Serotonin and Threat:
from Gene to Behaviour

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This dissertation is submitted for the degree of

Doctor of Philosophy

To my parents,

for encouraging my curiosity from an early age.

And to Bambi, my dear dog,

for being a lovely companion in a lonely childhood.

Preface

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text

It does not exceed the prescribed word limit for the relevant Degree Committee.

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Abstract

Anxiety and fear are emotions provoked by threatening situations and shape adaptive behaviours, but excessive and uncontrollable anxiety and fear form core symptoms of anxiety disorders. High trait anxiety, an individual's disposition to feel anxious, is associated with greater risk of developing depression and anxiety disorders. This raises the question: why are some more vulnerable to experiencing negative emotions associated with threat than others? As serotonin has been implicated as a key neuromodulator of emotion, the thesis addresses this question by adopting a multi-systems approach to investigate the link between serotonin and both threat-driven behaviour and trait anxiety with the common marmoset as a model.

Firstly, factors underlying threat-related behaviour modelled with an exploratory factor analysis revealed a relationship between a predominantly avoidant fear coping style and an increased propensity for anxiety, establishing a link between specific fear-driven behavioural patterns and anxiety.

After characterising anxiety and fear-driven behaviours, mRNA quantification of brain regions implicated in anxiety revealed serotonergic gene expressions corresponding to anxiety and fear-driven behaviours. Most notably, amygdala serotonin transporter expression was positively associated with anxious behaviour and was differentiated by the serotonin transporter polymorphism. Based on this association, the hypothesis that increased amygdala serotonin transporter expression may contribute to the high trait anxious phenotype was tested. Consistent with this hypothesis, blockade of amygdala serotonin transporters via local infusions of a selective serotonin reuptake inhibitor (SSRI), citalopram reduced key characteristics of the high trait anxious phenotype: high state anxiety, and both the behavioural and physiological expression of conditioned fear.

Anatomically, high anxious animals showed reduced basolateral amygdala (BLA) volume in adulthood. Moreover, BLA volume in adulthood was differentiated by the serotonin transporter polymorphism. During development, high anxious animals showed a delayed BLA growth trajectory. These findings demonstrate morphological changes in the BLA across different developmental timepoints predictive of high anxiety in adulthood.

Taken together, findings here provide evidence of amygdala serotonin's role in trait anxious expression, and propose behavioural, genetic, molecular and anatomical factors that may contribute to an individual's vulnerability to anxiety.

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List of Abbreviations

5-HT	5-hydroxytryptamine, serotonin
5-HTTLPR	Serotonin-transporter-linked polymorphic region
ACC	Anterior cingulate cortex
ACTH	Adrenocorticotropic hormone
AP	Anteroposterior
ATD	Acute tryptophan depletion
BA	Basal nucleus of the amygdala
BAI	Beck anxiety inventory
BCNI	Behavioural and clinical neuroscience institute
BLA	Basolateral amygdala
BNST	Bed nucleus of the stria terminalis
CBT	Cognitive behavioural therapy
CeA	Central nucleus of the amygdala
CI	Confidence interval
CIS	Chronic immbolisation stress
CRF	Corticotropin-releasing factor
CRS	Chronic restraint stress
CS	Conditioned stimulus
CSF	Cerebrospinal fluid
CUS	Chronic unpredictable stress
dlPFC	Dorsolateral prefrontal cortex
DNA	Deoxyribonucleic acid
DOI	2,5-Dimethoxy-4-iodoamphetamine
DRN	Dorsal raphe nucleus
DSM	Diagnostic and statistical manual of mental disorders
DTI	Diffusion tensor imaging
EFA	Exploratory factor analysis
fMRI	Functional magnetic resonance imaging
GAD	Generalised anxiety disorder
GM	Grey matter
HCl	Hydrochloric acid
Hipp	Hippocampus
HPA	Hypothalamic–pituitary–adrenal

LA	Lateral nucleus of the amygdala
LM	Lateromedial
LTD	Long term depression
LTP	Long term potentiation
MDD	Major depressive disorder
mPFC	Medial prefrontal cortex
MRI	Magnetic resonance imaging
MRN	Median raphe nucleus
MSA	Measure of sampling adequacy
NAcc	Nucleus accumbens
OFC	Orbitofrontal cortex
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PD	Panic disorder
PET	Positron-emission tomography
PTSD	Post-traumatic stress disorder
OCD	Obsessive compulsive disorder
RNA	Ribonucleic acid
ROI	Region of interest
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
SAD	Social anxiety disorder
SD	Standard deviation
SEM	Standard error of the mean
SSRI	Selective serotonin reuptake inhibitor
STAI	State-trait anxiety inventory
TIV	Total intracranial volume
TSAF	Time spent at the front
US	Unconditioned stimulus
vlPFC	Ventrolateral prefrontal cortex

Chapter 1: General Introduction

The study of how we regulate emotions has achieved substantial progress over the recent decades. Studies into how the brain and body responds to threat has been particularly successful due to advancements in neuroimaging techniques and significant contributions from animal models. But basic research on emotion regulation has not translated to significant progress in our ability to treat pathological forms of threat processing, with the absence of substantial improvements in treatment options within the last decade. Issues arising from current pharmacological interventions range from side effects, delayed onset of action, and low efficacy leading to premature treatment discontinuation and nonadherence (Taylor, Abramowitz and McKay, 2012). In order to advance clinical approaches to anxiety disorders, we need to develop a better understanding of the neural substrates underlying threat-driven emotion processing, and anxious and fear-driven behaviour in animal models. Moreover, the study of the fundamental process underlying threat behaviour will advance our understanding of emotion regulation. This chapter will consist of an introduction to emotions, the threat circuit, anxiety disorders, trait anxiety, and the role of serotonin in modulating threat processing.

1.1 Emotions

Emotions serve an adaptive role in our lives. Positive emotions drive us towards rewarding behaviours such as deriving pleasure from eating calorically dense food; whereas negative emotions help shape behaviour to avoid harm, for example, fear motivating us to run away from a predator. In Darwin's classic treatise, *The Expression of the Emotions in Man and Animals*, he proposed that emotions were a result of natural selection with two particular major insights: emotions were expressed similarly across the world in different cultures, and emotions were expressed similarly across closely-related animal species. Indeed, if emotions did not have a net positive effect on human well-being, survival and reproduction, we would expect emotion to be phased out and not phylogenetically conserved.

Ekman (1972) proposed that there were six distinct basic emotions (anger, disgust, fear, happiness, sadness, and surprise) that were expressed universally, innate to specific neural circuits, and were evolved to drive behaviours that increased survival. However, Barrett (2006a, 2006b) challenged the view that there were emotions that were natural kinds (definite real categories not dependent on human description) on the basis that neuroimaging studies in humans report similar brain areas showing activation in response to stimuli linked to different basic emotions, and human basic emotions were different than those identified in animals. Barrett's theory of constructed emotion posits that emotions

are phenomena that emerge as a product of the brain categorising interoceptive signals into emotion concepts from one's own culture (Barrett, 2016). LeDoux disputes Barrett's rebuttal of the classical view of emotions as evidence from neuroimaging studies do not have the resolution to truly conclude that similar neural circuits underlie different emotions as different neuronal subpopulation may mediate these different emotions within each region. In contrast, LeDoux argues for the study of emotion from the perspective of survival circuits. He proposes that the innate circuits within the mammalian brain mediate not emotions per se, but vital life-sustaining survival processes, such as defence and energy maintenance (LeDoux, 2012).

1.1.1 LeDoux's survival circuits and threat

Ledoux posits that the function of survival circuits is to coordinate physiological changes and behavioural interactions with the environment in the presence of opportunities and challenges, and only indirectly influences feelings. An example of a survival circuit is the circuit for threat processing. All organisms from the simplest to the most complex of life forms needs to be able to process information of potential threats and react to threat effectively in order to survive and defend one's self in a complex environment. Although the overall complexity of the brain varies across mammals, threat processing has been well studied and shown to be conserved within the mammalian brain (Janak and Tye, 2015). The amygdala, a critical structure in threat processing, is well conserved across species (figure 1.1). As different animals face different environments and occupy different ecological niches, the behavioural responses initiated by these circuits will be species-specific, even though the circuit for threat processing may be species-general.

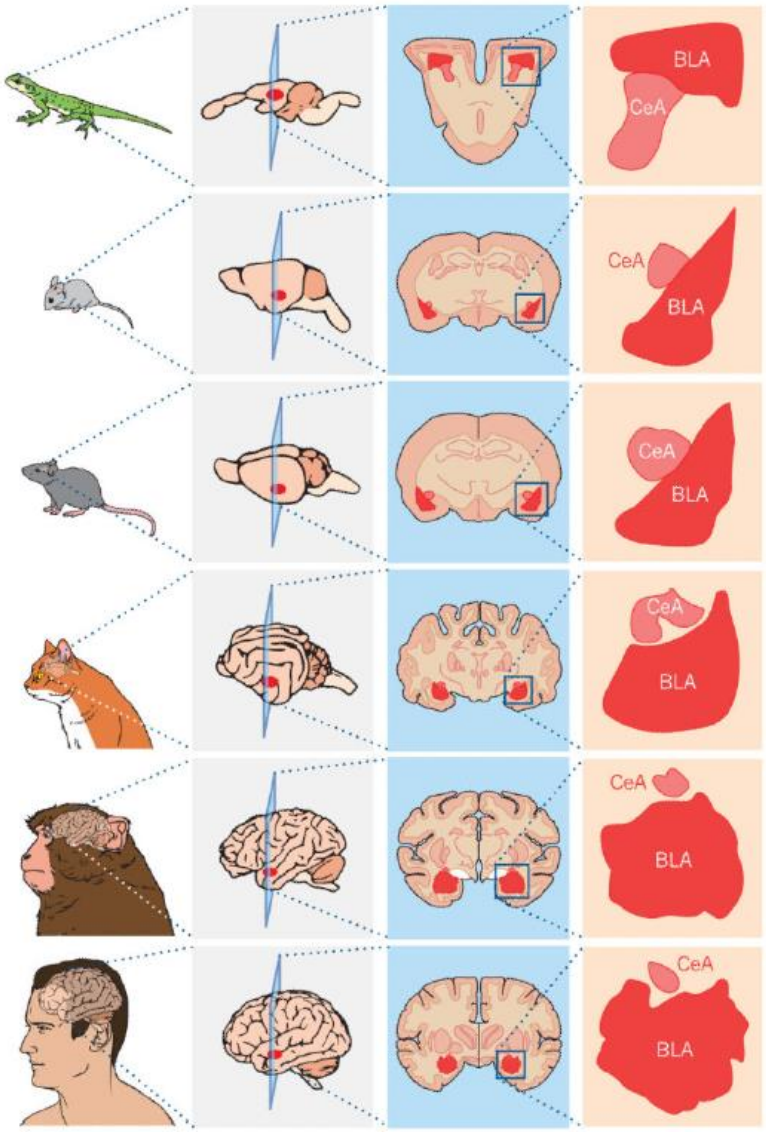


Figure 1.1: Conservation of the primary amygdala nuclei across species by Janak & Tye (2015). The basolateral nuclei of the amygdala (BLA) and central nucleus of the amygdala (CeA) or analogues of lizards, mice, rats, cats, monkeys and humans (top-to-bottom).

Activation of survival circuits not only lead to the initiation of innate behavioural responses, but also generalized arousal. The generalized arousal response encompasses neurotransmitter release modulating neural activity, the release of hormones in the body, and physiological changes such as those within the cardiovascular and metabolic domain.

The serotonin system has been implicated in the mediation of threat sensitivity (trait anxiety) and threat-related behaviours (Cools *et al.*, 2005; Fisher and Hariri, 2013; Bocchio *et al.*, 2016). Serotonergic neurons from both the dorsal and median raphe nuclei innervate the corticolimbic circuit, modulating activity of regions in the circuit for fear and anxiety (Jacobs and Azmitia, 1992).

Stressors in the environment in the form of a predator or signals associated with potential harm also elicit the release of corticotropin-releasing factor (CRF) from the hypothalamus. The CRF initiates the release of adrenocorticotropic hormones (ACTH) from the pituitary which in turn initiates the secretion of glucocorticoids (the primary glucocorticoid released is cortisol in humans and nonhuman primates and corticosterone in most other animals) from the adrenal cortex (Smith and Vale, 2006). The hypothalamic-pituitary-adrenal (HPA) axis forms the central neuroendocrine stress response and the glucocorticoids released in systemic circulation mediate the body's ongoing response to stress (cardiovascular, metabolic, immune, etc.) and activate glucocorticoid receptors expressed in particularly high concentrations in brain regions implicated in emotion regulation: the hippocampus, the amygdala, and the prefrontal cortex (Gray and Bingaman, 1996; Joëls, 2001; McKlveen *et al.*, 2013). Survival circuits instantiate substantial changes in the brain-body state, with the action of neurotransmitters being fast-acting, while the action of hormones in the peripheral system are slow-acting and form a more long-term response to threat-relevant stimuli.

Motivation like feelings of hunger and sexual drive, aren't traditionally perceived as emotions but constitute a critical part of our inner lives and are sensations whereby the object of appraisal is internal. Emotions, in contrast, are induced following cognitive appraisal of external stimuli. LeDoux points out that survival circuits engage the motivational system to increase the chance that goal-directed behaviour is reinforced and motivated. For example, activation of the fear circuit via an aversive trigger such as being bitten after encountering a dog, will lead to the learning (reinforcement) and internal drive (motivation) to avoid dogs, and the re-activation of the fear circuit upon seeing a dog. Thus, activation of survival circuits and circuits for motivation and learning (conditioning) are intertwined.

LeDoux posits that emotions related to survival circuits, such as fear, manifest as a product of: 1) survival circuits and corresponding behavioural pathway activation, 2) generalised arousal (neuromodulatory, hormonal, and physiological), 3) activation of motivational systems, and finally the combination of these states and other subcomponents is appraised and labelled in the cognitive workspace representing our consciousness (figure 1.2). More complex emotions such as shame and guilt are modulated by a separate more complex system that may not involve survival circuits. Insofar

as animals have survival circuits and are conscious beings possessing a cognitive workspace, we may infer that animals have constructs such as emotions as well. However, as there are differences in the brains of humans and animals, the neural circuits giving rise to conscious representation are likely to be different too. By extension, the emotions or conscious feelings arising from humans and animals would likely be different as well. This is reminiscent of the concept of qualia as described by Nagel in "*What Is it Like to Be a Bat?*": it is impossible to know the mental state of a bat (Nagel, 1974). Similarly, it is impossible to know the fear of a rat, or the anxiety of a monkey. Therefore, we should study survival circuits that are emotionally significant without assuming that the phenomena studied are fundamentally the same to the phenomena experienced by people when describing emotions.

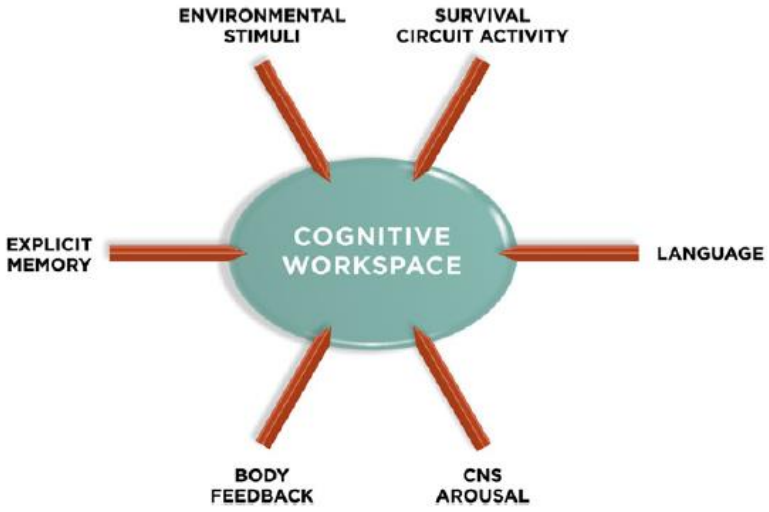


Figure 1.2: Components forming emotions (subjective feelings) within the cognitive workspace by LeDoux (2012).

Moving forward, when the term anxiety or fear is referred to within this thesis, the underlying processing of threat within their respective neural circuits are being referred to, not the subjective feelings that may manifest. Not only is there no way of knowing if the animal is experiencing any conscious feeling at all, but even if they do, whether these conscious feelings are similar to that experienced by humans remains unknown. With this approach we will discuss and further explore the threat circuit underlying anxiety and fear in animal models without anthropomorphising animal behaviour and affect.

1.2 Threat: Anxiety and Fear

Fear and anxiety are terms that are often used interchangeably but refer to distinct threat states. An anxiety-provoking trigger is a context or stimulus that signals potential or uncertain threat. For example, a sound coming from the bushes acts as an anxiety-provoking trigger as it may be from a predator or a non-threatening animal like a passing deer. As an animal is anxious, the anxiety circuit directs attention towards the uncertain threat stimuli and the animal displays vigilant behaviour. The stress response initiates physiological and metabolic events such as increased heart rate, vasoconstriction, mobilisation of energy stores and decreasing motility of the digestive system to prepare for the rapid execution of different potential fear responses.

In an experimental setting, anxiety-measuring paradigms in rodents focus on the behavioural aspect of anxiety. Among the most commonly used anxiety assays, the elevated plus maze (similar to the elevated T-maze) and open field test are examples of approach-avoidance conflict task capitalizing on the rodents innate conflicting drive to explore novel spaces and to avoid exposed areas where they might be vulnerable when anxious (Calhoon and Tye, 2015). Anxious animals therefore, tend to avoid the open arms of the elevated plus maze and open centre of the open field test, and prefer the enclosed arms or the walled sides of the respective tests. Other paradigms also take advantage of behaviours suppressed by high levels of anxiety. For example, in the novelty suppressed feeding test, rodents display hyponeophagia, whereby initiation of feeding is suppressed in anxious animals because there is an uncertainty of threat in a novel environment. Similarly, inhibition of social interaction with an unfamiliar conspecific is measured as anxiety-induced suppression of normal behaviour in the social interaction test.

In humans, anxiety induced by a specific paradigm (e.g. threat of shock) is often assessed via self-reported questionnaires such as the state-trait anxiety inventory (STAI). The STAI evaluates two subscales: state anxiety, the participants current state feelings of anxiety, e.g. feelings of nervousness and worry; and trait anxiety, the participant's trait proneness to feelings of anxiety. In contrast, the Beck Anxiety Inventory (BAI) focuses on the somatic symptoms of anxiety, and has been designed specifically to distinguish between symptoms of clinical anxiety from symptoms of depression (Beck *et al.*, 1988). As the BAI evaluates symptoms occurring over the last week, compared to the subscales of the STAI, the BAI assesses persistent state anxiety. Self-reported measures may however, be less reliable than objective measures of behaviours or autonomic readings as it is affected by an individual's reporting biases and may not accurately measure instances where nonconscious processing of threat is involved.

The physiological component of anxiety in both humans and rodents are often studied by measuring autonomic arousal in the form of increased heart rate or blood pressure, or by measuring levels of stress hormones (cortisol). As anxiety increases the activity of sweat glands, the skin conductance response is used as a measure of stress and anxiety as well.

Furthermore, automatic behaviours such as the startle reflex may also be used as a measure of an animal's anxious or fearful state. The startle reflex such as the jump response of rats in response to the sudden onset of an intense neutral sound stimulus is potentiated when the animal is in the presence of a conditioned aversive cue (fear-potentiation) or in the context where an aversive stimuli was received (anxiety-potentiated) (Siepmann and Joraschky, 2007). The startle reflex is also measured in humans, for example, the eyeblink component of the startle reflex in humans was potentiated by the presentation of an aversive conditioned cue previously paired with an electric shock (Grillon *et al.,* 1991).

In contrast to anxiety, a fear-provoking trigger is a stimulus that directly signals threat. An example would be seeing the stripes of a tiger passing behind a bush. Predators such as snakes and spiders are examples of innate triggers that elicit a fear response without prior association and conditioning (Ohman, 1986; Hoehl *et al.*, 2017). Innate fear triggers are vital as often animals don't survive multiple harmful encounters with a predator, and thus hard-wired responses to specific threats are vital to survival. Experimental paradigms measuring the fear response often expose participants to stimuli representing innate fear triggers, such as images of a snake.

Fear circuits are also flexible to associative learning. As demonstrated by classical conditioning paradigms, animals may acquire conditioned fear responses to a previously neutral stimuli or context by pairing the neutral stimuli or context with the presentation of an innate fear trigger, such as an electric shock (Flor and Birbaumer, 2001). The fear conditioning paradigm has been used in humans, rodents and nonhuman primates to test both unconditioned (innate) and conditioned (learned) fear responses (Milad and Quirk, 2012; Wallis *et al.*, 2017).

The adaptive value of different fear responses is dependent on a combination of complex factors such as the threat type, threat distance, and possibility for escape (Blanchard *et al.*, 2011). The classic fight-or-flight response describes two general strategies to cope with imminent threat: 'fight' e.g. confront and prepare to defend oneself against the threat, or 'flight' e.g. distance one's self from the threat and attempt to flee. Either responses are valid in different scenarios. Fighting would only be effective against a threat that can be effectively overcome or intimidated, but would lead to harm against a threat that one cannot fend off. Whereas, fleeing is effective against threats that cannot lead a successful pursuit but leaves you more vulnerable against a threat that is more mobile.

Fear responses also need to change adaptively to the evolving circumstance. For example, if attempts to fight/intimidate are unsuccessful and the threat maintains close proximity to the individual, a switch to the flight response to regain distance from the threat is necessary to survive the encounter. Thus, the

appropriate activation and switching between fear responding pathways is vital for adaptively responding to threats, and inappropriate fear responding styles are likely to have consequences on the fitness of individuals.

1.2.1 The Threat Circuit

A meta-analysis of positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) studies in emotion activation showed that threat-related stimuli consistently engaged the amygdala, supporting the view that the amygdala plays a central role in threat processing (Phan *et al.*, 2002).

The serial model of amygdala function posits that incoming sensory information of both certain (fear) and uncertain (anxiety) threat converge from the thalamus and sensory regions to the basolateral amygdala (BLA). Long-term synaptic plasticity within these sensory inputs in the BLA (particularly the lateral nucleus of the amygdala, LA) has been implicated in the acquisition of conditioned fear responses (Chapman *et al.*, 1990). This is supported by evidence from rodents showing NMDA receptor-dependent long-term potentiation (LTP) after fear conditioning (McKernan and Shinnick-Gallagher, 1997; Quirk, Armony and LeDoux, 1997; Rogan, Stäubli and LeDoux, 1997; Rodrigues, Schafe and LeDoux, 2001; Goosens and Maren, 2004). Subsequent work in BLA-lesioned rodents support the view that the BLA plays a key role in the encoding and storage of fear memory (Gale *et al.*, 2004).

Information from the BLA is then passed to the CeA via direct and indirect pathway connections between the regions. Bilateral lesions of the CeA cause primates to display less fear-related behaviour in response to a snake and less anxiety-related behaviour in response to a human (human intruder test) (Kalin, Shelton and Davidson, 2004). Lesions of CeA also blocked fear-potentiated startle (Hitchcock and Davis, 1986, 1991; LeDoux *et al.*, 1988). The CeA projections to the hypothalamus and periaqueductal grey (Amaral *et al.*, 1992; Davis, 2000) initiates the expression of defensive physiological and behavioural reactions respectively. For example, lesions of the lateral hypothalamus affects blood pressure but not freezing, whereas lesions of the periaqueductal grey affects freezing but not blood pressure (LeDoux *et al.*, 1988). CeA projections are also involved in different fear coping responses. Gozzi et al. (2010) reported that projections of CeA neurons to cholinergic forebrain neurons mediated the switch between active (digging, exploring, rearing) and passive (freezing) fear behaviour in rodents in a conditioned fear paradigm.

However, sensory cortices and the thalamus also project to the central nucleus of the amygdala (CeA) (LeDoux, Farb and Ruggiero, 1990; Turner and Herkenham, 1991; Mcdonald, 1998), supporting the parallel model of amygdala function, which posits that sensory information enters the amygdala via both the CeA and LA, and projections from the LA and CeA drive distinct behaviours. Consistent with this, lesion studies in rats demonstrated that LA lesions and CeA lesions mediated different fear-conditioned behaviour, suggesting that the information flow from the LA and CeA may differentially

drive different components of threat-driven behaviour (Killcross, Robbins and Everitt, 1997; Manassero *et al.*, 2018). Thus, although the LA receives relatively substantial afferent innervation relative to the CeA, the amygdala does not process sensory information via the LA exclusively. The functional roles of the amygdala subnuclei should be further explored in nonhuman primates to elucidate how the LA and CeA may mediate dissociable cognitive processes within the amygdala microcircuit.

Information is also passed from the BLA towards the nucleus accumbens (NAcc) of the striatum. Dopamine signalling in the nucleus accumbens has been implicated in the expression of avoidance behaviour in response to threat (McCullough, Sokolowski and Salamone, 1993; Levita, Hoskin and Champi, 2012; Wenzel *et al.,* 2018). Findings from neuroimaging studies also point to the striatum's role in the coding of aversive prediction errors (Delgado *et al.*, 2008). Taken together, the evidence suggests that the striatum plays a role in aversive conditioning and that the nucleus accumbens is crucial to the expression of avoidance behaviour.

The BLA also releases CRF, stress hormones from projections to the bed nucleus of stria terminalis (BNST). Evidence from acoustic startle reflex in rodents suggest that the medial CeA mediates the fear response whereas lateral CeA corticotropin-releasing hormone mediated excitation of the BNST mediates the anxiety response (Grillon, 2008; Davis *et al.,* 2010). The BNST, along with the insular cortex and lateral prefrontal cortex, were more greatly recruited in individuals with greater trait anxiety (individual disposition to anxiety) and activity in the BNST corresponds to greater tracking of threat proximity, implicating the region in mediating anxious response (Somerville, Whalen and Kelley, 2010). Behavioural and physiological reaction to threat is initiated via projections from the CeA and the BNST to the hypothalamus and brainstem (Herman and Cullinan, 1997; Berntson, Sarter and Cacioppo, 1998). Stimulation of the oval nucleus of the BNST in mice increased open-arm time in the elevated plus maze test (low anxiety) and reduced respiratory rate, whereas stimulation of the anterodorsal BNST reversed these effects implicating opposing roles of BNST subregions in the modulation of anxious behaviour and physiology. Native spiking of anterodorsal BNST neurons also differentiated between safe and anxiogenic environments (Kim *et al.,* 2013).

Defensive responses and extinction/downregulation of threat circuit activation is mediated via reciprocal connections between the BLA, and both the ventral medial prefrontal cortex (vmPFC) and the ventral hippocampus (vHipp). The balance of activity between two subpopulation of basal amygdala (BA) neurons with distinct vmPFC and vHipp projection patterns modulate the extinction of conditioned fear responses in mice. BA neurons projecting to the mPFC and receive inputs from the vHipp mediated increased freezing (fear neurons), while BA neurons with reciprocal connections to the mPFC but do not project or receive inputs from the vHipp mediated extinction of the freezing response (extinction neurons) (Herry *et al.*, 2008). A distinct of subpopulation of BLA neurons projecting to the

vHipp were also shown to modulate anxious behaviour, with inhibition of BLA-vHipp synapses reducing anxiety and activation increasing anxiety (Felix-Ortiz *et al.*, 2013).

Evidence from studies where the threatening stimuli is masked suggest that threat processing in the amygdala is non-conscious. In masked stimuli paradigms, the threatening stimulus is masked by interfering with attentional mechanisms (attentional unawareness) or the stimulus is presented below sensory detection thresholds (sensory unawareness) (Diano *et al.*, 2016). Neuroimaging studies reported that the stimulus evoked responses in the amygdala even when the emotional stimuli were not attended to (Vuilleumier *et al.* 2001; Anderson *et al.* 2003; Bishop *et al.* 2004; Williams *et al.* 2005). Thus, amygdala activation may reflect the non-conscious bottom-up component of threat circuitry while other structures such as the vmPFC acts as conscious top-down modulatory components.

In summary, the amygdala plays a central role in the threat circuitry mediating anxiety and fear responses. The combination of bottom-up activation and top-down modulation of the amygdala within the limbic circuit is vital for functional threat processing and emotional learning (figure 1.3).

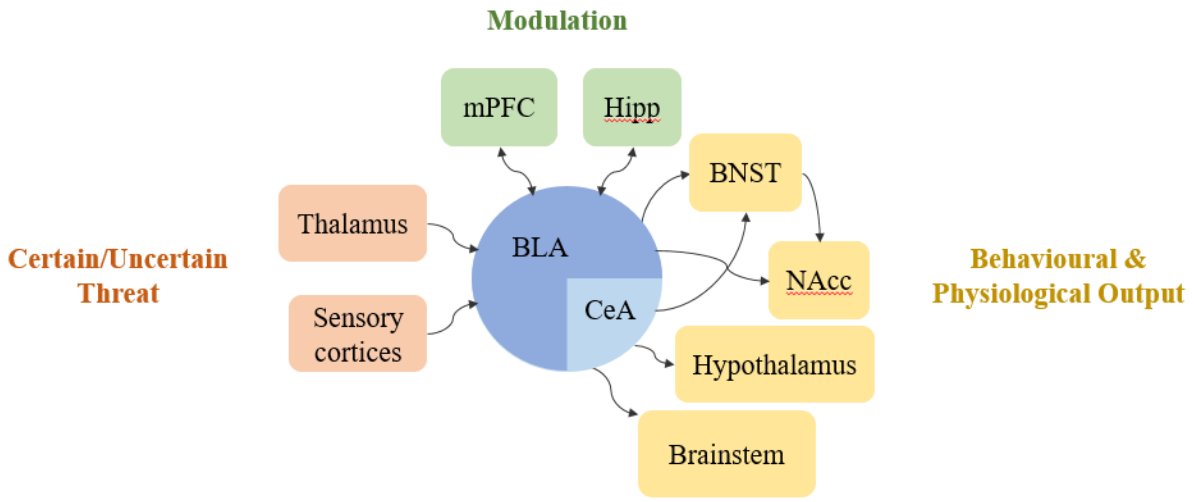


Figure 1.3: Core threat circuit underlying anxiety and fear responses. Schematic of the core threat circuit with the amygdala at the centre. Information of the perception of certain and uncertain threat flows from the sensory cortices and thalamus to the BLA (basolateral amygdala). Subsequently, mPFC (medial prefrontal cortex) and Hipp (hippocampus) modulate amygdala microcircuits of threat information. Finally, projections from the BLA and CeA (central nucleus of the amygdala) to downstream regions such as the BNST (bed nucleus of the stria terminalis), NAcc (nucleus accumbens), hypothalamus, and areas of the brainstem mediate various behavioural and physiological outputs of threat processing.

1.3 Disorders of Anxiety and Fear

Anxiety and fear are adaptive but when normal functioning within this circuit is altered, normal threat processing is disrupted and the individual suffers from pathological fear and anxiety. The International Classification of Diseases (ICD-11) and the Diagnostic and Statistical Manual (DSM-5) diagnostic systems distinguishes types of anxiety disorders, such as generalised anxiety disorder (GAD), panic disorder (PD), specific phobia, and social anxiety disorder (SAD) (World Health Organisation, 2011; American Psychiatric Association., 2013). It's worthwhile to note that posttraumatic stress disorder (PTSD) and obsessive compulsive disorder (OCD) were categorised out of anxiety disorders in both the ICD-10 and DSM-5. This follows the re-conceptualization of PTSD as a "trauma and stressor-related disorder" (DSM-5) and "reaction to severe stress and adjustment disorder" (ICD-10), and OCD into its own category (ICD-10 and DSM-5). Dysregulated anxiety and/or fear form the core symptomology of anxiety disorders.

GAD is highly comorbid with major depressive disorder, the most prevalent mood disorder, indicating a link between chronic anxiety and severe disruptions to mood (Hirschfeld, 2001; Conway *et al.*, 2006). Among individuals with both mood disorders and anxiety disorders, most developed anxiety disorders at an earlier age (Regier *et al.*, 1998; Kessler *et al.*, 2007). Thus, high anxiety early in life may predispose individuals to greater vulnerability to mood disorders.

According to a large population-based survey, approximately one in eight individuals (12.9%, 95%CI: 11.3%-14.7%) will suffer from an anxiety disorder over their lifetime worldwide (Steel *et al.*, 2014). Moreover, among the common mental disorders assessed, anxiety disorders were more prevalent than mood disorders and substance use disorders (Steel *et al.*, 2014).

Anxiety disorders are not only prevalent but also chronic and debilitating, leading to substantial impairment of workplace performance to the individual and imposes a major economic burden on society (Greenberg *et al.*, 1999; Ahola *et al.*, 2011; Wedegaertner *et al.*, 2013). Anxiety disorders were estimated to have cost the European Union in excess of 41 billion Euros (Andlin-Sobocki and Wittchen, 2005). The total cost of anxiety disorders on society (including service costs and lost employment cost) in the UK was approximately £8.9 billion in 2007 and expected to grow to £14.2 billion in 2026 (McCrone 2008). Anxious individuals are also at risk of developing coronary heart disease (Roest *et al.*, 2010). Patients with anxiety disorders were more likely to present with suicidal ideation and more likely to attempt suicide (Khan *et al.*, 2002; Sareen *et al.*, 2005; Nepon *et al.*, 2010). Clearly, anxiety disorders pose a significant public health problem and research into more effective treatment interventions should be a greater priority.

1.3.1 Neural mechanisms of dysregulated threat processing

Based on the amygdala's central role in the processing of threat stimuli and the finding that patients with anxiety disorders show hyperreactivity to threat in the form of excessive anxiety and fear, we would expect the amygdala to display hyperactivity in individuals with anxiety disorders. Indeed, meta-analysis of functional imaging studies observed amygdala hyperactivity in SAD, specific phobia and PTSD patients when processing emotionally significant stimuli, similar to amygdala activation in healthy subjects during fear conditioning (Etkin and Wager, 2007). Consistent with this, symptom provocation studies report hyperactivity in the amygdala in patients of other anxiety disorders such as PD (van den Heuvel *et al.*, 2005; Pfleiderer *et al.*, 2007) and GAD (McClure *et al.*, 2007).

Although symptoms of different anxiety disorders are heterogeneous, amygdala hyperactivity may reflect the exaggerated engagement of anxiety and fear circuitry resulting in dysregulated threat processing common to different anxiety disorder patients (Shin and Liberzon, 2010). Similarly, major depressive disorder patients also show hyperactivity in the amygdala when processing threat-related stimuli, suggesting that hyperreactivity of the amygdala to threat may be the common underlying mechanism mediating different pathological threat-related emotional processes (Peluso *et al.*, 2009; Yang *et al.*, 2010).

Etkin & Wager (2007) also found hyperactivity in the insula of anxiety disorder patients during emotional processing. The insular cortex is heavily interconnected with other regions of the threat circuit, the amygdala, the hypothalamus, and the periaqueductal grey (a region of the brainstem) (Paxinos and Mai, 2004). The insula has been implicated in general emotion processing and regulating autonomic function (Oppenheimer *et al.*, 1992; Phan *et al.*, 2002; Barrett and Wager, 2006; Lamm, Decety and Singer, 2011). The insula has also been implicated in conscious mental representations with damage in the mid-insula associated with anosognosia for hemiplegia, where the patients are unaware of their own motor deficits (Karnath, Baier and Nägele, 2005; Spinazzola *et al.*, 2008). (Bud) Craig (2009) has proposed that the insula may play a central role in awareness and the mental representation of emotions although the latter has been proposed to occur in anterior cingulate cortex in the theories of Damasio *et al.* (2000). Furthermore, the insula has also been implicated in the interoception of ascending homeostatic feedback from the body, such as processing sensory information of stress-induced increases in cardiorespiratory activity and other visceral sensations (Craig, 2014; Strigo and Craig, 2016). Taken together, increased insula activation may reflect more intense mental representations of threat, greater sensitivity to the body's physiological response to stress, or at the very least, more intense bodily responses among anxiety disorder patients.

Beyond the amygdala and insula, the prefrontal cortex has also been heavily implicated in the literature of anxiety disorders, although there exist substantial heterogeneity in the areas reported between studies (Shin and Liberzon, 2010). For example, when reading criticisms about themselves, SAD patients showed greater activation of the dmPFC (Blair *et al.*, 2008). Whereas, neuroimaging studies with specific phobia patients have implicated upregulation of the anterior cingulate cortex, ACC (Straube *et al.*, 2006; Straube, Mentzel and Miltner, 2006; Goossens *et al.*, 2007), hyperactivity in the medial OFC (Schienle *et al.*, 2007) and hypoactivity in the mPFC (Hermann *et al.*, 2007) when the patients were presented with phobia-relevant stimuli. Youths with GAD showed greater activation in the ventral prefrontal cortex and the ACC while attending to personal fear-provoking stimuli (McClure *et al.*, 2007). Variable involvement of prefrontal cortical regions in symptom provocation studies of different anxiety disorders have revealed differential prefrontal activation patterns corresponding to disorder-relevant information processing.

In summary, functional neuroimaging studies with anxiety disorder patients widely implicate the amygdala and insula hyperactivity, providing evidence that these structures may underlie the increased threat reactivity and negative affect shared across different anxiety disorders. In contrast, variable involvement of other cortical structures reported across the different individual subtypes of anxiety disorders likely represents the distinct top-down factors underlying dysregulated threat processing and cognitive mechanisms leading to the heterogeneity in symptomatology observed across different anxiety disorders.

1.3.2 Trait Anxiety, stress and cognitive biases

To understand the development of pathological emotion regulation, it is important to understand the altered neural mechanisms underlying trait anxiety. This is because high trait anxiety is a key risk factor for the development of anxiety disorders and depression (Mundy *et al.*, 2015; Jeronimus *et al.*, 2016)

In the discussion of anxiety, it is important to recognise the distinction between trait and state anxiety. Trait anxiety refers to an individual's enduring disposition for feelings of anxiety, and prevails along time and across different contexts. In contrast, state anxiety refers to an individual's transitory emotional state of feeling anxious in the presence of a stress-provoking stimulus. That state may differ when exposed to the same stressor over time.

Stress plays a critical role in mediating lasting effects on the brain and behaviour across an individual's development (Lupien *et al.*, 2009). The in utero environment has shown to have a significant effect on trait anxiety as evidence by stress exposure during the prenatal period in both humans and rodents leading to programming effects culminating in increased stress reactivity later in life (Barker, 1991; Koehl *et al.*, 1999; Weaver *et al.*, 2004; Seckl, 2007; Kapoor, Petropoulos and Matthews, 2008). Childhood and adolescents are also a critical period for the development of emotion regulation as evidenced by the association between compromised parental care and altered glucocorticoid secretion and risk of depressive symptomatology (De Bellis *et al.*, 1999; Heim *et al.*, 2000; Lupien *et al.*, 2000; Halligan *et al.*, 2007; Gunnar *et al.*, 2009; McGowan *et al.*, 2009). In contrast, the effects of stress exposure on neuronal anatomy in adulthood have been shown to be reversible in rodents after cessation of the stressor (Conrad *et al.*, 1999; Shansky *et al.*, 2009). In human adults, chronic exposure to increased glucocorticoids were associated with depression while decreased levels were associated with PTSD (Heim *et al.*, 2000; Yehuda, Golier and Kaufman, 2005). As stress has been shown to drive epigenetic change in the HPA axis and stress-responsive brain regions, stress-driven epigenetic change across different developmental periods may interact with genetic factors to contribute to vulnerability to dysfunctional emotional regulation (Hunter and McEwen, 2013; Bartlett, Singh and Hunter, 2017).

The predominant etiological model accounting for the relationship between trait anxiety, anxiety disorders and depression are that early life experiences, epigenetic and genetic factors lead to the formation of the high trait anxiety phenotype which subsequently, in response to stressful life events can lead to the development of pathological emotion regulation in the form of anxiety disorder and depression (figure 1.4) (Sandi and Richter-Levin, 2009). Understanding the neural mechanism underlying the high trait anxious phenotype will therefore enable us to develop better interventions preventing the manifestation of disorders of emotion dysregulation.

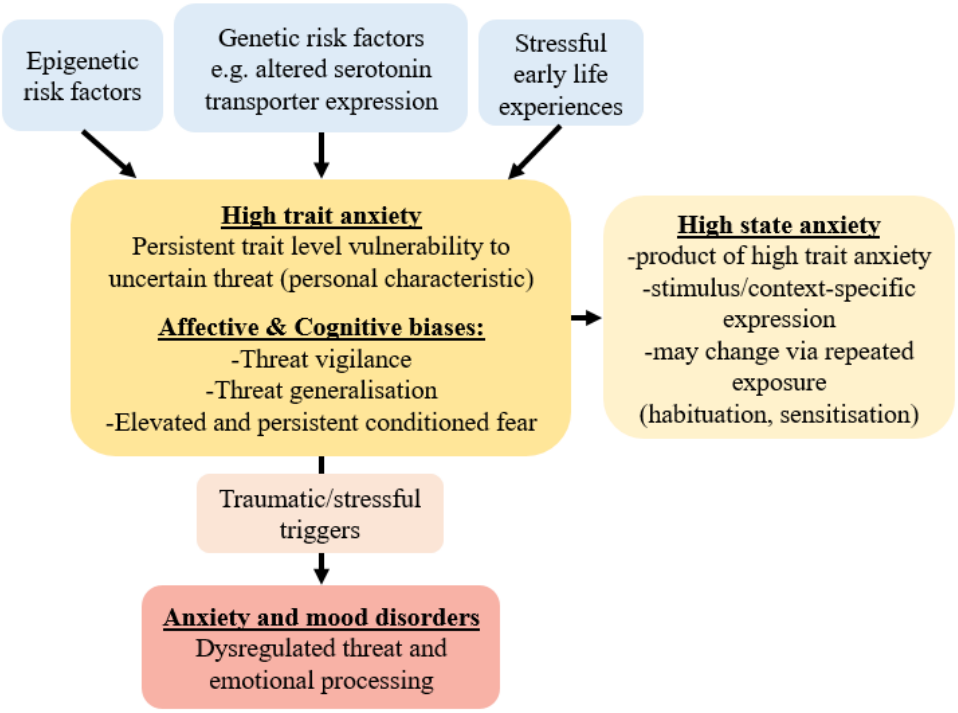


Figure 1.4: Trait anxiety: from early life factors to pathology. Genetic and environmental risk factors define the high trait anxiety phenotype, and subsequent traumatic events and chronic stress act on the vulnerability conferred to induce anxiety disorders and depression. High trait anxiety is characterised by an increased attentional bias to threat (threat vigilance), increased threat interpretation of emotionally ambiguous stimuli (threat generalization), altered conditioned fear response, and manifests as high state anxiety in anxiety-provoking context.

High trait anxious individuals are associated with altered neurocognitive threat processes: increased attentional bias towards threat (threat vigilance) and increased threat interpretation of emotionally ambiguous stimuli (threat generalization). Meta-analysis of studies with high trait anxious individuals and individuals with anxiety disorder report a reliable bias towards threat-related stimuli in different experimental paradigms, and even when the stimuli are beyond conscious perception (Bar-Haim *et al.*, 2007). This attentional bias has been proposed to be a result of bottom-up threat evaluation mechanisms biasing attentional competition in favour of threat-related stimuli (Mathews and Mackintosh, 1998). Upon longer stimuli presentation duration, high trait anxious individuals show attentional avoidance of the threat-related stimuli, indicating both threat vigilance and avoidance as components of the high trait anxious phenotype (Mogg, Bradley, *et al.*, 2004; Koster *et al.*, 2005a, 2006). Moreover, Bishop reported that high trait anxious individuals had impoverished recruitment of dorsolateral prefrontal cortex (dlPFC) for attentional control to inhibit distractions even in the absence of threat-related stimuli, suggesting that high trait anxious individuals may exhibit general attentional deficits as well (Bishop, 2009).

When presented with emotionally ambiguous stimuli, high trait anxious individuals are biased to making negative interpretations (Hirsch and Mathews, 1997; Richards *et al.*, 2002). High trait anxious individuals also present a negative interpretive bias towards predictions made about risk and chance, and about future life events (Eysenck and Derakshan, 1997; Stöber, 1997). As trait anxious individuals present with negative interpretive biases in response to emotional neutral stimuli (threat generalization) and negative prediction biases when estimating uncertain future events, the culmination of both may explain the high worrying symptomatology in anxiety disorder patients (Brown, Antony and Barlow, 1992).

High trait anxious individuals also show altered prefrontal-amygdala functioning in the context of conditioned fear. More specifically, high trait anxious individuals showed impoverished recruitment of ventral prefrontal cortex (vPFC) and amygdala hyper-reactivity to conditioned fear (Indovina *et al.*, 2011; Sehlmeyer *et al.*, 2011). Behaviourally, high trait anxious individuals also show deficits in safety learning, with delayed and incomplete extinction of conditioned fear, as measured by self-reported distress and startle responses (Gazendam, Kamphuis and Kindt, 2013). Thus, the impaired regulation of conditioned fear responses has been implicated as a key component of the high trait anxiety phenotype.

The key features of trait anxiety may lead to significant alterations in the way threat is processed and regulated. Labelling non-threatening stimuli as threatening when threat generalizing, may lead to the prolonged activation of threat circuitry and lead to high trait individuals pessimistically predicting uncertain outcomes and perceiving neutral conditions as threat-laden. Similarly, as high trait individuals are more vigilant towards potentially threatening stimuli, the attentional bias towards threatening stimuli may lead to overactivation of anxious and fear responses and the inability to effectively manage emotional distractors. Threat generalization and threat vigilance together may lead to high trait individuals experiencing chronic stress in non-threatening or mildly threatening environments and the acquisition of inappropriate conditioned fear responses. In turn, the disposition for exaggerated conditioned fear responses leads to hyperarousal and distress when individuals with high trait anxiety are threatened and may lead to maladaptive fear coping behaviours. Thus, these cognitive biases may explain why high trait anxious individuals are more vulnerable to the development of anxiety disorders compared low trait anxious individuals.

1.3.3 Current treatments for anxiety disorders

Individuals suffering from anxiety disorders are treated with medication, psychotherapy or both. Both psychotherapies and medications have led to greater clinical improvement than psychological placebos and placebo pills respectively (Bandelow *et al.*, 2015), and have been shown to effectively alleviate high trait anxious cognitive biases (Mogg *et al.*, 1995; Mogg, Baldwin, *et al.*, 2004).

Psychotherapies such as cognitive-behavioural therapy (CBT), mindfulness therapy and applied relaxation have the benefit of not having side effects and potential drug-drug interactions compared to pharmacological interventions, but has been shown to be less efficacious than medication for the treatment of GAD, PD, and SAD in a meta-analysis by Bandelow *et al.* (2015). CBT is often recommended as the first line psychological intervention for a wide range of psychological disorders including anxiety disorders, particularly in youths (Benjamin *et al.*, 2011). The combination of CBT and medication showed a marked increase from CBT/other psychotherapies alone, but do not show a substantial increase in effect size beyond medication alone (Bandelow *et al.*, 2015). The specific treatment components of CBT vary depending on the specific anxiety disorder but include a combination of problem-focused interventions to decrease maladaptive and increase adaptive coping behaviours, and modify maladaptive cognitions and beliefs (Craske, 2010).

First-line drug treatment for these disorders is currently a selective serotonin reuptake inhibitor (SSRI) (Bandelow *et al.*, 2008; National Institute for Health and Clinical Excellence, 2011; Baldwin *et al.*, 2014). SSRIs have "broad spectrum efficacy" and have been shown to be more effective than placebos in the treatment across different anxiety disorders (Bandelow *et al.*, 2008). If SSRIs or other members of the drug class are ineffective, alternate medication such as serotonin–norepinephrine reuptake inhibitors (SNRIs), pregabalin (α2δ subunit-containing calcium channel modulator), or monoamine oxidase inhibitors may be prescribed dependent on symptomatology and contraindications. SSRIs (and SNRIs) are associated with a delay in onset of clinical effect, and a transient increase in anxiety and risk of suicidal thinking during the first few weeks of treatment (Teicher, Glod and Cole, 1990; Sinclair *et al.*, 2009). Other side effects of SSRI such as nausea, weight gain, and sexual dysfunction may jeopardise compliance and contribute to discontinuation (Bandelow *et al.*, 2008). SSRI's putative mechanism of action is to inhibit serotonin transporters, leading to reduced reuptake of extracellular serotonin and consequently upregulating local serotonin signalling. SSRI's efficacy in treating anxiety disorders and as an antidepressant suggest that serotonin plays a significant role in modulating negative mood.

Alternatively, benzodiazepines may be prescribed for the rapid relief of anxiety without the worsening of symptoms observed with serotonin-targeting medication. Benzodiazepines enhances the effect of

GABA, the primary inhibitory neurotransmitter of the brain, by acting as a positive allosteric modulator at $GABA_A$ receptors (Brady *et al.*, 2006). Benzodiazepine has been proposed to exhibit anxiolytic properties due to its effect on the amygdala inhibitory network (Gauthier and Nuss, 2015; Babaev, Piletti Chatain and Krueger-Burg, 2018). However, enthusiasm for targeting the GABA-nergic system in the development of new treatment options have been tempered by benzodiazepine's strong sedative effects due to central nervous system depression, and its strong risk for dependence in patients. As SSRIs remain the safest and most effective option for long term treatment, future drug interventions may benefit from the short-term use of benzodiazepines combined with more long-term use of a drug targeting the serotonin system.

1.4 Serotonin

Serotonin (5-HT, 5-Hydroxytryptamine) is a monoamine neurotransmitter that is evolutionarily conserved, being present in all bilateral animals, and even found in invertebrates (Jonz *et al.*, 2001). Serotonin has been shown to be involved in regulating a wide range of physiological functions, including emotion, feeding, social behaviour, sexual behaviour, and the sleep/wake cycle (Lucki, 1998; Portas, Bjorvatn and Ursin, 2000; Hull, Muschamp and Sato, 2004; Kiser *et al.*, 2012; Voigt and Fink, 2015). Serotonergic neurons primarily from the dorsal raphe nucleus, but also the median raphe nucleus, project to regions across the corticolimbic circuitry and the serotonin released is responsible for the modulatory effects of serotonin on threat processing (Jacobs & Azmitia 1992).

Notably, dorsal raphe serotonergic neurons were reported by Ren *et al*. (2018) to be anatomically defined with segregated locations dependent on projection regions in rodents. Frontal-cortex projecting serotonin neurons were activated by reward and inhibited by punishment, and has been reported to promote active coping behaviours in stressful situations (forced-swim test). In contrast, amygdala-projecting serotonin neurons were activated by both reward and punishment and promoted anxious behaviour as measured by the open field test and elevated plus maze. These findings emphasise that serotonergic neurons have different projection patterns whose activation may differentially modulate anxious and coping behaviour.

Serotonin receptors are grouped into 7 families and 14 total known receptors consisting of primarily G protein-coupled receptors (GPCRs) except for 5-HT_3 receptors, which are ligand-gated ion channels (Nestler & Hyman 2009). The serotonin 1A (5-HT_{1A}), 2A (5-HT_{2A}), and 2C (5-HT_{2C}) receptors have been particularly implicated in the regulation of anxious and fear related behaviour.

The 5-HT_{1A} receptor is expressed as both an autoreceptor and post-synaptic receptor throughout the brain (Barnes and Sharp, 1999; Riad *et al.*, 2000). In the context of anxiety disorders, reduced 5-HT_{1A} have been found in patients suffering from panic disorder and social anxiety disorder (Neumeister *et al.*, 2004; Lanzenberger *et al.*, 2007; Nash *et al.*, 2008). 5-HT_{1A} receptors in the dorsal and median raphe nuclei are somatodendritic autoreceptors that act to downregulate activation of serotonergic neurons and thus regulate serotonergic signalling in projection areas (Hjorth and Sharp, 1991). Lower 5-HT_{1A} availability in the dorsal raphe nuclei is associated with greater threat-related amygdala reactivity, suggesting that weaker auto-inhibition of dorsal raphe serotonergic neurons may underlie greater sensitivity to threat (Fisher *et al.*, 2006). 5-HT_{1A} receptors are also localized in frontal and limbic regions innervated by serotonergic neurons as postsynaptic receptors on pyramidal and GABAergic interneurons (Santana *et al.*, 2004; Palchaudhuri and Flügge, 2005). In rodents, preferential activation of 5-HT_{1A} autoreceptors reduced conditioned fear learning but preferential activation of 5-HT_{1A}

heteroreceptors did not, emphasising potentially different roles of 5-HT_{1A} receptors dependent on localization (Zhao *et al.*, 2018). As activation of 5-HT_{1A} autoreceptors at the raphe nuclei inhibits serotonergic projections and thus downregulates serotonergic signalling across the brain, 5-HT_{1A} autoreceptors may play a more significant role compared to 5-HT_{1A} heteroreceptors in the modulation of the threat circuitry.

In contrast to the inhibitory 5-HT_{1A} receptor, 5-HT_{2A} and 5-HT_{2C} receptors are excitatory GPCRs. Although 5-HT_{2A} and 5-HT_{2C} receptors share strong similarities in their primary sequences and have overlapping expression in the layer V of the cortex, both receptors generally exhibit differing localization across other parts of the brain on both pyramidal and GABAergic interneurons (Bombardi, 2014; Shukla, Watakabe and Yamamori, 2014). 5-HT_{2A} receptor signalling has been linked to increased anxiety in humans, with neuroticism (measure of trait anxiety) positively associated with frontolimbic 5-HT_{2A} receptor binding (Frokjaer *et al.*, 2008). Consistent with this, 5-HT_{2A} receptor knockout mice displayed low anxiety and was normalized after cortical restoration of 5-HT_{2A} receptors (Weisstaub *et al.*, 2006). Similarly, transgenic mice with disruption to 5-HT_{2C} receptor expression exhibited a low anxious phenotype while mice overexpressing 5-HT_{2C} receptors display increased anxious behaviour (Kimura *et al.*, 2009). Taken together, these findings implicate cortical 5-HT_{2A} receptors and general 5-HT_{2C} receptors in the upregulation of anxious behaviour.

The serotonin transporter reuptakes extracellular serotonin into the presynaptic terminals where the neurotransmitter may be re-released or degraded and thus plays a key role in regulating serotonin availability and signalling (Hirano *et al.*, 2005). Notably, the variable number tandem repeat (VNTR) polymorphism occurring in the promoter region of the serotonin transporter gene on chromosome 17 in humans (5-HTTLPR) has been found to affect transcription of the serotonin transporter gene. Lesch *et al.* (1996) first reported that individuals with the short form of the 5-HTTLPR had higher scores of neuroticism, a measure of trait anxiety on the NEO Personality Inventory. The short allele of the 5-HTTLPR is also associated with reduced serotonin transporter expression and greater amygdala activity in response to threat-related stimuli (Lesch *et al.*, 1996; Little *et al.*, 1998; Mortensen *et al.*, 1999; Hariri *et al.*, 2002). Subsequently, Caspi *et al.* (2003) reported that individuals with the short allele of the 5-HTTLPR exhibited more depressive symptoms and be suicidal in relation to the occurrence of stressful life events and childhood maltreatment, implicating the 5-HTTLPR in the gene-environmental interaction leading to the development of psychopathology. In rhesus macaques, individuals with an analogous "short" allele polymorphism and reared with peers (instead of parents) have high levels of anxiety-related responses during separation, indicating that the 5-HTTLPR may interact with early life experience to affect trait anxiety (Barr *et al.*, 2004; Spinelli *et al.*, 2007). Following these key findings, large numbers of studies have investigated the relationship between the 5-HTTLPR and affective pathology. The following is a summary of meta-analyses of studies conducted on the 5-HTTLPR and different aspects of high anxious pathology (table 1.1):

Study	Effect	Subject
Pergamin-Hight *et al*. 2012	Significant	Attentional bias to negative stimuli
Munafò *et al.* 2008	Significant	Amygdala activation
Murphy *et al*. 2013	Significant	Amygdala activation
Miller *et al.* 2013	Significant	Cortisol stress reactivity
Schinka *et al*. 2004	Significant	Neuroticism
Sen *et al*. 2004	Significant	Neuroticism
Willis-Owen *et al*. 2005	Not Significant	Neuroticism and major depression phenotype
Munafò *et al.* 2009a	Not Significant	Neuroticism and harm avoidance
Gressier *et al*. 2013	Significant	Trauma exposure and risk of PTSD
Munafò *et al.* 2009b	Not Significant	Stress and risk of depression
Risch *et al.* 2009	Not Significant	Stressful life events and risk of depression
Uher & McGuffin 2010	Significant	Environmental adversity and risk of depression
Karg *et al*. 2011	Significant	Stress and risk of depression
Sharpley *et al.* 2014	Significant	Stress and risk of depression
Culverhouse *et al.* 2017	Not Significant	Stress and risk of depression
Bleys *et al.* 2018	Significant	Stress and risk of depression
Lasky-Su *et al*. 2005	Not Significant	Major depressive disorder or bipolar disorder
Lotrich & Pollock 2004	Significant	Major depressive disorder
Kiyohara & Yoshimasu 2010	Mixed	Major depressive disorder in Caucasians but not Asians
Clarke *et al.* 2010	Significant	Major depressive disorder
Blaya *et al.* 2007	Not Significant	Panic disorder
Zhao *et al.* 2017	Significant	Stress and risk of PTSD
Navarro-Mateu *et al.* 2013	Not Significant	PTSD
Lin 2007	Significant	OCD
Walitza *et al.* 2014	Significant	Early-onset OCD
Mak *et al.* 2015	Not significant	OCD
Li & He 2007	Significant	Suicidal behaviour
Fanelli & Serretti 2019	Significant	Risk of violent suicide attempt
Smits *et al*. 2004	Significant	SSRI efficacy in depressed patients
Serretti *et al*. 2007	Significant	SSRI efficacy in depressed patients
Taylor *et al.* 2010	Not Significant	SSRI and other antidepressant response in depressed patients
Porcelli *et al.* 2012	Mixed	SSRI efficacy and remission rate in Caucasians but not Asians

Table 1.1: Meta-analyses of studies involving the relationship between 5-HTTLPR and subjects relating to high anxious pathology.

Overall, the studies generally report a small effect of the serotonin transporter polymorphism on characteristics ranging from negative attentional bias and other high trait anxious phenotypes to stress mediated risk of depression and SSRI efficacy. However, conflicting findings among some meta-analyses has resulted in the 5-HTTLPR polymorphism remaining a contentious topic in affective pathology. Differences in findings likely reflect differences in the choice of included studies. Difference in the analytical method used may also affect results from the meta-analysis, for example Munafò *et al.* (2009b) found no significant effect with summary statistics, which is more conservative, while Karg *et al*. (2011) used a broader Z-score method to collate findings and found a significant effect (Taylor and Munafò, 2016). Differences in findings particularly among different ethnic groups may also be due to

the mediation of different environments and cultural attitudes towards specific coping behaviours. Thus, the serotonin transporter polymorphism is likely one among many other genetic and environmental factors with a moderate-to-small effect size underlying different neural mechanisms contributing to the high trait anxious phenotype and stress reactivity.

1.4.1 Serotonin manipulation and threat processing

Associations between serotonergic components and anxiety have advanced our understanding of how the serotonin system underlies activation of the threat circuit. Following from that, manipulations of the serotonin system within both humans and animal models have provided further insight into how alterations to the serotonin system may affect anxious behaviour.

Acute Tryptophan depletion (ATD)

To manipulate brain serotonin levels, acute tryptophan depletions (ATD) have been widely implemented in humans. ATD lowers serotonin synthesis rates by diminishing the availability of the precursor of serotonin, tryptophan via a combination of a low-protein diet with a tryptophan-deficient protein load of competing amino acids for brain uptake. ATD has been shown to reduce brain serotonin content and function in animals (Moja *et al.*, 1989; Young *et al.*, 1989), and reduce CSF tryptophan and serotonin metabolite (5-HIAA) in humans (Carpenter et al. 1998; Williams et al. 1999). ATD depletions in healthy volunteers interacts with individual threat sensitivity to increase amygdala reactivity (Cools *et al.*, 2005) and altered PFC-amygdala functional connectivity when responding to threat-related stimuli (Passamonti *et al.*, 2012). ATD also increases CRF, a key part of the body's early stress response, measured in cerebrospinal fluid (Tyrka *et al.*, 2004). Taken together, ATD studies in healthy volunteers indicate that lowered brain serotonin signalling increases vulnerability to anxiety. Robinson *et al.* demonstrated that ATD increased anxiety-potentiated startle but not fear-potentiated startle (2012). As serotonin inhibits BNST activation via 5-HT_{1A} receptors (Levita *et al.*, 2004), Robinson *et al.* (2012) proposes that ATD-mediated depletion of serotonin leads to increases in anxiety via the disinhibition of the anxiety-linked BNST, but does not interfere with BNST-independent fear mechanisms.

In a clinical cohort of recovered patients with PD and SAD anxiety disorders treated by SSRI or CBT, ATD increased both physiological (blood pressure) and psychological (self-reported anxiety levels) indices of anxiety in response to a stress challenge (Davies *et al.*, 2006). In particular, ATD induced a panic attack in remitted patients with PD when used in combination with a panicogenic challenge (flumazenil) (Bell *et al.*, 2002). Thus, ATD studies inducing relapse in treated patients of anxiety disorders suggest that remittance may be mediated by increased serotonin signalling.

Notably, ATD have shown mixed behavioural changes in rodents, with most studies showing no behavioural change (Lieben *et al.*, 2004; Uchida *et al.*, 2007; van Donkelaar *et al.*, 2009), while some have showed success with repeated ATD (Blokland, Lieben and Deutz, 2002) and an effect only in

specific strains (Jans and Blokland, 2008), suggesting the need for further refinement of ATD methodology in rodents.

Blockade of serotonin reuptake via SSRIs

Consistent with its effects in anxiety disorder and depressed patients, acute SSRIs have been shown to increase both anxiety and fear-potentiated startle in healthy participants (Grillon, Levenson and Pine, 2007). In rats, acute SSRIs similarly lead to high anxiety-like behaviour as measured by the light/dark test, where anxious rodents prefer dark compartments compared to exploring illuminated areas. (Arrant *et al.*, 2013). Acute SSRI administration in healthy men was also reported to result in increases in amygdala reactivity to general salient stimuli (angry, fearful, surprised and neutral faces) (Bigos *et al.*, 2008). Subsequently, Murphy *et al.* (2009) reported that an acute dose of an SSRI reduced amygdala response to fearful faces. As Bigos *et al*.'s (2008) study included only men, a small sample (N = 8), and reported an effect to a mixed series of facial expressions, it is difficult to compare it with the results obtained by Murphy *et al*.'s (2009) more rigorously conducted study (counterbalanced number of both genders, N = 26, only fearful faces). Thus, studies on the acute effects of SSRIs on threat-related amygdala reactivity warrant further investigation and replication. However, following sub-chronic administration (7 days), healthy participants not only showed reduction in amygdala reactivity, but also showed reduced mPFC and hippocampal reactivity to masked threat cues, suggesting that the treatment efficacy of longer term chronic SSRI may be mediated by an overall reduction in threat circuit reactivity and unconscious threat processing (Harmer *et al.*, 2006).

The increased endogenous serotonin from acute SSRIs may paradoxically lead to overall inactivation of serotonergic projections in the forebrain via stimulation of homeostatic raphe nuclei 5-HT_{1A} autoreceptors, which ultimately results in the enhanced anxiety and fear observed. After chronic administration, 5-HT_{1A} autoreceptors are desensitised and serotonergic release is restored, consequently SSRIs effectively upregulates serotonin signalling across the brain by reducing reuptake of extracellular serotonin (Blier *et al.*, 1998). 5-HT_{1A} autoreceptors desensitisation has been proposed to be a product of decreased levels of G_i proteins (Li, Muma and van de Kar, 1996). Consistent with this theory, co-administration of pindolol (5-HT_{1A}/β-adrenoceptor antagonist), accelerated the therapeutic effects of an SSRI and induced rapid improvement in treatment-resistant patients (Artigas *et al.*, 1996).

Bypassing systemic SSRI's potential effect on the dorsal raphe with intra-BLA SSRI infusions, induced reductions in anxiety as measured by reduced conditioned freezing to a context associated with footshock (Inoue *et al.*, 2004). In contrast, intra-BNST infusions of SSRI prior to fear conditioning enhanced fear memory and conditioned freezing (Ravinder *et al.*, 2013). Taken together, these findings provide support for the theory that systemic SSRI's acute effect is mediated by dorsal raphe serotonin action, and that serotonin has differential action in specific forebrain serotonergic projection regions.

Serotonin receptor activation and blockade via agonists and antagonists

Infusions of pharmacological manipulations within regions of the threat circuit allow us to further probe the role of specific serotonergic components in threat processing.

5-HT$_{1A}$. Systemic administration of a 5-HT$_{1A}$ receptor agonist exhibit anxiolytic and antidepressant-like properties in rodents (Nunes-de-Souza *et al.*, 2000; Jastrzębska-Więsek *et al.*, 2018). Similarly, 5-HT$_{1A}$ receptor agonist infusions in the BLA reduced anxious behaviour on the elevated T-maze (Zangrossi, Viana and Graeff, 1999) but increased anxious behaviour in the social investigation test (Gonzalez, Andrews and File, 1996). It should be noted that in Gonzalez *et al.*'s (1996) study, a substantially lower dose of 5-HT$_{1A}$ receptor agonist (8-OH-DPAT) was used (appx. 0.15–0.6 nmol) compared to that in Zangrossi *et al.* (1999) (8–16 nmol). Taken together, these results suggest that 5-HT$_{1A}$ receptor activation reduces anxiety but may have differing effects in the BLA either as a function of dose or behavioural paradigm (non-social vs. social).

5-HT$_{2A}$. Systemic administration of a 5-HT$_2$ agonist (2,5-Dimethoxy-4-iodoamphetamine, DOI) induced a reduction in fear-driven behaviour (passive avoidance) on the four-plates test (Nic Dhonnchadha *et al.*, 2003; Ripoll, Hascoët and Bourin, 2006). In the four-plates test, fearful animals avoid crossing the "plates" of the test area after they have experienced a foot shock doing so. Similarly, DOI induced an anxiolytic effect on the elevated plus maze. DOI's reduction of fear behaviour and anxiolysis was attenuated by a 5-HT$_{2A}$ antagonist but not a 5-HT$_{2B}$ antagonist or 5-HT$_{2C}$ antagonist (Nic Dhonnchadha *et al.*, 2003; Ripoll, Hascoët and Bourin, 2006). These findings indicate that DOI's reduction of fear and anxious behaviour is mediated by 5-HT$_{2A}$ receptor activation, and that 5-HT$_{2A}$ receptor may play a general role in downregulating threat reactivity.

5-HT$_{2C}$. Systemic administration of a 5-HT$_{2C}$ agonist increased anxious behaviour on the elevated plus maze (Pockros-Burgess *et al.*, 2014). Consistent with this, intra-BLA administration of 5-HT$_{2C}$ receptor agonist or serotonin increased anxious behaviour on the elevated T-maze. In contrast, intra-BLA administration of 5-HT$_{2C}$ receptor antagonist had the opposing effect and blocked the anxiogenic effect caused by intra-BLA serotonin and systemic administration of SSRIs (Vicente and Zangrossi, 2012). Intra-BLA 5-HT$_{2C}$ receptor antagonists also attenuated mixed 5-HT$_2$ agonist anxiogenesis on the open field test (Campbell and Merchant, 2003). Similarly, intra-BLA 5-HT$_{2C}$ receptor antagonists prevented uncontrollable stress-potentiated anxiety measured on a variant of the social investigation test (Christianson *et al.*, 2010). 5-HT$_{2C}$ receptors have been posited to produce anxiogenic effects by enhancing NMDA receptor function in the BLA (Jiang *et al.*, 2011). These findings indicate that activation of 5-HT$_{2C}$ receptors in the BLA lead to increased expression of anxious behaviour.

5-HT$_4$. Mendez-David *et al.* (2014) recently demonstrated that 5-HT$_4$ receptor agonists not only induced anxiolytic action on the elevated plus-maze, open field test, and novelty suppressed feeding test, but also induced antidepressant effects on depression-like measures of learned helplessness.

Notably, 5-HT_4 receptor agonist induced early onset of brain changes typically observed after chronic SSRI treatment: desensitisation of 5-HT_{1A} autoreceptors, increased tonic activation of hippocampal pyramidal neurons, and neurogenesis in the hippocampus (Lucas *et al.*, 2007). However, use of 5-HT_4 receptor agonists as a rapid action anxiolytic has been hampered by its disruption of normal gastrointestinal tract function (Tonini and Pace, 2006).

As a whole, serotonergic manipulations by changing overall serotonin signalling or altering activation of specific serotonin receptors have provided valuable insight into the specific serotonergic components relevant to the expression of anxiety. ATD-induced serotonergic depletion increased vulnerability to anxiety. Acute blockade of serotonin reuptake via SSRI administration increased behavioural measures of threat reactivity putatively due to its effect on 5-HT_{1A} autoreceptors but have shown potential to reducing threat reactivity when administered sub-chronically, implicating time-dependent neural plastic changes underlying SSRI treatment efficacy. For the serotonin receptors, 5-HT_{1A} receptor activation via agonists resulted in anxiolysis, potentially due to its effects on 5-HT_{1A} heteroreceptors. Furthermore, 5-HT_{2A} receptor activation reduced anxiety and fear, 5-HT_4 receptor activation was likewise anxiolytic, while 5-HT_{2C} receptor activation was linked to increased anxiety. Preclinical work in animals have provided valuable insight and more direct evidence of serotonin pathway's involvement in anxiety and has been a critical part of progress in the understanding of threat processing. However, as serotonin receptors are expressed on different neuronal subpopulation in different microcircuits, the effect of alteration in local serotonin levels and the role of specific receptor subtypes within regions of the threat circuit remains poorly explored.

1.5 Summary and aims

Substantial evidence in the literature implicates the serotonin system in the activity of the threat circuitry. Furthermore, alterations to this system have been associated with vulnerability to pathological fear and anxiety. Converging evidence suggest that a general increase in serotonergic tone, overall, plays a modulatory role to reduce vulnerability to anxiety. However, serotonin action across different regions of the threat circuit may play opposing roles dependent on the local serotonin receptor subtype activated (excitatory vs inhibitory) and the neuron type (e.g. principal neurons vs interneurons) involved. Although much progress has been made about serotonin's general involvement in regulating emotion, further understanding of the precise role of serotonin subcomponents within specific regions of the corticolimbic circuitry underlying anxious behaviour and trait anxiety remains to be elucidated. Progress in this area will enable us to improve treatments and clinical interventions for anxiety and mood disorders. To approach this question, the common marmoset makes an excellent model animal.

The common marmoset, *Callithrix Jacchus* is being increasingly used as a research model as the scientific community recognises the importance of nonhuman primates as a model for human disease. The common marmoset offers distinct advantages above both rodent models and other nonhuman primate models such as the rhesus macaque. As regions of the prefrontal cortex play a role in the modulation of the threat circuit, the high degree of similarity in cortical structures shared between the common marmoset and humans compared to rodents may aid in the translatability of this thesis's findings (figure 1.5). Compared to macaques, the relatively small size of marmosets makes them easier to handle and house in accommodations that meets their needs (Kishi *et al.*, 2014; Jennings *et al.*, 2016). In addition, their short gestation period (5 months) and relatively short period of post-natal development before they reach adulthood (2 years), makes them an ideal species for studying the aetiology of developmental disorders. Thus, marmosets provide an ideal balance of both sample size and behavioural complexity for research into the brain mechanisms of human cognition and emotion.

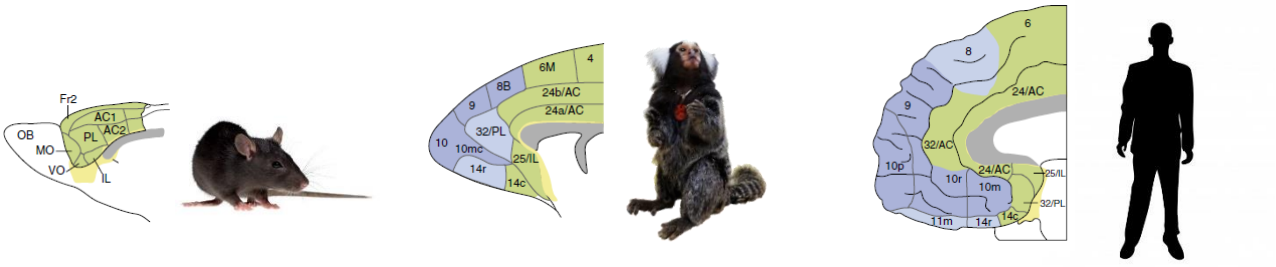


Figure 1.5: Cortical structure comparison. Sagittal view of the medial frontal cortical architecture revealed by histological staining in rodents, marmosets and humans (left-to-right). Brain figures by Kaiser & Feng (2015).

To study the role of serotonin in the regulation of threat processing from gene to behaviour using a multi-systems approach, this thesis is composed of 4 key studies:

i) Firstly, threat-related behaviour in the common marmoset was characterised. Anxious and fear-related behaviour in the common marmoset was statistically modelled on a population level. Furthermore, the relationship between anxious and fear-related behaviour was determined.

ii) After establishing a representation of anxiety and fear-driven behaviour in the marmoset in (i), potential alterations in serotonergic gene expression across different brain regions that may underlie individual differences in vulnerability to anxiety (trait anxiety) were determined. Moreover, the potential effect of the serotonin transporter polymorphism on regional gene expression was determined.

iii) To move from correlation to causation, region-specific serotonergic components corresponding to anxious behaviour in (ii) were targeted via pharmacological manipulations to attempt to alter the high trait anxious phenotype.

iv) Finally, beyond the (ii) genetic and (iii) neurochemical mechanism explored, potential anatomical changes in the developing and developed brain that corresponds to high anxiety later in life were studied.

Chapter 2: Anxiety and fear response in the common marmoset

Introduction: The common marmoset displays a diverse repertoire of behaviours in anxiety-provoking and fear-provoking contexts. The aim of the current study is to characterise the contribution of individual behavioural measures of the common marmoset towards underlying factors reflecting anxiety, as measured on the human intruder test and fear, as measured on the rubber snake test and to study the association between these related but distinct constructs.

Methods: An exploratory factor analysis (EFA) was conducted on behavioural data collected from animals screened for emotionality on the human intruder test (N=171) and rubber snake test (N=151) over an 8-year period. The consequent factors were tested for internal consistency via Cronbach's Alpha test. The correlations between the factors were investigated with animals tested on both tests (N=134).

Results: The human intruder test EFA revealed a factor with variable loadings reflecting avoidance behaviour (low time spent at the front, high time spent at the back, high average height), and vigilance (low locomotion and high head-bobbing and tse-egg calls). The rubber snake test EFA revealed a factor loaded with attention and mobbing calls (factor 1: high stare duration and stare count, and high tsik and tsik-egg calls) towards the snake, and an additional factor with variable loadings representing behavioural avoidance and passivity (factor 2: high distance from the snake, and low stare duration and locomotion). The factor in the human intruder test was negatively associated with engagement of the snake (factor 1) and positively associated with avoidance (factor 2) on the rubber snake test. When animals were grouped based on their rubber snake test factor scores, animals with a high factor 2 but low factor 1 score, had a higher human intruder test factor score.

Discussion: The factor driving vigilance and avoidance in the presence of the human intruder is interpreted as reflecting an animal's anxiety level. In the rubber snake test, factor 1 underlying attention towards and mobbing of the rubber snake reflects an animal's active fear response, whereas factor 2 underlying avoidance reflects an animal's avoidant fear response. The factors also revealed an association between fear responding style and anxiety implicating a link between a predominantly avoidant fear responding style and higher levels of anxiety, suggesting that an individual's trait disposition for high anxiety is associated with a predominantly avoidant relative to active coping strategy to fear. With the increasing popularity of the common marmoset for studies in fear and anxiety, our findings provide the basis for the use of unitary factor scores reflecting anxious and fearful behaviour for marmosets. findings here suggest that active coping should be an integral part of behavioural therapy to reduce vulnerability to anxiety.

2.1 Introduction

Anxiety and fear are key components of human emotion and are adaptive defensive responses to uncertain threat and certain, proximal threat respectively, but excessively high levels of threat responsivity serve as core symptoms of anxiety disorders and many mood disorders. Anxiety disorders adversely affect an estimated 1 out of 14 individuals across their lifetime globally (Baxter *et al.*, 2013). Fully characterising anxious and fearful behaviour in animal models advances our understanding of the behavioural expression of emotion regulation in the context of uncertain and certain threat. One such target animal model is the common marmoset.

The human intruder test and rubber snake test are commonly used in non-human primates to assess anxiety and fear behaviour respectively (Meunier *et al.*, 1999; Kalin *et al.*, 2001; Barros *et al.*, 2002; Kalin and Shelton, 2003; Izquierdo and Murray, 2004; Izquierdo, Suda and Murray, 2005). The human intruder test involves measuring the animal's behavioural response to an unfamiliar human, the 'human intruder' standing in front of the animal's home-cage and maintaining eye contact with the animal. Since animals bred in the laboratory have prior positive and negative experiences with human encounters, e.g. receiving food treats or being restrained for husbandry or experimental purposes, the unfamiliar 'human intruder' acts as an uncertain threat and creates an anxiety-provoking context. The rubber snake test can also be conducted in the home-cage and involves recording the animal's behavioural response to a rubber snake which acts as an inherent predatory stimulus, provoking an innate fear response (Barros *et al.*, 2002; Cross and Rogers, 2006).

The human intruder and snake tests are particularly popular as they require no specialised equipment (only standard recording devices) and can be performed in the home-cage without having to habituate the animal to a testing apparatus. They also elicit a wide range of the animal's behavioural repertoire e.g. vocalisations and specific avoidance strategies. Both human and animal studies of coping styles to highly stressful situations categorize responding into two broad dimensions representing cognitive and behavioural activity either towards (active) or away (avoidant) from the threat, commonly simplified as fight-or-flight (Koolhaas *et al.*, 1999). An animal's defensive response consists of various general components: e.g. attentional attendance towards or away from the threat, attempts to confront or avoid the threat, and behavioural disinhibition of aggression or behavioural inhibition of locomotion/exploration.

Nonhuman primates are social animals and vocalisations as specific cues to conspecifics are also key components of their threat response. Many vocalisations are commonly suppressed if an animal is isolated from its conspecifics (Coe *et al.*, 1982). As an arboreal species, testing in the home-cage also allows the animal to escape upwards and away from a threat, a response that can't be employed within

a constrained testing space. Therefore, the human intruder and snake tests also provide a more ethological approach to the testing of anxious and fear behaviour.

Early usage of the human intruder and rubber snake tests involved the measurement of single or 2-3 variables to reflect anxiety or fear. Time spent at the front of the cage and average distance from the "human intruder" during the human intruder test was shown to be sensitive to pharmacological manipulations used in the treatment of anxiety disorders such as diazepam and citalopram (Costall *et al.*, 1988; Carey *et al.*, 1992; Santangelo *et al.*, 2016). However, the use of just a few variables to represent anxiety may not be reliable as they may be driven by multiple underlying constructs to differing extents and influenced by individual trait variations. For example, a measure like the time spent at the front of the cage may be driven by other underlying factors such as an animal's territoriality or their sociability in the case of the human intruder, and not anxiety per se. Misattributing the effects observed leads to problems of interpretation when trying to translate to human findings. On the other hand, if we take a more comprehensive approach and account for the full behavioural repertoire the animal is displaying in the experimental paradigm by looking at effects on multiple measures independently, the difficulty encountered is the comparison of varying effects on multiple scales and the problem of multiple underlying constructs driving the measures observed remains unresolved. An early solution to overcome these problems is to reduce the data from multiple measures with the use of a principal component analysis (PCA) to obtain a single composite score.

The primary aim of the PCA is to simplify multi-measure data to derive a principal component score representing linear trends in the data. Our lab had previously conducted a PCA with data from a large cohort of animals tested on the human intruder test and the rubber snake test (Agustín-Pavón *et al.*, 2012; Shiba *et al.*, 2014) . By deriving a simplified score representing the behaviour observed, subsequent studies investigating the effects of a manipulation could determine a test animal's PCA-derived score using the coefficients and parameters from the initial larger group, as demonstrated by Agustín-Pavón *et al.* (2012) and Shiba *et al.* (2014), previous members of our lab. However, a limitation of the PCA is that it obtains a composite score by simplifying the data into its linear components but does not determine the latent variables driving the observed changes within the data. Where the PCA attempts to explain all the variance (common, specific and error variance) to simplify the data, the exploratory factor analysis (EFA) derives a mathematical model of the underlying constructs a.k.a. factors to explain the common variance driving the variation in the measured variables. The EFA is widely utilised in validation studies of psychological tests and has recently been used to uncover the latent variables affecting the behavioural response of rhesus macaques in the human intruder test (Gottlieb and Capitanio, 2013). Since anxiety and the fear response are critical focal points of this thesis, the aim of this study is to take advantage of the expanded dataset of animals screened on the human intruder test and rubber snake test at the University of Cambridge Roberts lab marmoset colony in the intervening years since the original PCA was performed and apply EFA to characterise the underlying

factors driving the common marmoset's behaviour within the human intruder and rubber snake tests. Although anxiety and fear are distinguishable cognitive constructs, both are key emotional responses to threat and share overlapping neurocircuitry. Thus, the potential underlying associations between animals' anxious and fearful behaviour was determined. The factors uncovered will elucidate the significance of the individual behaviours measured in the tests towards potential underlying constructs and enable us to interpret the animal's behaviour more meaningfully.

2.2 Methods

Subjects

171 common marmosets (*Callithrix jacchus*; male = 90, female = 81; age in years: 2.32 ± 0.62) were tested with the human intruder and 151 common marmosets (male = 77, female = 74; age in years: 2.51 ± 0.68) were tested with the rubber snake. Of these, 134 were tested on both human intruder test (age: 2.29 ± 0.62) and rubber snake test (age: 2.5 ± 0.68) and so scores obtained from the exploratory factor analysis were used to correlate the factor scores of both tests (male = 71, female = 63).

The animals are housed as male-female pairs in cages with quadrants of dimensions: 92cm (high) x 60 cm (wide) x 98cm and 73cm (sides). The animals were housed at the Innes Marmoset Colony (Behavioural and Clinical Neuroscience Institute, BCNI). Temperature (22 ± 1 °C) and humidity (50 ± 1%) conditions were controlled and a dawn/dusk-like 12 h-period was maintained. They were provided with a balanced diet and water ad libitum. All procedures were performed in accordance with the project and personal licenses held by the authors under the UK Animals (Scientific Procedures) Act 1986.

Human Intruder test

The procedure for the human intruder test is based on the method used by Costall et al. (1988). The test is carried out in the animal's home-cage (figure 2.1). Cameras and microphones are routinely present in the room for recording purposes such that all animals are habituated to the presence of recording equipment. Before the testing session begins, a camera and microphone are setup in front of the animal's home-cage. During a testing session, the animal is separated from their cage mate and restricted to the upper right-hand quadrant of their home cage for 8 minutes of baseline behaviour. Subsequently, an experimenter (unfamiliar to the animal) wearing a set of standard lab coat and trousers enters the room as the 'human intruder'. The 'human intruder' then stands 40cm from the front of the cage and maintains eye contact with the animal while maintaining a rigid posture and minimising movement for 2 minutes (intruder phase). Subsequently, the intruder leaves the room with recording continuing for 5 min to observe the recovery of normal behaviour (recovery phase). Only behaviour and vocalisations during the intruder phase are scored.

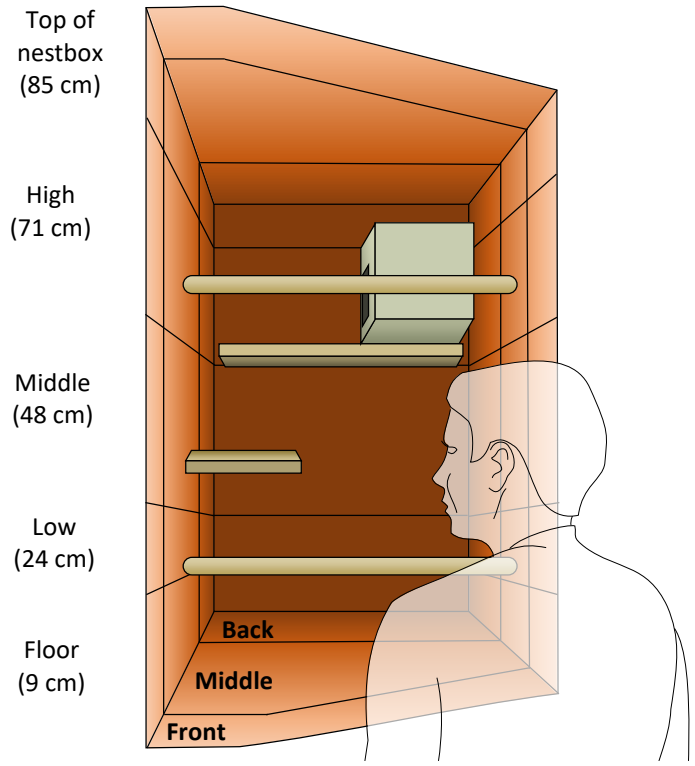


Figure 2.1 Human Intruder test setup. Schematic of relevant zones for the measurement of average height, time spent at the front and time spent at the back. Height of mid-point of different zones indicated.

Rubber snake test

Protocol: A snake model made of rubber (approximately 27cm tall) was used as the stimulus. The stimulus is modelled after a coiled and rearing snake with dark brown coloration and black stripes. The rubber snake was placed in an opaque white Perspex prism box (26 × 26 × 29.5 cm triangle sides × 30 cm high) with a sliding door. The placement of the box is designed to obstruct vision of the rubber snake from other animals in the room and only expose the snake to the test animal when the sliding door is removed (figure 2.2). Test animals are not exposed to the rubber snake model or the box containing the snake prior to testing.

The procedure of the rubber snake test is based on the methods developed in Shiba *et al.* (2014). Before the testing session begins, a wireless camera is placed on the top of the cage to provide a top-down view of the cage and a camera placed from the front of the cage for a frontal view. A microphone is also placed at the front of the cage for audio recording. During a test session, the animal is separated from their cage mate and restricted to the upper right-hand quadrant of their home cage. The 20-minute test session is divided into four 5-minute phases: a separation phase, where only the camera and microphone

were present; a pre-snake phase, where an empty box without the snake is placed in the test quadrant; a snake phase, where the empty box from the previous phase is replaced with the box containing the rubber snake (the sliding door is removed to expose the rubber snake once the box is in position); and a post-snake phase, where the empty box from the pre-snake phase is replaced in the test quadrant again.

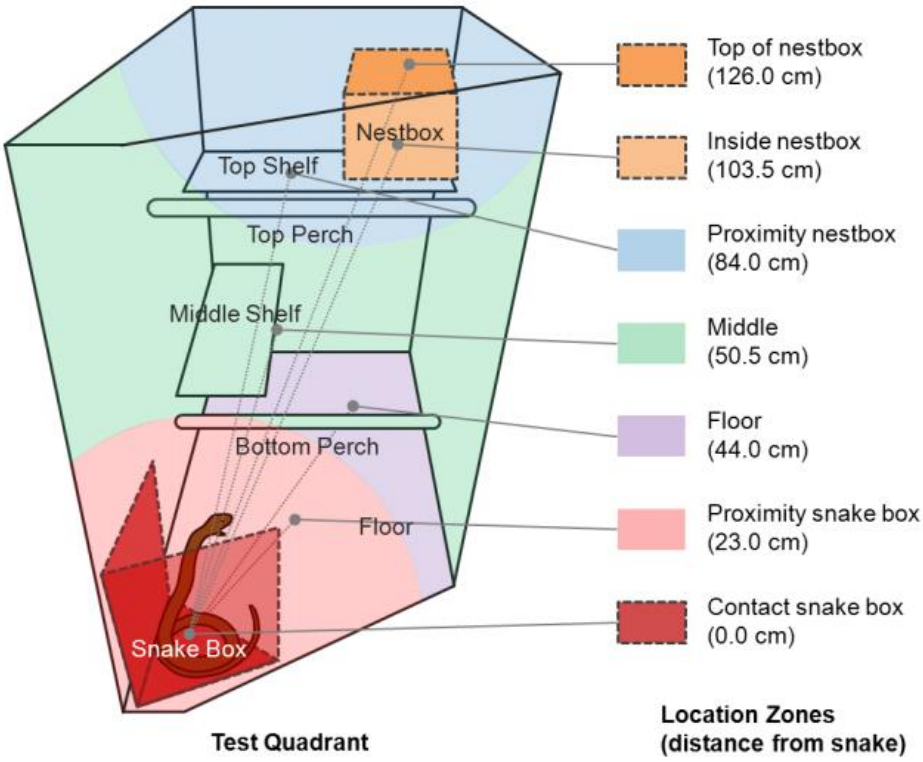


Figure 2.2 Rubber snake test setup. Schematic of a division of the home-cage with the addition of the contact snake box. Zones are depicted in different colours indicating the mean distances those zones represent relative to the rubber snake. Figure from Shiba *et al.* (2012).

Behavioural Scoring

The animal's observable behaviour was scored using the program JWatcher V1.0 (http://www.jwatcher.ucla.edu/). Average distance was used in the rubber snake test but not the human intruder test because the position of the threat: the rubber snake can be reduced to a single point relative to different positioning of the animal. In the human intruder test, the 'human intruder' facing the animal covers a larger area and the animal's position relative to the 'human intruder' is better represented by depth and height instead. The animal's vocalisations were extracted from the video files using Audacity, an audio editing software (Audacity, ver. 1.3.13, http://audacity.sourceforge.net/) and subsequently visualised in the form of sonograms using Syrinx, a sound analysis software. Classification of vocalisations are based on identifications from Bezerra & Souto's observation of wild common

marmosets (2008). Although other calls such as phee, twitter, and bark were observed, they occurred very few times and only in a small subset of the population which lead to their exclusion from this study. For the purposes of scoring, the test quadrant was divided into multiple zones represented by different depths and heights (more in section: 'Human Intruder test: Behavioural Measures'). Percentage time spent at the front of the cage and back of the cage was used as a measure of approach-avoidance behaviour instead of average depth due to studies showing the sensitivity of time spent at the front to anxiolytic manipulations (Carey *et al.*, 1992).

Human Intruder test: Behavioural measures

Time spent at the front (TSAF): Percentage time spent at the front of the cage reflects approach behaviour towards the human intruder. For the purposes of scoring, the test quadrant was divided into 3 zones: front, middle, and back. These different zones represent the depth of the zone relative to the "human intruder" (shown in figure 2.1).

Time spent at the back: Percentage time spent at the back of the cage reflects avoidance behaviour away from the human intruder. Scored similarly to 'Time spent at the front'.

Average height: Average height of the marmoset in the home-cage throughout the test period in centimetres. Positioning high in the cage and closer to the nestbox may reflect the common marmoset's innate flight response upwards as an arboreal species. For scoring purposes, the test quadrant is divided into 5 different zones: top of the nestbox, high, middle, low, floor. These different zones represent the height of the zones relative to the bottom of the test quadrant (shown in figure 2.1).

Locomotion: Percentage time spent changing locations within the home-cage.

Head-bobbing: Frequency of the animal bobbing its head to the side while staring at the object of interest and is often followed with vocalisation. Head-bobbing is often observed in the presence of an unfamiliar human and may be an alarm behaviour intended to signal potential threats to conspecifics.

Rubber snake test: Behavioural measures

Average distance: Average distance of the marmoset from the rubber snake throughout the test period. For scoring purposes, the test area was divided into seven zones based on their proximity to the rubber snake (shown in figure 2.2). Each zone is represented by the distance of the mid-point of that zone from the snake. The average distance is calculated by obtaining the sum of the multiplication of the percentage time spent in each zone with the distance of the respectively zones.

Locomotion: Percentage time spent changing locations around the home-cage.

Stare duration: Percentage time the animal spent maintaining eye and head orientation directly towards the rubber snake.

Stare count: Number of times the animal spends directing its attention towards the snake. Multiple counts indicate looking away and back towards the rubber snake, and reflects an animal repeatedly averting its gaze away from the snake but clearly pre-occupied with the snake.

Head-cock: Frequency of the animal cocking its head sideways while maintaining its attention towards the rubber snake. Head-cocks have been described as an observational behaviour when presented with a novel stimulus and occur during visual inspection (Menzel, 1980; Barros *et al.*, 2002).

Vocalisations in the human intruder and rubber snake tests

Egg calls: A short call with a few harmonics. May be uttered singly, in series, or in continuous combination after tse or tsik calls. Egg calls have been associated with vigilance behaviour, for instance when an unknown human approaches the group or when the calling marmoset is on the ground with sparse vegetation (Souto *et al.*, 2007). Primarily heard in response to human intruder and seldom heard in response to snake.

Tsik calls: Tsik calls are uttered as a mobbing call and have been observed being made by captive and wild common marmosets against conspecifics from other social groups, unfamiliar humans, and potential predators (Epple, 1968; Bezerra *et al.*, 2009). Tsik calls have also been observed being made by captive common marmosets in response to the stimulus presentation of a predator (Hook-Costigan and Rogers, 1998; Cross and Rogers, 2006).

Tsik-egg calls: Although not clearly characterised in the wild, tsik-egg calls of common marmosets have been associated with isolation in a novel environment and have been shown to be sensitive to an anxiogenic drug treatment (Kato *et al.*, 2014).

Tse calls: Sounds similar to tsik calls but distinguishable via sonogram. The lower frequency and end frequency of tse calls are higher than tsik calls. The frequency range in tse calls are also lower than tsik calls (Bezerra and Souto, 2008).

Tse-egg calls: A vocalisation consisting of a single utterance of tse followed by a single or a series of egg calls. Tse-egg calls are the primary call type uttered during vigilance behaviour (89.2% and 80.4% of total calls during vigilance in adults and juveniles respectively) (Bezerra and Souto, 2008).

Exploratory factor analysis (EFA)

All statistical analyses covered were conducted with SPSS (version 24; IBM Corp., Armonk, NY). An exploratory factor analysis (EFA) with a principal axis factoring extraction method was performed on the data obtained from the human intruder and rubber snake tests separately. The principal axis factoring extraction method was used as the variables revealed violations of normal distribution (shown in figure 2.3 and 2.4) and principal axis factoring does not assume multivariate normal distribution.

Pre-factor extraction tests. Before factor extraction, the Kaiser-Meyer-Olkin measure of sampling adequacy (MSA) was used as a measure of the proportion of common variance among the variables that may be driven by underlying factors. The Barlett's test of sphericity was used to evaluate if there were sufficient correlations between the variables such that the factor analysis is able to meaningfully model underlying constructs driving these correlations.

Post-factor extraction. After factor extraction, the communality of a variable is the extent to which that variable correlates with all other variables in the analysis. If the average communality of the variables is more than .7 after extraction, the Kaiser's criterion (eigenvalue > 1) should be used to determine the number of factors to extract, otherwise the scree plot's points of inflexion should be referred to instead (Field 2009). The scree plot shows the eigenvalue, which reflects the amount of variance explained, of each individual factor.

Rotation. If more than 1 factor is extracted, the factors are rotated to improve the interpretability of the resulting factors by maximizing the loadings of each variable to a specific factor and minimizing loading on other factors. A direct oblimin method (oblique rotation) was used to allow for correlations between the factors as there are no theoretical grounds to assume the independence of the factors.

After the factors are extracted and rotated, the factor loadings can be referred to as a measure of each variable correlation with the extracted factor. Factor loadings are considered significant above |.4| (Stevens, 1992). To measure the goodness-of-fit for the extracted factor model, a correlation matrix is constructed based on the model and the difference (residuals) between the reproduced correlation matrix and the original correlation matrix is computed. The proportion of nonredundant residuals with absolute values greater than .05 should be below the recommended value of 50% to suggest that the factor model doesn't have issues with poor fit (Field 2009). The factors scores were estimated with a regression method, preserving any existing correlation between the factors.

The internal consistency of the factors with significantly loading variables was examined using Cronbach's alpha. Cronbach's alpha evaluates how consistently the factor scores reflect the construct it is measuring (Cronbach, 1951).

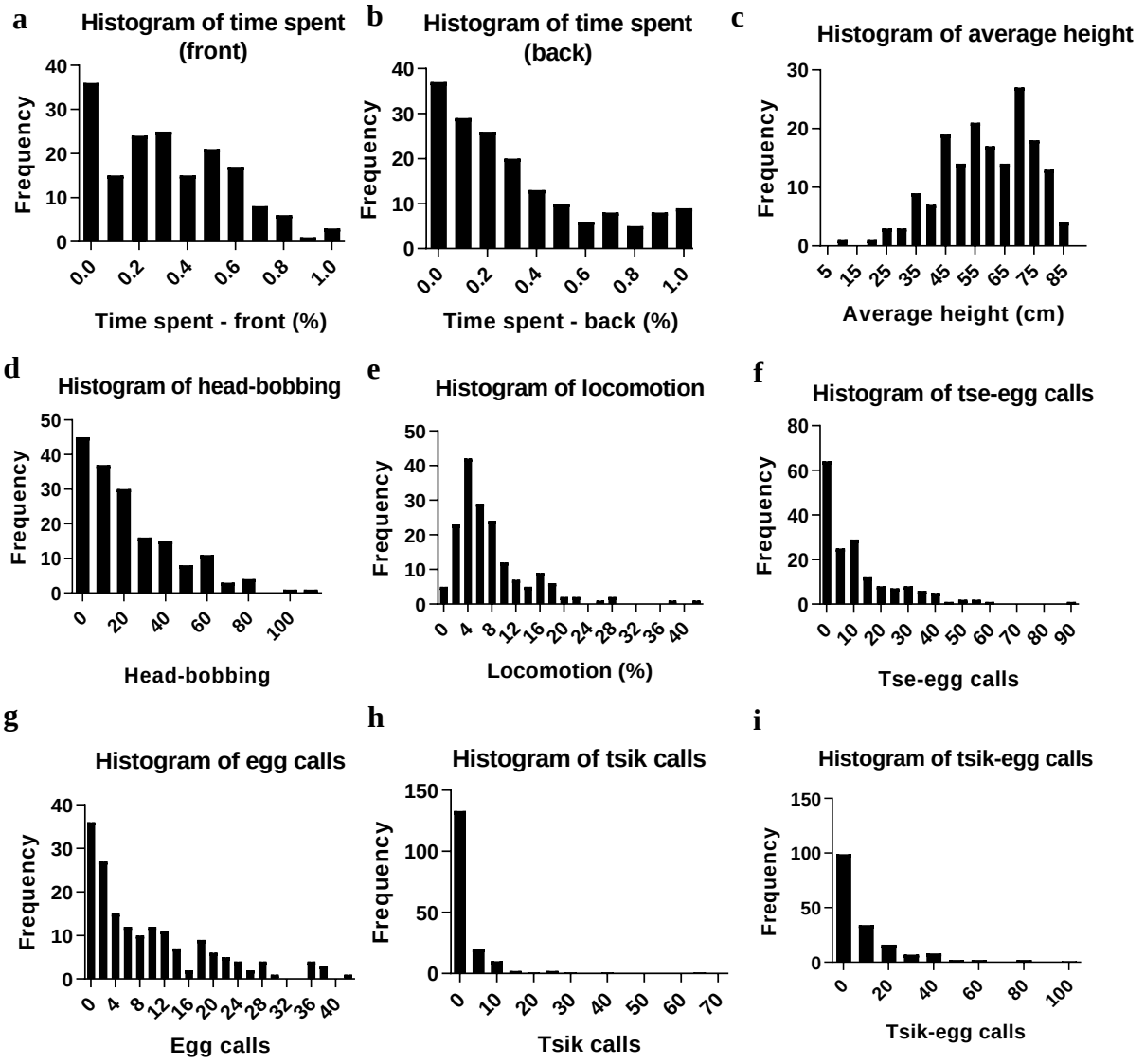


Figure 2.3: Histogram of human intruder test behavioural measures in the EFA. a) time spent at front, b) time spent at back, c) average height, d) head-bobbing, e) locomotion, f) tse-egg calls, g) egg calls, h) tsik calls, and i) tsik-egg calls. Variables display non-normal distribution.

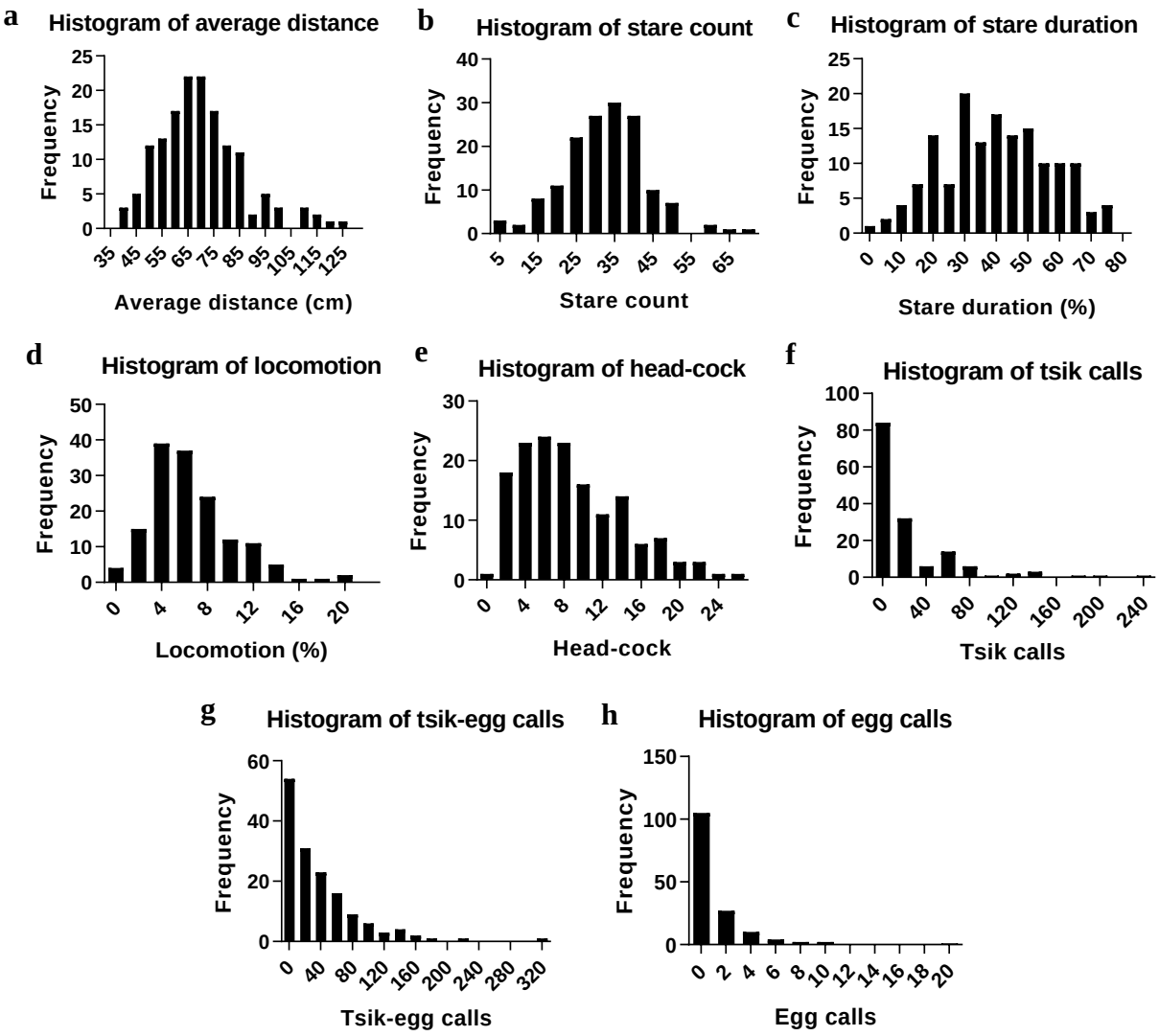


Figure 2.4: Histogram of rubber snake test behavioural measures in the EFA. a) average distance, b) stare count, c) stare duration, d) locomotion, e) head-cock, f) tsik calls, g) tsik-egg calls, and h) egg calls. All variables display substantial non-normal distribution.

Correlation between factor scores

The resulting factor scores of the human intruder test and rubber snake test were correlated with Pearson's product-moment correlation coefficient or Spearman rank correlation coefficient if the assumption of normality was severely violated ($p < .001$). Data are presented as mean ± SEM, standard error of the mean. Effect sizes of correlations are reflected in the correlation coefficients, r and r_s (Cohen, 1988, 1992).

2.3 Results

EFA reveals a single factor in the human intruder test

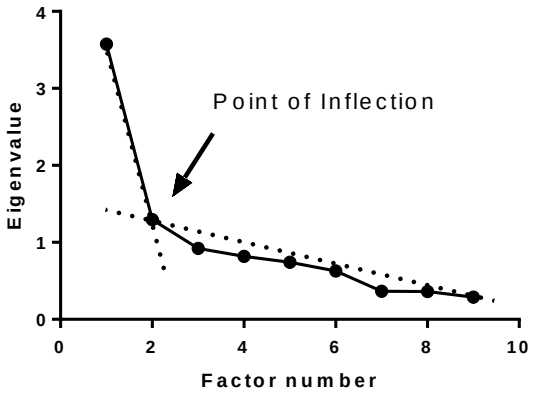
Initial runs of the exploratory factor analysis included: time spent at the front, time spent at the back, average height, locomotion, head-bobs, egg calls, tsik call, tsik-egg calls, tse calls, and tse-egg calls. The variable with the lowest measure of sampling adequacy that was below the standard of .5 defined by Field (2013), tse calls (MSA = .42) was removed from the EFA. Subsequently, the KMO measure of sampling adequacy for the final model indicated sufficient common variance for the factor analysis, KMO = .82, well above the recommended threshold of .6 (Kaiser, 1974). Bartlett's test of sphericity was significant ($\chi^2(36) = 460.8$, $p < .001$), indicating that correlations between items were sufficiently large for a factor analysis. Due to the low level of communalities, reflecting low inter-variable correlations, after extraction (figure 2.5a), the scree plot was consulted to decide the number of factors to extract instead of using Kaiser's criterion. Only 1 factor was extracted based on the point of inflection on the scree plot (shown in figure 2.5b). This factor accounted for 39.7% of the total variance. There were 16 (44.0%) nonredundant residuals, reflecting the sufficient fit of the one-factor model. The factor loadings are shown in the factor matrix of figure 2.5c. The variables that contributed greatest to the factor were the time spent at the front and back of the cage, average height and head-bobbing. Locomotion and tse-egg calls also contributed just above and below 0.5 (figure 2.5d). The highest score was associated with greater avoidance (more time spent at the back of the cage and relatively high up) and increased vigilance (making little movement, performing greater number of head-bobbing and tse-egg calls). The factor coefficient matrix estimated from the final output of the EFA and descriptive statistics of the sample is shown in figure 2.5c.

The factor with 6 significantly loading items had moderate reliability, Cronbach's alpha = .64. Kline (2000) notes that psychological constructs with Cronbach's alpha below .7 should be realistically expected. Eliminating any variables from the factor would not yield substantial increases to the alpha measure.

a

Variables	Communalities	
	Initial	Extraction
Average height	.55	.67
Head-bobbing	.54	.59
Time spent (back)	.52	.47
Tse-egg calls	.22	.17
Locomotion	.31	.32
Time Spent (front)	.59	.62
Egg calls	.19	.11
Tsik calls	.09	.01
Tsik-egg calls	.23	.10

b



c

Variables	*M*	*SD*	Factor loading	Score coefficient
Average height	58.69	15.37	**.82**	0.319
Head-bobbing	22.09	22.37	**.77**	0.295
Time spent (back)	31.02	30.07	**.69**	0.110
Tse-egg calls	11.74	14.78	**.42**	0.048
Locomotion	7.90	6.54	**-.57**	-0.100
Time Spent (front)	31.24	25.26	**-.79**	-0.288
Egg calls	9.13	10.03	.33	0.026
Tsik calls	2.73	7.48	-.09	-0.017
Tsik-egg calls	9.65	16.39	.32	0.008

d

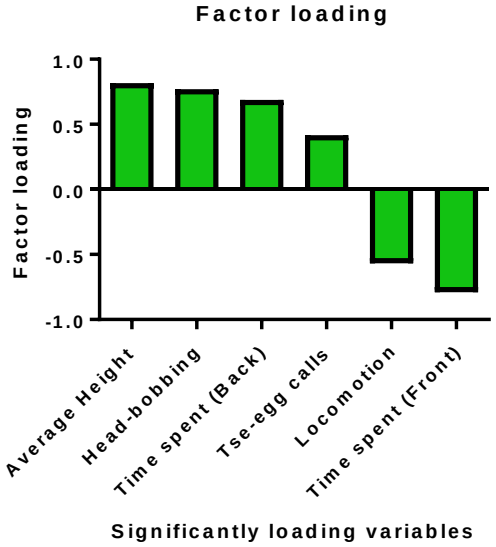


Figure 2.5: Human Intruder test exploratory factor analysis. (a) Table of communalities. (b) Scree plot showing the eigenvalue as a measure of explained variance for the number of factors extracted. Point of inflection shown. (c) Table of factor loadings and factor score coefficients for the variables in the human intruder test. Significant factor loadings (>|.4) in bold and green. Mean (*M*) and standard deviation (*SD*) of variables from the cohort (N=171). (d) Factor loading for variables loading significantly on the factor.

Rubber snake test EFA reveals two negatively correlated factors for behaviours in a fear-provoking context

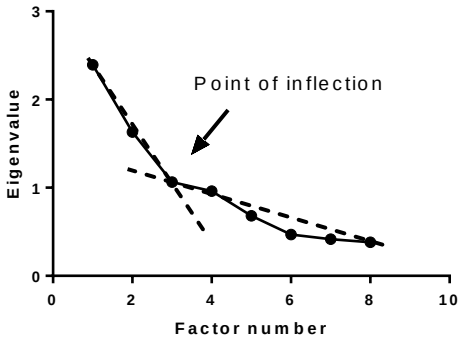
Initial runs of the exploratory factor analysis included: average distance, locomotion, stare duration, stare count, head-cocks, egg calls, tsik call, tsik-egg calls, tse calls, and tse-egg calls. The variable with the lowest measure of sampling adequacy that was below the criterion of .5 defined by Field (2013), tse calls (MSA = .32) were removed from the exploratory factor analysis. Tse-egg calls were removed in the subsequent run under the same criterion (MSA = .46). The KMO measure of sampling adequacy for the final model indicated sufficient common variance for the factor analysis, KMO = .63, just above the recommended threshold of .6 (Kaiser, 1974). Bartlett's test of sphericity was significant (χ^2 (28) = 233.1, $p < .001$), indicating that correlations between items were sufficiently large for a factor analysis. Due to the low level of communalities after extraction (figure 2.6a), the scree plot was consulted to decide the number of factors to extract instead of using Kaiser's criterion (Field 2009). Two factors were extracted based on the point of inflection on the scree plot (shown in figure 2.6b). These factors accounted for 50.3% of the total variance. The factor loadings after rotations from the pattern matrix are shown in figure 2.6c. There were 10 (35.0%) nonredundant residuals, indicating that the two-factor model does not have issues of poor fit. The first factor is characterised by (factor loading > .4) frequent mobbing calls (tsik-egg and tsik calls) and actively attending to the snake (longer durations spent staring at the rubber snake and higher frequencies of re-attending to the rubber snake after looking away) (figure 2.6d). The second factor is characterized by behavioural and attentional avoidance of the rubber snake: maintaining a further distance from the rubber snake, spending less time staring at the rubber snake, and remaining stationary (figure 2.6d). The factor score coefficients estimated from the final output of the EFA and descriptive statistics of the sample are shown in figure 2.6c.

Factor 1 with 4 significantly loading items had moderate reliability, Cronbach's alpha = .61. Factor 2 with 3 significantly loading items had relatively lower reliability, Cronbach's alpha = .52. As Cronbach's alpha tends to decrease as a product of a scale's lower number of items, the factor for avoidant fear responding's low number of items is likely to have contributed to the scale's relatively lower reliability.

a

Variables	Communalities	
	Initial	Extraction
Tsik-egg calls	.39	.64
Tsik calls	.31	.35
Stare count	.38	.45
Stare duration	.38	.50
Locomotion	.33	.40
Average distance	.36	.50
Head-cock	.16	.07
Egg calls	.07	.04

b



c

Variables	*M*	*SD*	Factor loadings		Score coefficient	
			1	2	Active	Avoidant
Tsik-egg calls	38.11	47.05	**.81**	.23	0.480	0.150
Tsik calls	23.55	40.00	**.60**	.19	0.177	0.064
Stare count	32.56	10.81	**.60**	-.20	0.257	-0.135
Stare duration	40.12	17.00	**.47**	**-.44**	0.223	-0.275
Locomotion	6.58	3.66	-.09	**-.65**	-0.026	-0.310
Average distance	69.90	16.37	-.02	**.70**	-0.045	0.423
Head-cock	8.41	5.40	.26	-.04	0.061	-0.001
Egg calls	0.93	2.30	.16	-.11	0.033	-0.038

d

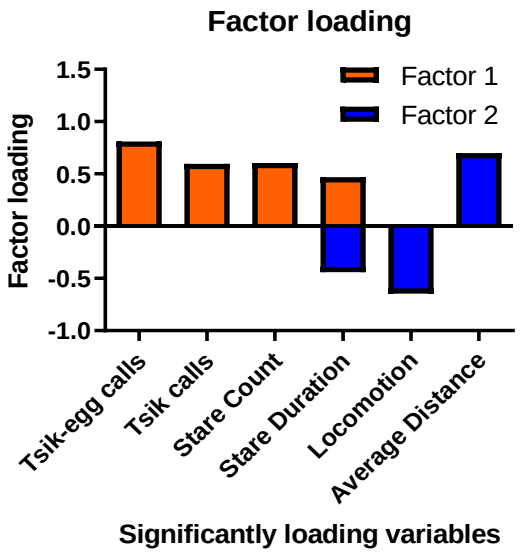


Figure 2.6 Rubber snake test exploratory factor analysis. (a) Table of communalities. (b) Scree plot with eigenvalues as a measure of explained variance for the number of factors extracted. Point of inflection shown. (c) Table of factor loadings from the pattern matrix and factor score coefficients for the variables in the rubber snake test. Significant factor loadings (>|.4) in bold (factor 1: orange) (factor 2: blue). Mean (*M*) and standard deviation (*SD*) of variables from the cohort (N=151). (d) Factor loading for variables loading significantly on factors 1 (in orange) and 2 (in blue).

The factor loading avoidant and vigilance behaviour in the human intruder test is negatively correlated with factor 1 (mobbing and attending the rubber snake) and positively correlated with factor 2 (avoiding the rubber snake) of the rubber snake test.

After deriving the factor scores corresponding to the factors extracted from the human intruder test and the rubber snake test, we evaluated the association between the individual factor scores of animals tested on both tests. Although calculation for Pearson's correlation coefficient is robust against violations of normality, factor 1 score of the rubber snake test severely violated the assumption of normality (all animals: $W(151) = 0.93$, $p < .001$; just animals scored on both tests: $W(134) = 0.92$, $p < .001$) (histogram shown in figure 2.7). Therefore, the nonparametric Spearman's rank-order correlation was used to determine the relationship between factor 1 of the rubber snake test and both factor 2 of the rubber snake test and the factor of the human intruder test. Factor 1 was significantly negatively correlated (nonlinear) to factor 2 ($r_s = -.25$, $p = .002$) with a small to medium effect size ($.1 < |r| < .3$) (figure 2.8a), indicating that behaviours corresponding to actively attending to the rubber snake are negatively associated with avoidant behaviours towards the rubber snake. Although the fan-shaped distribution of the scatterplot suggests heterogeneity of variance, homoscedasticity is not an assumption of Spearman's rank-order correlation and thus does not affect interpretation of the correlations.

There was a significant small to medium ($.1 < |r_s| < .3$) negative nonlinear correlation between the factor driving increased mobbing and greater attendance towards the rubber snake (rubber snake test factor 1) and the factor driving avoidance and vigilance of the human intruder (the human intruder test factor) ($r_s = -.18$, $p = .04$) (figure 2.8b). The marginal significance of the correlation between rubber snake test factor 1 and the human intruder test factor may be mediated by the more significant correlation of both factors with factor 2 of the rubber snake test.

Pearson's correlation coefficient was calculated for the association between factor 2 of the rubber snake test and the factor scores of the human intruder test though as they did not severely violate assumptions of normality ($p > .05$). There was a significant positive linear correlation between the factor driving greater avoidance of the rubber snake (rubber snake test factor 2) and the factor driving greater avoidance and vigilance of the human intruder (human intruder test factor) ($r = .31$, $p < .001$) (figure 2.8c). Cohen's criteria for the correlation coefficient ($.3 < |r| < .5$) suggested an effect size of medium to large practical significance (Cohen, 1988, 1992).

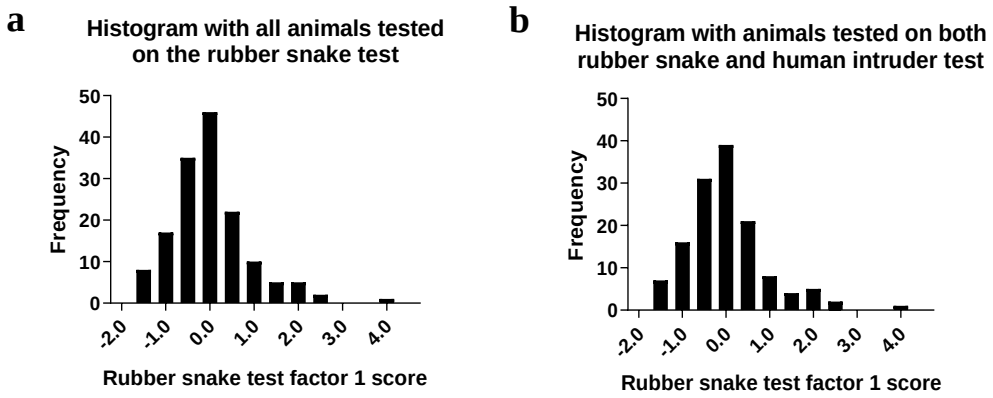


Figure 2.7: Histogram of rubber snake test factor 1 scores. Frequency distributions for rubber snake test factor 1 scores with (a) all animals tested on the rubber snake test (N = 151) and (b) only animals tested on both the rubber snake test and human intruder test (N = 134). Both histograms are positively skewed, depicting significant violations of normality ($p < .001$).

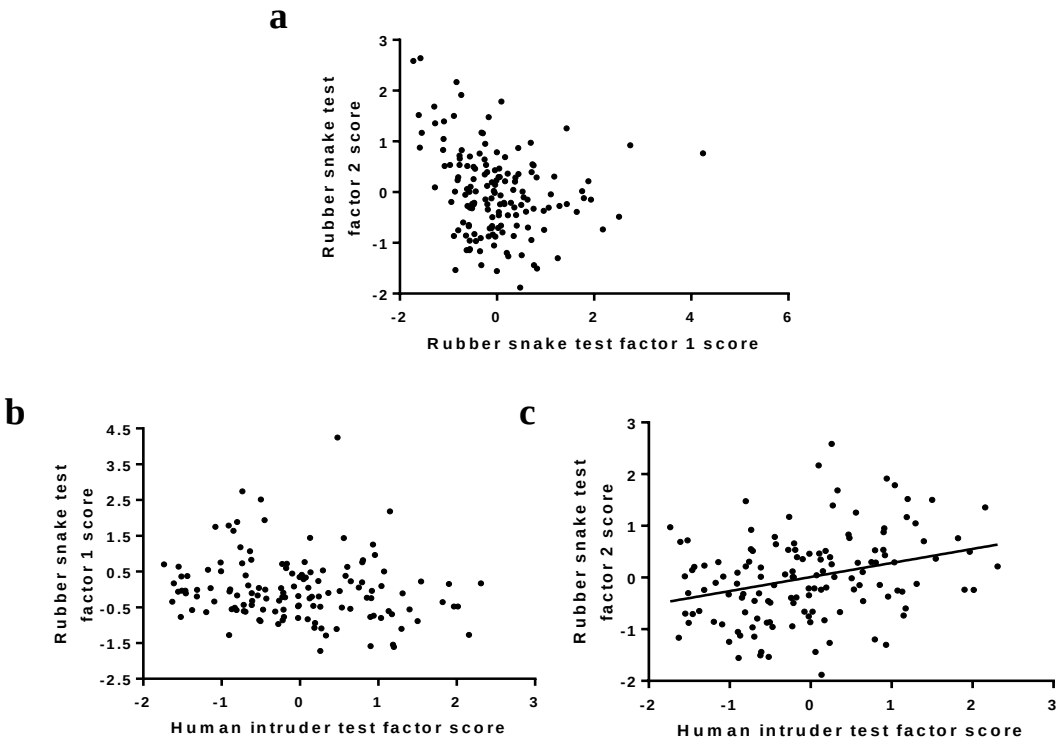


Figure 2.8: Association between factors from the human intruder test and rubber snake test. (a) Rubber snake test factor 1 and 2 scores are significantly negatively correlated (Spearman's, $p < .005$). Human intruder test factor scores are (b) significantly negatively correlated with rubber snake test factor 1 scores (Spearman's, $p < .05$) (c) and significantly positively correlated with rubber snake test factor 2 scores (Pearson's, $p < .001$). Regression line only shown for linear relationships measured by Pearson's product-moment correlations.

Animals with a low factor 1 but high factor 2 score (avoidant fear responding style) are more anxious compared to animals with other fear responding styles.

As lower mobbing (factor 1) but higher avoidance (factor 2) of the rubber snake was associated with greater human intruder test anxiety score, animals were grouped to determine if an avoidant fear responding style corresponded with increased anxious behaviour as measured by the human intruder test. Animals with the top 50% of factor 1 scores but the bottom 50% of factor 2 scores were classified as having an active fear responding style (N = 39), while animals with the bottom 50% of factor 1 scores and top 50% of factor 2 scores were classified as having an avoidant fear responding style (N = 39). Animals with both top 50% of factor 1 and 2 scores were classified as having a mixed fear responding style (N = 28). Finally, animals with bottom 50% of factor 1 and 2 scores are animals that showed low fear reactivity towards the rubber snake and were therefore not included in subsequent analysis (N = 28). As expected, animals grouped for active and avoidant fear responding styles had significantly different factor 1 and factor 2 scores (active: $t(38) = 10.7$, $p < .001$; avoidant: $t(38) = -10.1$, $p < .001$) (figure 2.9a).

There was a significant effect of fear responding style ($F(2,103) = 3.91$, $p = .023$). Indeed, a Dunnett's test revealed that animals with an avoidant fear responding style ($M = .33$, $SEM = .14$) had a higher anxiety score on the human intruder test compared to animals with an active ($M = -.17$, $SEM = .13$) and mixed ($M = -17$, $SEM = .20$) fear responding style (figure 2.9b).

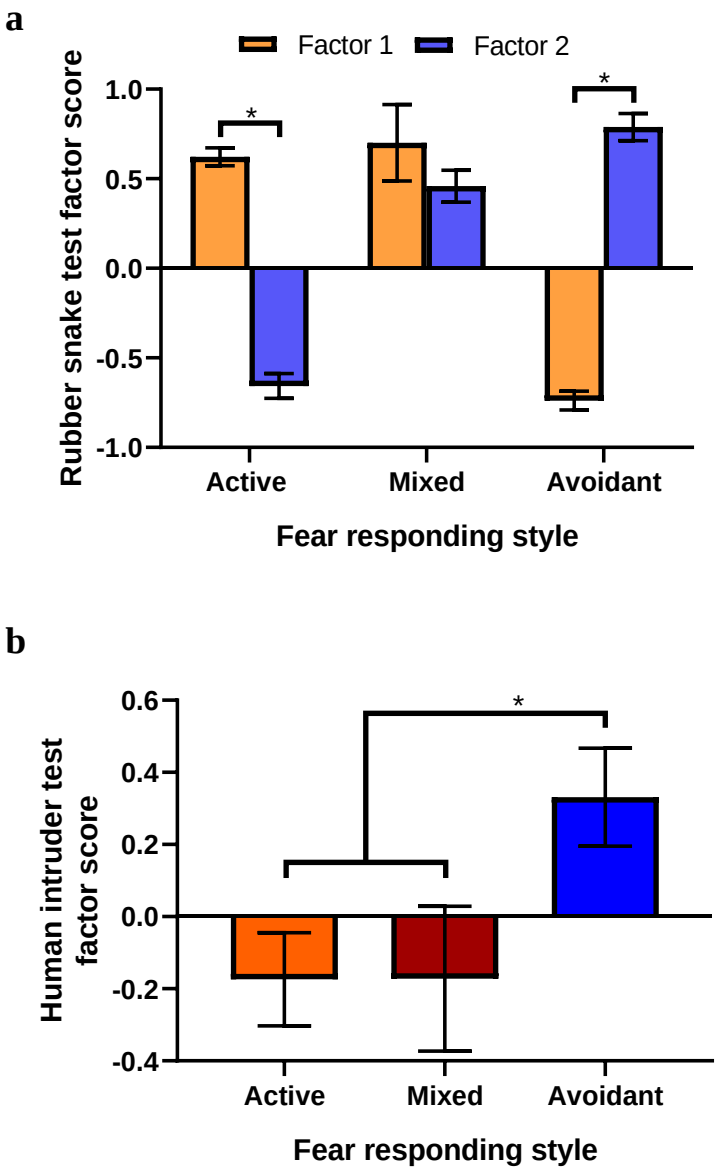


Figure 2.9 Fear responding style and corresponding factor scores. (a) Animals were grouped based on fear responding style: Active (high factor 1 scores, but low factor 2 scores; *$p < .05$), Mixed (high factor 1 and 2 scores), and Avoidant (low factor 2 score, but high factor 2 scores; *$p < .05$). (b) Animals with an avoidant fear responding style in the rubber snake test had higher levels of anxious behaviour as measured by the human intruder test factor score (*$p < .05$).

2.4 Discussion

Although a composite score to reflect anxiety and fear in the common marmoset have been derived previously using PCA, here I've derived a multi-measure score by modelling the underlying factor instead and studied the relationship between anxious and fear-driven behaviour.

Anxiety underlying behavioural responding on the human intruder test

Exploratory factor analysis of data from 171 marmosets confronted by an unknown human yielded one underlying factor driving the behaviours measured. This factor is interpreted as reflecting the animal's anxious temperament as it drives behaviour typically associated with high levels of anxiety. The behaviours that significantly load on the factor are (in descending order of significance): average height, time spent at the front, head-bobbing, time spent at the back, locomotion and tse-egg calls. An animal with a high anxiety factor score is characterised by marked avoidance behaviour: spending more time at positions further away from the human intruder (higher up and at the back of the cage), less time at positions close to the human intruder (the front of the cage) and spending more time stationary. Moreover, there is marked vigilance behaviour: performing more head-bobbing and vigilance calls (tse-egg calls). The uncertainty and anticipation model of anxiety posits that behavioural and cognitive avoidance and increased threat vigilance are among the key psychological processes central to the increased threat expectancies of subclinical and clinical anxiety (Grupe and Nitschke, 2013). Taken together, the anxiety factor in the human intruder test reflects classic components of anxiety: avoidance behaviour and active vigilance (figure 2.10).

Although animals make tsik, tsik-egg, and egg calls in the human intruder test, these variables did not load significantly on the factor for anxiety. One explanation may be that the mobbing calls, tsik and tsik-egg, may be driven specifically by an animal's fear (as evidenced by the call's loading in the rubber snake test) instead of anxiety. This indicates that although the "human intruder" is intended to induce anxiety, a low extent of fear may also be present in some animals during this task. If an additional factor was extracted, the additional factor only has tsik calls as a significant loader (table 2.1). The failure of egg calls to load significantly on either the human Intruder test factor for anxiety or on the two snake test factors suggests that egg calls may be a more general call made in response to mild stress, compared to the other calls (tse-egg, tsik, and tsik-egg) which are driven by more specific emotional states. Egg calls were also made at a relatively lower frequency than the other calls in the rubber snake test. Contrary to findings here linking tse-egg calls to anxiety, Kato *et al.* (2014) found that common marmosets emitted tsik-egg calls in the anxiety-provoking context (isolation in an unfamiliar context) and after anxiogenic drug (FG-7142) treatment. Unfortunately, a comparison between our studies is

difficult as tse-egg calls were not included in their study. The frequency of tse-egg calls may also have been diminished due to Kato *et al*.'s small sample size (N = 6; 2014). Tse-egg calls and tsik-related calls (tsik and tsik-egg) may be useful for distinguishing if a common marmoset is either anxious or fearful respectively.

Active and avoidant fear behavioural response underlying the rubber snake test

The exploratory factor analysis of data from the rubber snake test yielded two underlying factors driving the behaviours measured. The two factors may be interpreted as reflecting the animal's active and avoidant fear response. The behaviours that significantly load on rubber snake test factor 1 reflecting an active fear response are (in descending order of significance): tsik-egg calls, stare counts, tsik calls and stare duration. The active fear response factor drives higher frequencies of mobbing calls in the presence of the predator stimuli, tsik-egg calls and tsik calls. Mobbing calls serve to alert conspecifics of a potential predator and drive predators away (Epple, 1968). Tsik calls have also been associated with reduced cortisol levels, implicating mobbing behaviour in the reduction of physiological stress (Cross and Rogers, 2006). The factor representing an active fear response also underlies increased attention towards the predatory stimuli: longer durations of staring at the rubber snake and higher levels of re-diverting attention towards the snake (measured by stare count). The active fear response factor consists of a series of active attentional and vocalisation behaviours that may underlie the animal's attempt to confront and overcome a threat (figure 2.10).

In contrast to the factor for active fear responding, the cluster of behaviours significantly loading on the factor for avoidant fear responding (rubber snake test factor 2) serve to avoid contact between the animal and the threat (rubber snake) and avoid drawing attention to the animal. The behaviours loading significantly on the factor representing an avoidant fear response are (in descending order of significance): average distance, locomotion and stare duration. The factor representing an avoidant fear response corresponds to the animal's behavioural and attentional avoidance of the predatory stimuli: higher distance from the rubber snake, lower locomotion, and spending less time staring at the rubber snake (measured by stare duration).

Only head-cocks and egg calls did not load significantly on either fear responding factors. This may be due to an animal's head-cocks being associated to the animal's response to the novelty of the stimulus (rubber snake) (Menzel, 1980; Barros *et al.*, 2002) independent of the emotional response that may be elicited. Tilting one's head during a head-cock may be an attempt to shift the visual field and perspective to better examine a novel/unfamiliar object (the rubber snake).

Our findings of factors representing distinct fear responses in the common marmoset is consistent with coping inventories and questionnaires delineating active and avoidant coping strategies in humans

(Herman-Stabl, Stemmler and Petersen, 1995; Seiffge-Krenke and Klessinger, 2000; Frydenberg and Lewis, 2009; Pineles *et al.*, 2011). For example, in the most well-known coping questionnaire "*the ways of coping questionnaire*" by Folkman & Lazarus (1980), factor analyses have generally shown two coping style groups: approach-oriented coping and avoidant coping. Items that load highly on the factor for approach-oriented coping include items that address the source of stress e.g. "I try to talk about the problem with the person concerned", while items that load highly on the avoidant coping factor include items withdraw from or ignore the problem e.g. "I behave as if everything is alright" (Seiffge-Krenke and Klessinger, 2000). Natural defensive behaviours in rodents and other animals also indicate the existence of distinct proactive and reactive defensive behaviours (Koolhaas *et al.*, 1999; Blanchard, Griebel and Blanchard, 2001). For example, "proactive" rats display more aggressive behaviour in response to an intruder and spend more time actively burying a shock probe in the home cage, whereas "reactive" rats display less aggressive behaviours to an intruder and spend more time being immobile in the defensive burying test (Koolhaas *et al.*, 2010).

The negative association between active and avoidant fear responding is consistent with reports in the rodent literature of a negative association between active behaviour and freezing in response to conditioned fear (Gozzi *et al.*, 2010; Metna-Laurent *et al.*, 2012). Our finding is also consistent with the finding that although most people use both active and avoidant coping strategies in response to stressful situations, individuals vary in the tendency to use one type over the other as coping patterns (Folkman and Lazarus, 1980). Evidence from rodent studies manipulating amygdala signalling demonstrate the switching between the use of active or avoidant fear responding, implicating differential underlying neurological mechanisms and further supporting the view that these behaviours in a fear-provoking context are distinct groups of defensive behaviours (Gozzi *et al.*, 2010; Lázaro-Muñoz, LeDoux and Cain, 2010; Metna-Laurent *et al.*, 2012).

The literature on fear conditioning consists heavily of passive fear responses such as freezing, similar to avoidant fear responses described here (low locomotion) and not active expressions of fear. This is due to the propensity to freeze when rodents are fearful, in which most conditioned fear studies have been done but also because of the inescapable context in which classical fear conditioning is conducted which limits the viability of active behaviours to avoid or overcome the threat. It has been demonstrated that, in response to the conditioned stimulus (CS) predicting foot-shock, rodents show not just avoidant behaviours such as freezing, but also active behaviours after initial exposure to the CS such as digging and rearing (Metna-Laurent *et al.*, 2012). Alternative rodent conditioned fear paradigms have evidence that distinct fear responding patterns may be modulated by differential neural circuits: basal amygdala output and prefrontal-striatal circuit has been implicated in modulating an active avoidant fear response system (escape shuttling) and central amygdala output modulating a passive fear response system (freezing) (Amorapanth, LeDoux and Nader, 2000; Choi, Cain and LeDoux, 2010; Bravo-Rivera *et al.*, 2014, 2015). Work with the rodent shock-probe defensive burying test has also found that chronic stress

induces a shift from active to passive coping that can be reversed with an SSRI or extinction training (Jett *et al.*, 2015; Fucich, Paredes and Morilak, 2016; Hatherall, Sánchez and Morilak, 2016; Fucich *et al.*, 2018).

It appears that there may be 3 different fear-driven behaviours at play here: active (fight), avoidant (flight), or passive (freeze) responses. Freezing as a passive fear behaviour in rodents is easily quantified, but the emphasis on passive fear responding in the literature may confound our efforts to advance our understanding of human defence responses elicited by threat from rodent studies of fear. The avoidant factor defined in the marmoset here appear to combine elements of passivity (low locomotion) and avoidance (distancing oneself from the threat) and more closely mirrors the bimodal coping styles in humans (Coping Across Situations Questionnaire, CASQ: Seiffge-Krenke & Klessinger 2000; Adolescent Coping Scale, ACS: Frydenberg & Lewis 2009; Coping Strategies Inventory, CSI: Pineles *et al.* 2011). Our finding emphasises the need to view fear responding as not simply a unitary construct, but for more work to evaluate mechanisms underlying both active and avoidant components of fear behaviour and emotion regulation.

It's worth noting that while active and avoidant behaviours are split into two factors, the single factor underlying anxiety in the human intruder test consist of both active and avoidant behaviours. More specifically, vigilant behaviours to actively attend to the potential threat: head-bobbing and tse-egg calls, but also avoidant behaviours: staying at positions further away from the potential threat, and spending more time being still. Taken together, the factor's behavioural loading may indicate that when the animal is confronted by a stressor that may or may not be a threat (uncertain), a combination of both active (increased attention to the threat: vigilance) and avoidant (maintaining a safe distance and remaining still) behaviours are optimal responses. When the threat is ascertained however, and an animal moves into a fearful state, impulses for differing strategies to resolve the threat drives the observed active (fight: appearing threatening and alert conspecifics at the risk of drawing attention to self) and avoidant (flight: flee and staying still to avoid notice) fear responses captured by the two factors (figure 2.10).

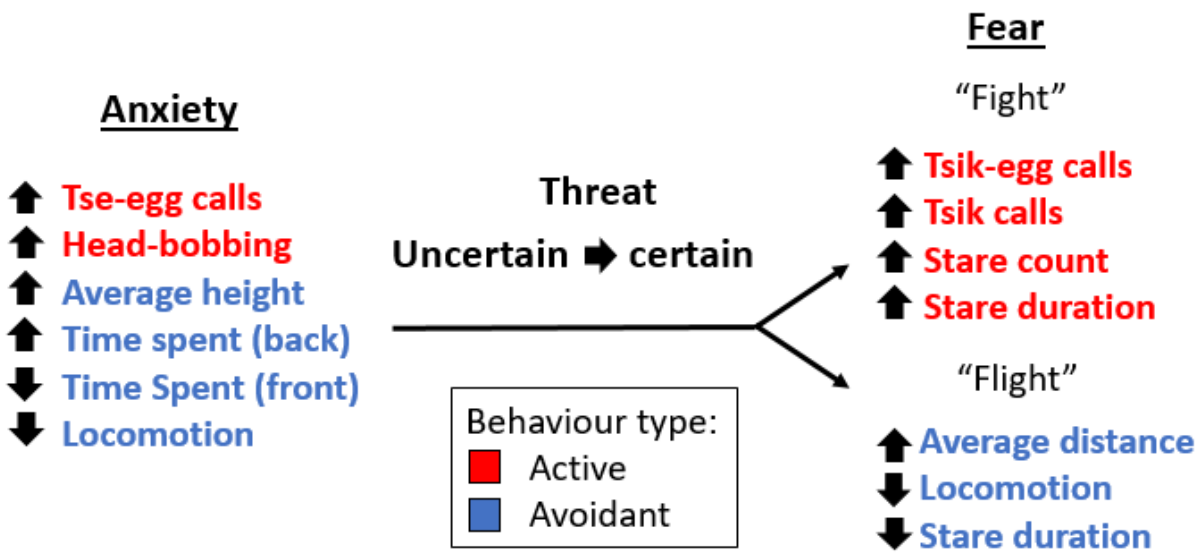


Figure 2.10 The transition of behaviours from anxiety to fear. As an animal's appraisal of threat goes from uncertain to certain, an animal's behavioural pattern shifts from a combination of both active (red) and avoidant behaviours (blue) to either a "fight" response characterised by active behaviours to confront the threat or a "flight" response characterised by attempts to avoid confrontation with the threat. Direction of arrows for variables indicate direction of factor loading.

Fear responding may be separated by active or avoidant behaviour as the optimal behaviour may be dependent on the predator. In a group situation, variation in individual responding styles when confronted by the same predator may allow for higher chances for individuals to survive dependent on whether the predator is deterred by mobbing (predators relying on remaining unnoticed, e.g. snakes) or aren't deterred by mobbing, and therefore the right course of action would be to avoid and flee (Crofoot and Crofoot, 2012). Individual responding styles are also associated with different costs: adopting an active response would lead to more immediate stress in the short term, but overcoming the threat may lead to less stress in the future; whereas adopting an avoidant response would lead to less immediate stress in the short term, but may lead to prolonged stress as the source of the stress is not overcome.

Increased anxiety on human intruder test is associated with an avoidant fear responding style.

The anxiety factor score derived from the human intruder test has a strong positive association with the avoidant fear response factor score and a negative association with the active fear response factor score derived from the rubber snake test. The association between anxiety and the respective fear responding styles suggests that individuals with a predominant coping pattern to disengage and avoid instead of engaging and confronting a threat may be predisposed to have a higher level of anxiety. Subsequent

analysis in which animals were grouped according to their factors scores on the distinct fear responding styles confirmed that indeed, avoidant fear responders had higher anxiety compared to both active and mixed fear responders. Consistent with this, the tendency to adopt an avoidant coping strategy (similar to avoidant coping) has been linked to anxiety and depressive symptoms during adolescence, and increased post-trauma PTSD symptom severity (Chan, 1995; Herman-Stabl, Stemmler and Petersen, 1995; Seiffge-Krenke and Klessinger, 2000; Gomez and McLaren, 2006; Pineles *et al.*, 2011). Avoidance behaviour has also been found to moderate an increase in general anxiety in women with specific phobias (Rudaz *et al.*, 2017).

Inversely, individuals prone to higher levels of anxiety may be predisposed to a predominantly avoidant coping style to fear. In support of this, although individuals with higher trait levels of anxiety show an attentional bias to the detection of threatening stimuli, they also tend to avoid attending to these stimuli if the threatening stimuli persists (Koster *et al.*, 2005b). It is worthwhile to note that although there are mixed fear responders (high on both measures of avoidant and active fear responding), that among the animals that were reactive towards the rubber snake, 73.6% were either active or avoidant fear responders, supporting the notion that most animals tend to have a predominant fear responding style. Taken together, a predominantly avoidant behavioural pattern to fear may be indicative of a maladaptive coping response that impedes the resolution of threat uncertainty via threat engagement/confrontation, leading to an increased vulnerability to anxiety that may in turn feed back into the tendency to avoid and not confront a threat.

EFA vs PCA

Previous efforts to simplify the variables within the human intruder test and rubber snake test with the common marmoset using a PCA derived two factors hypothesised to reflect an emotionality component and a coping strategy component (Agustín-Pavón *et al.*, 2012; Shiba *et al.*, 2014). For the human intruder test, the behaviours loading on the component interpreted as representing emotionality are similar to those derived from the EFA factor interpreted as representing anxiety, but the vocalisations are loading more significantly in the PCA analysis by Agustín-Pavón *et al.* (2012) (table 2.1). The low level of communality in the animal's vocalisations here (figure 2.5a) indicate that the difference in the vocalisation's loading in the PCA and EFA may reflect substantial specific and error variance, variance excluded from the EFA. The component interpreted as representing coping strategy from the human intruder test analysis by Agustín-Pavón *et al.* (2012) has vocalisations (egg, tsik, and tsik-egg calls) as significant loaders, variables that did not load significantly on the EFA (table 2.1). To investigate if the variables that did not load significantly on the current "anxiety" factor may load significantly on a second factor and correspond to the PCA "coping strategy" component, the EFA was repeated with a 2-factor extraction. We found that the second factor only has tsik calls as a significant loader (table 2.1),

but factors without multiple significant loaders are not meaningfully interpretable beyond the single measure.

With respect to the rubber snake test, the behaviours loading on the "emotionality" component based on Shiba *et al.*'s (2014) PCA is similar to the factor for avoidant fear responding but surprisingly, head-cocks, a behavioural response to novelty, loaded significantly in the PCA (table 2.2). The behaviours loading on the "coping strategy" component by Shiba *et al.* (2014) is similar to the factor for active fear responding, but stare duration did not load significantly on the component, this is attributable to stare duration's loading heavily weighted to the "emotionally" component (table 2.2).

Human Intruder test variables	EFA factors			PCA components*	
	1-factor extraction	2-factor extraction		2-component extraction	
	"Anxiety"	Factor 1	Factor 2	"Emotionality"	"Coping Strategy"
Average distance	-	-	-	**.86**	-.14
Average height	**.82**	**.81**	.01	-	-
Head-bobbing	**.77**	**.77**	-.02	**.74**	-.34
Time spent (back)	**.69**	**.69**	.09	-	-
Tse-egg calls	**.42**	**.41**	-.23	**.50****	.01**
Locomotion	**-.57**	**-.56**	.08	**-.84**	.06
Time spent (front)	**-.79**	**-.81**	-.16	-	-
Egg calls	.33	.32	-.27	**.45**	**-.63**
Tsik calls	-.09	-.06	**.52**	-.07	**.83**
Tsik-egg calls	.32	.37	**.37**	**.55**	**.54**
Tse calls	-	-	-	**.50****	.01**

Table 2.1: Human Intruder test EFA and PCA. Table of human intruder test EFA factor loadings from a 1-factor extraction and a 2-factor extraction. *Human intruder test PCA variable loadings on components from Agustín-Pavón *et al.* (2012) for comparison. **Tse calls and tse-egg calls were calculated as a single variable in the PCA (Agustín-Pavón *et al.*, 2012). Significant loaders (>|.4) in bold and yellow.

Rubber snake test variables	EFA factors		PCA components*	
	"Active fear responding"	"Avoidant fear responding"	"Emotionality"	"Coping strategy"
Tsik-egg calls	**.81**	.23	.04	**.90**
Tsik calls	**.60**	.19	.11	**.91**
Stare count	**.60**	-.20	-.26	**.73**
Stare duration	**.47**	**-.44**	**-.85**	.03
Locomotion	-.09	**-.65**	**-.71**	-.04
Average distance	-.02	**.70**	**.92**	.12
Head-cock	.26	-.04	**-.64**	.16
Egg calls	.16	-.11	-	-

Table 2.2: Rubber Snake test EFA and PCA. Table of rubber snake test EFA factor loadings and *PCA factor loadings from Shiba *et al.* for comparison (2014). Significant loaders (>|.4) in bold and yellow.

Although differences in the magnitude of loadings are observed between the PCA and EFA due to underlying methodological differences, overall the underlying constructs identified by the EFAs is supported by the trends identified by the previous PCAs. Besides the substantially expanded sample size the factors derived by the EFA improve from the previous PCAs by modelling the underlying constructs instead of only simplifying the data. This leads to composite scores derived that are more meaningfully defined and parameter estimates that are more reliable and generalizable (Widaman, 1993). The EFA also determined that there's only one substantial factor underlying the behaviours in the human intruder test, instead of two suggested by the components from the PCA. In terms of the rubber snake test, we more specifically interpret the significant factors in the context of differing coping strategies in response to fear. Moving forward, subsequent work should be performed to further validate the factors identified with a new cohort via a confirmatory factor analysis (CFA).

To conclude, factors characterising anxious behaviour and fear responding were identified in the common marmoset on the human intruder and rubber snake tests. Parameter estimates from this study can be used in subsequent studies to estimate the anxiety or fear responding factors scores of animals, instead of relying on single measure to represent an animal's anxiety or fear levels. With the factors identified, we found that higher levels of anxious behaviour in the human intruder test is associated with a primarily avoidant fear responding style on the rubber snake test, implicating a link between an animal's sensitivity to uncertain threat and coping strategy under certain threat. Our findings of distinct factors representing avoidant and active fear responding support the bimodal theory of defensive behaviour under high stress and threatening context. These findings demonstrate the importance of

analysing an animal's full repertoire of behaviour when trying to study specific underlying constructs. Not only are the factor scores more reliable than single behavioural measures but taking an analytical approach to modelling behaviour can lead to novel findings neglected when only evaluating single behavioural measures.

Chapter 3: The relationship between serotonergic gene expression and anxiety and fear behaviour

Introduction: The serotonergic system has been implicated in the regulation of anxious and fear behaviour. To elucidate the role of the serotonin system in threat-related responses, serotonergic gene expression in specific regions of interest that have been implicated in anxious behaviour and fear response was investigated. In addition, this study also studies the effect on serotonin brain function of the recently discovered marmoset serotonin transporter gene (*SLC6A4*) polymorphism, which is functionally homologous to the human 5-HTTLPR polymorphism, and has been associated with anxiety, gene expression and serotonin receptors binding.

Methods: In a cohort of 12 animals, qRT-PCR was used to measure RNA levels of target genes within the medial prefrontal (mPFC), orbitofrontal (OFC), ventrolateral (vlPFC), and dorsal anterior cingulate cortices (dACC), amygdala, and the dorsal and median raphe nuclei, the latter where serotonergic cell bodies lie. We targeted genes of serotonergic components implicated in anxiety: the serotonin transporter (*SLC6A4*) and 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors (*HTR1A, HTR2A, HTR2C*). The relationship between expression of these genes of interest and anxiety score on the human intruder test of anxiety and threat responses displayed on the rubber snake test was determined. The potential effect of the serotonin transporter polymorphism on *SLC6A4* gene expression within the regions of interest was also investigated.

Results: *SLC6A4* expression in the right amygdala and right vlPFC correlated positively with anxiety, and right mPFC *HTR2A* expression correlates positively with avoidant fear responding. These associations were driven by an overall change in anxiety and fear responding behaviours and not driven by a minority of loading behaviours. Moreover, *SLC6A4* expression in the right amygdala was higher in the AC homozygotes compared to CT homozygotes of the serotonin transporter polymorphism.

Conclusion: High *SLC6A4* expression in the amygdala and vlPFC corresponding with high anxiety supports the theory that low serotonin in critical regions of the brain lead to high anxiety. The finding that higher *HTR2A* expression in the mPFC corresponded to higher levels of avoidant fear response suggests upregulation of *HTR2A* expression as a compensatory mechanism to avoidant fear behaviour. Finally, the serotonin transporter polymorphism may play a role in the differential expression of *SLC6A4* in the amygdala associated with anxious behaviour. These findings emphasised the role of serotonergic genetic mechanisms in threat-related behaviours.

3.1 Introduction

Anxiety and fear are evolutionary adaptive emotional responses towards an uncertain threat in a hostile environment, but why are some less able to regulate this response despite being in non-threatening situations, leading to pathological forms of these responses in the form of anxiety disorders? Some anxiety disorders are characterised by excessive anxiety e.g. generalized anxiety disorder (GAD) and obsessive compulsive disorder, whereas other anxiety disorders such as phobic disorder, social anxiety disorder and post-traumatic stress disorder are characterised by both a heightened level of anxiety and fear (Forster *et al.*, 2012). These neuropsychiatric disorders are associated with dysfunction in an overlapping variety of brain regions. Specifically, the serotonergic brain circuitry has been heavily implicated in aversive emotional processing in both humans and animals (Cools, Roberts and Robbins, 2008).

The 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors and the serotonin transporter have been particularly implicated in the regulation of threat-related emotional response as discussed in *"1.4 Serotonin"*. Our lab recently found that marmosets with high trait anxiety show reduced extracellular serotonin levels in the amygdala in response to SSRIs, implicating potential alterations in the release of serotonin in the expression of high trait anxiety (Mikheenko *et al.*, 2015). The human and macaque serotonin transporter gene (*SLC6A4*) polymorphism, 5-HTTLPR has been implicated in trait anxiety and reduced serotonin transporter expression and high anxiety in humans and macaques. Recently, Santangelo *et al*. (2016) identified a double nucleotide polymorphism (−2053AC/CT) and two single-nucleotide polymorphisms (−2022C/T and − 1592G/C) within the marmoset *SLC6A4* repeat upstream region. The AC/C/G haplotype was associated with lower *SLC6A4* expression in blood lymphocytes and a high anxiety score on the human intruder test, with the CT/T/C haplotype showing the opposite. These results characterise a serotonin transporter promoter polymorphism in marmosets associated with differential gene expression and anxiety levels, acting as a functional analogue to the human and macaque 5-HTTLPR polymorphism.

At the neural level, the "short" allele of the 5-HTTLPR is linked to stronger amygdala activation to aversive stimuli and greater coupling between the amygdala and the medial prefrontal cortex (mPFC) (Heinz *et al.*, 2005; Madsen *et al.*, 2016). "Short" allele carriers also showed reduced grey matter in the pregenual anterior cingulate cortex (pgACC) and amygdala, and significantly less functional coupling between these regions (Pezawas *et al.*, 2005). These results suggest a link between serotonin transporter expression and functional and anatomical alterations in the prefrontal-cingulate-amygdala circuitry. Prefrontal-cingulate-amygdala circuitry is heavily implicated in emotional regulation as demonstrated by emotional task-specific functional connections of the amygdala with regions including the medial, orbital, and ventrolateral sections of the prefrontal cortex in humans (Banks *et al.*, 2007; Prater *et al.*,

2013; Gold, Morey and McCarthy, 2015). Similarly, impoverished ventral PFC recruitment and increased amygdala responsivity were linked independently with high trait anxiety (Indovina *et al.*, 2011). The dorsal medial prefrontal cortex (dmPFC) and dorsal anterior cingulate cortices' (dACC) connectivity to the amygdala has also been implicated in an "aversive amplification" circuit of anxiety, with negative bias to fearful stimuli under serotonin precursor tryptophan depletion corresponding with an increase in functional connectivity in the circuitry (Robinson *et al.*, 2013).

Taken together, these findings support the proposed model of dysfunction of the frontal-amygdala circuit in high trait anxiety (Indovina *et al.*, 2011). The model proposes that high anxiety is primarily a result of reduced top-down (prefrontal cortices) and enhanced bottom-up (amygdala) emotion regulation (Bishop *et al.*, 2004; Bishop, 2007). Alterations in this circuit have also been demonstrated in clinically anxious populations (Shin, Rauch and Pitman, 2006; Phillips, Ladouceur and Drevets, 2008; Tromp *et al.*, 2012; Robinson *et al.*, 2014).

The study of the serotonergic system underlying the expression of anxiety in these critical regions of the emotion regulation circuit will therefore expand our understanding of pathological anxiety and thus, help to improve treatment of mood disorders. Using the anxiety factor score derived from the exploratory factor analysis on the human intruder test, potential associations between expression of *SLC6A4* and several other critical serotonergic genes implicated in threat-related behaviour and anxiety-driven behaviour was investigated, to study the role of the serotonin system in anxious behaviour. Considering serotonin's involvement in not only anxiety but also fear, the fear responding factor scores derived in the rubber snake test was also used to explore associations between the target serotonergic genes and fear behaviour. Our target genes include *SLC6A4*, the 5-HT_{1A} receptor (*HTR1A*), the 5-HT_{2A} receptor (*HTR2A*), and the 5-HT_{2C} receptor (*HTR2C*). This project also seeks to extend findings on the marmoset serotonin transporter polymorphism using a cohort of marmosets with measures of anxious behaviour on the human intruder test from Shiba *et al.* (2014). Our brain regions of interest include: the medial prefrontal cortex (mPFC), the orbital frontal cortex (OFC), the ventral lateral prefrontal cortex (vlPFC), the dACC, the amygdala, and the dorsal and median raphe nuclei (DRN and MRN), the latter where serotonergic cell bodies lie.

3.2 Methods

Subjects

The cohort consisted of twelve common marmosets, *Callithrix Jacchus* (age: 3.82 ± 0.55 years; gender: 5 females and 7 males). Animals were genotyped for the serotonin transporter polymorphism by Santangelo *et al.* (2016). The animals were grouped into three serotonin transporter polymorphism groups, (i) AC homozygotes (animals with only AC/C/G alleles, N = 4), (ii) heterozygotes (animals with a AC/C/G allele and a CT/C/G or CT/T/C allele, N = 4), and (iii) CT homozygotes (animals with CT/T/C and CT/C/G alleles, N = 4). Tissue from the left dACC (female, heterozygote) and DRN (male, CT homozygote) of 1 animal respectively were not included as they were used for other tests prior to the initiation of this study. Prior to the extraction of their brain tissue, all animals of this cohort underwent the same series of behavioural testing. The cohort underwent testing on the human intruder test and rubber snake test as part of the population's screening procedure. The animals also had a telemetry device implanted and underwent pavlovian discriminative conditioning and cognitive flexibility tests as part of Shiba *et al.*'s cohort (2014).

The animals were housed at the Innes Marmoset Colony (Behavioural and Clinical Neuroscience Institute, BCNI) with temperature (22 ± 1 °C) and humidity (50 ± 1%) conditions controlled and a dawn/dusk-like 12 hour-period maintained. The animals were housed as male-female pairs (males were vasectomised) in cages of dimensions: 92cm (high) x 60 cm (wide) x 98cm and 73cm (sides) and were provided with a balanced diet and water ad libitum. Their cages contained a variety of environmental enrichment, including suspended ladders, wooden branches and ropes to climb and swing on and boxes. All procedures were performed in accordance with the project and personal licenses held by the authors under the UK Animals (Scientific Procedures) Act 1986 and the local AWERB policies.

Genotyping

Animals were genotyped using hair follicles plucked from the animals' back by Andrea Santangelo. Hair follicles were used as DNA collected from these samples showed lower levels of chimaerism and more accurately reflects brain genotype compared to tissues derived from the hematopoietic lineage (Sasaki *et al.*, 2009; Sweeney *et al.*, 2012; Santangelo *et al.*, 2016). DNA was extracted from the samples via the QIAamp DNA Micro kit. Subsequently, the product of polymerase chain reaction (PCR) with primers flanking the *SLC6A4* repeat region was used to enable the isolation of the targeted DNA segment in an agarose gel and purified before being sent for sequencing (Source BioScience, Cambridge, UK). The primer sequences can be found in Santangelo *et al.*(2016).

Expression assay

RNA extraction. Brain tissue samples from Shiba *et al.'s* (2014) cohort were dissected, snap frozen with liquid nitrogen and preserved in a -80 °C freezer. Total RNA was extracted with the RNeasy Plus Universal Mini Kit from QIAGEN in accordance to the manufacturer's protocol. Dissection of the regions of interest was conducted based on neuroanatomical landmarks and illustrated in figure 3.1. The brain tissue samples were weighed and then disrupted and homogenized with a TissureRuptor (QIAGEN). After separating the phases and a series of washes, the total RNA was eluted with 100 uL of RNase-free water. The RNA was kept in a -80 °C freezer and diluted in aliquots before use. Dilutions were determined based on the lowest total RNA concentration extracted among the samples as measured by spectrophotometry.

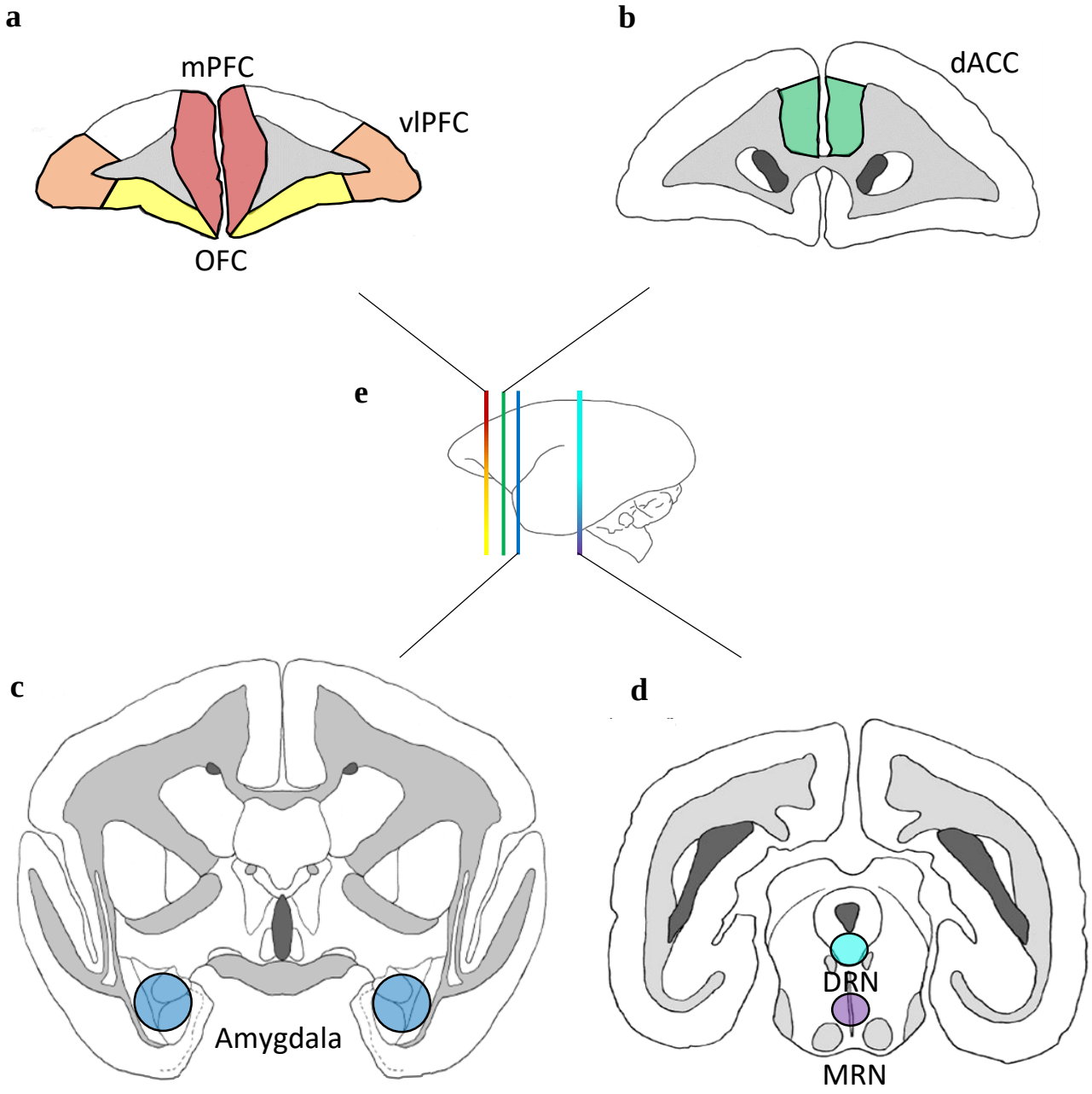


Figure 3.1: Brain regions of interest. Brain tissue dissection of the regions of interest (coronal view) with respective anterior/posterior coordinates: (a) AP = +15.8mm: mPFC (red), vlPFC (orange), OFC (yellow); (b) AP = +13.2mm: dACC (green); (c) AP = +9.6mm: amygdala (blue); (d) AP = +2.0mm: DRN (teal), MRN (purple).

qRT-PCR. The relative expression of *HTR1A, HTR2A, HTR2C, and SLC6A4* were quantified using qRT-PCR with Brilliant II SYBR Green qRT-PCR Master Mix Kit, 1-Step by Agilent Technologies and the Bio-RAD CFX96 Touch Real-Time PCR Detection System. Protocol of primer design for the target genes and the four reference genes used is discussed below. The optimal primer concentration was determined at 100 nM, which presented no primer-dimers formation while retaining high cycle thresholds (Ct) for the amplification of the target gene. All reactions were performed in duplicates for each individual samples and controls (triplicates for inter-run calibrators). The qRT-PCR program used: cDNA synthesis (30 min at 50 °C), RT-polymerase inactivation and DNA polymerase activation step (10 min at 95 °C), 40 two-step amplification cycles (denaturation for 30 s at 95 °C and combined annealing/extension for 1 min at 60 °C), and a final incubation step (temperature increased from 55 °C to 95 °C in 0.5 °C increments) for the construction of the melting curves.

Development of target serotonergic primer and reference primer validation. Primers for the target genes were designed with the aid of Primer-BLAST, a software tool from the National Centre for Biotechnology Information (NCBI) (Ye *et al.*, 2012). The predicted mRNA sequence used to design the primers were obtained from the NCBI's Reference Sequence (RefSeq) database or the Ensembl database (Pruitt *et al.*, 2012; Flicek *et al.*, 2014). The primer parameters for the Primer-BLAST was set to a product size of 90 – 110 bases. The melting temperature was set to a minimum of 55 °C, a maximum of 65 °C, an optimal temperature of 57 °C, and a maximum melting temperature difference between the forward and reverse primer of 3 °C. One of the primer pairs was specified to span an exon-exon junction for the specific amplification of RNA. Primer annealing sites were also selected to be located on spans of RNA sequence identical on all transcript variants. A minimum of 7 bases must anneal to exons at the 5' side of the junction and 4 bases at the 3' side of the junction. Candidate primers were selected from the output based on optimal G/C content, low self-complementarity, low 3' self-complementarity, and no off-target products. Subsequently, suitable candidate primers were ordered from Sigma-Aldrich and suspended before being kept in a -80 °C freezer in aliquots of 100 µM.

The specificity and efficiency of the candidate primers were tested via quantitative real-time polymerase chain reaction (qRT-PCR) with Brilliant II SYBR Green qRT-PCR Master Mix Kit, 1-Step by Agilent Technologies and the Bio-RAD CFX96 Touch Real-Time PCR Detection System. For the qRT-PCR run protocol, refer to the section: qRT-PCR reaction above. Primers were considered specific if they produced a single melting peak and were further validated via gel electrophoresis (3% agarose gel) to produce a single band at the estimated band size. The efficiency of the primers was calculated by the construction of standard curves derived from 5-point serial dilutions. Only primers with an efficiency of approximately 85%-115% were selected.

Four marmoset-specific reference genes were selected based on their expression stability in published papers evaluating their use as qRT-PCR reference genes with marmoset brain tissue: *ACTB* (β-Actin), *TBP* (TATA-box binding protein), and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) (Shimamoto *et al*. 2013); and *SDHA* (succinate dehydrogenase complex, subunit A) (Fujii *et al.*, 2013; Shimamoto *et al*., 2013). The specificity and efficiency of the reference genes were tested as described above. Details of the primers used for the target genes and reference genes are shown in Table 3.1 and 3.2 respectively.

Gene symbol	Gene name	Accession number	Primer pairs	Product size (bp)	Efficiency (%)
HTR1A	Serotonin receptor 1A	XM_008992005.1	F: CATGCACCATTAGCAAGGAC R: GGAATATGCGCCCATAGAGA	102	101.4
HTR2A	Serotonin receptor 2A	ENSCJAG00000009349	F: GCAGAATGCCACCAACTATT R: CGGTATCCATACAGGATGGT	105	99.0
HTR2C	Serotonin receptor 2C	XM_002763170.2	F: TCGTTCCTTGTGCACCTAAT R: CCACCATCGGAGGTATTGAA	104	100.2
SLC6A4	Serotonin Transporter	XM_008997143.1	F: GTTCTACGGCATCACTCAGTTC R: GCTGATGGCCACCCAGCAGATC	94	91.4

Table 3.1: Target genes and primers. Sequences of primers used to target the genes of interest in the expression assay. F: forward primer; R: reverse primer.

Gene symbol	Gene name	Accession number	Primer pairs	Product size (bp)	Efficiency (%)
ACTB	β-Actin	DD279463	F: AGCAGTCGGTTGGAGCGAGCAT R: TGGCTTTTGGGAGGGCAAGGGA	139	99.9
TBP	TATA box binding protein	ENSCJAT00000040537	F: GCCCGAAATGCCGAATATAA R: TTCTTCACTCTTGGCTCCTGTG	126	93.3
GAPDH	Glyceraldehyde-3-phosphate	XM_002759682	F: TAAGACCCCCTGGACCATCAGCC R: GGGGCAATTCGGTGTGGTGA	106	104
SDHA	Succinate dehydrogenase complex, subunit A	XM_002745154	F: TGGGAACAAGAGGGCATCTG R: CCACCACGGCATCAAATTCATG	86	101.8

Table 3.2: Reference genes and primers. Sequences of primers used to target the reference genes. F: forward primer; R: reverse primer.

EFA scores – Anxiety, active fear responding and avoidant fear responding

EFA scores were derived using an exploratory factor analysis with 171 common marmosets (*Callithrix jacchus*; male = 90, female = 81; age: 2.29 ± 0.62) screened for their behaviour on the human intruder test and 151 common marmosets (male = 77, female = 74; age: 2.51 ± 0.68) screened on the rubber snake test. A factor representing anxious behaviour was extracted from the array of behavioural variables measured in the human intruder test and factors representing active and avoidant threat responding were extracted from behavioural variables measured in the rubber snake test. Full description of EFA scores included in the chapter 2: "*Anxiety and fear response in the common marmoset*". Factor scores and behavioural scores of individual animals shown in table 3.3 and 3.4.

Animal	Anxiety score	**Average Height (cm)**	**Time spent at front (%)**	**Time spent at back (%)**	**Locomotion (%)**	**Head-bobs**	**Tse-egg calls**	Egg calls	Tsik-egg calls	Tsik calls
1	2.31	**81.97**	**2**	**98**	**1.77**	**100**	**52**	3	23	0
2	2.02	**82.32**	**0**	**99**	**0.3**	**76**	**41**	1	40	0
3	1.82	**80.33**	**0**	**99**	**1.54**	**63**	**58**	1	8	2
4	0.96	**67.49**	**11**	**38**	**7.85**	**64**	**4**	3	6	0
5	0.94	**76.22**	**23**	**59**	**7.43**	**53**	**7**	1	0	0
6	0.56	**68.35**	**48**	**38**	**4.8**	**55**	**22**	12	0	0
7	-0.44	**54.84**	**32**	**38**	**17.66**	**9**	**4**	1	2	7
8	-0.74	**41.81**	**49**	**16**	**10.48**	**20**	**6**	2	3	12
9	-0.74	**45.31**	**38**	**12**	**20.15**	**19**	**2**	0	2	14
10	-1.46	**34.00**	**77**	**11**	**16.25**	**9**	**0**	2	0	0
11	-1.55	**51.21**	**74**	**0**	**37.62**	**1**	**0**	0	0	0
12	-1.63	**35.03**	**67**	**19**	**28.46**	**0**	**0**	0	0	9

Table 3.3: Anxiety factor. Anxiety factor scores of individual animals derived from behavioural scores in the human intruder test. Behaviours that load significantly on the factor scores in bold.

Animal	Active fear score	Avoidant fear score	**Average Distance (cm)**	**Stare Count**	**Stare Duration (%)**	**Locomotion (%)**	Head-cock	**Tsik-egg calls**	**Tsik calls**	Egg calls
8	2.74	0.92	**70.6**	**62**	**23.6**	**3.6**	8	**210**	**138**	0
6	1.43	1.26	**83.4**	**45**	**21.8**	**3.0**	9	**151**	**79**	0
4	0.96	-0.37	**53.7**	**36**	**31.4**	**10.1**	21	**93**	**82**	2
9	0.73	0.55	**75.7**	**39**	**31.3**	**4.8**	25	**73**	**61**	0
11	0.64	-0.70	**47.2**	**39**	**37.9**	**9.0**	15	**85**	**8**	0
1	0.17	0.21	**72.1**	**31**	**46.0**	**3.4**	20	**44**	**2**	2
7	0.00	0.78	**85.9**	**35**	**33.8**	**3.4**	13	**14**	**80**	0
10	-0.04	0.14	**62.6**	**27**	**28.1**	**6.4**	22	**27**	**70**	0
12	-0.35	-1.16	**50.3**	**34**	**35.3**	**13.8**	15	**5**	**14**	0
3	-0.36	0.76	**73.0**	**27**	**22.2**	**2.7**	8	**35**	**29**	1
2	-0.48	-0.24	**55.6**	**27**	**45.2**	**3.2**	11	**0**	**0**	0
5	-0.74	1.91	**115.4**	**34**	**17.1**	**0.7**	5	**14**	**0**	1

Table 3.4: Fear responding factor. Active and avoidant fear responding factor scores of individual animals derived from behavioural scores in the rubber snake test (arranged in descending order of active fear score). Behaviours that load significantly on the factor scores in bold and coloured according to corresponding factor scores (Red: active fear; Blue: avoidant fear; Purple: both active and avoidant fear).

Statistical analyses

All statistical analyses were performed using SPSS (version 24; IBM Corp., Armonk, NY). Correlations between factors were calculated with Pearson product-moment correlation coefficient and differences between group means were calculated using one-way ANOVA. Multiple comparisons for the exploratory correlations between each target gene and factor score were accounted for using the Šidák correction for the number of areas (12) with adjusted alpha levels of α_{adj} = .0043. Effect sizes for significant findings were computed based on Cohen's (1988, 1992) method.

3.3 Results

SLC6A4 in the right amygdala and right vlPFC correlates with anxiety. Right mPFC *HTR2A* expression correlates with avoidant fear responding.

First, it was determined if the target gene's expression was associated with the factor scores for anxious and fearful behaviour on the human intruder and rubber snake test respectively. Pearson product-moment correlation coefficient was computed to analyse the relationship between expression of target genes in the brain regions of interest and the anxiety score from the human intruder test and threat responding scores from the rubber snake test (tables 3.5, 3.6 and 3.7).

	Target gene expression correlation with anxiety score			
Region	***HTR1A***	***HTR2A***	***HTR2C***	***SLC6A4***
Right mPFC	r = -.030 p = .925	r = .191 p = .552	r = -.182 p = .571	r = -.309 p = .328
Left mPFC	r = .340 p = .280	r = -.216 p = .499	r = -.046 p = .887	r = .353 p = .260
Right OFC	r = .109 p = .737	r = -.344 p = .273	r = -.001 p = .998	r = .586 p = .045
Left OFC	r = -.358 p = .253	r = -.755 p = .0045	r = .060 p = .854	r = .661 p = .019
Right vlPFC	r = .139 p = .667	r = .058 p = .857	r = .059 p = .855	**r = .806** **p = .002**
Left vlPFC	r = -.282 p = .374	r = -.394 p = .205	r = .184 p = .568	r = -.315 p = .318
Right dACC	r = .334 p = .289	r = -.145 p = .652	r = .587 p = .045	r = -.016 p = .960
Left dACC	r = -.210 p = .536	r = -.580 p = .061	r = .392 p = .233	r = .457 p = .158
Right Amygdala	r = -.195 p = .543	r = .647 p = .023	r = -.516 p = .086	**r = .809** **p = .001**
Left Amygdala	r = -.026 p = .936	r = .244 p = .445	r = -.210 p = .513	r = .005 p = .987
Dorsal Raphe Nucleus	r = -.032 p = .926	r = .464 p = .151	r = .622 p = .041	r = .168 p = .622
Median Raphe Nucleus	r = -.129 p = .689	r = -.258 p = .419	r = .249 p = .436	r = -.343 p = .276

Table 3.5: Correlation between target gene expression in each region of interest and anxiety score on human intruder test (correlation coefficients, *r* and unadjusted p-values, *p*). *SLC6A4* expression in the right vlPFC and right amygdala were significantly correlated with anxiety scores on the human intruder test (**, bold, $p < .0043$, $p_{corr} < .05$).

	Target gene expression correlation with active fear responding score			
Region	***HTR1A***	***HTR2A***	***HTR2C***	***SLC6A4***
Right mPFC	*r* = .206 *p* = .520	*r* = -.316 *p* = .317	*r* = .449 *p* = .144	*r* = .376 *p* = .229
Left mPFC	*r* = -.230 *p* = .472	*r* = -.129 *p* = .689	*r* = .508 *p* = .092	*r* = .545 *p* = .067
Right OFC	*r* = .129 *p* = .691	*r* = .075 *p* = .817	*r* = .290 *p* = .360	*r* = -.092 *p* = .776
Left OFC	*r* = .027 *p* = .932	*r* = -.029 *p* = .928	*r* = .605 *p* = .037	*r* = .201 *p* = .530
Right vlPFC	*r* = -.076 *p* = .815	*r* = -.319 *p* = .312	*r* = -.316 *p* = .317	*r* = -.159 *p* = .621
Left vlPFC	*r* = -.145 *p* = .653	*r* = .390 *p* = .210	*r* = -.118 *p* = .716	*r* = .701 *p* = .011
Right dACC	*r* = -.528 *p* = .077	*r* = -.374 *p* = .231	*r* = -.271 *p* = .395	*r* = .502 *p* = .097
Left dACC	*r* = -.198 *p* = .559	*r* = -.248 *p* = .463	*r* = .026 *p* = .940	*r* = -.085 *p* = .803
Right Amygdala	*r* = -.252 *p* = .430	*r* = -.265 *p* = .405	*r* = .450 *p* = .143	*r* = -.212 *p* = .509
Left Amygdala	*r* = -.285 *p* = .370	*r* = -.158 *p* = .624	*r* = -.015 *p* = .963	*r* = -.274 *p* = .388
Dorsal Raphe Nucleus	*r* = .309 *p* = .355	*r* = -.537 *p* = .088	*r* = .047 *p* = .891	*r* = .443 *p* = .172
Median Raphe Nucleus	*r* = -.195 *p* = .543	*r* = -.316 *p* = .316	*r* = -.440 *p* = .152	*r* = .285 *p* = .370

Table 3.6: Correlation between target gene expression in each region of interest and active fear responding score on rubber snake test (correlation coefficients, *r* and unadjusted p-values, *p*). None of the target gene expression levels were significantly correlated with active fear responding scores on the rubber snake test (p > .0043, pcorr > .05).

	Target gene expression correlation with avoidant fear responding score			
Region	***HTR1A***	***HTR2A***	***HTR2C***	***SLC6A4***
Right mPFC	r = .243 p = .447	**r = .787** **p = .002**	r = .031 p = .923	r = .129 p = .689
Left mPFC	r = .143 p = .657	r = .029 p = .929	r = .285 p = .369	r = -.034 p = .916
Right OFC	r = .573 p = .051	r = -.503 p = .095	r = -.230 p = .472	r = .259 p = .417
Left OFC	r = -.251 p = .432	r = -.360 p = .250	r = .127 p = .695	r = .697 p = .012
Right vlPFC	r = .098 p = .763	r = -.112 p = .728	r = .055 p = .865	r = .424 p = .170
Left vlPFC	r = .172 p = .593	r = -.075 p = .817	r = .215 p = .502	r = -.136 p = .673
Right dACC	r = .252 p = .430	r = -.034 p = .915	r = .369 p = .237	r = .260 p = .415
Left dACC	r = -.079 p = .818	r = -.292 p = .384	r = .303 p = .366	r = -.083 p = .809
Right Amygdala	r = .068 p = .835	r = .428 p = .165	r = -.008 p = .980	r = .319 p = .312
Left Amygdala	r = .112 p = .729	r = .188 p = .558	r = .293 p = .355	r = -.137 p = .670
Dorsal Raphe Nucleus	r = .291 p = .385	r = .382 p = .247	r = .231 p = .494	r = .265 p = .430
Median Raphe Nucleus	r = .072 p = .824	r = -.127 p = .694	r = -.231 p = .470	r = .362 p = .248

Table 3.7: Correlation between target gene expression in each region of interest and avoidant fear responding score on rubber snake test (correlation coefficients, *r* and unadjusted p-values, *p*). HTR2A expression in the right mPFC was significantly correlated with avoidant fear responding scores on the rubber snake test (**, bold, $p < .0043$, $p_{corr} < .05$).

SLC6A4 expression in the right amygdala (r = .809, p = .001, $p_{corr} < .05$) and right vlPFC (r = .806, p = .002, $p_{corr} < .05$) is significantly positively correlated with anxiety with a large effect size according to Cohen's criteria ($r > .50$) (figure 3.2ai and 3.2aii) (1988, 1992). *HTR2A* expression in the right mPFC is significantly positively correlated with avoidant fear responding (r = .787, p = .002, $p_{corr} < .05$) with a large effect size ($r > .50$) (figure 3.2b).

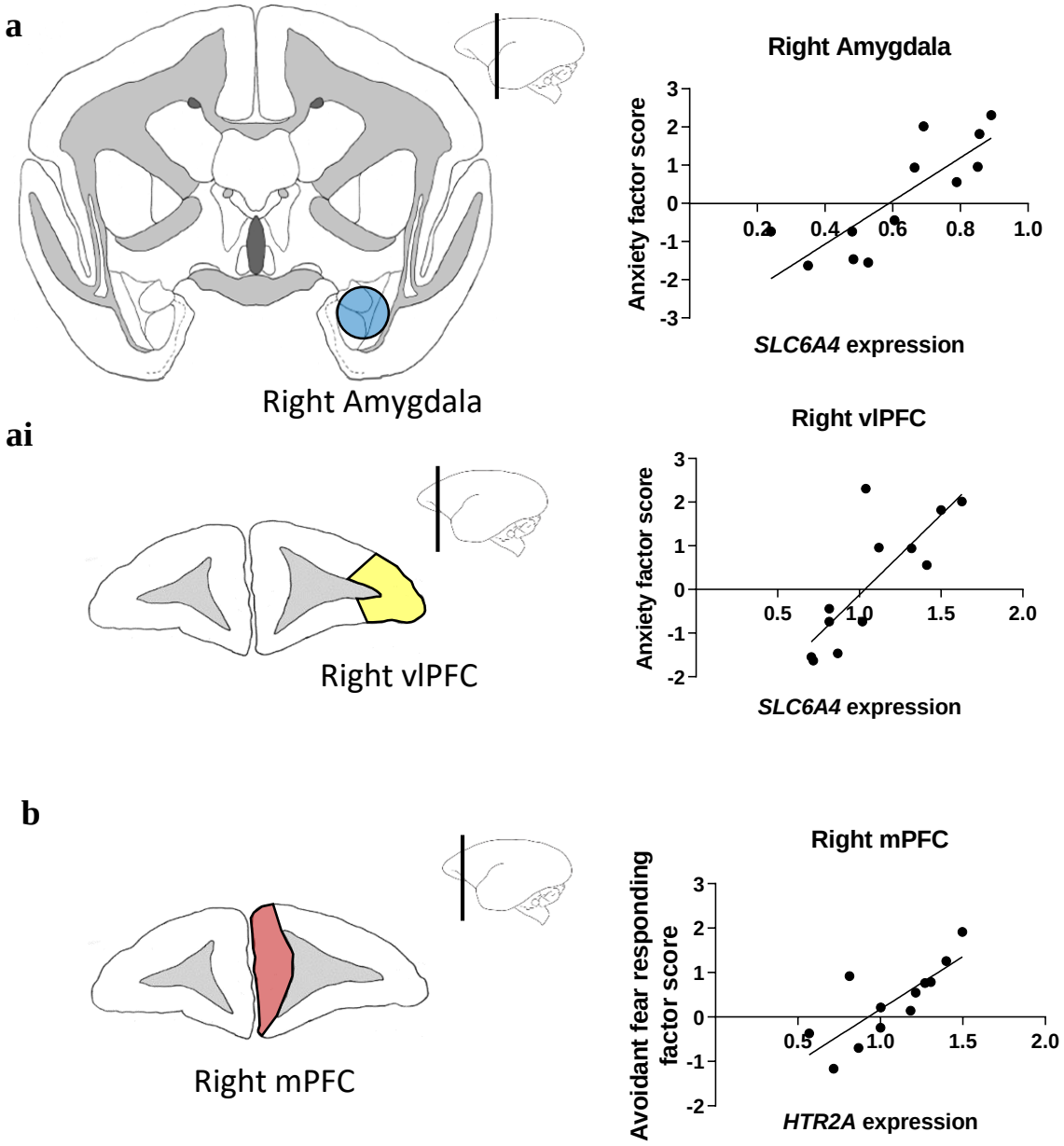


Figure 3.2: Region specific association of gene expression and anxious behaviour. Significant correlations were found between (a) anxiety and both (i) right amygdala and (ii) and right vlPFC *SLC6A4* expression, and between (b) avoidant threat responding and right mPFC *HTR2A* expression ($p < .0043$, $p_{corr} < .05$).

Factor-associated expression is driven by an overall change in behaviour.

To determine if the significant correlations found between the target genes and factor scores were generally associated or driven by only a specific subset of the underlying variables, the correlation between the right amygdala *SLC6A4* expression, right vlPFC *SLC6A4* expression, and right mPFC *HTR2A* expression, and respective significantly-loading variables were determined. As shown in table 3.8, right amygdala and vlPFC SLC6A4 expression were both correlated with all variables that load significantly on the anxiety factor score ($p < .05$). In contrast, right mPFC was correlated with two out of three variables that load significantly on the avoidant fear responding factor (average distance and locomotion, $p < .05$). This may be due to stare durations relatively lower factor loading (|.44|) compared to average distance (|.70|) and locomotion (|.65|) (table 3.9).

Anxiety factor score variables	*SLC6A4* expression	
	Right Amygdala	Right vlPFC
Average Height	$r = .858^{**}$ $p < .001$	$r = .794^{**}$ $p = .002$
Time spent at front	$r = -.687^{*}$ $p = .014$	$r = -.683^{*}$ $p = .014$
Time spent at back	$r = .714^{**}$ $p = .009$	$r = .756^{**}$ $p = .004$
Locomotion	$r = -.695^{*}$ $p = .012$	$r = -.841^{**}$ $p = .001$
Head-bobs	$r = .768^{**}$ $p = .004$	$r = .743^{**}$ $p = .006$
Tse-egg calls	$r = .660^{*}$ $p = .020$	$r = .687^{*}$ $p = .013$

Table 3.8: Correlation between right amygdala and vlPFC *SLC6A4* expression, and anxiety factor score variables (correlation coefficient, *r*; p-value, *p*). Right vlPFC and right amygdala *SLC6A4* expression is correlated with all the variables that load significantly on the anxiety factor score (*, $p = .05$; **, $p = .01$).

Avoidant fear responding variables	Right mPFC *HTR2A* expression
Average distance	r = .807**
	p = .002
Locomotion	r = -.756**
	p = .004
Stare duration	r = -.468
	p = .125

Table 3.9: Correlation between right mPFC *HTR2A* expression and avoidant fear responding variables (correlation coefficient, *r*; p-value, *p*). Among the variables that load significantly on the avoidant fear responding factor, right mPFC *HTR2A* expression was correlated with average distance and locomotion (**, $p < .01$) but not stare duration ($p > .05$).

Differences in right amygdala *SLC6A4* expression and anxious behaviour is associated with the serotonin transporter polymorphism

Given that *SLC6A4* expression in the amygdala and vlPFC is correlated with anxious behaviour and genetic factors affecting *SLC6A4* expression are associated with high trait anxiety in both humans and marmosets it was determined whether the recently discovered marmoset serotonin transporter polymorphism would be associated with this differential amygdala and vlPFC *SLC6A4* expression.

The animals were grouped according to the double nucleotide polymorphism (−2053AC/CT): i) AC homozygotes (AC/C/G), ii) heterozygotes (AC/C/G & CT/C/G and AC/C/G & CT/T/C), and iii) CT homozygotes (CT/T/C and CT/T/C & CT/C/G). Since the assumption of homogeneity of variance, as revealed by Levene's F test, was not met for right amygdala ($F(2,9) = 14.78$, $p = .001$) and right vlPFC ($F(2,9) = 4.90$, $p = .036$) SLC6A4 expression, the Welch's F test was used. There was a significant difference between right amygdala *SLC6A4* expression of the serotonin transporter polymorphism groups ($F_w(2,5.4) = 20.93$, $p = .003$, $p_{corr} < .05$) but not right vlPFC ($F_w(2,5.3) = 4.85$, $p = .06$, $p_{corr} > .05$). The Games-Howell post-hoc procedure revealed that amygdala *SLC6A4* expression was significantly higher in AC homozygotes (1.75 ± .19) compared to CT homozygotes (.80 ± .20), $p = .001$) (figure 3.3a). Cohen's effect size value ($d = 4.83$) suggested an effect of high practical significance ($d > 0.8$). *SLC6A4* expression in other regions of interest that did not correlate with anxiety were also not statistically different between the serotonin transporter polymorphism groups ($p_{corr} > .05$) (table 3.10).

Brain region	*SLC6A4* expression of AC homozygotes, heterozygotes, and CT homozygotes
Right mPFC	$F(2,9) = 1.54$ $p = .27$
Left mPFC	$F(2,9) = 0.86$ $p = .46$
Right OFC	$F(2,9) = 4.70$ $p = .04$
Left OFC	$F(2,9) = 1.60$ $p = .26$
Right vlPFC	$F_w(2,5.3) = 4.85$ $p = .06$
Left vlPFC	$F(2,9) = 0.25$ $p = .79$
Right dACC	$F(2,9) = 0.89$ $p = .44$
Left dACC	$F(2,8) = 5.63$ $p = .03$
Right Amygdala	**F_w (2,5.4) = 20.93** **p = .003**
Left Amygdala	F_w (2,5.3) = 1.07 $p = .41$
Dorsal Raphe Nucleus	$F(2,8) = 0.49$ $p = .63$
Median Raphe Nucleus	$F(2,9) = 1.43$ $p = .29$

Table 3.10: Potential genotype-associated difference in *SLC6A4* expression across regions of interest. Results of one-way ANOVA (F) or Welch test (F_w) (when the assumption of homogeneity of variance was violated) determining if SLC6A4 mRNA levels in each region of interest were significantly different between serotonin transporter polymorphism groups with p-values, p (unadjusted). *SLC6A4* expression in the right amygdala was significantly different between the different serotonin transporter polymorphism groups (bold, $p < .0043$, $p_{corr} < .05$).

Consistent with findings here of the correlation between right amygdala *SLC6A4* expression and anxiety and replicating previous findings from the lab by Santangelo *et al.* (2016), there was a statistically significant difference between the anxiety score of the serotonin transporter polymorphism groups as determined by one-way ANOVA ($F(2,9) = 8.16$, $p = .01$). A Tukey post-hoc test revealed that the anxiety score was significantly higher in AC homozygotes (1.55 ± .71, $p = .007$) compared to CT homozygotes (-1.16 ± .49), $p = .007$, $d = 4.44$ (Figure 3.3b).

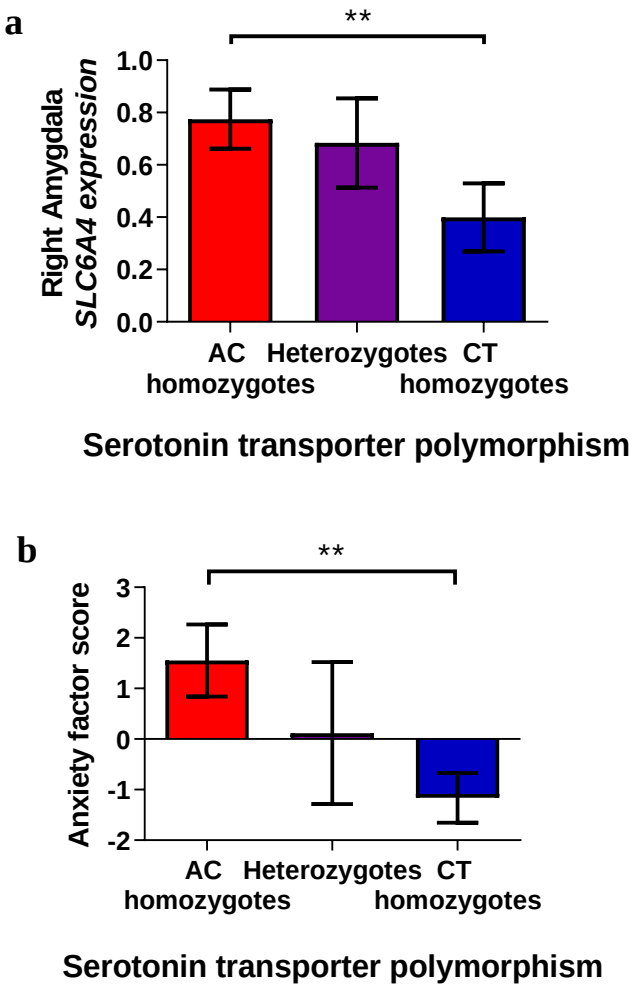


Figure 3.3 Genotype-associated differences in expression and behaviour. a) Right amygdala *SLC6A4* expression and b) anxiety score for the serotonin transporter polymorphism groups (AC homozygotes, heterozygotes, and CT homozygotes). **, $p < .01$. Error bars represent SD.

3.4 Discussion

Although the serotonergic system has been implicated in threat-related responses, the region-specific link between the expression of key serotonergic components and negative emotion regulation has not been extensively explored. The exploratory gene study here found that *SLC6A4* expression in the right amygdala and right vlPFC correlates with anxiety on the human intruder test, and *HTR2A* expression in the mPFC correlates with avoidant fear responding on the rubber snake test. Subsequent analysis revealed that these region-specific expressions were also correlated with all loading variables of the respective factor scores (except for the weakest significant loader of avoidant fear responding), indicating that the associations between expression and factor were likely due to a general effect on overall behaviour and not only driven by specific measures. The involvement of *SLC6A4* expression instead of specific serotonin receptor subtypes in anxiety may be an indicator that regulation of overall non-specific serotonergic signalling in the amygdala and vlPFC plays a more important role than the activation of specific receptor subtypes in anxious behaviour. In direct contrast, the involvement of specifically *HTR2A* expression in avoidant fear behaviour points towards the importance of the expression of excitatory serotonin 2A receptor in the mPFC compared to the other serotonin receptor subtypes investigated. Further analysis showed that anxiety-associated individual differences in right amygdala *SLC6A4* expression was also associated with the serotonin transporter polymorphism. More specifically, there was higher amygdala *SLC6A4* expression in high anxious AC homozygotes compared to low anxious CT homozygotes. The key findings at a glance shown in figure 3.4.

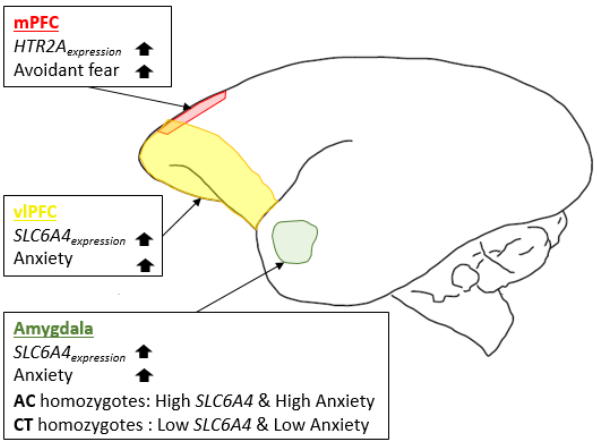


Figure 3.4: Summary of main results. mPFC *HTR2A* expression was positively correlated with passive fear coping, whereas vlPFC and amygdala *SLC6A4* expression were positively correlated with anxiety. Furthermore, AC homozygotes of the serotonin transporter polymorphism group is linked to higher amygdala *SLC6A4* expression and higher anxiety compared to CT homozygotes. Brain outline by Paxinos et al. (2012).

The finding that lower *SLC6A4* expression in the amygdala is correlated with lower anxiety scores, is consistent with the anxiolytic effect of chronic SSRIs that block *SLC6A4* reuptake of serotonin in the brain (van der Kolk *et al.*, 1994; van Vliet, den Boer and Westenberg, 1994; Boyer, 1995). This suggests that the maintenance of low serotonin levels in the amygdala in an individual, as mediated by predisposed high *SLC6A4* expression may play a role in an individual's high trait-level vulnerability to anxiety. These findings are also consistent with those of Mikheenko *et al.* (2015) of lower extracellular serotonin in the amygdala of high-anxious marmosets and with high amygdala SLC6A4 availability in high trait anxious rhesus monkeys, as measured by positron emission tomography (PET) (Oler *et al.*, 2009). Together, they suggest that amygdala *SLC6A4* expression may affect serotonin signalling in the amygdala and play a role in the inter-individual variation observed in trait anxiety. Studies with depressed patients and amygdala serotonin transporter PET binding have not been consistent with our findings: lower amygdala serotonin transporter binding was linked to high state anxiety (Reimold *et al.*, 2008) and higher amygdala reactivity (Schneck *et al.*, 2016). Consequently, subsequent work directly comparing mRNA expression and PET binding potential of amygdala serotonin transporters within depressed and healthy cohorts would elucidate the relationship of findings between these different modes of study. Furthermore, it emphasised the importance of validating our associative findings here with an investigation into the causal implications of alteration in amygdala serotonin transporter functioning in chapter 4.

Alongside the right amygdala, increased *SLC6A4* expression in the vlPFC corresponding with increased anxiety scores is also consistent with the low serotonin hypothesis of high anxiety. The vlPFC has been implicated in the top-down regulation of threat-related attention, with high anxious individuals showing reduced recruitment when expecting threat-related distractors (Bishop *et al.*, 2004). Reduced activation in the vlPFC was also observed in social anxiety disorder patients when performing a fear-evoking task (verbal fluency task) and has been shown to correlate with increased social avoidance (Yokoyama *et al.*, 2015). Taken in context, the results suggest that reduced serotonin as a product of increased SLC6A4 expression in the vlPFC may lead to reduced activation of the vlPFC and affect its ability to regulate threat-related attentional behaviour in an anxiety-provoking context.

Not so consistent with available evidence is the finding of increased mPFC HTR2A expression associated with increased avoidant fear responding which contradicts studies showing that serotonin 2A receptor activation is linked to reduced fear in rodents (Hughes, Tran and Keele, 2012; Zhang *et al.*, 2013). Serotonin 2A receptor binding potential in the pregenual PFC and subgenual PFC has also been linked to reduced threat-related amygdala reactivity, implicating serotonin 2A in the top-down regulation of amygdala reactivity to threat (Fisher *et al.*, 2009). However, the increased mPFC HTR2A expression shown here may be a compensatory product of increased avoidant fear instead of the neural substrate leading to avoidant fear responding. As serotonin 2A receptors are excitatory and primarily localized to pyramidal neurons of the PFC, increased mPFC *HTR2A* expression may act as a genetic

compensatory mechanism to upregulate activity in the region in response to an animal's experience of fear and avoidant coping style (Jakab and Goldman-Rakic, 1998; Aghajanian and Marek, 1999). However, subsequent work will have to specifically test and elucidate the validity of this hypothesis.

The finding that right amygdala *SLC6A4* expression and anxiety scores differed between the serotonin transporter polymorphism groups suggests that the latter may contribute to the individual variation in *SLC6A4* expression and anxiety. Specifically, AC homozygotes were associated with high right amygdala *SLC6A4* expression and high anxiety scores whilst CT homozygotes were associated with low right amygdala *SLC6A4* expression and low anxiety scores. These results replicate those of Santangelo *et al.* (2016) in terms of the serotonin transporter polymorphism's association with anxiety scores, but show opposing effects with respect to the polymorphisms effect on *SLC6A4* expression in lymphocytes versus *SLC6A4* expression in the amygdala. The high anxious AC homozygotes show reduced *SLC6A4* expression in lymphocytes, consistent with that shown in humans with the short allele (Lesch *et al.*, 1996), but increased *SLC6A4* expression in the right amygdala compared to CT homozygotes. The latter finding suggests that the marmoset serotonin transporter polymorphism may have a differential impact on mRNA levels within the brain, (e.g. amygdala) and lymphocytes. Further investigation revealed that *SLC6A4* expression in the dorsal and median raphe nucleus, where the serotonergic projections of the brain originate were not differentiated by the serotonin transporter polymorphism (table 3.10), suggesting that the serotonin transporter polymorphism may have a differential effect on *SLC6A4* mRNA expression at the level of the terminals in the amygdala but not the cell bodies.

Although *SLC6A4* mRNA is primarily localised in the cell body of the serotonergic neurons (close to its origin of synthesis, the nucleus), the *SLC6A4* mRNA measured at serotonergic projection areas may represent mRNA localised in the projection terminals or astrocytes (Hirst *et al.*, 1998). The localisation of mRNA to presynaptic nerve terminals has been postulated to enable local translation of synaptic proteins beyond the cell body (Akins, Berk-Rauch and Fallon, 2009). Previous studies have consistently reported the presence of *SLC6A4* mRNA in sites innervated by serotonergic neurons (Lesch *et al.*, 1993; Hernandez and Sokolov, 1997; Sun *et al.*, 2001; Beliveau *et al.*, 2017). Local translation in the presynaptic nerve terminals has been implicated in activity-dependent synaptic plasticity (Liu *et al.*, 2003; Si *et al.*, 2003; Akins, Berk-Rauch and Fallon, 2009). Recently, Younts *et al.* (2016) provide evidence of the presence of ribosomal proteins, a critical component for protein synthesis, at presynaptic terminals and evidence of presynaptic expression's involvement in plasticity. The importance of presynaptic translation in plasticity taken together with the results showing anxious behaviour correlated to *SLC6A4* expression in the amygdala but not the raphe nuclei, implicates locally regulated *SLC6A4* translation within the presynaptic nerve terminals or astrocytes, or both, across cortical and limbic regions as a mechanism for neural plasticity, in contrast to soma-dependent long term potentiation or depression (LTP or LTD), to modulate serotonin reuptake and consequently anxious

behaviour. Subsequent work should be dedicated to understanding the precise localization of the anxiety-related SLC6A4 mRNA and the mechanism through which the serotonin transporter polymorphism may affect nerve terminal *SLC6A4* mRNA expression.

The lateralization to the right hemisphere of findings here is consistent with previous findings of hemispheric asymmetry towards emotional tasks and stimuli, with the predominant theory that the right hemisphere is dominant for the perception and expression of emotion (Borod *et al.*, 1998). An alternative theory for the lateralization of emotional processing is the valence hypothesis which postulates that positive emotion processing is primarily lateralized to the left hemisphere and negative emotion processing to the right, but results in this field are far from consistent (Davidson, 1992; Canli *et al.*, 1998; Wager *et al.*, 2003).

Findings from the current study is limited by the sample size of the cohort. Subsequent work investigating subtler effects will benefit from the greater statistical power of an expanded sample size. Our current results of threat-associated gene expression would also be complemented by further work investigating potential threat-associated serotonergic protein density via immunoreactivity or positron-emission tomography (PET).

In summary, alterations in *SLC6A4* expression in the amygdala and vlPFC corresponding to anxious behaviour were identified, supporting the hypothesis that altered serotonin signalling in this area contributes to altered vulnerability to anxiety. Moreover, mPFC *HTR2A* expression was associated with avoidant fear responding, implicating upregulation of HTR2A expression as a compensatory mechanism to avoidant coping to fear. Finally, evidence that the serotonin transporter polymorphism may be driving the differential amygdala *SLC6A4* expression and corresponding high anxious behaviour were presented. The current results not only extend findings in regards to the serotonergic system's role in anxiety, but also creates the foundation for subsequent work involving the specific pharmacological manipulations of brain circuits with a new cohort.

Chapter 4: Role of amygdala serotonin transporter in the expression of trait anxiety

Introduction: High trait anxiety, an individual's disposition to feel anxious, is associated with higher risk of developing anxiety disorders and altered activity across cortical and amygdala circuitry. Since alteration in local serotonin signalling may play a key role in an individual's level of trait anxiety, I investigated region-specific serotonergic manipulation that may underlie the high trait anxious phenotype.

Methods: Animals cannulated in the dorsal anterior cingulate cortex (dACC) and amygdala were infused and tested on baseline changes in cardiovascular reactivity, changes in anxious behaviour on the human intruder test, and changes in conditioned fear on the fear extinction paradigm.

Results: Inhibition of serotonin reuptake in the amygdala via infusion of high dose citalopram reduced anxious behaviour and expression of physiological and behavioural measures of conditioned fear, but did not affect cardiovascular activity (heart rate, HR and mean arterial pressure, MAP) in a neutral condition. Inhibition of amygdala 5-HT_{2a} receptor binding via infusions of M100907 (5-HT_{2a} receptor antagonist) and dACC 5-HT_{2c} receptor binding via infusions of SB242084 (5-HT_{2c} receptor antagonist) did not affect baseline cardiovascular activity and anxious behaviour.

Conclusion: These results provides evidence that an acute dose of SSRI in the amygdala alters key characteristics of trait anxious expression by reducing anxious behaviour and conditioned fear expression. These findings elucidate the potential mechanism underlying SSRI's drug action and support the hypothesis that lowered amygdala serotonin signalling regulates threat-related emotion processing and may contribute to the high trait anxiety phenotype.

4.1 Introduction

The previous chapter explored potential genetic correlates to inter-individual expression in anxious behaviours, providing a potential basis for the inter-individual variability in trait anxiety. Building upon that, this chapter seeks to determine if the specific associations between serotonergic expression and high anxiety is indicative of altered region-specific serotonin signalling in high trait anxiety.

Trait anxiety is commonly measured in humans with questionnaires via the State-Trait Anxiety Inventory (STAI) (Spielberger, 1983). The STAI Trait assesses how responders "generally feel" about themselves on the basis of a 4-point Likert scale (from "not at all" to "very much"), e.g. "I lack self-confidence" or "I am a steady person". However, as questionnaires are confounded by an individual's perceptual biases, changes in an animal's trait anxiety are best assessed via changes in key characteristics of the high trait anxious phenotype.

As high trait anxious individuals are more prone to feelings of anxiety, state anxiety as measured by anxious behaviour is an unsurprising proxy to measure an individual's trait anxiety level. Anxious behaviour as captured by the EFA-derived anxiety score reflects an animal's vigilance and avoidance behaviour in the presence of a "human intruder", as an anxiety-provoking context (discussion in chapter 2: "*Anxiety and fear response in the common marmoset*"). Threat vigilance in the form of increased attentional bias towards threat, has been consistently associated with high trait anxious individuals and across anxiety disorders (Mathews and Mackintosh, 1998; Bar-Haim *et al.*, 2007; Cisler and Koster, 2010). Anxious behaviour on the human intruder test has also shown test-retest consistency, indicating that the behaviours displayed are consistent over time and may be indicative of trait levels of vulnerability to anxiety (Mikheenko *et al.*, 2015). Thus, the human intruder test anxiety score captures multiple components of anxious expression that have been associated with trait anxiety and may reflect an animal's enduring disposition for anxiety.

High trait anxiety is also associated with elevated and persistent conditioned fear responses. Indovina *et al.* (2011) reported that high trait anxious individuals show increased amygdala reactivity to conditioned fear stimuli, cues predictive of the occurrence of an aversive stimuli. Biases in fear learning and responding may underlie the excessive fear characteristic in most anxiety disorders. The fear conditioning paradigm has been used in humans, rodents and nonhuman primates to isolate the neural substrates of learned fear responses by dissociating the participant's response to the conditioned fear stimuli from the unconditioned fear stimuli (Milad and Quirk, 2012; Wallis *et al.*, 2017). Crucially, the fear conditioning paradigm also enables us to evaluate potential alterations in the participant's fear (un)learning process during extinction sessions and provides insight into adaptive fear coping processes.

This chapter seeks to determine if the association between high serotonergic transporter mRNA levels in the amygdala and high anxiety reported in the previous chapter are indicative of low local serotonin signalling leading to characteristics of the high trait-anxious phenotype: high anxiety reflected by avoidant behaviour and threat vigilance in the human intruder test, and conditioned fear expression measured physiologically and behaviourally in the fear extinction paradigm (Wallis *et al.*, 2017). As the drug's potential non-specific effect on physiology may affect an animal's anxious state, the drug's effect in a neutral condition was evaluated as well. Previously, it has only been demonstrated that direct infusion of a selective serotonin reuptake inhibitor (SSRI) in the amygdala and more specifically the basolateral amygdala (BLA) reduces conditioned fear freezing in rats (Inoue *et al.*, 2004; Kitaichi *et al.*, 2014). However, the causal role of amygdala serotonin transporter inhibition on anxious and conditioned fear behaviour in marmosets has not been investigated.

Amygdala 5-HT_{2a} receptor expression and dACC 5-HT_{2c} expression was only significantly associated with anxious behaviour before correcting for multiple comparisons. This may be due to a combination of both the limitation of the statistical power from the sample size used and the large number of brain regions corrected for. 5-HT_{2a} and 5-HT_{2c} receptor have been implicated in the regulation of anxiety but received less recognition compared to the serotonin 1A receptor in the wider literature (reviews: Bourin & Dhonnchadha 2005; Jiang *et al.* 2011; Heisler *et al.* 2007). As 5-HT_{2a} and 5-HT_{2c} receptors are expressed in abundance in the dACC and amygdala, manipulations of these receptor's signalling may have an impact on anxious behaviour (Shukla, Watakabe and Yamamori, 2014). Indeed, activation of 5-HT_{2c} receptor in the rodent BLA have shown anxiogenic effects (Campbell and Merchant, 2003; Christianson *et al.*, 2010; Vicente and Zangrossi, 2012). Thus, to take advantage of the study's cohort, the potential effect of region-specific serotonergic 2a and 2c receptor antagonism on anxious behaviour was also studied as a part of this chapter to explore the implications of specific serotonin receptor activation in regions of interest.

4.2 Methods

Subjects and Housing

The cohort consists of nine experimentally naïve marmosets (*Callithrix jacchus*) with respective test participation listed in table 4.1. The animals taking part in the experiment were bred on-site at the University of Cambridge Marmoset Breeding Colony. Animals joined the study after being screened on the human intruder test and rubber snake test (age: 38.6 ± 14.6 months). All animals were implanted with a telemetry probe and cannulated before behavioural testing (figure 4.1).

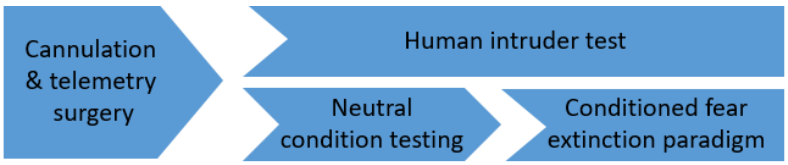


Figure 4.1: Timeline of surgery and behavioural testing. Animals underwent cannulation and telemetry surgeries before being tested on the human intruder test (conducted in the homecage) in conjunction with behavioural testing in the behavioural apparatus.

The marmosets were housed in male/female pairs (males were vasectomised), in a controlled environment (temperature: 22 ± 1°C; humidity: 50 ± 1%) and a dawn/dusk-like 12 hour-period was maintained. Their cages contained a variety of environmental enrichment, including suspended ladders, wooden branches and ropes to climb and swing on and boxes. The animals were fed a varied diet including fruit, rusk, malt loaf, peanuts, eggs, sandwiches and treats and they had ad libitum access to water. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and the local AWERB policies.

Human intruder test

Celery (F)			Annis (M)			Whitebeam (M)			Greedo (F)			Nix (F)		
25-11-16	Amygdala	Saline	10-04-17	Amygdala	M100907 .5 µg/site	10-02-17	Amygdala	Saline	10-11-17	Amygdala	PBS	23-02-17	Amygdala	citalopram 6 µg/site
09-12-16	Amygdala	citalopram 6 µg/site	28-04-17	Amygdala	PBS	24-02-17	Amygdala	M100907 .5 µg/site	08-12-17	Amygdala	M100907 .5 µg/site	09-03-17	Amygdala	Saline
03-03-17	Amygdala	M100907 .5 µg/site	19-05-17	Amygdala	citalopram 6 µg/site	10-03-17	Amygdala	PBS	09-02-18	Amygdala	Citalopram 30 µg/site			
17-03-17	Amygdala	PBS	16-06-17	Amygdala	Saline	23-03-17	Amygdala	citalopram 6 µg/site	09-03-18	Amygdala	Saline	**Watto (F)**		
25-08-17	Amygdala	Citalopram 30 µg/site	17-08-17	dACC	HPBCD	27-07-17	Amygdala	Saline	06-04-18	Amygdala	citalopram 6 µg/site	08-06-18	Amygdala	Saline
20-09-17	Amygdala	Saline	14-09-17	dACC	SB242084 1 µg/site	24-08-17	Amygdala	Citalopram 30 µg/site	04-05-18	Amygdala	Saline	20-06-18	Amygdala	Citalopram 30 µg/site
27-10-17	dACC	SB242084 1 µg/site	13-10-17	Amygdala	Saline	21-09-17	dACC	HPBCD	01-06-18	dACC	M100907 .5 µg/site			
01-12-17	dACC	HPBCD	24-11-17	Amygdala	Citalopram 30 µg/site	20-10-17	dACC	SB242084 1 µg/site	15-06-18	dACC	PBS			
04-05-18	dACC	PBS	20-06-18	dACC	M100907 .5 µg/site	31-05-18	dACC	PBS						
18-05-18	dACC	M100907 .5 µg/site	04-07-18	dACC	PBS	14-06-18	dACC	M100907 .5 µg/site						

Baseline testing

Celery (F)			Annis (M)			Whitebeam (M)			Greedo (F)			Nix (F)		
15-12-16	Amygdala	Saline	19-04-17	Amygdala	M100907 .5 µg/site	09-03-17	Amygdala	PBS	28-11-17	Amygdala	Saline	14-03-17	Amygdala	citalopram 6 µg/site
16-12-16	Amygdala	citalopram 6 µg/site	21-04-17	Amygdala	PBS	14-03-17	Amygdala	M100907 .5 µg/site	30-11-17	Amygdala	citalopram 6 µg/site	16-03-17	Amygdala	Saline
21-12-16	Amygdala	PBS	25-04-17	dACC	HPBCD	16-03-17	dACC	HPBCD	05-12-17	dACC	SB242084 1 µg/site	22-03-17	Amygdala	M100907 .5 µg/site
22-12-16	Amygdala	M100907 .5 µg/site	03-05-17	dACC	SB242084 1 µg/site	17-03-17	dACC	SB242084 1 µg/site	07-12-17	dACC	HPBCD	23-03-17	Amygdala	PBS
25-01-17	dACC	SB242084 1 µg/site	05-05-17	Amygdala	citalopram 6 µg/site	22-03-17	Amygdala	Saline	13-12-17	Amygdala	PBS			
31-01-17	dACC	HPBCD	09-05-17	Amygdala	Saline	28-03-17	Amygdala	citalopram 6 µg/site	15-12-17	Amygdala	M100907 .5 µg/site	**Anx (M)**		
												05-07-18	Amygdala	Saline
						Appo (M)			**Sidious (F)**			06-07-18	Amygdala	Citalopram 30 µg/site
						01-02-19	Amygdala	Citalopram 30 µg/site	15-01-19	Amygdala	Saline	02-08-18	Amygdala	citalopram 6 µg/site
						05-02-19	Amygdala	Saline	22-01-19	Amygdala	Citalopram 30 µg/site	08-08-18	Amygdala	Saline

Fear Extinction paradigm

Celery (F)			Annis (M)			Whitebeam (M)			Greedo (F)		
16-11-17	Amygdala	Citalopram 30 µg/site	15-02-18	Amygdala	Citalopram 30 µg/site	02-11-17	Amygdala	Saline	18-01-18	Amygdala	Saline
08-03-18	Amygdala	Saline	08-03-18	Amygdala	Saline	23-11-17	Amygdala	Citalopram 30 µg/site	15-02-18	Amygdala	Citalopram 30 µg/site

Table 4.1: Animal and respective test participation. Animal's gender (M: male; F: female), date of test participated in, target brain region and drug infusion received are listed. Cells are colour-coded for drug-control pairs.

Surgical Procedures

All animals underwent two surgical procedures: a surgery to implant intracerebral cannulae targeting the amygdala and dACC, and a surgery to implant a telemetric blood pressure monitor into the descending aorta. Both surgeries were completed before the animals started behavioural testing.

Cannulation surgery. Marmosets were premedicated with ketamine hydrochloride (Vetalar; 0.05 mL of a 100-mg solution, i.m.; Amersham Biosciences and Upjohn) before being given a long-lasting nonsteroidal anti-inflammatory analgesic agent (Carprieve; 0.03 mL of 50 mg/mL carprofen, s.c.; Pfizer). They were intubated and maintained on 2.0–2.5% isoflurane in 0.3 L/min O_2 and placed into a stereotaxic frame modified for the marmoset (David Kopf). Pulse rate, O_2 saturation, breathing rate, and CO_2 saturation were monitored by pulse oximetry and capnography (Microcap Handheld Capnograph; Oridion Capnography), and core body temperature was monitored by a rectal thermometer (TES-1319 K-type digital thermometer; TES Electrical Electronic). Cannulae (Plastics One) were implanted into the amygdala (single 15.0mm long cannula, anteroposterior (AP) +9.6, lateromedial (LM) ±5.6), and the dACC (double 3.5mm long cannulae 2mm apart, AP +13.2, LM ±1.0). After the animals have fully recovered, the animal was given an analgesic meloxicam (0.1 mL of a 1.5 mg/mL oral suspension; Boehringer Ingelheim) and each day for three days after surgery. Cannulae were cleaned, and dummy cannulae and caps were replaced weekly. Representative histology sections with cannulae placement shown in figure 4.2.

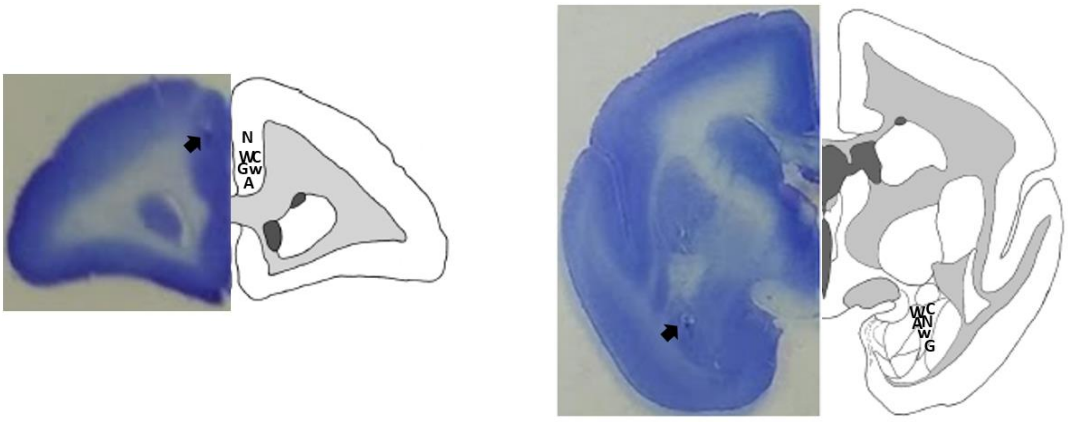


Figure 4.2: Cannulae placements. Representative (a) dACC and (b) amygdala histological sections with arrows marking the position of the cannula tip. Cannulae tip positions of individual animals shown on the right (C: Celery; W: Whitebeam; N: Nix; A: Annis; w: Watto; G: Greedo)

Telemetry Probe Surgery. Animals were anesthetized as described before. The probe catheter was implanted into the aorta near the aortic bifurcation and the telemetric blood pressure transmitter (Data Sciences International) was placed within the abdomen following procedures described previously (Schnell and Wood, 1993). All monkeys received meloxicam as described before in addition to prophylactic treatment with amoxicillin and clavulanic acid (Synulox; 50 mg/mL solution; Pfizer), for one day before and six days after surgery.

Drug treatment.

For infusions, the animal was held in the hand of an assistant while the experimenter removed the cannulae caps and wiped the surface of the cannulae with an alcohol swab. The experimenter then inserted a sterile injector (Plastics One) connected to a 2-μL gas-tight syringe in a syringe pump via infusion tubing down the guide cannulae before starting the infusion. After the infusion was completed, the position of a pre-loaded air bubble that had been marked was checked, movement of the air bubble beyond the mark indicates the successful infusion of the drug down the tubing. One minute is allocated before the removal of the injector following the completion of the infusion such that the drug has time to diffuse across the target region, reducing the risk of the drug being drawn up the guide due to the removal of the injector (Zeredo *et al.*, 2019). New sterile cannula caps were replaced before the animal is returned to the home cage. Testing was initiated 10, 5, and 0 minutes after citalopram, M100907, and SB242084 infusions respectively.

Drugs. Citalopram hydrobromide (Sigma-Aldrich/Tocris) was dissolved in sterile saline in 6 μg/μL (low dose) or 30 μg/μL (high dose) and infused in the amygdala at a rate of 0.5 μL/min for 2 minutes. M100907 (5-HT_{2a} receptor antagonist. $Ki_{5\text{-}HT2AR}$: 0.31 nM; $Ki_{5\text{-}HT2CR}$: 13.0 nM) was prepared by being dissolved in 40 μl 1M hydrochloric acid, HCL before being topped with 0.01M phosphate-buffered saline, PBS. 1 μg/μL M100907 (Sigma-Aldrich) was infused in the amygdala or dACC at a rate of 0.25 μL/min for 2 minutes. The 5-HT_{2c} receptor antagonist, SB242084 ($Ki_{5\text{-}HT2CR}$: 1.0 nM; $Ki_{5\text{-}HT2AR}$: 158.5 nM; Sigma-Aldrich) was dissolved in 10% 2-hydroxypropyl-beta-cyclodextrin (HPBCD, Sigma-Aldrich) solution before being infused at a concentration of 2 μg/μL and at a rate of 0.25 μL/min for 2 minutes. Dosage for the low dose citalopram, M100907 and SB242084 were based on effective brain infusions performed by Inoue *et al.* (2004), Pehek *et al.* (2001), and Alsiö *et al.* (2015) respectively.

Behaviour testing procedures

1. Human Intruder test

Anxious behaviour was assessed using the human intruder test. Full description of the human intruder test in Chapter 2: "Anxiety and fear response in the common marmoset". Briefly, the animal's behavioural response to an unknown "human intruder" maintaining eye contact for 2 minutes in a section of the homecage was recorded and scored. The behavioural measures scored included: time spent at the front of the cage, time spent at the back of the cage, average height, head-bobbing, locomotion, and vocalizations (egg, tsik, tsik-egg, tse and tse-egg calls). Different realistic masks (Greyland Film) were used for different sessions within-animals and for specific infusion between-animals, e.g. the "Chris" mask is used for the low dose Citalopram infusion for animal 1 and not used again for any other infusion in animal 1 and not used as a mask in the low dose Citalopram infusion of other animals in the human intruder test. Each saline-citalopram pair session was conducted as separate blocks to avoid the confounding effects of shifting baselines.

Anxiety factor score. Behavioural scores from the human intruder test for each individual variable were standardised to obtain z-scores using the mean and standard deviations of the population the EFA was derived from. Between-individuals and within-individual standard deviations are assumed to not be significantly different. The anxiety factor score is the sum of the product of all the variables' standardised score multiplied by their respective factor score coefficients.

Behavioural testing apparatus

Neutral condition and fear extinction tests took place within a sound-attenuated testing apparatus (figure 4.3). Animals were transported to the behavioural testing apparatus from the home cage via a transparent Perspex (Lucite) carry box. The marmoset remains inside the Perspex test box within the testing chamber at all times during testing. The test chamber was illuminated by houselights located on the top of the chamber and contained a computer-controlled speaker through which auditory stimuli could be played. The apparatus was controlled by the Whisker control system and in-house software (Cardinal and Aitken, 2010). Three video cameras and a microphone were positioned in the testing apparatus such that the behaviour of the animal could be monitored by the experimenter during testing and recorded for post-test analysis (Power Director; CyberLink).

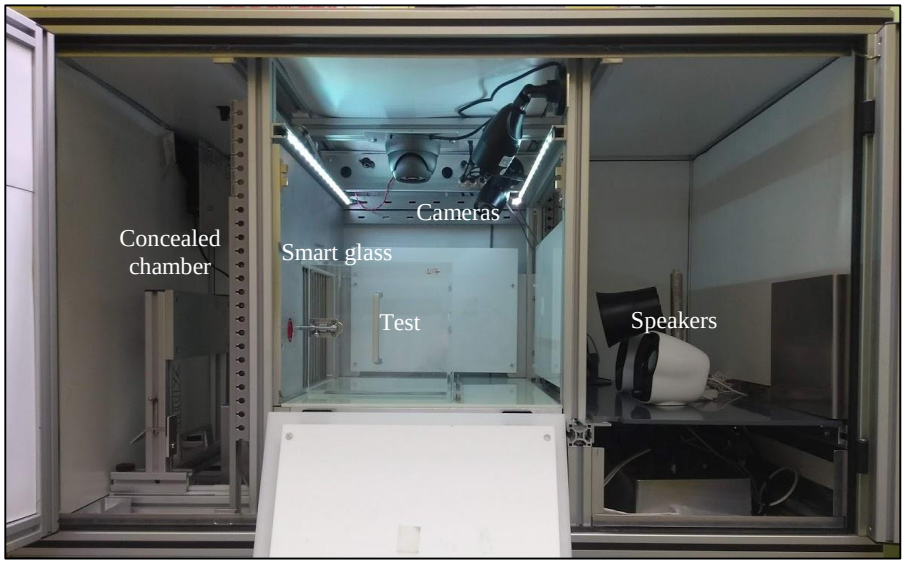


Figure 4.3: Testing apparatus. Picture of the testing apparatus with the concealed chamber, smart glass, the test box, cameras and speakers labelled.

2. Neutral condition testing

Monkeys were habituated to the testing apparatus for 15-minute sessions until their mean heart rate and mean systolic blood pressure remained stable across subsequent sessions (range: 11-20 sessions) before neutral condition testing commenced and subsequently testing on the fear extinction paradigm.

Neutral condition testing consists of 15-minute sessions with no active tasks. The first minute of each session is omitted from the analysis of cardiovascular reactivity to avoid the confounding effect of the animal's initial reaction to being transferred to the testing apparatus.

3. Conditioned fear and extinction paradigm

The protocol used is an adaptation of the classic rodent fear conditioning and extinction paradigm, with the foot shock replaced with the sight of a rubber snake model as an innate fear stimulus. Each fear extinction test consists of five sessions over five consecutive days: two sessions of habituation to the context, followed by a fear conditioning session, and lastly an extinction session (figure 4.4).

Habituation to the context. For two sessions, the animals were placed inside the Perspex test box and testing apparatus with context panels placed in. The context panels consist of differing black-and-white patterned panels used to distinguish the testing context between subsequent testing cycles. During these sessions, the animals are also habituated to the opaque smart glass turning clear and exposing an empty

chamber (US^-). The smart glass turns clear for 12 presentations of 5 seconds at 110-130 second intervals.

Fear conditioning. The fear conditioning session consists of nine trials: three trials of CS-US^- presentation and six trials of CS-US^+ presentation. Trials are presented at 160-180 second intervals. For the first three CS-US^- trials of the session, the auditory CS (70db) is presented for 20 seconds. The US^- is initiated during the last 5 seconds of the CS presentation and consist of the smart glass turning clear to expose an empty chamber. The rubber snake model was placed inside the chamber, unseen by the animal via a side hatch after the end of the final CS-US^- trial. For the following six CS-US^+ trials, when the smart glass turns clear in the final 5 seconds of the CS presentation, the animal is exposed to the sight of the rubber snake in the chamber (US^+). If an animal does not show a behavioural response to the sight of the rubber snake, the animal is re-tested in a new context and the US^+ for the fear conditioning session is changed to the presentation of the rubber snake with the test box in darkness (the chamber the rubber snake is in remains illuminated such that the rubber snake remains visible). Darkness has been demonstrated to facilitate the fear response of marmosets to a rubber snake (Shiba *et al.*, 2017).

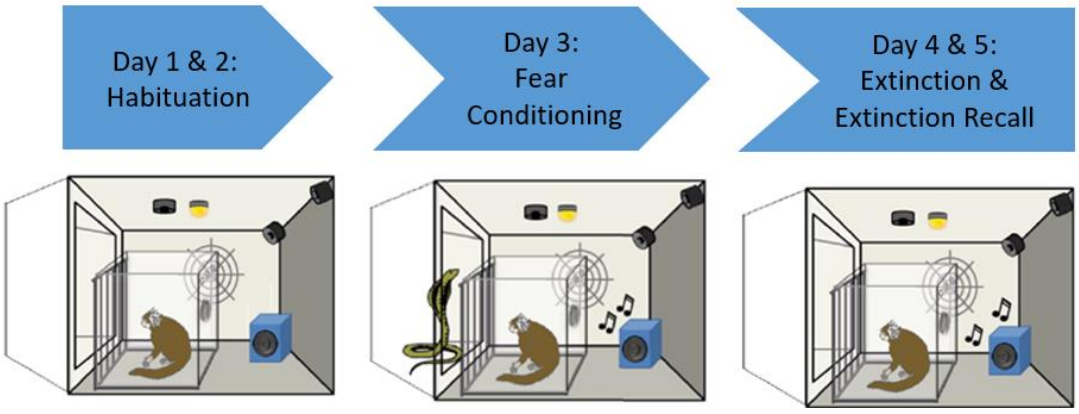


Figure 4.4: Conditioned fear and extinction paradigm. Animals are habituated to the context and the smart glass turning for the first two days of testing. On the third day, animals acquire the conditioned fear response by learning that the conditioned stimulus (CS) is paired with the unconditioned stimulus (US^+). On the following and final day, the animal is exposed to repeated presentations of the CS without the US^+.

Extinction. The infusion was carried out prior to the initiation of the extinction session. 20 CS-US^- pairings were presented at 60-80 second intervals. The CS is presented for 20 seconds. During the final 5 seconds of the CS presentation, the US^- is presented (the smart glass turns clear to expose an empty chamber).

Extinction recall. Similar to the extinction session, but the animal is exposed to only 12 repeated presentations of the CS and the smart glass exposing an empty chamber (CS-US$^-$) at 60-80 second intervals.

Fear extinction – behavioural and physiological scoring

Hypervigilant behaviour. Hypervigilant behaviour was scored as the sum of periods spent performing rapid head-swings and periods spent maintaining a completely still and hunched body posture. Slow and deliberate head movements that reflected normal vigilant or orienting behaviour were not scored. Durations of hypervigilance were scored using video recordings of the extinction sessions for the pre-CS period (15 seconds before the onset of the CS) and the CS period (during 15 seconds of the CS presentation).

Mean arterial pressure (MAP). The animal's cardiovascular response during the session were monitored remotely using the PhysioTel Telemetry System (Data Sciences International, DSI) to derive the animal's systolic and diastolic blood pressure. MAP = [(2 x diastolic blood pressure) + systolic blood pressure]/3. MAP, instead of heart rate, was used to represent the physiological aspect of the animal's stress response as it was less variable. One animal (Whitebeam) showed substantial distortion of the blood pressure reading when it spent short periods of time grooming its tail, these periods were omitted from the final analysis.

Data analysis and statistics

The freely moving animal's cardiovascular activity is transmitted via the implanted telemetry probe to a receiver in real-time and analysed post-test using Spike2 (version 8.11; CED). Any outliers and recording failures in the data were removed (blood pressure values >200 mm Hg or <0 mmHg or other abnormal spikes). Data collection was reliable overall, but data gaps of less than 0.4 s were replaced by cubic spline interpolation and gaps of more than 0.4 s were treated as missing values. Systolic and diastolic blood pressure events were extracted as local maxima and minima for each cardiac cycle.

All statistical analyses were performed using SPSS (version 24; IBM Corp., Armonk, NY). Differences between groups means were calculated using dependent t-tests for human intruder test anxiety scores. For the fear extinction paradigm, the drug's effect on the extinction process was determined via an interaction term in a linear regression model. Repeated measures two-way ANOVA was used to determine potential differences in the acquisition and extinction recall sessions prior and after animals received infusions on the extinction sessions respectively (factor: treatment and trial blocks; potential interaction).

4.3 Results

High dose SSRI infusion in the amygdala has an overall anxiolytic effect as measured by the anxiety score on the human intruder test.

The previous chapter established a link between amygdala serotonin transporter expression and anxiety. To investigate if amygdala serotonin transporter mediated expression of trait anxiety, we first studied the effect of central amygdala infusion of SSRI on anxious behaviour in the human intruder test. Low dose amygdala infusion of citalopram (6 µg/site) did not significantly affect anxiety scores ($t(4) = 1.741$, $p = .16$) (figure 4.5a). Whereas, inhibition of serotonin reuptake in the amygdala via local Infusion of high dose of SSRI, citalopram (30 µg/site) significantly reduced anxious behaviour as measured by the anxiety factor score in the human intruder test ($t(4) = 2.793$, $p = .049$) (figure 4.5b).

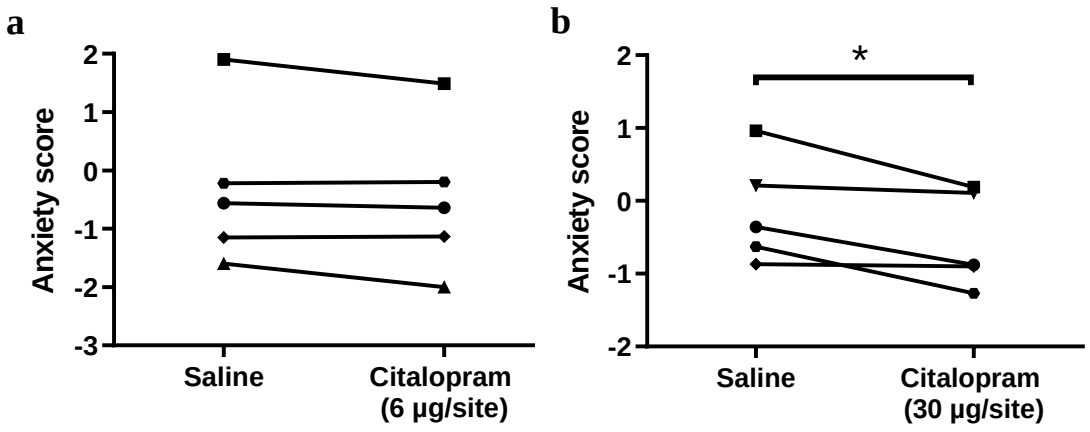


Figure 4.5: Effect of amygdala SSRI infusion on anxious behaviour in individual animals. There was no significant effect of (a) low dose infusion of citalopram in the amygdala on anxiety ($p > .05$), but (b) high dose infusion of citalopram in the amygdala significantly reduced general levels of anxious behaviour as measured by the anxiety factor score ($*p < .05$). (●: Celery; ■ : Whitebeam; ▲ :Nix; ◆ : Annis; ⬣ : Greedo; ▼ :Watto)

After finding that high dose infusions of citalopram in the amygdala had a general anxiolytic effect as measured by the anxiety factor score, we subsequently determined which of the individual measures that load significantly on the anxiety factor score were affected by SSRI inhibition. We found that high dose amygdala infusions of citalopram did not significantly affect individual measures that load significantly on the anxiety score (average height: $t(4) = 1.30$, $p = .264$; time spent at front: $t(4) = -1.89$, $p = .131$; time spent at back: $t(4) = 1.05$, $p = .355$; locomotion: $t(4) = 0.63$, $p = .56$; head-bobbing: $t(4) = .92$, $p = .41$; tse-egg calls: $t(4) = 1.14$, $p = .32$), indicating that its general effect on the anxiety factor score was not driven by only a specific subset of measures (figure 4.6).

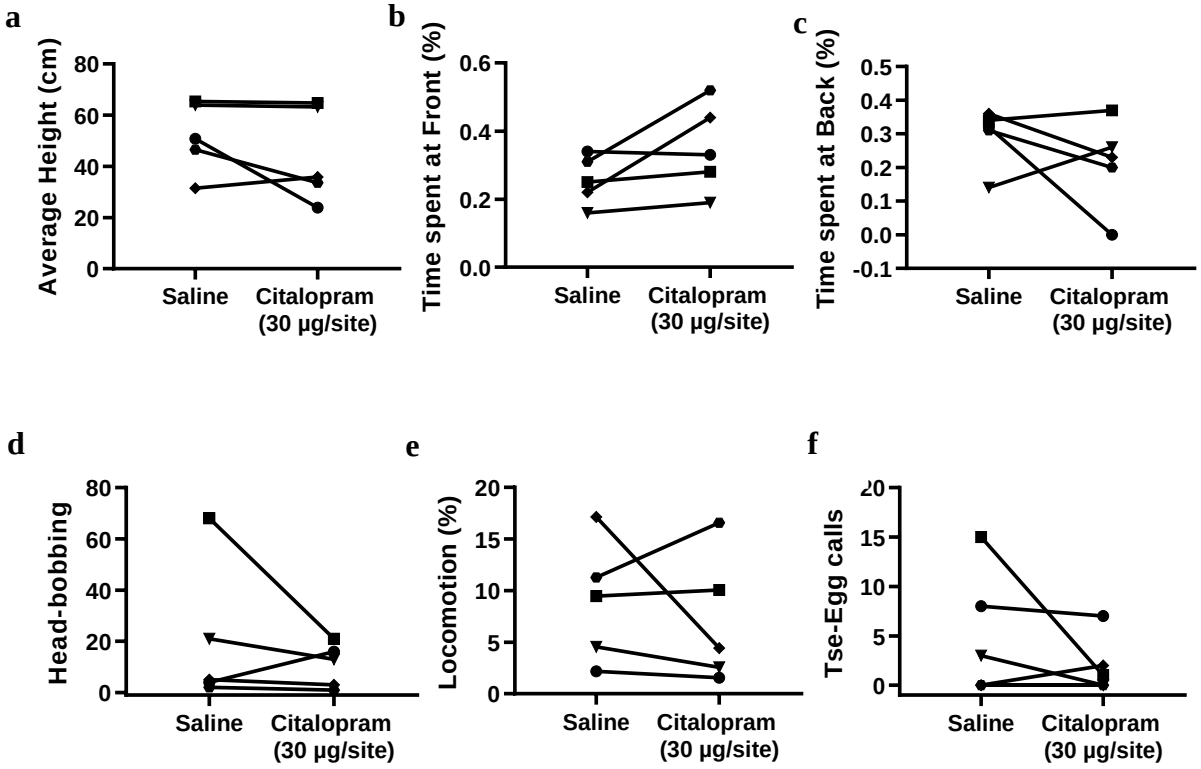


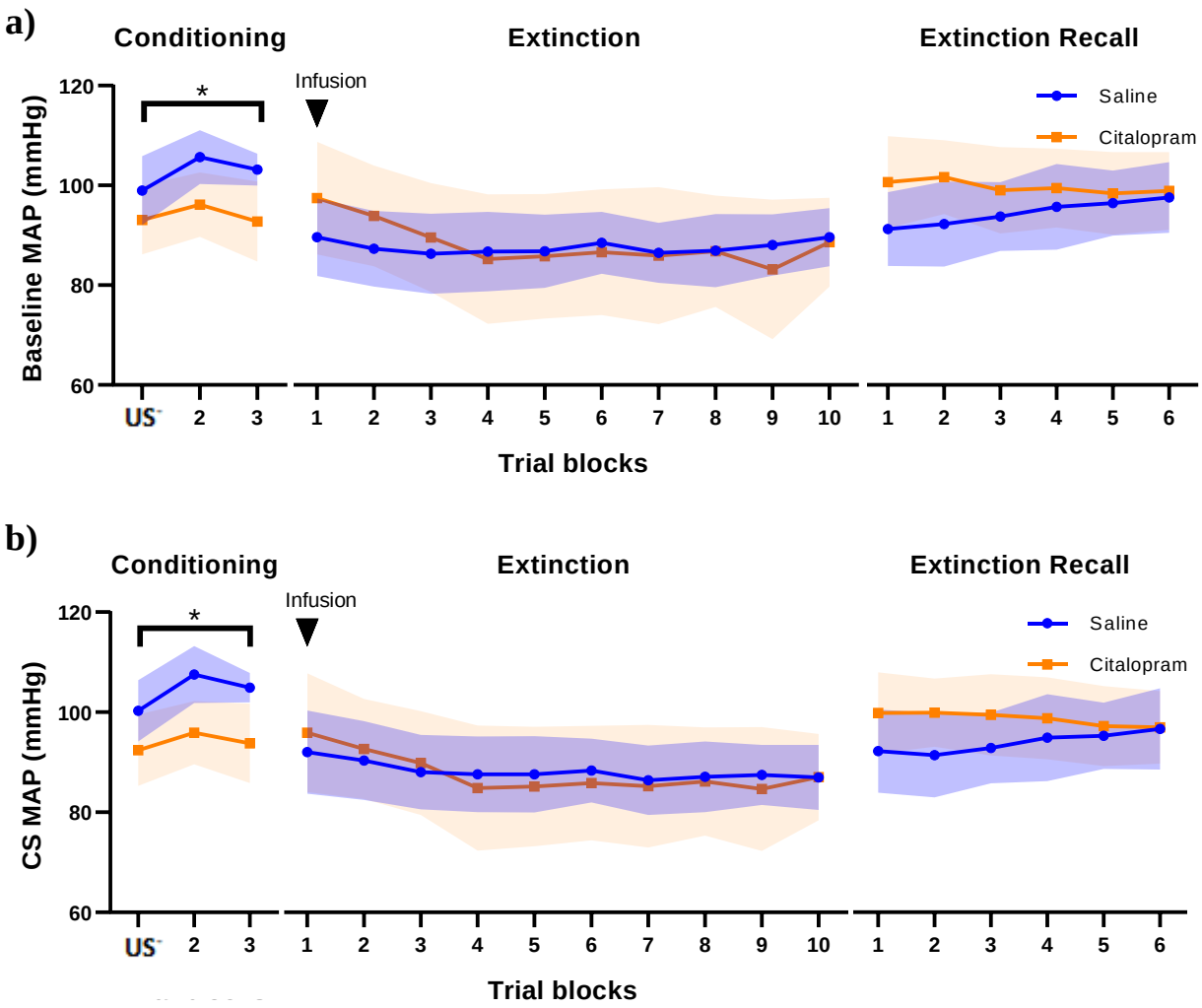
Figure 4.6: Effect of high dose amygdala infusion of SSRI on individual measures of anxious behaviour. There was no significant effect ($p > .05$) of high dose citalopram infusion in the amygdala on individual measures that load significantly on the anxiety factor score: a) average height, b) time spent at front, c) time spent at back, d) head-bobbing, e) locomotion (percentage time spent) and f) tse-egg calls. (●: Celery; ■ : Whitebeam; ◆ : Annis; ⬣ : Greedo; ▼ :Watto)

Inhibition of serotonin reuptake in the amygdala reduces expression of physiological and behavioural measures of conditioned fear.

To assess if inhibition of serotonin reuptake in the amygdala affected conditioned fear, animals underwent fear conditioning and were infused with citalopram or saline before the fear extinction session. To control for potential variability in the animal's baseline cardiovascular activity, CS-directed measures were obtained by subtracting measures during the initial 15 seconds of the CS period (as the US is presented during the remaining 5 seconds of the CS presentation) from the 15 seconds before the CS period (baseline). Although order of infusions were counterbalanced, animals due to receive amygdala infusions of citalopram showed lower baseline and CS MAP in conditioning prior to receiving the infusion (baseline: $F(1,3) =$, 16.15 $p = .028$; CS: $F(1,3) = 20.76$, $p = .02$), potentially indicating varied baseline levels of arousal on the day of conditioning (figure 4.7a and 4.7b). However, the CS-directed MAP in conditioning did not differ ($F(1,3) = 5.739$, $p = .096$), indicating that all animals did not differ in the learning of the CS-specific response (figure 7c). The animals did not show differences in baseline and CS MAP in extinction after receiving infusions (baseline: $F(1,3) = .009$, $p = .932$; CS: $F(1,3) = .004$, $p = .953$) and the next day in extinction recall (baseline: $F(1,3) = .198$, $p = .687$; CS: $F(1,3) = .181$, $p = .699$) (figure 4.7a and 4.7b).

The change in CS-directed MAP across trial blocks during extinction was significantly affected by amygdala serotonin reuptake inhibition (regression, treatment x trial blocks interaction: $\beta = .900$, $p < .001$). In the control condition (saline), animals show a classic extinction of elevated CS-directed MAP ($F(1,38) = 20.967$, $p < .001$), but not after amygdala infusion of citalopram ($F(1,38) = .909$, $p = .347$). Specifically, animals with amygdala infusions of citalopram (30 µg/site) do not show a conditioned fear response to the CS at the start of the extinction session and as such do not show an extinction trend across trial blocks (figure 4.7c). The drug effect on extinction was not followed by differences in extinction recall (ANOVA, treatment: $F(1,3) = .07$, $p = .809$; trial blocks: $F(1.392, 4.175) = .697$, $p = .498$; treatment x trial blocks: $F(1.507,4.521) = .967$, $p = .419$).

Behaviourally, all animals showed a startle response when first presented with the US^+ during the fear conditioning session. The animal's hypervigilant behaviour during extinction was scored to assess if the drug effect on extinction CS-directed MAP was reflected in behaviour. Indeed, the change in CS-directed hypervigilant behaviour across trial blocks during extinction was significantly affected by amygdala serotonin reuptake inhibition (regression, treatment x trial blocks interaction: $\beta = .504$, $p = .038$). The animals show a linear decline of CS-directed hypervigilant behaviour ($F(1,38) = 8.653$, $p = .006$) across trial blocks during the control extinction condition, but not after amygdala infusion of citalopram ($F(1,38) = 1.191$, $p = .282$) (figure 4.7d).


a)
Conditioning
Extinction
Extinction Recall
Infusion
Saline
Citalopram
Baseline MAP (mmHg)
120
100
80
60
*
US
2
3
1
2
3
4
5
6
7
8
9
10
1
2
3
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Trial blocks
b)
Conditioning
Extinction
Extinction Recall
Infusion
Saline
Citalopram
CS MAP (mmHg)
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Trial blocks

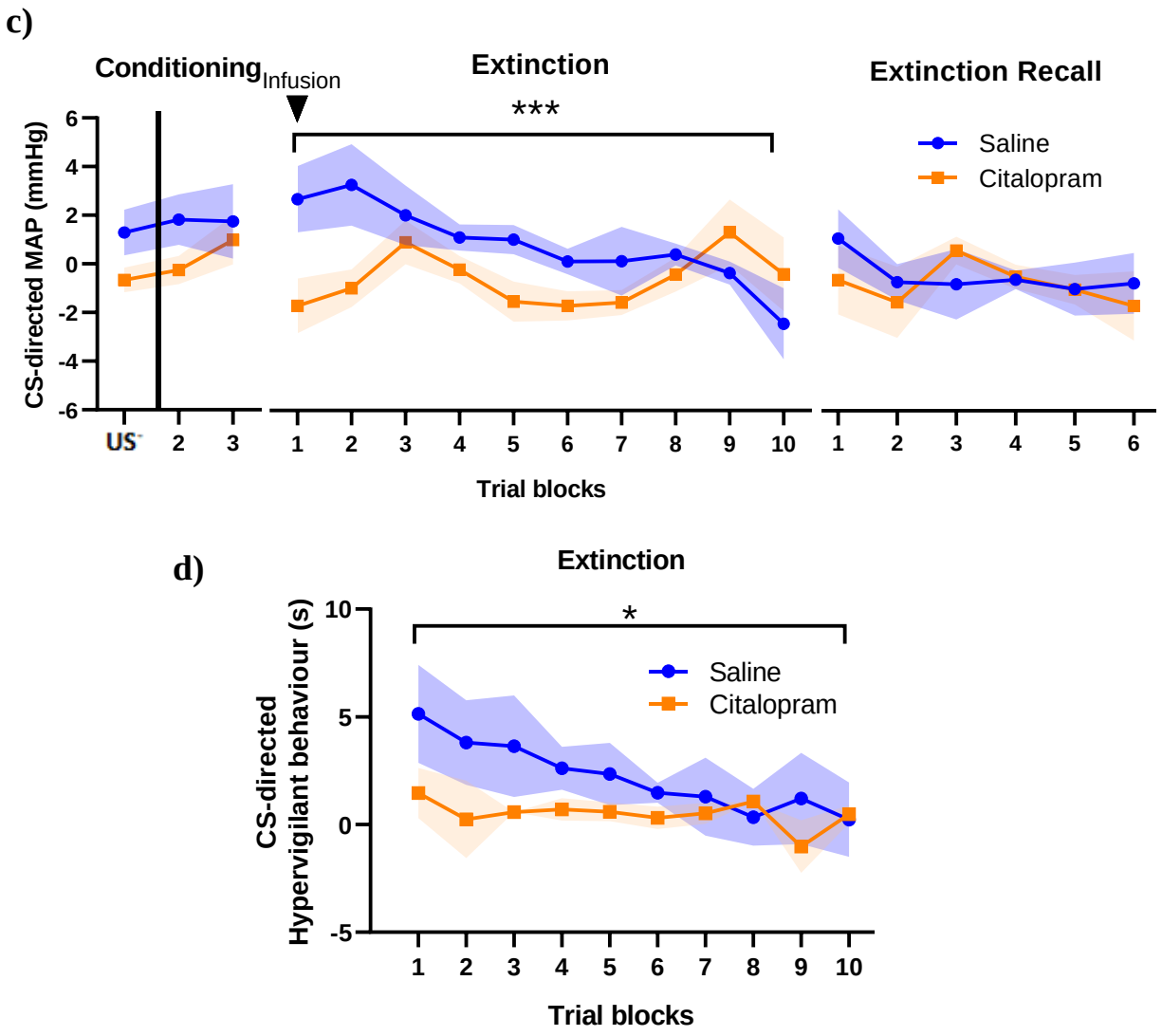

Figure 4.7: Effect of citalopram in the amygdala on the fear extinction paradigm. In the conditioning session prior to receiving infusions, animals' MAP differed during the (a) baseline and (b) CS period, but (c) CS-directed MAP did not differ depending on treatment groups. Amygdala infusions of citalopram (n = 4) significantly affected the extinction process (treatment x trial blocks slope interaction: (c) CS-directed mean arterial pressure (MAP): ***$p < .001$ and (d) hypervigilant behaviour: *$p < .05$). Animals' CS-directed MAP did not differ significantly the next day on extinction recall ($p > .05$). Error bands represent SEM.

Inhibition of amygdala serotonin reuptake did not affect cardiovascular activity (MAP and heart rate) in a neutral condition.

Cardiovascular activity reflects an animal's stress response. To determine if the SSRI's effect on MAP in the fear conditioning paradigm may be due to a general effect on physiological measures of emotion regulation, SSRI's effect on cardiovascular activity in the absence of emotional stimuli and task activity was determined. Both low dose (heart rate: $t(5) = .965$, $p = .38$; MAP: $t(5) = .024$, $p = .98$) and high dose (heart rate: $t(2) = 1.32$, $p = .32$; MAP: $t(2) = -1.94$, $p = .19$) infusions of citalopram in the amygdala did not significantly affect heart rate and MAP in neutral condition testing. Thus, citalopram's effect on MAP in the fear extinction paradigm and anxious behaviour in the human intruder test was not due to a more general effect on cardiovascular activity of inhibited amygdala serotonin reuptake (figure 4.8).

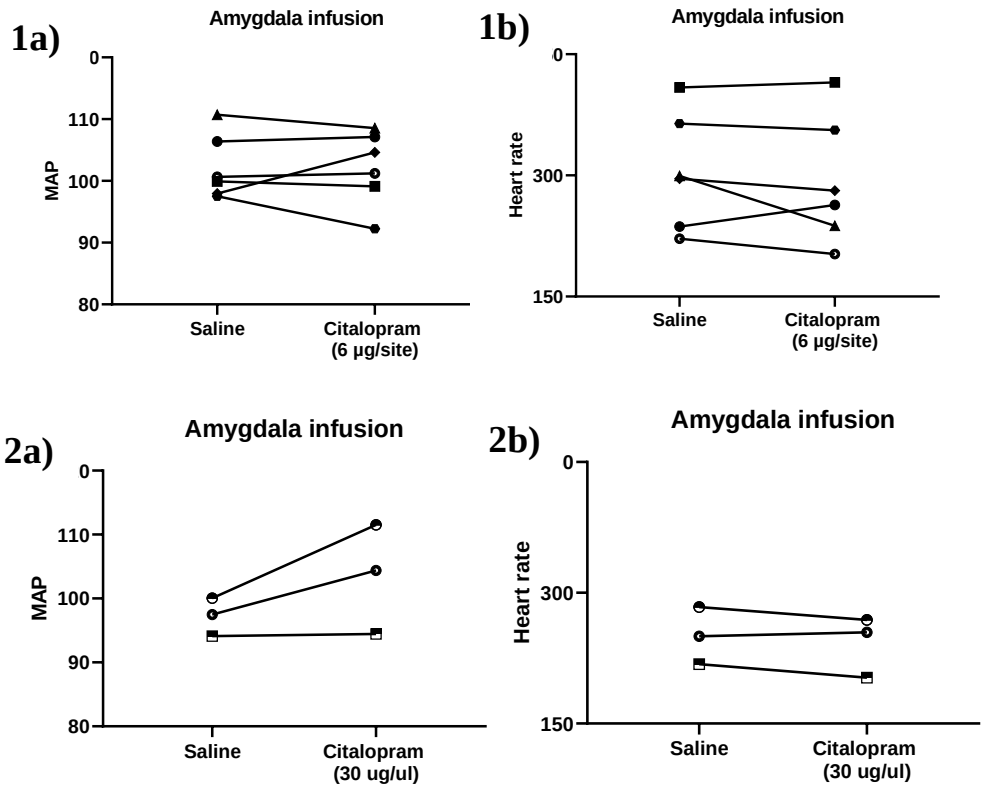

Figure 4.8: Effect of amygdala blockade of serotonin reuptake on neutral condition testing.
Infusions of (1) low and (2) high dose of citalopram in the amygdala did not affect (a) MAP and (b) heart rate in neutral condition testing. (●: Celery; ■ : Whitebeam; ▲ :Nix; ◆ : Annis; ⬣ : Greedo; ⭘ : Anx; ◓ : Appo; ⬒ : Sidious)

Inhibition of 5-HT_{2a} and 5-HT_{2c} receptor binding in the amygdala and dACC did not affect anxious behaviour.

Central infusions revealed that blockade of 5-HT_{2a} receptors via a 5-HT_{2a} receptor antagonist (M100907) in the amygdala and dACC did not alter anxious behaviour (amygdala: $t(2) = -2.79$, $p = .108$; dACC: $t(3) = -0.47$, $p = .674$) (figure 4.9a and 4.9b). Data of one animal receiving amygdala infusion of M100907 was omitted as the animal showed a persistent freezing behaviour throughout the "intruder phase" of the human intruder test. As the human intruder test anxiety factor score is derived with behaviour from the population, a highly atypical behaviour such as freezing is not properly modelled and significantly distorts the final score. Blockade of 5-HT_{2c} receptors in the dACC via a 5-HT_{2c} receptor antagonist (SB242084) did not alter anxious behaviour as well ($t(2) = 1.36$, $p = .307$) (figure 4.9c).

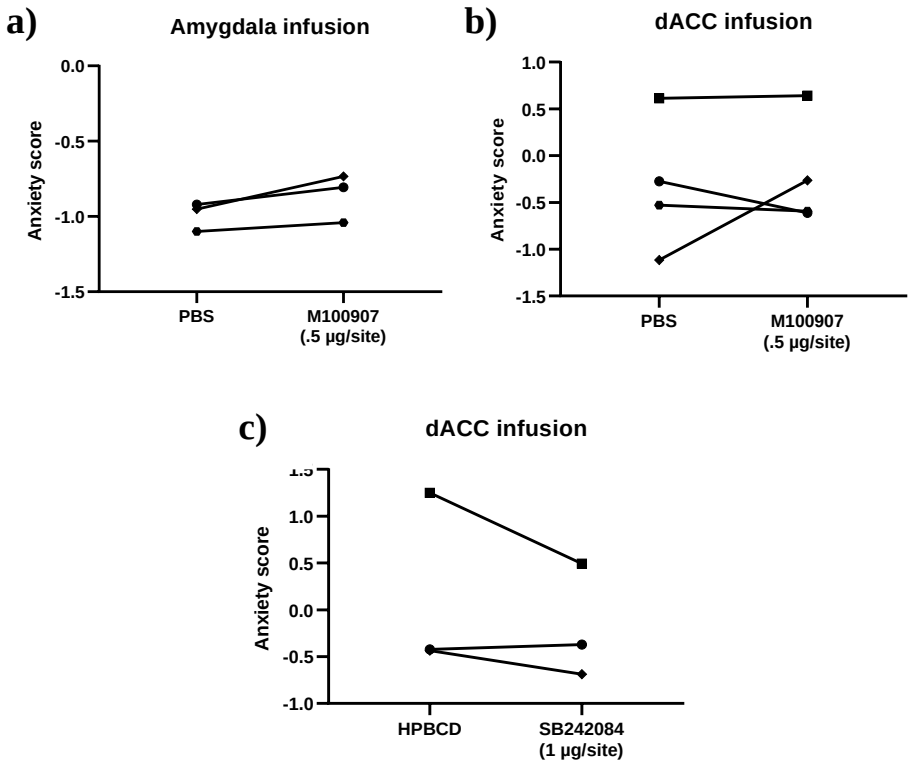

Figure 4.9: Effect of 5-HT_{2a} and 5-HT_{2c} antagonist in the amygdala and dACC on anxious behaviour. All infusions ((a) amygdala infusion of M100907; (b) dACC infusion of M100907; (c) dACC infusion of SB242084) did not have a significant effect on anxious behaviour ($p > .05$). (●: Celery; ■ : Whitebeam; ◆ : Annis; ⬣ : Greedo)

In a neutral condition. inhibition of amygdala 5-HT_{2a} receptor binding increased heart rate but did not affect MAP. Inhibition dACC 5-HT_{2c} receptor binding did not affect cardiovascular activity (heart rate and MAP).

Next, whether serotonergic manipulations in the dACC and amygdala would have a general effect on physiological measures of emotion regulation in a neutral condition was assessed. Animals were given the central infusion in a neutral context. Inhibition of amygdala 5-HT_{2a} receptor binding via a 5-HT_{2a} receptor antagonist (M100907, .5 µg/site) significantly increased heart rate but did not have an effect on MAP (heart rate: $t(4) = -3.657$, $p = .022$; MAP: $t(4) = -2.036$, $p = .11$) (figure 4.10.1). In contrast, inhibition of dACC 5-HT_{2c} receptor binding (SB242084, 1 µg/site; heart rate: $t(3) = .009$, $p = .99$; MAP: $t(3) = -.655$, $p = .56$) did not significantly affect heart rate and MAP in neutral condition testing (figure 4.10.2).

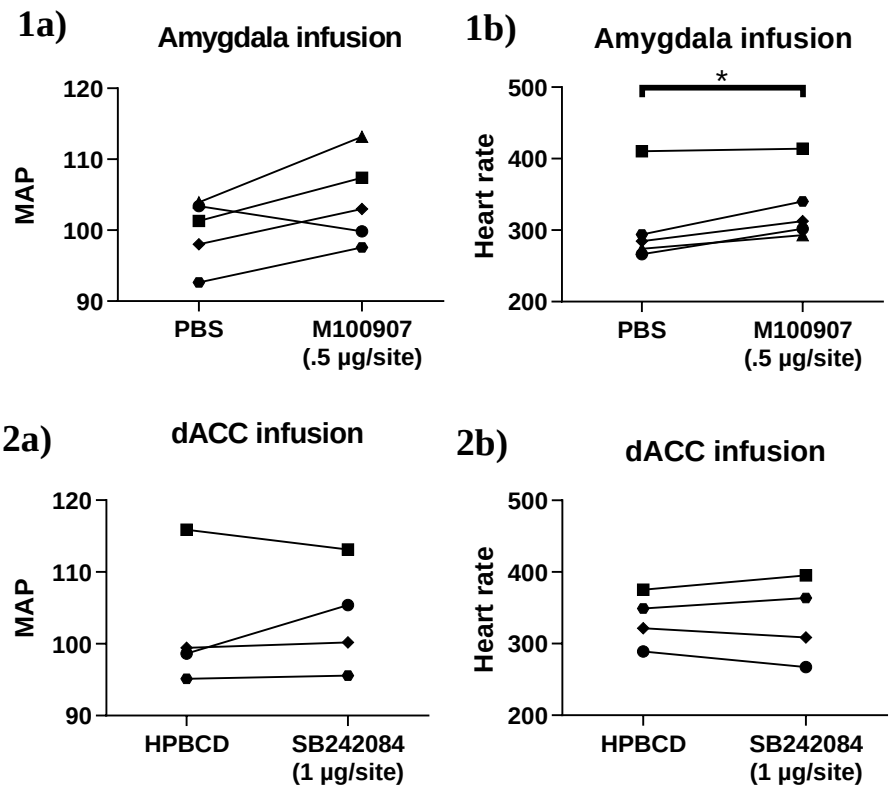

Figure 4.10: Effect of serotonergic manipulations on neutral condition testing. 1) Amygdala infusion of M10907 did not affect (a) MAP but increased (b) heart rate (*p < .05). 2) dACC infusions of SB242084 did not significantly affect a) MAP or b) heart rate ($p > .05$). (●: Celery; ■ : Whitebeam; ▲ :Nix; ◆ : Annis; ⬣ : Greedo)

4.4 Discussion

As predicted by the results of the previous chapter whereby higher levels of amygdala serotonin transporter mRNA expression predicted higher levels of anxious behaviour, acute inhibition of amygdala serotonin transporter via infusions of citalopram reduced indices of high trait anxiety assessed: anxious behaviour and conditioned fear expression. Reduction in the physiological measure of conditioned fear (MAP), in addition to the behavioural measure of conditioned fear (hypervigilance), indicates that alteration of amygdala serotonin signalling is capable of not only leading to behavioural change but may also play a role in the brain-body interaction of anxiety's somatic symptoms. My findings provide evidence in a primate model of trait anxiety for amygdala serotonin transporter's causal role in anxiety and conditioned fear, suggesting that serotonin signalling in the amygdala mediates the expression of the high trait anxiety phenotype. My findings also provide some insight into the mechanism underlying the anxiolytic action of SSRIs.

Contrary to our findings here, Johnson *et al.* (2015) reported that downregulation of amygdala serotonergic signalling via local injections of selective serotonergic neurotoxin (5,7-DHT) injections reduced anxious behaviour in the social interaction test but not the open field test, and reduced conditioned fear expression. However, it should be noted that although extracellular serotonin was reduced in the BLA, there was no clear post mortem evidence of loss of serotonergic terminals, indicating that the serotonergic neurotoxin may not have been completely effective. It should also be noted that destruction of serotonergic neurons may also have led to compensatory mechanisms and microcircuit level changes to the local serotonergic system over time, potentially explaining the conflicting findings reported by Johnson *et al*. (2015) when experimental testing was conducted 6-7 days post-BLA microinjection of 5,7-DHT. Compared to Johnson *et al.* (2015), serotonin was manipulated here by acute blockades of the serotonin transporter instead of serotonergic terminal destruction, and animals were tested after infusions with only a short wait time (10 minutes). Overall, the findings from Johnson *et al*. (2015) do not convincingly contradict the findings reported here.

Although SSRIs are the first line drug treatment for most instances of anxiety disorders, the neural substrates for the drug's efficacy remains largely unclear and their therapeutic effects are hugely variable across individuals. Chronic treatment using SSRIs have demonstrated anxiety symptom reduction across different anxiety disorder patients (van der Kolk *et al.*, 1994; van Vliet, den Boer and Westenberg, 1994; Boyer, 1995). However, SSRI treatment's efficacy can also be associated with an exacerbation of symptoms acutely before the delayed onset of anxiolysis (Teicher, Glod and Cole, 1990; Grillon, Levenson and Pine, 2007; Bigos *et al.*, 2008). Similarly, acute and chronic SSRIs have opposing effects on conditioned fear. Acute SSRIs were shown to enhance fear acquisition, whereas chronic treatment reduces fear acquisition, suggesting both anxiety and conditioned fear behavioural

responses are similarly modulated by alterations in systemic serotonin reuptake inhibition (Burghardt *et al.*, 2004). Taken together with findings of acute systemic SSRI enhancing anxiety and conditioned fear, findings here provide support for the hypothesis that SSRIs have conflicting effects in different regions of the brain that lead to a net anxiogenic effect when administered peripherally, e.g. downregulating serotonergic neurons via activation of somatodendritic serotonin 1A autoreceptors in the raphe nuclei, but an anxiolytic and conditioned fear reducing effect when infused centrally in the amygdala (Gartside *et al.*, 1995; Hajós, Gartside and Sharp, 1995). However, an acute SSRI has also shown anxiolytic effect when administered peripherally in low anxious marmosets with the CT/T/C haplotype (Santangelo *et al.*, 2016), and anxiogenic effect when infused in the BNST of rodents, providing further evidence that upregulated serotonin signalling may have differential genotype-dependent and region-specific effects and warrant further investigation (Marcinkiewcz *et al.*, 2016).

Serotonin transporter-positive fibers are distributed throughout the subnuclei of the amygdala (O'Rourke and Fudge, 2006). Serotonergic innervation of the amygdala from the dorsal raphe nucleus projects strongly to the basal amygdala, moderately to the lateral, basomedial and centromedial nuclei and only weakly to the centrolateral nucleus (Steinbusch, 1981; Sur, Betz and Schloss, 1996; Muller, Mascagni and McDonald, 2007). Serotonergic projections from the dorsal raphe innervate both principal neurons and interneurons in the BLA, the target of the cannulations and the main receiving nuclei of the amygdala which also receives converging inputs from cortical regions, the thalamus and the hippocampus (Aggleton, Burton and Passingham, 1980; Aggleton, 1986; Carmichael and Price, 1995; Ghashghaei and Barbas, 2002; Muller, Mascagni and McDonald, 2007). An electrophysiological study of the rat BLA demonstrated that serotonin in the BLA evoked depolarization of GABAergic interneurons (via $5HT_2$ receptor) which in turn drives the downregulation of BLA principal neuron activity (Rainnie 1999). SSRIs inhibit serotonin transporter leading to reduced reuptake of extracellular serotonin and increased sustained local serotonergic signalling. Taken together, the downregulation of BLA activity may underlie findings here of decreased trait anxiety expression in response to BLA infusions of an SSRI. Subsequent work identifying receptor subtype activation within specific amygdala subnuclei in response to serotonin will further elucidate the role of serotonin within the amygdala microcircuitry.

Inhibition of 5-HT_{2a} receptor binding in the amygdala increased heart rate in the neutral condition but did not have an effect on the human intruder test anxiety score, indicating that amygdala 5-HT_{2a} receptor activation may play a role in general cardiovascular reactivity but does not significantly affect behavioural responses to anxiety-provoking stimuli. Although preliminary investigation here of the effects of 5-HT_{2a} and 5-HT_{2c} receptor antagonism within the dACC and amygdala on anxiety did not yield a significant effect on anxious behaviour, it should be noted that manipulations of the 5-HT_{2a} and 5-HT_{2c} receptor target a specific subpopulation of neurons with those corresponding receptors, whereas a blockade of the serotonin transporter will affect all neurons regardless of serotonin receptor subtype.

Thus manipulations of the 5-HT_{2a} and 5-HT_{2c} receptor may have a subtler effect on behaviour compared to the serotonin transporter. More animals must be tested with differing doses in subsequent studies to form a more certain conclusion in regards to the role of 5-HT_{2a} and 5-HT_{2c} receptors within the dACC and amygdala on anxiety.

In conclusion, the series of behavioural analyses here demonstrated that inhibition of amygdala serotonin transporter leads to reduction in key threat-processing characteristics of the trait anxious phenotype: anxious behaviour and conditioned fear expression. These findings extend results from the previous chapter showing that amygdala serotonin transporter expression correspond to differing levels of anxious behaviour, by providing direct evidence that alteration in amygdala serotonin signalling may contribute to an individual's level of trait anxiety. Contrary to the anxiogenic effect of acute systemic SSRIs, it has been demonstrated here that an acute SSRI has an anxiolytic effect when infused in the amygdala. This suggest the anxiolytic effect of SSRIs as an effective antidepressant may be confounded by anxiogenic effects of the SSRI acting on other regions of the brain.

Chapter 5: Predictors of adulthood anxiety across development

Introduction: Diagnoses of anxiety disorders correspond to morphological changes in the amygdala of adolescents and adults. The basolateral nuclei of the amygdala (BLA) in particular has been shown to be sensitive to stress. Studying genetic factors that affect the structural change of the BLA and the developmental trajectory of the BLA will elucidate the changes that occur over an individual's lifetime that may underlie adulthood threat sensitivity.

Methods: The cohort of adult animals consist of 25 animals that underwent magnetic resonance imaging (MRI) with the BLA region of interest (ROI) subsequently drawn. The developing cohort consist of 24 animals MRI scanned at multiple time-points before and after puberty. The correlation between adulthood anxiety and BLA volume during adulthood and BLA volume change before and after puberty was determined. Furthermore, potential differences in adulthood anxiety among serotonin transporter polymorphism homozygotes was investigated.

Results: Correlational and trajectory analyses revealed that developing animals with higher anxiety display a delayed decline in BLA volume after puberty. In adulthood, higher levels of anxiety were associated with reduced general grey matter volume and left BLA volume, whereas reduced active coping in response to fear was associated with reduced right BLA volume. The high anxiety-associated AC homozygotes showed relatively smaller bilateral BLA volume compared to low anxiety-associated CT homozygotes.

Discussion and Conclusion: Our results show evidence of developmental and structural mechanisms underlying anxious and fear responding behaviour in adulthood. Moreover, I demonstrate a link between the newly identified marmoset serotonin transporter polymorphism and BLA volume after maturation.

5.1 Introduction

The first signs of mental illness occur early in life with nearly half of adults with a mental disorder having an onset age of 14 years and about 3 quarters before age 25 years (Kessler *et al.*, 2005). Anxiety disorders in particular have one of the earliest median onset age of 11 years. Adolescence is a critical period of development characterized by substantial cognitive, emotional, physiological, and behavioural changes. Although stress can lead to functional adaptive behaviours, chronic and severe stressors during critical periods of development by traumatic experiences may disrupt the brain's normal pathway for development and lead to life-long mental disorders. Consistent with this, both human and primate research has shown that juveniles experiencing early life stress and adversity present with enlarged amygdalas (Mehta *et al.*, 2009; Tottenham *et al.*, 2010; Lupien *et al.*, 2011; Whittle *et al.*, 2013; Coplan *et al.*, 2014).

Stressful stimuli elicit the release of corticotropin-releasing factor (CRF) which in turn plays a central role in regulating the hypothalamic-pituitary-adrenal (HPA) axis and initiates a cascade of events leading to the release of glucocorticoids from the adrenal cortex (Smith and Vale, 2006). The glucocorticoids released mediate the body's ongoing stress response (cardiovascular, metabolic, immune, etc.) and activate glucocorticoid receptors expressed in particularly high concentrations in brain regions implicated in emotion regulation: the hippocampus, the amygdala and the prefrontal cortex (Gray and Bingaman, 1996; Joëls, 2001; McKlveen *et al.*, 2013). This sequence of neuroendocrine events act as the mechanism in which traumatic life experiences lead to long term functional dysregulation and morphological change across the brain which may culminate in the characteristics of high trait anxiety (Christoffel, Golden and Russo, 2011). Indeed, a single acute dose of corticosterone, inducing an acute but substantial stress response, was sufficient to produce dendritic hypertrophy in the BLA and behavioural change within adult rodents (Mitra and Sapolsky, 2008).

In rodent models of stress, chronic restraint stress (CRS) was shown to differentially affect adolescents and adults, with CRS increasing number of spontaneously firing BLA neurons without affecting firing rate in adolescent rats, but increasing firing rate without increasing number of spontaneously firing neurons in adult rats (Zhang and Rosenkranz, 2012). Rodent models with juveniles and adults have also shown that chronic immobilisation stress (CIS) and CRS induce increases in spine density and dendritic arborization of BLA principal neurons accompanied by increased anxious behaviour and depressive-like behaviour (Vyas *et al.*, 2002; Vyas, Pillai and Chattarji, 2004; Mitra *et al.*, 2005; Eiland *et al.*, 2012). In contrast, chronic unpredictable stress (CUS) induced atrophy of BLA bipolar neurons and reduction of synapse density in the lateral amygdala of adults (Vyas *et al.*, 2002; Zhang *et al.*, 2014).

Taken together, rodent studies in developed animals provide evidence that stress differentially affects the BLA in developing and developed animals, stress mediates morphological change in BLA neurons and that BLA neuron structural change correspond to behavioural changes, and that the nature of the stressor may differentially affect the nature of the neuronal re-organization.

In humans, previous work has found that anxiety symptoms and greater threat reactivity were associated with greater amygdala (and more specifically BLA) volume in adolescents (De Bellis *et al.*, 2000; van der Plas *et al.*, 2010; Qin *et al.*, 2014). Young adults (~20 years) show a similar link between amygdala volume and threat vulnerability as adolescents do, consistent with the amygdala's continued development from birth to early adulthood (Machado-de-Sousa et al. 2014; Tian et al. 2016; review: Lupien et al. 2009). Although the contrary has also been shown (Milham *et al.*, 2005; Park *et al.*, 2015). The mixed findings in developing individuals may be due to the heterogeneity in the age range of participants and behavioural measure/symptom criteria selected. In contrast, adult studies have primarily found that both pathological anxiety and phobic (fear-based) disorders were linked to reduced amygdala volume (Massana *et al.*, 2003; Karl *et al.*, 2006; Hayano *et al.*, 2009; Rogers *et al.*, 2009; Irle *et al.*, 2010; Fisler *et al.*, 2013; Foell *et al.*, 2019). Rodent studies have mirrored human studies with accelerated amygdala development in adolescents after early-weaning, and reduced amygdala volume in matured animals with high fearfulness and stress reactivity (Yang *et al.*, 2008; Kikusui and Mori, 2009).

Sawiak *et al.* (2018) recently demonstrated that the marmoset BLA show an inverted-U shaped trajectory across development with an initial increase followed by a steady decline during puberty. This pattern of change in grey matter similarly occurs throughout the brain and reflects initial growth as a product of dendritic outgrowth and synaptogenesis followed by synaptic pruning and increased axon myelination (Giedd *et al.*, 1999; Paus, 2005; Gogtay and Thompson, 2010). As I was interested in different changes during development that may lead to long lasting changes in threat vulnerability, I investigate potential structural markers across the developing and developed brain predictive of anxious and fear-driven behaviour in adulthood. Specifically, using structural magnetic resonance imaging (MRI) data from a common marmoset cohort of developing animals scanned at pre-puberty (6-12 months) and post-puberty (12-21 months) and a separate cohort of animals scanned in adulthood (>21 months), I determine whether BLA volume is associated with emotion regulation and threat-related behaviour. The threat-related behaviour evaluated consists of anxious and fear-driven behaviour from animals screened on the human intruder test and rubber snake test respectively in adulthood. As the human and macaque serotonin transporter polymorphism was linked to altered amygdala volume, I was also interested in determining if the recently discovered marmoset serotonin transporter polymorphism mirrored this effect (Pezawas *et al.*, 2005; Santangelo *et al.*, 2016).

5.2 Methods

Subjects

The developing cohort consisted of 24 animals (Table 5.1: Male = 8; Female = 16. Pre-puberty scan age: 6-12, 7.88 ± 1.5 months; Post-puberty scan age: 12-21, 16.9 ± 2.7 months). The developing cohort consisted of a subset of Sawiak *et al*.'s (2018) cohort with data from the human intruder test and rubber snake test. The adult cohort consisted of 22 animals (Table 5.2: M = 14; F = 8. Age: >21, 31.4 ± 7.1 months). Only homozygotes were included as there were an insufficient number of adult heterozygotes scanned in the adult cohort (AC homozygotes, N = 11; M = 6, F = 5; Age: 32.8 ± 8.0. CT homozygotes = 11; M = 8; F = 3. Age: 29.9 ± 6.1). There was an insufficient number of AC homozygotes in the developing cohort to accurately evaluate the polymorphism's effect before adulthood. The developmental trajectory of the BLA was constructed with scans from 34 animals scanned a total of 125 times with an average of 3.7 scans per animal (Table 5.3: range 2-6 scans; n = 5, 13, 8, 4, 4 with 2, 3, 4, 5, 6 scans respectively).

No.	Name	Gender	Pre-puberty scan age (month)	Post-puberty scan age (month)	Human intruder test age (month)	Serotonin transporter polymorphism
1	Bernie	M	6.7	17.4	19.8	CT homozygote
2	Bomb	F	9.0	15.2	20.4	CT homozygote
3	Bullseye	M	8.9	20.5	28.9	Heterozygote
4	Chunk	F	8.9	20.5	29.3	AC homozygote
5	Deneb	M	7.7	15.3	22.8	CT homozygote
6	Eve	M	9.0	14.0	25.1	CT homozygote
7	Frangipane	F	6.2	12.6	21.0	Heterozygote
8	General	M	6.2	12.2	22.4	CT homozygote
9	Graves	M	6.2	16.9	19.1	Heterozygote
10	Heka	F	7.8	15.3	23.0	CT homozygote
11	Macintosh	M	8.8	20.0	34.6	Heterozygote
12	Manny	M	6.7	17.4	19.8	CT homozygote
13	Merida	F	11.9	20.0	27.2	Heterozygote
14	Referee	M	7.3	15.1	22.4	CT homozygote
15	Rex	F	9.1	20.2	20.5	AC homozygote
16	Roll	M	9.0	20.2	21.6	CT homozygote
17	Sarge	M	6.3	16.9	22.3	CT homozygote
18	Sheriff	F	7.3	15.1	22.7	Heterozygote
19	Slim	M	6.5	17.4	22.8	Heterozygote
20	Squibbles	M	9.1	14.8	24.8	CT homozygote
21	Syllabub	F	6.2	12.6	21.2	Heterozygote
22	Trixie	M	8.9	18.2	19.8	Heterozygote
23	Tuck	M	9.0	17.2	16.5	CT homozygote
24	Wall-e	M	6.5	20.5	20.3	AC homozygote

Table 5.1: Developing animal cohort. Detail of animals that were MRI scanned before and after puberty (12 months).

Marmosets were all born and reared at the Innes Marmoset Colony (Behavioural and Clinical Neuroscience Institute, BCNI). Animals of the developing cohort were housed in family groups in rooms with temperature (22 ± 1°C) and humidity (50 ± 1%) controlled and a dawn/dusk-like 12 hour-period was maintained. The animals of the adult animal cohort were housed in male/female pairs (males were vasectomised). All animals were provided with a balanced diet and water ad libitum. Besides MRI scans as detailed below, marmosets in this study did not undergo any other experimental procedures while being scanned for this study. Marmosets were regularly assessed for health as part of the normal colony procedures. No adverse health events were recorded for animals of this study. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and the local AWERB policies.

No.	Name	Gender	Scan age (month)	Human intruder test age (month)	Rubber Snake test age (month)	Serotonin transporter polymorphism
1	*Rose	M	36.5	35.1	-	AC homozygote
2	Merry	M	31.6	29.6	33.5	AC homozygote
3	Copper	F	22.8	21.0	24.5	AC homozygote
4	Windermere	F	21.8	18.1	22.0	AC homozygote
5	Cinders	F	50.0	46.6	50.4	AC homozygote
6	Bakerloo	M	29.9	29.6	30.5	AC homozygote
7	Axel	M	38.8	34.8	39.1	AC homozygote
8	Waterloo	F	30.4	29.5	31.0	AC homozygote
9	Sally	M	36.1	31.7	42.4	AC homozygote
10	Thissle	F	27.0	24.3	29.9	AC homozygote
11	Rhubarb	M	35.8	31.7	35.4	AC homozygote
12	Mace	M	24.9	23.5	27.2	CT homozygote
13	Riley	M	31.1	29.8	33.3	CT homozygote
14	Jetsam	M	23.6	20.8	24.5	CT homozygote
15	Micky	M	25.5	22.2	26.0	CT homozygote
16	Bob	M	30.0	29.7	30.8	CT homozygote
17	Elise	F	35.5	35.5	36.3	CT homozygote
18	Air	F	22.7	19.2	32.3	CT homozygote
19	Zoom	M	31.2	28.1	40.8	CT homozygote
20	Nettle	M	36.0	24.4	26.0	CT homozygote
21	Cabbage	F	26.6	21.3	24.5	CT homozygote
22	Birch	M	42.0	26.8	28.2	CT homozygote

Table 5.2: Adult animal cohort. Detail of animals that were MRI scanned in adulthood. *Rose was euthanised 2 months for respiratory difficulties after being screened on the human intruder test but before being screened on the rubber snake test.

On the day of scanning, animals were fasted. Marmosets were sedated with ketamine (20 mg/kg; Vetalar solution 100 mg/mL Pfizer, Kent, UK) and intubated for isoflurane anaesthesia (2.5% in 0.25–0.40 L/min O2). Throughout scanning, respiration rates were monitored with a pressure sensor over the upper abdomen (SA Instruments, Stony Brook, NY) and the isoflurane dose was adjusted between 1% and 3% to maintain respiratory rates in the normal range. A rectal thermometer was used to monitor temperature and a flowing-water heating system was adjusted as necessary. Marmosets were returned to their home cages once recovered.

	Low Anxious	Anxiety score	Age (months)	BLA volume (mm^3)	Adjusted volume (mm^3)
1	Eve	-0.2223	8.9	32.93	34.98
2	Eve	-0.2223	12.6	35.14	37.18
3	Eve	-0.2223	13.9	36.39	38.43
4	General	-0.23	3.7	33.51	35.39
5	General	-0.23	6.1	32.6	34.49
6	General	-0.23	12.1	37.04	38.93
7	Sarge	-0.24	3.5	36.4	33.62
8	Sarge	-0.24	6.2	39.03	36.25
9	Sarge	-0.24	11.5	42	39.22
10	Sarge	-0.24	14.3	37.82	35.04
11	Sarge	-0.24	16.7	38.63	35.86
12	Roz	-0.24	12	37.89	37.53
13	Roz	-0.24	20.1	33.64	33.28
14	Roz	-0.24	23	35.27	34.91
15	Bullseye	-0.26	8.8	37.02	38.92
16	Bullseye	-0.26	11.8	35.13	37.03
17	Bullseye	-0.26	20.3	29.63	31.53
18	Trixie	-0.43	3.2	34.91	33.78
19	Trixie	-0.43	8.8	38.19	37.06
20	Trixie	-0.43	11.5	38.64	37.51
21	Trixie	-0.43	14.4	36.49	35.36
22	Trixie	-0.43	16.2	37.52	36.38
23	Trixie	-0.43	18	35.71	34.58
24	Sheriff	-0.45	7.2	34.19	37.47
25	Sheriff	-0.45	12.5	33.57	36.85
26	Sheriff	-0.45	14.9	32.62	35.9
27	Macintosh	-0.47	8.7	35.19	35.51
28	Macintosh	-0.47	11.8	36.19	36.52
29	Macintosh	-0.47	17.3	35.36	35.68
30	Macintosh	-0.47	19.8	33.43	33.76
31	Macintosh	-0.47	24.2	33.42	33.74
32	Bernie	-0.5	3.6	39.13	37.21
33	Bernie	-0.5	6.6	40.02	38.1
34	Bernie	-0.5	11.7	39.92	38
35	Bernie	-0.5	14.4	37.7	35.78
36	Bernie	-0.5	17.2	33.56	31.64
37	Bernie	-0.5	21.1	36.68	34.76
38	Syllabub	-0.52	3.2	36.69	33.48
39	Syllabub	-0.52	6.2	41.74	38.52
40	Syllabub	-0.52	12.4	40.37	37.15
41	Emperor	-0.57	11.9	36.85	37.11
42	Emperor	-0.57	14.7	36.42	36.69
43	Emperor	-0.57	20.2	34.58	34.84
44	Emperor	-0.57	23	32.51	32.78
45	Tuck	-0.66	8.9	38.96	37.39
46	Tuck	-0.66	11.7	38.22	36.66
47	Tuck	-0.66	17	36.46	34.89
48	Ewok	-0.89	3.4	31.94	34.4
49	Ewok	-0.89	6.2	34.35	36.81
50	Roll	-1.01	8.9	42.03	37.76
51	Roll	-1.01	11.7	40.05	35.78
52	Roll	-1.01	17	39.09	34.82
53	Roll	-1.01	20	37.89	33.61
54	Yoda	-1.08	3.4	35.17	35.77
55	Yoda	-1.08	6.2	35.22	35.82
56	Bomb	-1.09	8.9	33.75	36.93
57	Bomb	-1.09	12.6	33.1	36.28
58	Bomb	-1.09	13.8	34.41	37.6
59	Bomb	-1.09	15	32.99	36.17
60	Frangipane	-1.51	3.2	33.04	34.86
61	Frangipane	-1.51	6.1	35.19	37.01
62	Frangipane	-1.51	12.4	34.69	36.5

	High Anxious	Anxiety score	Age (months)	BLA volume (mm^3)	Adjusted volume (mm^3)
1	Rex	0.93	9	34.71	35.64
2	Rex	0.93	11.9	34.84	35.76
3	Rex	0.93	17	34.57	35.49
4	Rex	0.93	20	34.31	35.23
5	Catapult	0.9	12	38.68	36.55
6	Catapult	0.9	14.5	39.02	36.89
7	Catapult	0.9	19.9	37.32	35.19
8	Greedo	0.79	3.7	34.95	35.12
9	Greedo	0.79	6.1	37.77	37.94
10	Deneb	0.53	7.6	37.29	35.62
11	Deneb	0.53	8.9	38.08	36.41
12	Deneb	0.53	12.1	39.67	38
13	Deneb	0.53	15.1	37.96	36.29
14	Trebuchet	0.33	11.9	38.71	36.22
15	Trebuchet	0.33	14.6	39.83	37.34
16	Trebuchet	0.33	19.9	37.4	34.9
17	Wall-e	0.29	6.4	38.17	37.5
18	Wall-e	0.29	9.2	38.74	38.07
19	Wall-e	0.29	14.8	36.94	36.26
20	Wall-e	0.29	17.1	36.75	36.07
21	Wall-e	0.29	20.3	30.38	29.71
22	Blake	0.13	12	36.95	37.24
23	Blake	0.13	20.1	33.58	33.88
24	Blake	0.13	23	34.01	34.3
25	Manny	0.13	3.6	37.2	36.04
26	Manny	0.13	6.6	38.26	37.1
27	Manny	0.13	11.7	37.96	36.8
28	Manny	0.13	14.4	37.92	36.76
29	Manny	0.13	17.2	35.81	34.65
30	Manny	0.13	21.1	33.48	32.32
31	Merida	0.07	11.8	33.45	35.88
32	Merida	0.07	17.3	33.1	35.53
33	Merida	0.07	19.8	32.35	34.78
34	Merida	0.07	24.2	30.8	33.23
35	Snowball	0.06	11.9	38.62	37.88
36	Snowball	0.06	16.9	37.01	36.27
37	Dicker	-0.02	3.2	33.15	33.6
38	Dicker	-0.02	5.8	37.37	37.82
39	Slim	-0.05	3	34.26	34.57
40	Slim	-0.05	6.4	37	37.31
41	Slim	-0.05	11.6	36.79	37.09
42	Slim	-0.05	14.3	37.03	37.34
43	Slim	-0.05	17.2	31.47	31.78
44	Graves	-0.08	3.6	37.85	35.84
45	Graves	-0.08	6.2	36.32	34.31
46	Graves	-0.08	11.5	40.45	38.44
47	Graves	-0.08	14.3	38.58	36.57
48	Graves	-0.08	16.7	36.69	34.68
49	Graves	-0.08	21.2	37.86	35.85
50	Referee	-0.18	7.2	34	36.23
51	Referee	-0.18	8.9	33.48	35.71
52	Referee	-0.18	12.5	34.88	37.11
53	Referee	-0.18	14.9	35.35	37.58
54	Heka	-0.2	7.7	35.55	36.44
55	Heka	-0.2	8.9	32.49	33.39
56	Heka	-0.2	12.1	37.04	37.93
57	Heka	-0.2	15.1	37.68	38.58
58	Chunk	-0.21	8.8	36.42	37.78
59	Chunk	-0.21	11.8	36.43	37.79
60	Chunk	-0.21	20.3	30.82	32.18
61	Squibbles	-0.2218	9	32.97	34.89
62	Squibbles	-0.2218	13.9	37.36	39.28
63	Squibbles	-0.2218	14.7	33.84	35.77

Table 5.3: Animals included in modelling the developmental trajectory of the BLA. Adjusted values of BLA volume were offset for the effect of gender and the random effect of subject. Individuals arranged in descending order of anxiety score.

Image Acquisition and Processing

Animals were scanned as part of Sawiak *et al.*'s (2018) developmental study using a Bruker PharmaScan 47/16 MRI system (Bruker, Inc., Ettlingen, Germany) at a field strength of 4.7 T. A custom 6 cm birdcage coil was used for signal transmission and reception. Structural images were acquired using a rapid acquisition with relaxation enhancement (RARE) sequence (parameters: TR/TEeff 11 750/23.5ms, 125 slices of 250 μm thickness, echo train length 4 and 3 averages) in 21m 44 s. The field of view was 64mm × 50mm yielding an isotropic resolution of 250 μm.

Images were processed using SPM8 (Wellcome Trust Center for Neuroimaging, UCL, UK) with the SPMMouse toolbox for animal data (Sawiak, Picq and Dhenain, 2014). Brains were aligned with tissue probability maps derived from marmosets from the same colony (Mikheenko *et al.*, 2015) and segmented into grey matter, white matter, and cerebrospinal fluid (GM, WM, and CSF). DARTEL (Ashburner, 2007) was used for image registration and to create population templates. The BLA ROI was drawn using Analyze 8 (Mayo Clinic) by an expert reviewer (Angela Roberts) with reference to a histological atlas (Paxinos *et al.*, 2012) (figure 5.1). Regional volumes were estimated by integrating Jacobian determinants from the DARTEL transformations over each region mask. No filtering based on tissue segmentation was used in this process. Total intracranial volume (TIV) was obtained as the sum of grey matter (GM), white matter (WM), and cerebrospinal fluid (CSF) volume.

Figure 5.1: BLA ROI. Schematic illustrating the BLA within the marmoset brain by Sawiak *et al.* (2018).

Developmental trajectory model

To illustrate trajectory of BLA, developmental data of animals from Sawiak *et al.*'s (2018) original cohort of animals from 3-21 months were split by the median human intruder test anxiety score. An additive mixed model was used to model these volumes using a spline estimation approach with GraphPad Prism ver.8 (Alexander-Bloch *et al.*, 2014). A restricted cubic spline curve was used to model the smooth but non-linear change in BLA volume across time. Each individual knot are points at which the individual polynomial spline function join. Five knots were used to model the date as recommended by Stone (1986). Total amygdala volume was adjusted for the effect of gender and the random effect of subject (inclusion of an additive constant varying by subject but not time).

5.3 Results

Reduced BLA volumetric decline post puberty is associated with higher anxiety

Anxiety was not associated with TIV, GM, WM, and BLA volume in pre-puberty or post-puberty ($p > .05$) (table 5.4). However, given that Sawiak *et al.* (2018) found that BLA volume follows a decline post-puberty, we investigated if the change in amygdala volume pre- and post-puberty corresponds to anxiety levels in adulthood. Greater decline in BLA volume post-puberty corresponds to lower anxiety score in adulthood (left: $r = .49$, $p = .032$; right: $r = .46$, $p = .047$). Difference in age between the pre-puberty and post-puberty scan, and the age of the post-puberty scan were included as covariates to remove the potential confound of variation in scanning age (figure 5.2a). This relationship was specific between the BLA and anxiety and not related to general threat reactivity as the change in the BLA during development was not associated with fear responding in adulthood (figure 5.2c and 5.2d. $p > .05$). To illustrate BLA developmental difference from the correlation, data of animals from Sawiak *et al.*'s (2018) original cohort of animals from 3-21 months were split by the median anxiety score to model the developmental trajectory of BLA volume. The trajectory pattern suggests that increased anxiety score's association with post-puberty difference may be due to a combination of high anxious animals tending to have lower BLA volume before puberty and delayed decline after puberty (figure 5.2b).

No.	Name	Anxiety score	TIV (mm^3)		Grey matter (mm^3)		White matter (mm^3)	
			Pre-puberty	Post-puberty	Pre-puberty	Post-puberty	Pre-puberty	Post-puberty
1	Bernie	-0.50	9567.5	8795.4	7495.4	6476.0	1575.8	1756.2
2	Bomb	-1.09	8461.1	8330.2	6729.7	6257.8	1350.0	1597.4
3	Bullseye	-0.26	9251.4	8491.9	7337.0	6209.4	1517.2	1693.1
4	Chunk	-0.21	9082.5	8464.3	7258.7	6042.0	1519.9	1729.8
5	Deneb	0.53	9032.9	8950.3	7166.9	6739.3	1602.9	1856.8
6	Eve	-0.22	8693.1	8862.1	6826.0	6832.0	1405.6	1665.2
7	Frangipane	-1.51	8882.4	8806.8	7119.9	6617.9	1425.8	1703.1
8	General	-0.23	8797.5	9145.5	6982.0	6875.8	1425.0	1846.0
9	Graves	-0.08	9677.9	9302.3	7715.0	7129.5	1475.4	1680.5
10	Heka	-0.20	9219.6	9242.4	7259.3	6697.2	1579.0	2006.3
11	Macintosh	-0.47	8839.2	8180.0	6920.0	6212.1	1418.5	1482.5
12	Manny	0.13	9309.0	8866.9	7292.8	6613.6	1534.6	1784.5
13	Merida	0.07	8457.6	7847.6	6467.3	5949.7	1480.2	1480.1
14	Referee	-0.18	8783.0	8729.2	6843.4	6424.3	1490.7	1820.7
15	Rex	0.93	8407.5	8058.5	6821.0	6027.5	1223.3	1410.8
16	Roll	-1.01	9968.2	9211.2	7866.2	6812.9	1816.1	1974.2
17	Sarge	-0.24	9443.0	9149.2	7537.4	6937.4	1523.5	1802.6
18	Sheriff	-0.45	8366.4	8096.9	6604.6	5968.1	1379.3	1639.9
19	Slim	-0.05	8557.2	8314.8	6807.0	6268.1	1348.0	1508.4
20	Squibbles	-0.22	9063.9	9055.2	6920.7	6736.7	1596.4	1686.5
21	Syllabub	-0.52	10054.2	10165.8	7893.7	7562.4	1737.6	1956.0
22	Trixie	-0.43	8954.5	8930.5	6980.6	6542.4	1496.2	1754.7
23	Tuck	-0.66	9135.1	8600.6	7406.1	6517.5	1471.7	1652.9
24	Wall-e	0.29	9498.7	8693.0	7626.9	6288.6	1504.4	1833.3

Table 5.4: General brain volume before and after puberty and individual adulthood anxiety score.

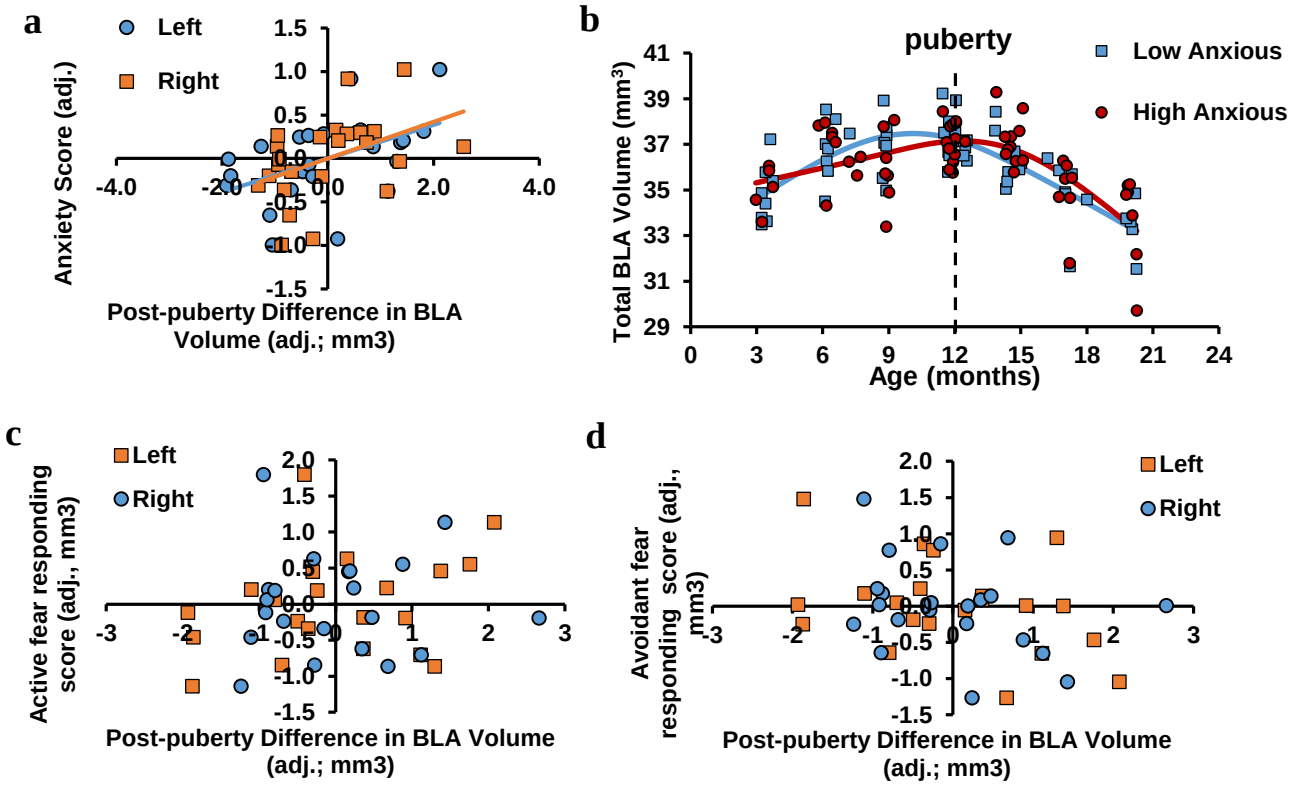

Figure 5.2: Adulthood anxiety is associated with pattern of BLA volume change post puberty. a) Partial regression plot and trendline illustrating that animals with a greater decline in BLA volume post-puberty (negative difference) had lower human intruder test anxiety scores in adulthood. b) Developmental trajectory of low and high anxious animals illustrating delayed BLA reduction post-puberty. This effect was specific to anxious behaviour as fear responding (c & d) did not correspond to BLA volume change after puberty. (Difference = [post-puberty BLA volume] – [pre-puberty BLA volume]). Adj.: adjusted.

Subsequently, I assessed if anxiety and fear responding style, measured by the human intruder test and rubber snake test factor scores respectively, were associated with variation in general brain structural changes whilst controlling for age and gender.

Anxiety score associated with reduction in grey matter and left BLA volume in early adulthood.

Human intruder test anxiety score was negatively correlated with GM volume (r = -.49, p = .033) but not correlated with WM volume (r = .32, p = .19) or TIV (r = -.35, p = .14) (figure 5.3a). Controlling for the anxiety score's association with GM volume and the potential effect of age and gender, whether anxious behaviour had a more specific effect, beyond that on grey matter volume, specifically on the structural volume of the BLA was assessed. Human intruder test anxiety score was negatively correlated with left BLA volume (r = -.65, p = .005), but not right BLA volume (r = -.20, p = .44) (figure 5.3b).

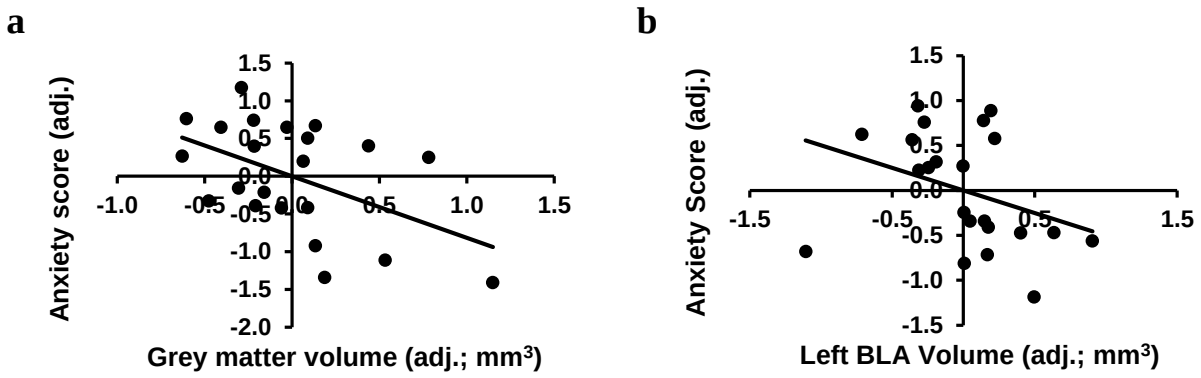

Figure 5.3: Anxious behaviour's association with brain volumetric differences in adults. Partial regression plots and trendline illustrating that anxious behaviour measured in the human intruder test in adulthood corresponds to reduction in general grey matter volume (p < .05) and b) a more specific reduction in left BLA volume (p < .01). Adj.: adjusted.

Active fear responding is associated with reduction in right BLA volume in adults.

Active and avoidant fear responding were not correlated with any measure of brain volume (Active: GM: r = .42, p = .07; WM: r = .38, p = .11; TIV: r = .31, p = .20; Avoidant: GM: r = -.19, p = .44; WM: r = -.21, p = .38; TIV: r = -.20, p = .41) (table 5.5). As fear responding style scores did not correspond to general volumetric variation, I subsequently assessed if fear responding styles had a specific effect on BLA volume whilst only controlling for age and gender. In adult animals, only higher active fear responding scores (r = .47, p = .049) was correlated with greater right BLA volume (figure 5.4), but not left BLA volume (r = .33, p = .18). Lower avoidant fear responding scores was not correlated with BLA volume (left: r = -.35, p = .16; right: r = -.45, p = .061).

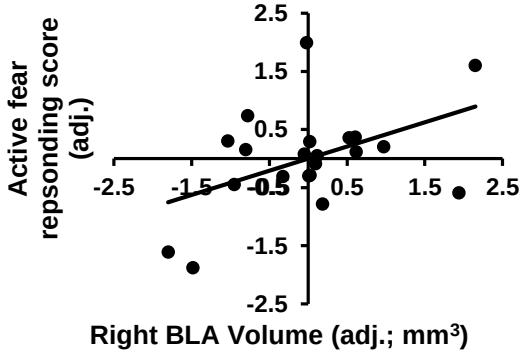

Figure 5.4: Fear-driven behaviour's association with brain volumetric differences in adults. Right BLA volume was positively associated with active fear responding in adulthood (p < .05). Adj.: adjusted.

		Rubber snake test fear behaviour		General brain volume (mm^3)			BLA volume (mm^3)	
No.	Name	Active score	Avoidant score	TIV	GM	WM	Left	Right
1	Rose	-	-	7181.1	4972.6	1389.7	13.23	16.02
2	Merry	-1.55	1.17	7417.1	5000.8	1593.0	13.27	15.32
3	Copper	-0.56	0.70	7710.2	5436.6	1615.1	13.84	16.82
4	Windermere	0.09	1.79	8567.8	6095.9	1668.1	15.19	17.75
5	Cinders	0.82	0.29	7243.3	5152.9	1435.3	13.86	16.18
6	Bakerloo	-0.77	0.54	8109.9	5696.9	1648.9	14.83	17.30
7	Axel	-1.61	1.52	7447.8	5192.2	1511.6	13.47	15.66
8	Waterloo	-0.48	0.50	7631.7	5359.7	1579.9	13.74	16.21
9	Sally	2.18	-0.74	8076.3	5572.9	1740.1	13.67	17.11
10	Thissle	0.07	-0.06	7993.6	5785.4	1540.9	14.88	18.13
11	Rhubarb	-0.10	0.19	8059.9	6133.8	1363.4	14.36	17.16
12	Mace	-0.05	-0.01	7970.3	5677.0	1680.3	14.32	17.06
13	Riley	0.41	-0.66	7858.8	5396.9	1711.2	14.94	17.65
14	Jetsam	-0.25	0.35	7532.6	5307.8	1471.3	14.03	17.20
15	Micky	0.05	0.46	7746.4	5453.0	1638.6	14.18	16.31
16	Bob	0.76	0.53	7613.3	5392.3	1579.6	14.30	17.07
17	Elise	0.22	0.36	7798.0	5777.6	1371.9	13.71	16.34
18	Air	-0.20	0.39	8035.2	5747.5	1660.1	15.61	18.72
19	Zoom	0.34	-0.87	7819.4	5545.9	1647.8	15.02	17.80
20	Nettle	1.79	-0.12	9132.1	6749.8	1589.2	14.94	17.26
21	Cabbage	-0.44	0.01	7927.4	5840.6	1440.3	14.96	17.14
22	Birch	-0.24	-0.15	8738.0	6376.3	1618.8	15.08	17.54

Table 5.5: Individual fear behaviour score on the rubber snake test, general brain volume and BLA volume.

High anxious AC homozygotes associated with smaller BLA volume in adulthood.

Following findings here that changes in BLA volume during and after development corresponding with adulthood anxiety, I assessed if the anxiety-linked serotonin transporter polymorphism affected amygdala volume in adulthood by conducting an ANCOVA whilst controlling for the potential effects of individual gender and age. TIV, grey matter, or white matter volume were not included as covariates as they did not differ among the homozygotes ($p > .05$; gender and age controlled). It was found that BLA volume of high anxiety-associated AC homozygotes (Left: 14.0 ± .7; Right: 16.7 ± .9) were significantly lower than CT homozygotes (Left: 15.0 ± 1.0; Right: 17.5 ± 1.0) in the left hemisphere, but only at a trend level on the right hemisphere (Left: $F(1,18) = 8.07$, $p = .011$; Right: $F(1,18) = 4.25$, $p = .054$) (figure 5.5).

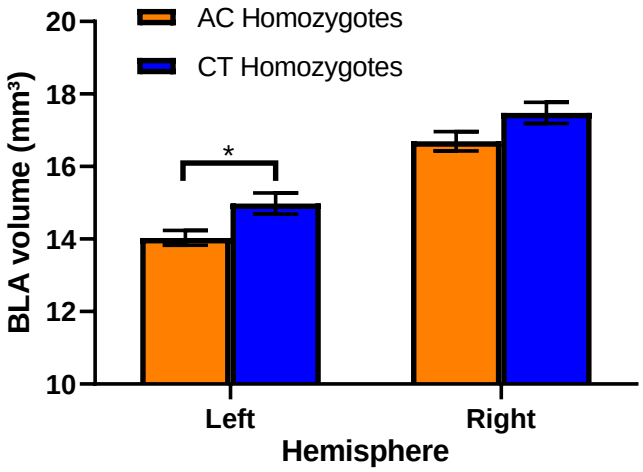

Figure 5.5: Differential BLA volume in adult homozygotes. Controlling for the effect of gender, age and TIV, the difference in the volume of the BLA between AC homozygotes compared to CT homozygotes was statistically significant in the left hemisphere, but not the right hemisphere. (*, $p < .05$)

5.4 Discussion

Although previous human and rodent studies have provided evidence of altered amygdala volume in the context of threat reactivity and pathological anxiety, this chapter uses a nonhuman primate model to demonstrate distinct morphological and genetic predictors of adulthood anxiety within the BLA during different stages of development. Animals with a delayed decline in BLA volume after puberty tended to have higher anxiety in adulthood, but after maturation, animals with lower BLA volume in adulthood were more anxious and expressed less active fear behaviour. Moreover, animals with the high anxious serotonin transporter polymorphism allele were predisposed to have higher anxiety in adulthood.

Disruption in the developmental pattern of the BLA may underlie aspects of the high threat vulnerable phenotype. Here, correlational and trajectory analysis indicates that individuals that have higher levels of anxiety in adulthood tend to have lower BLA volume before puberty and relatively delayed decline in BLA volume. Consistent with the attenuated growth observed pre-puberty here, childhood maltreatment was associated with a decrease in the normal pattern of growth or "flatter" growth of the amygdala from early to mid-adolescence in humans (Whittle *et al.*, 2013). Whittle *et al.* (2013) also reported lower amygdala volume in early adolescence but higher amygdala volume mid adolescence in juveniles with psychopathology, potentially corresponding to the shift in relative amygdala volume of high vs low anxious animals before and after puberty (figure 5.6). Previous studies have also implicated abnormal cortical maturation patterns in other mental disorders such as schizophrenia (Alexander-Bloch *et al.*, 2014). However, it is unclear as to whether the altered developmental pattern of the BLA in higher anxious individuals is (i) a compensatory product of higher stress reactivity in adolescence, (ii) reflects smaller BLA volume resulting in reduced efficacy in modulating threat-related stimuli, or (iii) a combination of both in the developing brain.

As high anxious adolescents tend to have lower BLA volume but also a delayed decline in volume post-puberty, the findings here potentially provide some insight into why studies relating adolescent BLA volume and anxiety disorders in the literature have been mixed. Studies with younger adolescents may find high anxiety linked to reduced BLA volume, while studies with adolescents in the period after low anxious individuals but not high anxious individuals have started BLA volume decline may find relatively increased BLA volume in high anxious adolescents (figure 5.6). Subsequent work evaluating the association between threat vulnerability in early life and BLA developmental trajectory will shed further light to disentangle the intricate relationship between adolescent development and life-long vulnerability to emotion dysregulation.

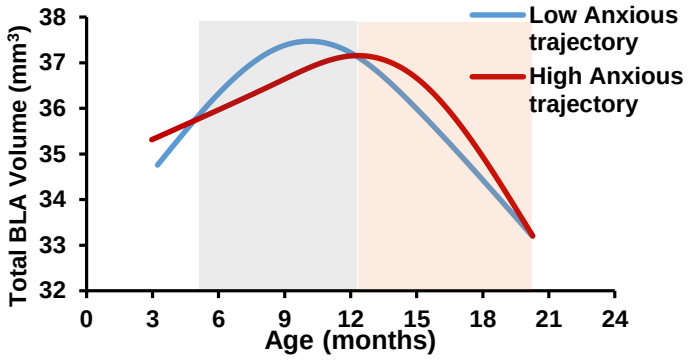

Figure 5.6: BLA developmental trajectory of high and low anxious animals. Developmental trajectory of BLA volume with period in which low anxious animals had greater total BLA volume relative to high anxious animals in grey (shaded), and period in which high anxious animals had greater total BLA volume relative to low anxious animals in orange (shaded).

After maturation, higher levels of anxiety were associated with lower general grey matter volume providing further evidence that higher levels of anxiety and consequently stress, lead to diffuse cross-regional changes. Reduced general grey matter in adulthood is consistent with evidence of diffuse grey matter reduction in PTSD patients (Li *et al.*, 2014). In contrast, fear reactivity (both active and avoidant fear responding) was not associated with general grey matter change. As fear is dependent on threat exposure and tends to be an acute response, anxiety may mediate general grey matter lost via prolonged stress as high trait anxious animals maladaptively experience anxiety in the absence of threat in a neutral environment. While controlling for anxiety's general effect on grey matter, anxiety had a more specific relationship with BLA volume. Specifically, greater anxiety was associated with lower left BLA volume, similar to the effect seen in animals before puberty. The lateralised effect observed is consistent with evidence from some human studies showing reduced left amygdala in patients with PTSD, panic disorder, and phobic disorder, but may also be a statistical artefact as a limitation of the sample size (Karl *et al.*, 2006; Hayano *et al.*, 2009; Rogers *et al.*, 2009; Fisler *et al.*, 2013). Lower active fear responding was linked to reduced right BLA volume, however this association may be mediated by the correlation between active fear responding and anxiety reported in chapter 2: "*Anxiety and fear response in the common marmoset*". Taken together, findings here point to the potential involvement of the BLA's anatomical reduction in increased anxious and lower active coping behaviour among matured individuals.

Current findings are limited by technical limitations to distinguish the cellular change underlying the observed structural change. Grey matter MRI signal reflects cell bodies, synapses, dendrites, terminals, glial cells, and unmyelinated axons, whereas the white matter signal primarily reflects myelinated axon tracts. The shift in grey/white matter volume observed may be due to changes in grey matter volume,

white matter volume, synaptic reorganization, or any combination of those changes. Current findings will be supplemented by a subsequent longitudinal study with high resolution structural scans differentiating grey matter and white matter within the amygdala. Distinguishing grey/white matter balance within the amygdala will further elucidate the developmental trajectory of neuronal change and its association with threat reactivity.

Our findings of reduced bilateral amygdala volume in AC homozygotes of the marmoset serotonin transporter polymorphism is consistent with evidence that serotonin plays a critical role during development. The serotonin transporter polymorphism's effect on amygdala volume is likely a downstream effect of altered amygdala serotonin transporter expression discussed in chapter 3: "*The relationship between serotonergic gene expression and anxiety and fear behaviour*", with changes in local serotonin signalling affecting neurogenesis, axon branching, and dendritogenesis (Gaspar, Cases and Maroteaux, 2003). Our findings are also consistent with evidence of smaller amygdalas in short allele carriers of the human serotonin transporter polymorphism, 5-HTTLPR even though the 5-HTTLPR is a length polymorphism and the marmoset serotonin transporter polymorphism is a double nucleotide polymorphism (Pezawas *et al.*, 2005).

To conclude, three factors predictive of high adulthood anxiety were determined: delayed decline of BLA volume after puberty, reduced left BLA volume in adulthood and carriers of the AC allele of the marmoset serotonin transporter polymorphism. These findings provide evidence for the view that genetic differences in the serotonin system affects the processing of stress which in turn lead to disruptions in the development of the BLA and contribute to BLA morphological changes in adulthood. While previous studies have explored potential markers for vulnerability to pathological anxiety of morphological change in adolescents and adulthood, findings presented here provide insight into the trajectory of BLA volume change from early life to adulthood that may underlie an individual's disposition to trait vulnerability to anxiety and fear. Findings here emphasise the need to further explore structural markers of trait anxiety and identify periods at which interventions may be effective at reversing these developmental and structural changes.

Chapter 6: General Discussion

Anxiety and fear are fundamental human emotions. However, a substantial proportion of society are struggling to cope with uncontrollable and excessive amounts of these normally adaptive emotions. Individuals with high trait anxiety are more vulnerable to developing mood and anxiety disorders. But, why are some more vulnerable to threat-related negative emotions than others?

Neuroimaging studies and experimental work in both humans and animals have identified features of the threat circuit preserved across species. Pharmacological work has identified serotonin as a key neuromodulator of threat processing and the serotonin transporter polymorphism, 5-HTTLPR has been implicated in mediating the impact of stress on the risk for not only depression, but also in an individual's vulnerability to anxiety. Furthermore, SSRIs have emerged as the first-line drug treatment for anxiety disorders. However, SSRIs transiently increase symptoms of anxiety during the onset of treatment and the role of serotoninergic components in specific regions of the threat circuit remain poorly understood, emphasising the need to advance our understanding of the specific role of serotonergic components in the regulation of threat.

The thesis addresses these issues by exploring the link between serotonin and threat behaviour with the common marmoset as a model. This chapter will summarise the results of these studies with a schematic of the key findings (figure 6.1). A discussion of the key research questions of serotonin's role in trait anxiety and the relationship between fear coping and anxious behaviour, and future work follows.

6.1 Summary of Results

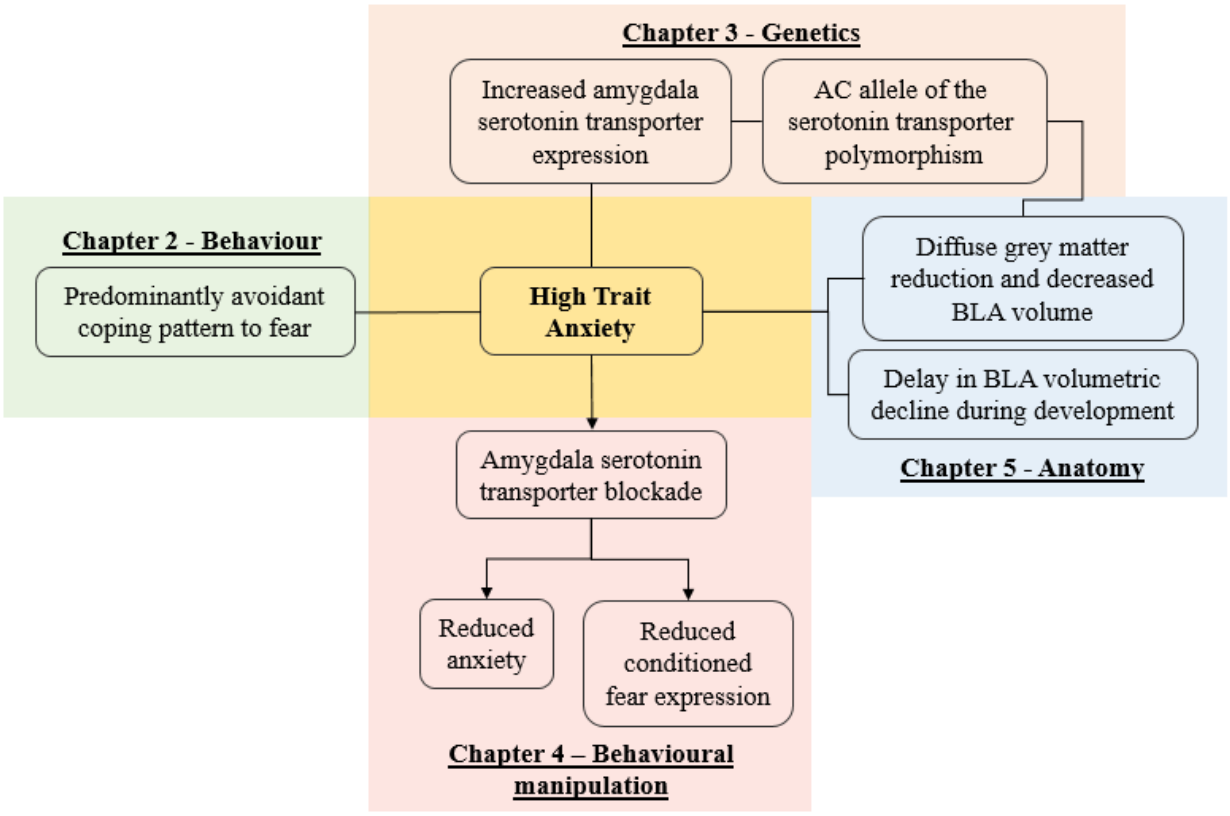

Figure 6.1: Key findings and associations of the thesis.

Firstly, chapter 2 explored the link between behaviours under different threatening situations to model anxious and fear-coping behaviours in the marmoset and establish the link between anxiety and fear-driven behaviours. Exploratory factor analysis revealed that anxious behaviour in the common marmoset was characterised by both active vigilance and avoidant behaviours when confronted with an unknown human in the human intruder test. However, fear was found to drive two negatively associated patterns of behaviours in the rubber snake test: active fear-coping behaviour characterised by actively attending to the snake and vocalising; and avoidant fear-coping behaviour characterised by behavioural avoidance and passivity. Animals with a high avoidant fear coping score but low active fear coping score had higher anxiety scores as measured on the human intruder test, suggesting that a predominantly avoidant fear coping style is linked to a greater vulnerability to anxiety.

Subsequently, chapter 3 investigated the potential associations between both the anxious and fear-coping behaviours modelled, and serotonergic gene expression within regions of interest. Serotonin transporter expression in the right amygdala and right vlPFC were associated positively with anxiety, and right mPFC 5-HT_{2A} receptor expression was associated positively with avoidant fear responding.

These findings identified potential genetic mechanisms underlying an individual's vulnerability to anxiety and tendencies for specific fear coping behaviours. Moreover, the recently discovered serotonin transporter polymorphism of the common marmoset differentiated serotonin transporter expression in the right amygdala, with AC homozygotes showing higher amygdala serotonin transporter expression compared to CT homozygotes, potentially driving the higher serotonin transporter expression in high anxious animals.

Building on the correlational findings of the previous chapter, chapter 4 explored if amygdala serotonin transporter played a causal role in two key characteristics of the high trait anxious phenotype: high state anxiety and exaggerated conditioned fear responding. We also examined the effect of the pharmacological manipulations on cardiovascular activity in a neutral condition to control for the pharmacological manipulation's non-specific effect on cardiovascular activity. Serotonin transporter blockade via an SSRI locally into the amygdala lead to reductions in anxious behaviour in the human intruder test. Furthermore, it also reduced both the physiological and behavioural conditioned response to threat. Inhibition of amygdala serotonin reuptake did not affect cardiovascular activity in a neutral condition, indicating that amygdala SSRI's only disrupted threat-provoked cardiovascular responses and not cardiovascular responses at baseline or at 'rest'. These findings support the theory that amygdala serotonin signalling as mediated by local serotonin transporters plays a role in the expression of the high trait anxious phenotype.

As the 5-HT_{2A} and 5-HT_{2C} receptors have been implicated in the regulation of anxiety, chapter 4 also studied the effect of 5-HT_{2A} and 5-HT_{2C} receptor antagonism in the amygdala and dACC on anxiety. Inhibition of amygdala 5-HT_{2A} receptor binding and both amygdala and dACC 5-HT_{2C} receptor binding did not affect anxious behaviour. In a neutral condition, inhibition of amygdala 5-HT_{2A} receptor binding increased heart rate but did not affect blood pressure. Inhibition of dACC 5-HT_{2C} receptor binding did not affect cardiovascular activity at all. These findings suggest that 5-HT_{2A} receptor activation does not significantly affect behavioural responses to anxiety-provoking stimuli, but may play a role in general cardiovascular reactivity. Whereas, 5-HT_{2C} receptor antagonism in the amygdala and dACC without manipulations of other systems were not sufficient to affect anxiety and general cardiovascular activity.

Anxiety disorders often have their onset during development. Thus, considering the amygdala's role as the central locus of the threat circuit and the fact that BLA morphology has been shown to be sensitive to stress, chapter 5 compared the volumetric changes of the BLA measured via MRI in the developing and developed brain of high and low anxious animals. In development, grey matter initially increases in volume but at some point during, or after puberty, grey matter volume apparently declines, probably reflecting changes in myelination as well as synaptic re-organisation. However, the decline in BLA volume post puberty was delayed in animals characterised as having high anxiety at the start of adulthood. Moreover, in adulthood, higher levels of anxiety were associated with reductions in global

grey matter volume and left BLA volume, and reduced active fear coping behaviour was associated with reduced right BLA volume. When the potential effect of the serotonin transporter genotype on BLA morphology was investigated, correlational analysis revealed that the high anxiety-associated AC homozygotes exhibited relatively decreased bilateral BLA volume compared to the low anxiety-associated CT homozygotes. These findings provide evidence of anatomical changes corresponding to the behavioural variance observed.

6.2 Amygdala serotonin transporter regulates trait anxiety

The primary goal of this thesis was the determination of potential serotonergic mechanisms underlying trait vulnerability to anxiety. A multi-systems approach was adopted to provide evidence for a potential mechanism of amygdala serotonin transporter's role in trait anxiety (figure 6.2).

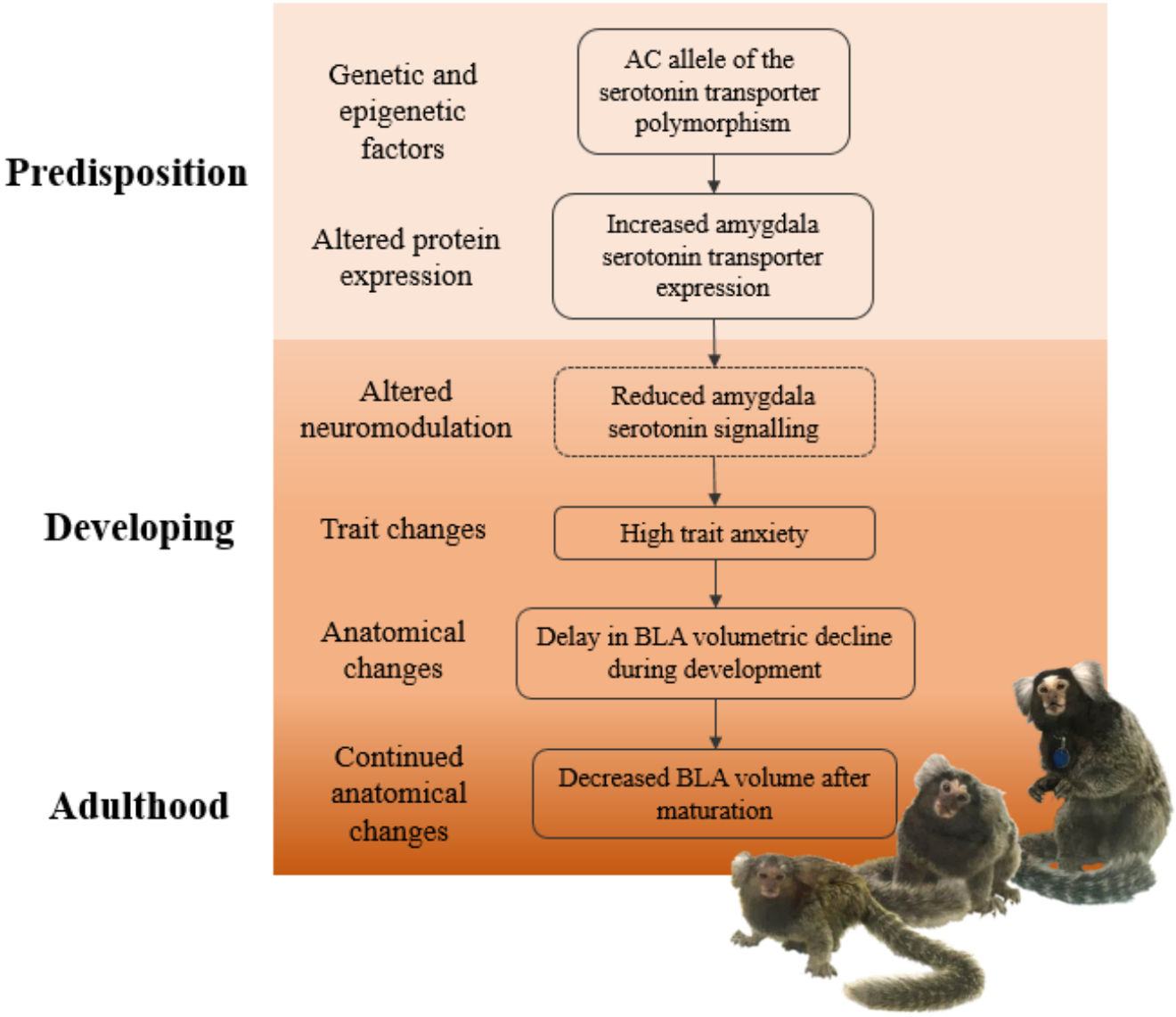

Figure 6.2: Potential pathway contributing to the high trait anxious phenotype. Altered genetic and epigenetic factors, such as the serotonin transporter polymorphism, contribute to differential expression in the serotonin system and may underlie individual differences in serotonin's modulatory effect on threat circuit reactivity. As the animal develops, stress reactivity interacts with stressors in the environment to alter the developmental trajectory of the amygdala. These changes likely contribute to life-long changes in anatomy and behaviour without intervention. **Marmoset picture taken by Christian Wood.*

As demonstrated by the human serotonin transporter polymorphism, 5-HTTLPR, genotypic variation may contribute to differential expression of key genes and underlie an individual's trait vulnerabilities. Consistent with this, the common marmoset serotonin transporter polymorphism was associated with differential levels of amygdala serotonin transporter mRNA levels (chapter 3). These differential levels of expression were predictive of individual variation of anxiety, with higher levels of serotonin transporter mRNA associated with higher levels of anxiety. Since the serotonin transporter downregulates serotonin signalling via reuptake of synaptic serotonin, increased serotonin transporter levels supports the lab's previous finding of reduced amygdala extracellular serotonin in high trait anxious marmosets (Mikheenko *et al.*, 2015). Taken together, these findings provide a genetic mechanism underlying the individual heterogeneity in vulnerability to anxiety, with AC homozygotes contributing to increased expression of amygdala serotonin transporter and leading to reduced local serotonin signalling. These findings are consistent with increased serotonin transporter availability reported in the amygdalohippocampal area associated with increased anxious temperament in macaques (Oler *et al.*, 2009).

As serotonin leads to direct activation of GABAergic interneurons and indirect inactivation of projection BLA neurons (Rainnie, 1999), increased serotonin reuptake from increased serotonin transporter expression may lead to reduced serotonin signalling and disinhibition of the BLA, and contribute to high anxiety. Future work studying other genetic and epigenetic mechanisms underlying altered serotonin signalling in high anxious individuals will supplement findings here.

To determine if amygdala serotonin transporters played a causal role in the expression of the trait anxiety phenotype beyond being simply associated with state anxiety, chapter 4 employed pharmacological manipulations and found that local blockade of serotonin transporters in the amygdala reduced both state anxiety expression and conditioned threat expression, effectively reducing key characteristics of high trait anxiety. Anxiety and fear/certain threat are commonly studied as separate constructs, but trait fear and trait anxiety are positively associated (Sylvers, Lilienfeld and LaPrairie, 2011). Differences in amygdala serotonin signalling may be a key modulator of threat circuit activation and more fundamentally affect vulnerability to threat.

The AC homozygotes were also associated with decreased BLA volume in adulthood, implicating the high anxious haplotype with not only changes in gene expression, but also anatomical changes in maturation. As stress has been shown to alter BLA neuron morphology and increase reactivity to anxiety (Vyas, Pillai and Chattarji, 2004; Mitra and Sapolsky, 2008; Eiland *et al.*, 2012; Zhang and Rosenkranz, 2012), the gene-expression changes corresponding to the AC homozygotes may lead to increased anxiety and stress during the animal's lifetime and lead to the anatomical changes observed in chapter 5: delay in BLA volumetric decline before maturation but decreased BLA volume after maturation. Alternatively, anatomical alterations may precede changes in anxious behaviour, with abnormality in

amygdala morphology reflecting some extent of neuronal loss-of-function and leading to dysfunctional threat processing. Further work will be required to better disentangle the cause-and-effect of altered amygdala morphology and developmental trajectory observed in high trait individuals.

Taken together, findings from these series of experiments track the potential effects of the serotonin transporter polymorphism and alteration in amygdala serotonin signalling on the anatomical and behavioural characteristics of high trait anxious animals. As amygdala hyperreactivity is consistently implicated across high trait anxious individuals and patients with anxiety disorders, findings here suggest that individuals may possess a relatively disinhibited amygdala and consequently, a greater vulnerability to threat-related negative emotions due to downregulations in local serotonin signalling. Furthermore, findings here also provide evidence of the region-specific effect of SSRI action and suggest that typical SSRI medication processed systemically in patients may have nontherapeutic/undesirable effects due to its whole-brain upregulation of serotonin. Future treatment interventions should attempt to selectively aim to upregulate serotonin signalling in the amygdala, avoiding the conventional downfalls of SSRI prescriptions: the initial worsening of symptoms and the side effects from off-site increases in serotonin signalling (weight gain, sleep disruption, sexual dysfunction, etc.). The clinical implication of findings here suggesting the involvement of the genetic polymorphism in developmental changes paired with findings in the literature of the early onset of anxiety disorder emphasise the need for targeted early interventions to develop resilience in vulnerable young children before the development of affective and anxiety disorders.

6.3 Avoidant coping patterns and anxiety

High trait anxious animals adopted a predominantly avoidant coping strategy in response to fear (chapter 2). This is consistent with the prevalence of avoidant behaviour in individuals struggling with anxiety and stress e.g. individuals with social anxiety disorder tend to avoid social situations, individuals with agoraphobia avoid leaving the house, or individuals with PTSD avoid uncontrollable intrusive memories (Brewin and Holmes, 2003). In the short-term, an avoidant coping strategy can reduce symptoms of fear or anxiety by avoiding engagement with the threat or avoiding the context in which the threat may occur. In the long-term however, the individual may tend to experience greater anxiety as the source of threat and stress are not confronted/overcome. In line with this, individuals show heightened amygdala responses to cues leading to the avoidance of an aversive stimuli, suggesting that even if avoidance behaviour may eliminate presence of the threat, the avoidant behaviour itself may lead to activation of threat circuitry (Schlund and Cataldo, 2010). Furthermore, natural processes that serve to reduce anxiety and conditioned fear responses such as desensitisation or fear extinction are inhibited by an individual's predominantly avoidant coping strategy. Consequently, the avoidant coping strategy is reinforced via negative reinforcement and may gain predominance over active coping impulses. Avoidant coping responses to fear may thus act as a maladaptive coping strategy sustaining high anxiety.

Specific fear responding behaviours were associated with different biological systems. Avoidant fear responding was positively associated with 5-HT_{2A} receptor expression in the mPFC, suggesting that 5-HT_{2A} receptor activation of cortical neurons may underlie expression of avoidant coping behaviour (chapter 3). On the other hand, active fear responding was positively associated with right BLA volume, suggesting that active coping behaviour may interact with BLA neuron morphology (chapter 5). These findings provide preliminary evidence of the biological basis of different fear coping strategies. Taken together, these findings suggest that although avoidant and active fear responding may have overlapping neural mechanisms at the level of the CeA (Gozzi *et al.*, 2010), differential neural pathways within the limbic system may contribute to the expression of active and avoidant coping behaviours.

The findings here emphasise the importance of CBT in the treatment of anxiety disorders. Specifically, the combination of exposure therapy and encouraging the use of active coping strategies instead of avoidance to engage with the source of distress, may play a significant role in improving individual resilience to stressors. These CBT strategies taken in conjunction with SSRI treatment to alleviate stress reactivity may be key to helping treatment-resistant individuals, suffering from excessive anxiety and fear, to develop healthy coping patterns in everyday life.

6.4 Strengths, limitations and future work

The primary strength of the thesis is the multi-systems approach adopted. By demonstrating alteration in amygdala serotonin corresponding to genetic, anatomical and behavioural changes with consistent findings among the different systems, insight into how changes in one system may translate into another enabled the development of a potential explanatory model. In contrast, a key limitation of the study is not being able to identify what underpinned the differential expression of serotonin transporter in the amygdala. The changes could be driven by mRNA in the serotonergic terminals, amygdala astrocytes, or both. The challenge of addressing this issue is that serotonin transporters are expressed at a very low level in the amygdala and conventional RNA fluorescence in situ hybridization (RNA-FISH) may not have the resolution necessary to localise differential expression corresponding to variation in anxious behaviour. Furthermore, the regions of interest included in chapter 3 were not exhaustive. Other regions implicated in anxiety such as the hippocampus and subgenual cingulate cortex should be investigated in a future study.

Another limitation of the study is the limited number of animals involved in the behavioural manipulation study. The primary constraining factor was the time required to test animals on various paradigms. Additionally, animals had to be selected to show avoidance of the human intruder and reactivity towards the snake in screening tests such that changes in anxious or fear-driven behaviours can be observed after the pharmacological manipulations.

Furthermore, due to time and technical constraints, work studying the effect of the serotonin transporter polymorphism on aspects of serotonin signalling could not be undertaken. Studying if the serotonin transporter polymorphism was associated with altered amygdala serotonin signalling in the form of differential release of serotonin via microdialysis, or differential principal neuron activation via single-unit recordings will shed further light on the potential effect of genetic factors on signalling changes relevant to threat processing.

A significant longitudinal study is currently underway with animals in the colony scanned with a high-resolution MRI scanner providing substantially more information to study the brain changes corresponding to behavioural changes across different developmental periods. Diffusion tensor imaging (DTI) diffusivity data will enable us to track and identify brain structural changes that correspond to different levels of anxiety and coping behaviours. These high-powered scanners will also enable us to distinguish regional grey and white matter of much greater resolutions and better disentangle local grey/white matter changes.

It’s important to keep in mind that the amygdala serotonin transporter is likely one of many key factors that contribute to the high trait anxious phenotype. Differences in receptor expression did not

correspond with differences in levels of anxious behaviour, but serotonin receptors may contribute to different levels of vulnerability to anxiety via different mechanisms. For example, the 5-HT_{2A} receptor and 5-HT_{2C} receptor protein ratio in the mPFC has been associated with motor impulsivity (Anastasio *et al.*, 2015). Moreover, sexual arousal in the hypothalamus-pituitary-testicular axis was facilitated by 5-HT_{2A} receptor antagonism but suppressed by 5-HT_{2C} receptor antagonism, further suggesting that the 5-HT_2 receptors possess differential roles in the modulation of behaviour (Popova and Amstislavskaya, 2002). Studying the 5-HT_{2A}:5-HT_{2C} receptor ratio instead of the serotonin receptors in isolation may be the key to identifying the involvement of serotonin receptors in anxiety.

Additionally, as serotonin also modulates sleep and sleep disturbances are prevalent in patients with anxiety disorders (Portas, Bjorvatn and Ursin, 2000; Staner, 2003), data of sleep/wake cycle and activity patterns throughout the day could potentially be collected from the colony's animals via wrist-worn accelerometer devices. Analysis of this dataset may provide further insight into the potential disruptive effects of altered serotonin signalling on an animal's general wellbeing.

6.5 Concluding remarks

As the results of this study have shown, the high trait anxious phenotype is associated with complex changes across different systems. Using only a handful of these factors to aid diagnoses of pathological forms of emotion regulation are unlikely to yield reliable results as many different factors contribute to an individual's trait anxiety level and risk of anxiety disorders and depression. Moving forward, it is critical that a concerted effort across different disciplines is made to identify these underlying factors and incorporate them as part of a predictive model that will enable us to prescribe more effective treatment strategies and move towards personalised medicine.

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