MASTER OF PHILOSOPHY

THESIS

Individual differences in coping style influence acute endocrine and neurobiological responses to psychosocial stress.

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B Biomed Sci

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Statement of Originality

This work contains no material, which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to the best of my knowledge and belief. The work embodied in this thesis does form the basis of a scholarly work of which I am a joint author, and I have included a statement endorsed by my supervisor clearly outlining the extent of my contribution to the joint publication. I give consent to this copy of my Thesis being made available for loan and photocopying, when deposited in the University Library, subject to the provisions of the Copyright Act 1968.

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Acknowledgement of authorship/collaboration

I hereby certify that the work embodied in this Thesis is the result of original research, the greater part of which was completed subsequent to admission to candidature for the degree and comprises the major contribution of behavioural testing and analysis, tissue collection and processing, immunohistochemistry, hormone detection assays, brain imaging, Fos detection, statistical analysis, and academic writing to the joint scholarly work.

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Thesis Synopsis

The psychosocial stress of social conflict contributes to the development of depression and anxiety in those individuals vulnerable to its effects, yet the factors that contribute to vulnerability remain unclear. Researchers investigating factors such as behaviour and physiology have used the animal resident/intruder social conflict model whereby a young male rodent (intruder) is placed into the home cage of an older male (resident) that is trained to attack and defeat all intruders. Findings reported previously have shown that defeated intruders displayed medium to longer-term stress-related changes in behaviour and physiology, with considerable variability in the severity of these changes reported from one individual to another. Interestingly, a reduction in severity of behavioural and physiological changes was associated most significantly with intruders that deployed 'active coping' behaviours during the social defeat interaction than animals that deployed 'passive coping'. However, these findings do not describe the short-term effects, raising the question; does coping style also influence the short-term stress response?

We investigated the relationship between coping behaviour adopted by intruders during a 10 minute social conflict culminating in defeat and both acute peak plasma corticosterone (CORT) stress hormone levels and number of cells expressing Fos protein in eight brain regions. Our investigations revealed that higher levels of fight and guard behaviours were associated with lower peak plasma CORT levels compared to ready submission, and that higher levels of fight were associated with fewer numbers of Fos-ir cells in prefrontal cortex (PFC), amygdala (Am), and paraventricular nucleus (PVN) brain regions. In general terms, these findings indicate that coping behaviour deployed *during*

social conflict influences the endocrine and neurobiological elements of the acute phase of the HPA axis response to psychosocial stress. Intruders that deploy an ‘active’ coping style including fight behaviours display significantly smaller physiological and neurobiological alterations in the acute response than intruders that deploy a ‘passive’ coping style during social conflict. These results demonstrate that the vulnerability to the effects of psychosocial stress are ameliorated by actively engaging with the perpetrator rather than passively taking the attack, and that adopting the behaviour fight is most protective. Further elucidation of the neural mechanisms that underpin the reduction in stress-induced effects is warranted.

Abbreviation	Definition
ABC	Avidin:Biotinylated enzyme Complex
ACEC	Animal Care and Ethics Committee
ACTH	adrenocorticotrophic hormone
aHyp	anterior hypothalamus
AHipA	amygdalohippocampal area
Am	amygdala
ANOVA	analysis of variance
AUC CORT	area under the curve for plasma corticosterone
AVP	arginine vasopressin
Base CORT	plasma corticosterone levels prior to defeat encounter
BNST	bed nucleus of the stria terminalis
BSA	bovine serum albumin
CA	catecholamine
CeA	central amygdala
CG	cingulate gyrus
CN	cuneiform nucleus
CORT	corticosterone
CRF	corticotrophin-releasing factor
DAB	diaminobenzidine
dBNST	dorsal bed nucleus of the stria terminalis
dmH	dorsomedial hypothalamus
dR	dorsal raphe
fMRI	functional magnetic resonance imaging
h	hour
HPA	hypothalamic-pituitary-adrenal
IEG	immediate early gene
IL PFC	infralimbic medial prefrontal cortex
ITF	inducible transcription factor
LC	locus coeruleus
lHyp	lateral hypothalamus
lPAG	lateral periaqueductal grey
lPOA	lateral preoptic area
LS	lateral septum
MeA	medial amygdala
mPFC	medial prefrontal cortex
mPOA	medial preoptic area
mR	medial raphe
mRNA	messenger ribonucleic acid
NSRI	noradrenaline serotonin reuptake inhibitor
NTS	nucleus of the solitary tract
OT	oxytocin
PACAP	pituitary adenylate cyclase-activating polypeptide
PAG	periaqueductal grey
PBS	phosphate buffered saline
Peak CORT	maximum plasma corticosterone level 15 or 30 min after onset of the defeat encounter
PFC	prefrontal cortex
PL PFC	prelimbic medial prefrontal cortex
PVN	paraventricular nucleus

S	septum
s	second
SEM	standard error of the mean
SHypN	septohypothalamic nucleus
SNS	sympathetic nervous system
SON	supraoptic nucleus
SSRI	selective serotonin reuptake inhibitor
TH	tyrosine hydroxylase
vBNST	ventral bed nucleus of the stria terminalis
VLM	ventrolateral medulla
vlPAG	ventrolateral periaqueductal grey
vLS	ventral lateral septum
vmHyp	ventromedial hypothalamus

Individual differences in coping style influence acute endocrine and neurobiological responses to psychosocial stress.

CHAPTER 1.

GENERAL INTRODUCTION

The impact of stress on health

Stress occurs when a challenge overwhelms, or is assessed by the brain as being likely to overwhelm, the body's normal homeostatic response mechanisms (Amat et al., 2005; Day, 2005). Early stress research focused on hypothalamic-pituitary-adrenal (HPA) and sympathetic nervous system (SNS) responses, whereas we now recognise that these are just two parts of a multi-system response that involves changes in cognitive, behavioural, immune, endocrine, and autonomic systems (Cannon, 1929; Selye, 1976; Sapolsky, 1988; Rodrigues et al., 2009).

In the short-term, changes are adaptive, but if exposure is prolonged, or the response is exaggerated, the alterations can become maladaptive, and initiate or exacerbate pathologies such as cardiac disease, autoimmune disease, diabetes, depression, and anxiety, to mention a few (McEwen and Sapolsky, 1995; Koob and Le Moal, 1997; Bjorntorp and Rosmond, 2000; Korte et al., 2005; Grillon et al., 2007). In addition to the deleterious effect on individuals, the economic burden to the community of the effects of stress is onerous. For example, a 1995/96 study from the UK reported that workplace stress affected one in five people at a cost of 3.8 billion pounds per year (UK Health and Stress Executive, 2005). A decade later, estimates from the US

reported that workplace stress and associated absenteeism cost around $300 billion (Wolfe and Walker, 2007).

Limiting the impact of stress

Of all the illnesses linked to stress, anxiety and depression are perhaps the most commonly recognised, with average global prevalence conservatively estimated between 2% and 5% (Fischer, 1999; Wong and Licinio, 2001). Anxiety and depression are currently treated with drugs that, when first introduced into clinical practice, were actually intended to treat maladies such as tuberculosis, gastrointestinal disorders, and premenstrual disorders, among others. Serendipitously, these drugs, which include benzodiazepines, tricyclic antidepressants, selective serotonin reuptake inhibitors (SSRIs), and noradrenaline reuptake inhibitors (NSRIs), were found to be even more valuable as remedies for mood disorders (Aneshensel and Stone, 1982; Coco et al., 1992; Levine et al., 2001). However, despite the claims of selectivity that are now made when such drugs are advertised, it is well understood by most neurobiologists that they all alter signalling cascades in multiple systems and, for this reason, can have considerable adverse side-effects with, in some cases, only relatively small safe therapeutic dose ranges (Mitchell et al., 1997; Roose, 1999; Montgomery et al., 2005). Moreover, a fundamental issue with all these pharmaceuticals is that none are the result of ‘rational design’, meaning that none of them was expressly designed to target stress control systems. In fact, rational design of appropriately selective drugs requires a deep understanding of the stress control pathways (Rasmussen, 2006). In this

regard, considerable progress has occurred over the last two decades in terms of our detailed knowledge of these pathways (Hausler et al., 1985; Cullinan et al., 1993; Bhatnagar and Meaney, 1995; Veenema and Neumann, 2007). However, our knowledge is still far from complete and new insights are needed (Rasmussen, 2006). The identification of novel targets will provide new avenues for treatments and potentially enhance the development of new pharmaceutical remedies. One promising new avenue of discovery that has gained prominence in recent years involves the investigation of the factors associated with individual differences in responses to stress and, by extension, susceptibility to stress-related illness (McEwen, 2000; Hamann and Canli, 2004; Sgoifo et al., 2005; McEwen, 2008).

Individual differences in responses to stress

It has long been recognised by clinicians, and researchers, that individuals vary enormously in their responses to potentially stressful situations (McQuitty, 1950; Thorndike, 1950; Goldstein, 1973; Meaney et al., 1991; Nielsen et al., 1999; Walker et al., 2007). It is ironic, then, that pre-clinical studies concerning stress have routinely reported data that has been standardised by elimination of the 'noise' of individual variation inherent in animal populations (Koolhaas et al., 2007). However, that is starting to change. Increasingly, it is recognised that individual variation that exists across a population, rather than simply reflecting random differences, likely has an important role in population dynamics and survival. Of particular note is the genetic variation for characteristics such as aggression, which means that

different sub-groups of a population have differences in 'fitness' that enable them to deal successfully with different environmental changes (Koolhaas et al., 1999). Now that researchers have begun to accept the idea that individual differences have biological significance, the wider implications are also being appreciated. With regard to stress research, it has been proposed that identifying the neural mechanisms that underpin differences in the responsiveness of individuals to stress may provide new information about stress control mechanisms in the brain, and thereby help us to identify new and potentially critical targets suitable for rational design of 'anti-stress' drugs (McEwen, 2008).

Approaches to the study of individual differences in neural mechanisms that underpin responses to stress

In determining a suitable approach for the investigation of the neural mechanisms that underpin individual differences in responses to stress, several inter-related matters need to be considered, namely: i) whether such studies should be clinical or pre-clinical; ii) the selection of neural mapping techniques most suitable for identifying critical brain circuitry; and iii) the selection of the most appropriate stressor(s) to test for differences in responses to stress.

i) Should studies be clinical or pre-clinical?

Ideally, studies directed at the identification of neurocircuitry that confers vulnerability or resilience to stress related illness would be conducted on humans. However, clinical studies are costly and complex, and do not provide

identification of relevant neurocircuitry with the necessary resolution (Schneider et al., 1996; O'Neil and Moore, 2003). With regard to the issue of resolution, it is notable that several functional magnetic resonance imaging (fMRI) studies have been conducted using human subjects to measure regional neural activity in the brains of different individuals exposed to the same stressor (Ueda et al., 2003; Northoff et al., 2004; Straube et al., 2007). However, while this is a great advance, the regional information that it provides is inadequate in detailing the neurobiological systems that underpin differences at a neurocircuit level. In contrast, however, studies conducted on non-human animals are able to employ techniques that do permit this level of resolution, suggesting that pre-clinical studies using non-human animals as subjects would be the investigative method of choice.

ii) Which neural mapping techniques are suitable for identifying critical brain circuitry?

One of the most useful advances in pre-clinical neural imaging techniques in the past four decades has been the development of our ability to map neural patterns of inducible transcription factor (ITF) expression in the brain, such as the phosphoprotein product Fos, of the immediate early gene (IEG) c-*fos* (Sagar et al., 1988). The discovery that extracellular stimuli, especially those associated with different types of stressor, elicit rapid and transient IEG expression followed by ITF production, and that evidence of such activation can be visualised at the level of the individual cell, has resulted in a surge of pre-clinical studies over the last few decades detailing neural activation

patterns associated with responses to stressful stimuli (Sheehan et al., 2000); (Sagar et al., 1988); (Cullinan et al., 1995); (Martinez et al., 1998); (Sawchenko et al., 2000); (Calfa et al., 2007); (Dayas et al., 2001). Interestingly, many studies have combined neural mapping with the localisation of phenotype-specific neuronal markers, thereby further enhancing our knowledge of critical neural circuits. One example of this is the combination of Fos detection with immunohistochemical detection of transmitter-specific enzymes such as tyrosine hydroxylase (Smith and Day, 1993). If one accepts that the need to map relevant stress control systems in great detail dictates the use of cellular detection techniques in non-human animals, the next issue to decide upon is the identity of the stressor(s) that would be most suitable for probing individual differences in stress responses.

iii) Which stressor(s) should be used to assess individual differences in responses to stress?

To date, pre-clinical studies concerning stress have used an extremely wide range of stressors. This was not considered to be an issue for quite some time, as most researchers assumed that the neural circuitry involved in generating stress responses was likely to be the same regardless of the identity of the stressor (Cannon, 1929; Selye, 1998). However, studies conducted over the past decade have provided unequivocal evidence that different circuits can be involved in generating responses to different types of stressors (Day et al., 1999; Dayas et al., 2001; Buller et al., 2003). Accordingly, it is now appreciated that care must be taken to select stressors appropriate to the question being

addressed. With regard to the human experience of the effects of stress, it is generally agreed that a majority of individuals suffer most stress-related illness as a consequence of psychosocial stressors (McEwen and Seeman, 1999); (Schneiderman et al., 2005). In particular, negative social interactions that lead to 'social conflict' have been identified as extremely potent sources of stress that can lead to disorders such as depression and anxiety (Bjorkqvist, 2001; Notelaers et al., 2005; Cavigelli et al., 2007). Fortunately, a relevant pre-clinical model of psychosocial stress arising from negative social interactions is available. The 'resident/intruder' model has high face, construct, and predictive validity as a representation of human social conflict and defeat, and has been used and validated with a variety of species, the most commonly used being the rat (Kollack-Walker et al., 1997; Martinez et al., 1998; Miczek et al., 1999; Fuchs et al., 2004; Nikulina et al., 2004; Frank et al., 2006).

A pre-clinical model of psychosocial stress - the resident/intruder model

The 'resident/intruder' model can be used to replicate the natural tendency of a male rat 'resident' to establish a territory that he will aggressively defend against unfamiliar male 'intruders' (Koolhaas et al., 1980; Treit, 1985; Korte et al., 1995; Sgoifo et al., 1999; Veenema and Neumann, 2007). Intruders introduced into the resident's home-cage are invariably attacked and usually defeated by the resident, as signalled by the submissive behaviour of the intruders. Defeated intruders display an acute stress response, including SNS and (HPA) axis activation, altered body temperature, and behaviour suggestive of anxiety-like effects. Over subsequent days and weeks a

substantial number of defeated intruders go on to display a succession of longer-term, physiological and behavioural alterations that can include weight loss, markedly reduced social and non-social exploratory activity, reduced locomotor activity, and disruption of immune function and circadian heart rhythms, suggestive of depression-like effects (Berton et al., 1999; Blanchard et al., 2001b; Meerlo et al., 2002; Bhatnagar et al., 2006; Calfa et al., 2007). However, these longer-term effects are not universal; intruders appear to display differences in their responsiveness to social conflict interactions. As to the possible basis of these differences, it is notable that two studies have reported that intruders that readily submit to the resident actually suffer more of the longer-term physiological and behavioural effects than do those intruders that engage more actively with the resident (Stefanski, 1998; Meerlo et al., 1999). Hence, Stefanski (1998) reported that, although all defeated intruders displayed acute anomalies in immune function and body weight, only animals that readily submitted to the resident still had elevated B lymphocyte numbers and reduced body weight one week after their last conflict interaction. Similarly, Meerlo et al (1999) reported that, although all defeated intruders displayed decreases in the amplitude of their diurnal heart rate, body temperature, and locomotor activity rhythms, intruders that did not attempt to counter the resident's attacks displayed much larger and longer lasting decreases than intruders that did attempt to resist and fight back. These studies are important for three reasons: i) they demonstrate that we can observe significant individual differences in stress responses in a laboratory animal model; ii) these

differences appear to be meaningfully organised rather than random; and iii) the behaviour adopted at the time of social conflict appears to determine the medium to long-term effects that ensue (Bohnen et al., 1991; Ramaekers et al., 1998; Berton et al., 1999; Davidson, 2000). With regard to the latter point, it is noteworthy that psychologists would describe the behaviour displayed during a period of challenge, such as that which corresponds to an episode of conflict, as coping behaviour or coping style. Therefore, it has been suggested that individual differences in responses to stress may be understood in terms of individual differences in coping style (Holahan and Moos, 1987); (Koolhaas et al., 2007).

Individual differences in coping style

Coping style can be defined as the approach or strategy that an individual deploys, consciously or unconsciously, in his/her attempt to master a potentially harmful challenge (Koolhaas et al., 1999; Folkman and Moskowitz, 2004). In regards to the psychological and physical effects of stress, coping style can be thought of as the adaptive mechanism used by an individual to deal with changes in the environment, and can comprise trait-dependent and state-dependent elements (Lazarus and Folkman, 1984). Trait-dependent coping strategies can be thought of as patterns of behaviour that endure over time in a range of settings, whereas state-dependent coping strategies can be thought of as behaviour patterns that change as settings alter (Hino et al., 2002). Importantly, pre-clinical studies can be designed to elicit predominantly state-dependent coping by presenting an unambiguous challenge such as

haemorrhage (Barton and Passingham, 1982), or to elicit predominantly trait-dependent coping by presenting an ambiguous challenge such as social conflict (Blanchard et al., 2001a); (Koolhaas et al., 2007).

Humans exist in dynamic environments that present a variety of challenges of an ambiguous nature, which suggests that trait-dependent coping strategies may be particularly relevant to individual differences in the development of stress-related illness (McEwen, 2008). When dealing with a challenge, humans can display a wide repertoire of coping strategies such as acceptance, avoidance, escapism, or emotional-blunting, to name a few (Rauch et al., 2007). In fact, some researchers have argued that these specific coping strategies can be categorised, based on the behavioural clusters deployed, into two basic adaptive styles; 'active' or 'problem-focused' coping, and 'passive' or 'emotion-focused' coping (Hino et al., 2002; Matheson and Anisman, 2003; Folkman and Moskowitz, 2004). Broadly-speaking, active coping strategies characteristically involve a problem-solving approach, or direct efforts to address the challenge, whereas passive coping strategies characteristically involve avoidance of, or detachment from, the challenge (Folkman and Moskowitz, 2004).

Like humans, non-human animals can adopt different coping strategies when faced with stressful situations, the characteristics of which appear to model the 'active' and 'passive' styles adopted by humans (Bandler et al., 2000; Keay and Bandler, 2001; Ebner et al., 2005a). An active coping style is characterised in non-human males by displays of territorial control and

aggression, whereas passive coping is generally characterised by immobility, decreased reactivity, and low aggression (Koolhaas et al., 1999). Notably, it has been reported that, when allowed a 'free choice' of response to various ambiguous threats, individual male rats from wild-type or minimally domesticated populations consistently adopt either a cluster of behaviours that can be considered as an active coping response, or a cluster of behaviours that can be considered as a passive coping response. Moreover, the distribution of these trait-dependent coping styles within a population is generally bimodal, with about half the population adopting an active coping style and half adopting a passive coping style (Koolhaas et al., 1999). However, this bimodal distribution does not necessarily occur in domesticated animal populations (Neophytou et al., 2000). For example, strains of inbred laboratory rat populations have been observed to primarily display an innate preference for a passive coping style, whereas others predominantly display an innate preference for active coping style (Koolhaas et al., 1999). The skewing of the distribution pattern within domesticated populations is thought to reflect changes in the naturally-occurring selection pressures that maintain genetic variability in wild-type populations such as territorial competition or predators, thereby allowing the characteristics of the behavioural clusters observed in wild-type populations to drift, or inadvertently constrict, into a narrower range in domesticated animals (Koolhaas et al., 1999; Cohen and Zohar, 2004; Veenema and Neumann, 2007). Pursuant to this, therefore, studies concerned with investigating differences in coping style adopted in 'free-choice' situations

should probably attempt to use animals derived from outbred strains that are as close as possible to a wild-type, and thus, more likely to display a more naturalistic range of coping behaviours. Thus, the pre-clinical model of choice for the assessment of individual differences in trait-dependent coping style response would most likely be one that uses an ambiguous threat situation.

Using the concept of coping style to explain individual differences in the response to social conflict interactions

As mentioned earlier, of the pre-clinical studies that have investigated the consequences of social conflict using the rat resident/intruder model, two have reported that intruders that engage in fighting with the resident suffer fewer and/or more transient physiological and behavioural disturbances in the days following defeat than those that do not counter the attacks (Stefanski, 1998; Meerlo et al., 1999). Based upon the discussion in the preceding section, this can now be conceptualised in terms of differences in coping styles. Thus, intruders that engage in fighting with the resident could be described as displaying active coping, this style being associated with fewer and more transient physiological and behavioural effects than passive coping, a style that, in this context, is characterised by submission to the resident. However, while the data supports this interpretation in the case of studies that have dealt with the longer-term (days to weeks) effects of social defeat, the same is not true of studies that have assessed the acute (minutes to hours) effects of social conflict interaction. In particular, three studies have examined whether coping style modulates the effects of social conflict on perhaps the most ‘classic’ element of

the acute stress response, i.e. HPA axis activation, and their findings have been contradictory. Two of the studies yielded data interpreted as indicating that neither active nor passive coping styles had effect on the immediate HPA axis response to social conflict interaction (Sgoifo et al., 1996; Ebner et al., 2005a), whereas the third study reported that social defeat elicited enhanced HPA axis responses in association with active coping (Frank et al., 2006). These studies are contradictory. However, it is notable that, in contrast to the longer-term studies reported by Meerlo et al (1999) and Stefanski (1998), the three studies concerning acute HPA axis responses to conflict interaction all assessed coping style based on behaviour displayed either *before* or *after* the conflict interaction episode, rather than assessing coping style based on behaviour adopted *during* the conflict interaction. This is the critical difference between the longer-term studies and those that report acute effects, which provides a potentially significant area of investigation.

Neural activation patterns associated with stress

For several years, neurobiologists have attempted to pinpoint the neurocircuitry associated with the stress response. As a result, it has become evident that a relatively restricted number of brain regions are consistently and prominently activated during the generation of a stress response, perhaps the most notable being the prefrontal cortex (PFC), bed nucleus of the stria terminalis (BNST), amygdala (Am), lateral septum (LS), dorsomedial hypothalamus (dmH), paraventricular nucleus (PVN), periaqueductal grey (PAG) and, in the brainstem, the catecholamine (CA) cell groups of the nucleus

of the solitary tract (NTS) and the ventrolateral medulla (VLM) (Campeau et al., 1991; Deutch et al., 1991; Cullinan et al., 1995; Sagar et al., 1995; Matsuda et al., 1996; Day et al., 1999; Keay and Bandler, 2001; Spencer et al., 2004).

Since the 1980s neuronal activation mapping studies involving detection of changes in Fos expression have helped to build a picture of the complex sequence of events that occur in the initiation of a typical stress response. With the exception of olfactory cues such as predator odour, an initial response involves the rapid relay of sensory information to the thalamus for consolidation and distribution to other areas of the brain with the initiation of a rapid activation of the relay from the thalamus to the amygdala (Iwata et al., 1986). Subsequently, simultaneous activation and regulation of distinct elements such as the central amygdala (CeA) in the case of a physical stressor, or the medial amygdala in the case of a psychological stressor, are mediated via projections to a range of structures involved in the regulation of both HPA axis activation and SNS responses, such as those to the PVN in the generation of HPA axis responses, and to the dmH in the generation of SNS responses (Dayas et al., 1999; DiMicco et al., 2002) to name just two. Meanwhile, sensory information relayed simultaneously from the thalamus to the neocortex is processed in the PFC which then modulates relevant subcortical pathway activation via its projections to the BNST, Am and LS (Spencer et al., 2004; Spencer et al., 2005). Simultaneously, visceral information related to the context of the challenge, for example cardio-respiratory status, is communicated by inputs from brainstem catecholamine neurons in such regions as the NTS and VLM,

which can further modify the activity of relevant cortical and sub-cortical neurocircuitry (Day, 1989; Dayas et al., 1997; Dayas et al., 2001), thereby adjusting behavioural, physiological and other stress response elements relevant to that individual brain's interpretation of the challenge. Thus, in regards to the neural mechanisms involved in the generation of stress responses, this brief overview highlights the complexity and interconnectedness of the neurocircuitry that initiates, interprets, and responds to stressors in keeping with each individual brain's functional pattern.

Interestingly, one of the major highlights to come out of c-*fos* expression mapping studies has been that physical stressors elicit somewhat different neural activation patterns to those associated with psychological stressors, suggesting that the brain itself categorises stressors and uses partially separate subsections of central stress neurocircuitry to elicit responses to stressors drawn from different categories (Cullinan et al., 1995; Kollack-Walker et al., 1997; Martinez et al., 1998; Dayas et al., 1999; Yokoyama and Sasaki, 1999; Dayas et al., 2001; Keay and Bandler, 2001). For example, physical stressors such as haemorrhage are associated with the activation of the central amygdala, whereas psychological stressors such as restraint are associated with activation of the medial amygdala (MeA) (Dayas et al., 2001). The detection of c-*fos* expression using techniques such as immunohistochemistry for its ITF product Fos protein enables researchers to assess changes in the ubiquitous neural activation mechanism within brain regions. However, the functional change associated with the activation detected can not be defined, as

the cell could be, for example, glutamatergic and therefore excitatory (Sun and Guyenet, 1986), or GABAergic and therefore inhibitory (Nicoll et al., 1980). However, cell phenotype can be determined more precisely if other techniques are employed such as the immunohistochemical approach to detect catecholaminergic cells in the brainstem by detecting tyrosine hydroxylase, the rate-limiting enzyme in the production of dopamine (Smith and Day, 1993). As has been noted previously in this Introduction, these findings have led to a new appreciation of the care that must be taken to select stressors and assessment techniques relevant to the issues being considered in pre-clinical studies (Mitchell and Redfern, 2005). This is particularly relevant to designing investigations into individual differences in coping style and stress responses, the effects of which may be attributable to a variety of contributing factors (Noble, 2005).

Neural activation patterns associated with acute social conflict

Three studies have used the rodent resident/intruder model to investigate c-*fos* expression patterns associated specifically with the stress of social conflict interactions, albeit without consideration of individual differences (Kollack-Walker et al., 1997; Martinez et al., 1998; Kollack-Walker et al., 1999). A partial summary of the brain regions reported by Martinez (1998), and Kollack-Walker (1997); (1999) to have been activated as a result of an intruder's defeat is contained in Table 1.1.

Martinez (1998) reported that, within 60 minutes of defeat, rats exposed to one social conflict episode displayed c-*fos* activation, as indicated by

increased numbers of Fos-ir positive cell numbers in the basal forebrain regions including the LS, BNST, LPO, lHyp, PVN, CeA and MeA, and in brainstem regions such as the cingulate gyrus (CG), dorsal raphe (dR), medial raphe (mR), locus coeruleus (LC) and NTS (Table 1.1). It was further reported that, at the conclusion of nine subsequent social defeats, different activation was apparent. Areas such as S, lHyp, lPOA, CeA, LC and NTS no longer expressed c-*fos*, whereas regions such as BNST, PVN, MeA, CG and Raphe displayed persistent c-*fos* expression. These differences were thought to reflect cellular adaptation in response to a persistent stressor.

Kollack-Walker (1997) examined c-*fos* mRNA in brains of male Syrian hamsters exposed to both acute and chronic social defeat and found that, although activation levels were increased in many areas examined (Table 1.1), this was a selective neural activation pattern depicting habituation of the ITF to the stressor in chronically defeated males compared to acutely defeated males. There was significant reduction in PVN, SON, septohypothalamic nucleus (SHypN), intermediate subdivision of the LS, CeA, and amygdalohippocampal area (AHipA) activation associated with chronic stress, whereas c-*fos* expression in the regions anterior hypothalamus (aHyp), vmHyp, dPAG, dR, cuneiform nucleus (CN) and LC did not differ between groups. In concert with these findings plasma cortisol levels were reported to be similar in all groups despite the changes in neural activation; it was proposed that this reflected adaptability at a molecular level capable of altering neurotransmitter events within the limbic-HPA axis. Another study by Kollack-Walker (1999) reported a

significant relationship between c-*fos* mRNA levels and the fight behaviour adopted by subdominant male Syrian hamsters when confronted by a dominant male. Increased c-*fos* mRNA levels were detected in regions such as CC, LS, BNST, mPOA, Hyp, CeA, AHipA, dPAG, dR, CN and LC (Table 1.1) in subdominant males, whereas increased c-*fos* mRNA expression was detected in only the SON in dominant males.

The findings of these three studies support the notion that neural activation differs in accord with exposure to acute vs. chronic social stressors, and that ITF detection is an appropriate tool to assess the neural activation pattern differences between groups. However, the techniques used in each study to detect c-*fos* expression differ, which makes direct comparisons difficult. Also, and more significantly for the purposes of the study being reported here, only the study by Martinez (1998) reports on the cellular activation patterns associated with the acute response to social conflict. The relationship between cellular activation patterns and social defeat are based on differing factors such as acute versus chronic social defeat or dominant versus subdominant behaviour in the reports from Kollack-Walker (1997) (1999). However, it is evident that both rats and hamsters display enhanced c-*fos* expression in brain regions associated with stress pathways when exposed to the stress of social conflict, and that the activation patterns are remarkably similar to those described by other studies that investigated exposure to psychological stressors such as immobilisation, restraint, forced swim and white noise (Cullinan et al., 1995; Campeau and Watson, 1997; Dayas and Day, 2002; Trneckova et al.,

2006; Valles et al., 2006). These reports suggest then that, in general terms, social conflict is a stressor that involves activation of many brain regions, although they stop short at reporting an association between the behaviour adopted *during* the social conflict and the neural activation patterns of individuals.

Individual differences in coping style influence acute endocrine and neurobiological responses to psychosocial stress.

Table 1.1. Summary of neural Fos expression patterns reported in rodent intruders exposed to acute social defeat (Legend: + denotes increase relative to controls; - denotes not reported).

	Martinez et al.	Kollack-Walker et al.	
	(1998b)	(1997)	(1999)
	Rats	**Hamsters**	
Cingulate cortex	ND	+	+
Lateral Septum	+	+	+
BNST	+	+	+
Preoptic area	+	+	+
Hypothalamus	+	+	+
Paraventricular n	+	+	+
Arcuate n.	ND	+	+
Supraoptic n.	ND	+	+
Amygdala			
Medial	+	+ [1]	ND
Central	+	+	+
PAG			
Dorsal	ND	+	+
Ventrolateral	+	ND	ND
Raphe nuclei			
Dorsal	+	+	+
Medial	+	-	ND
Locus coeruleus	+	+	+
NTS	+	ND	ND

[1] Note: in the study by Kollack-Walker et al (1997), quantification of c-*fos* expression indicated that activation of the medial amygdala was greater than that of the central amygdala. This observation is noteworthy because it is consistent with previous reports concerning other types of psychological stressors, which also found increased c-*fos* expression in the MeA than CeA e.g. Dayas et al 2001.[1]

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Neural regions of potential importance to the adoption of different coping strategies

Our understanding of the neural pathways that underpin individual differences in response to social conflict, and how these relate to differences in coping style, is inadequate. However, if one puts aside the issue of the stress responses associated with social conflict, and simply focuses on the question of which brain regions might be associated with the adoption of active vs. passive coping style, there are several potential regions of interest.

One such region is the periaqueductal grey (PAG). A number of studies have reported two preferentially activated PAG subregions associated with active vs. passive coping responses to physical stressors, these being the lateral PAG (lPAG) when active coping is displayed, and the ventrolateral PAG (vlPAG) when passive coping is displayed (Bandler et al., 2000; Keay and Bandler, 2001). Moreover, when chemically stimulated, the lPAG and vlPAG were found to be associated with active coping behaviours, and passive coping behaviours, respectively (Bandler et al., 2000). However, it is interesting to note that the preferential activation of the lPAG and vlPAG that was evident after exposure to different physical stressors was not so evident when a psychological stressor was utilised (Keay and Bandler, 2001). Therefore, the PAG is perhaps best thought of as an integrating relay centre that moulds behavioural, autonomic, and endocrine elements of the stress response into coherent patterns, rather than a region that gives rise to innate preferences in coping styles.

Other subcortical structures that may also function as integrating relay centres are the lateral septum (LS), bed nucleus of the stria terminalis (BNST) and amygdala (Am). The lateral septum is a region once thought to be the centre of 'stress behaviour' and has long been known to be associated with the modulation of behaviours that would be construed as being linked to active coping (Kasper, 1964; Miller and Mogenson, 1971; Ebner et al., 2008). Lesions of the LS have been found to alter levels of aggression in both mice and rats and, more recently, it has been demonstrated that release of vasopressin and substance P in the septum enhance and inhibit, respectively, active coping behaviour (Koolhaas et al., 1998; Ebner et al., 2008; Frank and Landgraf, 2008). The bed nucleus of the stria terminalis (BNST) is thought to be associated with modulation of active coping behaviours, with lesions of BNST reported to reduce mobility in a repeated swim test in rats (Schulz and Canbeyli, 2000), and treatment with an aromatase inhibitor was shown to be associated with ventral BNST (vBNST) activation and reduced aggressive behaviour in mice (Trainor et al., 2006).

The amygdala (Am) has long been thought of as the 'fear and anxiety' centre (Etkin et al., 2006), and, as mentioned previously, two of its sub-regions, the medial Am (MeA) and the central Am (CeA), are reported to be differentially activated in response to psychological and physical stressors, respectively (Dayas et al., 2001). Lesions of the Am have been associated with the abolition of the bradycardia and the immobility associated with conditioned fear in rats, an outcome that suggests the Am may play a role in promotion of a passive

coping strategy (Roozendaal et al., 1990, 1991). Even more interesting, however, are reports that the release within the Am of corticotrophin-releasing factor (CRF) and pituitary adenylate cyclase-activating polypeptide (PACAP) reduce, while oxytocin (OT) enhances, active coping, respectively (Heinrichs et al., 1992; Ebner et al., 2005b; Legradi et al., 2007). Notably, animals bred for trait-anxiety behaviours displayed elevated CeA activation associated with active coping (Veenema and Neumann, 2007; Frank and Landgraf, 2008), while increased arginine vasopressin (AVP) release from the MeA was associated with reduced aggression, i.e. active coping behaviour, in mice and rats (Koolhaas et al., 1998).

Another brain region considered likely to be important in the adoption of active vs. passive coping behaviour is the medial prefrontal cortex (mPFC). Recent reports suggest that mPFC sub-regions infra-limbic (IL PFC) and pre-limbic (PL PFC) mediate suppression of passive coping indirectly by facilitating active coping (Shah and Treit, 2003), with lesions of IL PFC, in particular, shown to be associated with the modulation of exploration, which could be construed as active coping behaviour (Jinks and McGregor, 1997). Additionally, human fMRI studies report that mPFC left-biased activation is associated with approach behaviour and positive affect (thought to reflect an active coping style), and right-biased activation is associated with withdrawal, shyness and defensive behaviours (thought to suggest a passive coping style) (Gainotti, 1984; Silberman and Weingartner, 1986).

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Physical and psychological stressors are known to elicit catecholamine cell activation in the ventrolateral medulla (VLM) and the nucleus of the solitary tract (NTS) in the brainstem, the effects of which are directly mediated via signals to the central nervous system in the case of physical stressors, or via higher neuraxis relay network structures such as the MeA and PVN in the case of psychological stressors (Dayas et al., 2004). Although there are no studies that assess the direct association of catecholamine activation and coping style in individuals exposed to social conflict, previous studies have assessed CA cell activation in response to physical and psychological stressors using immunohistochemical techniques sensitive to the catecholamine synthetic enzyme tyrosine hydroxylase (TH), and reported CA involvement in stress responses (Dayas et al., 2001).

Thus, examination of neural activation levels in brain regions considered important in the selection of coping style may further elucidate the contribution of individual differences to the acute responses elicited by psychosocial stress.

Impact of coping style on conflict-induced neural activation patterns

Of the three studies that reported changes in neural activation levels associated with both acute and chronic social conflict interactions, none addressed the relationship between individual changes in neural activation and the associated differences in coping-related behaviours. However, there is one other study that has reported that active coping behaviour adopted during a social conflict interaction episode is associated with *reduced* neural activation in a number of brain regions and *enhanced* acute HPA axis activation (Frank et

al., 2006). This study requires careful appraisal, as the findings reported differ from those of Kollack-Walker (1997), which suggested that fight behaviour is associated with reduction of both neural activation and HPA axis responses, the latter being assessed on the basis of changes in cortisol levels. They also differ from the findings of studies that assessed the longer-term effects of social defeat and found that an active coping style, as determined by fight behaviour, was associated with fewer and more transient affects on immune system function, body weight (Stefanski, 1998), heart rate, body temperature, and locomotor activity (Meerlo et al., 1999)

Unlike previous studies by Meerlo (1999) and Stefanski (1998) the study by Frank et al (2006) examined the consequences of differences in coping style of two strains of rats selectively bred over a number of generations for behavioural differences associated with 'active' vs. 'passive' coping styles. Specifically, the strain referred to as being characterised by 'high anxiety behaviour' (HABs) were selectively bred to display behaviours such as reduced locomotor activity and reduced aggression, which are generally associated with a 'passive coping' style, while the strain referred to as being characterised by 'low anxiety behaviour' (LABs) were specifically bred to display behaviours such as increased self-grooming and rearing, which are behaviours generally associated with an 'active coping' style (Frank et al., 2006). Importantly, however, previous studies had suggested that, in addition to differences in anxiety-related behaviour, HABs and LABs also displayed different coping responses to a range of stressors (Keck et al., 2003).

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In principle it is not unreasonable to assume, as Frank et al did, that comparing the two selectively bred lines of animals could potentially provide useful information about coping-related differences in neural and HPA axis responses of social conflict interactions. The results produced unexpected findings, however, that included *reduced* neural activation and *enhanced* HPA axis activation in active coping intruders instead of the expected result of reductions in both neural and HPA axis activation levels. Consequently, it is reasonable to wonder if the selectively bred animals might be unrepresentative of a 'normal' population in more ways than anticipated. Consistent with this view, it has been reported previously that LABs displayed a greater increase in body temperature and locomotor activity responses to acute stress than HABs (Liebsch et al., 1998) and, more recently that the LAB line of rats also display unexpected anomalies in HPA axis responsiveness to a range of stressors (Veenema and Neumann, 2007). Accordingly, it seems reasonable to suggest that it would be of considerable interest to investigate the neural activation patterns linked with coping styles adopted by intruders from a more natural population of animals that have not been selected for any particular behaviour or other factor.

Study aims and general approach

In broad terms, the research described in this thesis aimed to improve our understanding of the relationship between coping behaviour adopted during social defeat and the corresponding acute HPA axis and neural activation responses. In contrast to previous studies using the rodent 'resident/intruder'

model to examine acute effects on defeated intruders, we investigated the occurrence of individual differences in responses. This assessment required the use of a relatively large number of animals (n=59) and a form of data analysis that involved the application of behavioural 'cut-off' criteria (Cohen and Zohar, 2004; Jezova et al., 2004). This technique identified specific sub-populations from which meaningful individual differences could be determined and for which the assumption of homogeneity was inappropriate, and was used to assess both the HPA axis activation (as an indicator of the acute stress response) and the changes in patterns of Fos-like immunoreactivity (as a marker of alterations in brain activity).

The specific aim of this research was to assess the validity of the hypothesis that the coping behaviour deployed at the time of social conflict interactions has the potential to moderate the acute stress response. In particular, two predictions arising from that hypothesis were derived:

1. That the peak plasma corticosterone responses elicited by social conflict would vary in accord with the coping behaviour deployed during the conflict episode, with active coping being associated with smaller responses.
2. That differences in the coping behaviour deployed during a conflict episode would be accompanied by differences in the patterns of neural activation observed in stress-sensitive regions of the brain.

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CHAPTER 2.

Impact of coping behaviour adopted during social conflict on acute HPA axis response.

INTRODUCTION

Social conflict is a powerful form of psychological stress that can lead to depression and anxiety (Bjorkqvist, 2001; Gilbert, 2001). As with other forms of psychological stress, individuals appear to vary in their vulnerability to the impact of social conflict interactions. Pre-clinical studies addressing this issue have used the 'resident/intruder' paradigm to model social conflict by placing a naïve male 'intruder' into the territory of an aggressive male 'resident' (Miczek et al., 1990; Huhman, 2006). Accordingly, a number of studies have reported that the medium to longer-term effects of social conflict interactions on a male intruder vary in accord with the behaviour adopted by the intruder in response to the resident attacks during social conflict (Tornatzky and Miczek, 1993; Meerlo et al., 1996; Koolhaas et al., 1997; Walker et al., 2007). Most notably, intruders that engaged in fighting with the resident suffered fewer physiological and behavioural effects in the ensuing days and weeks than intruders who readily submitted – in spite of reports that all intruders were defeated (Stefanski, 1998; Meerlo et al., 1999). One interpretation of this outcome is that 'active' coping (characterised by aggression and territorial control) moderates the stress response associated with social conflict interactions more effectively than

'passive' coping behaviour (characterised by immobility, decreased reactivity, and low aggression) (Sgoifo et al., 1996; Ebner et al., 2005a). However, studies concerning *acute* responses to social conflict have yielded less definitive results. Of the three relevant studies, two reported that 'passive' and 'active' intruders displayed indistinguishable HPA axis hormone responses to social conflict interactions (Sgoifo et al., 1996; Ebner et al., 2005a), while the third reported that 'active' intruders displayed *higher* HPA axis hormone responses to social conflict interaction than did 'passive' intruders (Frank et al., 2006). Notably, in contrast to the longer-term studies, these three studies assessed the intruder's coping style based on behaviours displayed *before* or *after* the conflict interaction episode, rather than *during*. In fact, Sgoifo et al (1996) assessed coping style based on behaviour adopted *before* exposure of the intruder to the social conflict interaction, whereas Frank et al (2006) and Ebner et al (2005a) both assessed coping style on the basis of behaviour adopted during a period of separation of the resident and intruder *after* one defeat. Consequently, we hypothesised that perhaps it is the coping behaviour deployed by an individual *during* a social conflict interaction episode that has the potential to moderate its stressfulness for that individual.

To address this issue, the present study assessed both the coping style adopted by intruders *before* a social conflict interaction (by means of a defensive burying test) and the coping style adopted by intruders *during* a social conflict interaction (by assessing behaviour adopted during 10 mins full contact between the resident and intruder). These assessments of coping behaviour

were then related to the corresponding acute HPA axis response to the conflict interaction (assessed by measuring plasma corticosterone).

METHODS

Study design

We wanted to investigate the relationship between coping behaviour adopted by intruders during a 10 minute social conflict culminating in defeat and associated changes in both peak plasma corticosterone (CORT) stress hormone levels and cell numbers expressing inducible transcription factor (ITF) Fos protein in eight stress pathway brain regions. To achieve these outcomes it was necessary to first establish certain techniques that were not yet part of the laboratory's pre-existing repertoire. Most notable among these were the following;

1. Design, build and trial a number of enriched environment cages for resident males and their tubally-ligated female companion.
2. Surgical fallopian tube ligation of each female resident companion.
3. Develop attack behaviour training for each resident male, with only those that defeated each intruder retained in the study.
4. Develop the defensive burying pre-test after designing and commissioning build of the probe.
5. Design and test in-house behavioural analysis software specific for each protocol undertaken, in accord with data management best practice.

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Animals and housing

The University of Newcastle Animal Care and Ethics Committee (ACEC) approved experimental protocols. The Sprague Dawley rats used were obtained from the University of Newcastle Animal Services Unit, and housed in an animal facility maintained at 21^{o}C ± 1^{o} with a 12:12 h light cycle (lights on 14:00, at maximum 60 lux) with standard rat chow and water available *ad libitum*. All animals, including male intruders, and male and female residents, were acclimatised to being handled during weekly cage cleaning and weighing in the animal facility for one week prior to and after entry into the study at 8 weeks of age. Intruder males were the focus of the study and were housed in individual red acrylic 40 x 25 x 25 cm cages for the duration of the study, and were surgically prepared and tested in accord with protocols outlined in Table 2.1.

Table 2.1. Timeline of intruder activity from entry into study at 8 weeks old

Intruder activity regime	
	Entry into study at 8 wks old
Day 1	Acclimatisation to facility for 1 week in group housing
Day 7	Transfer to individual cage
Day 10	Defensive burying test in home cage
Day 11	Jugular catheterisation surgery
Day 18	10 min familiarisation in resident cage with resident pair removed
Day 19	Collect blood 90 min prior to and at 15 min intervals for 60 min following 10 min social conflict episode

Individual differences in coping style influence acute endocrine and neurobiological responses to psychosocial stress.

Defensive burying test

A defensive burying test was conducted to determine each individual intruder rat's innate coping style based on the notion that the behaviours 'immobility' and 'bury', reflect 'passive' and 'active' coping, respectively (Sgoifo et al., 1996). After entry into the study intruders were acclimatised to individual housing for three days then each was subjected to one 10 min test that involved placing an electrified 7 x 2 cm probe wrapped with parallel strands of uncoated wire on the cage bedding; this probe delivered a 1mA shock when touched by the intruder. The episode was videotaped with infrared cameras over a 10 min period from the time that the intruder received the first shock from the probe and three behaviours were scored for duration and frequency including:

a) explore – locomotion activity including stand directed at exploring the cage or the probe;

b) bury – animal used front or hind limbs to shift bedding material toward the probe; and

c) immobile – animal remained motionless and attenuated the probe.

Surgery

An indwelling catheter was implanted into each intruder's left jugular vein one day after the defensive burying test. Intruders received Carprofen (5mg/kg, s.c.) analgesic, were anaesthetised (Ketamine 75 mg/kg and Xylazine 10mg mL/kg, i.p.), and then aseptically implanted with a silastic catheter (ID: 0.64 mm; OD: 1.19 mm; Dow Corning) into the left jugular vein. Polyethylene tubing (ID: 0.58 mm; OD: 0.96 mm; Australian Scientific) was attached to the silastic and

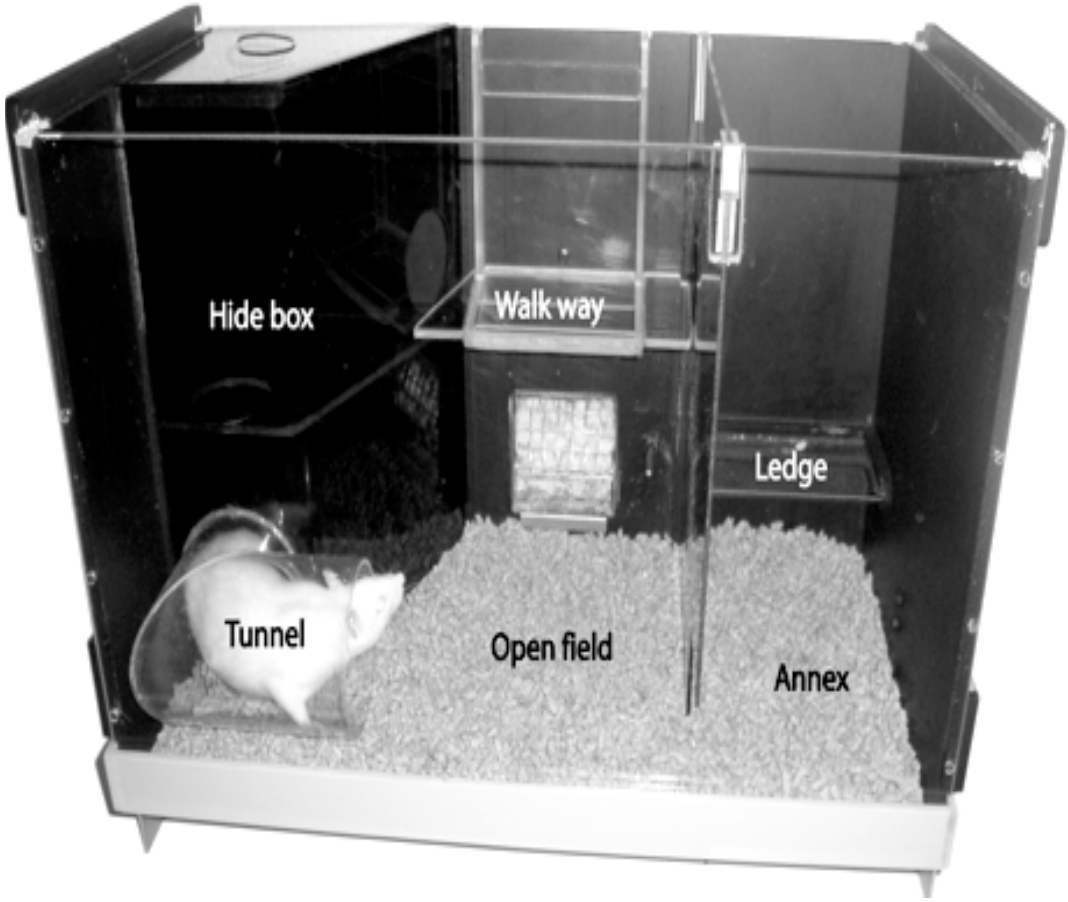
tunnelled subcutaneously to the back of the neck. The catheter was then exteriorised, filled with heparinised saline (25 IU/mL), and sealed. Xylazine was reversed with Antisedan (1mg/kg i.p.) after surgery (Jongen and Norman, 1987) and each animal's recovery monitored and recorded for six hours post-surgery.

Resident/intruder model preparation

Resident Sprague Dawley rat pairs consisted of a male (500-700 g) and a tubally-ligated female (~250 g) (Navori et al., 1952) housed together in a large, purpose-built, multi-compartment cage 60 x 30 x 40 cm (Figure 2.1) modelled on a natural burrow system (Dielenberg et al., 2006). Use of an enriched cage is thought to optimise the potential strength and range of agonistic behaviour displayed, thereby ensuring an environment appropriate for the assessment of coping styles (Blanchard et al., 1995; Blanchard et al., 2001c).

Individual differences in coping style influence acute endocrine and neurobiological responses to psychosocial stress.

Figure 2.1. Multi-compartment cage of a resident used for social conflict.



Resident training and selection

After four weeks undisturbed co-habitation with a tubally-ligated female companion each resident male received two weeks of aggressive defence training, with one 20 min session per day conducted on each third day with a test animal age (~ 9 weeks old) and weight (300-400 g) matched to an intruder; only those residents that displayed short attack-latency (within 60 sec) and consistently defeated training animals were retained in the study.

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Intruder preparation

Seven days after indwelling catheter implantation and one day prior to social conflict each intruder was exposed to a resident's cage for 10 min while the resident pair was absent. This was undertaken to reduce the possible confound in neural activation associated with perceptual novelty due to exposure to the temporal, olfactory, auditory, and visual elements of an unfamiliar cage (Martinez et al., 1998).

Social conflict interaction protocol

Females were removed from the resident home cage 15 min before the social conflict episode. Each intruder (300-400 g) was placed into the resident cage used for familiarisation and allowed to engage with the trained resident in an unseparated social conflict for 10 min. The interaction was digitally recorded using infrared cameras for later analysis.

Social conflict behaviour observations

Each intruder's behaviour was analysed for duration (time spent) and frequency (number of events) for eight behaviours detailed in Table 2.2 including; fight, guard, upright, walk, flight, explore, submit, and immobile. All intruders displayed at least one event of the characteristic defeat behaviour 'submit' and were therefore considered to have been defeated by the resident (Grant and Mackintosh, 1962). Images of four intruder behavioural stances are shown in Figure 2.2; A) immobile; B) submit; C) guard; and D) fight.

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Table 2.2 Characteristics that defined intruder behaviours analysed.

Fight	aggressive action adopted by the intruder and directed towards the resident during active aggression by resident toward the intruder
Guard	positioning at any doorway or other area of the resident cage to non-aggressively block the advance of the resident which variously involved the intruder blocking a doorway directly with its flank or, alternatively, orientating itself so that it was directly facing the doorway with its nose either sitting in the plane of the doorway or advanced slightly through the entrance
Upright	rigid hind or fore-limbs directed toward the resident in static resistance to an approach or attack by the resident
Walk away	slow movement away from the resident after a guard, submit, immobile, upright or fight event
Flight	rapid movement away from the resident after a guard, submit, immobile, upright or fight event;
Explore	non-specific locomotion directed at exploring the cage; neither away from nor toward the resident;
Submit	lying on the back or side with no sign of resistance while the resident sniffed the ano-genital or ventral surface of the intruder;
Immobile	rigid stance with all legs in contact with the ground, adopted during contact by the resident.

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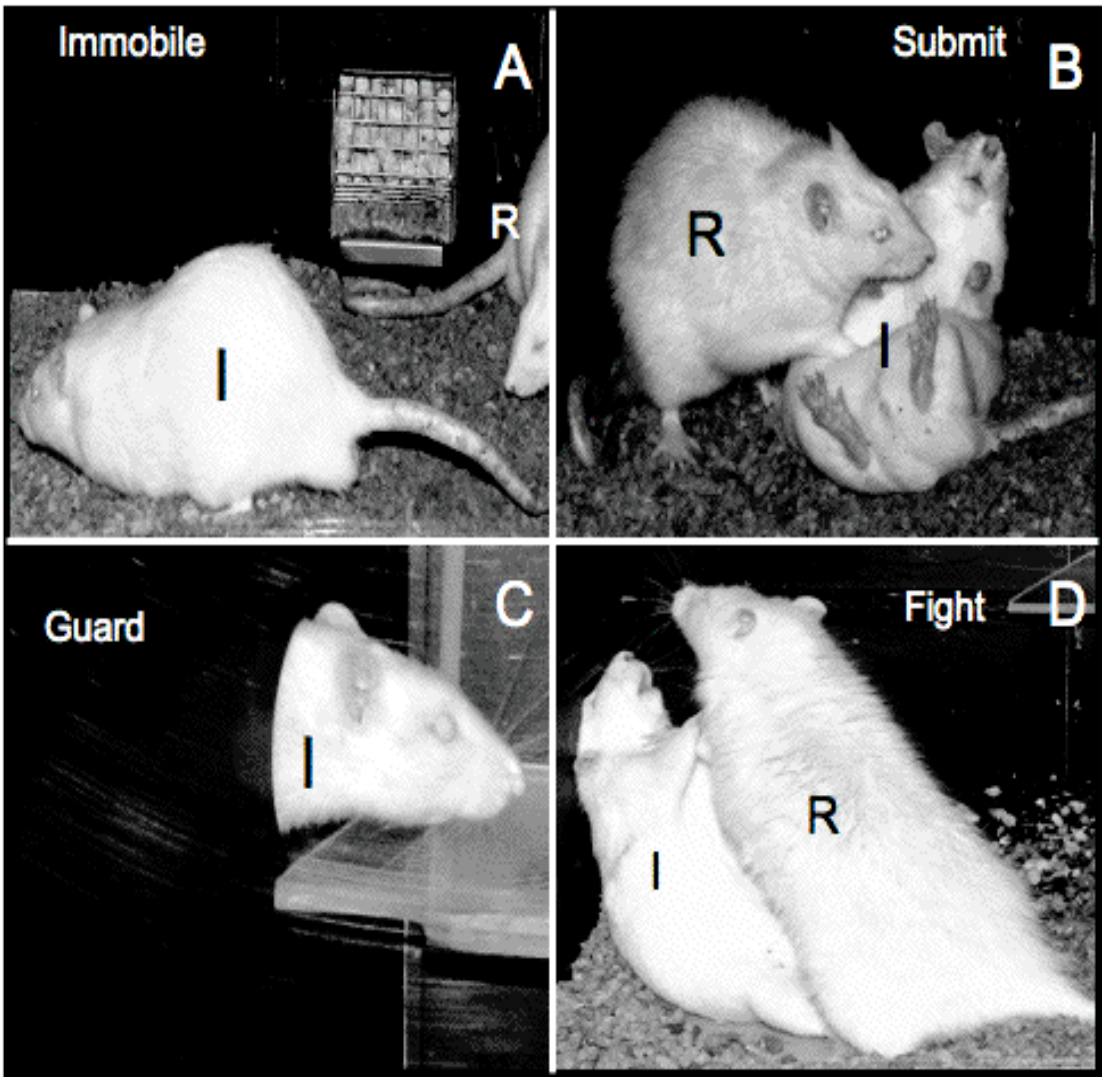
Figure 2.2. Images of four intruder behavioural stances adopted during social conflict interactions; **A.** Immobile **B.** Submit **C.** Guard and **D.** Fight:[2].



[2] I = Intruder, R = Resident.

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Plasma sample collection

Blood samples were taken from each intruder on the day of social conflict in accord with the timetable outlined in Table 2.3. Briefly, two hours before the social conflict episode the indwelling catheter was unsealed and attached to a length of polyethylene tubing (50 cm) that led to a sampling syringe at the outside of the cage (to least disturb the subject during sample collection). A baseline blood sample was drawn 30 min later, then the sampling syringe and tubing removed, and the catheter resealed. Immediately after the 10 min social conflict episode the tubing and sampling syringe were reattached to the indwelling catheter of the intruder, and the animal then returned to his home cage. Blood samples were taken at 15, 30, 60 and 90 min time-points after the start of the conflict episode (Darlington et al., 1986). All samples were of 0.65 mL and were immediately replaced with an equivalent volume of heparinised saline (25 IU/mL). Blood was centrifuged (10,000 *g*, 4°C, 10 min) in untreated tubes and the supernatant collected and stored at -20^{o}C. Plasma corticosterone (CORT) levels were assayed using a commercial radioimmunoassay kit (MP Biomedicals Inc, Cost Mesa, CA) from which the recovery of exogenous CORT was 100% and intra and inter-assay coefficients of variation were less than 8% and 10%, respectively.

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Table 2.3. Intruder blood sample collection timetable on day of social conflict

Day 19 –timeline of blood sample collection and social conflict episode	
- 120 minutes	attach syringe to catheter
- 90 minutes	take baseline blood sample and remove syringe
0 to + 10 minutes	social conflict episode
+ 15 minutes	attach syringe, take blood sample
+ 30 minutes	blood sample
+ 60 minutes	blood sample
+ 90 minutes	blood sample
+ 120 minutes	transcardial perfusion, harvest brain

Data analysis

A number of discussions were held to determine the most meaningful method of analysis, with a variety of techniques considered such as the use of Bayesian statistics, which is one method thought to best detect differences between individuals. However, it was decided that the utility of behavioural cut-off criteria would best determine the populations most suitable for investigation with more standard methods of analysis. We chose to use repeated measures analysis of variance (ANOVA) to analyse relationships between dependent and independent variables and to test potential correlations with Pearson Product Moment analysis. The data was analysed with SPSS v 14. Pearson product moment correlations were used to examine whole group inter-test associations i.e. defensive burying measures vs. social conflict measures, defensive burying vs. plasma CORT, and social conflict measures vs. plasma CORT. Three different measures of CORT levels were considered: Base CORT - the level

prior to the social defeat episode; Peak CORT - the peak level achieved after defeat whether 15 or 30 min after the start of the social defeat episode; and area under the curve (AUC) CORT - an estimate of average hormone levels based on area under the curve calculated via the trapezoidal method using GraphPad Prism 4.0.

Behavioural 'cut-off' criteria analysis was used to asses where meaningful individual differences may exist in the data and thus the assumption of homogeneity may be inappropriate (Cohen and Zohar, 2004). Accordingly, where a defensive burying or social conflict behavioural test measure was significantly correlated with CORT levels across the entire sample, two contrasting sub-groups were created by selecting intruders from the lower and the upper 20^{th} percentile with regard to scores on that behavioural measure. CORT levels were then calculated separately for each sub-group and differences determined using Independent Samples T-Tests, with differences to $p<0.05$ taken as significant.

RESULTS

Behaviours displayed during social conflict interaction episodes

All intruders displayed at least one clear episode of submission during the social conflict interaction and were therefore considered to have been defeated. Of the eight behaviours adopted by intruders and detailed in Table 2.4, the behaviours ‘fight’ and ‘guard’ were adopted the most frequently, with ‘guard’ occurring for the longest duration.

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Table 2.4. Intruder social conflict interaction behaviour scores; average number of events ±SEM; behaviour event as percentage of total events; average time in seconds ±SEM; and time spent as percentage of total time.

Behaviour	Mean number of events ±SEM	% total events	Mean total duration (s) ±SEM	% total duration
Explore	3 ± 2	9.3	80 ± 9	13.4
Fight	11 ± 1	30.1	23 ± 3	3.8
Upright	3 ± 0	7.8	28 ± 8	4.6
Submit	4 ± 0	11.0	99 ± 19	16.5
Walk	2 ± 0	6.8	7 ± 1	1.1
Flight	1 ± 0	3.1	2 ± 0	0.3
Guard	10 ± 1	27.7	294 ± 21	49.2
Immobile	5 ± 1	13.7	67 ± 12	11.2

Relationship between social conflict behaviour and plasma corticosterone

Relative to baseline levels, intruders (n=48) displayed significantly elevated plasma CORT levels ($F(4,188) = 37.9$, $p<0.01$) (Figure 2.3) with post-hoc analysis revealing significant elevation at each post-social conflict episode time-point assessed; 15 min ($t(47) = 11.3$, $p<0.01$); 30 min ($t(47) = 8.6$, $p<0.01$); and 60 min ($t(47) = 3.5$, $p<0.01$). The amount of time subjects engaged in any one behaviour was not related to changes in plasma CORT levels. However, significant negative correlations were found between Peak CORT and the number of 'fight' events ($r^2 = -0.29$, $p<0.05$), and between Peak CORT and the number of 'guard' events ($r^2 = -0.31$, $p<0.05$) (Table 2.5).

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Figure 2.3. Mean plasma CORT at pre- and post-social conflict time-points 15, 30, 60, and 90 min; baseline level ~100ng/mL was similar to levels at 90 min post social conflict, with Peak CORT ~335ng/mL at 15 min post social conflict.

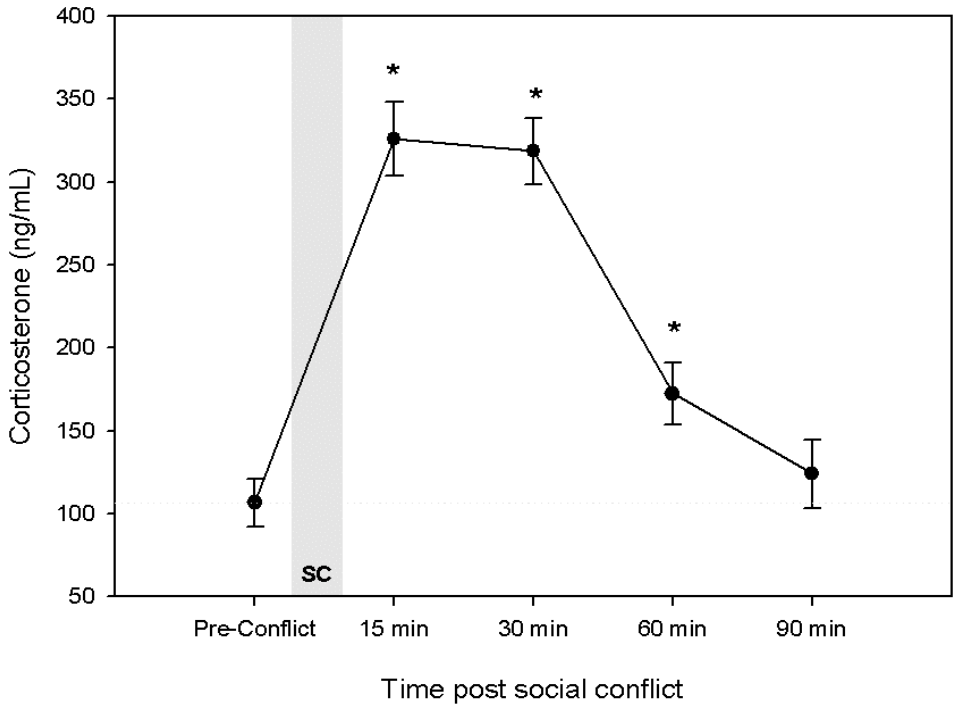


Table 2.5. Pearson product (r-square) analysis was used to determine the relationships between social conflict behaviour events and plasma CORT levels.

	Peak Cort	**AUC Cort**	**Explore**	**Fight**	**Upright**	**Submit**	**Flight**	**Guard**	**Walk**	**Immobile**
Base Cort	**0.39****	**0.40****	-0.16	-0.08	0.13	-0.25	-0.12	-0.13	0.13	0.17
Peak Cort		**0.74**.**	0.01	**-0.29***	-0.05	-0.14	0.07	**-0.31***	-0.03	-0.03
AUC Cort			-0.11	-0.13	0.04	-0.02	0.18	-0.19	0.02	0.16

* Correlation significant at the 0.05 level (2-tailed)

** Correlation significant at the 0.01 level (2-tailed)

BASE CORT = plasma CORT levels 90 minutes prior to conflict

PEAK CORT = maximum plasma CORT level after social conflict, whether at +15 or +30 minutes

AUC CORT = area under the curve for plasma CORT.

Individual differences in coping style influence acute endocrine and neurobiological responses to psychosocial stress.

Relationship between defensive burying, social conflict interaction behaviours, and post-conflict interaction plasma corticosterone levels.

Intruders were exposed to a 10 min defensive burying (shock probe) test 7 days prior to social conflict and the behaviours bury, explore, and immobile, were measured for duration and frequency. On average, 145 (±8) behavioural events were displayed, comprising; 40 (±3) bury events; 31 (±2) immobile events; and 74 (±8) explore events. Analysis of the relationship between defensive burying and social conflict behaviours revealed several significant and positive correlations (Table 2.6), the most interesting in view of the CORT and social conflict interaction behaviour relationship described previously being the significantly positive correlation between the number of social conflict 'fight' events and both the defensive burying total events (bury plus explore plus immobile) and the number of defensive burying 'explore' events (r^2 = 0.34, p<0.05 in both instances). Despite this correlation, however, neither Peak CORT nor AUC CORT levels were correlated with any of the defensive burying behaviours. A separate analysis based upon *time* rather than number of behavioural *events* revealed no significant correlations between duration of defensive burying behaviours and social conflict behaviours or plasma CORT levels.

Individual differences in coping style influence acute endocrine and neurobiological responses to psychosocial stress.

Table 2.6. Relationships between number of events of defensive burying and social conflict behaviours.

Defensive burying test behaviours	**Social conflict interaction behaviours**							
	Fight	**Explore**	**Upright**	**Submit**	**Flight**	**Guard**	**Walk**	**Immobile**
Bury	0.10	-0.28	-0.10	0.11	0.02	-0.01	0.05	-0.06
Immobile	-0.05	0.21	0.11	0.38*	0.17	0.02	-0.16	0.42*
Explore	**0.34***	-0.09	0.01	0.28	-0.11	0.27	0.39*	0.29
Total events	**0.34***	-0.13	0.00	-0.10	-0.04	0.25	0.33*	0.12

* Correlation significant at 0.05 level (2-tailed).

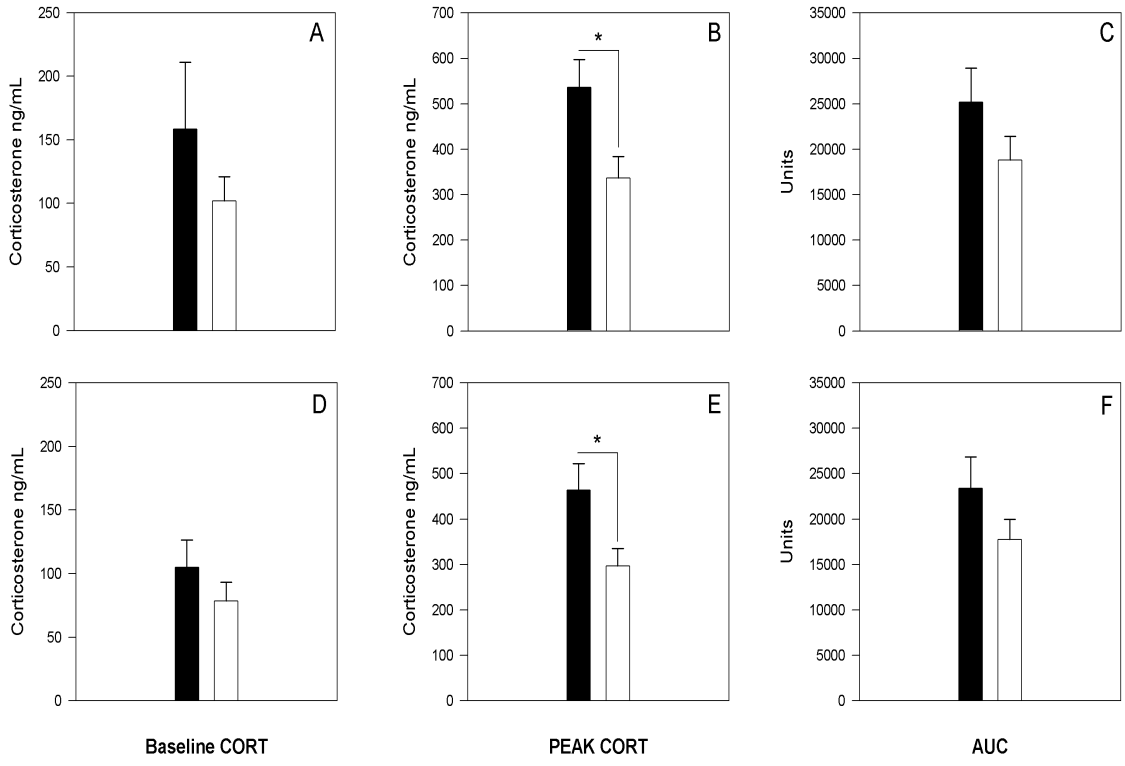
Analysis of corticosterone responses based on social conflict behaviour 'cut-off' criteria

As mentioned previously, the number of 'fight' and 'guard' behaviour events were significantly correlated with peak plasma CORT levels, therefore 'cut-off' behavioural analysis was conducted and the upper and the lower 20th percentile of 'fight' and 'guard' behavioural distributions determined. Four groups, each of n = 10 were derived, and included 'high fight' (16-21 fights), 'low fight' (0-6 fights), 'high guard' (15-22 guard events), and 'low guard' (0-5 guard events). Mean plasma CORT levels for each of these four groups was then calculated at each time point and Independent Samples T-Tests revealed that there was a significantly lower post-defeat Peak CORT level for both 'high fight' and 'high guard' cohorts than the corresponding 'low fight' and 'low guard' cohorts (Figure 2.4). These findings, in addition to the high frequency of events of both behaviours and an inverse relationship between Peak CORT levels of

Individual differences in coping style influence acute endocrine and neurobiological responses to psychosocial stress.

fight and guard suggested a significant relationship between the two behaviours, which further analysis indicated was a strong and positive correlation ($r^2 = 0.69$, $p<0.01$).

Figure 2.4 Mean Baseline, Peak, and AUC CORT levels of high and low fight, and high and low guard cohorts.



A, B and C depict high and low fight groups,
D, E and F depict high and low guard groups.
Black bars indicate lowest 20th percentile group, white bars indicate highest 20th percentile group.
*Significance at 0.05 confidence level (2-tailed).

Individual differences in coping style influence acute endocrine and neurobiological responses to psychosocial stress.

DISCUSSION

There have been three previous reports concerning the relationship between coping style and acute HPA axis response to social conflict culminating in defeat of the intruder. Two of these studies found no relationship between coping style and acute HPA axis activation in response to social conflict (Sgoifo et al., 1996; Ebner et al., 2005a), and the third found that 'active' copers displayed enhanced HPA axis responses to this form of stress (Frank et al., 2006). These outcomes were at odds with earlier reports that suggested that the adoption of an 'active' coping style during a social conflict interaction reduced the extent and duration of longer-term effects significantly more than the adoption of a 'passive' coping style during a social conflict interaction (Meerlo et al., 1996; Stefanski, 1998). Accordingly, we wondered whether the reason for this discrepancy might be related to the fact that the different studies assessed coping style at different times, with only the longer-term studies assessing coping style during the conflict episode. In particular, we hypothesised that the coping behaviour adopted *during* a social conflict interaction would be relevant to the modulation of acute HPA axis responses to the social conflict interaction. Ergo, we report that those intruders that engaged in more 'fight' or 'guard' behaviour events with the resident during an acute unseparated social conflict interaction displayed less elevated acute HPA axis activation than those intruders that engaged in lower numbers of 'fight' or 'guard' behaviour events. In terms of the relationship between coping style adopted *during* a social conflict interaction and the corresponding acute effects

this study has provided data showing that the acute corticosterone response to social conflict varies in accord with the coping style displayed during the social conflict episode, but is unrelated to the coping behaviour displayed in a defensive burying test conducted prior to the conflict interaction.

High levels of fight and guard behaviours are associated with a reduced adrenocortical response to social conflict interaction.

The present findings provide evidence that intruder male rats displayed *acute* adrenocortical response to social conflict that varied in accord with the behaviour displayed *during* the encounter. In particular, the initial analysis of the data showed that Peak CORT responses to social conflict were inversely correlated with the frequency with which intruders either engaged in fights with the resident, or engaged in 'guarding' by using the fixtures of the arena to control their exposure to the resident. Further analysis of the data using the relatively new ‘cut-off’ behavioural criteria approach (Cohen and Zohar, 2004) identified sub-populations of intruders with ‘fight’ or ‘guard’ scores below the 20^{th} or above the 80^{th} percentile, thus deriving four groups of intruders: i) 'low fight'; ii) 'high fight'; iii) 'low guard'; and iv) 'high guard'. Subsequent analysis revealed that both fight and guard behaviours moderated acute plasma CORT responses, with average Peak CORT levels of both 'high fight' and 'high guard' groups significantly lower than the corresponding 'low fight’ and 'low guard' group. Further exploration of the relationship between fight and guard behaviours revealed that, in addition to both behaviours occurring the most

frequently and being inversely correlated with Peak CORT, they were highly correlated with each other.

Previous reports concerning the effect of coping behaviour on responses to social conflict interaction.

The finding that high levels of fight and guard behaviours were associated with smaller adrenocortical responses to social conflict interactions is consistent with previous reports that 'active' coping behaviour - generally characterised by aggression and territorial control - is associated with a reduction in the longer-term (days to weeks) effects of social conflict on a defeated intruder, with such effects including alteration of immune function and biorhythms, and enhanced fear responses (Stefanski, 1998; Meerlo et al., 1999). However, as mentioned previously, the present findings appear to contradict three previous studies that dealt directly with the impact of intruder coping behaviour on acute HPA axis responses to social conflict. These three studies provided data suggesting that; i) 'active' coping had no effect on the CORT response to social conflict interaction (Sgoifo et al., 1996); ii) 'active' coping had no effect on the adrenocorticotrophic hormone (ACTH) response to social conflict (Ebner et al., 2005); and iii) 'active' coping was associated with increased CORT and ACTH responses to social conflict (Frank et al., 2006). However, a key difference exists between these previous studies and the present one: only the present study assessed coping on the basis of behaviour displayed *during* the conflict interaction. Of the three previous studies, one inferred coping behaviour on the basis of behavioural pre-tests (Sgoifo et al.,

1996), one inferred coping behaviour based on displays of freezing behaviour *after* the intruder was separated from the resident (Ebner et al., 2005), and one inferred coping behaviour on the basis of membership of selectively bred animal lines reported to differ in their tendency to display active vs. passive coping when challenged (Frank et al., 2006). We suggest that the most prudent explanation of the apparent discrepancy between the present findings and those of the three earlier studies is; it is behaviour deployed *at the time* of the conflict interaction that has primacy in determining the acute HPA axis response to social conflict.

Failure of the defensive burying test to predict the adrenocortical response to social conflict.

Consistent with the view that it is behaviour deployed *at the time* of the conflict interaction that is most critical in determining acute HPA axis responses to social conflict, we found that behaviour displayed during defensive burying tests conducted prior to social conflict did not correlate with the plasma Peak or AUC CORT response to conflict interaction - an outcome that matches an earlier report from Sgoifo et al. (1996). However, the total number of behavioural events displayed during the defensive burying test, and the number of exploration events engaged in during that test, were both significantly and positively correlated with both the number of fight events and guard events scored by the intruder during the social conflict interaction. Of additional interest were the significantly positive correlations found between defensive burying test immobility behaviour with immobility and submissive behaviour

during the social conflict episode, and defensive burying test explore and total behaviour events with walk behaviour during the social conflict interaction. Collectively, these results speak to the utility of the defensive burying test as an indicator of coping style, even though it did not predict the acute HPA axis response to social conflict. This said, in previous studies that used the defensive burying test as an indicator of innate coping style, it was the duration of immobility or burying behaviour, rather that the number of behavioural events, that was associated with active vs. passive coping; an association not found in the present study.

Observation of guard behaviour during social conflict interaction.

While the resident/intruder paradigm has been extensively used over the past three decades, we believe that this study provides one of the first reports of guard behaviour in this context. The behaviour itself has been described previously in both the visible burrow system - a habitat providing burrows and an open area for investigation of rat colonies - and other ethological investigations (Blanchard et al., 2001b). The primary purpose for the deployment of the behaviour has been suggested as defensive; effectively by reducing the opportunity of the opponent to engage. The fact that this behaviour has not been described within the resident/intruder literature is somewhat surprising, but several explanations are apparent. Firstly, a majority of studies investigating the resident/intruder paradigm have employed a separation procedure whereby the intruder and resident are physically separated by a mesh divider following the first instance of submissive

behaviour, effectively eliminating the opportunity for the display of guard behaviour. Secondly, previous investigations have utilised the resident/intruder paradigm in environments devoid of any object except the walls that confine the animals, again eliminating the opportunity for the display of guard behaviour.

Limitations of the current study

In the present study, acute HPA axis activity was assessed only on the basis of determinations of plasma CORT levels; samples were collected for determination of ACTH levels but perished due to storage issues. Nevertheless, we contend that determination of plasma CORT levels is the most critical measure of HPA axis activation, as glucocorticoids mediate the majority of its physiological effects. Moreover, it is well understood that the three levels of the HPA axis, the hypothalamic CRF cells, the pituitary corticotrophs, and the adrenocortical cells of the zona fasciculata, do not operate in lock-step with each other. Ideally, therefore, indicators of activity at all three levels would be collected, but adrenocortical output cannot be reliably inferred on the basis of activity at either of the higher levels.

Conclusions

A number of previous studies have shown that 'active' coping behaviour deployed *during* social conflict can moderate the medium to longer-term (days to weeks) effects on a defeated individual exposed to this potent psychosocial stressor. However, there has been confusion as to whether the same is true with regard to the acute HPA axis response. The present study appears to

resolve this issue, demonstrating that when coping is assessed on the basis of behaviours displayed *during* the conflict interactions, 'active' coping intruders do indeed display smaller rises in plasma corticosterone responses to an acute social conflict interaction.

Individual differences in coping style influence acute endocrine and neurobiological responses to psychosocial stress.

CHAPTER 3.

Impact of coping behaviours adopted during a social conflict interaction on neural activation

INTRODUCTION

The findings described in the preceding chapter are consistent with the view that the behaviour, i.e. coping style, adopted during social conflict interactions can modulate the stress response associated with the social defeat that occurs during that episode. Specifically, it was demonstrated that individual intruders that engaged in fights or guarding behaviour during their interaction with the resident displayed smaller acute HPA axis responses than intruders that did not. Importantly, these findings form the basis from which to address the question of whether the behavioural and hormone response differences observed correspond to differences in neural activation in the brain.

To date, four studies have assessed neural activation associated with the stress responses of social defeat (Kollack-Walker et al., 1997; Martinez et al., 1998; Kollack-Walker et al., 1999), although only one has assessed the association between individual differences in coping style and neural activation patterns (Frank et al., 2006). However, interpretation of the data presented in that report is difficult. Firstly, the study contrasts selectively bred lines of animals thought to differ in both their anxiety behaviour and coping styles, but, as discussed in Chapter 1, it is not clear that these selectively bred lines of

animals truly represent the extremes of normal distribution in the way assumed by the authors. Secondly, the study by Frank et al (2006) assessed coping behaviour on the basis of two factors; i) membership of a selectively bred strain of rats (HABs that preferentially adopted 'passive' coping behaviours, and LABs that preferentially adopted 'active' coping behaviours), and ii) the behaviour observed immediately after social conflict, during a period of separation of the resident and intruder. With regard to the latter issues, the data presented in Chapter 2 clearly showed that coping behaviours deployed during a social conflict interaction provided a meaningful gauge of an individual's likely response to that form of stress. Therefore, when all issues concerning the generalisabilty of the findings presented by Frank et al (2006) were considered, it was concluded that there would still be significant value in investigating the relationship between coping style, social conflict, and neural activity, in a 'natural' population of animals, specifically an outbred strain of rat.

Fortunately, this was possible to achieve by using those same animals as used for the experiments described in Chapter 2. In that experiment, the final blood sample required for corticosterone determinations was collected 90 minutes after the commencement of the conflict episode. The optimal time-point for the expression of stimulus-induced ITF Fos has been shown to occur approximately 2 hours after stimulus onset (Dragunow and Faull, 1989), so it was possible to sacrifice the animals 30 minutes after the last blood collection and at the two-hour time-point to then harvest the brains and process for Fos-like immunoreactivity.

Individual differences in coping style influence acute endocrine and neurobiological responses to psychosocial stress.

Consequently, the present study aimed to determine whether coping style adopted during a social conflict episode involving social defeat was associated with modification of neural activation patterns in brain structures previously demonstrated to participate in the regulation of response to psychological stress. Consistent with the findings presented in the preceding chapter, we were particularly interested to examine the relationship between neural activation patterns and displays of fight and guard behaviour during social conflict.

METHODS

Animals, housing, defensive burying, surgery, and social conflict

The subjects used for the present study were the same animals used to derive the data described in Chapter 2, with the last remaining cohort of animals assigned to the study used as control animals (n=4) that were age and weight-matched to intruders. Each animal was exposed to all protocols in accord with the 'Intruder activity regime' outlined in Table 2.1 except for social conflict, at which time they were exposed to the social conflict arena only. Subjects were sacrificed two hours after acute social conflict, or an equivalent time for controls, perfused with fixative solution and brains harvested then immunolabelled as described below.

Tissue fixation and sectioning

Two hours after social conflict for intruders, or exposure to the resident cage for controls, animals were deeply anaesthetized using Pentobarbitone

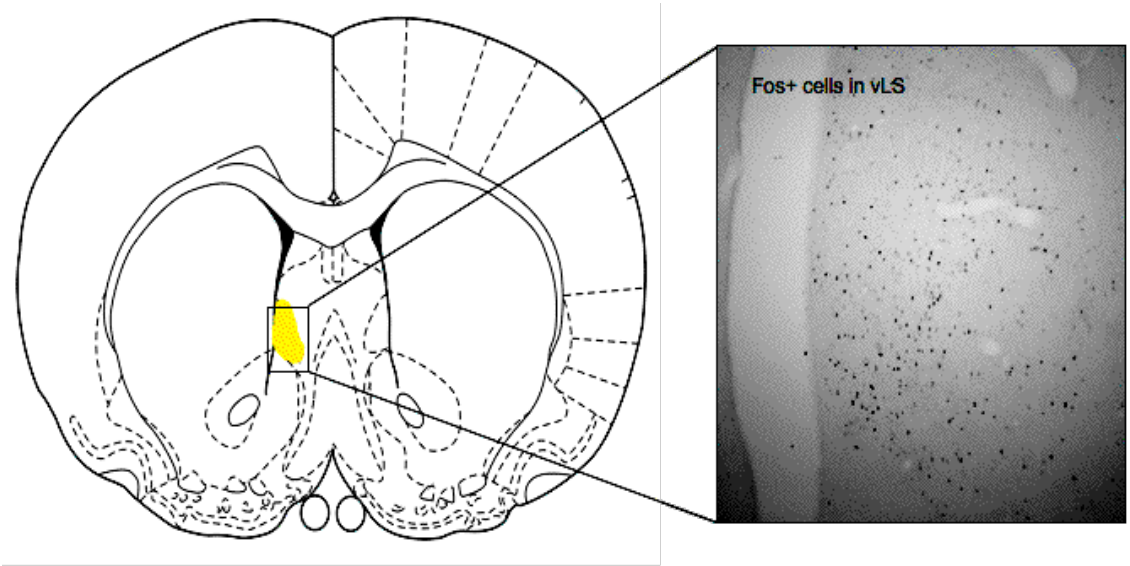
Sodium (80mg/kg i.p.) and transcardially perfused. The perfusion proceeded in two stages; i) tissue was cleared with ~100mLs 2% sodium nitrite (pH 6.0) in 0.1M phosphate buffered saline (PBS), and ii) perfused with ~500mL 4% paraformaldehyde in 0.1M PBS pH 7.4 at room temperature. The brain was then harvested, post-fixed in 4% paraformaldehyde for one hour, then sectioned at the junction of the pons and mid-brain, and post-fixed for a further hour before cryoprotection in 10% sucrose (in 0.1M PBS pH 7.4 at 4°C) overnight. Serial coronal sections were cut at 40μm using a freezing microtome (Leica SM2000R) with the stage being cooled using a Peltier device (physitem BFS-30TC ER controller) set between -30° and -20°C and collected in 4 x 4 section trays, then stored in antifreeze solution (30% sucrose, 60% ethylene glycol in 0.05M sodium phosphate buffer pH 7.4) at 4°C until required for immunohistochemistry.

Immunohistochemistry

To minimize variability in immunohistochemical processing tissue samples from multiple subjects were batch processed at the same time for the immunodetection of Fos using an immunoperoxidase treatment previously described (Smith and Day, 1993). Primary antibody omission was conducted on a number of sample sections to confirm the specificity of the immunoreactivity. Briefly, sections were rinsed in 0.1M PBS, permeabilised, endogenous peroxidases cleared in 3% hydrogen-peroxide methanol, blocked in 3% bovine serum albumin (BSA) in 0.1M PBS pH7.4, and incubated in primary Fos antibody (rabbit anti-c-*fos* 1:10,000 Santa Cruz Biotechnology CA)

for 72 hours at 4°C. Sections were rinsed in PBS, incubated for 2 h in secondary antibody (donkey anti-rabbit 1:500, Amersham Biosciences USA), rinsed in PBS, and incubated in Avidin:Biotinylated enzyme Complex (ABC Vectastatin kit 6μL/mL from Australian Lab Services Aust). To visualise horseradish peroxidase complex activity sections were incubated in nickel diaminobenzidine (DAB) and the reaction stopped once an optimal intensity was obtained (Figure 3.1). Sections were serially mounted on chrome-alum slides, dehydrated in a series of alcohols, cleared in xylene, then coverslipped.

Figure 3.1 Cells that displayed a dark-black stain were counted as Fos-positive in the eight forebrain regions such as the vLS.



Brain regions and Fos immunoreactivity quantification.

Brain regions were selected for investigation of neural activation based on the factors; i) the region was considered to be influential in a psychosocial stress response; or ii) the region had been shown previously to be consistently

involved in the response to acute social defeat (Table 1.1). The eight forebrain regions examined included infralimbic medial prefrontal cortex (IL PFC), prelimbic medial prefrontal cortex (PL PFC), vBNST, dBNST, CeA, MeA, vLS, and PVN and cells counted as Fos-ir if they displayed clearly discernible dark-black nuclei. Fos-ir cells were counted manually using a Zeiss Axioskop microscope set at 10 X magnification attached to a digital camera (Insight SPGT SciTech from Diagnostic Instruments Inc Model 18.2 Colour Mosaic). The boundaries of the regions counted were determined in accord with cytoarchitectural structures identified using a stereotaxic rat brain atlas (Paxinos and Watson, 2005). Each region was counted over a number of sections that spanned the approximate caudal to rostral extent of each region (Table 3.1)

Table 3.1 List of brain regions examined, numbers of sections counted, and the approximate corresponding rostrocaudal level relative to bregma.

Brain region	**Number of sections counted**	**Approximate bregma loci**
PL PFC	4	+2.2 to +3.4 mm
IL PFC	4	+2.2 to +3.4 mm
CeA	5	-3.2 to -1.2 mm
MeA	5	-3.2 to -1.2 mm
dBNST	4	+0.12 to +0.48 mm
vBNST	4	+0.12 to +0.48 mm
PVN	3	-2.2 to -0.9 mm
vLS	19	-0.4 to +1.60 mm

Each brain region examined was identified according to relevant cytoarchitectural landmarks defined in a stereotaxic rat brain atlas (Paxinos and

Watson, 2005). The mPFC was taken as the region medial to corpus callosum (Hurley et al., 1991); the region lateral to the ventral tip of the internal capsule was defined as CeA and the region lateral to the supraoptic nucleus was defined as the MeA (Dayas and Day, 2002); BNST regions above the anterior commissure were defined as dorsal BNST (dBNST) and those below the anterior commissure as ventral BNST (vBNST) (Spencer et al., 2005); the PVN was taken to include both the medial parvocellular and magnocellular divisions.

Data analysis

The experiments reported in Chapter 2 showed a significant correlation between intruders' Peak CORT levels and both fight and guard event scores. Moreover, the analysis of the data also showed significant differences in the Peak CORT levels of intruders in the lower versus the upper 20th percentile with regard to either fight or guard event scores. Therefore, it was decided that, rather than analyse Fos-positive cell counts from every animal used in Experiment 1, Fos-ir cell counts would be done on sub-groups of animals identified using these same cut-off criteria. Accordingly, Fos-positive cell counts were only conducted on brain tissue from defeated intruders in (a) the lower or upper 20th percentile in terms of fight event scores, or (b) the lower or upper 20th percentile in terms of guard event scores. This produced four sub-groups, which we refer to as 'low fight' (n = 10), 'high fight' (n = 10), 'low guard' (n = 10), and 'high guard' (n = 10). For each of these 'treatment groups' and the control group (n = 4) we counted Fos-positive cells in every 4th section along the rostrocaudal axis in the eight forebrain regions of interest.

Individual differences in coping style influence acute endocrine and neurobiological responses to psychosocial stress.

Separate repeated measures analyses of variance (ANOVA), using 'treatment group' as the between subjects variable and 'rostrocaudal level' as the within subjects factor were then performed to investigate differences in Fos-positive cell numbers for each brain region. With regard to our investigation of treatment group effects, two separate sets of repeated measures analyses were conducted, one comparing the two fight groups and the control group, and a second comparing the two guard groups and the control group. Tukey's HSD was then used to perform post-hoc analyses, where appropriate. Differences with $p<0.05$ were taken as significant.

RESULTS

Relationship between social conflict behaviour and c-*fos* activation

Fos data was obtained for one group of control animals (n = 4) and four groups of intruders (all n = 10): high fight (16-21 fights), low fight (≤6 fights), high guard (15-22 guard events), and low guard (≤5 guard events). Occasional problems with fixation, storage, or sectioning of brain tissue meant that reliable Fos data could not be obtained for every animal included in the high fight, low fight, high guard, and low guard groups that had provided the CORT data described in the previous section. In such cases (n = 6) we substituted the brains of animals that met the same behavioural criteria.

Eight forebrain regions were assessed for Fos-ir cells over a range of sections (Table 3.1), with cells displaying dark-black nuclei considered to be Fos-ir. Repeated Measures ANOVAs performed on Fos-ir counts for each fight,

guard, and control group across each brain region examined revealed there were no significant 'treatment group' by 'rostrocaudal level' interactions. We report, therefore, only the treatment group and rostrocaudal level *F* statistic for each region examined and mean Fos-ir counts ± SEM collapsed across all rostrocaudal levels (Table 3.2).

Table 3.2. Mean Fos-ir cell counts in selected brain regions of control, 'high fight', 'low fight', 'high guard', and 'low guard' groups ± SEM.

Region	Control	Hi fight	Lo fight	Hi Guard	Lo Guard
PL PFC	137±58	629±168	1210±197 *	932±192	992±214
IL PFC	40±19	277±76	547±78 * †	437±87	453±102 ‡
CeA	2±1	94±30	259±48 * †	125±27	155±35 ‡
MeA	95±37	280±68	540±85 * †	412±66Δ	404±73 ‡
dBNST	61±18	113±24	206±33 *	170±30	202±37
vBNST	138±41	197±50	450±85 * †	320±66	383±61
PVN	116±51	199±48	475±85 * †	212±50	397±56 ‡
vLS	523±47	464±138	1364±193 * †	945±180	1169±194

Confidence levels considered significant; * = $p<0.05$ for low fight vs. controls; † = $p<0.05$ low fight vs. high fight; Δ= $p<0.05$ high guard vs. controls; ‡= $p<0.05$ low guard vs. control (Tukey's HSD).

Differences between 'high fight', 'low fight', and control groups

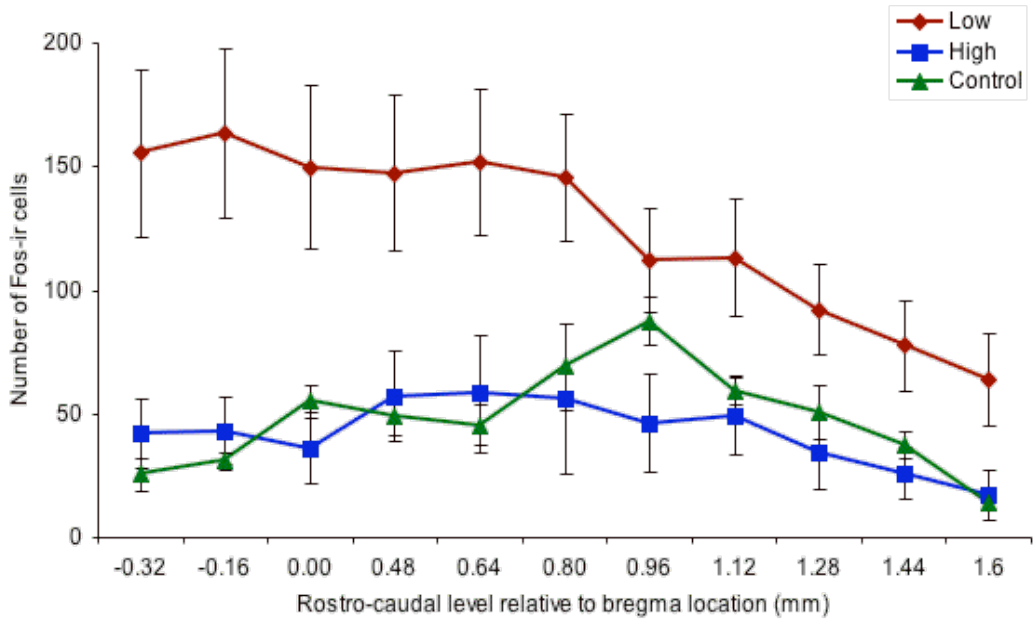
Repeated measures ANOVAs used to assess differences between 'high fight', 'low fight', and control groups in eight forebrain regions counted revealed a significant main effect of 'treatment' group in all regions examined (Table 3.2). The significant *F* statistic for each of the treatment group differences for each of the eight brain regions was: PL FPC $F(2,21) = 6.5$, $p<0.05$; IL PFC $F(2,21) = 8.1$, $p<0.05$; CeA $F(2,21) = 9.5$, $p<0.05$; MeA $F(2,21) = 6.9$, $p<0.05$; dBNST

$F(2,21) = 5.3$, $p<0.05$; vBNST $F(2,21) = 5.1$, $p<0.05$; PVN $F(2,21) = 5.8$, $p<0.05$; and vLS $F(2,21) = 9.5$, $p<0.05$. Tukey's post-hoc analysis indicated that the 'low fight' group had significantly higher Fos-ir levels compared to either 'high fight' or control groups in all regions except PL PFC and dBNST, where 'low fight' differed significantly with control group only.

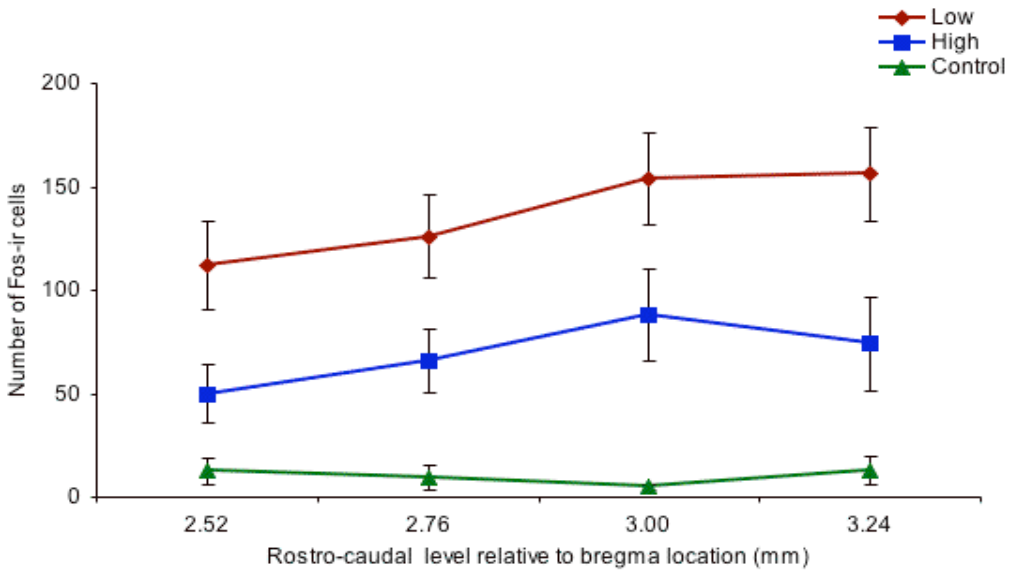
Repeated measures ANOVAs also revealed a significant difference in Fos-ir counts along the rostrocaudal extent of the vLS ($F(10,210) = 2.9$, $p<0.05$) and the IL PFC ($F(3,63) = 3.7$, $p<0.05$), with counts being higher at the caudal pole of the vLS (Figure 3.2 A.) and at the rostral pole of the IL PFC (Figure 3.2 B.).

Figure 3.2 Average Fos-ir cell numbers for low (red) and high (blue) fight, and control (green) groups at each bregma level across the rostrocaudal extent of vLS (Panel A.) and IL PFC (Panel B.).

A.



B.



Differences between 'high guard', 'low guard', and control groups

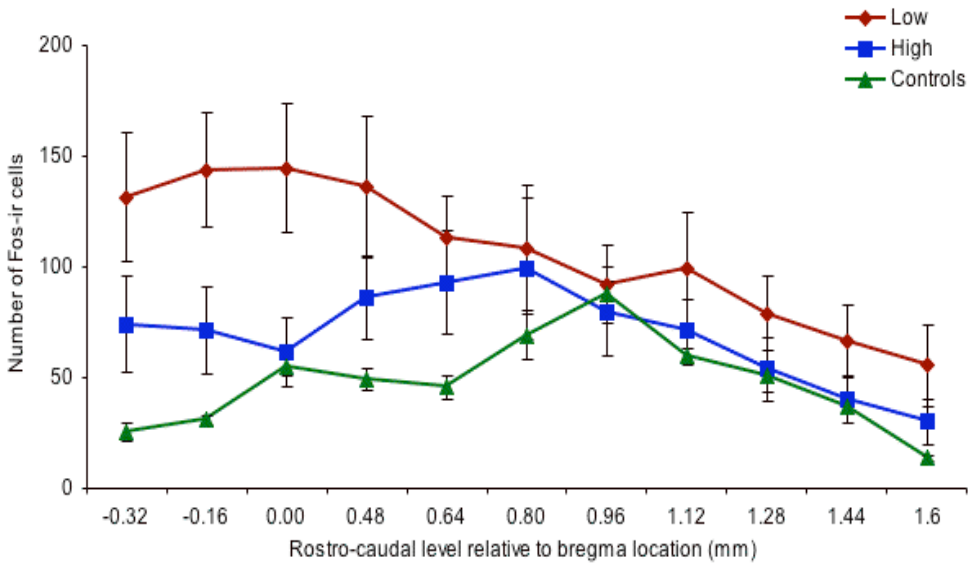
Repeated measures ANOVAs used to assess the differences between 'high guard', 'low guard', and control group counts for eight forebrain regions revealed a significant main effect of 'treatment group' in four forebrain regions examined (Table 3.2). The significant *F* statistic for the treatment group differences for each region was: IL FPC $F(2,21) = 3.5$, $p<0.05$; CeA $F(2,21) = 4.3$, $p<0.05$; MeA $F(2,21) = 3.9$, $p<0.05$; and PVN $F(2,21) = 3.9$, $p<0.05$. Tukey's post-hoc analysis indicated that the 'low guard' group had significantly higher Fos-ir cell counts compared to controls in the four regions IL PFC, CeA, MeA, and PVN whereas high guard group was significantly higher than control Fos-ir cell counts in MeA.

Repeated measures ANOVAs also revealed a significant difference in Fos-ir counts along the rostrocaudal extent of the vLS ($F(10,210) = 4.1$, $p<0.05$) and the IL PFC ($F(3,63) = 4.0$, $p<0.05$), with counts being higher at the caudal

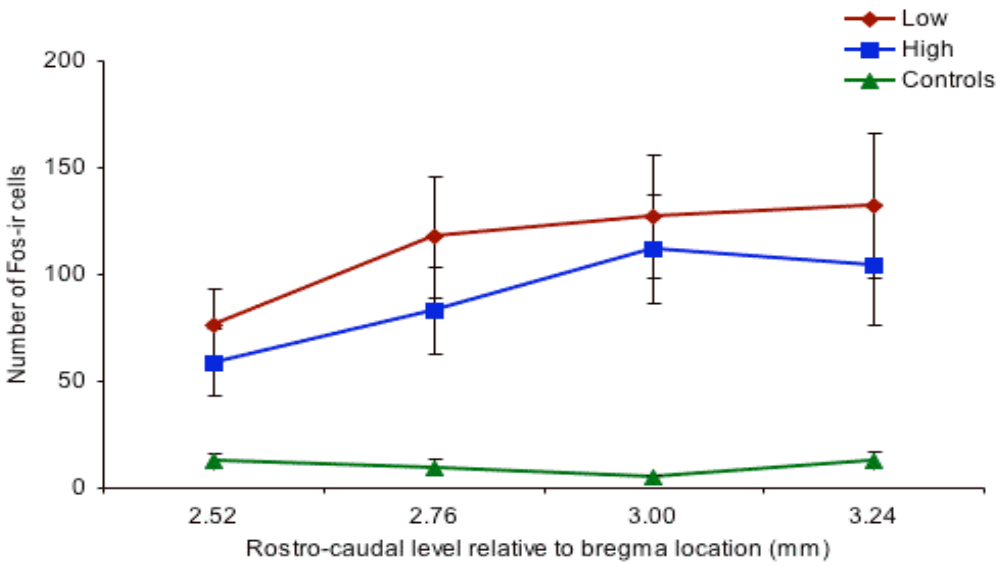
aspect of the vLS (Figure 3.3 A.) and at the rostral aspect of the IL PFC (Figure 3.3 B.) .

Figure 3.3 Average Fos-ir cell numbers for low (red) and high (blue) guard, and control (green) groups at each bregma level across the rostrocaudal extent of vLS (Panel A.) and IL PFC (Panel B.).

A.



B.



Individual differences in coping style influence acute endocrine and neurobiological responses to psychosocial stress.

DISCUSSION

This study assessed the association between coping behaviours adopted by defeated individuals during an acute, unseparated social conflict encounter and corresponding neural activation levels within eight forebrain regions. In general terms, our findings suggest that there was a relationship between the numbers of Fos-ir cell populations and fight and guard behaviour adopted during social conflict. Specifically, a low level of fighting was associated with significantly higher Fos-ir levels in all forebrain regions compared to controls that did not engage in social conflict, whereas high levels of fighting were associated with much lower Fos-ir levels in six of the eight regions examined. Low levels of guard behaviour were associated with significantly elevated Fos-ir levels compared to controls in four of the eight brain regions examined whereas high guard was associated with elevation in one brain region. These findings suggest that, although intruders that engaged in social conflict with a resident suffered social defeat, the differences detected in Fos-ir levels varied in accord with the frequency of fighting or guarding by the intruder during the social defeat encounter. Interestingly, the behaviour fight has been associated with territorial control (de Boer et al., 2003) and the behaviour guard with an active avoidance strategy (Blanchard et al., 2001a), which, when considered in terms of the results we report, suggest that the adoption of active coping behaviours is associated with lower levels of neural activation.

Neural activation levels detected using immunohistochemistry provide an informative insight into the brain pathways that are involved with the acute

response to an event, which in this instance is the defeat of a social conflict. What is not apparent, however, is the type of activation that has occurred. As discussed previously, there are many neural connections between the regions of the brain examined. Cellular activation is influenced by both excitatory and inhibitory inputs, the determination of which is not within the scope of the Fos-ir technique. Nuclear labelling such as that attained with this technique provides an insight into the cell numbers that have undergone nuclear activation, but does not identify the activation outcome nor the type of cell that has been activated. Elucidation in further studies of the identity of cell populations involved would be of considerable interest as this could provide useful candidates for directed drug treatments or other therapies. In addition to identifying the neurotransmitter type of the activated cells it would be of interest to identify their projections and their inputs, including the identity of the receptors they express, for example glucocorticoid and mineralocorticoid receptors.

Effect of social conflict on patterns of neural activation in the forebrain

In view of our findings (detailed in Chapter 2), which reported that displays of the active coping behaviours fight and guard during a defeat encounter were associated with a reduced corticosterone response to defeat, we were interested to ascertain whether there were corresponding differences in the defeat-induced activation of stress-sensitive forebrain structures, particularly those implicated in the regulation of HPA axis responses to stress (Day et al., 1999; Herman et al., 2003; Spencer et al., 2005). Importantly, this

undertaking was made possible by adopting the method of data analysis that we had utilised in our examination of the corticosterone data, i.e. the technique of applying behavioural 'cut-off' criteria to identify key sub-populations of subjects. Therefore, we were able to demonstrate that those that achieved a fight score below the 20th percentile, the 'low fight' intruders, had significantly higher numbers of Fos-ir neurons in the eight forebrain regions examined than control animals exposed only to an empty resident's cage. In contrast, forebrain Fos-ir numbers in the upper 20th percentile or 'high fight' group did not significantly differ from controls, although there were significant differences with ‘low fight’ Fos-ir counts in six of the eight forebrain regions examined; IL PFC, vBNST, PVN, CeA, MeA, and vLS, suggesting that 'high fight' behaviour is associated with significantly reduced defeat-induced neuronal activity in the forebrain. A comparable, although less distinct, result was observed in relation to the behaviour guard. Relative to controls, the lower 20th percentile or 'low guard' group displayed significantly elevated Fos-ir numbers in four of the eight forebrain regions: CeA, IL PFC, PVN, and MeA. In contrast, the upper 20th percentile or ‘high guard' group was significantly elevated compared to controls in only one region: MeA. Hence, the behaviour guard, like the behaviour fight, appears to be associated with reductions in defeat-related neuronal activity in the forebrain.

Fos-ir activation data was collected over a number of rostrocaudal serial sections for the eight forebrain regions of interest, and analysis of this collapsed data revealed no significant interactions between ‘treatment group’ by

'rostrocaudal level', suggesting that even in instances where the behaviour displayed during the defeat encounter was associated with a modulation of Fos expression, the overall effect was uniform along the rostrocaudal extent of the particular brain region. However, there was a significant difference in Fos-ir cell counts along the rostrocaudal axis of the vLS and the IL PFC of defeated animals, smaller numbers being observed at the caudal poles of the IL PFC and the rostral pole of the vLS. The biological relevance of this finding is open to speculation, with one simple explanation being that there are simply less stress-responsive cells in those regions of the IL PFC and the vLS. Another, more speculative explanation, is that there is a differential response based on the activation of vasopressinergic (VP) receptors within these regions (Koolhaas et al., 1998), which are innervated by VP fibres from the BNST and MeA in response to social challenges. This LS VP system is highly variable between sexes and between individual males, and expression is dependent on circulating gonadal steroids. Interestingly, Koolhaas et al (Koolhaas et al., 1998) suggested that higher levels of VP network were associated with more passive behaviours than active behaviours. Activation within the VP system is one possible explanation for the differences reported between Fos-ir cell numbers in vLS regions. In fact, previous evidence suggests that different stressors can recruit different sub-populations of neurons in other brain regions - notably amongst brainstem catecholamine cells (Dayas et al., 2001). It could therefore be of interest in future studies to determine whether different stressors

elicit the same patterns of activation along the rostrocaudal axis of the IL PFC and the vLS as social defeat appears to.

Several previous studies have quantified the pattern of c-*fos* activation elicited in the brain by social defeat using the 'resident/intruder' test, but most have not assessed the impact of differences in coping behaviours. Thus, in studies conducted on hamsters, exposure to social conflict for 30 min significantly elevated c-*fos* expression in a number of regions generally associated with the control of responses to psychological stress, e.g. cortex, amygdala, septum, and hypothalamus (Kollack-Walker et al., 1997). Similarly, rats exposed to defeat during a 10 min unseparated social conflict displayed significantly elevated numbers of Fos-ir cells in regions such as the LS, BNST, PVN, MeA, and CeA (Martinez et al., 1998). In the present study we show that c-*fos* activation in the same regions identified by these two studies is associated with the response to social conflict but, unlike these studies, we also show that the responses vary in accord with the coping style adopted during the social conflict interaction. In a later study by Frank et al (2006) however, intruder rats were used that had been selectively bred for either high trait anxiety behaviours (HAB) or low trait anxiety behaviours (LAB). Importantly, HABs had previously been reported to display a preference for passive coping behaviour whereas LABs preferentially adopted active coping behaviour. In contrast to the results obtained in the present study, these two lines displayed similar defeat-induced elevation of Fos-ir cell numbers in most forebrain regions, the exception being

CeA and MeA, where LABs displayed lower Fos-ir activation than HABs (Frank et al., 2006).

Conclusion

An 'active' coping style is typified by increased displays of aggression or active avoidance whereas a 'passive' coping style is typified by decreased displays of aggression or active avoidance (Koolhaas et al., 1999). Therefore, if one considers the behaviour adopted by an intruder during social conflict in terms of displays of aggression or avoidance, it could be said that intruders that engaged with the resident in a high number of fight or guard events adopted an 'active' coping style, and intruders that engaged in lower numbers of fight or guard events adopted a 'passive' coping style. Our study reported that the relationship between coping behaviour adopted during social conflict interactions and the corresponding forebrain neural activation patterns identified two keys correlations: i) active copers displayed neural activation levels similar to individuals not exposed to social conflict, and ii) passive copers displayed neural activation levels significantly higher than active copers. In fact, our findings differ to the only other study that has investigated neural activation patterns in relation to coping style that found both active *and* passive coping styles were associated with significantly elevated neural activation (Frank et al., 2006). Interestingly, however, the association between coping behaviour adopted during social conflict interactions and neural activation determined in our study is also supported by our findings that acute HPA axis activation is less elevated in active copers than passive copers.

Individual differences in coping style influence acute endocrine and neurobiological responses to psychosocial stress.

CHAPTER 4.

General Discussion

Overview of the current findings

The objective of the research described in this Thesis was to consider the hypothesis that coping behaviour adopted during a social conflict interaction between a resident male rat and an intruder rat, culminating in defeat of the intruder, could moderate the resultant acute stress response of the defeated intruder. In particular, two predictions arising from that hypothesis were tested:

1. That the peak plasma corticosterone responses elicited by social conflict would vary in accord with the coping behaviour deployed during the conflict episode, and that active coping would be associated with smaller responses.
2. That differences in the coping behaviour deployed during the social conflict episode would be accompanied by differences in the patterns of neural activation observed in stress-sensitive regions of the brain.

The data presented in Chapters 2 and 3 demonstrate that the predictions were indeed accurate, and thus provide support for the hypothesis that coping behaviour deployed during social conflict moderates the resultant acute stress response in defeated intruders. These findings are consistent with previous studies that have assessed the longer-term physiological and behavioural effects of social defeat on defeated individuals. Those studies provided data that indicated that the coping style adopted during social conflict moderated the

behavioural and physiological consequences of the conflict, and that reduced responses were associated with deployment of an active coping style (Stefanski, 1998; Meerlo et al., 1999). In contrast, previous studies that focused on the acute physiological and behavioural effects of social defeat reported either that there was no moderating effect of coping behaviour (Sgoifo et al., 1996; Ebner et al., 2005a), or that there was an elevated response in active copers (Frank et al., 2006). Obviously these three latter reports are not only at odds with the earlier reports concerning longer-term effects of social conflict; they are also at odds with each other. A critical analysis of all of these previous reports led us to notice that, in the studies that had dealt with the effects of coping strategy on acute responses to social conflict, coping styles had been evaluated on the basis of behaviour observed either before or after the social defeat episode. Therefore, it became clear that a clarification of the relationship between coping style adopted during social conflict and the associated acute stress response to social conflict was still needed. The present findings provide that clarification.

Broader implications of the current findings

The findings of this study have served to fill a gap in our understanding of the acute effects associated with the stress of social conflict. Beyond these findings there are some broader issues worth noting.

A key element of this study was the use of the resident/intruder rodent model of social conflict, which has been used previously by a number of researchers to investigate the effects of psychosocial stress on both the

resident and/or the intruder (Bohus et al., 1993; Haller et al., 1996; Stefanski, 1998; Miczek et al., 1999; Walker et al., 2007). Our study, however, was undertaken utilising an adaptation of the resident/intruder model designed to simulate the naturalistic environment of wild-type rodents, by creating an enriched environment within the resident homecage by using cage dividers and shelves (Blanchard et al., 1995). As a consequence of this unique design, we observed the behaviour guarding, which until now, had only been reported in an open burrow system and not in a laboratory-style cage. This innovative approach demonstrates the utility of the resident/intruder paradigm as a rich model useful in assessing behavioural, endocrine, and neurological elements in a naturalistic setting.

The finding that the coping style adopted during social conflict can modulate the acute physiological and neural consequences of a complex form of stress advances our understanding of the contribution made by individual differences in vulnerability to stress-induced psychopathology. We report that the adoption of active coping strategies at the time of social conflict were associated with smaller acute effects, whereas the adoption of passive coping strategies at the time of social conflict were associated with larger acute effects. Other reports have suggested that the longer-term effects are also less severe in those individuals that adopt an active coping strategy, particularly when compared to some individuals that adopt passive coping strategies (Keay and Bandler, 2001; Ebner et al., 2005a; Salvador, 2005). The implications of these effects are enormous. If one considered the possibility that those individuals

that respond to a stressful situation with passive behaviours that result in pathologies such as depression (Muris et al., 2001), hormone imbalances (Theorell, 1992), or pain (Covic et al., 2000) for example, could somehow alter that response to that of an active coping strategy, the more severe outcomes could potentially be improved.

Future directions

This study has demonstrated that coping strategies adopted during a social conflict interaction modulate the acute effects of exposure to psychosocial stress, and that the degree of modulation differs between individuals. The critical challenge, from the perspective of a neurobiologist, is to identify those brain mechanisms that are critical to the occurrence of individual differences in the selection of coping strategy and, by extension, stress resilience.

Prior to conducting the Fos study, the possibility had existed that this neural activity mapping exercise might have revealed coping-specific “activity footprints” that, conceivably, might have helped to identify brain structures critical to style selection. However, this did not prove to be the case. Coping-related differences were seen across most stress-sensitive areas and the most parsimonious explanation is that this simply corresponds to a global reduction in stress responding. Accordingly the objective of identifying a “coping style selection centre” remains an elusive but desirable objective. One way forward, however, may be to examine the role of specific neurotransmitter or neuromodulator systems in the expression different coping styles. In this regard it would seem prudent to begin by considering neurotransmitter and

neuromodulator that have already been implicated, in various ways, in contributing to changes in stress resilience, for example CRH, adrenal steroids, testosterone, noradrenaline, neuropeptide Y, serotonin and dopamine, to name a few.

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