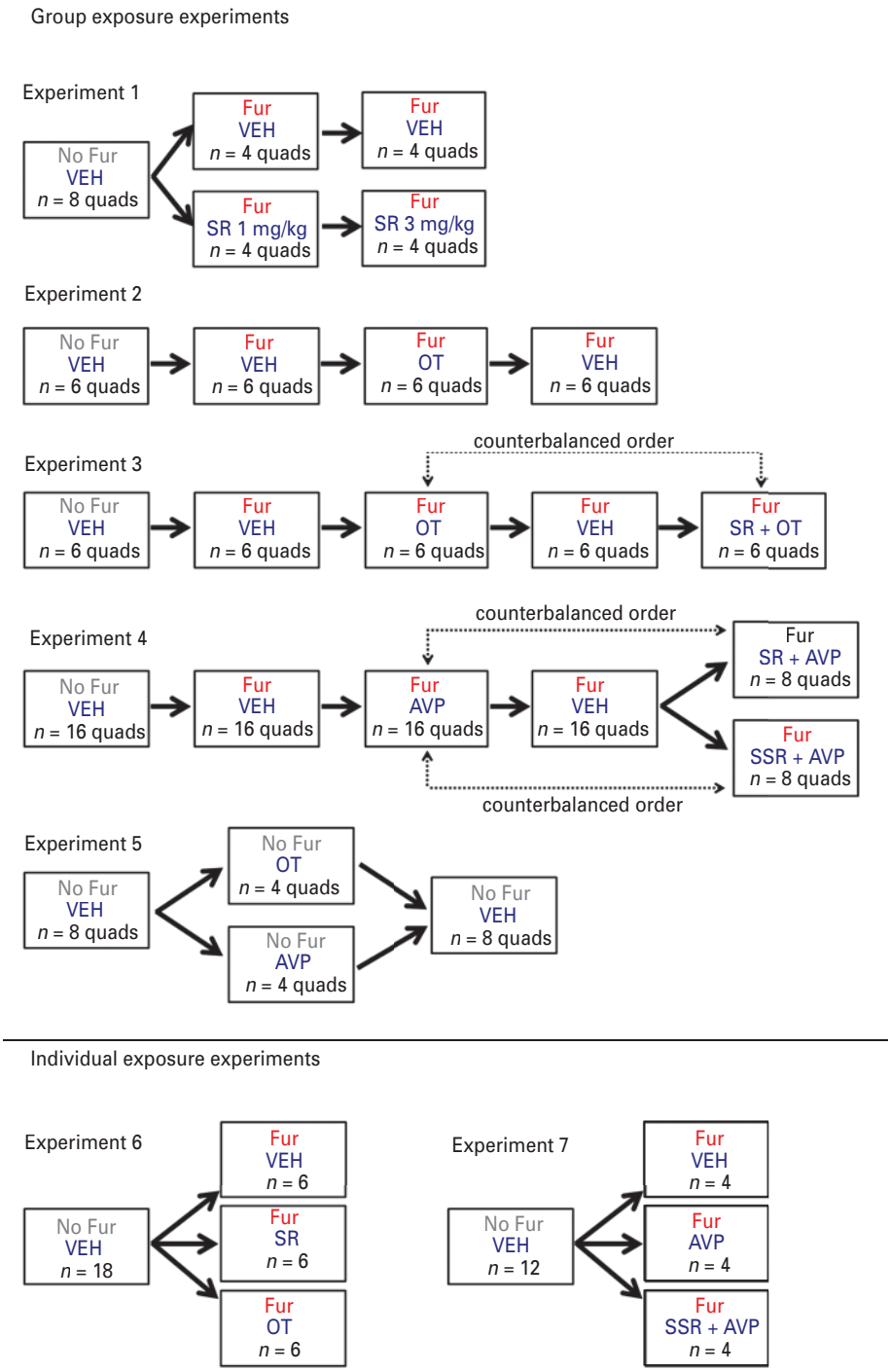

Fig. 1. Design of experiments. Experiment 1: On the first day of the experiment, rats were given a vehicle injection and then 5 min later placed in the test arena in the absence of cat fur for 30 min. On the next day half of the rats (n=4 quads) were injected with SR49059 (1 mg/kg, i.p.) 5 min prior to the 30 min fur exposure session, while the other half received an equivalent vehicle injection. After a one-week washout this procedure was repeated with the rats in the SR condition receiving a higher 3 mg/kg dose of SR49059 prior to the cat fur exposure session. **Experiment 2:** Using an ABA design to control for the effects of repeated fur exposure, 24 rats (n=6 quads) were given a VEH injection then 5 min later were placed into the testing arena in groups of four cage-mates for a 60 min session with no fur present. The next day proceeded as for day 1; however, a 2 g ball of cat fur was present in the arena. On the following day the rats were given OT (0.5 mg/kg, i.p.) prior to their fur exposure session. The following day they were given VEH prior to their exposure session to ensure responding returned to baseline levels. **Experiment 3:** Using a counterbalanced ABAC design, 24 rats (n=6 quads) were tested as for Experiment 2, with the addition of an exposure session in which rats were given an injection of SR49059 (3 mg/kg, i.p.) 5 min prior to their OT injection. **Experiment 4:** Using a counterbalanced ABAC design, 64 rats (n=16 quads) were tested as for Experiment 3 with the exception being that AVP (0.01 mg/kg, i.p.) was used instead of OT and half of the rats were given SR49059 (3 mg/kg, i.p.) 5 min prior to AVP during one of

Data analysis

Data for Experiment 1 were analysed using mixed model ANOVA and planned contrasts to examine simple main effects. Data for Experiments 2–5 were analysed using repeated measures ANOVA and planned contrast analysis. Data for Experiments 6 and 7 were analysed using mixed model ANOVA and follow up contrast analysis. Fisher's LSD procedure was used for contrasts; as such comparisons were only computed when the overall ANOVA was significant. For the sake of brevity, only the results of the focused contrast analyses are reported.

Results

In all experiments examining defensive aggregation in response to predator odour (Experiments 1–4), cat fur induced a pronounced increase in huddling and decrease in stimulus contacts, as reported in previous studies (Kendig et al., 2011; Bowen et al., 2012, 2013). In Experiments 2–4 we also examined the number of faecal boli deposited, which was significantly increased by cat fur, as previously reported (Bowen et al., 2013). Additionally, in Experiment 4 we examined average distance travelled by each rat, which was significantly decreased by cat fur, as reported previously (Bowen et al., 2012). The *p*-values for these comparisons are indicated in Figs. 2–4.

Experiment 1: the effect of SR49059 on defensive aggregation

Rats treated with SR49059 spent significantly less time huddling relative to vehicle-treated rats (Fig. 2*a–c*), $F(1,6)=9.34$, $p=0.022$. Simple main effect contrasts revealed that only the higher dose of SR49059 (3 mg/kg) significantly reduced huddling [3 mg/kg *vs.* VEH: $p=0.05$; 1 mg/kg *vs.* VEH: $p=0.357$]. Stimulus contacts were not affected by either 1 mg/kg ($p=0.845$) or 3 mg/kg SR49059 ($p=0.408$) relative to VEH treatment (Fig. 2*d*).

Experiment 2: the effect of OT on defensive aggregation

OT increased huddling relative to VEH (Fig. 3*a*) [$F(1,15)=13.92$, $p=0.014$] and also significantly reduced contacts with the fur stimulus [Fig. 3*b*, $F(1,5)=43.23$, $p=0.001$] and the number of faecal boli deposited in the arena [Fig. 3*c*, $F(1,5)=86.32$, $p<0.001$]. There was no significant difference between the VEH (baseline) sessions conducted before and after OT treatment in: huddling [$p=0.173$], contacts [$p=0.695$] or faecal boli [$p=0.513$].

Experiment 3: the effect of SR49059 on OT-induced increases in defensive aggregation

OT again increased huddling in rats relative to VEH treatment [$F(1,5)=9.49$, $p=0.027$] and this effect was prevented by SR49059 (Fig. 3*d*) [SR+OT *vs.* VEH: $p=0.184$]. OT again reduced contacts relative to VEH [$F(1,5)=39.26$, $p=0.002$] and this was prevented by SR49059 (Fig. 3*e*) [SR+OT *vs.* VEH: $p=0.183$]. OT again reduced the number of faecal boli relative to VEH [$F(1,5)=99.46$, $p<0.001$] but this effect was unaffected by SR49059 (Fig. 3*f*) [*vs.* VEH: $F(1,5)=35.25$, $p=0.002$]. There was no significant difference in huddling ($p=0.513$), stimulus contacts ($p=0.34$) or faecal boli ($p=0.726$) between the two baseline sessions conducted before and after OT treatment.

Experiment 4: the effect of AVP on defensive aggregation

AVP increased huddling during cat fur exposure (Fig. 4*a*), $F(1,15)=11.76$, $p=0.004$. This effect was not significantly altered by SR49059 pre-treatment [*vs.* AVP only: $p=0.970$]. Conversely, SSR149415 prevented the increased huddling caused by AVP [SSR+AVP *vs.* VEH: $p=0.598$].

AVP also reduced the number of contacts with the fur stimulus (Fig. 4*b*) [$F(1,15)=53.63$, $p<0.001$] and this effect was partly reversed by SR49059 [*vs.* AVP only: $F(1,7)=14.93$, $p=0.006$]. In contrast, SSR149415 did not alter this effect [*vs.* AVP only: $p=0.388$].

AVP reduced the number of faecal boli deposited during cat fur exposure (Fig. 4*c*) [$F(1,15)=128.46$, $p<0.001$] and this effect was not significantly altered by SR49059 [*vs.* AVP only: $p=0.486$]. Conversely, SSR149415 given with AVP significantly increased the number of faecal boli when compared to AVP only [$F(1,7)=9.34$, $p=0.018$].

The average distance travelled by each rat was significantly decreased by AVP (Fig. 4*d*), $F(1,15)=134.58$, $p<0.001$. SR49059 partially prevented this effect, relative to AVP only [$F(1,7)=17.14$, $p=0.004$]. Conversely, SSR149415 did not prevent the reduced distance travelled caused by AVP, [$p=0.256$].

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the exposure sessions and the other half of the rats were given SSR149415 (30 mg/kg, i.p.) prior to AVP. **Experiment 5:** The rats ($n=32$) were given a VEH injection then 5 min later were placed into the testing arena in groups of four cage-mates for a 60 min session with no fur present. The following day proceeded as for day 1 but with the half the rats ($n=4$ quads) were injected with AVP (0.01 mg/kg, i.p.) 5 min prior to the session and the other half were given OT (0.5 mg/kg, i.p.). The next day all rats were given a VEH injection 5 min prior to the test session. **Experiment 6:** The rats ($n=18$) were injected with vehicle and placed individually in the cat odour avoidance testing apparatus for 20 min with no fur present. On the following test day, rats received (according to group allocation) either OT (0.5 mg/kg, i.p.), SR49059 (3 mg/kg, i.p.) or vehicle and 5 min later were placed in the hide box apparatus with fur present. **Experiment 7:** Rats ($n=12$) were randomly assigned to one of three conditions: VEH+VEH; AVP+VEH; and SSR149415+AVP ($n=4$ per condition). These rats were tested as described for Experiment 6. On the test day, rats received (according to group allocation) either vehicle+AVP (0.01 mg/kg, i.p.), SSR+AVP (30 mg/kg+0.01 mg/kg, i.p.) or vehicle+vehicle.

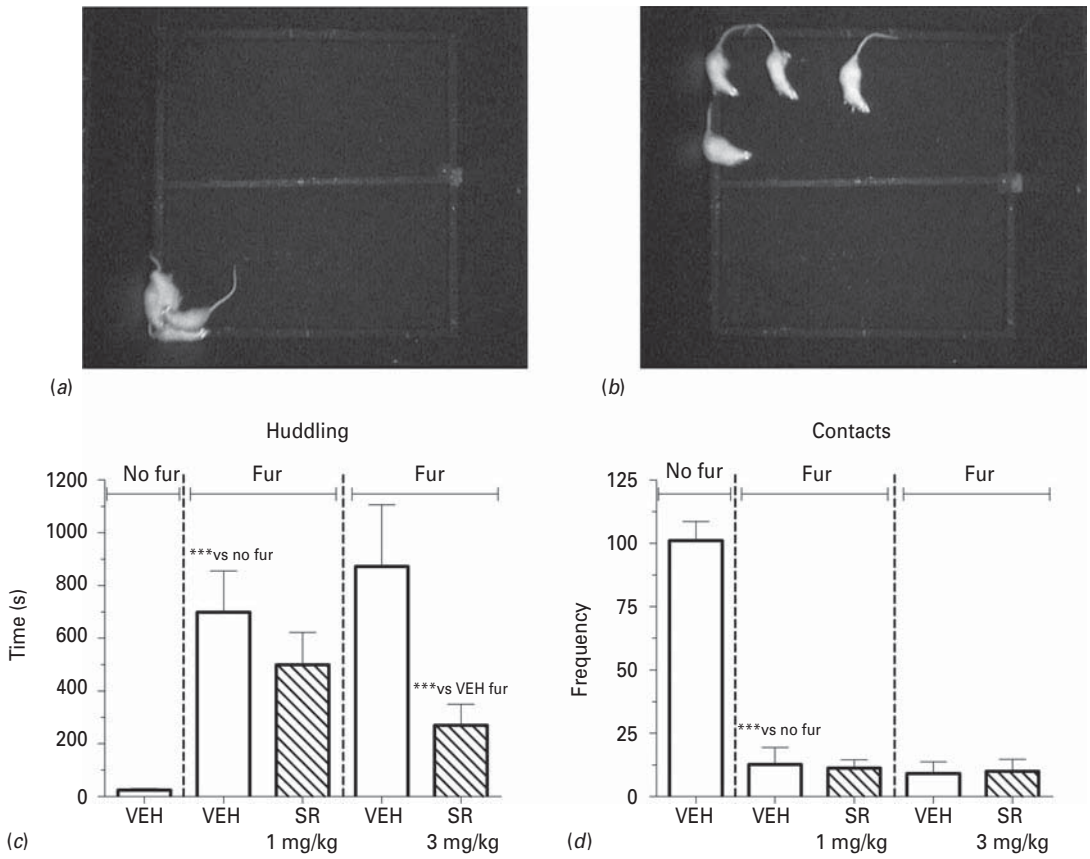

Fig. 2. The effects of the $V_{1A}R$ antagonist SR49059 on huddling induced by cat fur and the number of contacts with the cat fur stimulus. (*a*) Rats in a VEH quad engaging in the characteristic huddling response. (*b*): Rats in a quad pre-treated with 3 mg/kg SR49059 displaying avoidance of physical contact with other rats instead of the usual huddling response. Compared to VEH, 3 mg/kg SR49059, but not 1 mg/kg, inhibited huddling (*c*) and neither dose of SR49059 had any significant effect on the number of contacts with the cat odour stimulus (*d*). ***$p<0.001$.

There was no significant difference in huddling [$p=0.674$], stimulus contacts [$p=0.349$], faecal boli deposited [$p=0.575$] or average distance travelled [$p=0.475$] between the two baseline VEH sessions.

Experiment 5: the effect of OT and AVP on aggregation with no predator odour present

AVP significantly increased aggregation relative to VEH even when no fur was present in the arena (Fig. 5*a*) [$F(1,3)=29.44$, $p=0.01$] and also significantly reduced contacts with the empty fur holding apparatus [Fig. 5*b*, $F(1,3)=51.98$, $p=0.005$] and the number of faecal boli deposited in the arena [Fig. 5*c*, $F(1,3)=65.46$, $p=0.004$]. There was no significant difference between the VEH (baseline) sessions conducted before and after AVP treatment in: huddling [$p=0.171$], contacts [$p=0.873$] or faecal boli [$p=0.345$].

OT had no significant effect on huddling relative to VEH when no fur was present in the arena (Fig. 5*a*) [$p=0.229$], but did significantly reduce contacts with the empty fur holding apparatus [Fig. 5*b*, $F(1,3)=17.26$, $p=0.025$] and the number of faecal boli deposited in the arena [Fig. 5*c*, $F(1,3)=17.66$, $p=0.025$]. There was no significant difference between the VEH (baseline) sessions conducted before and after OT treatment in: huddling [$p=0.467$], contacts [$p=0.956$] or faecal boli [$p>0.999$].

Experiment 6: the effect of SR49059 and OT on the defensive response of individual rats

Cat fur significantly increased the amount of time individual rats spent hiding compared to baseline [$F(1,15)=78.62$, $p<0.001$]. This effect did not differ significantly between treatment conditions [all $p>0.590$] (Fig. 6*a*).

Cat fur also reduced the contacts rats made with the clip on which the fur was placed relative to baseline when no fur was present [$F(1,15)=49.26$, $p<0.001$], and significantly decreased the time spent approaching the clip [$F(1,15)=56.29$, $p<0.001$]. Again, these effects did not differ significantly between treatment conditions [contacts: all $p>0.095$; approach: all $p>0.785$] (Fig. 6*b*).

The distance travelled by rats was also reduced by cat fur relative to baseline [$F(1,15)=98.25$, $p<0.001$] with no difference between treatment conditions [all $p>0.081$] (Fig. 6*c*).

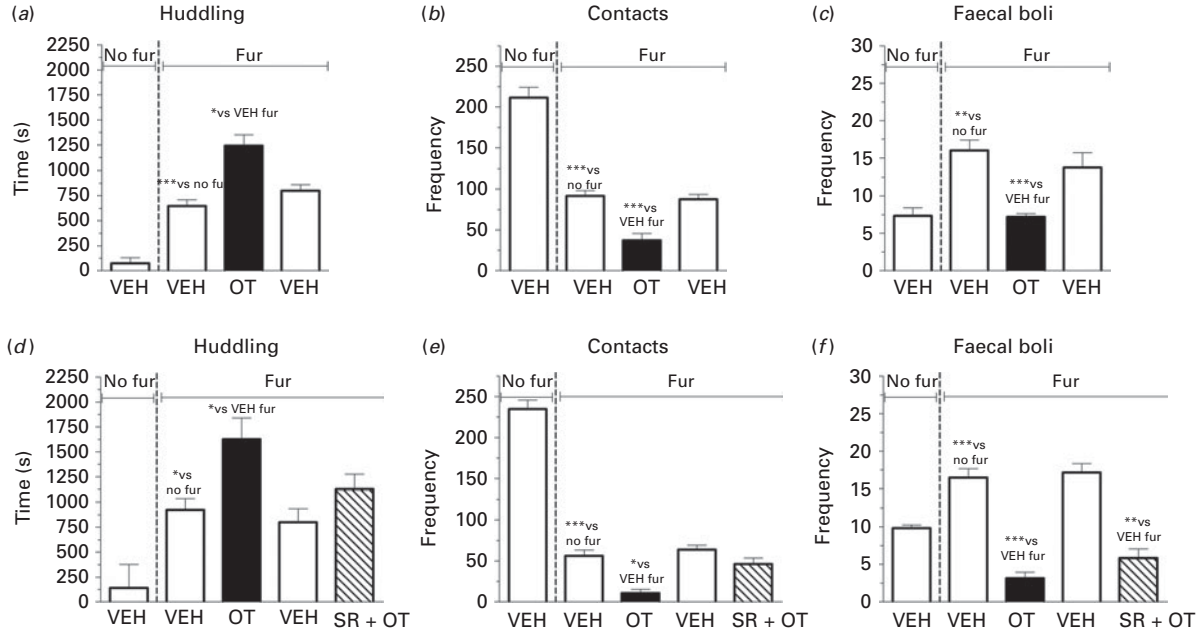

Fig. 3. The effect of oxytocin on huddling induced by cat fur, contact with the odour stimulus, and defecation and antagonism by SR49059. A 0.5 mg/kg injection of oxytocin significantly increased the amount of time quads spent huddling (*a*), decreased the number of stimulus contacts (*b*) and decreased their number of faecal boli (*c*). A 3 mg/kg injection of SR49059 given 5 min prior to an 0.5 mg/kg injection of oxytocin inhibited the oxytocin induced increase in huddling (*d*) and reduction in stimulus contacts (*e*), but had no effect on the decreased defecation (*f*). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

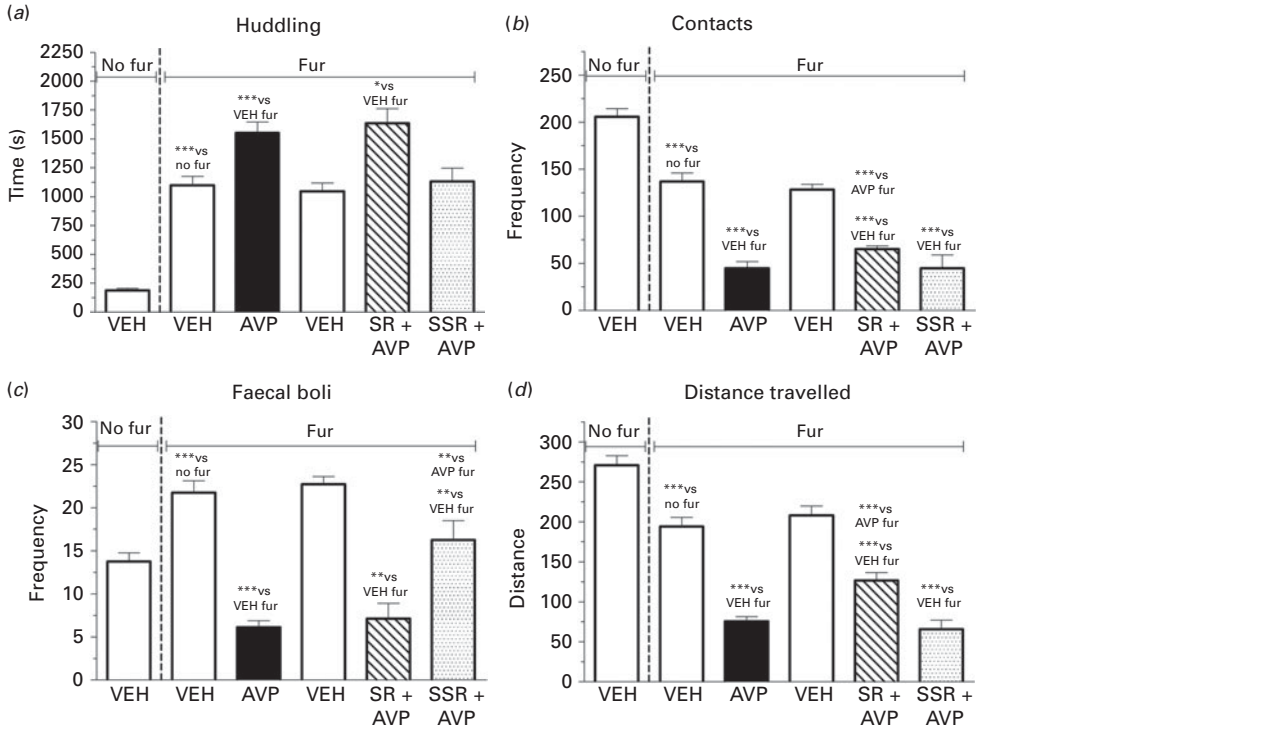

Fig. 4. The effect of AVP on huddling induced by cat fur, contact with the odour stimulus, defecation, distance travelled and antagonism by SR49059 and SSR149415. A 0.01 mg/kg injection of AVP significantly increased the huddling response to cat fur, an effect, that was blocked by 30 mg/kg SSR149415 but not by 3 mg/kg SR49059 (*a*). AVP accentuated fur-induced reductions in stimulus contacts, and this effect was partially blocked by SR49059 but was unaffected by SSR149415 (*b*). AVP caused a significant reduction in the number of faecal boli deposited in response to cat fur, and this reduction was partially blocked by SSR149415 but not by SR49059 (*c*). AVP augmented the reduction in distance travelled in response to cat fur, an effect, which was partially blocked by SR49059 but not SSR149415 (*d*). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

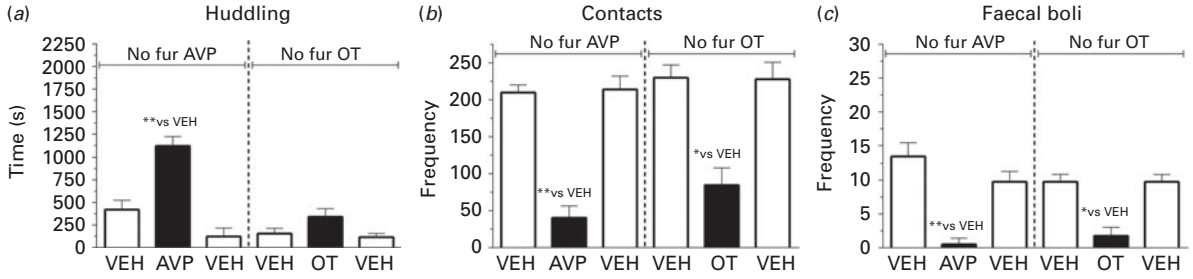

Fig. 5. The effect of oxytocin and AVP on huddling in the absence of cat fur, and on contact with the empty odour holding apparatus, and defecation. A 0.01 mg/kg injection of vasopressin significantly increased the amount of time quads spent huddling but a 0.5 mg/kg injection of oxytocin had no effect on huddling (*a*). Both oxytocin and vasopressin decreased the number of stimulus contacts (*b*) and decreased the number of faecal boli (*c*).

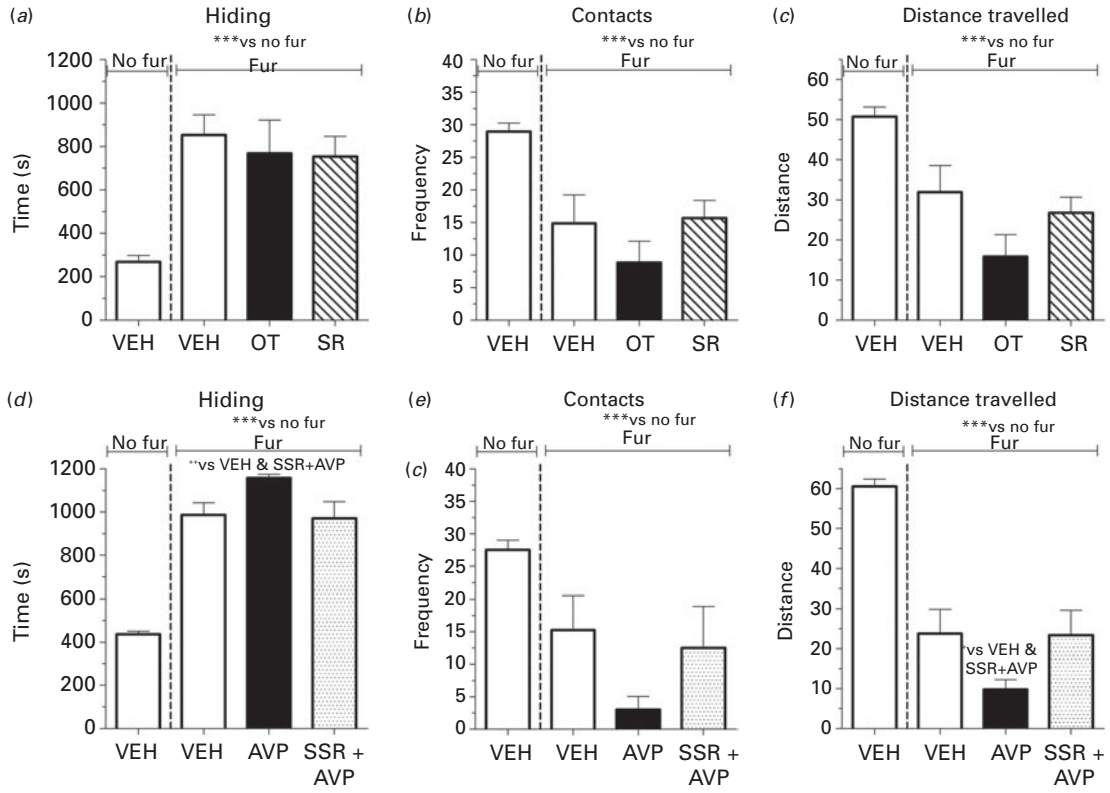

Fig. 6. The effects of oxytocin, SR49059, AVP and SSR149415 on hiding in rats individually exposed to cat odour. When rats were individually exposed to cat odour in the classic cat odour avoidance paradigm, 3 mg/kg SR49059 and 0.5 mg/kg OT had no significant effect on the cat-odour-induced increase in time rats spent inside the hide-box (*a*); decrease in the number of stimulus contacts (*b*); or reduction in distance travelled (*c*). Conversely, 0.01 mg/kg AVP: caused a significant augmentation of the elevation in time spent hiding in response to cat fur (to almost maximal levels), an increase that was blocked by 30 mg/kg SSR149415 (*d*); AVP (0.01 mg/kg) had no significant effect on the cat-fur-induced reduction in stimulus contacts (*e*); but caused a significantly more pronounced reduction in distance travelled, which was prevented by SSR149415 (*f*). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Experiment 7: the effect of AVP on the defensive response of individual rats to cat odour and the involvement of the AVP $V_{1B}R$

Cat fur again significantly increased the amount of time rats spent hiding relative to baseline [$F(1,9)=501.82$, $p<0.001$] and AVP augmented the magnitude of this hiding response [VEH *vs.* AVP: $F(1,9)=9.68$, $p=0.012$]. SSR blocked the augmenting effect of AVP on hiding [VEH *vs.* SSR+AVP: $p=0.874$; AVP *vs.* SSR+AVP: $F(1,9)=10.72$, $p=0.01$] (Fig. 6*d*).

Cat fur again reduced the number of contacts rats made with the clip relative to baseline (no fur) [$F(1,9)=27.79$, $p=0.001$] and significantly decreased the amount of time rats spent approaching the clip [$F(1,9)=194.44$, $p<0.001$]. The magnitude in this reduction in contacts

and approach did not differ significantly between treatment conditions [contacts: $p=0.203$; approach: $p=0.361$] (Fig. 6*e*).

Cat fur significantly decreased distance travelled relative to baseline, $F(1,9)=158.18$, $p<0.001$. This decrease was significantly more pronounced for rats given AVP [VEH *vs.* AVP: $F(1,9)=6.99$, $p=0.027$], an effect that was prevented by SSR pre-treatment [VEH *vs.* SSR + AVP: $p=0.978$; AVP *vs.* SSR + AVP: $F(1,9)=6.84$, $p=0.028$] (Fig. 6*f*).

Discussion

The current series of experiments examined the modulation of anti-predator defensive behaviour in groups of rats, and individual rats, given peripheral administration of OT and AVP. Both neuropeptides increased defensive aggregation in groups of rats subjected to predatory threat, while only AVP appeared to intensify the defensive behaviour of individual rats. AVP, but not OT, also increased aggregation of rats placed in the testing apparatus without the predatory stimulus present.

A role for V_{1A}Rs in the social response to threat was evident, arising from observations that the V_{1A}R antagonist SR49059 reduced huddling and also prevented the augmenting effects of OT on huddling. In contrast SR49059 did not prevent AVP enhancement of huddling, which was instead blocked by the V_{1B}R antagonist SSR149415. SSR149415 also prevented AVP intensification of defensive behaviours under individual exposure to predator threat. Overall, this suggests a more general role for V_{1B}Rs in anxiety, and a more selective role for V_{1A}Rs in the social response to threat.

The increased huddling observed with AVP and OT and opposing effects of SR49059 are consistent with previous studies involving other species. Central infusion of mesotocin, a non-mammalian analogue of OT, potently promotes flocking and flock size selection (forms of defensive aggregation) in Estrildids, while infusion of a V_{1A}R antagonist into the lateral septum inhibits these behaviours in this species (Goodson et al., 2009; Kelly et al., 2011). Interestingly, we recently found that huddling in rats was strongly correlated with activation in the ventral part of the lateral septum (Bowen et al., 2013). Defensive aggregation in zebrafish (shoaling) is similarly increased by both OT and AVP (Braida et al., 2012). Overall it appears that the role of OT and AVP systems in promoting defensive aggregation are remarkably well conserved across species.

OT is better known for its role in promoting social proximity under appetitive or affiliative conditions. Thus OT increases social and filial huddling in rat pups (Odya et al., 2002; Alberts, 2007; Kojima and Alberts, 2011), and passive close contact (adjacent lying) in adult male rats meeting for the first time (Ramos et al., 2013). In the present study, SR49059 resulted in virtually complete blockade of the OT-induced increase in huddling, indicating that this effect is likely to be primarily mediated by the V_{1A} receptor. However, some role for the OTR cannot be entirely ruled out based on the present study. Further support for OT acting at the V_{1A}R comes from previous studies. For instance, similar to the present study, the effects of OT on adjacent lying are also reversed by SR49059, although at a lower 1 mg/kg dose, but not by an OTR antagonist (Ramos et al., 2013). In a mouse model of autism, OT administration rescues social impairments in OTR-KO mice and this effect was blocked by SR49059 (Sala et al., 2011). Thus, OT acting on V_{1A}Rs appears to be a common element in both defensive social behaviours and positive affiliative behaviours induced by a number of proximal causes, including olfactory cues (e.g. the present study and Kojima and Alberts, 2011).

Both OT and AVP reduced the number of contacts with the predatory stimulus under group exposure conditions. The AVP effect is likely due to the increased generalised anxiety observed in AVP-treated rats. OT, in contrast, did not appear to affect any aspect of the defensive response in individual rats. Thus the reduced contacts under group conditions might be seen as OT inducing a bias towards a social response to threat (huddling) at the expense of individual exploration (stimulus contact) when the presence of conspecifics allows such a selection.

Given that OT appeared to be acting via AVP V_{1A}Rs to elicit its effect on defensive aggregation, we predicted AVP would be effective at increasing huddling at much lower doses than OT, and this was indeed demonstrated. AVP increased huddling at 1/50th the dose of OT that was used. However, while the effect of AVP on positive, appetitive social interaction in male rats was blocked by SR49059 in a previous study (Ramos et al., 2013), the increased threat-induced social behaviour in rats administered AVP in the present study was not affected by blockade of V_{1A}Rs. However administration of SSR149415 effectively reduced the AVP induced amplification of huddling.

In contrast to OT and SR49059, AVP also increased the anxiogenic response to cat fur in individual rats. Indeed, AVP treated rats spent almost the maximal amount of time in the hide box: 1158 s out of a possible 1200 s, on average. Importantly, SSR149415 prevented this augmented anxiogenic effect of AVP in individually exposed rats. The anxiogenic effects of AVP (Neumann and Landgraf, 2012) and anxiolytic effects of SSR149415 (Griebel et al., 2002, 2005) are well described and the modulation of social behaviours by V_{1B}Rs may be directly linked to this role in anxiety (Griebel et al., 2002, 2005; Blanchard et al., 2005). Thus the most obvious explanation of these findings is that the increased anxiety to the cat fur induced by AVP promotes defensive aggregation. Consistent with this notion, AVP, but not OT, increased huddling when no fur was present in the arena. This supports a more specific role for OT whereby the social response to threat is augmented without enhancing anxiety *per se*, while AVP is likely to increase huddling through a more general induction or exacerbation of the

stress response. Therefore, the reversal of AVP-induced huddling by SSR149415 may be related to the antagonist's ability to block the increased generalised anxiety induced by AVP. As such, administration of SSR149415 alone would also be predicted to have an anxiolytic effect, as it does in other paradigms (Griebel et al., 2002), and would subsequently be predicted to reduce the huddling and more general defensive response to cat fur.

Unlike Ramos and colleagues (2013), who reported close aggregation between two male rats pre-treated with OT before meeting for the first time, we did not observe any effect of OT on social behaviour in the absence of predatory threat. Ramos and colleagues reported that the Long-Evans/Hooded Wistar strain they used appeared particularly sensitive to OT-induced social effects. This could be due to the higher basal levels of anxiety in the Long-Evans strain (Boakes et al., 2000) combined with the mild anxiety induced by the social interaction paradigm (due to the presence of an unfamiliar conspecific) providing the optimal conditions for OT to exert a social aggregation response.

OT has relatively high affinity for the $V_{1A}R$ and very low affinity for the $V_{1B}R$ (Chini and Manning, 2007). In contrast, AVP has high affinity for both V_1Rs, with affinity being the highest for the $V_{1B}R$ (Chini and Manning, 2007; Manning et al., 2012). Given that only the $V_{1B}R$ antagonist blocked the AVP-induced increase in huddling (whereas the $V_{1A}R$ antagonist blocked OT-induced increases in huddling) it seems that enhanced activation of the $V_{1B}R$-mediated anxiety/huddling response overrides the more specific defensive aggregation response mediated via the $V_{1A}R$.

In the present study blockade of the $V_{1A}R$ did not appear to have any effect on generalised anxiety towards cat fur in individually exposed rats, and OT, which increased defensive aggregation primarily via the $V_{1A}R$, did not increase the individual defensive response to the cat fur. This contrasts with previous studies suggesting that the $V_{1A}R$ plays a direct role in anxiety, with greater $V_{1A}R$ activation sometimes being anxiogenic. However, results are inconsistent. For instance, one study reported that $V_{1A}R$ KO mice had lower anxiety than wild-types (Bielsky et al., 2004b) while a more recent study failed to replicate this (Wersinger et al., 2007). Another study reported that up-regulation of $V_{1A}R$ expression in the ventral forebrain of monogamous male prairie voles increased anxiety (Bielsky et al., 2004a), highlighting the importance of considering regional distributions of $V_{1A}Rs$ and potential differential effects of $V_{1A}R$ stimulation or antagonism at different loci. Additionally, studies of the $V_{1A}R$ in anxiety have often used ligands that lack selectivity over the V_{1B} and/or V2 and OT receptors (Bleickardt et al., 2009; Mak et al., 2012) making interpretation difficult. Our findings support a direct role of $V_{1B}Rs$ in anxiety whereby AVP acts at these receptors to increase generalised anxiety. Conversely, the present study suggests the $V_{1A}R$ plays a less direct role in regulating anxiety whereby activation of these receptors promotes a social response to the stressor, which may subsequently buffer individuals against anxiety (Bowen et al., 2013).

When extrapolated to human psychiatric disease, our study suggests there may be differential utility for OT and AVP in treating social withdrawal components of disorders such as schizophrenia and autism. Therefore, we would predict that OT and other drugs that target the $V_{1A}R$ system selectively over the $V_{1B}R$ system would not increase the anxiogenic response to threat but rather selectively increase adaptive social responding to threat. In a recent study we have shown that defensive aggregation has lasting benefits to rats in terms of reducing individual stress responses to predatory threat (social buffering) (Bowen et al., 2013). The present study might lead to a hypothesis that $V_{1A}R$ acting drugs could assist psychiatric dysfunction by directing people experiencing stress towards social support, rather than social isolation, allowing them to gain the benefits of social buffering during stressful events, which could, for example, help prevent the stressor from triggering psychotic episodes (Norman et al., 2005; Meyer-Lindenberg and Tost, 2012). Indeed, recent clinical trials point to the utility of intranasal OT in treating disorders characterised by social deficits and withdrawal, reporting it improves social cognitive deficits in both schizophrenia (Pedersen et al., 2011) and autism (Hollander et al., 2003, 2007). Finally, human studies indicate that social buffering in humans is enhanced by OT administration (Heinrichs et al., 2003). Our findings are consistent with the model proposed by Neumann (2009) in which OT promotes a variety of social behaviours that result in stress buffering while also directly modulating the physiological stress response at the level of the paraventricular nucleus of the hypothalamus and amygdala. Future studies may usefully explore the capacity of OT and $V_{1A}R$ agonists to promote social modulation of stress responses.

The present study does not conclusively determine whether the observed effects of the neuropeptides are centrally or peripherally- mediated. It is worth noting that one recent study demonstrated that both nasal and i.p. administration of OT resulted in significant increases in OT levels in microdialysates sampled from the amygdala and hippocampus of rats and mice (Neumann et al., 2013), supporting the notion that peripherally administered neuropeptides can cross the blood-brain barrier and reach brain regions relevant to behaviour and emotion. However, it might still be argued that effects observed in the present study are peripherally mediated: for example AVP might act via $V_{1B}Rs$ in the anterior pituitary that modulate the release of ACTH, and thus corticosterone (Meyer-Lindenberg et al., 2011), to affect the stress response. Overall, however, the present study joins other recent findings from our group (Bowen et al., 2011; Ramos et al., 2013) and others (e.g. Feifel et al., 2012; Bales et al., 2013) that peripherally

administered OT and AVP can modulate complex social behaviours.

The consistency of the findings of the present study with those conducted in other species suggests there may be some conservation of the basic mechanisms influencing the social response to threat across both mammalian and non-mammalian species. Furthermore, they indicate that dual systems have evolved to regulate the huddling response: an OT- and V_{1A}R-mediated effect that is specific to the social response to threat and unrelated to the more general anxiety response; and an AVP V_{1B}R-mediated effect that is directly linked to the more general anxiogenic response to the stressor. Pharmacological manipulation of the former system could provide potential benefits in treating disorders characterised by social withdrawal and isolation in the face of threatening or stressful situations by promoting adaptive social responding without enhancing the more general anxiety response.

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Statement of Interests

None

Supplementary material

For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145714000388.

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Chapter 5: Active coping toward predatory stress is associated with lower corticosterone and progesterone plasma levels and decreased methylation in the medial amygdala vasopressin system

NOTE: The supplementary methods referred to in the publication presented in this chapter can be found in Appendix 6

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Active coping toward predatory stress is associated with lower corticosterone and progesterone plasma levels and decreased methylation in the medial amygdala vasopressin system

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ABSTRACT

An active coping style displayed under stress – which involves proactive investigatory responses toward environmental threats – has been associated with reduced vulnerability to psychiatric illness. However, the neurobiological determinants of coping styles are not well understood. When rats are exposed to a naturalistic stressor (cat fur) in a group, some individuals in the group show robust active investigation of the stimulus while others show a passive response involving retreat, immobility and close aggregation with conspecifics. Here we explored endocrine and epigenetic correlates of these contrasting coping styles. Male Wistar rats ($n = 48$) were exposed to cat fur in groups of 4 and the passive and active responders were identified and assessed for endocrine and epigenetic differences. Three days after the final cat fur exposure, active responders had substantially lower plasma levels of corticosterone and progesterone than passive responders. Plasma and testicular testosterone levels did not differ between active and passive responders. Active responders had markedly less methylation of the AVP CGCG promoter region located at base 4970 in the posterodorsal region of the medial amygdala but did not differ in the methylation status of the CCGG sequence located at base 2243. This is in agreement with prior research suggesting that AVP and progesterone act in opposition within the medial amygdala to modulate stress-related behaviors. The present study reports striking endocrine and epigenetic differences between active and passive responders, providing insight into potential systems involved in the manifestation of differing coping styles.

Introduction

Individual animals exposed to the same stressful situation often exhibit diversity in their behavioral responses to that situation. A common difference relates to active and passive coping styles. Active response styles involve proactive investigatory responses toward environmental threats, a more aggressive phenotype toward conspecifics, and less pronounced neural and physiological stress responses (Koolhaas et al., 1999, 2010). Conversely, passive response styles involve avoidance of environmental threats, reduced aggression toward conspecifics, and a more pronounced neural and physiological stress response (Koolhaas et al., 1999, 2010). In humans, a more active coping style is associated with reduced vulnerability to anxiety disorders and depression as well as better physical health, particularly cardiovascular health outcomes, whereas a more passive coping style is associated with heightened risk of developing mood and anxiety disorders as well as cardiovascular problems (Chiavarino et al., 2012; LeDoux and Gorman, 2001; Russo et al., 2012).

In recent work we studied variability in the responses of rats exposed to a naturalistic stressor, cat fur, in a large arena (Bowen and McGregor, 2014; Bowen et al., 2012, 2013; Kendig et al., 2011). When rats were exposed to cat fur in groups of 4 cagemates we observed a clear distinction between active and passive responders (Bowen et al., 2013). Active responders exhibit more frequent approaches toward the predator stimulus and show less conditioned fear to a context associated with the predator odor. Conversely, passive responders avoid the stimulus and spend prolonged periods huddling together in one of the corners of the arena. These traits are highly consistent across repeated exposures to cat fur and generalize to other situations.

Our studies indicate the amygdala–lateral septum system may play an important role in the defensive response to cat odor (Bowen et al., 2013). The posteroventral medial amygdala (MePV), in particular, is strongly activated by cat odor and has been identified as a critical pathway in the defensive response to predator odors (e.g. risk assessment and inhibition of locomotor activity and grooming) (Dielenberg and McGregor, 2001; McGregor et al., 2004). Crosstalk between the MePV and the neighboring posterdorsal medial amygdala (MePD) appears to play an important role in some of the acute and more enduring behavioral

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responses to predator odor exposure, such as inhibition of mating (Apfelbach et al., 2005; Takahashi, 2014). The MePD has recently been linked to coping style, with early life stress associated with reduced activity in the MePD and an enduring passive coping style (Nishi et al., 2013). Pathways involving the neuropeptide AVP that are centered around the medial amygdala (MeA), bed nucleus of the stria terminalis (BNST) and lateral septum have received particular attention in studies on aggression and coping styles (Koolhaas et al., 1998, 2010; Veenema and Neumann, 2007).

There is a close relationship between aggression and coping strategy in both humans and animals, with a strong positive association existing between an active coping strategy and propensity to display offensive aggression (Koolhaas et al., 2010; Veenema and Neumann, 2007). As such, offensive aggression is often used as a measure of coping style (Koolhaas et al., 2010; Veenema and Neumann, 2007). Microinfusion of AVP into the cerebral ventricles (Winslow and Insel, 1993) or within the BNST or lateral septum increases offensive aggression in both hamsters and rats (Delville et al., 1996; Ferris et al., 1984; Irvin et al., 1990; Koolhaas et al., 1998). Denser AVP staining and greater receptor abundance are found in the lateral septum of more aggressive mouse strains (Bester-Meredith et al., 1999). Moreover, deletion of the AVP V1B receptor gene essentially blocks all offensive aggression but does not alter defensive aggression in mice (Wersinger et al., 2002, 2007).

It therefore appears that increased activity in the AVP system in the MeA may be associated with active coping toward threat. One mechanism through which differences in the activity of the MeA AVP system might be maintained is through the methylation status of the AVP promoter. Heightened testosterone results in reduced methylation of the AVP promoter CCGG sequence located at base 2243 (CpG site 1 henceforth) and the CGCG sequence at base 4970 on the AVP promoter (CpG site 2 henceforth), and a subsequent increase in activity in the AVP system (Auger et al., 2011). However, progesterone also plays an important and independent role in regulating AVP expression in the MeA (Auger and De Vries, 2002; Auger and Vanzo, 2006; Bychowski et al., 2013). Specifically, the CGCG sequence at base 4970 on the AVP promoter is in close proximity to progesterone response elements that may facilitate a role for progesterone in regulating the methylation status of the MeA AVP system (Auger et al., 2011; Mohr and Richter, 1990; Shapiro et al., 2000a).

Indeed, progesterone may be of particular relevance in regulating any relationship between coping style and methylation of the AVP promoter that may exist, as different coping styles do not appear to be associated with testosterone (Koolhaas et al., 2010). Conversely, the reduced anxiety and active coping style observed during the estrus phase in female rodents has been linked to lower circulating levels of progesterone (Babar et al., 2008; Gangitano et al., 2009). Another steroid hormone, corticosterone, has also been linked to coping strategy, with lower basal levels of corticosterone associated with an active coping style displayed across a number of tests, such as the resident intruder test and the defensive burying test (Korte et al., 1992, 1996).

Therefore, we examined whether active and passive coping to predator threat was associated with differences in the methylation status of AVP promoters in the MeA and whether these differences are associated with testosterone, progesterone and/or corticosterone levels.

Methods

All experimental procedures were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition, 2004) and were approved by the University of Sydney Animal Ethics Committee (approval number L29/7-2010/3/5360).

Subjects

Subjects were 48 adult male Albino Wistar rats (Animal Resources Centre, Perth, Australia), aged 12 weeks at the start of testing. After behavioral screening was completed, it was established that the mean body weight of active ($M = 390.6$, $SEM = 5.1$) and passive ($M = 386.8$, $SEM = 6.6$) responders did not differ ($p > 0.1$). All rats were housed in cages of four with *ad libitum* access to food and water in the home cage and were handled daily for 1 week prior to the start of testing.

Behavior and classification

The threat stimuli used were 2 g balls of cat fur (for more information see S1). Testing was conducted in two identical 1200 mm × 1200 mm × 900 mm ($l \times w \times h$) wooden framed arenas painted matte black, located adjacent to each other (Bowen et al., 2012, 2013; Kendig et al., 2011). The cat fur was placed flush against the center of one of the walls, underneath a wire mesh cylinder (80 mm diameter, 95 mm high, 2.5 mm^2 aperture). The cylinder was also present in the arena during habituation (no fur) sessions in the arena.

The back of each rat was uniquely marked with a Sharpie non-toxic permanent marker to allow identification of individual rats within a quad of four rats. Initially, quads of four rats from the same homecage were placed into the arena for a 50 min habituation session each day for three consecutive days. No cat fur was present during these sessions. Following this, they underwent three further daily test-sessions of 50 min duration with the cat fur present underneath the cylinder.

The number of times each rat made contact with the fur stimulus (defined as a rat placing its nose within 3 cm of the stimulus) during the three fur exposure sessions was used for the classification of rats as active (top 25%), neutral (middle 50%) or passive (bottom 25%). Video recordings of the sessions were made and the number of stimulus contacts made by each rat was scored for the purpose of classification. Classification resulted in 12 passive and 14 active responders. The larger number of active responders was due to 3 rats having the frequency of stimulus contacts corresponding to the 75th percentile.

Plasma and tissue collection

Three days after the final predator odor exposure rats were sacrificed by decapitation without anesthetic and the trunk blood, testicles and brains were collected. All rats were extensively habituated to the procedure leading up to decapitation in the days prior to the sacrifice to ensure minimal stress was induced prior to decapitation. Blood was centrifuged at 4 °C for 15 min at 3300 g, and plasma was collected for analysis. Tissue and plasma were snap frozen in liquid nitrogen and stored at −80 °C.

Methylation of AVP promoter regions in the MePD

The MePD (Bregma −2.76 to −3.24, interaural 6.24 to 5.76; Paxinos and Watson, 2007) was microdissected from snap frozen tissue (for further information on the microdisection procedure please see S1). Extracted gDNA was treated with Methylation Specific Restriction Enzymes (MSRE) *Hpa*II and *Bst*UI (protocol adapted from Auger et al., 2011). The extent of methylation was quantified by qPCR. Briefly, methylated DNA is protected from MSRE digestion, leaving more intact template DNA for the qPCR reaction. Relative expression with reference to no enzyme control was quantified using freely available REST software (http://www.REST.de.com) (Pfaffl et al., 2002). For a more detailed description of this procedure please refer to S1.

LC-MS/MS

Testosterone was extracted from the homogenized testicles (see S1) and the concentration of testicular testosterone measured by LC-MS/MS and expressed per gram of tissue. Testosterone, corticosterone and progesterone were extracted from the plasma (see S1) and the concentration of each of the three analytes per ml of plasma was determined using LC-MS/MS. For more details on the LC-MS/MS analysis, see S1.

Statistical analysis

Stimulus contacts were analyzed using mixed model ANOVA with contrast analysis used to examine the effect of fur and the differences between active and passive responders. For the sake of brevity, only the results of the contrast analysis are presented. Consistency of responder type classification was assessed by computing Cronbach's α and the intraclass correlation (ICC) with absolute agreement for the number of stimulus contacts across the 3 fur exposure sessions. The methylation status of the AVP promoter regions of interest in the MePD of active and passive responders was compared using REST 2009 (Pfaffl et al., 2002), with 10,000 iterations used for the bootstrap randomisation (more information on REST can be found in S1). Plasma and testicular hormone levels in active and passive responders were compared using independent samples t-tests. Cohen's *d* was computed as a measure of effect size for the contrast analyses conducted for analysis of the behavioral data and the t-tests conducted for the analysis of plasma hormone levels.

Results

Behavior

As shown in Fig. 1A, the distribution of stimulus contacts averaged over the three fur exposure days was used for classification of rats as active (top 25%), neutral (middle 50%) or passive (bottom 25%) responders. As shown in Fig. 1B, there was no difference between active and passive responders in the number of contacts made with the wire mesh cylinder when it had no cat fur underneath ($F(1,24) = 1.25$, $p = 0.274$, $d = 0.43$). Both active and passive responders engaged in significantly fewer contacts with the wire mesh holder when it had the cat fur underneath [active: $F(1,14) = 34.02$, $p < 0.001$, $d = 1.64$; passive: $F(1,12) = 78.16$, $p < 0.001$, $d = 2.91$]. However, the reduction in stimulus contacts when fur was present was significantly more pronounced for passive responders [$F(1,24) = 14.61$, $p < 0.001$, $d = 1.5$], with active responders engaging in significantly more stimulus contacts than passive responders across all three fur exposure sessions [exposure 1: $F(1,20) = 126.43$, $p < 0.001$, $d = 4.79$; exposure 2: $F(1,24) = 103.27$, $p < 0.001$, $d = 3.94$; exposure 3: $F(1,24) = 35.98$, $p < 0.001$, $d = 2.34$; Fig. 1B]. Responder type was highly consistent across the three exposure sessions [Cronbach's $\alpha = 0.905$, ICC = 0.899, $p < 0.001$]. Significantly more stimulus contacts were made on exposure session 3 compared to exposure session 2, averaged across responder type [$F(1,25) = 12.46$, $p = 0.002$, $d = 0.69$]. However, the magnitude of the increase in stimulus contacts did not differ between active and passive responders ($p = 0.663$). This increase from days 2 to 3 is thus unrelated to responder type and reflects habituation to the cat fur stimulus (Dielenberg and McGregor, 1999).

Hormone analysis

Three days after the final fur exposure, active responders had significantly lower plasma levels of corticosterone [$t(24) = 2.56$, $p = 0.017$, $d = 1.02$; Fig. 2A] and progesterone [$t(24) = 3.1$, $p = 0.005$, $d = 1.31$; Fig. 2B] than passive responders. There was no difference between active and passive responders in plasma testosterone [$p = 0.781$; Fig. 2C] or testicular testosterone at this time-point [$p = 0.634$; Fig. 2D].

Methylation of AVP promoter regions in the MePD

Methylation of AVP CpG site 1 (CCGG sequence) in the MePD did not differ between active and passive responders ($p = 0.317$; Fig. 3A). Methylation of AVP CpG site 2 (CGCG sequence) in the MePD was 4.16 fold greater in the passive responders relative to their active counterparts ($p = 0.032$; Fig. 3B).

Discussion

The present study confirms our previous description of active and passive coping styles in rats exposed to predatory threat in home cage groups (Bowen et al., 2013). As previously reported, we observed a highly consistent difference in predator approach behavior, whereby active responders engaged in far more contact with the cat fur than passive responders, who instead spend greater time engaging in defensive aggregation (tight social grouping in response to threat) (Bowen et al., 2013). Importantly, there was no difference between active and passive responders in the number of stimulus contacts when no fur was present in the arena, indicating the observed behavioral differences between responder types were not simply due to differences in general exploratory behavior under baseline (no threat) conditions. Extending on our behavioral characterization, here we show conspicuously divergent endocrine and epigenetic indices across these two responder types. Specifically, 3 days after the final fur exposure, passive responders had markedly higher levels of plasma corticosterone and progesterone

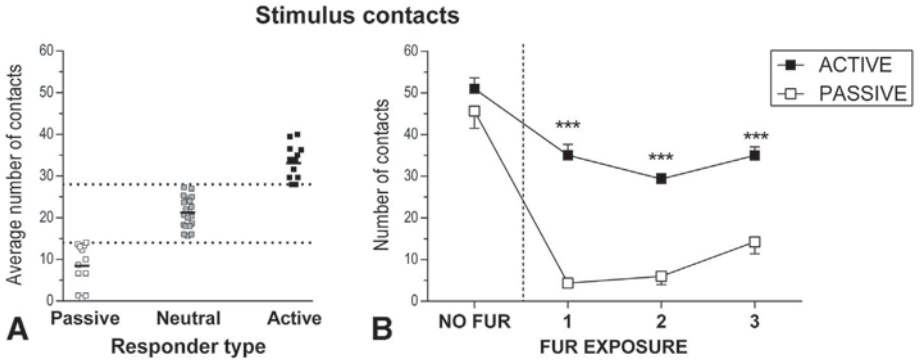

Fig. 1. Classification and stimulus contact behavior of active and passive responders. A: Classification of rats as passive, neutral or active responders was based on the average number of stimulus contacts made by each rat over the three 50 min fur exposure sessions. The top 25% of responders were classified as active and the bottom 25% as passive. The lower dotted line above represents the cut-off for passive responders and the higher dotted line represents the cut-off for active responders. B: Cat fur caused a significant decrease in stimulus contacts in both active and passive responders, however, the reduction was significantly more pronounced in passive responders. ***$p < 0.001$ vs passive.

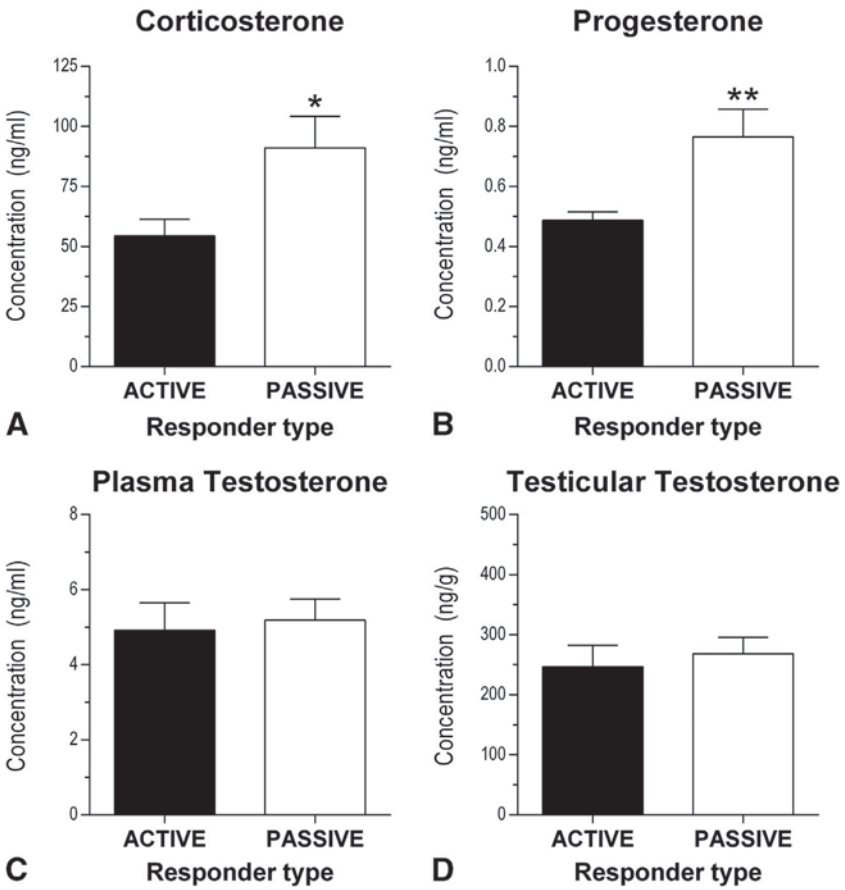

Fig. 2. Basal endocrine differences between active and passive responders. Active responders had significantly lower basal levels of plasma corticosterone (A) and progesterone (B). There was no significant difference between active and passive responders in plasma or testicular testosterone (C, D). *$p < 0.05$ vs active; **$p < 0.01$ vs active.

and showed greater methylation of AVP CpG site 2 in the MePD, suggesting less activity of this central AVP system.

The lower corticosterone plasma levels observed in active responders in the present study is consistent with some earlier studies in short attack-latency (SAL) and long attack-latency (LAL) mice. These mice show an active and passive coping strategy respectively across a number of tests such as the resident intruder test and the defensive burying test, and have low and high levels of corticosterone respectively (Korte et al., 1992, 1996). Furthermore, rats given subcutaneous injection of corticosterone show increases in passive responding and depression-like behavior on the forced swim test (Gregus et al., 2005). These studies suggest that even under conditions when an acute stressor is absent, elevated corticosterone may play a causal role in promoting passive styles of responding to stress.

It is unlikely that the elevated corticosterone in passive responders is a result of the predator stress exposure rather than a pre-existing characteristic as studies examining corticosterone release in response to cat fur have found that, unlike other stressors such as immobilization and foot shock, levels of corticosterone return to normal by 60 min post fur exposure (Muñoz-Abellán et al., 2008; Ottenweller et al., 1992). Furthermore, despite heightened anxiety-like behavior on the elevated plus-maze occurring as long as 7 days after fur exposure, no differences

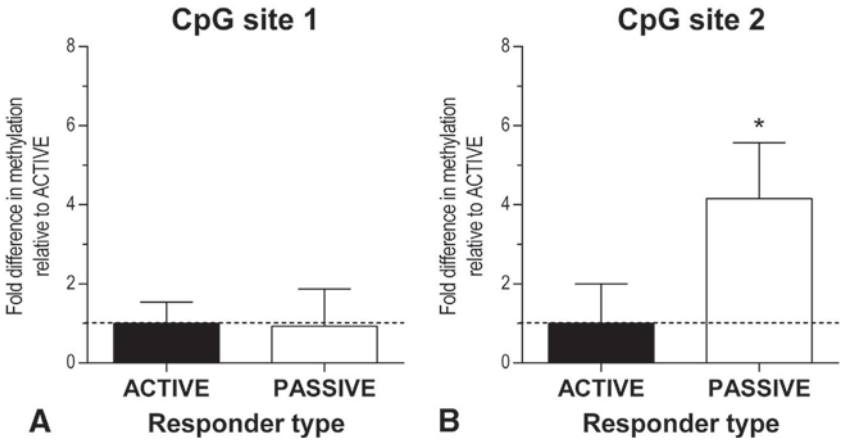

Fig. 3. Epigenetic differences between active and passive responders. A: Methylation of CpG site 1 in the MePD did not differ between active and passive responders. B: Methylation of CpG site 2 in the MePD was more than 4 fold greater in passive responders relative to active responders. * $p < 0.05$ vs active.

in corticosterone are observed beyond the acute increase during stressor exposure (Muñoz-Abellán et al., 2008). Finally, elevated basal corticosterone appears to facilitate contextual-fear conditioning (Pugh et al., 1997), which suggests the elevated corticosterone levels observed in passive responders may play a role in promoting their heightened contextual-fear conditioning to predator odor exposure (Bowen et al., 2013).

Consistent with previous studies (Koolhaas et al., 2010), we found no association between either plasma or testicular testosterone and coping strategy. Importantly, this study shows for the first time a link between higher progesterone levels in males and a passive coping strategy. This finding is consistent with previous reports linking lower circulating progesterone with the reduced anxiety and more active coping style observed during the estrus phase in female rodents (Babar et al., 2008; Gangitano et al., 2009). However, it is also possible that transient changes in hormone levels elicited during the 50 min test are involved in the expression of active versus passive coping during stressor exposure.

It is also of interest that progesterone release in response to stress appears to promote social contact as a coping strategy (Wirth, 2011). We have previously shown that passive responders in our paradigm engage in significantly more defensive aggregation (extended periods of tight social grouping with conspecifics) in response to predator threat (Bowen et al., 2013). In light of the present findings, it is possible that progesterone is acting to promote that behavior by switching animals from a more active response to threat to a more passive, affiliative response to the stressor (Bowen et al., 2013; Wirth, 2011).

Our present findings are consistent with numerous studies demonstrating that heightened AVP activity, especially in the system originating in the MeA and BNST and projecting to the lateral septum, is associated with offensive aggression (a commonly used measure of active coping, Koolhaas et al., 2010) in hamsters, rats and mice (Bester-Meredith et al., 1999; Delville et al., 1996; Ferris et al., 1984; Irvin et al., 1990; Koolhaas et al., 1998; Wersinger et al., 2002). Our findings draw particular attention to the MePD, where suppressed activity has previously been associated with enduring passive responding in rodents across a number of tests (e.g. the forced swim test and two-way active avoidance test) following early life stress (Nishi et al., 2013). Our study indicates reduced epigenetic inhibition of the AVP gene in this region in active responders, suggesting a mechanism that might underlie differences in AVP expression in specific brain systems in active and passive responders. Given that behavioral differences exist between active and passive responders on first exposure to predator threat, it is likely that pre-existing differences in AVP activity are associated with the expression of active and passive coping.

The genetically selected SAL mice and low-anxiety behavior (LAB) rats (the latter of which are bred to display lower anxiety-related behavior on numerous tests such as the elevated plus-maze, emergence test and open-field test) display heightened aggression and an active coping style relative to their passive-coping counterparts, LAL mice and high-anxiety behavior (HAB) rats (Beiderbeck et al., 2007; Koolhaas et al., 1999, 2010; Landgraf and Wigger, 2002; Liebsch et al., 1998; Ohl et al., 2001; Veenema et al., 2003a, 2003b, 2007). A small number of studies examining inter-strain differences in these lines reported that active coping was associated with *reduced* AVP activity in the amygdala-BNST-lateral septum system (Beiderbeck et al., 2007; Compaan et al., 1993). In contrast, many of the studies reporting an association between *increased* activity in specific AVP systems and active coping have examined intra-strain variations in coping style, the present study included. This emphasizes that the involvement of heightened AVP activity in active coping may be relatively consistent when intra-strain differences in coping style are examined, whereas the association between AVP and inter-strain and inter-species differences in coping style appears to be more complex.

In the present study, the enhanced methylation of the AVP promoter in the MeA of passive responders was selective to CpG site 2 (CGCG sequence), with no difference observed at CpG site 1 (CCGG sequence). CpG site 2 is located in close proximity to a number of binding sites for transcriptional regulators, one of which may be of particular importance to the present study: progesterone response elements (PREs; Auger et al., 2011; Mohr and Richter, 1990; Shapiro et al., 2000a). In contrast, CpG site 1 is not located in close proximity to such transcriptional regulators (Auger et al., 2011). Progesterone and AVP appear to have opposing effects in the BST-MeA-Septum system (Auger and Vanzo, 2006). Expression of progestin receptors on AVP cells appears to be unique to this system as none of the AVP cells in the suprachiasmatic, supraoptic and paraventricular nuclei exhibit progestin receptor immunoreactivity (Auger and De Vries, 2002). Further, progesterone treatment of adult male rats suppresses AVP expression in the BNST and MeA, and infusion of AVP into the LS rescues progesterone induced impairment in social recognition in adult male rats (Auger and Vanzo, 2006; Bychowski et al., 2013). Progesterone is thus an alternative mechanism to testosterone (levels of which did not differ between active and passive responders) by which this AVP system can be regulated, suggesting progesterone may act to influence behaviors via its regulatory action on AVP (Auger and Vanzo, 2006).

It is possible that the increased progesterone in the passive responders is acting at progestin sensitive transcriptional regulators in the MeA to selectively methylate the AVP CpG site 2, which is proximal to the PREs, unlike CpG site 1 (which did not differ between responder types). While the specific involvement of proximal PREs in AVP gene expression has not been explored, other HREs, such as estrogen response elements, that are proximal to the AVP promoter have been shown to play a functional role in regulating AVP expression (Shapiro et al., 2000b). As such, our study suggests that examination of a role for proximal PREs in regulating AVP gene expression through epigenetic mechanisms could be of interest in future studies.

Given the specificity of the methylation in passive responders to AVP CpG site 2, exploration of the downstream sites that are specifically affected by methylation of this promoter region could provide important insights into the neurobiology of coping. Furthermore, there may be merit in targeting the methylation status of CpG Site 2 in the MeA with novel treatments for psychiatric disorders associated with a passive coping style, such as depression and anxiety disorders. However, caution must be taken in interpreting the present findings until a more detailed analysis of the relationship between the methylation status of the promoter, gene expression and protein synthesis and release is conducted as there is a possibility that the gene is transcribed but the protein is not made, appropriate post-translational modification does not occur, or AVP is synthesized but not released.

The behavioral and biological differences between active and passive responders reported here cannot be explained as simple anosmia to the cat stimulus since we have previously shown that the differences between active and passive responders generalize to tests that do not involve an olfactory threat stimulus (Bowen et al., 2013). Furthermore, the defensive response to predator odor is still present in active responders, it simply differs in type and magnitude (Bowen et al., 2013). Finally, during fur exposure the active responders actually show greater activation in the accessory olfactory regions responsible for processing predator odors, likely due to their increased contact with the stimulus (Bowen et al., 2013). As such, it is safe to conclude that the difference between these responder types lies not in their ability to sense the predator stimulus, but rather in the way they respond once the threat has been identified.

This study demonstrated for the first time that there are marked epigenetic differences in the MePD AVP system between active and passive responders which suggest active responders have greater activity in this system. This provides further weight to the hypothesis that heightened AVP activity in the MeA plays an important role in active coping and provides evidence of an epigenetic mechanism through which the heightened activity in this system might be maintained in active responders. The epigenetic differences observed in the present study were not related to testosterone levels, suggesting another important mechanism exists for maintaining the methylation status of the AVP promoter region CGCG sequence in the MeA, with progesterone being a candidate worthy of

further consideration. Finally, our findings emphasize the importance of examining the association between circulating levels of the endocrine hormones progesterone and corticosterone and different coping styles.

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Appendix A. Supplementary data

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Chapter 6: General discussion

6.1. Chapter overview

The principle aims of this thesis were to: (1) develop a novel rodent model of defensive aggregation; (2) delineate individual differences in coping styles to environmental threats using this model; (3) explore the neural substrates of, and neuropeptide influences on, defensive aggregation and different coping styles towards predatory threat; and (4) explore the subtle psychological benefits that may arise from defensive aggregation or an active coping style toward predatory threat.

These aims were addressed by exposing groups of varying sizes (individual rats, groups of two, and groups of four rats) to predatory threat (cat fur) and conducting detailed behavioural assays, c-fos immunohistochemistry, pharmacological manipulations and analysis of endocrine and epigenetic differences. Pharmacological manipulations of OT and AVP systems enabled exploration of the role of these neuropeptides in the social response to threat and their more general role in anxiety. This provided preliminary information on the potential therapeutic value of OTR and $V_{1A}R$ based treatments of psychiatric disorders characterised by social isolation and withdrawal in the face of threat.

The introductory chapter of this thesis proposed seven hypotheses regarding defensive aggregation and coping styles towards predatory threat. These were:

1. Rats may aggregate in response to cat odour and other unconditioned stressors.

2. Defensive aggregation may allow social buffering of behavioural and neural responses to predatory threat.

3. There may be a clear and consistent distinction between active and passive coping styles in outbred rats exposed to predatory threat in groups.

4. The expression of an active coping style in response to predatory threat may depend upon the presence of conspecifics.

5. Passive coping styles may be associated with a greater social response to threat.

6. Active coping towards predatory threat may foster greater resilience during chronic stress and lower levels of anxiety and depression-like behaviour.

7. The neuropeptides OT and AVP may be involved in the social response to threat and in the mediation of active and passive coping styles.

The work presented in this thesis has, for the most part, provided support for these hypotheses. The first section of this concluding chapter summarises and discusses the main findings of each chapter and relates findings back to the above hypotheses. The second section of the chapter discusses the broader significance of the work presented in this thesis, its limitations, and potential directions for future research.

6.2. Summary of findings

6.2.1. Chapter 2. Huddling elicited in rats by cat fur and bright light

Chapter 2 (Bowen *et al.*, 2012) described the first laboratory model of defensive aggregation, which examines groups of laboratory rats' response towards predatory threat. While the visible burrow system of Blanchard and Blanchard (1989) reported elements of defensive aggregation in rats exposed to a cat, this was not the primary intent of their model. We showed that groups of four, but not two, rats exposed to either cat fur or bright light huddle closely together for long periods. This response was accompanied by other behavioural features that are customary in the reaction to unconditioned stressors, namely decreased locomotion, arrest of non-defensive behaviours, and, in the case of the cat odour, avoidance of the threatening stimulus (Apfelbach *et al.*, 2005; Walker and Davis,

1997). This study thus provided important proof of concept in demonstrating that defensive aggregation can be readily elicited in the laboratory by exposing laboratory rats to cat fur. This provided a novel opportunity to explore the underlying neurobiology and pharmacology of defensive aggregation in mammals. Arguably, defensive aggregation should be added to reduction of activity, arrest of non-defensive behaviours and movement to a safer location as a fourth fundamental "pillar" of the rodents' defensive behavioural response to predatory threat.

The findings of Chapter 2 emphasised the importance of overall group size in determining the expression of defensive aggregation. Huddling was only observed in groups of four, but not two rats exposed to cat odour or bright light. This is consistent with Hamilton's (1971) theoretical and mathematical framework which predicts that minimal immediate survival advantage is afforded by huddling in groups of only two animals. Subsequent experimental work examining fish predation on the marine insect *Halobates robustus* provided strong evidence that survival advantage promoted through the *dilution effect* does indeed have a strong positive relationship to group size (Foster and Treherne, 1981).

Chapter 2 showed that defensive aggregation was not only elicited by predatory threat, but also by another anxiogenic stimulus: bright light. This confirms previous anecdotal observations of huddling in response to non-predator related unconditioned stressors, such as lightning (Hamilton, 1971). It is worth considering the possibility that the huddling and more general anxiety-like response of rodents to bright light reported in this study may, however, be indirectly related to predator defence (Navara and Nelson, 2007). Aerial predators pose a special threat to rodents (Brachetta *et al.*, 2014), and these predators rely

heavily on vision to locate their prey. As such, fear and, if possible avoidance, of bright light, under which the vision of these predators is at its most acute, would promote survival. Consistent with this, rodents are nocturnal foragers and tend to remain in their burrows for most of the daylight hours (Fenn and Macdonald, 1995). However, rodents also alter their activity pattern in response to much more subtle changes in light. For example, desert-rats and deermice tend to remain in their burrows during the full moon and those that do not suffer increased risk of predation by nocturnal predators due to their heightened visibility (Clarke, 1983; Daly *et al.*, 1992).

6.2.2. Chapter 3. Huddling elicited by cat fur: coping styles, neural substrates and social buffering effects.

Active vs passive coping

Chapter 3 (Bowen *et al.*, 2013) identified a clear distinction between active and passive coping styles towards predator threat when a large cohort of rats was exposed to a cat fur stimulus in groups of four. The study found that coping styles (namely, active *versus* passive) are highly consistent across repeated exposures to predatory threat and generalise to other behavioural tests, with an active coping strategy associated with lower levels of generalised anxiety-like and depression-like behaviour.

These findings are largely consistent with the human literature which has found that passive coping individuals are more prone to mood and anxiety disorders than active coping individuals (Fletcher *et al.*, 2013; LeDoux and Gorman, 2001; Muris, 2002; Zhang *et al.*, 2014). Interestingly, while rats with a passive response style displayed conditioned fear in the context in which they had been exposed to cat fur, no such effect was evident in the active responders. Given that contextual fear conditioning to predator odour is a

preeminent animal model of PTSD (Berardi *et al.*, 2012; Siegmund and Wotjak, 2006), the striking difference in contextual fear conditioning between active and passive responders in our model speaks to its possible utility for exploring the relationship between coping strategies and PTSD. Clinical studies suggest that a passive coping style in the face of stress is a risk factor for developing PTSD following a traumatic event (Zhang *et al.*, 2014). In our study, rats showing a passive coping style displayed a pronounced increase in generalised anxiety-like behaviour following the chronic predatory stress exposure. Conversely, no such increase in anxiety-like behaviour was observed in active coping rats. This suggests that a passive coping style may be associated with greater susceptibility to the lasting negative impacts of chronic stress.

The second experiment reported in Chapter 3 showed that the expression of active coping toward predatory threat is at least somewhat dependent on the presence of other conspecifics. Again we observed the pronounced distinction between active responders, who engaged in a high number of contacts with the cat fur, and passive responders who avoided the cat fur stimulus and instead spent most of the session huddling with the other rats. None of the rats exposed to cat fur alone in the large arena displayed an active coping style toward the predatory threat, while there were both active and passive responders evident amongst those exposed in a group. Thus, the expression of active responding to predatory threat, which poses a salient and drastic consequence (death), may require the additional motivation provided by the presence of conspecifics. This is consistent with field and laboratory studies conducted in a number of species which indicate active responding to predatory threat has a social component of establishing and/or cementing social status

within the group (Blanchard and Blanchard, 1989; Mloszewski, 1983; Zahavi, 1990; Zahavi and Zahavi, 1997).

As mentioned above, Chapter 3 also made the important observation that passive responders spend much more time engaged in defensive aggregation than active responders. This is consistent with reports from studies of other animal species and from studies of humans that have found passive coping individuals tend to give preference to seeking out social contact/support as a coping strategy and that they gain greater benefit from that social contact/support than individuals with an active coping style (Nolen-Hoeksema and Davis, 1999; Reimert *et al.*, 2014). Our model thus provides a potentially useful new way of exploring the relationship between passive coping and a social response to threat.

Chapter 3 also reported that neural activation in several brain regions, as indicated by c-Fos expression, differed between rats showing active versus passive coping styles. Active responders showed greater activation of the AOB, likely due to their increased contact with the cat odour stimulus, and decreased activation in the anterior olfactory nucleus (AOL), ventral part of the lateral septum (LSV), AcbSH, somatosensory barrel cortex (BC), and medial caudate putamen (CPuM). The greater activation in the LSV in passive responders exposed to cat fur is of particular interest as higher levels of OT binding in this region is associated with heightened social behaviour in female mice (Curley *et al.*, 2009). It may be that the increased activation in this region in passive responders in response to the cat fur plays some role in their heightened social response to the threat and the possible involvement of OT warrants further exploration.

Social buffering

Exposure to predatory threat in a group of four rats, as opposed to exposure when alone, resulted in clear differences in the behavioural and neural response to the same stressor. Rats exposed in a group showed a less pronounced defensive response across all three of the primary defensive responses to predatory threat: reduction of activity; cessation of non-defensive behaviours; and avoidance of the threating stimulus. Specifically, group exposed rats showed less locomotor suppression, more grooming and greater levels of stimulus contact in response to cat fur. These findings provide important support for our hypothesis presented in the introduction that an important and hitherto unreported function of defensive aggregation is to provide social buffering of the stress response.

This acute social buffering would allow individual animals to more quickly return to non-defensive behaviours than if they were alone. This is reminiscent of the *group vigilance effect* whereby diminished vigilance by an individual animal within a group does not drastically reduce overall predator detection capabilities of the group (Elgar, 1989; Hamilton, 1971; Lima, 1995; Roberts, 1996; Rogovin *et al.*, 2004). Therefore, animals within a group can reengage in other activities important for survival, such as self-maintenance and foraging, without drastically compromising their ability to detect a predator. This is consistent with other studies demonstrating a balancing of risk and reward is an integral part of the response to predatory threat. For instance, mice placed in a predator risk-taking task will carefully balance their foraging and defensive behaviours based on the trade-off between the probability of being attacked (based on salience of the predatory cue) relative to the value of the reward (based on the quality and quantity of the food available) (Dent *et al.*, 2013).

Groups of rats exposed to cat fur also showed buffering of the neural response to predatory threat, with reduced Fos expression in a number of key regions relative to individually exposed rats. These included the lateral preoptic nucleus (LPO), CPuM, LHb and dorsomedial PAG (DMPAG). The increased activation in the posterior AOB seen in group-exposed rats is again likely due to the increased stimulus contact with the predator odour stimulus in these animals, as this region is critically involved in the sensory processing of cat odour (McGregor *et al.*, 2004; Staples *et al.*, 2008; Takahashi, 2014). The LPO and DMPAG are part of a distributed system previously implicated in the defensive response to cat odour (Apfelbach *et al.*, 2005; Takahashi, 2014). For example, lesions of the dorsal PAG interfere with both the cardiovascular and behavioural responses to cat odour exposure in rats (Dielenberg *et al.*, 2004). As such, the reduced magnitude of defensive responses in socially exposed rats is consistent with the attenuated neural activation observed in these brain regions involved in defensive behaviour.

6.2.3. Chapter 4. The role of oxytocin and vasopressin in social responding to threats

Chapter 3 established that defensive aggregation in the face of predatory threat is associated with a pronounced buffering effect at a behavioural and neural level. Chapter 4 (Bowen and McGregor, 2014) explored whether the social response to threat might be modulated by neuropeptides, and if OT or AVP might play a permissive role in the motivation to initiate social contact in response to stress. This motivational factor has not been subject to much previous analysis in the social buffering literature and could be of critical importance to the development of novel treatments for disorders characterised by social isolation and withdrawal in the face of threat (Wirth, 2011). We were particularly interested in OT and AVP as these neuropeptides play an important role in motivating

conspecifics toward social behaviour under appetitive or positive affiliative conditions (Neumann, 2008; Ramos *et al.*, 2013; Witt *et al.*, 1992; Young and Wang, 2004), but their role in promoting social affiliation in mammals in threatening situations had yet to be examined.

The experiments presented in Chapter 4 established that both OT and AVP increase defensive aggregation in response to predatory threat but that they appear to do so through different mechanisms. Blockade of V_{1A}Rs with SR49059 reduced huddling in response to predatory threat but had no effect on the defensive response of individual rats to predatory threat. OT increased huddling to predatory threat but did not affect aggregation under baseline conditions or alter the defensive response of individual rats toward predatory threat. The augmentation of the social response to threat by OT was prevented by co-administration of SR49059. This finding is consistent with a growing literature indicating that a number of the prosocial effects of OT appear to be mediated by actions at the V_{1A}R, for which OT also has relatively high affinity (Chini and Manning, 2007; Manning *et al.*, 2012), rather than the OTR (Ramos *et al.*, 2013; Sala *et al.*, 2011).

AVP also increased huddling in response to predatory threat. However, unlike OT, the increased huddling to predatory threat induced by AVP was not blocked by SR49059. Rather, the AVP augmentation of defensive aggregation was prevented by administration of the V_{1B}R antagonist SSR149415. Unlike OT, AVP also increased huddling under baseline (no predatory threat) conditions and increased the defensive response to predatory threat in rats individually exposed to cat fur, an effect that was also blocked by the V_{1B}R antagonist.

These findings suggest that OT, via an action at the V_{1A}R, causes a specific enhancement of social responding to threat without affecting the more general anxiety response to cat

fur. Conversely, AVP causes a more global enhancement of anxiety via its actions at the $V_{1B}R$ and this leads to huddling under baseline (non-threatening) conditions and magnifies the huddling response to predatory threat. This implies there may be differential utility of OT and $V_{1A}R$ agonists compared to AVP as treatments promoting social responding to environmental threats. Targeting the $V_{1A}R$ is of greater potential given that it does not apparently involve a global exacerbation of anxiety.

Chapter 4 also indicated that both OT and AVP may be involved in the normal processes of defensive aggregation in mammalian species. This is consistent with the demonstrated involvement of various analogues of OT and AVP in the defensive aggregation observed in fish and avian species (Braida *et al.*, 2012; Goodson and Kabelik, 2009; Goodson *et al.*, 2009; Kelly *et al.*, 2011). The similar role we identified for these neuropeptide families in rats suggests that the mechanisms driving social responding to threat appear to be highly conserved across species. Overall, this fits within the general framework that sees neuropeptides of the vasotocin family as highly conserved in both structure and function across mammalian and non-mammalian species (Hoyle, 1999).

6.2.4. Chapter 5. Endocrine and epigenetic correlates of active coping

The final experimental chapter in this thesis (Bowen *et al.*, 2014) explored neuroendocrine and epigenetic correlates of active and passive coping styles in rats confronted with predatory threat. The behavioural characterisation of active and passive coping styles toward predatory threat, first reported in Chapter 3, was confirmed in another large cohort of rats. Again, there was a temporally consistent difference in predator approach behaviour, whereby active responders engaged in far more contact with the cat

fur than passive responders, who instead tended to spend greater time engaging in defensive aggregation.

Endocrine analysis indicated that passive responders had markedly higher levels of corticosterone and progesterone than active responders three days after the final fur exposure. In humans, cortisol and progesterone levels are generally found to be positively correlated with stress and anxiety with some studies suggesting that increased cortisol may induce progesterone release (for a review see Wirth, 2011). As such, the observed elevations in both of these steroid hormones in passive responders is consistent with their higher baseline levels of anxiety-like behaviour reported in Chapter 3. Elevated basal corticosterone levels have been previously observed in passive responders in some earlier studies of active and passive coping involving other behavioural paradigms (Korte *et al.*, 1992; Korte *et al.*, 1996). Differences in progesterone are also consistent with previous demonstrations that progesterone treatment reduces aggression, a trait that is often used as a proxy for active coping. Lower offensive aggression is usually associated with a more passive coping style (Koolhaas *et al.*, 2010; Veenema and Neumann, 2007) in male rodents and hamsters (Fraile *et al.*, 1987; Frye *et al.*, 2006; Hull *et al.*, 1980; Miczek *et al.*, 2003). Consistent with previous studies of coping styles, we observed no difference between active and passive responders in either plasma or testicular testosterone levels (Koolhaas *et al.*, 2010).

Perhaps the most striking finding reported in Chapter 5 was the more than 4 fold greater methylation of a CGCG promoter region of the AVP gene in the MeA in passive responders. Overall, this implies that active responders towards predatory threat have much greater activity in this AVP system. Several previous studies illustrate greater activity

in the MeA-BNST AVP system (which projects to the lateral septum) in active compared to passive responders (Bester-Meredith *et al.*, 1999; Delville *et al.*, 1996; Ferris *et al.*, 1984; Irvin *et al.*, 1990; Koolhaas *et al.*, 1998; Nishi *et al.*, 2013; Wersinger *et al.*, 2002). Our findings suggest that epigenetic regulation of the AVP gene expression may be involved in maintaining the differential activity in this system which underpins different coping strategies.

6.3. Broader implications and future directions

6.3.1. Localisation of the OT and AVP systems involved in defensive aggregation

Chapter 3 reported the intriguing finding that the amount of Fos activation in the lateral septum was strongly correlated with defensive aggregation, while in Chapter 4, blockade of the $V_{1A}R$ by SR49059 inhibited huddling and peripheral administration of OT enhanced the huddling response via an action at $V_{1A}Rs$.

The peripheral route of administration used in the experiments presented in in Chapter 4 did not allow localisation of the effects of the peripherally administered peptides and antagonists to a specific brain region. However, the lateral septum is a region rich in $V_{1A}Rs$ and stimulation of the non-mammalian analogue of the $V_{1A}R$ in the lateral septum has been heavily implicated in defensive aggregation in estrildids (Goodson and Kabelik, 2009; Goodson *et al.*, 2009; Kelly *et al.*, 2011). Given the striking conservation of the vasotocin-family neuropeptide system in both structure and function across mammalian and non-mammalian species (Hoyle, 1999), and the profound conservation of defensive aggregation as a response to predator threat across species (Chen and Kolokolnikov, 2014; Foster and Treherne, 1981; Fryxell, 1995; Kirkwood and Robertson, 1999), the lateral septum appears to be a good starting point for future studies aimed at localisation of the neurobiological

systems driving defensive aggregation in mammals. As such, future studies might examine the effect of directly infusing OT or a $V_{1A}R$ antagonist into the lateral septum on defensive aggregation.

6.3.2. The role of the lateral habenula

Chapter 3 reported activation of the LHb by exposure to predatory threat and the subsequent reduction of that activation by social exposure. These findings were of particular interest since the LHb had not been considered in earlier studies of defensive behaviour toward predatory threat. We found that predatory threat activated the LHb to a similar extent as "classic" predator odour activated regions such as the MePV (Apfelbach *et al.*, 2005; McGregor *et al.*, 2004; Takahashi, 2014).

It has been proposed that the LHb connects the limbic system with the basal ganglia to control motoric programs involved in defensive responses (Hikosaka, 2010; Hikosaka *et al.*, 2008; Pobbe and Zangrossi Jr, 2010). In some ways the LHb can be thought of as a "central command station" receiving inputs from regions involved in detecting aversive stimuli and sending outputs to regions involved in coordinating the appropriate behavioural response (Hikosaka, 2010; Hikosaka *et al.*, 2008). For example, aversive situations activate the LPO which then projects to the LHb causing increased activity in LHb neurons (Hikosaka, 2010; Hikosaka *et al.*, 2008; Pobbe and Zangrossi Jr, 2010). Interestingly cat odour induced activation in the LPO was also inhibited by social exposure (Chapter 3).

Once receiving its inputs, the LHb then projects to the dorsal PAG via the DRN causing an increase in 5-HT mediated neurotransmission, resulting in immobility/freezing and cessation of non-essential activities such as grooming (Hikosaka, 2010; Hikosaka *et al.*, 2008; Pobbe and Zangrossi Jr, 2010). Again, it is interesting to note that cat fur induced activation

in the dorsal PAG was also inhibited by social exposure to the predatory threat (Chapter 3). The pathways connecting parts of the basal ganglia, such as the CPuM, to the LHb have been proposed as the primary controllers of the motoric response to failure and punishment, and are central to behavioural inhibition (Hikosaka, 2010). Specifically, it has been proposed that punishment increases neuronal activity in the basal ganglia which then projects to the LHb causing inhibition of dopamine neuronal activity and a subsequent decrease in dopamine release in regions of the striatum, resulting in avoidance of the action that led to punishment (Hikosaka, 2010; Hikosaka *et al.*, 2008).

Given the powerful activation of the LHb observed in the present study, future work might explore LHb involvement in controlling, in particular, motoric aspects of the defensive response to predator threat. This could be done by lesioning or pharmacologically deactivating (by, for example, muscimol infusion) the LHb. Based on the work above, this would be predicted to interfere with the motoric aspects of the defensive response to cat fur. Given the pronounced reduction in LHb activation in socially exposed animals the role of the LHb in social buffering also warrants further examination. This is further supported by work conducted by another group since we published the study presented in Chapter 3, which found that social isolation increases c-Fos activation in the LHb in post-weaning rats and subsequent social-play reduces c-Fos activation in this region (van Kerkhof *et al.*, 2013).

6.3.3. A possible role for the balance of AVP and progesterone in active, passive and maladaptive coping

Chapter 5 reported that passive coping toward predatory threat is associated with decreased AVP activity in the MeA, consistent with numerous previous studies, and increased progesterone. In Chapter 3, we showed that passive responders engage in far more aggregation in response to predator threat, consistent with previous studies

demonstrating that passive responders show greater preference for, and benefit from, social responding to threat (Nolen-Hoeksema and Davis, 1999; Reimert *et al.*, 2014). Interestingly, it has been argued that progesterone may promote an affiliative response to stressful situations (Wirth, 2011). For example, administration of progesterone or its metabolite allopregnanolone increases passive social contact (Frye *et al.*, 1998) and, as discussed above, reduces aggression (Fraile *et al.*, 1987; Frye *et al.*, 2006; Hull *et al.*, 1980; Miczek *et al.*, 2003). In humans, both exogenous administration of progesterone and higher endogenous progesterone are associated with greater motivation for affiliation (Schultheiss *et al.*, 2003).

Based on this information, it can be proposed that active coping involves greater AVP activity in the MeA and lower progesterone levels, which may be involved in promoting a heighted aggressive and proactive response to stressors as opposed to the social response more common in passive responders. Conversely, passive coping appears to involve lower AVP activity in the MeA which may influence their more reactive response to the stressor and their greater susceptibility to adverse consequences of the stressor exposure. However, passive responders appear to partly counteract this heightened susceptibility through their social response to the stressor, perhaps mediated by their higher progesterone levels, which results in buffering of the stress response. Maladaptive coping (see Fig 1) appears to comprise of the reactive components of the passive response style without the important social coping strategies that buffer the stress response.

Consistent with this hypothesis, people suffering from a number of psychological disorders (such as schizophrenia, depression, bipolar disorder and PTSD) often display a passive coping style combined with a maladaptive, anti-social response to threat (Chevallier

et al., 2012; Fletcher *et al.*, 2013; Lam and Wong, 1997). Furthermore, numerous studies have found levels of progesterone and/or its metabolite allopregnanolone are lower in persons suffering from these disorders (for a review see van Broekhoven and Verkes, 2003; Wirth, 2011). Interestingly, pharmacological treatment of depression with SSRIs or other antidepressants results in a normalising of progesterone or allopregnanolone levels over time as well as improvements in social functioning (Romeo *et al.*, 1998; van Broekhoven and Verkes, 2003).

Future studies might usefully explore how reciprocal interplay between AVP and progesterone activity in the MeA-BNST-lateral septum axis might lead to different coping styles (see Fig 1) via pharmacological manipulation of these systems. This might conceivably result in novel treatments for psychiatric disorders that promote either a more active response style or a social response to threat. Part of this approach would require manipulation of the social response to threat in rodents with social withdrawal-like behaviour that mimics that observed in persons suffering a number of psychiatric disorders.

Socially withdrawn rats could be produced through a number of means. Cumulative observations during the studies presented in this thesis suggest that there are a small number of rats within each cohort that avoid both the cat fur stimulus as well as contact with the other rats when they are socially exposed to cat odour. These rats could prove to be a very useful model of the maladaptive coping strategy described earlier in this Chapter (see Fig. 1).

Another useful approach would be to induce social avoidance using, for example, repeated phencyclidine (PCP) administration which causes profound social withdrawal in rats (Lee *et al.*, 2005). Interestingly, OT administration has been shown to rescue PCP social

impairments in rats under normal (i.e. non-heightened threat) conditions (Lee *et al.*, 2005). Another useful method for inducing social withdrawal in rodents is through social fear conditioning (Toth *et al.*, 2012b). This paradigm involves administering a foot shock to the rodent whenever they make contact with a conspecific kept inside a cage in the arena, eventually resulting in avoidance of conspecifics. OT administration (ICV) abolishes this conditioned social avoidance (Toth *et al.*, 2012a). Given the utility of OT in inducing a social response to threat in our model, and its ability to reverse social deficits in models of social withdrawal and social fear conditioning, OT may be particularly effective in enhancing social motivation during threatening situations in individuals with a maladaptive coping style.

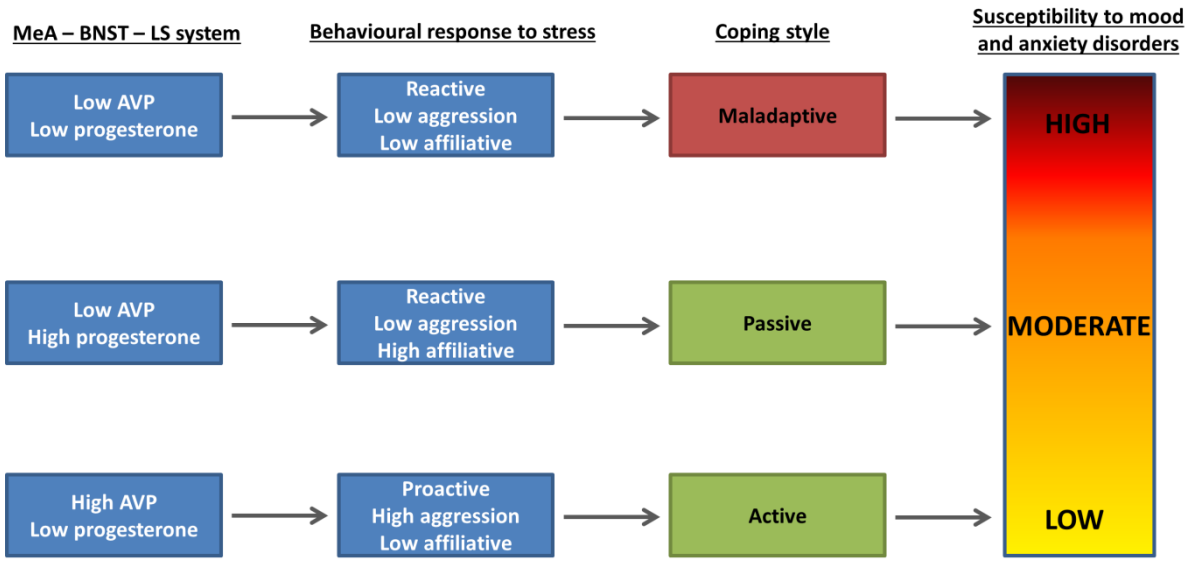

Figure 1. **A proposal of how balance of AVP and progesterone in the MeA – BNST – lateral septum system may influence coping style and susceptibility to psychological disorders.** High AVP and low progesterone in the MeA-BNST-LS system may promote an active coping style characterised by a proactive, aggressive response to stress rather than a reactive and affiliative response. This proactive and resilient response style affords a low risk of developing stress-related mood and anxiety disorders. Due to their more reactive or "helpless" response style, passive responders have an increased risk of developing stress-related mood and anxiety disorders. However, they counteract the increased risk brought about by their reactive coping strategy by seeking out social support in times of stress which subsequently buffers the stress response, counteracting some of the impact of their less resilient coping style. Finally, those with low AVP and low progesterone in the MeA-BNST-LS system have a reactive response to stress but do not counteract this with seeking out social support, and instead remained isolated and withdrawn during and after stressful events or periods. This results in a high susceptibility to mood and anxiety disorders.

6.3.4. A possible role for progesterone in mediating AVP CGCG promoter methylation in the MeA

Chapter 5 reported that a passive coping style was associated with greater methylation of the CGCG promoter region, but not the CCGG promoter region, for the AVP gene in the MeA. Previous work indicates that the methylation status of both the CGCG and CCGG promoter regions are maintained by testosterone, with higher testosterone levels associated with lower methylation and thus greater activity in this system (Auger *et al.*, 2011). However, the lack of plasma or testicular testosterone differences between active and passive responders (Chapter 5) suggests another mechanism is likely responsible for maintaining the differential methylation status of this promoter region in active and passive responders.

One possibility is that testosterone differences present only during early development are involved. This idea is supported by a small number of avian studies. Infusion of testosterone into Japanese quail eggs produces a more active coping phenotype in the offspring in an open-field test and restraint-stress task (Niall Daisley *et al.*, 2005). These effects of testosterone on coping style are usually short-lasting, suggesting it is unlikely that transient elevation during this developmental phase results in long-lasting epigenetic changes in the MeA AVP system (Koolhaas *et al.*, 2010). However, one study found injection of testosterone into the eggs of Black-headed gulls resulted in enhanced active responding that lasted up to one year following hatching, suggesting more enduring changes to coping style are possible following transient changes in testosterone levels during early development (Eising *et al.*, 2006). Future studies of active and passive coping using our model might monitor testosterone levels in the prenatal and early postnatal stage to

determine if any transient differences in testosterone in this early developmental epoch are correlated with different epigenetic profiles and coping styles later in life.

Perhaps a more interestingly possibility is that the different methylation status of active and passive responders reported in Chapter 5 is related to the robust differences in progesterone levels that we also found between active and passive responders. The CGCG promoter region, but not the CCGG promoter region, is in close proximity to progesterone response elements and these may play a role in regulating the methylation status of this promoter region (Auger *et al.*, 2011; Mohr and Richter, 1990; Shapiro *et al.*, 2000). Indeed, previous work demonstrated that progesterone and its metabolite, allopregnanolone, downregulate AVP gene transcription (Patchev *et al.*, 1996; Patchev *et al.*, 1994), although its ability to do this through methylation of AVP promoter regions has yet to be examined. In general, progesterone and AVP appear to have opposing effects in the BNST-MeA-lateral septum system (Auger and Vanzo, 2006). Expression of progestin receptors on AVP cells is unique to this system with AVP neurons in the suprachiasmatic, supraoptic and paraventricular nuclei showing no progestin receptor immunoreactivity (Auger and De Vries, 2002). Further, progesterone treatment of adult male rats suppresses AVP expression in the BNST and MeA and infusion of AVP into the lateral septum rescues progesterone-induced impairment of social recognition in adult male rats (Auger and Vanzo, 2006; Bychowski *et al.*, 2013).

Future studies might explore the possibility that progesterone regulates AVP activity in the MeA-BNST-lateral septum system through increasing methylation of the CGCG promoter region for the AVP gene. This could be done mechanistically by using progesterone implants which would increase progesterone levels and, if the aforementioned hypothesis is correct,

would subsequently increase methylation of the CGCG promoter region of the AVP gene in the MeA and potentially induce a more passive coping style.

6.3.5. Defensive aggregation in females

One limitation of the work in this thesis is that it examined defensive aggregation exclusively in male rats. This was a considered decision based on the complexity immediately added to studies that involve female subjects. When females are included, the stage of the estrous cycle has to be controlled, which drastically increases the required number of subjects when group exposure is involved or requires that ovariectomies be performed. As such, we made the choice to examine male rodents while establishing the model and conducting the preliminary studies. However, our understanding of the defensive aggregation response in male rats is now at a stage of maturity that would benefit from a complementary examination of female rats.

The first studies might examine defensive aggregation in all-female groups of rats exposed to cat fur. The field literature usually reports an even more pronounced aggregative response to threat in females. For example, when African Buffalo are under predatory threat they will huddle together with the females and their young at the centre of the huddle and the males surrounding them (Mloszewski, 1983). In the laboratory "visible burrow system" study by Blanchard and Blanchard (1989), the female rats were the last to return to the surface from the huddle in the burrow following the introduction of a live predator to the burrow surface (Blanchard and Blanchard, 1989). The *tend-and-befriend* theory argues that social responding to threat is of particular importance to females. It is argued that the typical *fight-or-flight* response is not always the most suitable response for females, who may need to protect offspring that are not yet capable of successfully

engaging with or fleeing from a predator (Taylor *et al.*, 2000). In the human literature, the *need for affiliation* is stronger in females and high levels of affiliative behaviour seem more related to mood and wellbeing in females than in males (Wong and Csikszentmihalyi, 1991). Therefore, we would predict that the social response to threat might be even stronger amongst groups of females exposed to predatory threat. It will also be interesting to explore variations in this response throughout stages of the estrous cycle, which sees large variation in circulating levels of progesterone.

Another important question is what differences in behaviour might be observed in mixed-sex groups of rats exposed to predatory threat in our model? The field literature indicates that one of the functions of active responding to predatory threat appears to be demonstration of reproductive fitness to potential mates. We might then predict that the presence of females would augment the behaviour of the males. In the field, the most active responding males usually take up the most dangerous positions, such as the perimeter of a herd of African Buffalo and the Sentinel position toward the top of the canopy in a group of Arabian Babblers (Mloszewski, 1983; Zahavi, 1990; Zahavi and Zahavi, 1997). Interestingly, these active responding males appear to enjoy enhanced reproductive success, which is particularly striking in populations of Arabian Babblers in which the dominant, active responding males produced 95% of the offspring in one study (Lundy *et al.*, 1998). These studies suggest that the active responding males' response to predatory threat may become even more pronounced with females present. We might also predict that the females would take up the more protective positions within the huddle, with the active responder type males on the perimeter.

Finally, characterisation of defensive aggregation and coping strategies in females would facilitate cross-fostering studies aimed at understanding the heritability of different coping strategies toward predatory threat. The genetically selected strains of active and passive coping mice and rats speak to the genetic heritability of active and passive coping (Koolhaas *et al.*, 2010). However, there may also be environmental components and, as indicated by our work in Chapter 5, epigenetic components that contribute to coping style that may be passed on through rearing. There have been few cross fostering studies conducted examining these various factors and those that do exist are somewhat conflicting. For instance, pups from the passive coping BALB/cByJ mouse strain raised by dams from the more active coping C57BL/6ByJ strain grow up to exhibit a less pronounced corticosterone response and lower PFC dopamine utilisation in response to a loud-noise stressor, similar to the response observed in the active coping C57BL/6ByJ mice (Prakash *et al.*, 2006). Interestingly, these effects were asymmetrical with no change in coping style observed in C57BL/6ByJ mice raised by BALB/cByJ dams. In contrast to this study, cross-fostering did not alter the behavioural phenotype of the active coping LAB rats or passive coping HAB rats (Wigger *et al.*, 2001). These studies have examined cross fostering using genetically selected strains and perhaps a more accurate indication of the various contributions of genetics, epigenetics and environment would be provided by using a cross fostering approach with a model such as ours, which examines natural variation in coping styles.

6.4. Conclusions

The research presented in this thesis has developed the first laboratory model of mammalian defensive aggregation. The core model shows that groups of four laboratory

rats exposed to an unconditioned stressor, bright light or cat odour, spend long periods tightly huddling together. This highly reliable and characteristic aggregation response to predatory threat was used as a platform to explore the neurobiological determinants of huddling and coping styles. Defensive aggregation might be considered a fourth fundamental behavioural response to predator threat in rodents alongside reduction in activity, cessation of non-threat related activities, and avoidance of the predator stimulus. Defensive aggregation provides hitherto uncharacterised acute social buffering of both the behavioural and neural response to predatory threat. Social buffering may have evolved as a means of promoting a swifter return to important non-threat related activities when the added safety of numbers ensures it will not drastically increase the probability of attack.

Peripheral administration of synthetic OT or AVP increased defensive aggregation in rats, albeit through different mechanisms. OT selectively increased the social response to threatening situations via actions at the $V_{1A}R$ but did not influence anxiety. Conversely, AVP enhanced anxiety-like behaviour via an action at the $V_{1B}R$ and subsequently induced a huddling response under baseline conditions and enhanced the huddling response to cat fur. These finding suggest that OT and $V_{1A}R$ agonists are of greater interest in the development of novel treatments for disorders characterised by dysfunctional social behaviour when under stress.

A clear distinction between active and passive responders to predatory threat was identified. However, expression of active coping toward predator threat appeared to be socially mediated being only expressed in the presence of conspecifics. In response to the predator threat, active responders engaged in less avoidance of the predator stimulus,

showed less immobility/freezing in response to the threat stimulus and no conditioned fear response to the context in which they were exposed to the threat.

Conversely, passive responders favoured defensive aggregation over risk assessment behaviour and showed pronounced conditioned fear responses when returned alone to the context in which they were exposed to the predatory threat. Active responders also had lower levels of generalised anxiety-like and depression-like behaviour compared to passive responders and were less susceptible to the negative behavioural effects of chronic predator stress exposure. Active responders showed less neural activation in response to predator threat in a number of limbic and brainstem regions involved in the expression of the anxiety and fear responses to predator threat. Furthermore, active responders had substantially lower baseline levels of corticosterone and progesterone and less epigenetic silencing of the MeA AVP system. These findings provide important targets for future studies examining the neurobiological underpinnings of different coping strategies.

The distinct active and passive coping styles identified in our studies are largely consistent with previous studies of coping strategies in rodents; however, the model presented in this thesis has several advantages over the paradigms used in earlier studies. In the first chapter of this thesis, we identified three prevalent issues with the most commonly used rodent models of active and passive coping: (1) use of unnatural stressors such as shock; (2) measurement of aggression as a proxy for active coping; (3) lack of equivalency in the outcomes of different response strategies; and/or (4) over reliance on genetically selected strains. The new model presented in this thesis overcomes these issues by: facilitating a rapid, clear and consistent identification of active and passive responding rats within a single outbred strain and in response to a naturalistic, ethologically valid stressor;

and ensuring that the primary outcome (avoidance of a predator encounter) is equivalent between active and passive responders.

The model presented in this thesis will hopefully be used in the future to further probe the neurobiology of social responses to threat and active and passive coping in mammals. It has demonstrated that more subtle benefits accrue from defensive aggregation than the immediate survival advantages identified in the field literature. Indeed, it is possible that *social buffering* evolved as a more subtle function of defensive aggregation that facilitates reengagement in important non-threat-related behaviours. Administration of OT or stimulation of V_{1A}Rs was identified as a means of selectively promoting social responding to threat without increasing anxiety-like behaviour. This is the first demonstration of OT enhancing social contact under threatening situations and our findings suggest that OT or V_{1A}R agonists could hold potential for treating disorders characterised by social withdrawal in the face of threat. Finally, we provide the first report of striking epigenetic differences in the MeA AVP system between active and passive coping rats, providing a potential mechanism through which the heightened activity frequently observed in this system in active responders might be maintained. It is hoped that the work presented in this thesis has served as a foundation for the future investigation of the neurobiological mechanisms driving, and adaptive benefits underlying, a social response to threat.

6.5. References

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Appendix 1: Rubbings deposited by cats elicit defensive behaviour in rats

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Rubbings deposited by cats elicit defensive behavior in rats

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ABSTRACT

Laboratory rats display pronounced defensive behaviors when confronted with a range of cat-derived stimuli, including collars worn by a cat, cloths rubbed on a cat, and cat fur. One possible explanation of this phenomenon (the "kairomone hypothesis") is that rats derive a survival advantage by eavesdropping on signals used by cats to communicate with each other. Cats are known to rub their bodies on objects at strategic environmental locations to signal their identity and mating potential to other cats. The current study assessed the sensitivity of laboratory rats to these body rubbings. In Experiment 1, food deprived Sprague–Dawley rats were trained to consume food pellets in one arm of a Y maze. On test day a damp cloth was placed near the food pellets that had been rubbed on a location (wall) where a cat had recently engaged in body rubbing. A control cloth and a collar worn by the cat were also tested. The presence of both the body rubbing residue and the cat collar increased latency to eat and decreased amount of food eaten. The disruption of consummatory behavior in the test environment was still evident 24 h later in the absence of odor stimuli. Experiment 2 tested the reaction of naïve Wistar rats to body rubbings using a paradigm in which rats were given the opportunity to hide. Relative to a control condition, rats exposed to a cotton pad wiped on a cat body rubbing location showed increased hiding behavior, decreased exploration and reduced stimulus approach and investigation. These defensive responses persisted for up to 4 days following a single stimulus exposure. These results suggest that rats eavesdrop readily on body rubbings cats use for identification purposes, providing further support for a kairomone hypothesis of predator odor avoidance.

1. Introduction

Detection of predator odors by prey species provides an important mechanism for anti-predator defense, alerting prey to the potential presence of a predator. Studies in both laboratory and field have shown multiple behavioral, endocrine, and reproductive effects of predator odors on prey, as well as persisting avoidance of locations where predator odors were encountered [1]. Recent experimental studies [e.g. 2] indicate that predator odors can activate prey accessory olfactory pathways associated with pheromone processing. This has led to suggestions that predator odors can serve as "kairomones": chemical signals that have a primary pheromonal communication function within the predator species but can be intercepted by a prey species, thereby increasing prey fitness relative to the predator [3,4]. This interception process is sometimes termed 'eavesdropping'.

The response of rats to cat odor is perhaps the predator–prey interaction most studied in laboratory settings, with a majority of early work in the Blanchard laboratory [5–8]. The exposure of rats to fabric collars worn by cats, cloth rubbed over a cat, or cat fur itself, produces immediate and long lasting defensive behavior [9–11]. Nonetheless, recent work [e.g. 11,12] indicates that our understanding of this phenomenon is not comprehensive, particularly with respect to the anatomical source and chemical profile of the effective odor stimulus. One potential odor source is cat cheek glands and related body rubbings. A "body rubbing" consists of a rubbing on an environment location, a person, or conspecific that starts from the cheek region and encompasses most of its flank [13,14]. The resultant residue appears to act as a feline social cue, conveying information about proximity, identity, familiarity, sex, and dominance of the individual [15–17]. However, it is presently unknown if body rubbing residue produces a defensive response in rats similar to that seen to a cat collar or cat fur. Given the frequency and ubiquity with which cats engage in rubbing behavior, it seems possible that rats eavesdrop on these markings and respond defensively.

The present research tested the hypothesis that body rubbing residue from a domestic cat acts as a kairomone for a domestic rat, leading it to withdraw from and avoid the environmental location associated with the odor. We tested this hypothesis, and the generality of any observed effects, by using two different experimental paradigms in two different laboratories using body rubbings from two different cats, and two different strains and sexes of laboratory rat. Experiment 1, conducted at Indiana University (USA), examined the response of female Sprague Dawley rats to a body rubbing residue,

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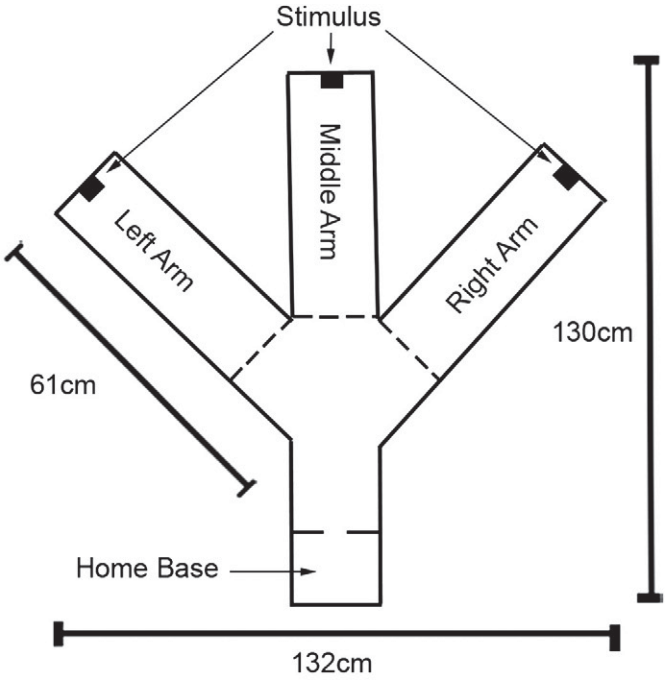
a cat collar or a control stimulus in a Y maze apparatus (Fig. 1). Hungry rats were trained to eat food pellets in this maze and were then exposed to one of the three test stimuli. We measured suppression of feeding in the presence of the stimulus and persistent defensive behavior in the test environment following its removal. We predicted that the cat collar, and perhaps the body rubbing residue, would reduce feeding and induce avoidance of the odor location in this paradigm [9,18].

Experiment 2, conducted at the University of Sydney (Australia), used a hide box paradigm [e.g. 2,10,19] (Fig. 2) to assess the response of male albino Wistar rats to body rubbing residue or to a control stimulus. It was hypothesized that the rats exposed to body rubbing residue would quickly show increased hiding, suppressed movement and reduced approach toward the stimulus location. We also predicted that on subsequent days these defensive responses would continue into the test context in the absence of the predator stimulus.



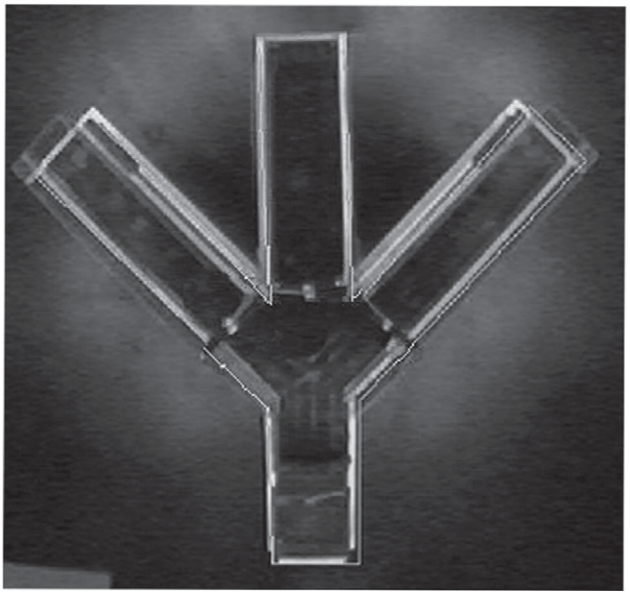


Fig. 1. A top view of the equiangular Y-maze used in Experiment 1 with areas demarcated via Ethovision tracking software. Each group had only one arm opened during a trial and the arm differed for each group.

2. Experimental design

2.1. Experiment 1

2.1.1. Subjects

The subjects were 22 female Sprague–Dawley rats (*Rattus norvegicus*) between 2 and 3 months old at the start of the experiment. The rats were housed in individual plastic cages in a colony room, initially under conditions of ad libitum food and water access. The colony room was light between the hours of 07:00 to 19:00 and dark during the remaining hours. Although we tested the animals during the light cycle for the convenience of the experimenter, their activity level was ensured by their circadian anticipation of receiving a daily food ration between 45 and 60 min after their test trial ended [20]. The colony room temperature was maintained at a range of 19–25 °C.

2.1.2. Screening

The Sprague–Dawley rat strain is commonly used in laboratory research. However, this strain has been found to be less reactive to cat odors than other common strains such as the Wistar or Long–Evans rat [9]. Pilot studies in our laboratory show that this strain contains sub-populations of "responders" and "non-responders" to cat odor, a finding similar to that reported in Lister rats [21]. We therefore screened for responders by presenting each rat in its home cage with a small plastic box, with 24 holes drilled in the walls, containing a clump of cat fur. The rats typically approached a box and manipulated and sniffed them. Within a minute or two responders would pause, look, and sniff outside their cage, then run to the opposite end of the cage and remain motionless for 5–10 min after the plastic box was removed. These behaviors are similar to those previously reported to the presentation of similar stimuli in home cages [22]. While the results of this screening procedure did not ultimately predict responding in the test apparatus with 100% accuracy, it increased the likelihood of responding from 50% to 70% of the subjects. In the present experiment 35 rats underwent the screening process and 22 were selected for further testing.

2.1.3. Food deprivation

Beginning 7 days before the experiment, each rat was given an amount of food suited for reducing and maintaining its weight at 85% of free feeding level. During the experimental phase the animals were weighed and fed after being returned to the colony room.

2.1.4. Odors

A standard nylon safety stretch cat collar stimulus was obtained from a 12 year old spayed female domestic house cat. The collar had been worn by the cat for the previous 9 months.

The body rubbing residue stimulus was obtained by rubbing a damp 7.6 cm × 10.2 cm square of clean cheese cloth against the corner of a kitchen refrigerator, where a 3 year old neutered male domestic house cat body rubbed daily, typically before feeding time.

A control stimulus was obtained by rubbing a damp piece of cloth on the refrigerator approximately 25–38 cm above the original marking location, an area out of reach for the cat. Both the collar and the marking samples were placed into plastic bags and stored at 2 °C when not in use. Before presenting the stimuli to the rats, the samples were warmed to room temperature by placing the bag under warm running water for 5 min.

2.1.5. Apparatus

The experiment took place in the stem and one arm of a wood multi-arm "Y-maze" (Fig. 1) with Plexiglas walls and pulleys for raising doors. Each arm was 61 cm in length and the height of the Plexiglas walls were 28 cm. Each arm and the release area had metal doors that could be opened or closed by 4 metal pull switches below the maze. On the end wall of each arm a small alligator clip was attached

at a height of 7.6 cm to allow easy presentation and removal of odor samples.

Each group was trained in a separate arm in order to reduce potential contamination effects among odors. Thus, the body-mark group had access only to the left arm, the control cloth group only the middle arm, and the cat collar group only the right arm. During each trial a total of 70 food pellets (97 mg weight, BioServ Corp.) were placed at the end of each "odor" arm on the floor directly below the alligator clips. Rats readily learned to move into the arm after relatively few daily exposures and typically ate 50 pellets during a 3 min trial.

A Panasonic video camera was hung from the ceiling at the center of the room. The camera was connected to a DVD recorder as well as to a monitor and computer, all located outside the experimental room. Sessions were recorded on the computer using the Ethovision tracking program (Ethovision 3.1, by Noldus Information Technologies, NL).

2.1.6. Procedure

Rats were randomly assigned to three groups; *a negative control group* to be exposed to the control marking stimulus (n = 6 rats), *a positive control group* to be exposed to the cat collar (n = 8), and *the experimental group* to be exposed to the body rubbings (n = 8). Groups were tested in a different order each day with trials run between the hours of 12:00 and 15:00.

The experiment consisted of three phases: a 5 day pre-exposure baseline, a 2 day odor exposure phase, and a 4 day post-odor exposure phase. During the pre-exposure baseline, a damp piece of clean cloth (for the negative control and experimental groups) or an unused piece of cat collar (for the positive control group) was attached to alligator clips at the end of one of the arm mazes (above the food source). During the odor exposure phase the clean cloth and unused collar pieces were replaced by the test stimuli. During the post-odor exposure phase both the stimuli and the alligator clips were removed.

For each trial, the experimenter placed a subject in the release area (Home Base, see Fig. 1). At the end of the 3 min trial the experimenter re-entered the room, collected the rat, and returned it to its compartment in the carrying cage. The experimenter then recorded the number of food pellets remaining, replenished them, and cleaned the maze using clean paper towels and an organic-acid cleaning solution (Rocal-D). The room was ventilated for a short period of time to allow the solution to dry fully and the odor to dissipate.

2.1.7. Measures and data analysis

Pellet consumption was measured by counting the number of pellets in the food arm before and after a trial. Using Ethovision, we also analyzed: average distance (cm) from the food/odor during a trial, latency to reach food (sec) after the trial began, and total distance traveled (cm) over the course of a trial. Latency to reach food was also measured.

The data from each phase (pre-exposure, exposure, and post-exposure) were analyzed in three separate analyses of variance (ANOVA). A one-way ANOVA involving all groups was completed for the final baseline day (Day 5). The exposure phase was analyzed using a 2 day repeated measures ANOVA and the post-exposure phases consisted of a 4 day repeated measures ANOVA. Each of these ANOVAs was used to determine if there was a main effect between groups during the exposure phases, effects of the exposure, and effects of subsequent exposure days. All ANOVAs used rejection criteria of $p<0.05$.

2.2. Experiment 2

2.2.1. Subjects

The subjects were 24 male Albino Wistar rats (Charles River, obtained from the Animal Resources Centre, Perth, Australia) weighing, on average, 256 g at the start of the experiment. Rats were housed 6 per cage and with ad libitum access to food and water. All testing took place during the dark half of the reverse light cycle (6:00–18:00) when the rats are most active. The colony room was maintained at 22 ± 2 °C.

2.2.2. Odors

Odor samples were collected from a 4 year old neutered male domestic house cat on Swisspers™ Cotton Wool Square Pads (McPherson's Consumer Products, Kingsgrove, NSW 2208, Australia) dampened with 0.5 ml of tap water prior to sample collection. The cat was an enthusiastic marker and samples were taken from 3 different doors around the house. The experimenter followed the cat and when he rubbed against a door the cotton pad was vigorously rubbed against the bottom of the door where he had just rubbed (active sample) and another cotton pad was rubbed similarly against the top of the door (control sample) in the same fashion.

After the samples were collected they were placed in a plastic specimen jar and stored at −20 °C. In total, 3 active samples and 3 control samples were collected. Prior to testing, the samples were heated in a scientific oven (Binder, Crown Scientific, Australia) at 40 °C for 30 min and in between each session the samples were returned to the oven for 5 min.

2.2.3. Apparatus

All testing took place inside one of 6 identical chambers measuring 60 × 25 × 35 cm with a red Perspex hidebox (14 × 23 × 22 cm) placed at one end (see Fig. 2). At the center of the wall at the opposite end of the chamber to the hide box there was a metal alligator clip to which the cotton pad could be secured. The chambers were thoroughly cleaned in between sessions with 30% ethanol v/v in water and allowed to dry. A given subject was placed in the same chamber throughout the experiment.

A miniature video camera with infrared LED lights (Jaycar Ltd, Sydney, model QC3468) was located above each test chamber and sent its signal to a MacMini computer running Trackmate 1.0 video tracking software (MotMen Lrd, Cook's Hill, NSW, Australia). The software automatically computed the time spent by the rat inside the hide box (s); the time spent in the approach zone of the chamber (s); the number of close approaches to the stimulus location (contact frequency); and the total distance traveled (mm).

2.2.4. Procedure

The procedure followed the standard "cat odor avoidance" test paradigm described in previous publications [2,9,19]. On a single baseline day subjects were placed in their chamber for 20 min with no sample present. Over the next 2 days subjects were placed into their chamber for one 20 min session on each day with half the subjects having an active sample present and the rest a control sample present (exposure phase). Over the next 4 days subjects were placed in their chamber for 20 min with no sample present (post-exposure phase) to test for conditioning to the chamber. Subjects were always placed inside the hide box of the chamber at the start of the session.

2.2.5. Data analysis

The independent variables were condition (control sample or cat sample), baseline, exposure day (2 days), and post-exposure day (4 days). The dependent variables were: the time spent inside the hide box (s); the time spent in the approach zone of the chamber (s); the number of close approaches to the stimulus (contact frequency); and the distance traveled (mm). An independent samples *t*-test was run on the data from the habituation day and two repeated measure ANOVAs were run, one with the 2 exposure days as the within subjects factor and the other with the 4 post-exposure days as the

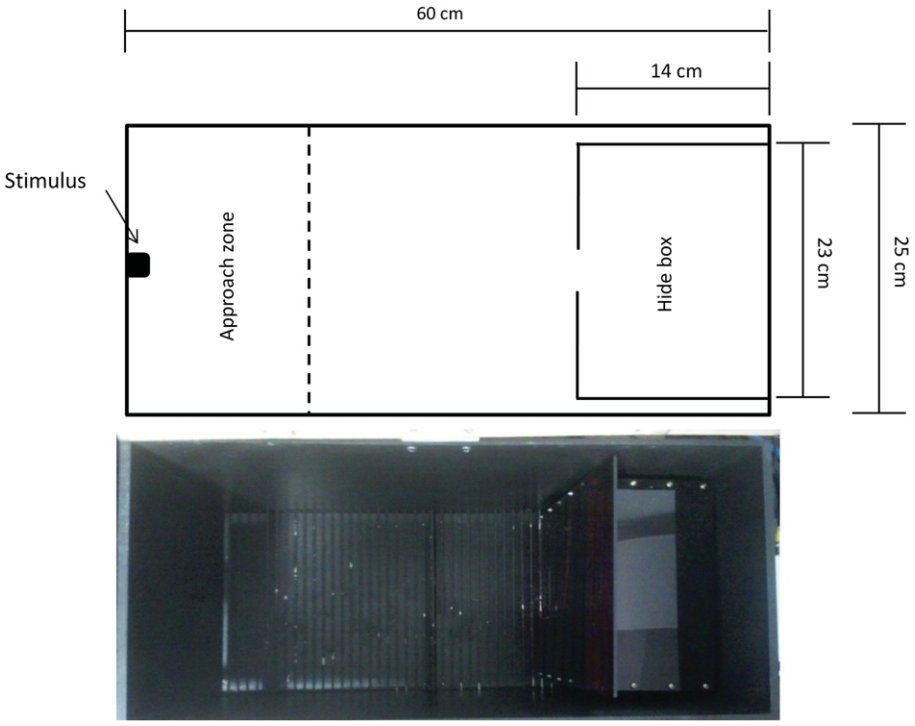


Fig. 2. A schematic of the test apparatus used in Experiment 2 (top) and a top-down picture of the apparatus (bottom). Whenever the subject was located in the hide box this was scored as hiding. When the subject was in the approach zone this was scored as approach and when the subject made contact with the stimulus with its nose this was scored as a stimulus contact.

within subjects factor. The between subjects factor on all tests was odor (cat or control).

3. Results

3.1. Experiment 1

3.1.1. Pellet consumption

During the first 5 days of training the rats increased their food consumption to a stable average (only the final day is displayed in Fig. 3) with no difference among groups on the fifth day ($F(2, 19) = 1.87$, $p > 0.05$). On the first exposure day, the cat collar and body rubbing groups significantly decreased food consumption and this effect persisted on the second day, with no change to the control group. There was a group effect ($F(2, 19) = 6.9$, $p < 0.01$) and no effect of days ($F(1, 19) = 0.46$, $p > 0.05$).

Across the 4 post-exposure days there were no significant effects of group ($F(2, 19) = 1.61$, $p > 0.05$) or days ($F(3, 57) = 1.88$, $p > 0.05$), but the suggestion of an interaction of group by days ($F(6,42) = 2.11$, $p = 0.066$). A one-way ANOVA showed a significant group effect during the first post-exposure day ($F(2, 19) = 3.83$, $p < 0.05$) with both the collar and rubbings groups still eating significantly less than the control group.

3.1.2. Average distance from food/odor

During the pre-exposure phase rats became accustomed to settling near the food source to eat with no difference between groups on the fifth day ($F(2, 19) = 2.02$, $p > 0.05$). On the exposure day both the cat collar group and the body-mark group immediately increased their average distance from the food source (Fig. 3B). There was a significant group effect ($F(2, 19) = 5.86$, $p = 0.01$), but no effect of days ($F(1,19) = 3.42$, $p > 0.05$). In the post-exposure phase, the average distance from the food/odor never completely returned to baseline after 4 days, shown as a significant effect of group ($F(2, 19) = 3.81$, $p < 0.05$), but no effect of days ($F(3, 57) = 1.5$, $p > 0.05$).

3.1.3. Latency to reach food/odor

After initial training all of the rats approached the food source within 6 s of release and with no difference among groups ($F(2, 19) = 2.99$, $p > 0.05$) on the fifth baseline day. On the first day of exposure to the stimuli, the control group latency to approach remained the same while the cat collar group and body-mark group increased their approach latency by an average of 93 s and 25 s, respectively. On the second exposure day the groups increased their average approach latency by 140 and 105 s, respectively. Across the two exposure days there was a significant effect of group ($F(2, 19) = 6.59$, $p < 0.01$) and days ($F(1, 19) = 7.13$, $p < 0.05$). In the post-exposure phase, the latency greatly diminished relative to the exposure phase for both the marking and collar group, but did not completely return to baseline as demonstrated by a significant groups effect ($F(2, 19) = 5.12$, $p < 0.05$), and the absence of a days effect ($F(3, 57) = 1.33$, $p > 0.05$).

3.1.4. Total distance traveled

During initial training, rats learned to quickly access and consume pellets leading to very low levels of distance traveled, with no significant group differences on the fifth day ($F(2, 19) = 2.9$, $p > 0.05$). On the first exposure day, both the collar and the body rubbings groups increased their average distance traveled leading to a significant group effect ($F(2, 19) = 6.16$, $p < 0.01$) and an effect of days ($F(1, 19) = 10.23$, $p = 0.005$). In the post-exposure phase there remained a significant group effect ($F(2,19) = 4.97$, $p < 0.05$) but no effect of days ($F(3,57) = 1.42$ $p > 0.05$) providing further evidence that the rats did not completely regain their baseline behavior after predator odor exposure.

3.2. Experiment 2

3.2.1. Time spent hiding

During baseline there was no significant difference in time spent hiding between rats in the rubbings condition and rats in the control condition, $t(22) = 0.43$, $p > 0.05$. On the two exposure days, rats

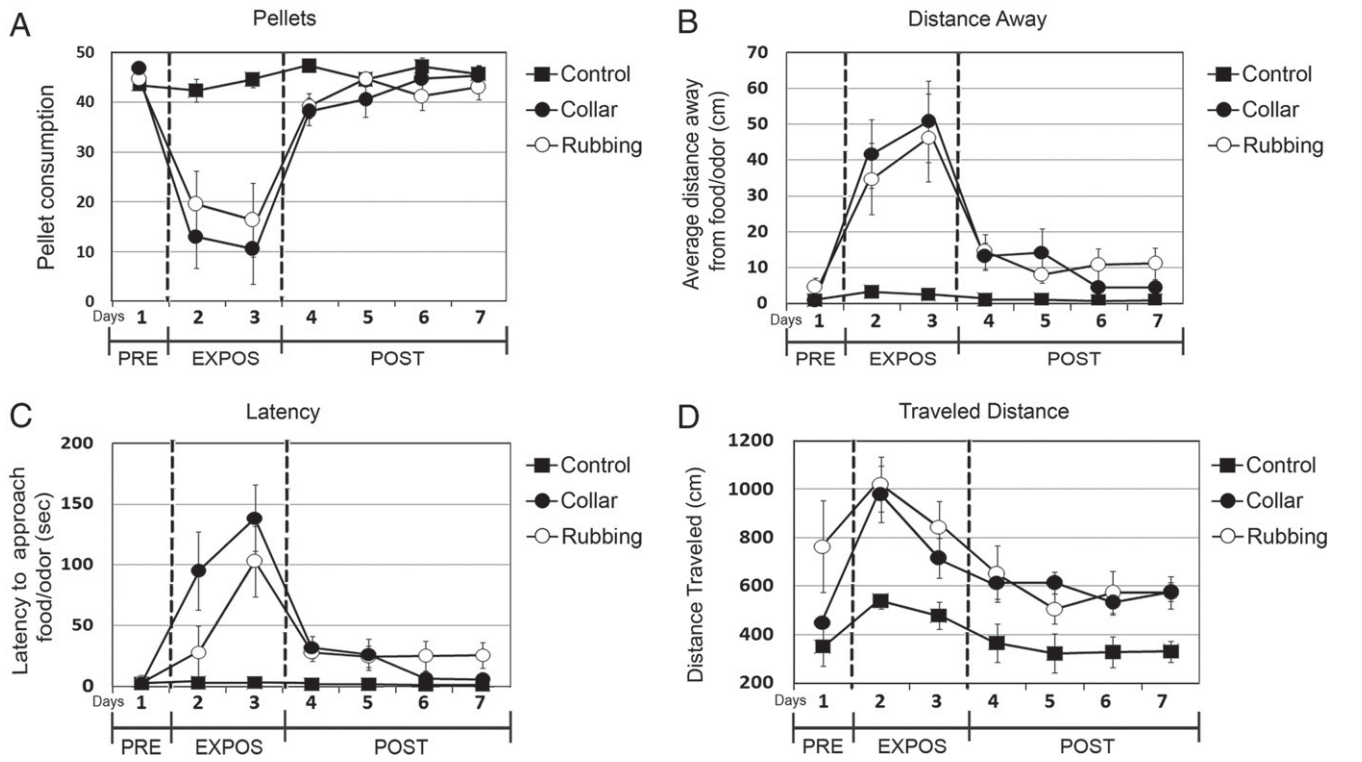


Fig. 3. Results from Experiment 1. There were no differences in any of the measures at baseline. Subjects exposed to cat body rubbings or a cat collar consumed less pellets (A), stayed a higher average distance away (B), had increased latency to approach the food source (C); and traveled more distance (D) than controls during exposure. After exposure, the subjects returned to baseline for pellet consumption but not for the other three measures. The abbreviations for the phases are PRE (pre-exposure), EXPOS (exposure), and POST (post-exposure).

exposed to body rubbings spent significantly more time hiding than controls ($F(1, 22) = 34.35$, $p < 0.0001$) with no effect of days ($F(1, 22) = 1.76$, $p > 0.05$) (Fig. 4A). During the 4 post-exposure days, Rats in the exposure condition also spent significantly more time hiding than did controls, $F(1, 22) = 13.62$, $p < 0.001$.

3.2.2. Time spent in the approach zone

During baseline there was no significant difference in time spent in the approach zone of apparatus between rats in the *cat rubbing* and *control* conditions ($t(22) = 1.14$, $p > 0.05$) (Fig. 4B). During the exposure phase, rats in the cat odor exposure condition spent significantly less time in the approach zone averaged over the two exposure days ($F(1, 22) = 26.84$, $p < 0.0001$) with no effect of day ($F(1, 22) = 0.03$, $p > 0.05$). Rats in the body rubbings condition spent significantly less time in the approach zone averaged over the four post-exposure days, $F(1, 22) = 14.72$, $p < 0.001$.

3.2.3. Number of contacts

At baseline there was no significant group difference in the number of visits to the contact area, $t(22) = 1.98$, $p > 0.05$ (Fig. 4C). In the exposure phase, rats in the body rubbings condition had significantly fewer visits to this zone than controls ($F(1, 22) = 18.81$, $p < 0.0001$) with no effect of day ($F(1, 22) = 0.541$, $p > 0.05$). In the post-exposure phase rats in the body rubbings condition continued to make significantly fewer visits to the contact zone ($F(1, 22) = 15.45$, $p < 0.001$).

3.2.4. Total distance traveled

At baseline there was no significant difference in the total distance traveled during the session between rats in the cat condition and rats in the control condition, $t(22) = 1.83$, $p > 0.05$ (Fig.4D). In the exposure phase, rats exposed to body rubbings traveled significantly less distance than controls averaged over the two exposure days ($F(1, 22) = 15.05$, $p < 0.001$). The distance traveled was significantly greater on the second exposure day ($F(1, 22) = 18.65$, $p < 0.0001$) and this increase in activity did not differ between the two conditions ($F(1, 22) = .162$, $p > 0.05$). Over the post-exposure phase rats in the cat condition traveled significantly less distance than controls ($F(1, 22) = 4.70$, $p < 0.05$), indicating lasting effects of exposure to cat stimuli.

4. Discussion

Overall, the two experiments reported here provide evidence that rats react defensively to the body rubbings that cats frequently deposit in their environment. Given that these body rubbings are most likely selected to provide important chemical signals to other cats, these results provide evidence for the "kairomone hypothesis" of predator odor effects in rats.

Experiment 1 demonstrated that female Sprague–Dawley rats respond in a similar fashion to a well-established predator odor stimulus (a worn cat collar) and to body rubbings (from the same cat). Exposure to either stimulus caused naïve rats to delay their subsequent entry to the feeding area where the odor stimulus was located, thus significantly increasing their overall distance from the feeding area, and decreasing their pellet intake. There was some evidence that the collar appeared to be more efficacious than the rubbings in retarding approach to the food during the first stimulus exposure, but this difference appeared to disappear by second exposure (Fig. 3). Other measures of stimulus efficacy in provoking defensive behavior were broadly similar for the collar and rubbings stimuli.

Odor exposed rats traveled farther during the exposure sessions, an effect explained by the very low levels of distance traveled by control rats in this paradigm in which rats spend the vast majority of their time consuming pellets in the static feeding location. Rats exposed to the previously worn cat collar or to the rubbings engaged in risk assessment behaviors and defensive approach and retreat when near the odor, leading to a greater overall distance traveled.

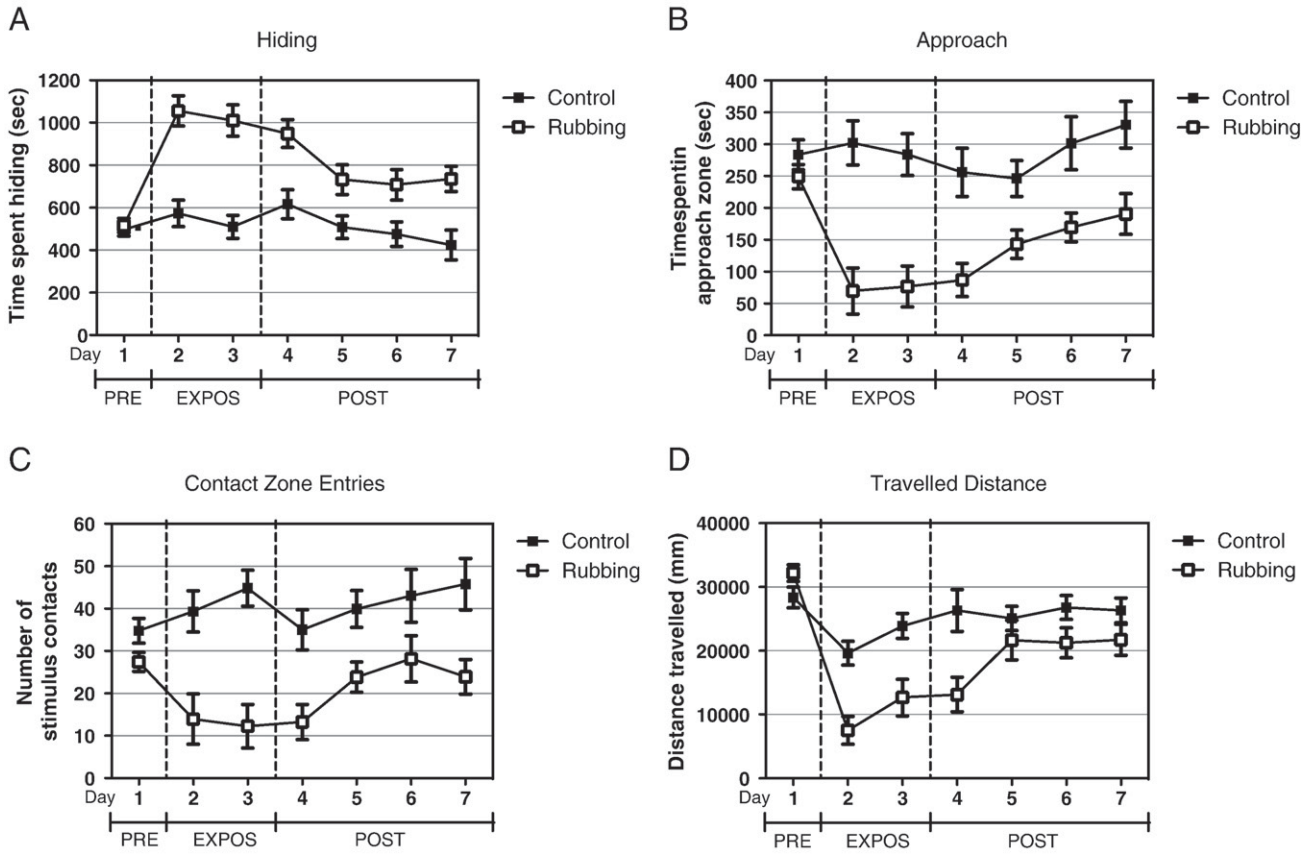


Fig. 4. Results from Experiment 2. There was no difference on any of the measures at baseline. Subjects exposed to cat body-mark residue spent longer hiding (A), and spent less time approaching the stimulus (B), had fewer contacts with the stimulus (C), and moved less (D) during exposure. After exposure, contextual conditioning was apparent on all measures with extinction occurring most rapidly in distance traveled. The abbreviations for the phases are PRE (pre-exposure), EXPOS (exposure), and POST (post-exposure).

In the post-exposure phase, food consumption returned to baseline with a minor decrement on the first post-exposure day. Other behavioral measures including latency to approach the food, average distance from the food, and distance traveled did not fully return to baseline across the post-exposure days suggesting subtle, yet persistent conditioned effects arising from exposure to cat stimuli.

Experiment 2, using the cat odor avoidance paradigm, showed that Wistar rats respond to feline body rubbings residue with a defensive repertoire similar to that previously observed with presentation of a cat collar [2,9]. A cotton pad wiped vigorously on a location where a cat had recently body-rubbed caused a major increase in hiding, with rats spending more than 80% of the test avoiding the stimulus in the hide box. In addition, rats spent less time approaching the stimulus, engaged in fewer stimulus contacts and showed less distance traveled in the test session, effects that are typical of those seen with a cat collar [2,9]. The body rubbings also produced powerful contextual conditioning in Wistar rats with increased defensiveness clearly identifiable on all of the relevant measures for much of the 4 day post-exposure phase.

Overall the contextual conditioning of the Sprague Dawley rats in Experiment 1 appeared weaker than that seen with the Wistar rats in Experiment 2 and to that reported previously in Wistar rats in response to both cat collar and cat fur [1,9,11]. This may be due to the overall low sensitivity of the Sprague–Dawley strain to cat odors compared to Wistar rats. In particular, our initial findings showed that contextual conditioning in Sprague–Dawley rats to cat odor are relatively weak [9,19]. However, the food-deprived status of the rats in Experiment 1 may have decreased the expression of conditioned defensive behavior in these rats during the post-exposure phase.

These observations that rats respond similarly to cat fur, a cat collar, and cat body rubbing residue raise the possibility that the same active ingredient is present in all three stimuli, and it is responsible for eliciting the defensive behaviors observed. Cats have a variety of scent glands including those in the tail, around the mouth and cheeks (where the cat often begins rubbing), the neck (where the collar is located), and the paws (which the cat uses to clean its fur by kneading their paws against objects and surfaces) [16,23].

Given that cats typically commence their marking behavior by rubbing with their cheeks, it is tempting to conclude that the "body rubbings" used in the present study are in fact better described as "cheek rubbings". However it should be acknowledged that the vigorous grooming behavior of cats using their paws and saliva means that scent from one anatomical location is likely spread across much of the surface area of the cat, and certainly to the paws. This raises the possibility that the effect of body rubbings in the current study could be caused by a substance (or substances) present at almost any location on the cat's body. Nonetheless, our use of rubbings collected from a surface immediately after the cat had displayed cheek rubbing behavior suggests a specific role for cheek-derived stimuli. Additional studies might usefully compare whether cat fur taken from an area of the cat where the scent glands are located (such as the cheek, neck or tail) is more effective at eliciting defensive behavior than fur taken from parts of the cat where there are fewer scent glands.

Chemical analysis of undomesticated cat-mark secretions has shown that they are high in lipids, and may include saliva [24,25]. The human allergen Fel d1, and fragments associated with the proteolyic cleaving of this large protein, are also present in high concentrations in washings taken from the feline cheek zone [26]. Large

individual variations in levels of Fel d1 and its associated fragments are measured in the cheek zones of individual cats and this may be linked to gender and behavior and could even provide an individualized fingerprint used for conspecific identification via markings [27]. In addition, Papes et al. [28] recently showed that Fel d4, another possible human allergen, and a chemical component of cat saliva, can strongly activate the accessory olfactory system and elicit defensive responses in mice. It would clearly be of interest then to determine the role of both Fel d1 and d4, and their fragments, as the key stimuli that mediate the defensive response of rats to cat body rubbings.

An important question is why predator-derived kairomones exist given that they would appear likely to decrease a predator's prey-catching fitness. Some have answered this question [1,3,18] in terms of multi-component optimization, whereby the benefit of intra-species communication obtained by marking behavior outweighs any cost imposed by inter-species eavesdropping. The tendency of domestic cats to rub their bodies against objects, other cats, and people they relate to socially [15] has been described as a type of social interaction [16] in which the animals provide individualized information that includes sex, age, and status [16,17]. For example, male and female estrous cats tend to rub more often in the presence of other estrous cats than in the presence of non-estrous females [16,29–31], also suggesting that these markings may have an important role in reproduction and mate selection. It seems likely then that body rubbings (and possibly cat odor in general) serve the primary beneficial purpose. If one observes where a cat rubs, it is often against inanimate objects, people, and other cats [15,31,32], seeming to gravitate toward edges and protrusions.

How do these characteristics factor into cat odor's relationship with rats? That rats can differentiate among cats based only on their collars suggests that cats produce an individualized marker, transferred by contact, that prey species can identify [2]. This is in line with recent research with invertebrates which suggests odors have both intra- and inter-species roles [33–35]. Based on this work, Sbarbati et al. [36] and Papes et al. [28] argued that the earliest functions of kairomones were for intra-species communication and self-recognition in predators. Presumably, these important functions outweighed any costs of alerting prey in natural selection. It is possible that the cat odor/rat alerting relationship may have evolved along similar lines. Rats that were sensitive to, and subsequently avoided such odors were more likely to survive and breed, thereby selecting for a combination of odor sensitivity and a behavioral tendency to avoid the locations of cat territorial odors.

5. Conclusions

The current experiments provide demonstrations that the residue of domestic cat rubbings provokes strong primary defensive responses in two different strains of rats in two different test paradigms in two different countries. In addition to provoking primary defensive responses, body rubbing residue elicited a secondary defensive response in the form of contextual conditioning. Both primary and secondary response to body rubbing residue are similar to the response elicited by cat collars and cat fur, suggesting these three odor sources might share a common molecular basis.

Importantly, these data suggest that rats are able to identify areas frequented by potential cat predators through recognition of their social odor markings (kairomones), which rats respond to by avoiding areas in which they were encountered. Thus, it appears that rats, like a number of invertebrate species, evolved to identify (eavesdrop on) the social pheromones of a species preying on them, and use such "kairomones" to locate and avoid areas frequented by the predator species. Documentation of such eavesdropping in vertebrates is rare, but the present data support the view that stimuli produced by individual cats for purposes of social communication and territorial demarcation can be intercepted by rats to avoid predation and thereby increase their chances of survival and reproduction.

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Appendix 2: Adolescent oxytocin exposure causes persistent reductions in anxiety and alcohol consumption and enhances sociability in rats

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Adolescent Oxytocin Exposure Causes Persistent Reductions in Anxiety and Alcohol Consumption and Enhances Sociability in Rats

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Abstract

Previous studies have suggested that administration of oxytocin (OT) can have modulatory effects on social and anxiety-like behavior in mammals that may endure beyond the time of acute OT administration. The current study examined whether repeated administration of OT to male Wistar rats (n = 48) during a key developmental epoch (early adolescence) altered their physiology and behavior in later-life. Group housed rats were given intraperitoneal injections of either 1 mg/kg OT or vehicle during early adolescence (post natal-days [PND] 33–42). OT treatment caused a transient inhibition of body weight gain that recovered quickly after the cessation of treatment. At PND 50, the rats pre-treated with OT displayed less anxiety-like behavior on the emergence test, while at PND 55 they showed greater levels of social interaction. A subgroup of OT pre-treated rats examined at PND 63 showed a strong trend towards increased plasma OT levels, and also displayed significantly increased OT receptor mRNA in the hypothalamus. Rats pre-treated with OT and their controls showed similar induction of beer intake in daily 70 min test sessions (PND 63 onwards) in which the alcohol concentration of beer was gradually increased across days from 0.44% to 4.44%. However, when given *ad libitum* access to beer in their home cages from PND 72 onwards (early adulthood), consumption of beer but not water was significantly less in the OT pre-treated rats. A "booster" shot of OT (1 mg/kg) given after 25 days of *ad libitum* access to beer had a strong acute inhibitory effect on beer intake without affecting water intake. Overall these results suggest that exogenous OT administered during adolescence can have subtle yet enduring effects on anxiety, sociability and the motivation to consume alcohol. Such effects may reflect the inherent neuroplasticity of brain OT systems and a feed-forward effect whereby exogenous OT upregulates endogenous OT systems.

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Introduction

The neuropeptide oxytocin (OT) regulates a number of critical behavioral repertoires in mammals including maternal behaviour [1–3], social interaction and social preference [4,5], sexual behaviour [6], and anxiety-like behaviour [2]. The anxiolytic properties of OT, and related OT receptor agonists, have been documented in many different rodent models of anxiety and with many different routes of administration [7–11]. OT can also reverse some of the anxiogenic effects of social separation. For example, social interaction deficits in prenatally stressed rat pups were reversed by administering OT into the central amygdala [12]. Central administration of OT also reduced distress ultrasonic vocalisation production in rat pups during social isolation, perhaps by mimicking the effects of social contact [13]. In a more recent study, socially isolated voles given OT peripherally showed fewer lasting adverse effects of long-term social isolation than controls [14]. Furthermore, chronic central OT administration in male rats increased the duration and frequency of non-sexual physical contact with female rats, irrespective of their oestrous cycle, which suggests chronic OT may facilitate nonsexual social interactions [15]. The anxiolytic and prosocial effects of OT evident in these animal models have led to worldwide interest in exogenous OT as a potential therapeutic for human psychiatric disorders [2,16–23].

In some published studies these effects of OT are more apparent with repeated, rather than acute, administration. Thus chronic, but not acute, centrally administered OT attenuated the pathological high anxiety of female rats selectively bred for high anxiety-related behaviour [24] while 3 days of peripheral OT treatment significantly reduced anxiety in rats following the induction of colitis [25]. In other studies, repeated peripheral OT treatment had lasting beneficial effects on blood pressure and pain tolerance [26,27] and causes long-lasting decreases in blood concentrations of corticosterone [10,28]. Even more striking, rats administered 1 mg/kg OT peripherally once per day from postnatal days 1–14 showed significantly reduced blood pressure in adulthood (aged 7–8 months) [29].

The ability of exogenous OT to cause enduring residual changes in behavioral and physiological traits may reflect the inherent plasticity of OT neural systems. Heightened stimulation of hypothalamic OT receptors (OTRs) triggers increased dendritic and peripheral release of OT which further stimulates OTRs

establishing a positive feedback loop resulting in hypertrophy of the oxytocinergic neurons, decreased astrocytic coverage of neurons, and a subsequent increase in juxtaposition of neurons at the level of somas and dendrites across the entire OT system (for a review see [30]). Ultimately, this process results in a lasting increase in the productivity and functionality of the OT system, the duration of which can last for months depending on the magnitude and length of the stimulation [31–33]. Importantly from a therapeutic perspective, these changes can be induced both *in vivo* and *in vitro* by administration of exogenous OT [34–37]. Of particular relevance to the present study, plasticity has been demonstrated in the hypothalamic magnocellular neurons of adolescent male rats [38] and robust changes in OTR density occurs in a number of brain regions during adolescent development [39].

Adolescence is a key developmental epoch in mammals during which sexual maturity is attained and adult behavioral repertoires rehearsed and consolidated. Many human psychiatric problems have their ontogeny in perturbations in adolescent development caused by trauma or drug and alcohol abuse [40,41]. Accordingly, the present study sought to characterize the potential of OT administration, when given chronically during adolescence, to modulate social behavior, anxiety, and alcohol consumption. Alcohol was of interest given the ample evidence that both centrally and peripherally administered OT can reduce tolerance, dependence and self-administration of various drugs of abuse through interaction with neural sites implicated in the development of drug addiction and craving [18,42–46]. Several studies report lower tolerance to and consumption of alcohol amongst breastfeeding mothers (in whom the central OT system is upregulated) compared to non-lactating mothers [47–49]. OT administered both centrally and peripherally during chronic ethanol treatment attenuates the development of tolerance to ethanol-induced hypothermic, myorelaxant and akinesic effects in mice [50,51], and decreases the severity of withdrawal symptoms in mice [52]. In rats, peripheral OT attenuated tolerance to alcohol-induced narcosis [53].

We therefore predicted that rats given repeated OT treatment during adolescence might exhibit altered alcohol self-administration in adulthood. To test this hypothesis we utilized the beer model, a well-established and ecologically valid model of alcohol consumption [54–57], which has been successfully used to test a number of pharmacotherapies for alcohol consumption and alcohol-related disorders [58–60]. Given that OT inhibits the development of ethanol tolerance (see above), and inhibits drug-induced activation of addiction-relevant brain regions [43], we predicted that OT pre-exposure might affect alcohol consumption. However, we hypothesized that differences in consumption might only emerge towards the end of a long period of continuous alcohol access as it can take several weeks for tolerance [61,62] and ethanol-induced neuroadaptations to occur [63] with voluntary consumption. We additionally predicted that OT pre-treatment might alter anxiety and social behavior, and be accompanied by long-term up-regulation of endogenous OT systems.

Methods

Subjects

All experimental procedures were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition, 2004) and were approved by University of Sydney Animal Ethics Committee (approval number L29/11-2009/3/5178). The subjects were 48 male Australian Albino Wistar (AAW) rats (Animal Resources Centre, Perth, WA, Australia). They were brought to our facility at PND 21 and handled extensively for 11 days prior to treatment. All rats were housed 8 per cage with *ad libitum* access to food and water. The rats were at PND 33 at the start of dosing, an age corresponding to early adolescence. At this time they weighed 127–177 g.

Drugs

OT was obtained from Auspep Ltd (Parkville, Victoria, Australia) and was dissolved in 0.9% saline vehicle at a concentration of 1 mg/ml and injected intraperitoneally (IP) at a dose of 1 mg/kg. This dose was chosen as several studies have demonstrated long lasting changes following repeated peripheral administration of this dose of OT [26–29].

Experimental design

An overview of the experimental protocol is presented in Figure 1. Drug administration was conducted between PND 33–42 as this is widely considered to correspond to the early adolescent period in the developmental life cycle of the rat [64,65]. This period was of particular interest as adolescence is considered a key developmental epoch in humans, during which many psychological and addictive disorders have their roots [40,41].

The eight day washout before the start of testing was implemented to allow the acute effects of the drug to completely dissipate and to allow the subjects to reach late adolescence. Late adolescence was of particular interest as it is the developmental period during which many psychological disorders, such as generalized and social anxiety become manifest in humans [40]. Furthermore, this washout period allowed the average body weight of the two conditions to return to the same level after transient inhibition of weight gain in the OT-treated rats. The social interaction test was conducted five to six days after the emergence test to minimize possible carry forward effects from one test to the other (see Figure 1).

The brain and plasma analysis was conducted on samples taken on PND 63 as this corresponds to early adulthood in the rat [64,65] and it was of particular interest to see if OT pre-treatment was capable of causing physiological and neural changes that would endure into adulthood. Alcohol induction also began at PND 63 as, in humans, young adulthood is the period during which excessive patterns of alcohol consumption usually develop [66]. Furthermore, previous research has identified this as a key phase for the development of excessive alcohol consumption in rats [65].

Drug administration phase

Rats were treated daily with OT 1 mg/kg (group referred to as OT, n = 24) or vehicle (group referred to as VEH, n = 24) for 10 days, from PND 33–42. Body weight was recorded four times during the drug administration phase to establish any effect of OT on weight gain. Subjects were also weighed before the start of the critical tests conducted in the post-drug administration phase to ensure that average weight did not differ significantly between conditions. Body weight was measured throughout the alcohol administration period to ensure there were no differences in body weight which might explain any differential consumption of alcohol.

Post –drug behavioral testing: Emergence test

After an 8 day washout period, all rats were tested in the emergence test. This was conducted as previously described [67]

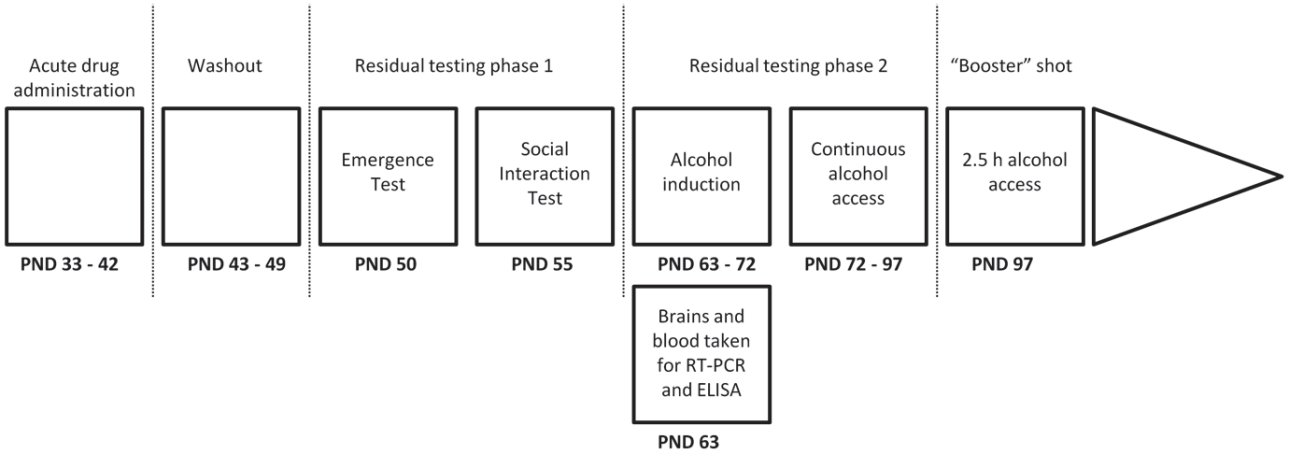

Figure 1. Experimental procedures. There were 24 VEH and 24 OT subjects for inital drug administration, washout and residual testing. Eight VEH and Eight OT subjects from the initial 48 subjects then underwent alcohol induction on PND 63–72 and were used in the subsequent experiments conducted between PND 72–97. Another five VEH and five OT subjects taken from the initial 48 subjects were culled on PND 63 and their brain and blood was later analysed. Abbreviations: VEH = vehicle; OT = oxytocin; PND = post natal day.
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in an arena measuring 200 (W)×200 (L)×80 (H) cm made of white wooden walls and a black floor. A red Perspex hide box measuring 23 (W)×22.5 (L)×14.5 (H) cm, with a single opening at its center, was placed flush against the center of one of the walls. Testing took place under two bright white spotlights shining directly onto the arena from opposite sides. A camera mounted to the ceiling directly above the apparatus fed video of the tests to a computer and monitor in an adjacent room where the sessions were scored electronically by automatic tracking software (TRACKMATE 1.0; MotMen Ltd, Cook Hill, NSW, Australia). The tracking accuracy of the software has been validated over several years of use in our laboratory with the version used for this study validated using a large cohort of rats (n = 64) and two experienced hand scorers.

The test commenced with individual rats being placed alone inside the hide box by the experimenter. Over a 5 min period the software automatically scored the distance travelled by each subject during the session as well as the time spent (a) in the hide box, (b) with head protruding outside the hide box, (c) in the open field, and (d) in the third of the arena farthest from the hide box (far zone). Additionally, the number of rats to emerge from the hide box in each condition was compared across the two groups. At the end of the 5 min the rat was returned to its home cage and the hide box and arena were thoroughly cleaned with 70% ethanol and allowed to dry before the next rat was tested.

Post –drug behavioral testing: social interaction test

Five to six days after the emergence test, and 13 days after the end of drug treatment, rats were tested in the social interaction test. The test arena measured 200 (W)×200 (L)×80 (H) cm and consisted of black walls and a black floor. Testing took place under red light. A camera mounted to the ceiling directly above the apparatus projected video of the tests to an external computer and monitor where the sessions were scored electronically by automatic social tracking software (TRACKMATE SOCIAL v. 0.9; MotMen Ltd, Cook Hill, NSW, Australia). The social tracking capability of this software has been validated using a large cohort of rats (n = 64) and two experienced hand scorers.

Social interaction testing was conducted on a single day when the rats were at PND 55. Each rat was matched with a rat of the same experimental condition (OT or VEH) and body weight (within 10 g) but from a different home cage, making a total of 12 pairs from each condition. Each pair was placed in the centre of the arena for 5 min and the amount of social interaction and distance between the two rats over time was automatically scored by the software. The dependent variables of interest were the time animals spent in close proximity to each other (within 1.5 body lengths), the number of active social contacts over the session (these include instances of following, head to head investigation, anogenital investigation and adjacent lying), the average distance travelled for each pair (travelled activity), and the average body movement while stationary for each pair (non-travelled activity). The arena was thoroughly cleaned with 70% ethanol and allowed to dry before the next pair was tested.

Post –drug behavioral testing: alcohol consumption

A total of 21 days after cessation of OT or VEH pre-treatment (PND 63), 16 rats, 8 per group, were randomly selected to test adult consumption of beer. Rats were initially introduced to alcohol using a lickometer system (as described in [57]) over 9 days of testing. Briefly, this apparatus contains 16 individual chambers that each contained two tubes through which solutions are delivered. The rat licked the tube a certain number of times to receive a predetermined quantity of the solution. In this case, a 0.07 ml drop of solution was provided after every 3 licks at a tube.

A step up procedure was used where the concentration of alcohol in the beer was gradually increased over days. The base solution used was Coopers Ultra-Light 0.44% Alcohol Beer (''near-beer''). Ethanol was then added to the ''near-beer'' to make 1.44%, 2.44%, 3.44%, and 4.44% ethanol containing beer. ''Near-beer'' was used as an initial low-alcohol control for any non-specific treatment effects on appetite or taste preference [58]. We have shown that even high levels of ''near-beer'' consumption do not produce behavioral changes indicative of intoxication [56]. Both tubes in each of the 16 lickometer cages were filled with: ''near-beer'' on day 1, 1.44% beer solution on day 2, 2.44% beer solution on day 3, 3.44% beer solution on day 4, 4.44% beer solution on days 5–9. Daily sessions in the lickometer ran for 70 min.

Following the ninth and final lickometer session, on PND 72, rats were placed in individual housing where they had 24 h access

to 4.44% beer solution, and *ad libitum* food and water for 25 days. Water and beer bottles were weighed and changed at the same time each day (in the middle of the dark cycle) and mls consumed were calculated.

Following the final measurement of intakes on day 25 (PND 97), rats in the OT group received a further 1 mg/kg "booster" injection of OT, while control rats received an equivalent saline injection. Ten minutes after injection rats were placed back in their home cage with access to 4.44% beer solution and water. Two-and-a-half hours later the mls consumed of water and beer were calculated so that the effects of the "booster" shot of OT on beer intake could be assessed.

Blood collection and brain dissection

At PND 63, 21 days after the end of drug treatment, 10 rats (5 OT & 5 VEH), that had not been exposed to alcohol, were decapitated using a guillotine. Immediately after decapitation, trunk blood for each rat was collected and each brain was dissected, and the hypothalamus was removed, snap-frozen in liquid nitrogen then stored at −80°C as previously described [68]. Trunk blood for each rat was placed into Lithium Heparin tubes and stored on ice before all samples were centrifuged at 1000× g for 10 min at 4°C. Blood plasma supernatant for each sample was collected from the spun tubes and stored individually at −80°C as previously described [69].

RT-PCR

Each of the 10 frozen hypothalamic tissue samples was homogenized using a rotor-stator homogenizer and RNA was extracted using the RNeasy Mini Kit (QIAGEN, Doncaster, VIC, Australia) according to the manufacturer's instructions. The quality and concentration of extracted RNA was determined using a Nanodrop 2000 spectrophotometer (ThermoScientific, Scoresby, VIC, Australia). Samples were stored at −20°C. RNA was reverse transcribed to cDNA for each sample according to protocols from Applied Biosystems (Mulgrave, VIC, Australia) in a 20 µl reaction containing: 2 µl of 5× buffer RT; 0.33 µl of DTT; 0.33 µl RNasin; 1 µl of 10 mM dNTP mix; 1 µl of 1 mg/ml random primers; 0.5–1 µg of template RNA (quantity dependent on concentration of RNA); and RNase free water (quantity dependent on concentration of RNA). Samples also contained 1 µl of reverse transcriptase whereas negative controls contained none. The reaction mix was incubated in a gradient thermal cycler at 37°C for 1 h then at 70°C for 15 min to inactivate reverse transcriptase. Samples were stored at −20°C.

cDNA was prepared for amplification according to protocols from Applied Biosystems (Mulgrave, VIC, Australia) on a 96 well reaction plate. Each 25 µl reaction contained: 12.5 µl of Taqman Universal PCR Master Mix, No AmpErase UNG (2×); 1.25 µl of 20× 18S Taqman Endogenous Control Mix; 1 µl of cDNA; 9 µl of water. Finally, each 25 µl reaction contained 1.25 µl of the relevant 20× Taqman Gene Expression Assay Mix: rat OT gene expression assay or rat OTR gene expression assay.

Following preparation, the plate was centrifuged at 10,000 rpm for 30 s at 25°C. Relative quantification was then performed using real time PCR on ABI PRISM 7000 detection system (Applied Biosystems, Mulgrave, VIC, Australia) using thermal cycling conditions consisting of an initial denaturation stage of 10 min at 95°C, then 40 cycles at 95°C for 15 s, annealing at 60°C for 1 min. Two negative controls were included, one containing no cDNA template, the other containing the negative control mix from the reverse transcription stage. Reactions for each biological sample and the negative controls were run in triplicates. Two plates were run in total, one for each target gene of interest: OT and OTR.

ELISA

Determination of plasma OT concentration was performed as using an Oxytocin Enzyme Immunoassay Kit (Assay Designs, Ann Arbor, MI, USA) according to manufacturer instructions. The only additional substance required was a protease inhibitor (Sigma-Aldrich, Castle Hill, Australia), which was added to the samples, and deionised water. All samples were run in duplicate and standards were prepared to allow determination of concentration. Prepared plates were analyzed using a microplate reader and analysis involved comparisons of the unknown samples with a standard curve generated from internal standards. Results were expressed as the plasma concentration of OT in pg/ml for each sample.

Statistics

Body weight data were analysed using mixed model ANOVA with trend analysis of the dosing period used to establish differences in weight gain and follow up contrasts in the post-treatment period used to verify that the two groups returned to and remained at the same weight after the cessation of treatment.

Data from the emergence test and social interaction tests were analysed using independent samples t-tests. Additionally, the number of rats to emerge from the hide box in each condition was compared using a Chi-Square Test of independence. Pattern of beer and water consumption over the 25 days home cage access were examined using mixed model ANOVA and planned contrasts were conducted to compare the intake between the two groups on each day. Given the number of comparisons required, a decision wise error rate (DER) was used as is recommended by Perneger [70]. Total beer and water consumed over the 25 days, the last week, and in the 2.5 h following the "booster" shot were examined using independent samples t-tests.

The analysis for the RT-PCR was conducted using ABI PRISM 7000 detection system software 7000 SDS version 1.3.1.21 (Applied Biosystems) to obtain CT values for gene expression and statistical analysis was conducted using Relative Expression Software Tool (REST) version 2.0.7 (Corbett Research Pty. Ltd.). Relative gene expression was analysed for statistical significance for each normalised target gene (OT and OTR) comparing the OT pre-treated samples to the VEH samples.

For the ELISA the average OT plasma concentration was compared across groups using an independent sample t-test.

Welch's correction for unequal variances was used wherever the assumption of homogeneity of variance was violated as indicated by a p-value$<$0.05 on Levene's test for homogeneity of variance.

Results

Body weight

At the start of dosing there was no difference in body weight between OT ($M = 150.37$, $SD = 8.90$) and VEH ($M = 150.83$, $SD = 11.29$) rats, $p = 0.877$. However over the dosing period weight increased at a significantly greater rate for VEH compared to OT rats, as indicated by the significant linear interaction trend, $F(1,46) = 7.34$, $p<0.01$. However, despite this difference in weight gain there was no significant difference in mean weight between OT ($M = 203.77$, $SD = 13.01$) and VEH ($M = 211.08$, $SD = 19.84$) rats on the last day of treatment, $p = .138$. There was no significant difference in body weight between OT ($M = 297.79$, $SD = 15.73$) and VEH ($M = 301.71$, $SD = 21.17$) rats at the start of behavioral testing, 8 days after the cessation of dosing, $p = 0.471$. Further-

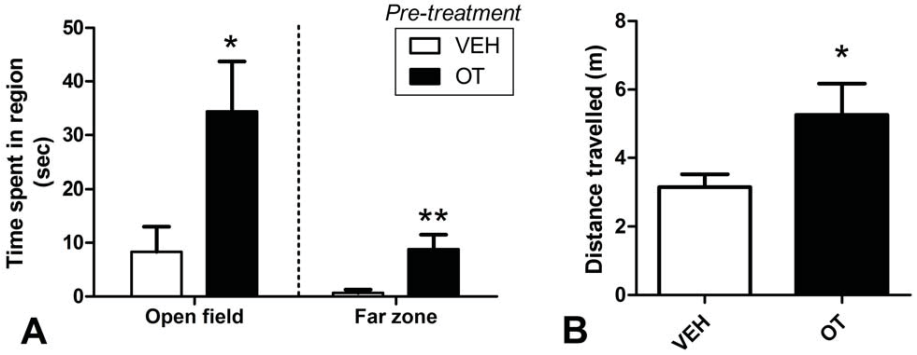

Figure 2. Emergence test. Results from the 5 min emergence test conducted 8 days after the cessation of 10 consecutive days of once per day treatments with either 1 mg/kg OT or VEH. **A.** Mean (+SEM) seconds spent in the open field and in the third of the open field farthest from the hide box (far zone). **B.** Mean (+SEM) metres travelled during the test. N = 24 per condition. * Significantly different to VEH, p<0.05 ** Significantly different to VEH, p<0.01.
doi:10.1371/journal.pone.0027237.g002

more, the groups did not differ significantly at any timepoint throughout the alcohol administration period (all *p-values*>0.05), with OT ($M = 442.50$, $SD = 30.90$) and VEH ($M = 450.62$, $SD = 20.37$) subjects equivalent in weight at the end of the experiment when the final "booster" shot was given $p = 0.546$.

Emergence test

The Chi-Square test of independence revealed significantly more OT pre-treated rats emerged from the hide box during the 5 min session (15 out of 24) compared to VEH treated rats (4 out of 24), $\chi^2(48) = 10.54$, p = 0.001. Rats pre-treated with OT spent significantly: less time in the hide box [$t(35.827) = 2.30$, $p = 0.027$]; more time in the open field (Figure 2A) [$t(34.256) = 2.51$, $p = 0.017$]; and more time in the far zone of the open field (Figure 2A), $t(24.995) = 2.96$ $p<0.01$. Furthermore, the OT pre-treated rats travelled significantly more distance during the session compared to the VEH pre-treated animals (Figure 2B), $t(28.89) = 2.12$, $p = 0.043$. There was no significant difference between OT and VEH animals in time spent with head out of the hide box, p>0.05.

Social interaction test

OT pairs spent significantly more time than VEH pairs in close proximity to each other (Figure 3A), $t(22) = 2.17$, $p = 0.041$. OT pairs also came into social contact significantly more times than VEH pairs (Figure 3B), $t(22) = 2.12$, $p = 0.045$. As seen in Figure 3C, OT rats travelled significantly more meters than VEH animals during the social interaction test, $t(22) = 2.68$, $p = 0.014$.

Consumption during alcohol induction and continuous access to beer

During the alcohol induction period in the lickometer apparatus, during which alcohol concentrations were gradually increased in daily 70 min sessions, there were no significant differences between OT and VEH pre-treated subjects in the consumption of any ethanol concentration of beer (see Figure 4): "near-beer" (.44%) ($p = 0.794$); 1.44% ($p = 0.556$); 2.44% ($p = 0.490$); 3.44% ($p = 0.320$); or 4.44% (all *p-values*>0.421).

Figure 5 illustrates the consumption for OT and VEH pre-treated subjects over the course of the continuous access period for beer and water under individual housing. There was no significant overall pre-treatment effect on beer or water consumption, both *p-values*>0.05. However, the OT pre-treated animals consumed significantly less beer than the VEH pre-treated animals on day 2 [$F(1, 14) = 9.55$, $p<0.01$], 13 [$F(1, 14) = 5.71$, $p = 0.031$], 20 [$F(1, 14) = 5.47$, $p = 0.035$], 21 [$F(1, 14) = 5.34$, $p = 0.037$], 23 [$F(1, 14) = 9.08$, $p<0.01$] and 25 [$F(1, 14) = 6.84$, $p = 0.020$] of continuous access.

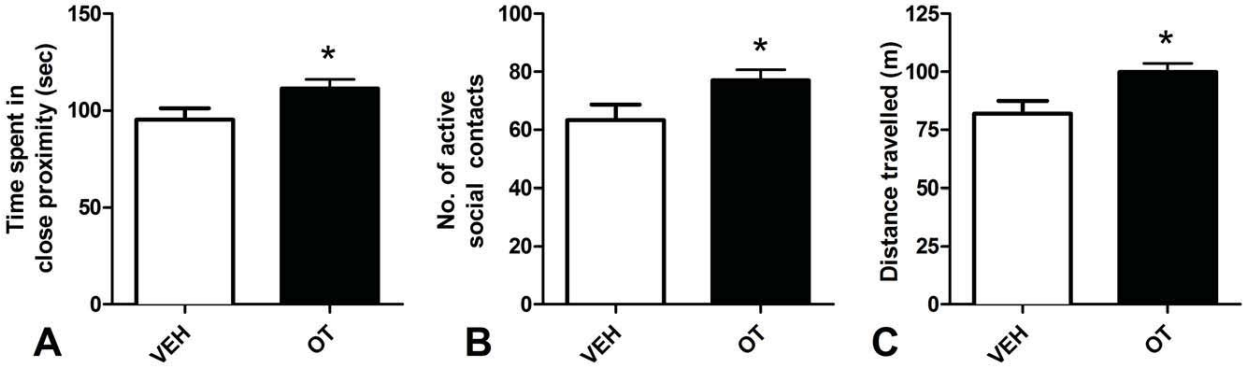

Figure 3. Social interaction test. Results from a 5 min social interaction test conducted 13 days after the cessation of 10 consecutive days of once per day treatments with either 1 mg/kg OT or VEH. **A.** Mean (+SEM) seconds the two subjects spent in close proximity (within one body length). **B.** Mean (+SEM) number of active social contacts between the subjects. **C.** Mean (+SEM) meters travelled by each pair of subjects (on average) during the social interaction test. N = 24 per condition. * Significantly different to VEH, p<0.05.
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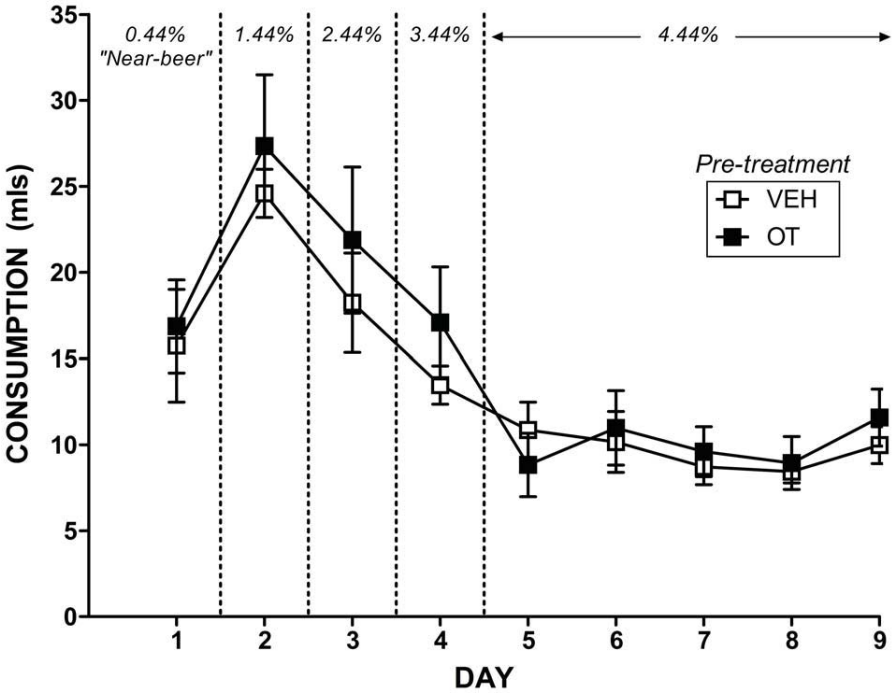

Figure 4. ''Near-beer'' and beer consumption during the 9 day alcohol induction phase in the lickometer apparatus. Mean (± SEM) consumption (in mls) of increasing concentrations of beer (from .44% ''near-beer'' up to 4.44% EtOH beer). The 9 day induction period began 21 days after the cessation of 10 consecutive days of once per day treatments with either 1 mg/kg OT or equivalent VEH saline. Subjects were placed in the lickometer for one 70 min session per day. N = 8 per condition.
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Furthermore, over the final seven days of continuous access OT pre-treated rats consumed significantly less beer in total than the VEH pre-treated rats, $t(14) = 2.24$, $p = 0.042$. This emerging difference was further reflected by VEH animals alcohol intake showing a significant linear increase over the 25 days [$F(1, 7) = 7.85$, $p = 0.026$] and OT animals showing no such increase, $F(1, 7) = 3.47$, $p = 0.105$. OT pre-treated animals consumed significantly more water than VEH pre-treated animals on day 17 [$F(1, 14) = 7.12$, $p = 0.018$] but there were no significant differences in water consumption on any other days, all *p-values*>0.05.

Effects of an OT ''booster'' shot on beer consumption

Figure 6 illustrates consumption of beer and water in the 2.5 h following a ''booster'' treatment with OT or VEH saline. Following the ''booster'' dose OT subjects consumed significantly less beer than VEH subjects, $t(14) = 3.13$, $p<0.01$. There was no significant difference in water consumption between OT and VEH subjects, $p>0.05$.

RT-PCR

All samples were clean and had adequate RNA concentration (data not shown). There was no amplification in negative controls, indicating expression in biological samples was not due to contamination. OTR mRNA was significantly up-regulated in OT animals to 1.35 fold higher expression than the VEH animals (Figure 7A), $p = 0.032$. OT mRNA expression was essentially equal in OT and VEH animals (fold difference = 0.958), $p>0.05$.

ELISA

In the cohort of adolescent Wistar rats, there was a strong trend towards significantly greater plasma OT concentration (pg/ml) (Figure 7B) in the OT compared to VEH animals, $t(8) = 2.22$, $p = 0.057$.

Discussion

The current results provide intriguing preliminary evidence that repeated exposure to exogenous OT in adolescence can have subtle yet significant effects that last into adulthood, effects that are potentially significant in a clinical sense. These effects include (1) reduced anxiety, (2) increased sociability, (3) decreased alcohol consumption, and (4) up-regulated OT in plasma and OTR mRNA in the hypothalamus. In addition, we demonstrated that a ''booster'' shot of OT can have an additional acute inhibitory effect on alcohol consumption. These effects are now discussed in turn.

Inhibited body weight gain over dosing period

Over the 10 day dosing period OT treated rats gained weight at a significantly slower rate compared to VEH treated rats. Previously reported effects of OT on body weight gain are somewhat contradictory with studies reporting both increases and reductions in weight gain after OT administration [71,72]. Reductions in weight gain or weight loss have been associated with an acute decrease in food intake for several hours following OT administration [72]. Increases in weight gain following OT administration are not associated with any change in food intake but rather with neuroendocrine changes that promote anabolic metabolism [71]. Most likely there are strain and possibly sex and developmental differences that dictate the effect of OT on weight gain which may depend on factors such as endocrine profile and sensitivity to OT. Weight quickly recovered and at the start of behavioral testing, one week after the cessation of OT adminis-

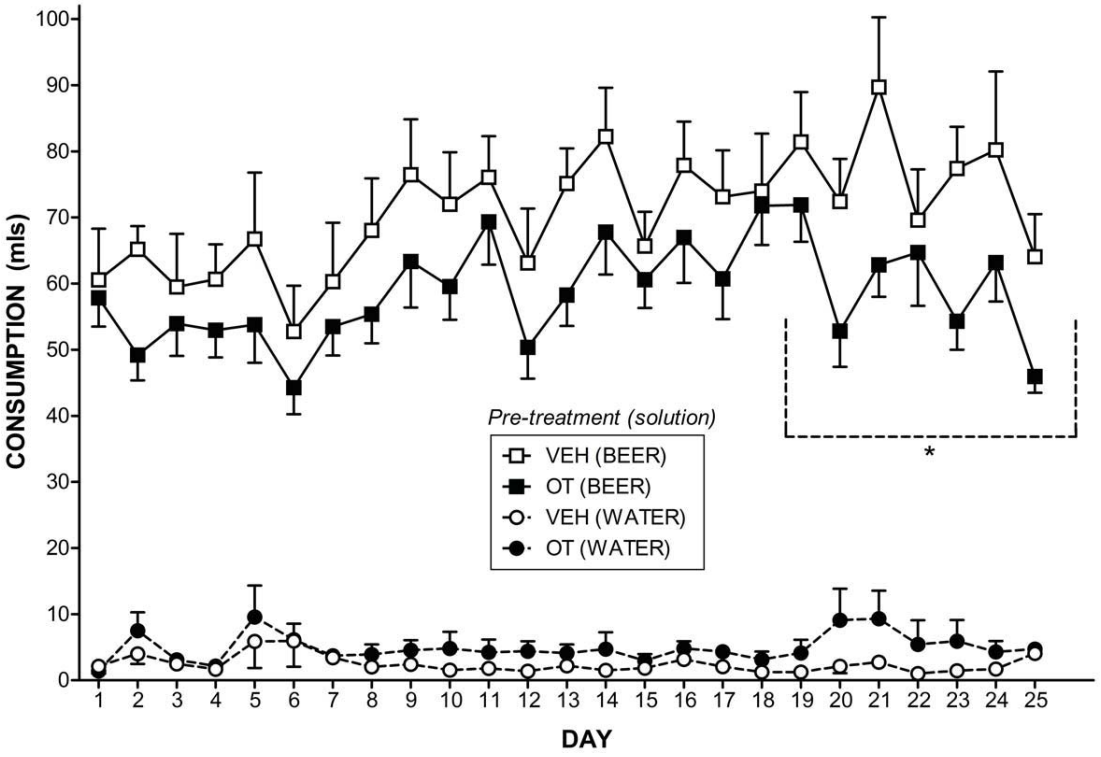

Figure 5. Alcohol and water consumption under continuous access. Mean (± SEM) 4.44% EtOH beer and water consumption (in mls) for each of the 25 days of continuous access to beer and water is illustrated. Continuous access began 30 days after the cessation of 10 consecutive days of once per day treatments with either 1 mg/kg OT or equivalent VEH. Subjects were housed individually and had 24 h access to both beer and water over the entire 25 days. N = 8 per condition. * indicates sig. difference in total beer consumption between OT and VEH over the final seven days of continuous access, $p<0.05$.
doi:10.1371/journal.pone.0027237.g005

tration, there was no weight difference between OT and VEH subjects and this lack of difference remained throughout the study. This suggests the effect of OT on weight gain is transient and caused by the direct effects of the exogenous OT [73].

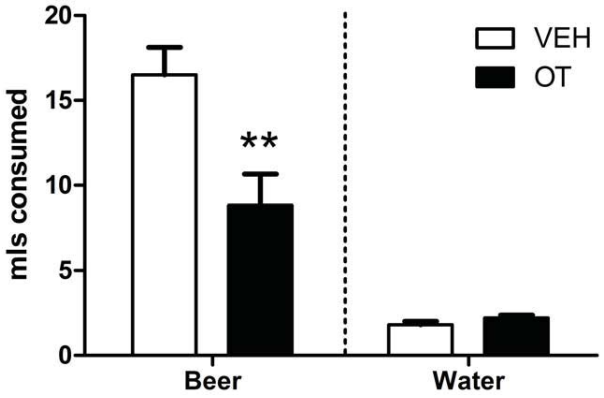

Figure 6. Alcohol and water consumption following the OT "booster" shot. Mean (+SEM) consumption (mls) of 4.44% EtOH beer and water over the 2.5 h following injection of a "booster" of either 1 mg/kg OT or equivalent VEH saline. The "booster" shot was administered after the final measurement of intakes on the final day of continuous access to beer and water. N = 8 per condition. ** Significantly different to VEH, $p<0.01$.
doi:10.1371/journal.pone.0027237.g006

Reduced generalized anxiety

More than one week following the cessation of pre-treatment the subjects given OT showed a marked reduction in generalized anxiety-like behavior in the emergence test. Additionally, over 2 weeks after cessation of pre-treatment, subjects given OT had increased sociability and activity in the social interaction test, with increases in locomotor activity in open-fields indicative of increased exploratory behavior and reduced general-anxiety [74,75]. OT pre-treated subjects also travelled significantly more distance in the emergence test which is to be expected given more of the OT pre-treated subjects emerged from the confines of the hidebox. However, given that an increase in locomotor activity was also observed in the social interaction test, an effect of OT pre-treatment on general locomotor activity cannot be ruled out.

The reductions in generalized anxiety-like behaviour observed in this study are in line with a variety of studies demonstrating anxiolytic properties of OT and OT receptor agonists [7,9,11,21]. However, perhaps of most interest, the present findings confirm that OT can cause sustained reduction in general anxiety-like behavior that remains well beyond the period during which the administered OT is present. Several studies have found an anxiolytic effect of OT only after chronic administration [24,25] and in light of the present study's findings it is plausible that the anxiolytic effect of OT is due as much to lasting behavioral and neuroadaptations as it is to the direct effects of administered OT, which are presumably present for only a short time.

This longer-term reduction in anxiety caused by the short term treatment with OT is consistent with previous reports of altered physiological functioning (such as decreased blood pressure and

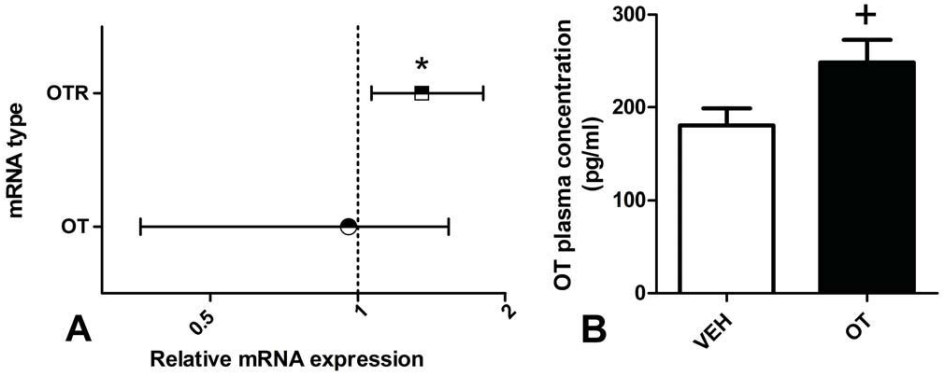

Figure 7. RT-PCR and ELISA. Results from the RT-PCR and ELISA conducted on the 10 rats (5 OT, 5 VEH) randomly selected 21 days after the cessation of 10 consecutive days of once per day treatments with either 1 mg/kg OT or equivalent VEH saline. **A.** Fold change (±SE) in OT and OTR mRNA expression in the OT pre-treated subjects compared to VEH. **B.** Mean (+SEM) blood plasma concentration of OT (pg/ml) in the OT and VEH pre-treated subjects. *Significant fold change, $p<0.05$. + Trend towards significant difference compared to VEH, $p=0.057$.
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corticosterone blood concentration) that lasted well beyond the duration of the treatment [10,26–29,71,76]. Interestingly, all of those physiological changes are associated with a relaxed condition [77–82]. This suggests OT may be having both a psychological and physiological impact that is conducive to a lasting state of reduced anxiety.

Increased sociability

More than two weeks after the cessation of pre-treatment the subjects given OT showed a subtle but significant increase in social behavior indicated by a greater frequency of active social contacts and an increased duration spent in close proximity in the social interaction test compared to VEH treated subjects. Consistent with the assertion that OT plays an important role in the formation and regulation of social bonds [83,84], these findings support previous research that indicated exogenous OT causes a reduction in social anxiety and lessens the impact of social isolation by perhaps mimicking social interaction itself [12–15]. Furthermore, the present study extends our understanding of the effects of exogenous OT on social behavior by providing initial evidence that peripherally administered OT can cause longer term adaptive behavioral changes that are conducive to reduced social anxiety and a subsequent increase in initiations of positive social contact.

Alcohol consumption

During the 9 day alcohol induction period there were no significant differences between OT and VEH pre-treated rats in consumption of varying concentrations of beer. Importantly, the lack of a significant difference in the consumption of the ''near-beer'' base solution establishes that the later diminution in the consumption of alcoholic beer as a result of OT pre-treatment is unlikely to be due to basic alterations in appetite or taste aversion as the ''near-beer'' differs from the higher concentration beer only in EtOH content [58]. Furthermore, the lack of a significant difference in weight between the groups throughout the alcohol induction and continuous access stage of testing provides further evidence that there was no difference in caloric intake between the two conditions.

During the 25 days of continuous access to beer and water in the home cage, which started 30 days after the cessation of drug administration, a significant difference emerged over the access period with OT pre-treated rats consuming significantly less alcohol over the final week of continuous access. In line with this, the alcohol consumption of VEH rats increased significantly over the course of the experiment, whereas there was no significant increase for OT rats. This provides preliminary evidence that short-term pre-treatment with OT can inhibit the development of alcohol consumption for at least 55 days after the cessation of the pre-treatment regime. This is particularly exciting given the short action of currently available treatments for alcohol abuse leads to a very high rate of relapse resulting in a great need for treatment options with improved long term efficacy, such as that demonstrated by OT in this study [54,85,86].

Interestingly, as we predicted, the emergence of a stronger, more convincing, difference in alcohol intake between VEH and OT rats from day 19 to 25 coincides with the time-course for development of tolerance to alcohol in standard strain and non-alcohol preferring rats (sometimes taking up to 24 days or longer) [61,62]. Previous studies have demonstrated OT and OT fragments are able to reduce or eliminate tolerance to a number of the behavioural and physiological effects of ethanol [50,51,53,87,88]. This suggests one possibility is that the emerging consummatory differences between VEH and OT rats in the present study may be due to absence of tolerance in the OT-treated subjects. Studies from the human literature also provide anecdotal support for this theory with several studies reporting lower tolerance to and consumption of alcohol amongst breastfeeding mothers, in whom the central OT system is up-regulated compared to non-lactating mothers [47–49]. Alternatively, the emerging difference in ethanol consumption observed in this study may be due to inhibition of addiction related neuroadaptations causes by ethanol in regions such as the Nucleus Accumbens. This is plausible given that it can take several weeks for ethanol induced neuroadaptaions in brain regions involved in addiction to occur [63] and OT has been shown to inhibit drug induced activation of these regions [43]. Future studies should explore the possibility that OT induced differences in ethanol consumption are due to inhibition of the development of tolerance and/or changes in the actions of ethanol in brain regions involved in addiction.

Of importance, this study established that OT can cause long-term reductions in alcohol consumption using a continuous access paradigm. Demonstrating such effects under continuous access is particularly challenging due to the constant availability of alcohol. However, it is also arguably the most valid model given the widespread current availability of alcohol in human society.

Furthermore, the access was provided in individual housing where there was no other source of stimulation for the rat. Such an environment is known to be stressful, and given the direct relationship between stress and alcohol consumption (for a review of the negative impact of isolation on stress levels and alcohol consumption in rats see [89]) this provided further ecological validity to the environment in which alcohol was provided. For example, it has been shown that socially isolated rats sometimes consume a significantly greater quantity of ethanol than group housed rats [90]. It is therefore possible that OT pre-treatment is augmenting alcohol intake by preventing stress induced increases in alcohol intake arising from the isolation housing. The fact that OT effects were found under a paradigm so conducive to alcohol consumption and so long after treatment is quite remarkable.

OT "booster" shot

In addition to the long-term suppression of alcohol consumption seen after chronic OT administration, the "booster" shot of OT at the end of the 25 day continuous access period (when consumption patterns were established) significantly inhibited consumption of alcohol for the 2.5 h following treatment. Furthermore, the "booster" treatment had no effect on the consumption of water over the 2.5 h period, which suggests it might be an effect that is selective to alcohol containing fluids. This provides evidence that acute OT could be an effective and selective treatment aimed at immediate reductions in alcohol consumption as well as an effective option for more long-term term reductions in consumption. However, the present study does not determine whether the pre-treatment is required for the more immediate effects of the "booster" shot to occur. The current study therefore provides impetus for more comprehensive future preclinical studies of OT as a possible therapeutic for alcohol use disorders. Future studies might utilize "near-beer" as a control solution (as described previously in [58]) to determine with greater precision how specific the consummatory suppression resulting from the "booster shot" is to alcohol.

Enduring upregulation of the endogenous OT system

As expected, there was evidence of long-term enhanced functioning of the central OT system in response to peripheral OT treatment. OTR mRNA was significantly, albeit moderately, upregulated and there was evidence of increased plasma levels of OT. This is not surprising given the essential role OTRs play in inducing and maintaining long-term neuroadaptations of the OT system [30]. For example, increased OT levels (both central and peripheral) and increased OTR binding, mRNA and density has been associated with the up-regulation of the OT system that occurs during gestation, parturition and lactation [30,91–94]. It is plausible that upregulation of OTR mRNA is at least partly responsible for the behavioral findings in this study given the association between increased OTR mRNA and reduced anxiety [95], and increased OT levels and OTR binding and reduced alcohol consumption [96,97].

There was a strong trend towards increased OT plasma concentration in OT pre-treated rats in this study and given blood collection took place 21 days after treatment, it is possible the effect was simply wearing off and would have been significant closer to treatment. Follow up analysis of the plasma from another study in which rats underwent a similar pre-treatment with OT or VEH found a highly significant increase in OT plasma levels in the OT pre-treated animals two weeks after the cessation of the pre-treatment period (Carson, Bowen & McGregor, unpublished findings). Furthermore, the plasma levels observed in that experiment were similar to those observed here, providing further support for the strong trend observed in the present study. This provides further evidence that chronic OT administration causes a lasting up-regulation of activity in the endogenous oxytocinergic system and is in line with a wealth of previous studies which demonstrate that this system is capable of undergoing morphological changes which create a positive feedback loop whereby increased OT levels stimulate further OT release (for a review see [30]).

Conclusion

This study has provided initial evidence that OT might have utility as a unique pharmacotherapy with both treatment and prophylactic utility. A brief administration of OT during early adolescence reduced generalized and social anxiety related behaviors in late adolescence and inhibited the development of excessive alcohol consumption in adulthood. These behavioral changes were associated with long-term up-regulation of the endogenous OT system. Finally, a "booster" dose of OT caused an immediate and marked reduction in alcohol consumption. These results suggest OT, or synthetic OT receptor agonists, could have the potential to be a beneficial and enduring treatment option for generalized and social anxiety disorders as well as alcohol use disorders. Current pharmacological treatment options for these disorders are plagued by lack of efficacy and intolerable side effects. The enduring nature of the behavioral and morphological changes seen in this study suggests that exogenous OT may have the capacity to do more than temporarily alleviate pathological symptoms.

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Author Contributions

Conceived and designed the experiments: MTB DSC AS JCA ISM. Performed the experiments: MTB DSC AS JCA ISM. Analyzed the data: MTB DSC AS JCA ISM. Contributed reagents/materials/analysis tools: MTB DSC AS JCA ISM. Wrote the paper: MTB DSC AS JCA ISM.

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Appendix 3: Adolescent exposure to oxytocin, but not the selective oxytocin receptor agonist TGOT, increases social behaviour and plasma oxytocin in adulthood

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Adolescent exposure to oxytocin, but not the selective oxytocin receptor agonist TGOT, increases social behavior and plasma oxytocin in adulthood

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ABSTRACT

There are indications that exposing adolescent rodents to oxytocin (OT) may have positive "trait-changing" effects resulting in increased sociability and decreased anxiety that last well beyond acute drug exposure and into adulthood. Such findings may have relevance to the utility of OT in producing sustained beneficial effects in human psychiatric conditions. The present study further examined these effects using an intermittent regime of OT exposure in adolescence, and using Long Evans rats, that are generally more sensitive to the acute prosocial effects of OT. As OT has substantial affinity for the vasopressin V1a receptor (V1aR) in addition to the oxytocin receptor (OTR), we examined whether a more selective peptidergic OTR agonist – [Thr4, Gly7]-oxytocin (TGOT) – would have similar lasting effects on behavior. Male Long Evans rats received OT or TGOT (0.5–1 mg/kg, intraperitoneal), once every three days, for a total of 10 doses during adolescence (postnatal day (PND) 28–55). Social and anxiety-related behaviors were assessed during acute administration as well as later in adulthood (from PND 70 onwards). OT produced greater acute behavioral effects than TGOT, including an inhibition of social play and reduced rearing, most likely reflecting primary sedative effects. In adulthood, OT but not TGOT pretreated rats displayed lasting increases in social interaction, accompanied by an enduring increase in plasma OT. These findings confirm lasting behavioral and neuroendocrine effects of adolescent OT exposure. However, the absence of such effects with TGOT suggests possible involvement of the V1aR as well as the OTR in this example of developmental neuroplasticity.

Introduction

A fundamental characteristic of humans and other mammals is a strong innate desire for social contact. Whether fleeting or enduring, social experiences have a complex capacity to influence our physical and psychological wellbeing (Neumann, 2009). While the regulation of social behavior involves multiple complex inputs, the neuropeptides oxytocin (OT) and vasopressin (AVP) appear to have key roles.

In general terms, OT appears to modulate the salience of social stimuli to facilitate social approach (Young, 2011). Indeed, oxytocin receptor (OTR) null mice (OTR−/−) lack the natural preference for social over non-social stimuli (Sala et al., 2011), while rodents pretreated with an OTR antagonist also show reduced social preference (Lukas et al., 2011). In tests of social interaction, chronic intracerebroventricular (ICV) OT in male rats increases the amount of time spent in direct physical contact with other rats in the absence of any changes in sexual behavior, locomotor activity, or core body temperature (Witt et al., 1992). OT also rescues normal social preference in rats made socially "phobic" via a social defeat experience (Lukas et al., 2011). Moreover, peripherally administered OT or AVP given to male Long Evans rats meeting for the first time, increased the amount of time spent in 'adjacent lying', a prosocial behavior during which two rats lie passively next to each other for prolonged periods (Ramos et al., 2013).

There is additional interest in the longer lasting residual effects of OT treatment on social behavior. Our group recently discovered that daily injections of OT (1 mg/kg, intraperitoneal (IP)) for ten days during early adolescence in rats lead to subsequent decreases in anxiety-like behavior and significant increases in sociability during adulthood (Bowen et al., 2011). These lasting social changes were accompanied by significant up-regulation of OTR mRNA in hypothalamic tissue and a trend towards increased plasma OT (Bowen et al., 2011). Such findings are consistent with physiological studies suggesting profound plasticity in hypothalamic OT systems, and a feedforward capacity whereby exogenous OT stimulation promotes endogenous OT release and hypertrophy of OT magnocellular neurons (Theodosis, 2002; Theodosis et al., 1986). These observations also agree with earlier work where chronic OT administration fostered a 'behaviorally calm' anti-stress state characterized by a lasting reduction in blood pressure and plasma corticosterone (Petersson et al., 1997, 1999).

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The present study further examined the effects of adolescent OT exposure on subsequent behavior in adulthood. Of specific interest was a comparison of the effects of OT with those of a more selective OTR agonist, the peptide analog of OT called [Thr4, Gly7]-oxytocin (TGOT). OT has relatively low selectivity for the OTR over AVP receptor subtypes, particularly the vasopressin 1A receptor, V1aR (Braida et al., 2012; Hicks et al., 2012; Terrillon et al., 2002). Thus it is feasible that OT acts at the V1aR to mediate some of its key behavioral effects (Busnelli et al., 2013; Ramos et al., 2013). For example, Sala et al. (2011) showed that OT and AVP administered ICV rescued the abnormal social exploration and social recognition of OTR −/− mice via an action on V1a receptors. TGOT is fundamentally similar to OT, but has very low affinity for the rat V1aR (>10,000 nM) and V1bR (8000 nM) relative to the OTR (0.8 nM) (Manning et al., 2012). TGOT thus permits the study of OTR-related effects with greater specificity than with OT itself which has affinities of 71.0 nM (rat V1aR), 294 (rat V1bR) and 1.0 nM (rat OTR) (Manning et al., 2012).

The acute behavioral effects of TGOT in rodents are not well characterized, particularly when administered peripherally. In a recent study, delivery of equivalent ICV doses of TGOT or OT rescued the social deficits seen in knockout mice heterozygous for the OTR gene (OTR +/−), suggesting dose equivalence in acute behavioral effects (Sala et al., 2012). Here we administered equivalent peripheral doses of TGOT and OT in adolescence and examined acute behavioral effects in models of social behavior and anxiety, as well as then examining any lasting residual effects in adulthood. Long Evans rats were used rather than the Albino-Wistar rats used in our earlier studies (Bowen et al., 2011; Hicks et al., 2012), given the high sensitivity of this strain to the prosocial effects of OT and AVP (Ramos et al., 2013).

The style of social interaction between adolescent and adult rats is qualitatively different (Spear, 2000). Social play behavior, characterized by 'rough-and-tumble' play, displays an inverted U-shaped curve across development, peaking in mid-adolescence at post-natal day (PND) 30 and disappearing in early adulthood at PND 60 (Pellis & Pellis, 2007; Trezza & Vanderschuren, 2008). During this period, robust reorganization in V1a and OT receptor expression is observed within several brain regions and is likely to be associated with the development adult-typical social behaviors (Lukas et al., 2010; Veenema et al., 2013). Thus by examining behavioral effects in adolescent rats that were acutely dosed with OT and TGOT we are able to discern, for the first time, the dose-dependent effects of OT and TGOT on social play.

In assessing lasting residual behavioral changes in adulthood resulting from OT and TGOT exposure, both social and anxiety-related behaviors were targeted. An additional interest was any persistent endocrine changes following repeated peripheral OT and TGOT that might underlie any observed changes in behavior. It was hypothesized that repeated adolescent exposure to OT, and perhaps TGOT, would produce enduring reductions in plasma corticosterone (Petersson et al., 1999) and an upregulation of plasma OT (Bowen et al., 2011; McGregor & Bowen, 2013), providing a 'boost' in the functionality of the OT system.

Methods

Subjects

The subjects were 32 experimentally naïve male Long Evans rats (*Rattus Norvegicus*) (Adelaide University, Adelaide, Australia). Upon arrival, rats were handled for six days prior to the start of experimentation (PND 28), generally accepted as the first day of adolescence in rats (Spear, 2000). The rats were maintained in a temperature- (22 ± 1 °C) and humidity-controlled colony room with a 12-hour reverse light cycle (lights on at 21:00 h). To control for litter effects, rats were randomly assigned to three treatment groups: OT (n = 10), TGOT (n = 10), and Vehicle (n = 12). Rats were housed in groups of eight per cage, counterbalanced for treatment, with food and water available *ad libitum* except during short testing procedures. All experimental procedures were approved by the University of Sydney Animal Ethics Committee.

Drugs and administration

OT and TGOT were obtained from Auspep Ltd. (Parkville, VIC, Australia) and stored at −20°C. Both drugs were dissolved in saline (0.9%) and injected IP at a dose of either 0.5 or 1 mg/kg every third day. Rats were assessed for various acute behavioral effects of some of these doses (see Fig. 1 for overview of schedule). The 0.5–1 mg/kg OT dose was chosen on the basis of a range of studies showing effectiveness in altering behavior and producing brain activation (Bowen et al., 2011; Carson et al., 2010; Hicks et al., 2012). Additionally, 0.5 mg/kg was of interest given that this dose strongly enhanced social interaction in Long Evans rats in a recent study (Ramos et al., 2013). Since OT and TGOT have very similar affinity for the OTR (Elands et al., 1988; Lowbridge et al., 1977) and appear to have similar behavioral potency when given ICV (Sala et al., 2012), we used equivalent doses of each peptide in this study.

Acute behavioral tests

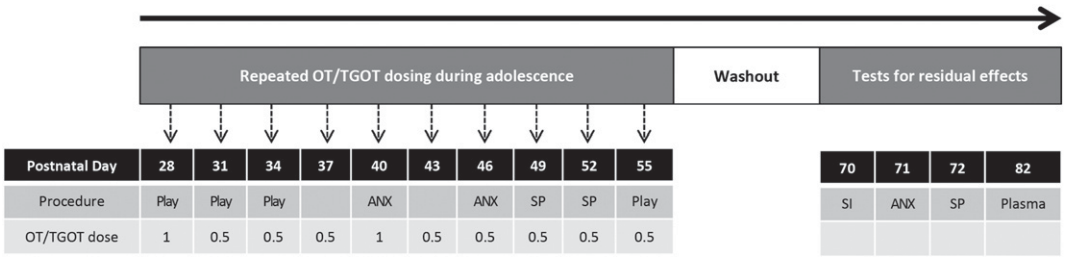
Social play

On PND 28, 31, 34 and 55, rats were assessed for social play behavior. Testing utilized four large black plastic arenas (77 × 51 × 47 cm) with a floor of absorbent bedding. The testing room was dimly lit with a red light (40 W) and the temperature was maintained at 22 ± 2 °C. Rats were individually habituated to the arenas the day prior to the test for 30 min to overcome unfamiliarity of the testing environment. On the day of testing, pairs of rats receiving the same treatment and of approximately the same body weight (≤10 g difference), but from different home cages (i.e. unfamiliar), were placed together into the arena and tested for 20 min. The test began 5 min post-injection. Novel partner combinations were created for each test, and the time of day of testing and test chamber allocated was counterbalanced across treatment conditions. To allow for some evaluation of dose–response effects, a higher (1 mg/kg) dose of OT or TGOT was administered on PND 28 followed by a lower (0.5 mg/kg) dose on PND 31, 34 and 55 (Fig. 1).

Video acquisition software (MotMen Trackmate Quad, v4.6, Motion Mensura, Cooks Hill, NSW, Australia) recorded these sessions and a rater blind to treatment conditions scored the behaviors. Social play was scored as the duration of dyadic interactions that involved pouncing, boxing, biting the nape of the neck and wrestling the partner rat into submission (for overview, see Trezza et al., 2010). Rearing was also scored and was defined as the animal standing on hind legs, including leaning against the wall of the arena in an attempt to investigate its surroundings (Lever et al., 2006). Rearing also served as a general index of any non-specific sedative effects of drug treatment.

Emergence test

On PND 40 and 46 the rats were assessed for anxiety-like behavior using the emergence test. Again to obtain something of a dose response function, distinct 1 mg/kg and 0.5 mg/kg doses were used on these two tests (Fig. 1). The test was conducted in a large white walled arena with a black wooden floor (120 × 120 × 60 cm). A black wooden hide box (40 × 24 × 17 cm) with a hinged red Perspex lid was placed against the center of one wall. Two spotlights (150 W) illuminated the arena to produce a bright open field that is aversive to rats. Each rat was individually tested for 5 min after being placed inside the hide box. Scored behaviors included time spent in: (a) the open field; (b) the hide box; (c) latency to emerge from the hide box; and (d) risk assessment (center of mass in the hide box with part or all of head in the open field).



Postnatal Day	28	31	34	37	40	43	46	49	52	55	70	71	72	82
Procedure	Play	Play	Play		ANX		ANX	SP	SP	Play	SI	ANX	SP	Plasma
OT/TGOT dose	1	0.5	0.5	0.5	1	0.5	0.5	0.5	0.5	0.5				

Fig. 1. Schematic representation of the experimental sequence and the behavioral tests conducted. Abbreviations: 'Play': Test of social play; 'ANX': Tests of anxiety-like behavior (emergence test and elevated plus-maze); 'SP': Social preference test; 'SI': Social interaction test; 'OT': oxytocin; 'TGOT': [Thr4, Gly7]-oxytocin.

Elevated plus-maze

Immediately after the emergence test on PND 40 and 46, rats were placed in the center of a standard elevated plus-maze (EPM) apparatus consisting of four 45 × 10 cm arms (two open without walls and two enclosed by 41 cm high red Perspex walls) arranged in a cross, and elevated 60 cm off the ground. All arms were constructed with a black plywood floor. The test was performed in a dim room illuminated by infrared light. The 5 min test began when the rat was placed in the center square of the maze, facing the open arms. Scored behaviors included: (a) time spent on the open arms; (b) time spent inside the closed arms; (c) time spent in risk assessment (the center of mass in the closed arms with part or all of the head in the open arms).

Social preference test

On PND 49 and 52, rats were tested for social preference using a paradigm adapted from Hicks et al (2012). The same black plastic arenas were used as for social play (see above), except that two small white rectangular cages (23 × 10 × 11 cm) were placed on either side of the arenas on the east and west walls. A novel 'prisoner' rat that the test rat was unfamiliar with was introduced into one of the two rectangular cages, whereas a toy rat of similar size and appearance was placed into the opposite rectangular cage. The location of the prisoner and toy rat was counterbalanced across treatment. The test rat was placed in the middle of the arena facing the north wall and its behavior was video recorded for 10 min. The time spent with the prisoner and toy rat was scored if the test rat was within 150 mm of the cage. Half of the rats were assessed in the social preference test on PND 49 while the other half were tested on PND 52. This was done so that half of the rats acted as drug- and weight-matched prisoner rats for the other half of the cohort and vice versa. The caged rat and the test rat were unfamiliar, of similar weight and received identical drug treatment 5 min prior to the test. The rectangular cages allowed for visual, auditory, and olfactory communication while avoiding potential confounds resulting from aggressive interaction or 'rough-and-tumble' play (Moy et al., 2004). The toy rat was used to control for the possibility of the test rat investigating the caged rat out of visual interest (Motbey et al., 2012). Scored behaviors were the time spent in close proximity to the live prisoner rat and the toy rat.

Post-treatment behavioral testing

A 14-day drug-free period ensued after the final injection during which all subjects remained in their home cages, and were briefly handled every three days. To assess any lasting residual behavioral effects of repeated OT or TGOT treatment in adolescence, subjects were tested again for some of the behaviors described above. Testing took place over three days (PND 70–72) in this order: 1) Social interaction; 2) emergence test and EPM; 3) social preference test (see Fig. 1). Certain adjustments to the post-treatment behavioral testing were made. For the test of social interaction, the same arenas were used as for social play however the adult social behaviors of mounting, anogenital sniffing, adjacent lying and close proximity (rats located within 1.5 body lengths of each other) were measured, in addition to rearing.

Due to constraints relating to the number of tests performed and the number of available partners in each treatment group, some rats were tested with previously encountered partners. However, such "familiar" partner involved a minimum two-week period since last meeting, a period which most likely exceeds the duration of social memory of rats (Moura et al., 2010).

Blood collection and assay

To assess the potential long-lasting endocrine changes arising from adolescent OT or TGOT exposure, all 32 rats were killed on PND 82. Approximately 5 mL of trunk blood was collected in pre-chilled heparinized tubes and plasma was separated by centrifugation at 1600 *g* for 15 min at 4 °C. Plasma was aliquoted and stored at −80 °C within 20 min after collection. Samples were thawed only at the time of assay.

At present there is much debate surrounding the measurement of peripheral OT using enzyme-linked immunosorbent assay (ELISA) and whether the samples must first undergo extraction. Szeto et al. (2011) argue that without extraction, the assay is inadvertently tagging molecules other than OT leading to an overestimation of actual OT quantity in the unextracted sample. These molecules displayed OT-immunoreactivity leading the authors to suggest that they may be degradation products of OT. However, whether these molecules reflect circulating levels of the peptide and are behaviorally active is yet to be determined (McCullough et al., 2013). As such, the current study examined both extracted and unextracted OT to compare results for both.

OT was assayed using a commercially available OT ELISA kit (ENZO Life Sciences, Ann Arbor, MI). Unextracted samples were diluted 1:4 times in assay buffer as previously described (Kramer et al., 2004; Szeto et al., 2011). This dilution allowed all unextracted samples to fit within a reliable portion on the standard curve. Plasma samples were also extracted via solid-phase extraction (SPE) as described in the ENZO Life Science OT protocol manual. Plasma (1 mL) was mixed with an equal volume of 0.1% trifluoroacetic acid (TFA) in water, centrifuged and applied to a 200 mg C18 Sep-Pak column pre-conditioned with 0.1% TFA in water. The columns were then washed four times with 3 mL of 0.1% TFA in water. Sample was eluted slowly by applying a solution comprised of 60% acetonitrile and 40% of 0.1% TFA in water. The solvent was evaporated to dryness using a centrifugal concentrator under vacuum (Genevac, EZ-2 Series). For immunoassay, the dried samples were reconstituted in 0.23 mL of assay buffer provided in the ELISA kit. This concentration step allowed the extracted samples to be detectable by the assay (i.e. fit within the limits of sensitivity). The OT assay had a lower limit of sensitivity of 15.6 pg/mL and an upper limit of 1000 pg/mL. Extracted and unextracted samples were separately assayed on a single plate. The intra-assay coefficient of variation was 2.1% for unextracted and 1.14% for extracted assays.

AVP was similarly assayed using the commercially available Arg8-vasopressin ELISA kit (ENZO Life Sciences, Ann Arbor, MI). Unextracted samples were diluted 1:4 times in assay buffer. The AVP assay had a lower sensitivity of 4.10 pg/mL and an upper limit of 1000 pg/mL. All samples were assayed on a single plate. The intra-assay coefficient of variation was 3.06%.

Corticosterone was assayed using a commercially available corticosterone ELISA kit (ENZO Life Sciences, Ann Arbor, MI). Samples were first diluted with 2.5 parts of steroid displacement reagent for every 97.5 parts of neat sample. Following this, the sample was then diluted 1:50 times in assay buffer provided in the ELISA kit. The corticosterone assay had a lower sensitivity of 26.99 pg/mL and an upper limit of 20,000 pg/mL. All samples were assayed on a single plate. The intra-assay coefficient of variation was 4.36%.

Statistical analysis

Social play behavior was compared across groups using two-way ANOVA (3 treatments × 4 time-points/2 doses) with the error rate controlled across pairwise comparisons using the Bonferroni procedure. Behaviors on the emergence and EPM tests were analyzed using repeated measures ANOVA (3 treatments × 2 time-points/doses) with the Bonferroni correction procedure again employed. The acute test of social preference was analyzed using a one-way ANOVA with Bonferroni correction.

The long-term residual tests were analyzed using one-way ANOVA with the Student–Newman–Keuls post hoc tests used to control the type 1 error rate across the pairwise comparisons. Plasma OT (extracted and unextracted), AVP and corticosterone levels were also analyzed in this way. A predetermined criterion excluded any samples from the neuropeptide plasma analyses that were above or below 2 standard deviations of the mean. All data were analyzed using SPSS 19.0 for Macintosh (SPSS Inc., Chicago, IL. USA). Adjusted *p*-values are shown with a statistical significance level of $p < 0.05$ set for all analyses.

Results

Acute behavioral effects

Social play

The results of the tests of social play are shown in Fig. 2. For social play behavior (Fig. 2a), there was a significant overall main effect of treatment, $F(2,51) = 16.59$, $p < 0.001$, but with no main effect of time (PND), $F(3,51) = 1.14$, $p = 0.342$, and no overall treatment by time interaction effect, $F(6,51) = 1.06$, $p = 0.40$. Post hoc pairwise comparisons using the Bonferroni correction showed that OT treatment significantly reduced the amount of time rats engaged in social play behavior relative to vehicle on PND 28 (1 mg/kg; $p < 0.01$), 34 (0.5 mg/kg; $p = 0.001$) and 55 (0.5 mg/kg; $p < 0.05$). OT treatment also reduced play relative to TGOT treatment on PND 34 ($p < 0.01$) and 55 ($p < 0.05$), both 0.5 mg/kg but not on PND 28 or 31 ($p > 0.05$). TGOT had no significant effects on social play behavior relative to vehicle treatment ($p > 0.05$).

With respect to rearing behavior (Fig. 2b), there was a significant main effect of treatment, $F(2,51) = 29.16$, $p < 0.001$, and time $F(3,51) = 3.31$, $p = 0.027$, but no significant overall treatment by time interaction effect, $F(6,51) = 1.04$, $p = 0.41$. Post hoc pairwise comparisons showed that OT significantly reduced rearing relative to vehicle at PND 28 ($p < 0.001$), 31 ($p < 0.05$), 34 ($p < 0.001$), and 55 ($p < 0.01$) and reduced rearing relative to TGOT treatment on PND 28 ($p = 0.001$) and 34 ($p < 0.01$) only. TGOT had no significant effects on rearing relative to vehicle treatment ($p > 0.05$).

Emergence test

Results from the emergence test at PND 40 (1 mg/kg) and PND 46 (0.5 mg/kg) are presented in Table 1. There was a significant main effect of treatment on risk assessment, $F(2,29) = 3.92$, $p = 0.031$. There was no significant main effect of treatment on time spent in the open field, $F(2,29) = 1.30$, $p = 0.289$, time spent inside the hide box, $F(2,29) = 2.12$, $p = 0.138$, risk assessment, $F(2,29) = 2.12$, $p = 0.138$, or latency to emerge from the hide box, $F(2,21) = 0.84$, $p = 0.447$. Averaged over treatment, rats spent significantly more time in the open field during

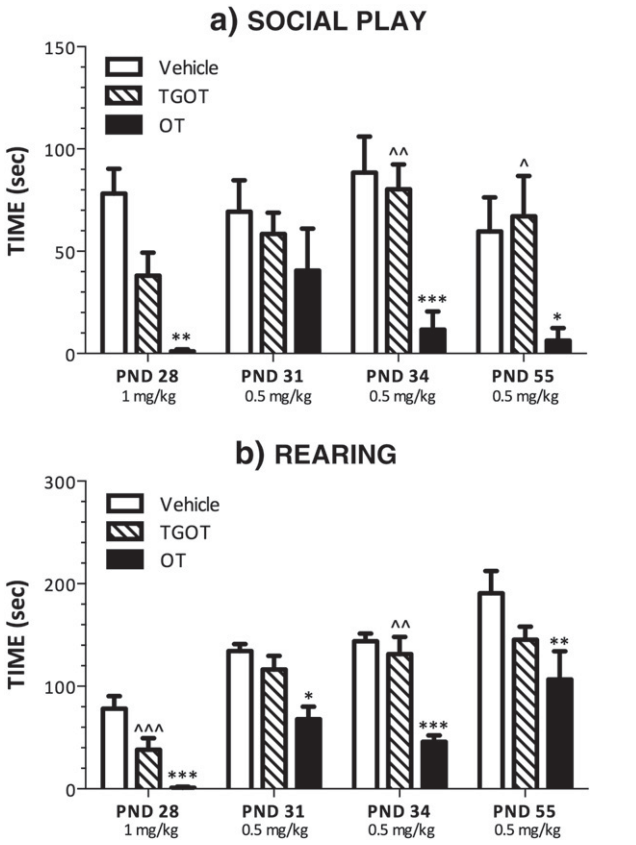


Fig. 2. Mean results for the time spent (sec) engaged in a) social play and b) rearing at PND 28 (1 mg/kg), 31 (0.5 mg/kg), 34 (0.5 mg/kg), and 55 (0.5 mg/kg) following an acute intraperitoneal injection of OT, TGOT, or vehicle. Data represent mean ± SEM. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; compared to vehicle. $^{\wedge}p \leq 0.05$; $^{\wedge\wedge}p \leq 0.01$; $^{\wedge\wedge\wedge}p \leq 0.001$, compared to OT. Abbreviations: 'PND': post-natal day; 'OT': oxytocin; 'TGOT': [Thr4, Gly7]-oxytocin.

Table 1
Acute effects of OT and TGOT on the emergence test and elevated plus-maze tests.

	TREATMENT CONDITION		
	Vehicle	TGOT	OT
	PND 40 (1 mg/kg)		
Emergence test			
Open field (sec)	21.8 (8.35)	18.1 (6.40)	16.3 (7.70)
Hide box (sec)	209.0 (12.04)	205.3 (10.24)	232.9 (13.30)
Risk assessment (sec)	69.1 (31.55)	76.7 (8.53)	49.8 (7.68)
Latency to emerge (sec)	144.5 (40.64)	141.0 (35.53)	171.7 (43.95)
Elevated plus-maze			
% open time	18.3 (13.4)	12.6 (9.7)	13.1 (18.8)
Open arm entries	3.3 (0.53)	2.4 (0.45)	2.3 (0.63)
Closed arm entries	4.7 (0.8)	7.9 (0.4)	4.8 (0.6)
Total arm entries	9.9 (1.2)	10.3 (0.6)	7.1 (1.1)
Risk assessment (sec)	56.7 (13.7)	64.9 (22.9)	68.5 (34.8)
	PND 46 (0.5 mg/kg)		
Emergence test			
Open field (sec)	59.9 (20.04)	44.0 (13.83)	21.5 (8.80)
Hide box (sec)	182.3 (18.31)	185.9 (18.43)	234.6 (21.73)
Risk assessment (sec)	57.8 (7.41)	66.61 (9.95)	43.9 (10.92)
Latency to emerge (sec)	164.4 (32.86)	124.43 (33.43)	251.4 (29.77)
Elevated plus-maze			
% open time	22.8 (3.88)	20.3 (5.25)	7.8 (3.46)
Open arm entries	4.7 (0.80)	2.7 (0.62)	1.5 (0.58)**
Closed arm entries	7.2 (0.9)	7.6 (0.43)	5.3 (0.73)
Total arm entries	11.8 (1.3)	10.3 (0.72)	6.8 (0.7)
Risk assessment (sec)	54.7 (7.08)	53.6 (4.78)	54.2 (14.1)

Note: Data represent mean (SEM).
** $p \leq 0.01$, compared to vehicle.
Abbreviations: 'OT': oxytocin; 'TGOT': [Thr4, Gly7]-oxytocin; 'PND': post-natal day.

their second emergence test, $F(1,29) = 7.51$, $p = 0.01$. Averaged over treatment, there was no significant effect of time (PND) on time spent inside the hide box, $F(1,29) = 2.54$, $p = 0.122$, risk assessment, $F(1,29) = 1.47$, $p = 0.234$, or latency to emerge from the hide box, $F(1,21) = 0.25$, $p = 0.627$. There was no significant time by treatment interaction effect for time spent in the open field, $F(2,29) = 1.23$, $p = 0.306$, time spent inside the hide box, $F(2,29) = 0.19$, $p = 0.828$, latency to emerge from the hide box, $F(1,21) = 1.13$, $p = 0.341$, or risk assessment, $F(2,29) = 0.05$, $p = 0.954$.

There were no other significant differences between treatment groups on any measures in the emergence test at PND 40 or 46, all $p > 0.05$.

Elevated plus-maze

Results from the EPM test, conducted immediately after the emergence test, on PND 40 (1 mg/kg) and PND 46 (0.5 mg/kg) are also shown in Table 1. There was a significant main effect of treatment on the number of open arm entries, $F(2,29) = 4.84$, $p = 0.015$, number of closed arm entries, $F(2,29) = 4.59$, $p = 0.018$, and total arm entries, $F(2,29) = 6.16$, $p = 0.006$. There was no significant main effect of treatment on the percentage of time spent in the open arms, $F(2,29) = 1.99$, $p = 0.155$, or risk assessment, $F(2,29) = 0.22$, $p = 0.807$. Averaged over treatment, there was no significant effect of time (PND) on the percentage of time spent in the open arms, $F(1,29) = 0.72$, $p = 0.405$, the number of open arm entries, $F(1,29) = 0.41$, $p = 0.526$, the number of closed arm entries, $F(1,29) = 0.44$, $p = 0.511$, the total arm entries, $F(1,29) = 0.66$, $p = 0.423$, or risk assessment, $F(1,29) = 2.21$, $p = 0.148$. There was no significant treatment by time interaction effect for time spent in the open arms, $F(2,29) = 0.72$, $p = 0.405$, number of open arm entries, $F(2,29) = 2.09$, $p = 0.142$, number of closed arm entries, $F(2,29) = 0.51$, $p = 0.607$, total arm entries, $F(2,29) = 1.16$, $p = 0.327$, or risk assessment, $F(2,29) = 0.375$, $p = 0.69$.

Post hoc pairwise comparisons using the Bonferroni correction revealed that on PND 46, OT treatment significantly reduced the number of open arm entries relative to vehicle treatment, ($p < 0.01$). OT treatment also indicated a trend towards decreased time spent on the open arms, ($p = 0.051$). OT treated rats had significantly fewer closed arm entries relative to TGOT treated rats ($p = 0.01$) at PND 40. At PND 46, OT treated rats had significantly fewer total arm entries relative to vehicle treated rats ($p < 0.01$). There were no other significant differences between treatment conditions on any measures on the EPM tests conducted on PND 40 and 46 ($p > 0.05$).

Social preference test

Results from the social preference test, conducted with half of the cohort of rats on PND 49 and the other half on PND 52 (dosed with 0.5 mg/kg), are shown in Fig. 3. Two rats each from the OT and the TGOT groups were excluded from the analysis due to a video recording failure. There was a significant main effect of treatment on time spent with the live rat, $F(2,25) = 4$, $p = 0.031$, and on time spent with the toy rat, $F(2,25) = 5.57$, $p = 0.01$.

Post hoc pairwise comparisons using the Bonferroni correction revealed that rats treated with OT spent significantly more time (sec) with the live rat ($p < 0.05$) and significantly less time with the toy rat relative to the vehicle group ($p < 0.05$). Rats treated with TGOT did not differ from vehicle treatment in these measures (all $p > 0.05$).

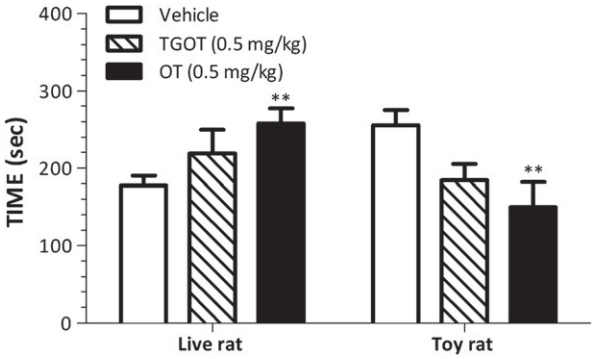


Fig. 3. Mean results for the time spent (sec) in close proximity with the live rat and toy rat following an acute intraperitoneal injection of OT, TGOT, or vehicle on the social preference test conducted across PND 49 and 52 (0.5 mg/kg). Data represent mean ± SEM. $^{**}p \leq 0.01$, compared to vehicle. Abbreviations: 'OT': oxytocin; 'TGOT': [Thr4, Gly7]-oxytocin.

Long-term residual behavioral effects

Social interaction test

The results of the social interaction test conducted two weeks after the final injection at PND 70 are shown in Fig. 4. There was a significant overall main effect of treatment on time spent in close proximity, $F(2,13) = 6.61$, $p = 0.01$, with post hoc tests indicating that the OT-pretreated pairs spent a significantly greater amount of time in close proximity (1.5 body lengths) with each other relative to vehicle- and TGOT-pretreated pairs, $p < 0.05$. There was no significant main effect of treatment on mounting behavior, $F(2,13) = 1.88$, $p > 0.05$, anogenital sniffing, $F(2,13) = 0.43$, $p > 0.05$, rearing, $F(2,13) = 1.94$, $p > 0.05$, or adjacent lying, $F(2,13) = 1.12$, $p > 0.05$; post hoc tests indicated no significant differences across groups for any of these behaviors in the social interaction test (all $p > 0.05$).

Emergence test

The results from the emergence test conducted at PND 71 are presented in Table 2. There was no significant main effect of treatment on time spent in the open field, $F(2,29) = 2.64$, $p > 0.05$, time spent inside the hide box, $F(2,29) = 1.42$, $p > 0.05$, risk assessment, $F(2,29) = 1.10$, $p > 0.05$, or latency to emerge from the hide box, $F(2,29) = 2.96$, $p > 0.05$.

Post hoc tests indicated that TGOT-pretreated rats took significantly less time to emerge from the hide box than OT-pretreated rats, $p < 0.05$. There were no significant effects of OT-pretreatment relative to vehicle on any measures in the emergence test (all $p > 0.05$).

Elevated plus-maze

The results for the EPM conducted immediately after the Emergence test on PND 71 are also presented in Table 2. There was no significant main effect of pretreatment on the percentage of time spent on the open arms, $F(2,29) = 2.62$, $p > 0.05$, number of open arm entries, $F(2,29) = 1.24$, $p > 0.05$, or risk assessment, $F(2,29) = 3.12$, $p > 0.05$.

Post hoc tests revealed no significant differences across groups on any of the above behaviors as measured in the EPM (all $p > 0.05$).

Social preference test

The results for the social preference test conducted at PND 72 are shown in Fig. 5. There was no significant main effect of treatment on

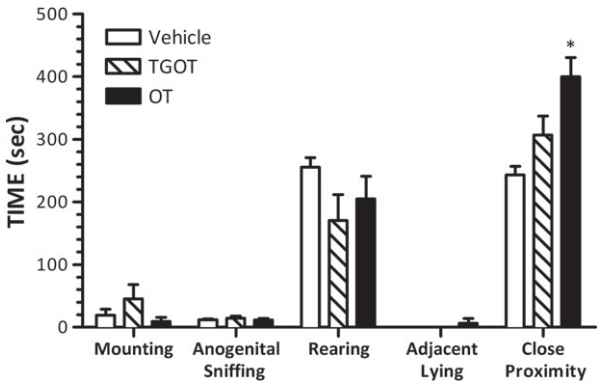


Fig. 4. Long-term residual effects observed in the social interaction test in OT, TGOT, and vehicle pretreated rats at PND 70. Data represent mean ± SEM. $^{*}p \leq 0.05$, compared to vehicle. Abbreviations: 'OT': oxytocin; 'TGOT': [Thr4, Gly7]-oxytocin.

Table 2
Long-term residual effects of OT and TGOT on the emergence test and elevated plus-maze tests.

	Pretreatment condition		
	Vehicle	TGOT	OT
	Residual effects (PND 71)		
Emergence test			
Open field (sec)	69.0 (17.14)	102.3 (20.93)	41.5 (16.36)
Hide box (sec)	127.6 (9.6)	116.8 (16.13)	149.2 (15.02)
Risk assessment (sec)	103.4 (14.14)	80.9 (13.04)	109.3 (15.06)
Latency to emerge (sec)	130.6 (31.0)	88.3 (31.75)^	198.6 (31.04)
Elevated plus-maze			
% open time	38.6 (5.35)	44.7 (4.67)	25.8 (7.09)
Open arm entries	5.8 (0.89)	6.6 (0.67)	4.7 (0.87)
Risk assessment (sec)	55.2 (7.12)	57.8 (7.8)	82.6 (10.5)

Note: Data represent means (SEM).
^ $p \leq 0.05$, compared to OT.
Abbreviations: 'OT': oxytocin; 'TGOT': [Thr4, Gly7]-oxytocin.

time spent with the live rat, $F(2,29) = 0.03$, $p > 0.05$, or time spent with the toy rat, $F(2,29) = 1.34$, $p > 0.05$. Post hoc tests did not indicate any significant differences across groups on any of the above behaviors as measured in the social preference test (all $p > 0.05$).

Plasma OT, AVP and corticosterone

Results for circulating unextracted and extracted plasma OT, AVP and corticosterone following OT and TGOT pretreatment are shown in Figs. 6a–d.

There was a significant main effect of treatment on unextracted plasma OT, $F(2,27) = 4.65$, $p < 0.05$, but not in extracted plasma OT, $F(2,29) = 0.05$, $p > 0.05$. Post hoc tests showed that OT, but not TGOT, pretreatment during adolescence resulted in a long-lasting increase in plasma OT concentration (pg/mL) in adulthood relative to the vehicle group, $p < 0.05$ (Fig. 6a). This difference was not present in extracted samples, $p > 0.05$ (Fig. 6b).

There was no significant main effect of treatment in plasma corticosterone levels, $F(2,29) = 1.39$, $p > 0.05$, or in plasma AVP levels, $F(2,26) = 0.04$, $p > 0.05$. Post hoc tests revealed no significant group differences in plasma corticosterone in the pretreated groups despite a downwards trend in both OT- and TGOT-pretreated groups relative to the vehicle group, $p > 0.05$ (Fig. 6c). Pretreatment groups did not significantly differ in plasma AVP, $p > 0.05$ (Fig. 6d).

Discussion

The present study compared the acute and long-term residual effects arising from repeated exposure to OT and TGOT during the adolescent period in male Long Evans rats. The acute effects of OT were generally greater than those of TGOT, with OT having an inhibitory

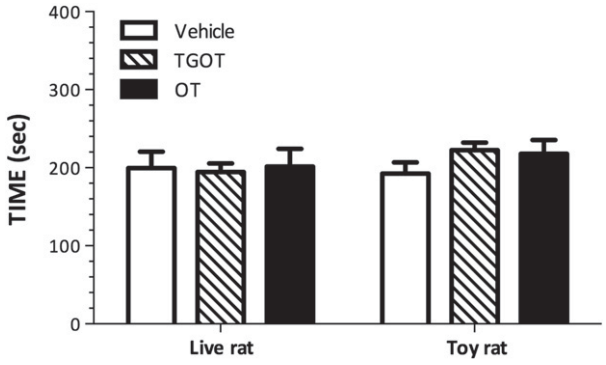


Fig. 5. Long-term residual effects observed in the social preference test in OT, TGOT, and vehicle pretreated rats at PND 72. Data represent mean ± SEM. Abbreviations: 'OT': oxytocin; 'TGOT': [Thr4, Gly7]-oxytocin.

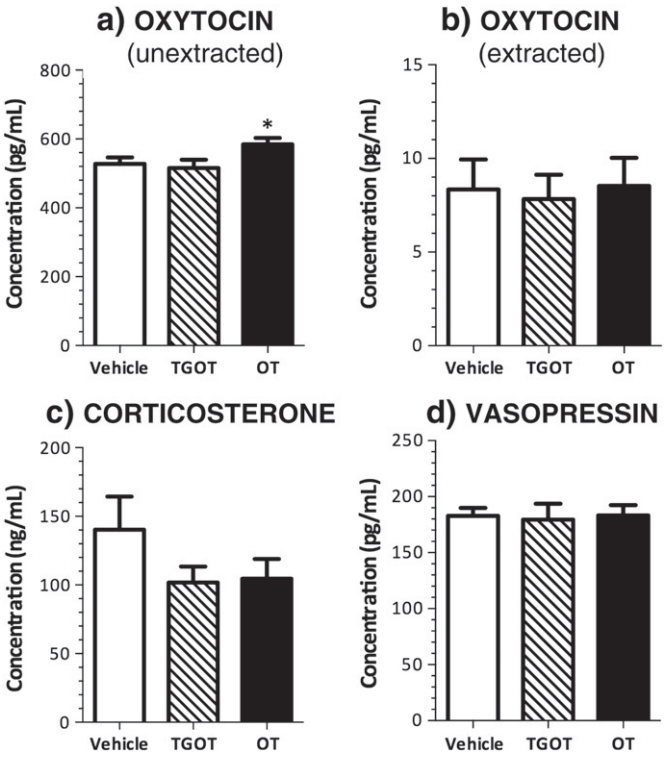


Fig. 6. Basal plasma levels of a) unextracted OT (pg/mL); b) extracted OT (pg/mL); c) corticosterone (ng/mL); and d) vasopressin (pg/mL) in OT, TGOT, and vehicle pretreated rats at PND 82. Data represent mean ± SEM. *$p \leq 0.05$, compared to vehicle. Abbreviations: 'OT': oxytocin; 'TGOT': [Thr4, Gly7]-oxytocin.

effect on social play and rearing, while increasing social preference. In terms of the long-term residual effects of repeated treatment, OT, but not TGOT, pretreatment resulted in greater long-lasting increases in social behavior on the social interaction test as well as increased basal plasma OT. These subtle OT-induced prosocial effects were not accompanied by any long-lasting changes in anxiety-related behavior as measured by the emergence test and the EPM.

Acute behavioral effects of OT and TGOT administration

OT produced a relatively consistent behavioral pattern in early adolescence, characterized by decreased social play behavior and rearing. OT acts to switch to an 'energy-saving' state where anabolic metabolism is promoted, pain threshold is increased, and blood pressure along with tail-skin temperature is decreased (Uvnäs-Moberg, 1998; Uvnäs-Moberg et al., 1992). Locomotor suppression is often observed following high doses of peripherally administered OT in rats (Carson et al., 2010; Uvnäs-Moberg et al., 1994). As such, after acute OT administration, the amount of intense and often rough play behavior exhibited by adolescent rats, such as pinning, pouncing, chasing, and biting the nape of the neck in order to pin the play-partner down into submission, was significantly reduced. The decrease in rearing behavior observed with OT was also indicative of sedative effects.

The finding that an acute injection of TGOT had a relatively modest influence on social play agrees with findings that the acute effects of peripherally administered OT may be mediated by the V1aR rather than the OTR. Ramos et al. (2013) showed that the OT-induced prosocial effects could be blocked by peripheral administration of a selective V1aR antagonist, SR49059. This mimics a previous study by Sala et al. (2011) who showed that ICV OT-induced rescue of the social deficits and cognitive impairment displayed by OTR −/− mice could also be blocked by ICV SR49059. Together, these findings suggest that exogenous OT may be mediating many of its effects via the V1a receptors. Moreover, this also agrees with recent findings by Veenema et al.

(2013) that AVP regulates social play in a sex- and gender-specific manner, and to a greater extent than OT. Given the crosstalk between OT with the AVP receptor subtypes and the prosocial role of the V1aR (Bielsky et al., 2005; Egashira et al., 2007; Landgraf et al., 2003), the lack of TGOT effects may be at least partly explained by its negligible affinity at the V1aR.

Neither OT nor TGOT produced acute anxiolytic effects on the EPM or emergence test during adolescence, a somewhat surprising result given other evidence for the centrally mediated anxiolytic actions of OT (Neumann, 2002; Uvnäs-Moberg et al., 1994). The majority of studies that have described the anxiolytic-like effect of OT involved either ICV administration (Windle et al., 1997) and/or the OT-induced reversal of elevated anxiety with OT in animals that were prenatally stressed or socially isolated (Grippo et al., 2009; Lee et al., 2007). OT-treated rats did display a reduction in the number of open arm entries and total arm entries relative to vehicle on the EPM at PND 46, as well as fewer closed arm entries relative to TGOT at PND 40; however this again perhaps reflects the sedation often seen after relatively high dose peripheral OT administration (Uvnas-Moberg et al., 1994).

In the social preference test conducted in late adolescence we found that OT, but not TGOT, significantly increased the amount of time spent in close proximity with a social stimulus (i.e. caged rat) relative to an object stimulus (toy rat), indicating a prosocial effect (Lukas et al., 2011). These observations in the social preference test are novel and extend, using this different paradigm, our observations of prosocial effects of peripheral OT in Long Evans rats assessed in the social interaction test. Up until now, OT was only observed to rescue the reduced social preference observed in Wistar rats subjected to social defeat, without having any intrinsic action in unstressed rats (Lukas et al., 2011). Similarly in Wistar rats, Hicks et al. (2012) found no effect of acute OT (1 mg/kg, IP) on social preference in an extended 30 min social preference test. Taken together, this tends to affirm the idea that greater prosocial effects of OT can be observed in Long Evans strain relative to Wistar rats. However, it should be noted that the relatively strong prosocial effect observed in the current study may have been the result of a cumulative effect developing from previous drug exposures during the adolescent period. While TGOT did not significantly increase social preference in the current study, it is notable that there were trends in the right direction, suggesting that further tests of social preference with higher doses of TGOT may be warranted. A positive outcome would be consistent with observations of a key role for the OTR relative to the V1aR in driving social preference (Lukas et al., 2011).

Lasting behavioral effects and neuroendocrine changes following intermittent OT and TGOT administration

The major aims of the current study were to replicate and extend the findings of Bowen et al. (2011) that repeated adolescence treatment with OT produces social and anxiety-related changes that last into adulthood. Consistent with these earlier findings, OT pretreated rats displayed a lasting facilitation of social behavior indexed by greater close-proximity with an unfamiliar rat in the social interaction test. This occurred without changes to the duration of other social behaviors such as anogenital sniffing, mounting and adjacent lying. These findings complement those noted in the introduction (Bowen et al., 2011; Witt et al., 1992) but using here a lower and more intermittent dosing regimen involving 0.5–1 mg/kg administered every three days across the extended adolescent period. This appears to have had a more potent effect, at least with respect to producing a significant residual increase in plasma OT levels observed in the present study.

Nonetheless, a discrepancy in the results following extracted and unextracted OT ELISA in the present study is notable (Figs. 6a and b), with a significant long-lasting increase in plasma OT in unextracted but not extracted samples. Some have argued that extraction removes a large portion of the measurable OT from the sample (Zhong et al., 2012), leading to a possible floor effect. However, without extraction, the assay may be erroneously tagging molecules other than OT, which may explain the multiple fold difference in circulating levels of OT in extracted and unextracted plasma (McCullough et al., 2013; Szeto et al., 2011). Given the uncertainty in the field at this point in time, it is worth investing effort into exploring other methods such as radioimmunoassay (RIA) (Kagerbauer et al., 2013; Neumann et al., 2013) and LC–MS/MS for analyzing OT in biological matrices. There were no significant pretreatment effects on adult AVP plasma levels, showing that intervention with OT or TGOT in adolescence was of no effect on this hormone, at least in the unextracted samples assessed here.

In contrast to previous results (Bowen et al., 2011), residual anxiolytic effects of OT pretreatment were not observed in the present study. Indeed, somewhat strikingly, TGOT pretreated rats exhibited less anxiety-like behavior than OT pretreated rats in adulthood, evidenced by the shorter latency to emerge from the hide box during the emergence test. One possible explanation for the absence of residual effects of OT on anxiety-like behavior in adulthood is the use of repeated testing with the emergence test and the EPM during adolescence, which might reduce the overall effectiveness of these tests in producing anxiety in adulthood. This effect was observed during acute dosing where rats, averaged across treatment, spent significantly more time in the open field on the second emergence test at PND 46. Notably, our previous study (Bowen et al., 2011) showed anxiolytic effects of OT pretreatment where rats were only tested once on the EPM.

Another possible limitation of the present study is the extent to which the selective OTR agonist, TGOT, crosses the blood–brain barrier (BBB). Very limited *in vivo* findings have been obtained with TGOT to date (Sala et al., 2012) making it somewhat difficult to put the findings observed in the present study into perspective. It is possible that the compound may be metabolically unstable once injected and is therefore rapidly broken down by peptidases before it is able to act on CNS regions known to mediate prosocial effects (McEwen, 2004; Busnelli et al., 2013). However, the fact that some behavioral effects were observed with TGOT such as the reduced latency to emerge from the hide box during the emergence test, suggests that it has some central actions. Overall, there is increasingly good evidence for CNS penetration of OT, as well as raised plasma levels, following peripheral administration (e.g. Neumann et al., 2013). It would clearly be of interest to also confirm this with peripherally injected TGOT.

The long lasting effects of repeated OT administration are yet to be fully examined in animal models and in humans. Several studies have found evidence for safe and effective use of chronic OT in treating social deficits, such as in children with autism spectrum disorder (ASD) (Kosaka et al., 2012; Tachibana et al., 2013) as well as in mouse models of ASD (Teng et al., 2013). However, other studies have brought to light a possible "darker side" where chronic OT administration disrupted partner preference formation in prairie voles (Bales & Perkeybile, 2012), caused an increase in agonistic behaviors and a dysregulated hypothalamic-pituitary–adrenal axis in pigs (Rault et al., 2013) and a lasting decline in sociability in mice (Huang et al., 2013). Given the plasticity of hypothalamic OT systems, it is important for preclinical studies to illustrate these possible detrimental effects of chronic administration in order to better inform human clinical trials on the efficacy of OT as a therapeutic treatment.

Conclusions

The present study suggests that adolescent exposure to OT, but not TGOT, can have subtle long-lasting effects on the adult social repertoire. Intermittent pretreatment with OT during adolescence had a pronounced positive long-term residual effect on social behavior and significantly elevated basal OT plasma levels. These findings suggest that OT has different acute and long-lasting residual effects compared to

the relatively selective OTR agonist, TGOT, and that the effect of OT on AVP receptors, such as the V1aR, may be relevant to explaining its capacity to produce acute and lasting effects on social behavior.

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Appendix 4: Supplementary methods for Chapter 3

NOTE: The numbered references (e.g. [1]) are referring to the reference list for the published manuscript presented in Chapter 3.

Detailed Immunohistochemistry methods

Tissue collection, preparation and staining

All rats were deeply anesthetized with sodium pentobarbital (Lethabarb, Virbac Pty Ltd, Milperra, NSW, Australia, 0.3 – 0.5 ml per rat), and then perfused transcardially with 200 ml of 0.1 M PBS, followed by 200 ml of 4% paraformaldehyde in PBS, pH 7.3. The brains were removed, blocked in the coronal plane, and placed in paraformaldehyde overnight at 4°C. They were then placed in 15% sucrose for 24 h, followed by 30% sucrose for 48 h. After this, the tissue blocks were placed on microtome stages, frozen to -17°C, and sectioned at 40 µm. The entire brain was sectioned coronally apart from one of the olfactory bulbs for each rat, which were sectioned in the saggital plane to allow clearer elucidation patterns of Fos in the AOB [18]. Consecutive sections were placed sequentially across four vials of 0.1 M phosphate buffer (PB).

Free-floating sections were incubated for 30 min in 1% hydrogen peroxide in PB and then for 30 min in 3% normal horse serum in PB. The sections were then incubated in primary c-Fos antibody (Ab) for 72 h at 4°C (rabbit polyclonal; reacts with c-Fos p62 of mous, rat, and human; non-cross-reactive with FosB, Fra-1, or Fra-2; Santa Cruz Biotechnology, Canta Cruz, CA). The primary Ab was diluted 1:2000 in phosphate buffered horse serum (PBH) (0.1% bovie serum albumin, 0.2% Triton X-100, and 2% normal horse serum in PB). Sections wree then washed for 30 min in PB at room temperature and incubated for 1 h at room temperature in secondary Ab (biotyinylated anti-rabbit IgG made in goat; diluted 1:500 in PBH; Vector Laboratories, Burlingame, CA). They were then washed in PB for an additional 30 min and then incubated for 1.5 hr in ExtrAvidin-horseradish peroxidase

(diluted 1:1000 in PBH; Sigma, St. Louis, MO). After this, they were washed three more times (30 min) in PB, after which horseradish peroxidase activity was visualized with nickel diaminobenzodine and glucose oxidase reaction as described previously [17]. This reaction was terminated after 10 min by washing in PB.

The sections were then mounted on subbed slides, dehydrated in ascending concentrations of ethanol, xylene cleared, and coverslipped.

Counting of labeled cells

The method described above produces a black oval-shaped immunoprecipitate confined to the cell nucleus of Fos-positive cells. This was quantified microscopically at 50 sites using a brain atlas for guidance [36] (see Table 1 for the regions and counts for areas where significant results were obtained). Only darkly labeled oval-shaped nuclei were counted. Three slightly different approaches to quantification were used relating to the following: (1) coronal sections for the entire brain; (2) saggital sections from the AOB; and (3) coronal sections from the main olfactory bulb (MOB).

Coronal sections Coronal sections were viewed under either a 20X or 40X objective and an optical graticule was used to manually quantify the number of Fos-positive neurons in each regions. The numbers of positive nuclei that fell within a 0.5 X 0.5 mm area (20X objective) or 0.25 X 0.25 area (40X objective) in each region of interest were counted from one section per rat by an observer who was blind to group assignment.

In several cases, the designated area to be counted was substantially larger than the boundaries of the graticule. In such cases, the graticule was placed in a fixed position within the region of interest relative to known anatomical landmarks. In other cases, the

designated area was smaller than the boundaries of the graticule. In such cases, only the region of interest, not the extraneous areas, were counted.

Accessory olfactory bulb The AOB was quantified microscopically (40X objective) in sagittal sections. The graticule was used to quantify Fos-positive cells in 12 subregions within the anterior and posterior glomerular, mitral and granule cell layers. Each region constituted a 0.25 X 0.25 mm square and was quantified by an observer who was blind to group assignment. For statistical analysis, counts from adjacent regions were summed to provide a total of six counts per rat for the AOB. For comparison purposes, an additional count from the mitral cell layer was made from coronal sections.

Main olfactory bulb For the MOB, a number (one to five) of representative coronal sections from each rat at a level of ~6.7 mm anterior to bregma were obtained. Fos expression in the entire glomerular layer of the MOB was counted microscopically for each of these sections by an observer who was blind to group assignment. For the purposes of analysis, the glomerular layer was subdivided into six separate sectors corresponding to the dorsomedial, dorsolateral, medial, lateral, ventromedial, and ventrolateral areas. A Fos count for each of these sectors was obtained for each rat by averaging counts for each sector from all the different sections counted.

For comparison purposes, two additional counts were made in the MOB in the granule cell layer using the approach described above for coronal sections.

Preparation of images

Digital images were made of representative pieces of tissue for illustrating the distribution of Fos-positive cells in key areas. The digitized images were produced with an

Olympus DP70 12.5 megapixel camera (Olympus, Tokyo, Japan) attached to an Olympus Optical BX51 light microscope (Olympus, Tokyo, Japan). Images were acquired with a desktop computer using custom software supplied with the camera system. All images were directly imported into Adobe Photoshop CS5 (Adobe Systems, San Jose, CA, USA) and reduced in size. The only post-production enhancements were conversion of color images to black and white, the uniform adjustment of brightness and contrast for printing purposes and the whitening of non-tissue surfaces using the eraser tool.

Appendix 5: Supplementary methods for Chapter 4

S1: Supplementary Methods

Cat odour stimulus

The predator stimuli used were 2 g balls of cat fur acquired from male cat carcasses kindly provided by *Australian Feral Pest Management*. Feral cats are routinely shot in Australia due to the threat they pose to native Australian wildlife. Deceased cats were stored at -20 °C. To prepare each predator stimulus, a 2 g ball of fur was shaved from the back and neck of the cat. The fur sample was stored in an airtight jar at -20 °C when not in use. Prior to use, the cat fur was heated in a scientific oven (Binder; Crown Scientific, Australia) at 40 °C for 30 min. In between testing sessions the ball was placed in the oven for 5 min at 40 °C to ensure the temperature was consistent across sessions. The heating process takes the temperature of the fur closer to the body temperature of a live predator.

Subjects and habituation

The subjects used for the Experiments were adult male Albino Wistar rats (Animal Resources Centre, Perth, WA, Australia) weighing between 425 and 635 g at the start of the experiment. They were housed in groups of 4 in a temperature controlled colony room (21 ± 2 °C) on a reverse light-dark cycle (lights on 21:00). Food and water were available *ad libitum* in the home cage and all behavioural testing took place during the dark cycle. Subjects were handled daily for 1 week prior to the start of testing. Quads were habituated to the testing arenas once per day for 30 min (Experiment 1) or 60 min (Experiment 2-5) for the three days prior to the start of testing, and were given saline injections prior to these sessions to habituate them to the injection procedure.

Drug solutions

SR49059 (Axon Medchem, Netherlands) was made up at 1 mg/ml by dissolving the compound in a 2% Tween 80, 15% DMSO, 83% saline (0.9% NaCl). OT (Auspep, Australia)

was made up at 0.5 mg/ml in saline. AVP (Auspep, Australia) was made up at 0.01 mg/ml in saline. SSR149415 (Axon Medchem, Netherlands) was made up at 10 mg/ml in 7.5% DMSO, 7.5% Tween 80 and 85% saline. The above solutions without the active compound were used for vehicle injections for the relevant conditions.

Trackmate Social: Kinect

In Experiment 4 we used our new custom tracking software *Trackmate Social: Kinect.* This software receives and analyses input from the Microsoft Xbox Kinect camera. The Kinect camera consists of an infrared laser projector and CMOS microchip sensor which together capture detailed real-time 3D data, allowing for full 3D motion capture capabilities. These 3D data are analysed by the *Trackmate Social: Kinect* software using a number of algorithms that allow for highly accurate real-time tracking of the rats in the environment.

Appendix 6: Supplementary methods for Chapter 5

Supplementary Methods

1. Cat odour stimulus

The predator stimuli used were 2 g balls of cat fur acquired from male cat carcasses kindly provided by *Australian Feral Pest Management*. Feral cats are routinely shot in Australia due to the threat they pose to native Australian wildlife. Deceased cats were stored at -20 °C. To prepare each predator stimulus, a 2 g ball of fur was shaved from the back and neck of the cat. The fur sample was stored in an airtight jar at -20 °C when not in use. Prior to use, the cat fur was heated in a scientific oven (Binder; Crown Scientific, Australia) at 40 °C for 30 min. In between testing sessions the ball was placed in the oven for 5 min at 40 °C to ensure the temperature was consistent across sessions. The heating process takes the temperature of the fur closer to the body temperature of a live predator.

2. LC-MS/MS

Testosterone was extracted from 1 g aliquots of testicular homogenate (25% testicle and 75% water) using Clean Screen® CSDAU503 solid phase extraction columns (500 mg/3 ml) from United Chemical Technologies (Bristol, PA, USA). Proteins were precipitated from testicular homogenate aliquots via addition of 1 ml sodium hydroxide (0.5 M) followed by 1 ml zinc sulphate (10 % w/v). Resulting supernatants were diluted and buffered to pH 6 with 3 mL phosphate buffer (0.1 M). SPE columns were preconditioned with 3 mL methanol, followed by 3 ml water, then 1 ml phosphate buffer (0.1 M, pH 6). Following sample application, columns were washed with 3 ml water and dried under vacuum (pressure 10 inches Hg) for 5 minutes before testosterone was eluted using 3 ml methanol.

Testosterone, corticosterone and progesterone were extracted from 0.25 ml plasma samples by liquid-liquid extraction with 1 ml methyl tert butyl ether. Plasma extracts were dried under nitrogen at 40^{o}C while testicle extracts were dried at 80^{o}C. Resulting residues were reconstituted in 100 μL initial mobile phase (0.1% formic acid in 10% methanol and 90% water) for LC-MS/MS analysis. Chromatographic separation was performed on a Kinetex XB-C18 column (100 mm x 2.10 mm, 1.7 μm) from Phenomenex (Lane Cove, NSW, Australia) via gradient elution at 0.2 ml/min using a Shimadzu Nexera™ ultra high performance liquid chromatograph (Shimadzu Corp, Kyoto, Japan). Analyte and internal standard ion transitions were acquired via multiple reaction monitoring using a Shimadzu 8030 triple quadrupole mass spectrometer operated in positive electrospray ionisation mode.

Testosterone, corticosterone and progesterone standards obtained from Cerilliant (TX, USA) were diluted to yield separate stock solutions for calibrator and quality control sample preparation. D3 testosterone, D4 cortisol and D9 progesterone internal standards were also obtained from Cerilliant and added to all samples, calibrators and quality controls prior to extraction. Calibrators produced a linear response at seven concentration levels ranging from 0.5 – 10 ng/mL for testosterone and progesterone in plasma, both 2 – 40 ng/mL and 10 – 200 ng/mL for corticosterone in plasma and 10 – 250 ng/g for testosterone in the testes. Samples with corticosterone levels above the initial quantification range (2 – 40 ng/mL) were re-analysed using a higher calibration range (10 – 200 ng/mL).

3. *Methylation of AVP promoter regions in the MePD*

3.1. *Microdisection of brain regions*

Prior to microdissection all apparatus used were either rinsed in DEPC water (Sigma-Aldrich, USA) and autoclaved or wiped with RNAzap (Sigma, USA). Samples were sectioned at 100 µm in a cryostat at -21°C and mounted onto autoclaved Superfrost glass slides (FischerScientific ,USA). The MePD was microdissected using autoclaved glass Pasteur pipettes to obtain micropunches that were transferred to lysis buffer for extraction. 1 µl of RiboLock (Thermo Scientific) was added per tube to ensure that the RNA does not degrade. In pilot studies cresyl violet staining was conducted to ensure the MePD was correctly and consistently identified for dissection using our method.

3.2. *gDNA extraction*

gDNA extraction was performed by the phenol chloroform isoamyl alcohol method. Briefly, tissues were microdissected, placed in lysis buffer and homogenized in phenol chloroform isoamyl alcohol (Sigma-Aldrich ,USA) and centrifuged at 8000 rpm for 15 min at 4°C. Ice cold isopropanol was added to the aqueous phase and incubated at room temperature for 15 min. Samples were centrifuged at 8000rpm for 15 min at 4°C. The pellets washed three times 75% ethanol and allowed to air dry at RT for ~15-30 min. They were resuspended in 30 µl MilliQ water and stored at -20°C for short time or -80°C for longer periods.

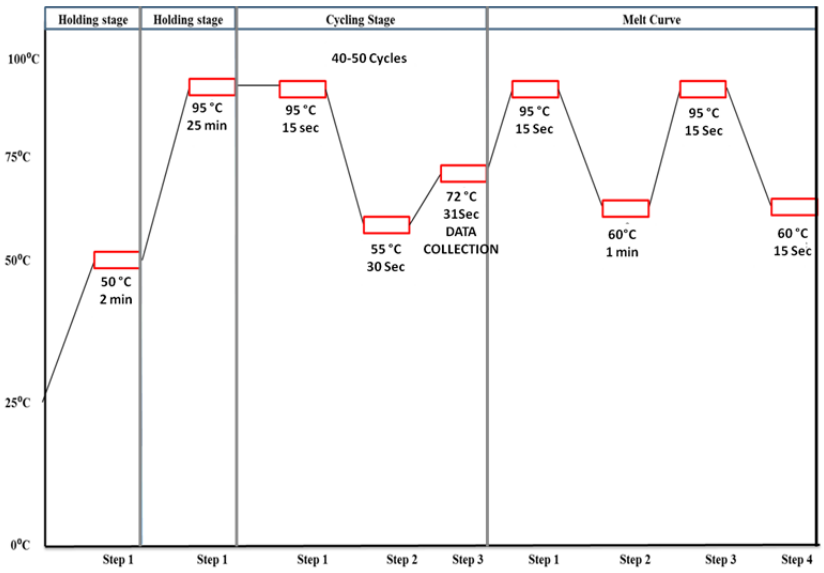
3.3. *Methylation Specific Restriction Digestion*

Methylation of AVP promoter was quantified using methylation sensitive restriction enzyme (MSRE) digestion in combination with qPCR quantification adapted from Auger et

al. 2011. Briefly, 600ng DNA from each rat was pipetted into two tubes: an enzyme treated and a no-enzyme control tube. These tubes were then processed using the same primers surrounding the targeted HpaII site (New England Biolabs, Ipswich, MA) or BstUI (Fermentas Inc., Glen Burnie, MD). HpaII was used to cleave DNA at unmethylated CCGG (titled promotor site 1) sites and BstUI at unmethylated CGCG (titles promotor site 2) sites. For promoter site 1 DNA from each animal was diluted in 1 µl of 'NEB buffer 1' in separate tubes. 2 µl of HpaII restriction enzyme was added and mixture was incubated for three hours at 37°C. For promoter site 2, DNA from each animal was diluted in 1 µl of 'buffer R' in separate tubes. 1 µl of BstUI restriction enzyme was added and mixture incubated at 37°C for one hour. Both enzymes were inactivated by treatment at 65°C for 20 min.

3.4. qPCR Reaction

Quantitative PCR reactions were run using an ABI 7500 machine. The run method is described in the figures below. All samples were run in triplicate.



Relative expression with reference to internal control was quantified using freely available Relative expression software tool (REST) (http://www.REST.de.com) (Pfaffl *et al.*

2002). 10000 randomizations were used for the bootstrap randomisation. This software uses PCR efficiency-calibrated model and randomization tests to obtain relative expression level and is based on the ΔΔCT method.

3.5. Primers Used

All primers were synthesized by AITBIOTECH, Singapore and adopted from Auger *et al*. 2011.

Primer Name	**Primer Sequence 5`-3`**
Promoter 1 forward (for CCGG CpG Site)	GTAGACCGCCACACCTGA
Promoter1 reverse (for CCGG CpG Site)	CCAGACATTGGTGTGTGACC
Promoter 2 forward (for CGCG CpG Site)	GGCCTTTGGCTCTATGTTC
Promoter 2 reverse (for CGCG CpG Site)	TTGAGGGTCACCTGGAAATC

PCR efficiency for each primer set was quantified using a series of sequentially diluted pooled samples that were quantified using qPCR in similar conditions. Primer efficiency was calculated using an online calculator available at:

http://www.thermoscientificbio.com/webtools/qpcrefficiency/.