The effects of losartan and diazepam on emotional processing

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Thesis submitted for the degree of Doctor of Philosophy
Trinity Term, 2021

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Abstract

Given the prevalence and substantial economic costs of anxiety disorders, and the shortcomings of current treatments, there is dire need for research that helps inform the development of new treatments and medications. The aim of this thesis was to further our understanding of the effects of two medications relevant to anxiety, losartan and diazepam, to help inform the use of existing treatments and lead to more effective ones.

Research has indicated that the angiotensin receptor antagonist losartan may potentially be a promising candidate to enhance the efficacy of exposure-based therapies. It however remains to be fully clarified how losartan affects some of the mechanisms relevant to exposure success.

In the first study, a single dose of losartan was shown to increase activation in the paracingulate gyrus, insular cortex, lingual gyrus, and fusiform gyrus in healthy, high trait-anxious volunteers, which possibly reflects modulation of higher-order visual processing. There was however no evidence found for an effect of losartan on neural responses in the hippocampus during non-emotional memory encoding. Losartan was also shown to increase positive attentional bias, which was reflected in attention being more firmly held by positive stimuli compared to neutral stimuli. Given that both greater reactivity in higher-order visual regions and positive valence training have been shown to be relevant for therapy success, these results may provide further support that losartan might potentially have synergistic effects with exposure therapy, but this remains to be tested directly.

The most common pharmacological treatments for anxiety disorders include selective serotonin reuptake inhibitors and benzodiazepines, but both groups of medications have limitations. A better understanding of how existing medications exert their anxiolytic effects may help guide development of new medications. As benzodiazepines are not effective in treating depression, researching their effects provide a means of teasing apart antidepressant and anxiolytic effects. A comprehensive understanding of the cognitive neuropsychological mechanisms behind their anxiolytic effects is still lacking.

In the second study, a 7-day treatment of diazepam was shown to lower connectivity between the amygdala and the pre- and post-central gyrus during cognitive reappraisal, and between limbic regions and the precuneous cortex in response to aversive pictures in healthy volunteers. The treatment also led to a decrease in activation in the right vlPFC during reappraisal, and to an increase in activation in the left vlPFC and right ACC in response to positive stimuli, without any subjective changes in mood and state anxiety. Diazepam may thus potentially be exerting its short-term anxiolytic effects by modulating activity within these brain areas.

Taken together, these findings provide valuable insights into potential mechanisms through which diazepam and losartan may exert their therapeutic effects. A better understanding of these mechanisms can hopefully help inform the development of future anxiolytics and combination treatments.

A brief summary of the impact of the COVID-19 pandemic on this thesis

Right before the university buildings closed due to COVID-19, I had worked on and gained ethical approval for a study that was meant to investigate the effects of losartan on reward processing, vigilance, and stress responses in healthy volunteers. It would have been a double-blind, randomised, placebo-controlled study, where 60 participants would have worked on a battery of computerized tasks, including the Avoidance-Cost Task, which measures how rewards are weighted against threat consequences when gambling for fictive money; the Gamified Foraging Task, which ecologically captures vigilance; and the Oxford Cognition Stress Task, which involves measuring changes in mood, heart rate, and salivary cortisol in response to challenging cognitive tasks and failure feedback. This study would have provided 3 data chapters for my thesis. Everything was ready, but as I couldn't recruit for it for over a year, I unfortunately ended up not being able to run it. I thus ended up analysing existing data sets related to another medication (diazepam) relevant to anxiety throughout the COVID-closures, which have provided me with 3 data chapters for my thesis, albeit about a different medication. My other losartan study was also put on pause during this time, which led to fewer participants being recruited than planned.

Acknowledgements

First, I would like to express my heartfelt gratitude to my outstanding supervisors, Dr Andrea Reinecke and Prof. Catherine Harmer, for their invaluable supervision and continuous encouragement throughout my time at Oxford. I am sincerely grateful to both of them for having taken on the supervision of my dissertation and for their mentorship. They have been such an inspiration. I have also greatly appreciated all their support, and their valuable insights, advice, and feedback during the preparation of this thesis. It has been a great honour being taught by such brilliant scientists!

I am also incredibly grateful for the guidance, support, and advice I received from Dr Marieke Martens in regard to the brain analysis. She was always there for me whatever problem I had, which I greatly appreciated. Her infectious enthusiasm, warmth, and academic intellect made working with her such an enjoyable experience! I would also like to thank my colleagues and friends at the PERL and OXITED groups for the support, engaging discussions, and inspiration. It has been a great privilege being part of such dynamic and vibrant groups! I am also grateful to all the fantastic people working at the Department of Psychiatry for providing such a warm and welcoming environment.

Finally, special thanks are reserved for my family and friends for supporting and encouraging me through the highs and lows of DPhil life and for always giving me a reason to smile. I would especially like to thank my mother for her endless support and for giving me the experiences that have made me who I am, this DPhil journey would not have been possible if not for her. I would also like to thank my late father for always having faith in my abilities and encouraging me to pursue my dreams – I wish you could have been with me through this journey, dad.

Declaration

I declare that this thesis is my own work, completed under the supervision of Dr Andrea Reinecke and Prof. Catherine Harmer. It has not been submitted, either partially or in full, for any other qualification at the University of Oxford or any other institution. All data was collected by me, except the pre-existing data sets used for the diazepam part of this thesis, which Prof. Harmer kindly provided me with during the COVID-19 pandemic disruption.

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List of abbreviations

ACC	Anterior cingulate cortex
ACS	Attentional Control Scale
Ang II	Angiotensin II
Ang IV	Angiotensin IV
ANOVA	Analysis of variance
ASI-R	Anxiety Sensitivity Index - Revised
ASL	Arterial spin labeling
BAS	Behavioral Activation Scale
BDI-II	Beck Depression Inventory II
BET	Brain Extraction Tool
BFS	Befindlichskeit Scale of Mood and Energy
BIS	Behavioral Inhibition Scale
BMI	Body mass index
BOLD	Blood-oxygen-level-dependent
CBT	Cognitive behavioural therapy
CNS	Central nervous system
COVID-19	Coronavirus disease-19
CR	Conditional responding
CS	Conditioned stimulus
DSM	Diagnostic and Statistical Manual of Mental Disorders
ecStroop	Emotional counting Stroop
EPI	Echo planar images
EPQ	Eysenck Personality Questionnaire
ERQ	Emotion Regulation Questionnaire
FAST	FMRIB's Automated Segmentation Tool
FEAT	fMRI Expert Analysis Tool
FILM	FMRIB's Improved Linear Model
FIX	FMRIB's ICA-based Xnoiseifier
FLAME	FMRIB's Local Analysis of Mixed Effects
FLIRT	FMRIB's Linear Image Registration Tool
fMRI	Functional magnetic resonance imaging
FOV	Field of view
FSL	FMRIB Software Library
GABA	Gamma-aminobutyric acid
GLM	General linear model
GM	Grey matter
HPA	Hypothalamic-pituitary-adrenocortical
IAPS	International Affective Picture System
ICA	Independent component analysis
M	Mean
MCFLIRT	FMRIB's Linear Image Registration Tool
MELODIC	Multivariate Exploratory Linear Decomposition into Independent Components
MNI	Montreal Neurological Institute
MRI	Magnetic resonance imaging
MTG	Middle temporal gyrus
n	Sample size
NART	National Adult Reading Test
OCMR	Oxford Centre for Clinical Magnetic Resonance Research
OHBA	Oxford Centre for Human Brain Activity
PANAS	Positive and Negative Affect Schedule
PCA	Principal component analysis
PE	Parameter estimate
PPI	Psychophysiological interactions
PTSD	Post-traumatic stress disorder
RAS	Renin-angiotensin system
ROI	Region of interest
RT	Reaction time
SBRef	Single-band reference
SCID	Structured Clinical Interview for DSM disorders
SD	Standard deviations
SEM	Standard error of the mean
SSRI	Selective serotonin reuptake inhibitor
STAI-S	State-Anxiety subscale of the State-Trait Anxiety Inventory
STAI-T	Trait-Anxiety subscale of the State-Trait Anxiety Inventory
STW-2	Spot the Word 2
SVC	Small volume correction
TE	Echo time
TMS	Transcranial magnetic stimulation
TR	Repetition time
US	Unconditioned stimulus
VAS	Visual analogue scales
vlPFC	Ventrolateral prefrontal cortex
vmPFC	Ventromedial prefrontal cortex

Chapter 1: General Introduction

1.1 Anxiety disorders and emotional processing

Anxiety disorders have been shown to be one of the most prevalent class of lifetime disorders in today's modern society (Kessler et al, 2005). They include several conditions such as social anxiety disorder, panic disorder, specific phobia, and post-traumatic stress disorder (PTSD), but common to all are excessive fear and worry that interfere with daily life (American Psychiatric Association, 2013). Anxiety disorders tend to be chronic illnesses, often characterized by remissions and exacerbations, with a more or less continuous and unrelenting course (Shader & Greenblatt, 1993). The majority of people with anxiety disorders experiences serious impairments in their daily lives by their symptoms, which include increased fear and emotional reactivity, negative attentional biases, and tendencies to experience emotions as aversive, and often result in dysfunctional emotion regulation and avoidant behaviour (Campbell-Sills, Ellard, & Barlow, 2014). Anxiety develops when a previously neutral stimulus or situational cue becomes associated with an aversive one and starts generating a conditioned fear response and feelings of hypervigilance, uncontrollability, and distress. In response to this acquired distress, the person engages in avoidance, such as physically avoiding the anxiety-provoking situation or using mental distraction, to escape and alleviate it. This in turn negatively reinforces the avoidance and leads to it persisting, as it prevents the person from experiencing opportunities that potentially could extinguish the conditioned response (McGuire, Lewin, & Storch, 2014). Given the prevalence (Kessler et al, 2005) and substantial economic costs (Kessler & Greenberg, 2002) of anxiety disorders, a great deal of effort has been put into researching the

cognitive and neural mechanisms that are thought to contribute to the development and maintenance of these disorders.

1.2 The cognitive neuropsychology of emotional processing and anxiety

Silvers, Buhle, & Ochsner (2014) developed a model of the processes and neural systems that support emotion generation and regulation, which proposes that prefrontal, cingulate, and parietal control regions modulate activity in affective appraisal regions, such as the amygdala and insula, as well as occipito-temporal regions involved in semantic and perceptual representations. Furthermore, they have suggested that people with anxiety disorders may have an inability to accurately appraise threat, or an inability to reappraise threat, or even both, which has been linked to abnormal activity in prefrontal control regions and affective appraisal regions (Silvers et al, 2014). It has been a long-held view that fear and anxiety are linked to hyper-activation in deep limbic structures (i.e., amygdala and insula; Etkin & Wager, 2007), where amygdalar hyperactivity has for example been observed during negative emotional processing in people with social anxiety disorder (Stein, Goldin, Sareen, Zorrilla, & Brown, 2002; Phan, Fitzgerald, Nathan, & Tancer, 2006), panic disorder (Van den Heuvel et al, 2005), specific phobia (Dilger et al, 2003; Straube, Mentzel, & Miltner, 2006), and PTSD (Rauch et al, 2000; Shin et al, 2005). Furthermore, hypo-activation in prefrontal areas (e.g., dorsolateral and dorsomedial prefrontal cortex) has been observed in anxiety disorders during emotion regulation (Ball, Ramsawh, Campbell-Sills, Paulus, & Stein, 2013), which involves the modification of emotional responses via the engagement of top-down control processes (Silvers et al, 2014). This hypo-activation is thought to contribute to the characteristic emotion dysregulation seen in anxiety disorders, thus possibly

reflecting insufficient top-down control (Ball et al, 2013). More recent neurobiological frameworks related to anxiety disorders have suggested that some prefrontal areas may also be hyperactive during emotional processing, which possibly reflects an increased utilisation of dysfunctional emotion regulation strategies (Reinecke et al, 2015). Increased ventrolateral prefrontal cortex (vlPFC) activity has for example been associated with increased use of regulatory strategies, such as cognitive avoidance, in response to anxious hyper-reactivity (Hofmann, Ellard, & Siegle, 2012). These dysfunctional strategies are thought to play a big part in maintaining anxiety disorders and are thus targeted during exposure-based cognitive behavioural therapy (CBT). It has also been shown that amygdala attenuation during threat processing is associated with decreased activity in prefrontal brain areas, such as the vlPFC, after CBT (Månsson et al, 2013). A neurocognitive model of emotional processing and anxiety is illustrated in Fig. 1.1.

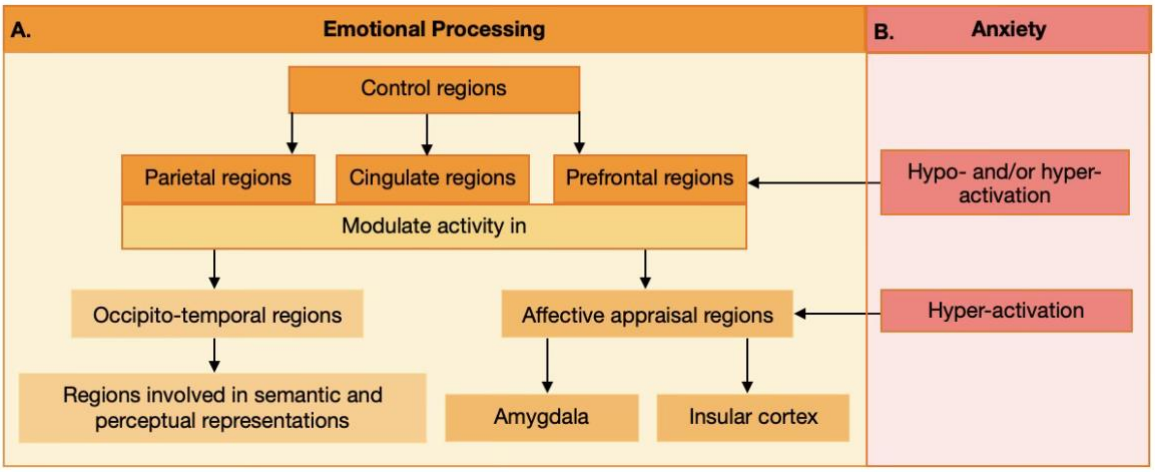

Figure 1.1 – *Neurocognitive model of emotional processing and anxiety*

A. Prefrontal, cingulate, and parietal control regions modulate activity in affective appraisal regions and occipito-temporal regions during generation and regulation of emotions. **B.** Anxiety has been linked to both hypo-activation in prefrontal control regions, such as the dorsolateral and dorsomedial prefrontal cortex, and hyper-activation in regions, such as the ventrolateral prefrontal cortex, as well as to hyper-activation in affective appraisal regions, which may reflect insufficient top-down control during emotional processing.

1.3 Use of pharmacological interventions to enhance psychological treatments for anxiety disorders

1.3.1 *Cognitive behavioural therapy for anxiety disorders*

Exposure-based CBT has been the most effective and widely employed treatment of anxiety disorders (Deacon & Abramowitz, 2004), where the core therapeutic component is exposure with response prevention (McGuire et al, 2014). Despite its effectiveness, overall treatment response rates are still only estimated to be around 50% across anxiety disorders, meaning that half of patients fail to respond (Loerinc et al, 2015). Researchers have thus begun looking for strategies to improve the treatment, which has involved identifying the mechanisms underlying its efficacy. Mechanisms such as attentional biases and fear extinction have been shown to be important for exposure success (Price, Tone, & Anderson, 2011; McGuire et al, 2014; Heinig et al, 2017). Cognitive models of anxiety have proposed that anxious individuals preferentially allocate attention to threat-related information and that this attentional bias may contribute to the development and maintenance of anxiety (Eysenck, 2014; Mathews, 1988). Moreover, CBT has been shown to reduce this attentional bias (Price et al, 2011; Pishyar, Harris, & Menzies, 2008; Lundh & Öst, 2001). CBT success has however been shown to be affected by the direction of attention biases, where biases away from threatening information (avoidant biases) have been associated with a poorer treatment response compared to biases toward threatening information (vigilant biases). A potential explanation for this poorer response is that attentional avoidance limits engagement with exposure and acquisition of extinction learning (Price et al, 2011). Extinction learning involves a systematic exposure to inappropriately feared stimuli or situations to extinguish anxious responses (Deacon & Abramowitz, 2004), and is thought to be one of the

main mechanisms underlying the efficacy of exposure-based CBT (McGuire et al, 2014; Heinig et al, 2017). During exposure-based CBT, patients are repeatedly exposed to triggering situations or internal states to elicit anxiety, while being instructed to resist engaging in avoidance, so that they can learn to habituate to the triggering state. This in turn teaches them that avoidance is not required to reduce anxious responses and that their anticipated aversive outcomes are inaccurate. A new fear learning thus occurs and symptoms decrease, as new associations emerge between the triggering stimuli and the non-feared outcome through repeated and graduated exposures (McGuire et al. 2014), whereby the previous association is gradually overwritten by a new association with safety (Craske, Treanor, Conway, Zbozinek, & Vervliet, 2014). Given that extinction is one of the main mechanisms underlying the efficacy of exposure-based CBT (McGuire et al, 2014; Heinig et al, 2017), augmenting fear extinction might be a promising way to increase clinical effects of the treatment.

1.3.2 *Cognitive behavioural therapy and fear extinction*

Fear extinction in a laboratory setting has been shown to be associated with outcomes of human exposure therapy (Forcadell et al, 2017) and is often thought of as an analogue to it (Reinecke et al, 2018). Extinction of fear conditioning in laboratory settings, which usually involve rodents, refers to a reduction in conditional fear responding (CR, usually freezing) after repeated presentation of a conditioned stimulus (CS, usually a tone) in the absence of an aversive unconditioned stimulus (US, usually a footshock) with which it was paired previously (Chang et al, 2009). The extinction decreases the CS' ability to evoke the CR, which was established during an initial phase where the CS and US were paired (CS-US association; fear

acquisition), as the rodent learns that the CS no longer predicts the aversive US, and a new inhibitory stimulus-response (CS-no US) association (extinction memory) is formed (Delamater, 2004). A considerable effort has been placed on understanding the neural circuitry underlying fear extinction in rodent models, as well as in humans. An essential brain circuit, including the prefrontal cortex, hippocampus, and amygdala, is thought to be involved, where the prefrontal cortex is thought to exert top-down control over subcortical structures to inhibit learned fear responses to conditioned fear cues (Giustino & Maren, 2015). Studies have also implicated the ventromedial prefrontal cortex (vmPFC) and the hippocampus in the consolidation of extinction memories in both animal and human studies (for a review, see Maren, 2011). Significant activations in these areas have been observed in response to extinguished vs. unextinguished stimuli in humans, where activations in the vmPFC and the hippocampus were also positively correlated with the magnitude of extinction memory, as well as positively correlated with one another during extinction recall, which supports their involvement in the recall of extinction memories (Milad et al, 2007). Furthermore, enhanced extinction recall has been shown to positively predict CBT outcome in anxiety disorders (Berry, Rosenfield, & Smits, 2009; Forcadell et al, 2017).

1.3.3 *Fear extinction and pharmacological interventions*

Recent research has indicated that drugs targeting glutamatergic mechanisms, such as D-cycloserine, may augment exposure-based CBT by enhancing fear extinction recall. These drugs however have the limitation of potentially enhancing fear reconsolidation during unsuccessful sessions (Mataix-Cols et al, 2017). Drugs targeting the renin-angiotensin system, such as losartan, have on the other hand

been shown to augment fear extinction recall, without augmenting fear acquisition in rodents (Marvar et al, 2014). This is important as it minimizes the risk of a drug-induced worsening of clinical symptoms after unsuccessful exposure, unlike D-cycloserine which may exacerbate it, thus making losartan a promising potential drug for anxiety disorders (Reinecke et al, 2018). The losartan group in the study by Marvar et al. (2014) exhibited significantly less fear responses (freezing) to the presentation of a feared stimulus during extinction recall, which involves re-exposure to the feared stimulus in the extinction context at a later time, suggesting that losartan might reduce fear memory through enhancing the memory consolidation of fear extinction (Marvar et al, 2014). Furthermore, a recent study showed that a single dose of losartan facilitated extinction learning in healthy humans by reducing psychophysiological threat responses and selectively enhancing vmPFC activation in response to threat signals, which exerts regulatory control over the amygdala and is thought to play a key role in extinction (Zhou et al, 2019). Given that recent findings have suggested a clear association between extinction-related vmPFC activity and exposure therapy success (Ball et al, 2017), and between enhanced extinction memory and better CBT outcomes in anxiety disorders (Berry et al, 2009; Forcadell et al, 2017), losartan may be a highly promising candidate to enhance the efficacy of exposure-based therapies.

1.3.4 *Losartan and the renin-angiotensin system*

Losartan acts as an angiotensin receptor antagonist on the renin-angiotensin system (RAS), a hormone system that regulates blood pressure, by preventing a docking of the protein angiotensin II (Ang II) to AT1 receptors; this then leads to a dilation of vessels and a reduction in blood pressure. AT1 receptors are located

widely in brain areas involved in memory, threat processing, and fear conditioning, such as the hippocampus and amygdala (McKinley et al, 2003), and it has been well established that the brain has its own intrinsic RAS, which has been implicated in the modulation of memory (Bild, Hritcu, Stefanescu, & Ciobica, 2013) and emotional processes related to anxiety (Braszko, Kulakowska, & Winnicka, 2003). Furthermore, administering Ang II has been shown to increase hippocampal oxidative stress and induce memory deficits in rats (Bild et al, 2013), as well as to induce anxiety-like effects and increase prooxidant status in the temporal cortical area, which includes the hippocampus and amygdala (Ciobica, Hritcu, Nastasa, Padurariu, & Bild, 2011). A reduction of central Ang II activity has on the other hand been shown to have both antidepressant- and anxiolytic-like effects, as well as to enhance learning and memory (for a review, see Gard, 2002). Moreover, it has been suggested that when AT1 receptors are blocked, Ang II may be more readily converted to angiotensin IV (Ang IV; Ongali et al, 2014; Royea, Zhang, Tong, & Hamel, 2017) that binds to AT4 receptors, which are also widely distributed in brain areas such as the hippocampus and amygdala and have been shown to improve learning and memory (Wright & Harding, 2008; Gard, 2008). Furthermore, it has been shown that Ang II modulates sympathoadrenal and hypothalamic-pituitary-adrenocortical (HPA) axis activation during stress via AT1 receptors (Jezova, Ochedalski, Kiss, & Aguilera, 1998). Losartan might thus possibly be modulating AT1-activation of the HPA axis and sympathoadrenal system, mitigating AT1-initiated oxidative stress, and enhancing cognitive performance via AT4 receptors (Jezova et al, 1998; Ongali et al, 2014).

1.3.5 *Cognitive enhancing and stress reducing properties of losartan*

Losartan crosses the blood-brain barrier and is well known for its anti-inflammatory and antioxidant properties in mouse models (Danielyan et al, 2010; Ongali et al, 2014). Increasing evidence is emerging that losartan enhances cognition (Fogari et al, 2003; Tedesco et al,1999), with even a single dose of 50mg being shown to improve memory function (Mechaeil, Gard, Jackson, & Rusted, 2011) and to enhance learning from positive relative to negative events (Pulcu et al, 2019) in healthy humans. A single dose of 50mg has also been shown to enhance early amygdala threat discrimination and to increase responsiveness in brain areas involved in threat processing, which has been suggested to potentially augment exposure in humans by facilitating safety learning and engagement with exposure (Reinecke et al, 2018). Furthermore, losartan has been shown to reduce anxious behaviours and stress responses in animal (Ranjbar, Aghaei, Moosazadeh, & Shabani, 2018; Kumar, Singh, Mishra, Sah, & Pottabathini, 2015; Üresýn, Erba, & Özek, 2004) and human studies (Shkreli et al, 2020), and regular angiotensin receptor blocker treatments have been shown to have protective effects on PTSD symptoms in people exposed to trauma (Khoury et al, 2012), which is in line with the accumulating evidence that the RAS and stress response regulation are interrelated (Üresýn et al, 2004; Aguilera, Kiss, Luo, & Akbasak, 1995; Saavedra et al, 2005).

Taken together, losartan has been shown to enhance both extinction and extinction recall in rodents (Marvar et al, 2014), to enhance vmPFC activation in response to threat signals (Zhou et al, 2019), to facilitate threat processing (Reinecke et al, 2018), to reduce stress responses (Shkreli et al, 2020), and to have protective effects on PTSD symptoms (Khoury et al, 2012), which are all relevant to

exposure therapy success. Furthermore, it has been shown to improve memory (Mechaeil et al, 2011) and to affect emotional processing by inducing a positive learning bias (Pulcu et al, 2019). Losartan may thus be a highly promising pharmacological candidate to enhance the efficacy of exposure-based therapies.

1.4 Pharmacological treatments for anxiety disorders

Apart from CBT, the most common therapies for anxiety disorders include selective serotonin reuptake inhibitors (SSRIs) and benzodiazepines, as they have been proven to have anxiolytic efficacy (Baldwin et al, 2005), but both groups of medications have limitations. Similar to CBT, a significant proportion of people fail to respond to SSRIs (Koen & Stein, 2011), and those who do respond often experience unpleasant side-effects including nausea, insomnia, and sexual dysfunction (Baldwin et al, 2005). Benzodiazepines on the other hand can cause troublesome sedation at higher doses in acute treatment, which can impair attention and memory, and cause dependence with long-term use (Baldwin et al, 2005). Given the frequency of anxiety disorders and the shortcomings of current pharmacological treatments, there is dire need for research that helps inform the development of new medications. A better understanding of how existing medications exert their anxiolytic effects may help guide this development. SSRIs have the advantage of being effective for both anxiety and depression, but this property makes it very difficult to distinguish between their anxiolytic and antidepressant effects. A potential way to gain a better understanding of the mechanisms behind these effects would be to compare SSRIs with medications that are effective for anxiety but not depression, such as benzodiazepines (Johnson, 1985).

1.4.1 *Therapeutic effects of benzodiazepines*

Benzodiazepines exert their therapeutic effects by producing allosteric changes that enhance the effect of the neurotransmitter gamma-aminobutyric acid (GABA) at the $GABA_A$ receptors. GABA is the most abundant inhibitory neurotransmitter in the central nervous system (CNS) and GABA receptors are the most important inhibitory receptors in the brain. Benzodiazepines produce these allosteric changes by increasing the GABA-induced frequency of opening of the chloride channels and thus increasing the apparent affinity of the receptor for GABA (D'Hulst, Atack, & Kooy, 2009), which results in anxiolytic, sedative, hypnotic, muscle relaxant, and anticonvulsant effects (Baldwin et al, 2013). $GABA_A$ receptor α_1-containing subtype is thought to mediate benzodiazepines' sedative/motor effects, while the α_2 and α_3 containing-subtypes are thought to mediate their anxiolytic effects (McKernan et al, 2000). Unlike SSRIs that can take up to several weeks, benzodiazepines exert their anxiolytic effects after administration of just a single dose and are usually prescribed for short-term relief for about two to four weeks. Although the pharmacological mechanisms of benzodiazepines are relatively well understood, a comprehensive understanding of the cognitive neuropsychological mechanisms behind their anxiolytic effects is still lacking.

It has been shown that SSRIs cause a reduction in amygdala activity amongst anxious patients (Faria et al, 2012) and that similar amygdala activity changes have been observed in relation to threat in healthy participants receiving an acute dose (Murphy, Norbury, O'Sullivan, Cowen, & Harmer, 2009) and after repeated administration for 7 days (Harmer, Mackay, Reid, Cowen, & Goodwin, 2006). Given that this reduction in brain activity happened in the absence of any pathophysiology and without any subjective changes in mood and anxiety, it

strongly suggests that these brain activity changes may play an important role in SSRIs' anxiolytic effects, as opposed to being just a by-product of being less anxious during the scan or testing period. A better understanding of the cognitive neuropsychological mechanisms behind benzodiazepines anxiolytic effects could thus potentially help researchers tease apart the anxiolytic and antidepressant effects of SSRIs and help inform the development of better anxiolytics.

1.4.2 *Benzodiazepines and emotional processing*

A number of studies have investigated acute effects of the long-acting benzodiazepine diazepam. A single dose of diazepam has been shown to reduce accuracy in detecting threat-related facial expressions (Blair & Curran, 1999; Zangara, Blair, & Curran, 2002; Del-Ben et al, 2012), and to reduce startle responses and increase attentional vigilance to masked happy faces compared to threatening or ambiguous ones at a non-sedating dose (Murphy, Downham, Cowen, & Harmer, 2008). Moreover, a non-sedating dose of diazepam has been shown to attenuate activation in the amygdala, insula, and orbitofrontal cortex in response to fearful faces, while also increasing activation in the anterior cingulate cortex (ACC), which could possibly be due to its top-down regulation of these structures (Del-Ben et al, 2012). Another study showed that a single dose of the benzodiazepine lorazepam attenuated activation in the amygdala and insula in response to emotional stimuli in a dose-dependent manner in healthy participants without any subjective changes in anxiety (Paulus, Feinstein, Castillo, Simmons, & Stein, 2005). Furthermore, a repeated administration of diazepam for 7 days has been shown to reduce vigilant–avoidant patterns of emotional attention (Pringle, Warren, Gottwald, Cowen, & Harmer, 2016), and to increase functional connectivity in brain areas of

emotional processing independent of task selection and clinical status (Pflanz et al, 2015). Benzodiazepines do thus appear to affect emotional processing in healthy participants without any subjective changes in anxiety, similar to SSRIs (Murphy et al, 2009; Harmer et al, 2006). Furthermore, $GABA_A$ receptor α_2 and α_3 subunits, which are thought to be responsible for benzodiazepines' anxiolytic effects (McKernan et al, 2000), are located in high concentrations in the amygdala and frontal regions (D'Hulst et al, 2009). Based on these findings, benzodiazepines' anxiolytic effects may be due to them modulating activity within the limbic and frontal areas related to emotional processing.

1.5 Aims and objectives

Given the prevalence (Kessler et al, 2005) and substantial economic costs (Kessler & Greenberg, 2002) of anxiety disorders, and the shortcomings of current treatments, there is dire need for research that helps inform the development of new treatments and medications. This thesis aims to investigate the effects of the two medications losartan and diazepam on memory and emotional processing in two different studies, to help inform the use of existing treatments and lead to more effective ones.

Research has indicated that the brain RAS may play an important role in the pathophysiology and extinction of anxiety (Braszko et al, 2003; Ciobica et al, 2011; Gard, 2002). The angiotensin receptor antagonist losartan may be a highly promising candidate to enhance the efficacy of exposure-based therapies, as it has been shown to enhance both extinction and extinction recall (Marvar et al, 2014), to enhance vmPFC activation in response to threat signals (Zhou et al, 2019), to facilitate threat processing (Reinecke et al, 2018), to reduce stress responses

(Shkreli et al, 2020), and to have protective effects on PTSD symptoms (Khoury et al, 2012), which are all relevant to exposure therapy success. Furthermore, it has been shown to improve memory (Mechaeil et al, 2011) and to affect emotional processing by inducing a positive learning bias, where losartan reduced the influence of negative outcomes on participants, while leaving the influence of positive ones unaffected (Pulcu et al, 2019). It however still remains to be fully clarified how losartan affects some of the mechanisms relevant to exposure success in humans, such as attentional biases (Price et al, 2011) and hippocampal functioning (Maren, 2011). Chapters 2 to 4 in this thesis will thus focus on a double-blind, randomised, placebo-controlled study, where the objective was to investigate the key effects of a single dose of losartan on hippocampal functioning during a memory encoding task in a functional magnetic resonance imaging (fMRI) scanner, and on attentional biases during a dot probe task outside of the scanner in healthy, high trait-anxious volunteers. A better understanding of losartan's effects on these mechanisms could potentially help lead to losartan and similar agents being used for the development of more effective and compact treatments of anxiety disorders.

Benzodiazepines appear to affect emotional processing without any subjective changes in anxiety in healthy participants (Del-Ben et al, 2012; Paulus et al, 2005), similar to SSRIs (Murphy et al, 2009; Harmer et al, 2006). As benzodiazepines are not effective in treating depression, researching their effects provide a means of teasing apart antidepressant and anxiolytic effects, since any effects observed can directly be related to their anxiolytic action, as opposed to SSRIs. $GABA_A$ receptor α_2 and α_3 subunits, which are thought to be responsible for benzodiazepines' anxiolytic effects (McKernan et al, 2000), are located in high concentrations in the amygdala and frontal areas (D'Hulst et al, 2009).

Benzodiazepines' anxiolytic effects may thus be due to them modulating activity within these areas. Most of the current research has focused on their acute effects; it may however be more clinically relevant to investigate if the effects are the same after short-term administration, as benzodiazepines are usually prescribed over a period of two to four weeks and there may be differences between acute and short-term treatments, as has been shown for SSRIs (Murphy et al, 2009). Furthermore, a short-term administration may elicit more reliable changes on emotional processing. Chapters 5 to 8 in this thesis will thus focus on a double-blind, randomised, placebo-controlled study looking at how a 7-day treatment of the benzodiazepine diazepam affects healthy volunteers during a battery of computerized tasks assessing emotional processing in a fMRI scanner. The objective of this study was to investigate whether diazepam modulates activity within the limbic and prefrontal areas related to emotional processing. A better understanding of the cognitive neuropsychological mechanisms behind benzodiazepines effects could potentially help researchers tease apart the anxiolytic and antidepressant effects of SSRIs and help inform the development of better anxiolytics.

Chapter 2: Overall methods for the losartan study

2.1 Introduction

The losartan study of this thesis was a double-blind, randomised, placebo-controlled study investigating the effects of a single dose of losartan on hippocampal functioning during a memory encoding task and on attentional biases during a dot probe task. Methods that the following two task chapters have in common will be presented here, while methods that only apply to each specific task will be presented separately in each chapter.

2.2 Methods

2.2.1 *Participants*

Ethical approval was granted by the Central University Research Ethics Committee of the University of Oxford. Thirty healthy, high trait-anxious volunteers, aged 18 to 50 years old, were recruited through adverts in local papers, via student mailing lists, and on posters in departments and colleges at the University of Oxford and Oxford Brookes University. Participants were excluded if they fulfilled criteria for a current DSM-IV Axis 1 psychiatric disorder (assessed using the Structured Clinical Interview for DSM disorders (SCID-IV Axis I; First, Williams, Spitzer, & Gibbon, 2007)); psychotropic medication use, illicit drug use, or use of a drug from another study during the last 6 weeks; were currently on blood pressure or other heart medication; had any medical or MRI contra-indication (e.g., metal in body); or were pregnant or breast-feeding. Participants were also required to have a score equal to or higher than 40 on the Trait-Anxiety subscale of the State-Trait Anxiety Inventory (STAI-T; Spielberger, Gorsuch, Lushene, Vagg, & Jacobs, 1983), be right-

handed, have a body mass index (BMI) between 18-30, be fluent in English, and be non- or light smokers (<5 cigarettes per day). The two groups were well matched on sociodemographic, clinical, and personality parameters (see Table 2.1).

Table 2.1 – Sociodemographic, clinical, and personality characteristics

	Placebo (n=14)	*Losartan (n=16)*
Gender		
Male	n = 3	n = 4
Female	n = 11	n = 12
Age	26.86 (2.52)	27.50 (2.72)
Trait Anxiety (STAI-T)	48.86 (2.34)	49.88 (1.51)
Verbal IQ (STW-2)	106.93 (2.99)	105.69 (2.65)
Attentional Control Scale (ACS)		
Total	50.36 (2.58)	51.81 (1.70)
Focusing	21.50 (1.27)	22.06 (0.89)
Shifting	28.86 (1.62)	29.75 (1.36)
Anxiety Sensitivity Index (ASI-R)	27.14 (5.66)	23.31 (3.90)
Behavioral Inhibition Scale (BIS)	13.14 (0.64)	13.06 (0.87)
Behavioral Activation Scale (BAS)	25.93 (1.31)	25.38 (1.58)
Beck Depression Inventory (BDI-II)	10.14 (2.45)	7.88 (1.52)
Eysenck Personality Questionnaire (EPQ)		
Neuroticism	12.57 (1.35)	11.81 (1.50)
Psychoticism	3.71 (0.68)	2.44 (0.53)
Extraversion	12.86 (1.22)	13.63 (1.40)
Lie/Social Desirability	8.50 (1.03)	8.94 (0.80)

Values are means (SEM)

2.2.2 *Procedure*

After passing an initial email screening, which included the STAI-T to make sure they had a score equal to or higher than 40, participants were invited to come to the Neurosciences Building at Department of Psychiatry for a more thorough medical and psychiatric screening, and to give informed consent. During this visit participants also filled in several questionnaires: Beck Depression Inventory (BDI-

II; Beck, Steer, & Brown, 1996) to measure depression; Spot the Word (STW-2; Baddeley & Crawford, 2012) to assess verbal intelligence; Anxiety Sensitivity Index (ASI-R; Taylor & Cox, 1998) to measure anxiety; Attentional Control Scale (ACS; Derryberry & Reed, 2002) to measure differences in attentional control; Behavioral Inhibition Scale (BIS) and Behavioral Activation Scale (BAS; Carver & White, 1994) to measure motivation to avoid aversive outcomes and to approach goal-oriented outcomes respectively; and the Eysenck Personality Questionnaire (EPQ; Eysenck & Eysenck, 1975) to assess personality traits. This visit lasted approximately 60 minutes.

After successfully passing the screening visit, subjects were invited for a 3-hour testing visit at the Department of Psychiatry and the Oxford Centre for Human Brain Activity (OHBA). They were instructed to have their last meal 2 hours before the visit and to avoid large amounts of alcohol on the night before. Participants were randomly allocated by the senior investigator, who had no direct contact with the participants, to receive either 50mg of losartan or a placebo. The tablets were over-encapsulated to make sure they looked identical. Physiological parameters (blood pressure and heart rate) were measured before capsule intake and at drug peak level (approx. 1 hour after intake), using a blood pressure machine, to monitor basic peripheral effects of the drug. Participants completed visual analogue scales (VAS), both before capsule intake and at drug peak level, to assess potential subjective physiological and mood effects of the drug, and also before and after going into the MRI scanner. The scales assessing potential transient effects of the drug included “anxious”, “sleepy”, “flushed”, “tearful”, “nauseous”, “hopeless”, “tremor”, “sad”, “dizzy”, “depressed”, “heart racing”, and “alert”, while the MRI scales included “happy”, “sad”, “hostile”, “alert”, “anxious”, and “calm”. Participants were asked to

indicate on each scale how they felt at the moment by drawing a vertical line on a 10cm horizontal line ranging from “not at all” to “extremely”. Intensity of the experienced effect was then measured using a ruler (1-100mm). At drug peak level, which was approx. 1 hour after intake, experimental testing commenced. The testing included a memory encoding task both before and during a fMRI scan, and a dot probe task outside of the scanner. Breathing, heart rate, and skin conductance were also measured throughout the scanning session using BIOPAC. Recognition of the photos presented in the memory encoding task was then tested after participants completed the dot probe task. After completing the testing, participants filled in side effects scales, where they were asked to indicate if they had noticed the following side effects since taking the capsule: “nausea”, “dizziness”, “dry mouth”, “headache”, “alert”, “agitation”. The effects were coded according to the intensity of the experienced side effect (0 = absent, 1 = mild, 2 = moderate, 3 = severe). They were then reimbursed with 50 GBP for their time. The timeline of the study is presented in Figure 2.1.

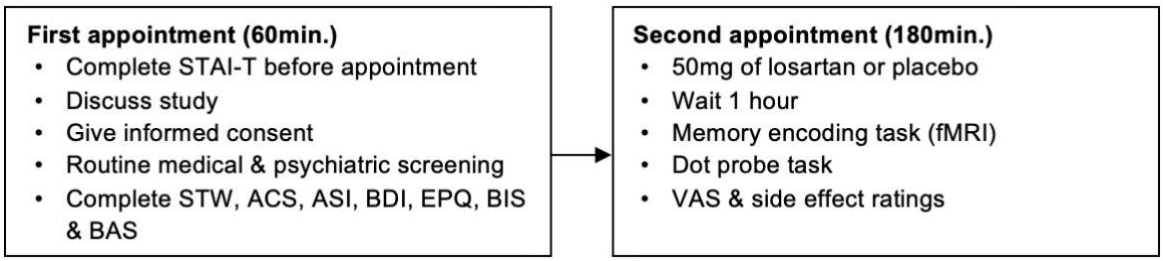

Figure 2.1 – *Timeline of the losartan study*

2.2.3 *Tasks*

The method sections of Chapter 3 and Chapter 4 describe the memory encoding task and the dot probe task respectively.

2.2.4 *Behavioural and MRI analyses*

The behavioural analysis was carried out using IBM SPSS Statistics for Windows, version 27 (IBM Corp., Armonk, N.Y., USA). To determine if there were any significant effects of treatment group on mood and physiological parameters, a 2×2 mixed analysis of variance (ANOVA) was conducted for the scores on each VAS, the blood pressure measures, and the heart rate measures. Treatment condition (losartan vs. placebo) was entered as the between-group factor and time (before drug intake vs. at drug peak level; pre- vs. post-MRI scan) as the within-subjects factor. Furthermore, to determine if there were any significant effects of treatment group on the side effect ratings, Mann-Whitney U tests were conducted for each side effect. The following two chapters include details about behavioural and MRI analyses related to each task.

Chapter 3: The effects of losartan on neural correlates of memory encoding

3.1 Introduction

As mentioned in Chapter 1, exposure-based cognitive behavioural therapy (CBT) has been the most effective and widely employed treatment of anxiety disorders (Deacon & Abramowitz, 2004), but overall treatment response rates are still only estimated to be around 50% across them (Loerinc et al, 2015). Researchers have thus begun looking for strategies to improve the treatment, which has involved identifying the mechanisms underlying its efficacy. One such mechanism is fear extinction, where systematic exposure to inappropriately feared stimuli or situations is used to extinguish anxious responses (Deacon & Abramowitz, 2004). Given that fear extinction is one of the main mechanisms underlying the efficacy of exposure-based CBT (McGuire et al, 2014; Heinig et al, 2017), augmenting fear extinction might be a promising way to increase clinical effects of the treatment.

A considerable effort has been placed on understanding the neural circuitry underlying fear extinction. An essential brain circuit, including the prefrontal cortex, hippocampus, and amygdala, is involved, where the prefrontal cortex is thought to exert top-down control over subcortical structures to inhibit learned fear responses to conditioned fear cues (Giustino & Maren, 2015). Studies have also implicated the ventromedial prefrontal cortex (vmPFC) and the hippocampus in the consolidation of extinction memories in both animal and human studies (for a review, see Maren, 2011). Significant activations in these areas have been observed in response to extinguished vs. unextinguished stimuli in humans, where activations in the vmPFC and the hippocampus were also positively correlated with the magnitude of extinction memory, as well as positively correlated with one another during

extinction recall, which supports their involvement in the recall of extinction memories (Milad et al, 2007). Furthermore, enhanced extinction recall has been shown to positively predict CBT outcome in anxiety disorders (Berry et al, 2009; Forcadell et al, 2017).

A study by Marvar et al. (2014) found that the angiotensin receptor antagonist losartan augmented fear extinction and reduced fear memory in rodents through enhancing the memory consolidation of fear extinction. A recent study also showed that a single dose of losartan facilitated extinction learning in healthy humans by selectively enhancing vmPFC activation in response to threat signals (Zhou et al, 2019). Given that recent findings have suggested a clear association between extinction-related vmPFC activity and exposure therapy success (Ball et al, 2017), and between enhanced extinction memory and better CBT outcomes in anxiety disorders (Berry et al, 2009; Forcadell et al, 2017), losartan may be a highly promising candidate to enhance the efficacy of exposure-based therapies.

Losartan acts as an angiotensin receptor antagonist on the renin-angiotensin system (RAS) by preventing a docking of the protein angiotensin II (Ang II) to AT1 receptors. AT1 receptors are located widely in brain areas involved in memory, threat processing, and fear conditioning, such as the hippocampus and amygdala (McKinley et al, 2003), and it has been well established that the brain has its own intrinsic RAS, which has been implicated in the modulation of memory (Bild et al, 2013) and emotional processes related to anxiety (Braszko et al, 2003). Furthermore, administering Ang II has been shown to increase hippocampal oxidative stress and induce memory deficits in rats (Bild et al, 2013), as well as to induce anxiety-like effects and increase prooxidant status in the temporal cortical area, which includes the hippocampus and amygdala (Ciobica et al, 2011). A

reduction of central Ang II activity has on the other hand been shown to have both antidepressant- and anxiolytic-like effects, as well as to enhance learning and memory (for a review, see Gard, 2002). Moreover, it has been suggested that when AT1 receptors are blocked, Ang II may be more readily converted to angiotensin IV (Ang IV; Ongali et al, 2014; Royea et al, 2017) that binds to AT4 receptors, which are also widely distributed in brain areas such as the hippocampus and amygdala and have been shown to improve learning and memory (Wright & Harding, 2008; Gard, 2008). Furthermore, it has been shown that Ang II modulates sympathoadrenal and hypothalamic-pituitary-adrenocortical (HPA) axis activation during stress via AT1 receptors (Jezova et al, 1998). Losartan might thus possibly be modulating AT1-activation of the HPA axis and sympathoadrenal system, mitigating AT1-initiated oxidative stress, and enhancing cognitive performance via AT4 receptors (Jezova et al, 1998; Ongali et al, 2014).

Losartan has been shown to improve memory function in animals (Raghavendra et al, 1998) and prospective memory in healthy humans (Mechaeil et al, 2011), but it remains to be clarified how losartan affects hippocampal functioning, which has been shown to be relevant to exposure success in humans (Maren, 2011). Furthermore, whether losartan's memory effects are due to it having an effect on memory encoding also remains to be explored. This study therefore sought to investigate the key effects of a single dose of losartan on hippocampal functioning in healthy, high trait-anxious volunteers, during a memory encoding task that has reliably been shown to preferentially activate the hippocampus (Filippini et al, 2009). As losartan has been shown to improve memory function (Raghavendra et al, 1998; Mechaeil et al, 2011), it was predicted that losartan would increase hippocampal activation during memory encoding.

3.2 Methods

3.2.1 *Participants & procedure*

Information about the participants and the procedure is presented in Chapter 2. In brief, this was a double-blind, randomised, placebo-controlled study investigating the effects of a single dose of losartan (50mg) on hippocampal functioning during a memory encoding task. 30 healthy, high trait-anxious volunteers were recruited, but 6 participants could not undergo fMRI due to MRI contra-indication (e.g., dental bridge, very recent tattoo). The analysis thus included 24 participants, with 11 in the placebo group and 13 in the losartan group.

3.2.2 *Experimental task*

The task used in the current study was a ‘‘novel vs. familiar’’ memory encoding paradigm adopted from Filippini et al’s (2009) study, with a slightly shorter image presentation during the scan (2000ms instead of 3250ms). This task has reliably been shown to preferentially activate the hippocampus. The task included 91 coloured pictures of either landscapes or animals (see Figure 3.1). All pictures were emotionally neutral and similar in brightness, complexity, and contrast. Presentation (Neurobehavioral Systems) was used to run the task and record responses in text files.

Before the fMRI scan, participants worked on a classification task which included 8 pictures (4 animals and 4 landscapes), which were displayed to each participant 8 times in a pseudorandom order on a laptop (picture presentation: 3250ms; intertrial interval: 500ms; 8 pictures in a block; fixation cross between blocks: 5000ms). Participants were asked to classify the pictures as either a picture

containing a landscape or an animal by using keys on a keyboard, and to try to remember the pictures for a subsequent task. Participants were then tested for memory of the images in a short pre-scan memory task, which included the 8 familiar pictures and 8 novel pictures, to ensure they had satisfactorily encoded the familiar ones. The pictures were presented in pseudorandom order and participants were asked to select between 2 keys whether they were familiar or novel (picture presentation: 3250ms; intertrial interval: 500ms).

Participants were then asked to categorise more pictures during the fMRI scan and to also try to memorise them for a subsequent memory task. The images presented during the scan included images the participants already memorised during the pre-scan classification task, so that brain activity in response to novel images could be compared to activity in response to familiar ones. The task included 12 picture blocks, where each block consisted of 8 trials and lasted 20s. In each trial, participants were presented with a picture of either a landscape or an animal and were instructed to categorise them using a button box. The pictures were presented for 2000ms in each trial, followed by an intertrial interval of 500ms. A fixation cross was presented between each block for 12 seconds. 6 blocks included the familiar photos and 6 included novel ones. The familiar and novel blocks alternated, and the order was counterbalanced. Images were displayed in pseudorandom order, where the familiar pictures were presented in a different order each time. The novel images were 48 in total (24 animals and 24 landscapes).

About an hour after the fMRI scan, participants were then tested again on a laptop for memory of the images viewed during the scan. 83 images were presented in pseudorandom order (picture presentation: 4000ms; intertrial interval: 1000ms),

where 56 were familiar and 27 were novel, and participants were asked to select between 2 keys whether the images had been seen inside the scanner or not.

Figure 3.1 – *Examples of pictures used during the memory encoding task*

3.2.3 *Behavioural analysis*

The behavioural analysis was carried out using IBM SPSS Statistics for Windows, version 27 (IBM Corp., Armonk, N.Y., USA). To determine if there were any significant effects of treatment group on classification accuracy during the scan, memory recognition during the pre-scan memory task, and memory recognition during the post-scan memory task, Mann-Whitney U tests and an independent t-test were conducted for each respectively. Furthermore, to determine if there were any significant effects of treatment group on heart rate, skin conductance, and

breathing throughout the scan (BIOPAC), Mann-Whitney U tests and an independent t-test were conducted for each respectively. For information about the analysis of the subjective ratings and physiological parameters please see section 2.2.4 of Chapter 2.

3.2.4 *Image acquisition*

A 3T Siemens Prisma scanner (Siemens, Erlangen, Germany) with a 32-channel head-coil, located at the Oxford Centre for Human Brain Activity (OHBA), was used to acquire T1-weighted structural images and T2-weighted transverse echo planar images (EPI). Functional imaging consisted of 60 T2-weighted EPI slices (TR = 800ms, TE = 30ms, flip angle = 52°, matrix size = 90 × 90, voxel dimension = 2.4mm isotropic, slice thickness = 2.4mm, field of view (FOV) = 192 × 192mm^2, 500 volumes, echo spacing = 0.51ms). Gradient-echo fieldmap images were acquired for distortion correction (60 slices, slice thickness = 2.4mm, voxel dimension = 2.4mm isotropic, FOV = 192 × 192mm^2, TR = 590ms, TE1 = 4.92ms, TE2 = 7.38ms, flip angle = 46°), and T1-weighted structural images were acquired for anatomical alignment (192 slices, slice thickness = 1mm, TR = 1900ms, TE = 3.97ms, flip angle = 8°, voxel dimension = 1mm isotropic, FOV = 192 × 192mm^2). Furthermore, a single-band reference (SBRef) image was acquired to improve image registrations (60 slices, slice thickness = 2.4mm, voxel dimension = 2.4mm isotropic, FOV = 192 × 192mm^2, TR = 800ms, TE = 30ms, flip angle = 52°).

3.2.5 *Image analysis*

MRI data were analysed using FSL (FMRIB Software Library v6.6) tools (https://fsl.fmrib.ox.ac.uk/fsl). Structural anatomical scans were brain extracted

using FSL's Brain Extraction Tool BET (Smith, 2002). Pre-processing consisted of brain extraction using the Brain Extraction Tool (BET; Smith, 2002), motion correction using FMRIB's Linear Image Registration Tool (MCFLIRT; Jenkinson et al, 2002), the use of a SBRef image to improve image registrations, distortion correction using gradient-echo fieldmaps, spatial smoothing using a Gaussian kernel of FWHM 5mm, grand-mean intensity normalisation of the entire 4D dataset by a single multiplicative factor, registration of the functional space template to the anatomical space and the Montreal Neurological Institute (MNI) 152 space using the FMRIB's Linear Image Registration Tool (FLIRT; Jenkinson & Smith, 2001; Jenkinson et al, 2002), and high-pass temporal filtering equivalent to 90s (Gaussian-weighted least-squares straight line fitting, with sigma = 45.0s). Time-series statistical analysis was then carried out using FMRIB's Improved Linear Model (FILM) with local autocorrelation correction (Woolrich et al, 2001) and a custom 3-column format convolved with a gamma hemodynamic response function, and its temporal derivative, was used to model the data. The main contrast of interest was novel vs. familiar, but estimates of familiar vs. novel, familiar vs. baseline, and novel vs. baseline were also obtained for reference, where fixation blocks were the baseline reference. Furthermore, motion traces detected by MCFLIRT were included in the model as nuisance regressors to account for motion.

The group level analysis was carried out using FMRIB's Local Analysis of Mixed Effects (FLAME; Woolrich et al, 2004). The general linear model (GLM) included the 2 groups (losartan and placebo), where group averages, average across both groups, and differences between groups for each contrast were tested. Significant activations were determined by cluster-based thresholding of $Z > 3.1$ and

a family-wise error-corrected cluster significance threshold of $p < 0.05$ (Worsley, 2001).

As this task only included 24 participants due to disruptions related to the COVID-19 pandemic, power to detect smaller effects was limited. A less stringent group level analysis was thus also carried out using FLAME (Woolrich et al, 2004), to investigate whether losartan had any effects that didn't reach significance due to loss of statistical power. The GLM included the 2 groups, where group averages, average across both groups, and differences between groups for each contrast were tested. Significant activations were determined by cluster-based thresholding of $Z > 2.3$ and a family-wise error-corrected cluster significance threshold of $p < 0.05$ (Worsley, 2001).

In addition to the whole-brain analysis, a small volume correction (SVC) was preformed using predefined anatomical regions of interest (ROIs), which included the left and right hippocampus. These regions were defined using the Harvard-Oxford Subcortical Structural Atlas and a 50% threshold. The small volume correction method restricts the search space for significant voxels only to those voxels within the mask, which reduces the number of multiple comparisons and boosts statistical power. A less stringent SVC analysis ($Z > 2.3$, $p < 0.05$) was also preformed using the ROIs, to investigate whether losartan had any effects that didn't reach significance due to loss of statistical power.

3.3 Results

3.3.1 *Sociodemographic, clinical, and personality characteristics*

Sociodemographic, clinical, and personality characteristics of participants included in the memory encoding task analysis are presented in Table 3.1. The analysis of this task consisted of 24 participants, as 6 participants (3 = losartan, 3 = placebo) could not undergo the fMRI scan due to MRI contra-indication (e.g., dental bridge, very recent tattoo). The analysis thus included 11 participants in the placebo group and 13 in the losartan group. The two groups were well matched on sociodemographic, clinical, and personality parameters.

Table 3.1 – Sociodemographic, clinical, and personality characteristics

	Placebo (n=11)	*Losartan (n=13)*
Gender		
Male	n = 2	n = 4
Female	n = 9	n = 9
Age	27.91 (3.10)	29.23 (3.17)
Trait Anxiety (STAI-T)	50.64 (2.73)	51.23 (1.63)
Verbal IQ (STW-2)	105.27 (3.31)	107.62 (3.01)
Attentional Control Scale (ACS)		
Total	48.27 (2.80)	50.08 (1.64)
Focusing	20.73 (1.53)	21.46 (1.01)
Shifting	27.55 (1.66)	28.62 (1.37)
Anxiety Sensitivity Index (ASI-R)	29.36 (7.02)	27.46 (3.94)
Behavioral Inhibition Scale (BIS)	13.09 (0.72)	12.62 (1.00)
Behavioral Activation Scale (BAS)	25.18 (1.58)	25.08 (1.95)
Beck Depression Inventory (BDI-II)	11.00 (3.06)	9.23 (1.63)
Eysenck Personality Questionnaire (EPQ)		
Neuroticism	13.36 (1.47)	13.08 (1.61)
Psychoticism	4.09 (0.84)	2.38 (0.66)
Extraversion	12.18 (1.34)	13.31 (1.73)
Lie/Social Desirability	9.27 (1.19)	8.77 (0.92)

Values are means (SEM)

3.3.2 *Subjective rating results and physiological parameters*

There was no significant main effect of treatment group on any of the VAS assessing potential transient effects of the drug (all *ps* > .260), nor on any of the MRI VAS (all *ps* > .082). Moreover, there were no significant interactions between group and the timing of any of the scales (before capsule intake vs. at peak level: all *ps* > .225; pre- vs. post-MRI scan: all *ps* > .110). There was also no significant main effect of treatment group on any of the side effect ratings (all *ps* > .358). Furthermore, there was no significant main effect of treatment group on physiological parameters (blood pressure and heart rate; all *ps* > .143), and no significant interactions between group and the timing of any of the measurements (before capsule intake vs. at peak level; all *ps* > .892). These results suggest that the analysis was not confounded by physiological, mood, or side effect differences between the two groups and confirms that participant blinding was maintained.

3.3.3 *Behavioural performance*

There were no group differences in accuracy in classifying the pictures during the scanning session (Z = -1.051, p = .293). Furthermore, there were no group differences in memory recognition during the pre-scan memory task (Z = -1.276, p = .202), nor in memory recognition during the post-scan memory task ($t(22)$ = -.320, p = .752; see Table 3.2).

Table 3.2 – Behavioural results of the memory encoding task

	Placebo	Losartan	*P*
Classification accuracy during scan	94.03%	98.72%	.293
Pre-scan recognition	99.43%	98.08%	.202
Post-scan recognition	72.84%	73.86%	.752

Values denote % of correct responses

Furthermore, there were no group differences in breathing ($t(22) = .069$, $p = .946$), heart rate ($Z = -.492$, $p = .622$), and skin conductance ($Z = -.550$, $p = .582$) throughout the scanning session (BIOPAC).

3.3.4 *Main effect of task*

Across both groups, increased activation was observed in the lateral occipital cortex extending into bilateral hippocampus, temporal fusiform cortex, parahippocampal gyrus, frontal areas, cerebellum, and thalamus in response to novel images compared to familiar images (novel>familiar, overall mean; see Figure 3.2 and Table 3.3), which is consistent with previous research (Filippini et al, 2009; Golby et al., 2005).

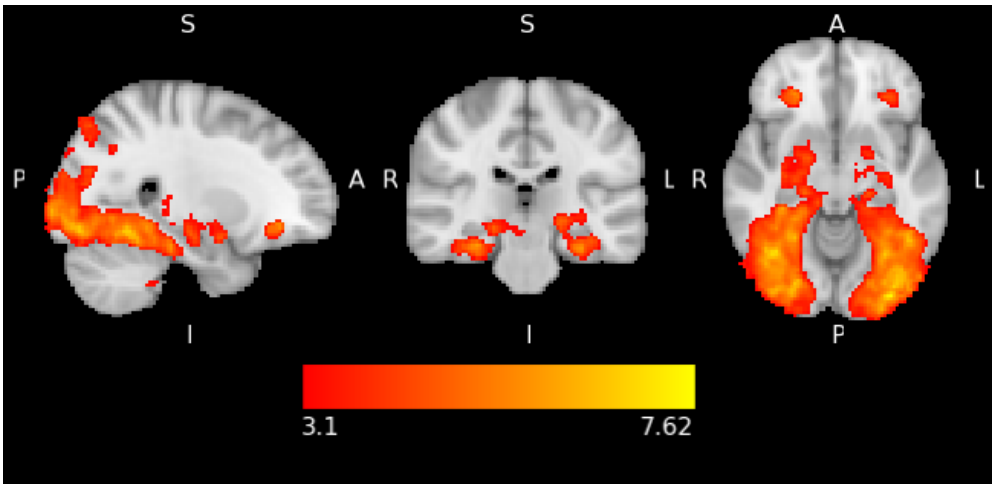

Figure 3.2 – *Whole-brain activation in response to the novel photos across groups*

Sagittal, coronal, and axial images depicting neural activation in response to the novel photos compared to the familiar ones across groups (losartan and placebo) during the memory encoding task. Images are thresholded at $Z > 3.1$ and $p < 0.05$ corrected.

Table 3.3 – Main effect of the memory encoding task across groups ($Z > 3.1$, $p < .05$)

Cluster	Brain area	Side	Cluster size (voxels)	MNI max (x, y, z)	*z*-score	*p*-value
Task activation (novel>familiar)						
1	Lateral occipital cortex extending into bilateral hippocampus, temporal fusiform cortex, & parahippocampal gyrus	L	22344	-34, -90, -10	7.55	<.001
2	Thalamus	L	377	-6, -2, 4	4.13	<.001
3	Middle frontal gyrus, precentral gyrus, & inferior frontal gyrus	L	297	-42, 10, 30	5.37	<.001
4	Superior frontal gyrus & paracingulate gyrus	L	242	0, 20, 50	4.08	<.001
5	Cerebellum	L	230	-22, -36, -42	5.39	<.001
6	Frontal pole & frontal orbital cortex	R	173	26, 36, -10	5.56	.003
7	Cerebellum	R	162	22, -40, -44	4.87	.004
8	Frontal orbital cortex & frontal pole	L	135	-36, 34, -14	4.75	.012
9	Cingulate gyrus, anterior division	R	127	6, 6, 26	4.74	.016

3.3.5 *Effect of treatment*

No group differences were found in BOLD activation for any of the contrasts in the whole brain analysis ($Z > 3.1$ thresholded, $p < 0.05$ corrected), nor in the left and right hippocampus in the small volume correction analysis. As this task only included 24 participants due to disruptions related to the COVID-19 pandemic, power to detect smaller effects was limited. A less stringent threshold was thus used to investigate whether losartan had any effects that didn't reach significance due to loss of statistical power. A less stringent whole brain analysis ($Z > 2.3$ thresholded, $p < 0.05$ corrected) revealed increased BOLD activation in the losartan group relative to placebo group in response to both novel and familiar photos in the paracingulate gyrus, insular cortex, lingual gyrus, and fusiform gyrus (see Figure 3.3 and Table 3.4). No group differences were found in BOLD activation for any of the contrasts in the less stringent small volume correction analysis of the left and right hippocampus.

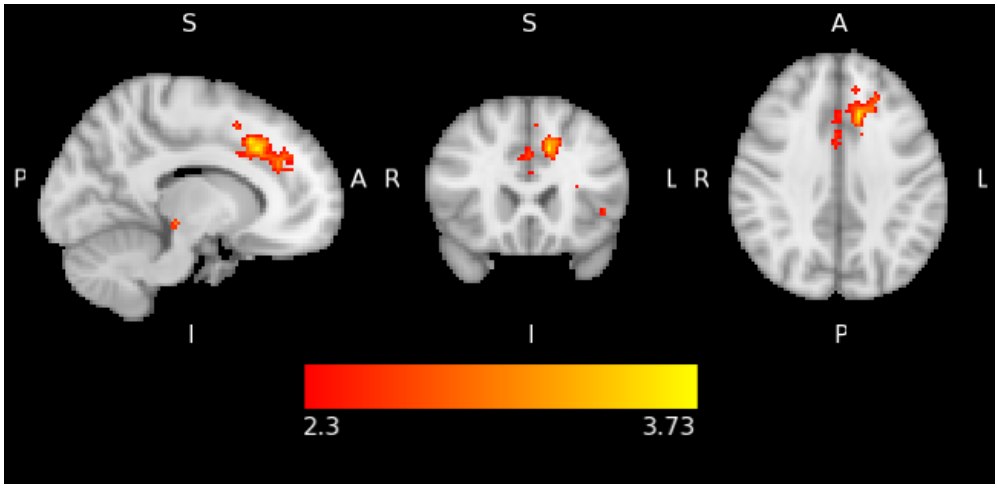

Figure 3.3 – *Whole-brain activation in response to both novel and familiar photos in the losartan group*

Sagittal, coronal, and axial images depicting neural activation in response to both novel and familiar photos in the losartan group compared to the placebo group during the memory encoding task. Images are thresholded at $Z > 2.3$ and $p < 0.05$ corrected.

Table 3.4 – Whole-brain group differences of losartan > placebo ($Z > 2.3$, $p < .05$)

Cluster	Brain area	Side	Cluster size (voxels)	MNI max (x, y, z)	*z*-score	*p*-value
Mean (familiar & novel photos)						
1	Paracingulate gyrus	L	854	-12, 22, 36	3.65	<.001
2	Insular cortex	L	583	-38, 8, 2	3.69	.001
3	Lingual gyrus & fusiform gyrus	R	334	8, -84, -12	3.48	.047

3.4 Discussion

Losartan has been shown to improve memory function in animals (Raghavendra et al, 1998) and prospective memory in healthy humans (Mechaeil et al, 2011), but it still remains to be clarified how losartan affects hippocampal functioning, which has been shown to be relevant to exposure success in humans (Maren, 2011). In this study, the key effects of a single dose of losartan on hippocampal functioning during a memory encoding task in healthy, high trait-anxious volunteers were investigated. As losartan has been shown to improve memory function (Raghavendra et al, 1998; Mechaeil et al, 2011), it was predicted that losartan would increase hippocampal activation during memory encoding. There was however no evidence found for an effect of losartan on neural responses in the hippocampus during memory encoding. Furthermore, there were no group differences in memory recognition during the pre-scan and post-scan memory tasks. Given the relatively small sample size used in this analysis due to the COVID-19 pandemic disruption, power to detect smaller effects was limited. A less stringent threshold was thus used to investigate whether losartan had any effects that did not reach significance due to loss of statistical power. The less stringent whole brain analysis ($Z > 2.3$ thresholded, $p < 0.05$ corrected) revealed an increased BOLD activation in the losartan group relative to placebo group in response to both novel and familiar photos in the paracingulate gyrus, insular cortex, lingual gyrus, and fusiform gyrus.

Increased activation was observed in the lateral occipital cortex extending into bilateral hippocampus, temporal fusiform cortex, parahippocampal gyrus, frontal areas, cerebellum, and thalamus in response to novel images compared to familiar ones in all participants, which is consistent with previous research (Filippini et al, 2009; Golby et al., 2005) and confirms that participants were successful in

completing the task. There were also no group differences in accuracy in classifying the pictures during the scanning session.

Visual analogue scales were given at baseline and at drug-peak level, to measure potential subjective physiological and mood effects of the drug, as well as before and after the scan. Blood pressure and heart rate were also measured at baseline and at drug-peak level to monitor basic peripheral effects of the drug. Side effects were then measured at the end of the testing visit. No group differences or interactions were found on any of the measures, which suggests that the analysis was not confounded by physiological, mood, or side effect differences between the two groups and confirms that participant blinding was maintained. Furthermore, there were no group differences in breathing, heart rate, and skin conductance throughout the scan (BIOPAC), which suggests that the analysis was also not confounded by group differences on basic physiological measures during the scan.

No evidence was found for an effect of losartan on neural responses in the hippocampus during non-emotional memory encoding, nor on memory recognition outside of the scanner. Power to detect smaller effects was limited given the relatively small sample size used in this analysis due to the COVID-19 pandemic disruption, which could potentially explain this lack of effect. Another possible reason for the lack of an effect on memory recognition performance may be that losartan might have more of an effect after a longer time of memory consolidation. In the current study, participants were scanned during memory encoding and then tested for recognition within one hour, which is a relatively short retention interval. Longer time intervals might thus potentially be needed for losartan to have an effect on memory performance. A very recent study for example found that losartan enhanced general memory recognition performance 24 hours after encoding (Xu et

al, 2021). Furthermore, given that losartan has been associated with enhanced extinction memory in rodents (Marvar et al, 2014) and that the magnitude of extinction memory has been shown to be positively correlated with hippocampal activation during extinction recall in humans (Milad et al, 2007), a future study should focus on hippocampal activity in relation to extinction recall, as losartan's effects on the hippocampus may potentially be more pronounced during recall and in response to threat signals, as has been shown with vmPFC activation (Zhou et al, 2019). A very recent study by Xu et al. (2021) for example used an emotional encoding task and found that losartan decreased hippocampal activation during encoding of negative stimuli, compared to neutral and positive, which indicates a valence-specific modulation of hippocampal activation. Moreover, Zhou et al. (2019) found that losartan facilitated threat memory extinction by augmenting threat-specific encoding in the vmPFC, they however did not look at the hippocampus or extinction recall. Further investigation of this is clearly warranted, given the association between enhanced extinction memory and better CBT outcomes in anxiety disorders (Berry et al, 2009; Forcadell et al, 2017).

The losartan group in the current study showed an increase in activation in the paracingulate gyrus, insular cortex, lingual gyrus, and fusiform gyrus in response to both novel and familiar photos in the less stringent analysis compared to the placebo group. These areas have been shown to be activated during a similar encoding paradigm (Machielsen, Rombouts, Barkhof, Scheltens, & Witter, 2000). However, since these areas were activated in response to both novel and familiar photos, as opposed to novel (encoding condition) vs. familiar photos (control condition), there is a possibility that the activity may be more related to higher-order visual processing of stimuli rather than encoding. The authors in Machielsen et al.'s

(2000) study for example mention that although they have consistently found activation in the parahippocampal area, most activation is usually found in areas known to be involved in higher-order analysis of visual stimuli, including the lingual and fusiform gyri. They also noted that they were unclear about the functional role of the paracingular area in relation to the task. Losartan might thus possibly be modulating activity within regions related to higher-order visual processing. This may be particularly relevant for CBT, as greater reactivity in higher-order visual regions to threat has been shown to predict CBT success (Klumpp, Fitzgerald, & Phan, 2013b). Future studies may want to include a visual processing task to further investigate this, as modulation of activity within higher-order visual regions might have synergistic effects with exposure therapy.

In summary, there was no evidence for an effect of losartan on neural responses in the hippocampus during memory encoding, nor on memory recognition outside of the scanner. A possible reason for this may be that losartan might have more of an effect after a longer time of memory consolidation. Longer time intervals might thus possibly be needed between encoding and recognition testing for an effect, as has been shown in previous research (Xu et al, 2021). Furthermore, given that losartan has been associated with enhanced extinction memory in rodents (Marvar et al, 2014) and given that the magnitude of extinction memory has been shown to be positively correlated with hippocampal activation during extinction recall in humans (Milad et al, 2007), losartan may have more of an effect on hippocampal activity during extinction recall rather than during encoding. Losartan's effects on the hippocampus may also potentially be more pronounced in response to threat signals, as has been shown with vmPFC activation (Zhou et al, 2019) and during encoding of negative stimuli (Xu et al, 2021). A less stringent

group analysis ($Z > 2.3$ thresholded, $p < 0.05$ corrected) revealed increased activation in the paracingulate gyrus, insular cortex, lingual gyrus, and fusiform gyrus in response to both familiar and novel photos in the losartan group compared to the placebo group. These regions have been shown to be activated during a similar encoding paradigm (Machielsen et al, 2000). However, as they were activated in response to both novel and familiar photos, the activity may be more related to higher-order visual processing of stimuli rather than encoding. Losartan might thus possibly be modulating activity within regions related to higher-order visual processing. This modulation may be relevant for CBT, as greater reactivity in higher-order visual regions to threat has been shown to predict CBT success (Klumpp et al, 2013b). Definite conclusions can however not be drawn until more participants have been recruited in this study.

Chapter 4: The effects of losartan on attentional biases

4.1 Introduction

As mentioned in the previous two chapters, exposure-based cognitive behavioural therapy (CBT) has been the most effective and widely employed treatment of anxiety disorders (Deacon & Abramowitz, 2004), but overall treatment response rates are still only estimated to be around 50% across them (Loerinc et al, 2015). Researchers have thus begun looking for strategies to improve the treatment, which has involved identifying the mechanisms underlying its efficacy. One such proposed mechanism is change in attentional bias (Price et al, 2011). Cognitive models of anxiety have proposed that anxious individuals preferentially allocate attention to threat-related information and that this attentional bias may contribute to the development and maintenance of anxiety (Eysenck, 2014; Mathews, 1988). This bias seems to reflect both facilitated attentional engagement to negative information (Broadbent & Broadbent, 1988; MacLeod & Mathews, 1988) and impaired attentional disengagement from negative information (Koster, Crombez, Verschuere, & De Houwer, 2006; Salemink, van den Hout, & Kindt, 2007), which have been shown to independently contribute to variation in anxiety vulnerability (Rudaizky, Basanovic, & MacLeod, 2014) and thus could be differentially implicated in alternative forms of anxiety dysfunction. Enhanced attentional engagement to negative information, where anxiety reactivity is increased, might thus contribute more to disorders such as panic disorder, while impaired attentional disengagement from negative information, where anxiety perseveration is increased, might contribute more to disorders such as generalised anxiety disorder (Rudaizky et al, 2014). Cognitive interventions could thus potentially be made more efficient by targeting each attentional bias for therapeutic change dependant on clinical

condition. CBT has been shown to reduce attentional biases (Pishyar et al, 2008; Lundh & Öst, 2001), but its success has been shown to be affected by the direction of the bias. Biases away from threatening information (avoidant biases) have been associated with a poorer treatment response compared to biases toward threatening information (vigilant biases). A potential explanation for this poorer response is that attentional avoidance limits engagement with exposure and acquisition of extinction learning (Price et al, 2011).

The angiotensin receptor antagonist losartan may potentially be a promising pharmacological candidate to enhance the efficacy of exposure-based therapies, as it has been shown to facilitate extinction learning (Marvar et al, 2014; Zhou et al, 2019) and to enhance early threat discrimination and threat processing (Reinecke et al, 2018). Furthermore, losartan has been shown to reduce stress responses (Shkreli et al, 2020) and to have protective effects on PTSD symptoms (Khoury et al, 2012). Increasing evidence is emerging that it also enhances cognition (Fogari et al, 2003; Tedesco et al,1999), with even a single dose of 50mg being shown to improve memory function (Mechaeil et al, 2011; Raghavendra et al, 1998) and to enhance learning from positive relative to negative events (Pulcu et al, 2019). It however still remains to be fully clarified how losartan affects attentional biases. This study therefore sought to investigate the effects of a single dose of losartan on attentional biases, measured using a dot probe task, in healthy, high trait-anxious volunteers. As losartan has been shown to improve early discrimination of negative versus positive stimuli (Reinecke et al, 2018) and to induce a positive learning bias (Pulcu et al, 2019), it was predicted that losartan would reduce negative attentional biases and increase positive ones.

4.2 Methods

4.2.1 *Participants & procedure*

Information about the participants and the procedure is presented in Chapter 2. In brief, this was a double-blind, randomised, placebo-controlled study investigating the effects of a single dose of losartan (50mg) on attentional biases during a dot probe task. 30 healthy, high trait-anxious volunteers were recruited, but 1 participant had to be excluded from the analysis of this task due to technical problems with response capture. The analysis thus included 29 participants, with 14 in the placebo group and 15 in the losartan group.

4.2.2 *Experimental Task*

The task used in the current study was a dot probe task adapted from Rudaizky et al's (2014) study, which has been shown to provide sensitive but independent assessments of attentional engagement with, and attentional disengagement from, negative information in high trait anxious participants. This adapted version also included positive stimuli, as losartan has been shown to be relevant to positive processing (Pulcu et al, 2019). A total of 768 images were included in the task, where half of the images were representational (128 negative, 128 neutral, 128 positive; selected from the International Affective Picture System (IAPS; Lang, Bradley, & Cuthbert, 2008)), and the other half were of an abstract nature (384; collected via an 'abstract art' Google Image Search). The task included 384 trials, where each image was presented only once and the order of presentation was randomised. After every 96 trials, participants were given a self-timed rest period. This task was designed to permit independent assessment of selective attentional engagement with, and selective attentional disengagement from, the differently

valenced representational images. Examples of each type of trial can be found in Figure 4.1. During the task, participants were instructed to indicate whether the orientation of a target probe matched that of a cue probe, i.e., whether the probes (5mm red lines) were both presented horizontally or vertically, indicating a match, or whether one was presented vertically and the other horizontally, indicating a mismatch. Before the presentation of the probes, participants saw two white square outlines, each measuring 80×80mm (subtending a visual angle of 7.59°), on alternate sides of the screen, indicating the two loci where images would be shown, and a smaller red square, measuring 20×20mm (subtending a visual angle of 1.91°), appearing on either side, indicating where the cue probe would briefly appear to fixate each participant's attention. After 1000ms the cue probe appeared within the red square for 200ms, before an image pair appeared, with one image being abstract and the other representational (negative, neutral, positive). The representational image appeared either in the distal locus from where the participant was attending, which constituted the engagement bias trials, or in the same locus, which constituted the disengagement bias trials, with equal frequency. The image display lasted for either 500ms or 1000ms. Both durations were included, as previous probe task variants have mostly employed either 500ms or 1000ms stimulus exposure durations. The screen then cleared and a target probe appeared in either of the two white square outlines, at which point participants indicated whether the orientation of the cue probe matched the target probe using the left and right buttons of a mouse. The screen cleared for 1000ms after each response before the next trial began. Participants were instructed to answer as quickly and accurately as possible.

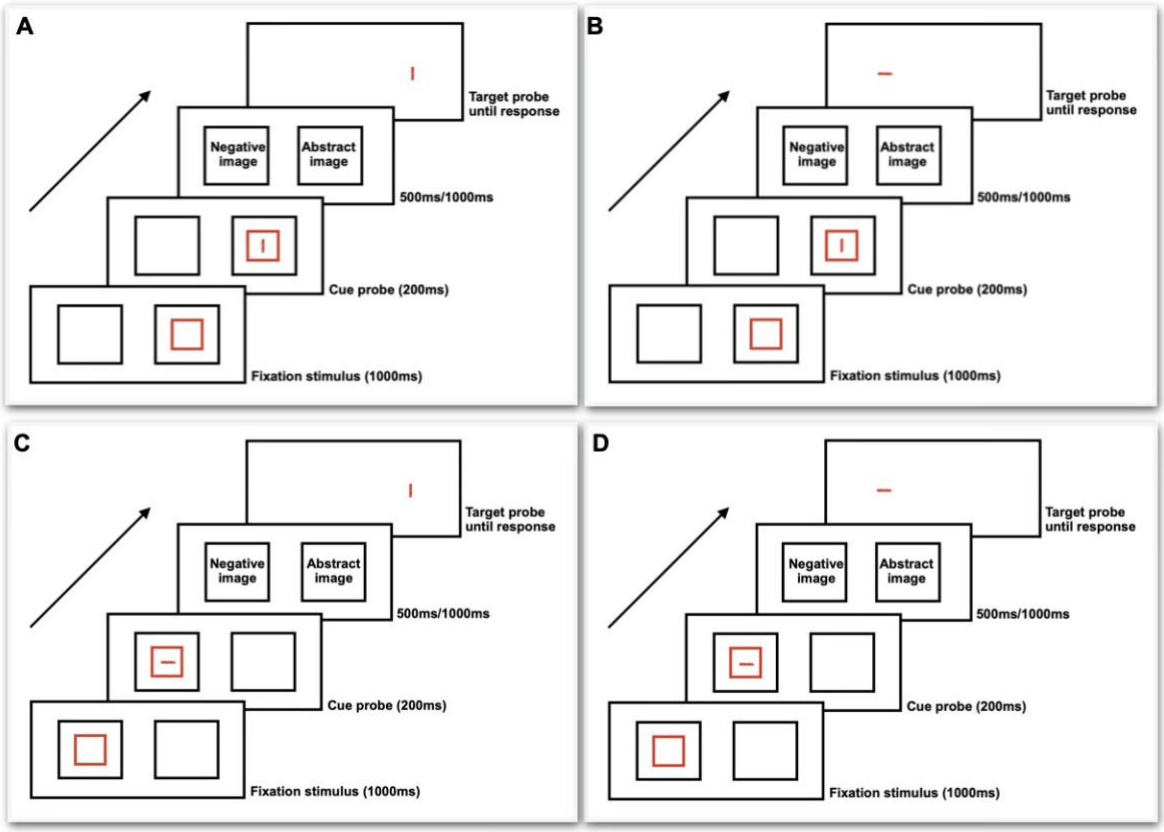

Figure 4.1 – *Examples of engagement and disengagement bias trials in the dot probe task*

The top two pictures (A & B) are examples of engagement bias trials, where attentional focus is initially anchored distally to the negative image (via fixation stimulus/cue probe). The top left picture (A) shows the target probe being distal to the negative image, while the right (B) shows the target probe being proximal to it. The bottom two pictures (C & D) are examples of disengagement bias trials, where attentional focus is initially anchored proximally to the negative image (via fixation stimulus/cue probe). The bottom left picture (C) shows the target probe being distal to the negative image, while the right (D) shows the target probe being proximal to it. Figure is adapted from Rudaizky et al. (2014).

4.2.3 *Behavioural analysis*

The behavioural analysis was carried out using IBM SPSS Statistics for Windows, version 27 (IBM Corp., Armonk, N.Y., USA). The mean reaction time for the engagement and disengagement trials of each experimental condition was computed for each participant. Incorrect trials were excluded, along with reaction times that deviated more than 2.58 standard deviations (SD) above or below the

corresponding mean (in total 6.96% of the data). The engagement bias indices were then calculated as follows using the trimmed means:

Negative Engagement Bias Index = (Cue probe distal to negative image in negative/abstract image pair: RT for target probe distal to negative image minus RT for target probe proximal to negative image) minus (Cue probe distal to neutral image in neutral/abstract image pair: RT for target probe distal to neutral image minus RT for target probe proximal to neutral image).

A higher score on the Negative Engagement Bias Index would represent selectively enhanced shifting of attention towards initially unattended distal images when these are negative rather than neutral.

Positive Engagement Bias Index = (Cue probe distal to positive image in positive/abstract image pair: RT for target probe distal to positive image minus RT for target probe proximal to positive image) minus (Cue probe distal to neutral image in neutral/abstract image pair: RT for target probe distal to neutral image minus RT for target probe proximal to neutral image).

A higher score on the Positive Engagement Bias Index would represent selectively enhanced shifting of attention towards initially unattended distal images when these are positive rather than neutral.

The disengagement bias indices were then calculated as follows using the trimmed means:

Negative Disengagement Bias Index = (Cue probe proximal to negative image in negative/abstract image pair: RT for target probe distal to negative image minus RT for target probe proximal to negative image) minus (Cue probe proximal to neutral image in neutral/abstract image pair: RT for target probe distal to neutral image minus RT for target probe proximal to neutral image).

A higher score on the Negative Disengagement Bias Index would represent a heightened tendency for attention to be held in the locus of initially attended proximal images when these are negative rather than neutral.

Positive Disengagement Bias Index = (Cue probe proximal to positive image in positive/abstract image pair: RT for target probe distal to positive image minus RT for target probe proximal to positive image) minus (Cue probe proximal to neutral image in neutral/abstract image pair: RT for target probe distal to neutral image minus RT for target probe proximal to neutral image).

A higher score on the Positive Disengagement Bias Index would represent a heightened tendency for attention to be held in the locus of initially attended proximal images when these are positive rather than neutral.

Accuracy of the treatment groups (losartan vs. placebo) was compared using a Mann-Whitney U test and performance was compared using independent t-tests based on the index scores. For information about the analysis of the subjective ratings and physiological parameters please see section 2.2.4 of Chapter 2.

4.3 Results

4.3.1 *Sociodemographic, clinical, and personality characteristics*

Sociodemographic, clinical, and personality characteristics of participants included in the dot probe task analysis are presented in Table 4.1. The analysis of this task consisted of 29 participants, as 1 participant from the losartan group had to be excluded due to technical problems with response capture. The analysis thus included 14 participants in the placebo group and 15 in the losartan group. The two groups were well matched on sociodemographic, clinical, and personality parameters.

Table 4.1 – Sociodemographic, clinical, and personality characteristics

	Placebo (n=14)	*Losartan (n=15)*
Gender		
Male	n = 3	n = 4
Female	n = 11	n = 11
Age	26.86 (2.52)	26.33 (2.63)
Trait Anxiety (STAI-T)	48.86 (2.34)	49.93 (1.61)
Verbal IQ (STW-2)	106.93 (2.99)	105.60 (2.84)
Attentional Control Scale (ACS)		
Total	50.36 (2.58)	51.27 (1.72)
Focusing	21.50 (1.27)	22.00 (0.95)
Shifting	28.86 (1.62)	29.27 (1.36)
Anxiety Sensitivity Index (ASI-R)	27.14 (5.66)	22.40 (4.05)
Behavioral Inhibition Scale (BIS)	13.14 (0.65)	13.33 (0.89)
Behavioral Activation Scale (BAS)	25.93 (1.31)	25.27 (1.69)
Beck Depression Inventory (BDI-II)	10.14 (2.45)	8.00 (1.62)
Eysenck Personality Questionnaire (EPQ)		
Neuroticism	12.57 (1.35)	11.40 (1.54)
Psychoticism	3.71 (0.68)	2.53 (0.56)
Extraversion	12.86 (1.22)	14.40 (1.25)
Lie/Social Desirability	8.50 (1.03)	8.87 (0.86)

Values are means (SEM)

4.3.2 *Subjective rating results and physiological parameters*

There was no significant main effect of treatment group on any of the visual analogue scales (VAS) assessing potential transient effects of the drug (all *ps* > .182). Moreover, there were no significant interactions between group and the timing of any of the scales (before capsule intake vs. at peak level; all *ps* > .118). There was also no significant main effect of treatment group on any of the side effect ratings (all *ps* > .259). Furthermore, there was no significant main effect of treatment group on physiological parameters (blood pressure and heart rate; all *ps* > .201), and no significant interactions between group and the timing of any of the measurements (before capsule intake vs. at peak level; all *ps* > .452). These results suggest that the analysis was not confounded by physiological, mood, or side effect differences between the two groups and confirms that participant blinding was maintained.

4.3.3 *Behavioural performance in the dot probe task*

The two groups did not differ in terms of accuracy (Z = -.065, p = .948; losartan: M = 93.30%, SD = 3.33; placebo: M = 92.93%, SD = 5.08). The mean response latencies obtained under each experimental condition for each group are shown in Table 4.2.

Table 4.2 – Mean response latencies obtained under each experimental condition for each group

Cue locus	*Valence*	*Target probe locus*	*SOA (ms)*	*Placebo M (SD)*	*Losartan M (SD)*
Distal (AET)	Negative	Distal	500	1032.20 (263.79)	1127.40 (343.95)
			1,000	1014.96 (234.34)	1072.39 (365.31)
		Proximal	500	1083.65 (244.87)	1210.28 (387.52)
			1,000	1014.91 (203.54)	1104.67 (295.38)
	Positive	Distal	500	1000.49 (203.66)	1072.72 (304.45)
			1,000	979.35 (215.19)	1065.72 (390.26)
		Proximal	500	1055.17 (220.17)	1102.89 (295.95)
			1,000	1007.60 (229.83)	1086.10 (315.69)
	Neutral	Distal	500	959.75 (181.16)	1076.50 (323.46)
			1,000	964.55 (217.39)	1058.64 (300.97)
		Proximal	500	1056.66 (275.38)	1132.18 (390.97)
			1,000	993.98 (223.00)	1076.53 (376.54)
Proximal (ADT)	Negative	Distal	500	1019.85 (247.37)	1098.50 (335.88)
			1,000	1005.85 (251.09)	1090.96 (472.66)
		Proximal	500	1077.40 (218.10)	1182.55 (344.93)
			1,000	1065.18 (254.14)	1097.97 (352.98)
	Positive	Distal	500	966.92 (218.86)	1056.40 (282.43)
			1,000	926.14 (185.18)	1054.96 (359.85)
		Proximal	500	1060.62 (278.61)	1111.64 (309.58)
			1,000	1075.26 (274.53)	1177.93 (461.43)
	Neutral	Distal	500	1013.83 (231.54)	1056.42 (297.88)
			1,000	978.11 (239.66)	1021.76 (318.75)
		Proximal	500	1013.15 (220.44)	1132.29 (396.91)
			1000	1029.34 (209.54)	1075.93 (270.29)

SOA = Stimulus onset asynchrony; AET = Attentional engagement trials; ADT = Attentional disengagement trials; values are mean reaction times in milliseconds.

There was a significant difference between the two groups on the Positive Disengagement Bias Index with the image exposure duration of 500ms, where the losartan group had higher attentional bias index scores than the placebo group ($t(27) = -2.569$, $p = .016$, $d = -.955$; see Figure 4.2).

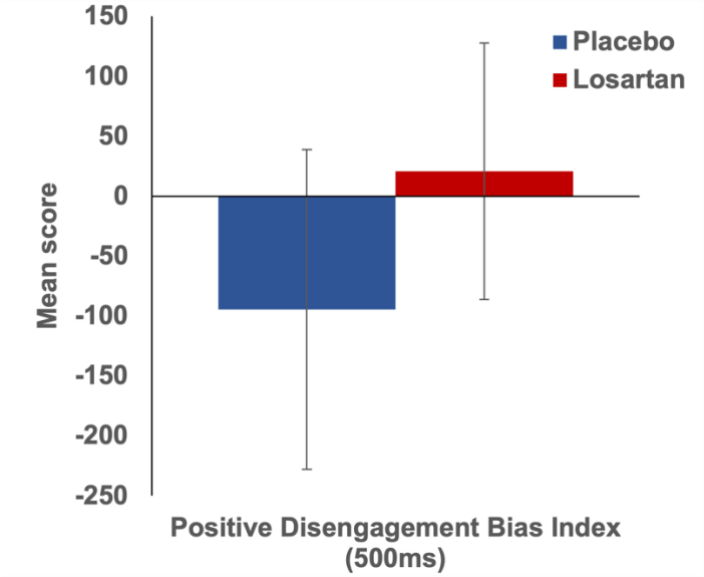

Figure 4.2 – *Positive Disengagement Bias Index scores for each group*

The losartan group (red) had significantly higher attentional bias index scores than the placebo group (blue) on the Positive Disengagement Bias Index with the image exposure duration of 500ms. Scores are expressed as mean ± SD.

No other group differences were found for any of the other indices (all *ps* > .295). The attentional bias indices obtained under each experimental condition for each group are shown in Table 4.3.

Table 4.3 – Means and standard deviations of attentional bias indices

			Placebo	*Losartan*	
Attentional bias type		*SOA (ms)*	*M (SD)*	*M (SD)*	*P*
Engagement bias indices	Negative	500	45.46 (161.08)	-27.20 (201.56)	.295
		1000	29.49 (163.00)	-14.39 (219.98)	.549
	Positive	500	42.22 (131.30)	25.51 (160.97)	.763
		1000	1.18 (139.42)	-2.49 (248.52)	.962
Disengagement bias indices	Negative	500	-58.23 (158.91)	-8.18 (205.74)	.472
		1000	-8.10 (174.00)	47.15 (168.37)	.393
	Positive	500	-94.37 (133.44)	20.64 (107.00)	.016
		1000	-97.90 (193.56)	-68.80 (283.35)	.751

SOA = Stimulus onset asynchrony

4.4 Discussion

It still remains to be fully clarified how losartan affects some of the mechanisms relevant to exposure success in humans, such as attentional biases (Price et al, 2011). In this study, the effects of a single dose of losartan on attentional biases were investigated, using a dot probe task, in healthy, high trait-anxious volunteers. As losartan has been shown to improve early discrimination of negative versus positive stimuli (Reinecke et al, 2018) and to induce a positive learning bias (Pulcu et al, 2019), it was predicted that losartan would reduce negative attentional biases and increase positive ones. The losartan group in the current study had higher scores than the placebo group on the Positive Disengagement Bias Index when the image exposure lasted 500ms, meaning that the losartan group's attention was more firmly held by the positive stimuli than by the neutral one. No other group differences were found on any of the other attentional bias indices. Furthermore, physiological parameters (blood pressure and heart rate) were measured and visual analogue scales administered at both baseline and drug-peak level to assess potential transient effects of the drug. Side effects were then measured at the end of the testing visit. No group differences or interactions were found on any of the measures, which suggests that the analysis was not confounded by physiological, mood, or side effect differences between the two groups and confirms that participant blinding was maintained.

The losartan group had higher attentional bias index scores than the placebo group on the Positive Disengagement Bias Index when the image exposure lasted 500ms. This means that the losartan group's attention was more firmly held by the representational image when it was positive rather than neutral in emotional tone, thus reflecting an increase in positive attentional bias. Losartan has been shown to

initiate a shift from aversive to appetitive learning, where the choices of participants who had received losartan were more influenced by positive outcomes than negative, indicating a positive learning bias, which, according to the authors, suggests that losartan may have the potential to also facilitate positive relative to negative learning during exposure therapy in humans (Pulcu et al, 2019). Similarly, a very recent study found that losartan shifted motivational and emotional salience away from social punishment towards social reward (Zhou et al, 2021). An increase in positive attentional bias, as seen in the present study, may potentially represent a mechanism by which losartan can induce a shift from negative to positive learning as described in previous research (Pulcu et al, 2019; Zhou et al, 2021). It has been suggested that adjunct positive valence training, which involves depicting a feared situation in a more positive light, may enhance the longevity of exposure treatment (Dour, Brown, & Craske, 2016). By enhancing positive attentional bias, losartan may thus possibly have the potential to more firmly focus people's attention to positive aspects of a feared situation, which could be beneficial during exposure therapy, but this of course requires further testing.

Power to detect smaller effects was limited given the relatively small sample size used in this analysis due to the COVID-19 pandemic disruption, which could potentially explain why no other group differences were found on any of the other attentional bias indices. Small sample sizes are usually thought to contribute to false negatives, which can conceal the presence of real effects, so definite conclusions cannot be drawn until more participants have been recruited in this study.

In summary, a single dose of losartan, in the absence of overall effects on heart rate, blood pressure, and mood, increased positive attentional bias, which was reflected in attention being more firmly held by positive stimuli compared to neutral

in the losartan group. An increase in positive bias may potentially represent a mechanism by which losartan can induce a shift from negative to positive learning, which has been described in previous research (Pulcu et al, 2019; Zhou et al, 2021). As it has been suggested that adjunct positive valence training may enhance the longevity of exposure treatment (Dour, Brown, & Craske, 2016), losartan may have the potential to enhance the treatment by more firmly focusing people's attention to positive aspects of a feared situation. No other group differences were found on any of the other attentional bias indices, which may have been due to lack of statistical power. Definite conclusions can thus not be drawn until more participants have been recruited in this study.

Chapter 5: Overall methods for the diazepam study

5.1 Introduction

The diazepam study of this thesis was a double-blind, randomised, placebo-controlled study done in 2012 that investigated the effects of diazepam on neural correlates of emotional processing during three tasks. Methods that the following three task chapters have in common will be presented here, while methods that only apply to each specific task will be presented separately in each chapter.

5.2 Methods

5.2.1 *Participants*

Ethical approval was granted by the NRES Committee South Central – Southampton B. 34 healthy volunteers, aged 18 to 34 years old, were recruited in 2012 through adverts in local papers, via student mailing lists, and on posters in departments and colleges at the University of Oxford and Oxford Brookes University. After passing an initial email screening, participants were invited to come to the Neurosciences Building at Department of Psychiatry for a more thorough medical and psychiatric screening, and to give informed consent, which lasted approximately 60 minutes. Participants were excluded if they had a current or past history of a DSM-IV Axis 1 psychiatric disorder (assessed using the SCID-IV Axis I; First, Williams, Spitzer, & Gibbon, 2007), a psychotropic medication use, a current or recent illicit drug use or use of a drug from another study (<3 months), a current or past history of drug or alcohol dependency or abuse, any medical or MRI contra-indication (e.g., metal in body), dyslexia, epilepsy, or were pregnant or breast-feeding. Participants were also required to be right-handed, have a BMI between

19-30, be fluent in English, and be non- or light smokers (<5 cigarettes per day). Furthermore, they were required to receive a physical examination by a medical doctor before participating, and each participant's general practitioner was informed of their involvement in the study before the beginning of the experiment. Sociodemographic, clinical, and personality characteristics are presented in Table 5.1. The two groups were well matched on sociodemographic, clinical, and personality parameters.

Table 5.1 – Sociodemographic, clinical, and personality characteristics

	Placebo (n=16)	*Diazepam (n=18)*
Gender		
Male	n=8	n=8
Female	n=8	n=10
Age	22.25 (1.00)	23.61 (0.90)
Verbal IQ (NART)	113.66 (1.41)	115.50 (1.68)
Beck Depression Inventory	1.44 (0.48)	1.54 (0.48)
Spielberger State and Trait Anxiety Inventories		
State-Anxiety	25.13 (0.98)	26.46 (1.62)
Trait-Anxiety	27.56 (1.30)	28.08 (1.77)
Eysenck Personality Questionnaire		
Neuroticism	4.75 (0.70)	5.54 (0.91)
Psychoticism	2.88 (0.64)	2.69 (0.61)
Extraversion	16.75 (0.93)	15.77 (1.02)
Lie/Social Desirability	7.31 (1.06)	7.08 (1.09)

Values are means (SEM)

5.2.2 *Procedure*

Participants were randomly allocated to receive either 15mg of diazepam or a placebo for 7 days. They were instructed to abstain from alcohol, to stick to their usual caffeine consumption, and not to cycle or drive during the treatment period. The treatment week was also scheduled such that it did not fall within the premenstrual week of female participants.

After successfully passing the screening visit, subjects were invited for a second visit at the Department of Psychiatry to receive the first half of the capsules and to fill in several questionnaires: Beck Depression Inventory (BDI-II; Beck, Steer, & Brown, 1996) to measure depression; National Adult Reading Test (NART; Nelson & Willison, 1991) to assess verbal intelligence; the State-Anxiety & Trait-Anxiety subscales of the State-Trait Anxiety Inventory (STAI; Spielberger, Gorsuch, Lushene, Vagg, & Jacobs, 1983) to measure anxiety, and the Eysenck Personality Questionnaire (EPQ; Eysenck & Eysenck, 1975) to assess personality traits.

For the next 6 days after their second visit, participants took 1 capsule in the morning after breakfast and 2 capsules in the evening after dinner. The capsules that contained diazepam had 5mg, so that subjects would receive 5mg in the mornings and 10mg in the evenings to minimise drowsiness. Before taking the medication each morning, participants completed a number of measures to assess side effects, mood and anxiety, and alertness for the previous 24 hours. These included side effects scales, the Befindlichskeit (BFS) scale of mood and energy (Von Zerssen, Strian, & Schwarz, 1974), the Positive and Negative Affect Schedule (PANAS; Watson, Clark, & Tellegen, 1988), and the Bond-Lader Visual Analogue Scales (VAS; Bond & Lader, 1974). The side effect scales included “drowsiness”, “lightheadedness”, “confusion”, “lack of coordination”, “forgetfulness”, “agitation”, and “muscle weakness”, and were coded according to the intensity of the experienced side effect (0 = none, 1 = mild, 2 = moderate, 3 = severe). The VAS included the following scales: “alert – drowsy”, “calm – excited”, “strong – feeble”, “muzzy – clear-headed”, “well-coordinated – clumsy”, “lethargic – energetic”, “contented – discontented”, “troubled – tranquil”, “mentally slow – quick-witted”, “tense – relaxed”, “attentive – dreamy”, “incompetent – proficient”, “happy – sad”,

"antagonistic – friendly", "interested – bored", "withdrawn – sociable". Participants were asked to indicate on each VAS how they felt at the moment by drawing a vertical line on a 10cm horizontal line between the two words. Half-way through the study week, participants returned to the Department of Psychiatry for a short visit to receive the second half of the capsules, and to monitor progress and any adverse side effects, as well as to minimise the potential for abuse of the drugs.

On the 7th day, participants were instructed to take their morning capsule 1 hour before coming to the Oxford Centre for Clinical Magnetic Resonance Research (OCMR) at the John Radcliffe Hospital for testing, which took approximately 120 minutes. No subject reported missing a dose. Before participants entered the scanner, they changed into metal free scrubs and a radiographer confirmed they were safe to go into the scanner. Once they were in the scanner, they completed an emotion regulation task, then a face classification task, and then finally an emotional counting Stroop task. The subjects also completed the State-Anxiety subscale of the State-Trait Anxiety Inventory (STAI-S) and the BDI again. After completing the testing, participants were unblinded by a third party and reimbursed with 100 GBP for their time. The timeline of the study is presented in Figure 5.1.

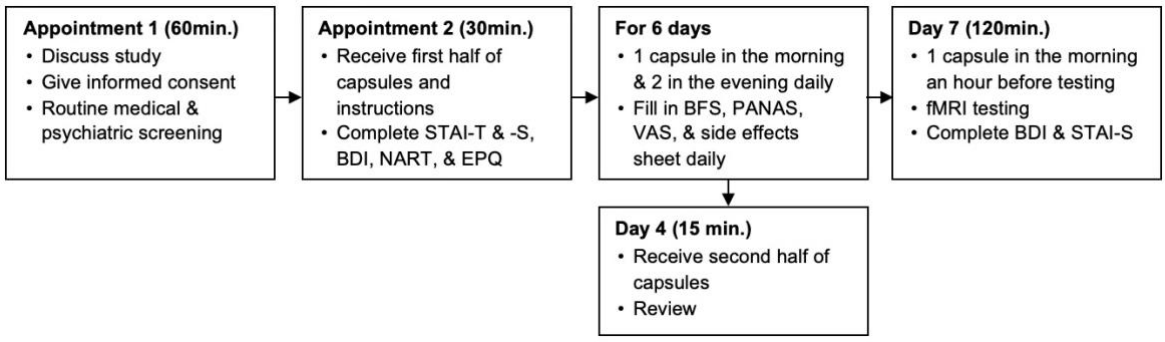

Figure 5.1 – *Timeline of the diazepam study*

5.2.3 *Tasks*

The method sections of Chapter 6, Chapter 7, and Chapter 8 describe the emotion regulation task, the face classification task, and the emotional counting Stroop task respectively.

5.2.4 *Behavioural analysis*

The behavioural analysis was carried out using IBM SPSS Statistics for Windows, version 27 (IBM Corp., Armonk, N.Y., USA). To determine if there were any significant effects of treatment group on the daily measures, a 7×2 mixed ANOVA was conducted for the scores on each VAS, the positive and negative subscales of the PANAS, the BFS, and the side effects. Treatment condition (diazepam vs. placebo) was entered as the between-group factor and treatment timing (day 1 to 7) as the within-subjects factor. Furthermore, to determine if there were any significant effects of treatment group on the measures participants completed during their first capsule pick-up day and their test day, a 2×2 mixed ANOVA was conducted for the scores on the STAI-S and the BDI. As before, treatment condition was entered as the between-group factor and treatment timing (first capsule pick-up vs. test day) as the within-subjects factor.

5.2.5 *MRI acquisition and analysis*

See each individual chapter for more details.

Chapter 6: The effects of diazepam on emotional processing during an emotion regulation task

6.1 Introduction

The majority of people with anxiety disorders experiences serious impairments in their daily lives by their symptoms, which include increased fear and emotional reactivity, negative attentional biases, and tendencies to experience emotions as aversive, which often result in dysfunctional emotion regulation (Campbell-Sills, Ellard, & Barlow, 2014). Emotion regulation is thought to be a multifaceted process where individuals modulate the intensity and direction of emotional responses via engagement of prefrontal, cingulate, and parietal top-down control regions to modulate activity in affective appraisal regions, such as the amygdala and insula (Silvers et al, 2014; Frank et al, 2014). Strategies to regulate emotions can be adaptive and maladaptive. Cognitive reappraisal is for example considered to be an adaptive strategy to regulate emotions across a variety of contexts and involves cognitively transforming an aversive situation or experience by generating neutral or positive interpretations or perspectives to lessen its emotional impact (Gross, 1998). In contrast, suppression of emotional expression and avoidance have long been thought of as maladaptive strategies that have been implicated in anxiety disorders (Dryman & Heimberg, 2018; Salters-Pedneault, Tull, & Roemer, 2004). Suppression of emotional expression involves inhibiting ongoing emotion-expressive behaviour to an emotionally aversive event and is thought to primarily modify the behavioural aspect of the response, without properly reducing the negative emotional experience, which may continue to linger and accumulate unresolved (John & Gross, 2004). Avoidance on the other hand includes physical and cognitive avoidance, where physical avoidance involves physically avoiding

aversive stimuli or situations, while cognitive avoidance involves suppressing or avoiding psychological experiences such as certain thoughts, emotions, and memories (Aldao, Nolen-Hoeksema, & Schweizer, 2010).

It has been suggested that people with anxiety disorders may have an inability to accurately appraise threat, or an inability to reappraise threat, or even both, which has been linked to abnormal activity in prefrontal control regions and affective appraisal regions (Silvers et al, 2014). Hyper-activation in deep limbic structures (amygdala and insula) has repeatedly been linked to fear and anxiety (Etkin & Wager, 2007), and hypo-activation in prefrontal areas (e.g., dorsolateral and dorsomedial prefrontal cortex) has been observed in anxiety disorders during emotion regulation (Ball et al, 2013). This prefrontal hypo-activation is thought to contribute to the characteristic emotion dysregulation seen in anxiety disorders, which has been related to under-utilization of adaptive emotion regulation strategies, such as reappraisal, and over-utilization of dysfunctional ones, such as cognitive avoidance, thus possibly reflecting insufficient top-down control (Ball et al, 2013). More recent neurobiological frameworks related to anxiety disorders have also suggested that some prefrontal areas, such as the ventrolateral prefrontal cortex (vlPFC), may also be hyperactive during emotional processing (Moon, Yang, & Jeong, 2015; Reinecke et al, 2015), which possibly reflects an increased utilisation of dysfunctional emotion regulation strategies (Reinecke et al, 2015). Increased vlPFC activity has for example been associated with increased use of regulatory strategies, such as cognitive avoidance, in response to anxious hyper-reactivity (Hofmann, Ellard, & Siegle, 2012). These dysfunctional strategies are thought to play a big part in maintaining anxiety disorders and are thus targeted during CBT. It has also been shown that amygdala attenuation during threat

processing is associated with decreased activity in prefrontal brain areas, such as the vlPFC, after CBT (Månsson et al, 2013).

It has been suggested that SSRIs may exert their effects by improving emotion regulation, as SSRI treatments have been associated with an increased use of cognitive reappraisal and a decreased use of suppression of emotional expression in both depressed and anxious patients (McRae, Rekshan, Williams, Cooper, & Gross, 2014; Feurer et al, 2021). However, as SSRIs are effective for both anxiety and depression, it can be difficult to distinguish between their anxiolytic and antidepressant effects. Benzodiazepines have more specific effects on anxiety, so any effects they exert on cognition and neural activation may be more directly related to their anxiolytic action, thus making it a good candidate to help tease apart the different components of SSRI action and to help inform the development of future anxiolytics. The relationship between benzodiazepines and emotion regulation has however not been well-explored, but benzodiazepines have been shown to affect brain regions relevant to adaptive emotion regulation, such as frontal control and affective appraisal regions. A non-sedating dose of the benzodiazepine diazepam has for example been shown to attenuate activation in the amygdala, insula, and orbitofrontal cortex in response to emotional material, while also increasing activation in the anterior cingulate cortex (ACC; Del-Ben et al, 2012), and similarly a single dose of the benzodiazepine lorazepam has been show to attenuate activation in the amygdala and insula in response to emotional stimuli (Paulus et al, 2005). Furthermore, $GABA_A$ receptor α_2 and α_3 subunits, which are thought to be responsible for benzodiazepines' anxiolytic effects (McKernan et al, 2000), are located in high concentrations in the amygdala and frontal regions (D'Hulst et al, 2009). Based on these findings, benzodiazepines' anxiolytic effects

may be due to them modulating activity within frontal and affective appraisal regions related to emotional processing and emotion regulation.

This study therefore sought to investigate whether diazepam modulates activity within the limbic and prefrontal areas in relation to adaptive emotion regulation. Furthermore, as it may be more clinically relevant to investigate a short-term administration, as benzodiazepines are usually prescribed over a period of two to four weeks, and as it may elicit more reliable changes as plasma drug levels may be more stable, a 7-day treatment was used in this study. In light of prior research on acute benzodiazepine administration (Del-Ben et al, 2012; Paulus et al, 2005), and $GABA_A$ receptor α_2 and α_3 subunits being located in high concentrations in the amygdala and frontal regions (D'Hulst et al, 2009), it was predicted that a 7-day treatment of diazepam would attenuate activation in the amygdala and insular cortex and possibly also modulate activity within the prefrontal areas in response to cognitive reappraisal.

6.2 Methods

6.2.1 *Participants & procedure*

For information about the participants and the procedure please see Chapter 5. In brief, this was a double-blind, randomised, placebo-controlled study done in 2012 that investigated the effects of a 7-day treatment of diazepam (15mg) on neural correlates of emotional processing. 34 healthy volunteers, aged 18 to 34 years old, were recruited, but 6 participants had to be excluded from the analysis of this task due to response capture, motion, and anatomical problems. The analysis thus included 28 participants, with 13 in the placebo group and 15 in the diazepam group.

6.2.2 *Experimental task*

In the emotion regulation task, participants were instructed to either passively view or reappraise aversive pictures during an fMRI scan. The stimuli were 40 coloured pictures from the International Affective Picture System (IAPS; Lang, Bradley, & Cuthbert, 1997), depicting aversive scenes, such as scenes with violence, death, and accidents. E-prime (version 2.0; Psychology Software Tools Inc., Pittsburgh, PA, USA) was used to run the task and record responses. The task included 8 picture blocks, where each block consisted of 5 pictures presented for 6 seconds each. A fixation cross was presented between each block for 30 seconds. For 4 of the blocks, participants were asked to passively view the aversive images without trying to change the emotional state (maintain blocks), while for the other 4 they were asked to decrease the negative affect by implementing cognitive strategies of reappraisal (reappraise blocks). The maintain and reappraise blocks alternated and the order was counterbalanced. Both conditions were also matched in respect to

content, arousal, and valence. Each block started with the word "Maintain" or "Suppress" (indicating reappraisal) displayed on the screen for 6 seconds, before the 5 images were presented, depending on the condition. Each block then ended with a rating scale ranging from 1 (neutral) to 4 (most negative), where participants were asked to indicate how they felt at the present moment using a button box. A diagram of the task is presented in Figure 6.1. Participants received training and had a practice run before the scan to make sure they understood the task. Participants then completed the Emotion Regulation Questionnaire (ERQ; Gross & John, 2003) after the scan to see if the groups were comparable in terms of everyday use of reappraisal and expressive suppression techniques.

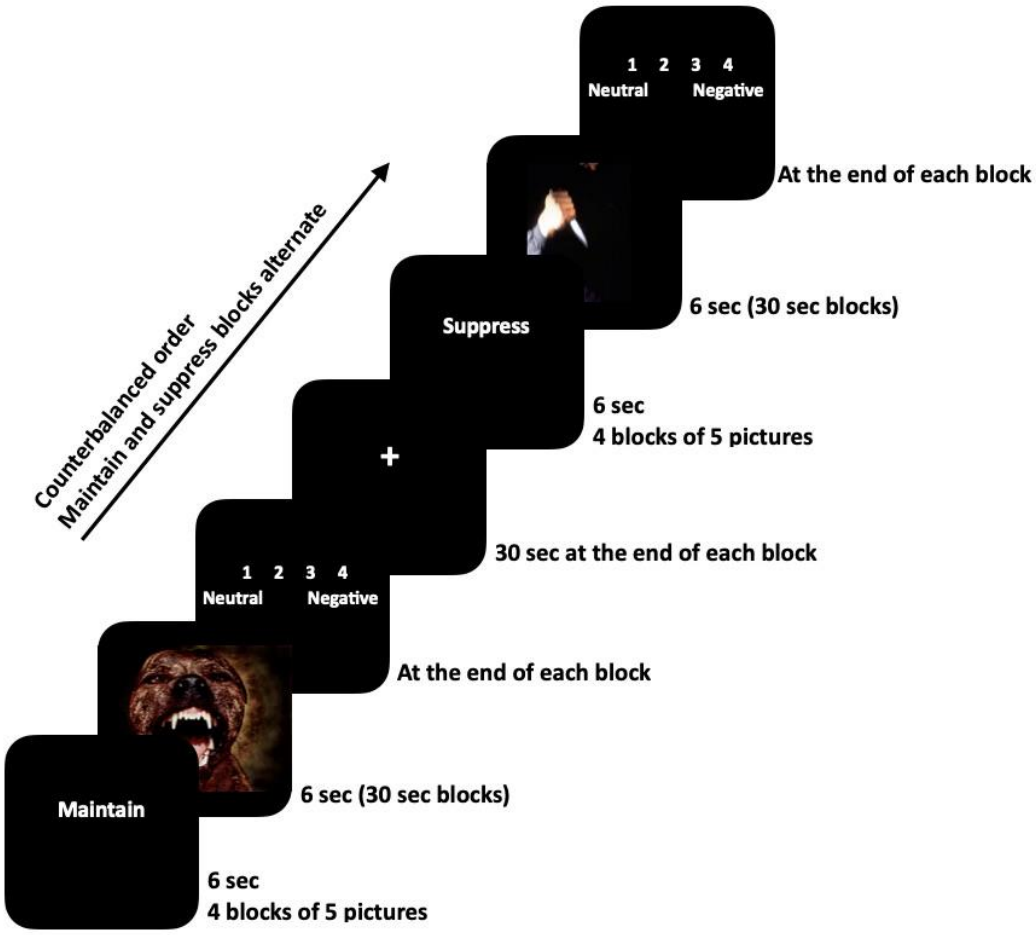

Figure 6.1 – *A diagram of the emotion regulation task*

6.2.3 *Behavioural analysis*

The behavioural analysis was carried out using IBM SPSS Statistics for Windows, version 27 (IBM Corp., Armonk, N.Y., USA). To determine if there were any significant effects of treatment group on everyday emotion regulation strategies (ERQ), a 2×2 mixed ANOVA was conducted. Treatment condition (diazepam vs. placebo) was entered as the between-group factor, and expressive suppression and reappraisal scores as the within-subjects factor. Furthermore, to determine if there were any significant effects of treatment group on negative affect ratings during the task, a 2×2 mixed ANOVA was conducted, where treatment condition was entered as the between-group factor, and reappraise and maintain block ratings as the within-subjects factor. For information about the analysis of the subjective ratings please see section 5.2.4 of Chapter 5.

6.2.4 *Image acquisition*

A 3T Siemens Magnetom Tim-Trio whole-body scanner system (Siemens, Erlangen, Germany) with a 32-channel head-coil, located at the OCMR, was used to acquire T1-weighted structural images and T2-weighted transverse echo planar images (EPI). Functional imaging consisted of 45 T2-weighted EPI slices (TR = 3000ms, TE = 30ms, flip angle = 90°, matrix size = 64 × 64, voxel dimension = 3mm isotropic, slice thickness = 3mm, FOV = 192 × 192mm^2, 183 volumes, echo spacing = 0.49ms). Gradient-echo fieldmap images were acquired for distortion correction (44 slices, slice thickness = 3.5mm, voxel dimension = 3.5mm isotropic, FOV = 192 × 192mm^2, TR = 488ms, TE1 = 5.19ms, TE2 = 7.65ms, flip angle = 60°), and T1-weighted structural images were acquired using an MP-RAGE sequence for

anatomical alignment (192 slices, slice thickness = 1mm, TR = 2040ms, TE = 4.7ms, flip angle = 8°, voxel dimension = 1mm isotropic, FOV = 192 × 192mm^2).

6.2.5 *Image analysis*

MRI data were analysed using FSL (FMRIB Software Library v6.6) tools (https://fsl.fmrib.ox.ac.uk/fsl). Structural anatomical scans were brain extracted using FSL's Brain Extraction Tool (BET; Smith, 2002). The fMRI analysis was carried out using the fMRI Expert Analysis Tool (FEAT) in FSL. Pre-processing consisted of brain extraction using BET (Smith, 2002), motion correction using FMRIB's Linear Image Registration Tool (MCFLIRT; Jenkinson, Bannister, Brady, & Smith, 2002), distortion correction using gradient-echo fieldmaps, spatial smoothing using a Gaussian kernel of FWHM 5mm, grand-mean intensity normalisation of the entire 4D dataset by a single multiplicative factor, registration of the functional space template to the anatomical space and the Montreal Neurological Institute (MNI) 152 space using the FMRIB's Linear Image Registration Tool (FLIRT; Jenkinson & Smith, 2001; Jenkinson, Bannister, Brady, & Smith, 2002), and high-pass temporal filtering equivalent to 90s (Gaussian-weighted least-squares straight line fitting, with sigma = 45.0s). Time-series statistical analysis was then carried out using FMRIB's Improved Linear Model (FILM) with local autocorrelation correction (Woolrich, Ripley, Brady, & Smith, 2001), and a custom 3-column format convolved with a gamma hemodynamic response function, and its temporal derivative, was used to model the data. The contrasts of interest were reappraise vs. maintain, maintain vs. reappraise, and the mean of maintain and reappraise (aversive pictures), whereas instruction/rating periods were excluded as regressors of no interest. Fixation cross blocks were implicit baseline. Motion traces

detected by MCFLIRT were included in the model as nuisance regressors to account for motion.

The group level analysis was carried out using FMRIB's Local Analysis of Mixed Effects (FLAME; Woolrich, Behrens, Beckmann, Jenkinson, & Smith, 2004). The general linear model (GLM) included the 2 groups (diazepam & placebo), where group averages, average across both groups, and differences between groups for each contrast of interest were tested (placebo>diazepam and diazepam>placebo). Significant activations were determined by cluster-based thresholding of $Z > 3.1$ and a family-wise error-corrected cluster significance threshold of $p < 0.05$ (Worsley, 2001). A further sensitivity group level analysis was done using the side effect of drowsiness as a covariate, to investigate whether the results were possibly confounded by differences in drowsiness between the two groups.

Grey matter (GM) images of each participant were extracted using FMRIB's Automated Segmentation tool (FAST; Zhang, Brady, & Smith, 2001). These were then registered to standard space, smoothed to match the intrinsic smoothness of the fMRI data (2.63mm), voxel-wise demeaned across all subjects, and added to the general linear model to remove any potential structural differences explaining the BOLD contrast differences.

In addition to the whole-brain analysis, a small volume correction (SVC) was preformed using predefined anatomical regions of interest (ROIs), which included the left and right amygdala and the left and right insular cortex. These regions were defined using the Harvard-Oxford Cortical and Subcortical Structural Atlases and a 50% threshold. As mentioned before, this method restricts the search space for significant voxels only to those voxels within the mask, which reduces the number of multiple comparisons and boosts statistical power.

Furthermore, a psychophysiological interactions (PPI) analysis was also done on the predefined left and right anatomical amygdala and insular cortex masks (seed ROIs) to investigate task-specific changes in the relationship between activity in different brain areas (O'Reilly, Woolrich, Behrens, Smith, & Johansen-Berg, 2012). For each participant, the standard space seed ROIs were registered to each subject's functional space to extract time-courses for each mask. These time-courses were then entered into first-level FSL PPI analyses, along with the psychological regressors (maintain and reappraise), the psychophysiological interaction regressors (maintain×time-courses and reappraise×time-courses), and the regressors of no interest (instructions and ratings). The individual contrast images were then entered into the group level, using a mixed-effects analysis across the whole brain, in order to identify brain areas that showed activity that covaried stronger with that of the seed ROIs in one of the two groups during reappraise blocks, maintain blocks, or aversive picture blocks in general. Significant results were then further explored by extracting parameter estimate (PE) values to visualise them.

6.3 Results

6.3.1 *Sociodemographic, clinical, and personality characteristics*

Sociodemographic, clinical, and personality characteristics of participants included in the emotion regulation task analysis are presented in Table 6.1. The analysis of this task consisted of 28 participants, as 6 participants had to be excluded due to technical response capture (placebo=2, diazepam=1), motion (diazepam=2), and anatomical (placebo=1) problems. The analysis thus included 13 participants in the placebo group and 15 in the diazepam group. The two groups were well matched on sociodemographic, clinical, and personality parameters.

Table 6.1 – Sociodemographic, clinical, and personality characteristics

	Placebo (n=13)	*Diazepam (n=15)*
Gender		
Male	n=7	n=6
Female	n=6	n=9
Age	21.31 (0.75)	23.36 (1.15)
Verbal IQ (NART)	113.45 (1.60	115.72 (1.98)
Beck Depression Inventory	1.69 (0.57)	1.45 (0.49)
Spielberger State and Trait Anxiety Inventories		
State-Anxiety	25.69 (1.12)	25.72 (1.44)
Trait-Anxiety	28.23 (1.53)	28.36 (1.90)
Eysenck Personality Questionnaire		
Neuroticism	4.92 (0.76)	5.55 (0.90)
Psychoticism	3.15 (0.74)	3.18 (0.62)
Extraversion	17.38 (0.90)	15.45 (1.17)
Lie/Social Desirability	7.00 (1.28)	8.00 (1.06)

Values are means (SEM)

6.3.2 *Subjective rating results*

There was no significant main effect of treatment group (diazepam vs. placebo) on the BFS, the PANAS, the STAI-S, or the BDI (all *ps* > .222), and there were no significant interactions between group and the timing of any of the measures (all *ps* > .110). Taken together, diazepam did not have a significant effect on subjective mood or state anxiety during the intervention week.

Furthermore, there were no significant main effects of treatment group on the lightheadedness, confusion, forgetfulness, and agitation side effect ratings (all *ps* > .051). There was however a significant main effect of group on the drowsiness ($F(1, 26) = 5.692$, $p = .025$, $\eta_p^2 = .180$), lack of coordination ($F(1, 26) = 7.261$, $p = .012$, $\eta_p^2 = .218$), and muscle weakness ($F(1, 26) = 4.464$, $p = .044$, $\eta_p^2 = .147$) ratings. Participants in the diazepam group were significantly drowsier and less coordinated, and experienced more muscle weakness throughout the study week compared to the placebo group, as shown in Figure 6.2, Figure 6.3, and Figure 6.4 respectively.

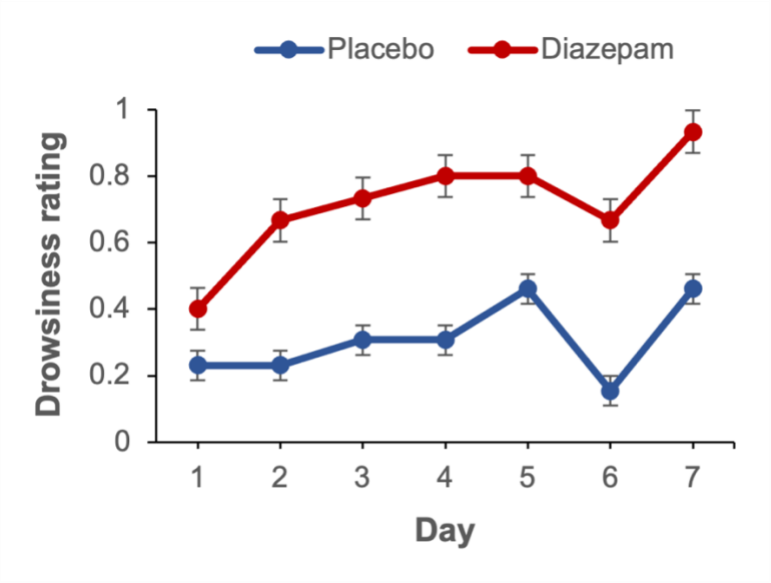

Figure 6.2 – *Drowsiness side effect ratings*

The diazepam group (red) was significantly drowsier throughout the intervention week compared to the placebo group (blue). Ratings are expressed as mean ± SEM.

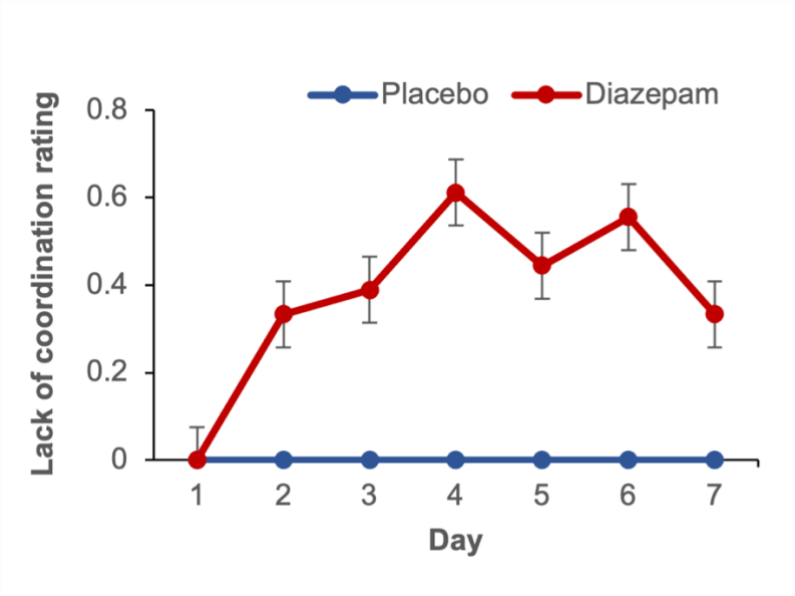

Figure 6.3 – *Lack of coordination side effect ratings*

The diazepam group (red) was significantly less coordinated throughout the intervention week compared to the placebo group (blue). Ratings are expressed as mean ± SEM.

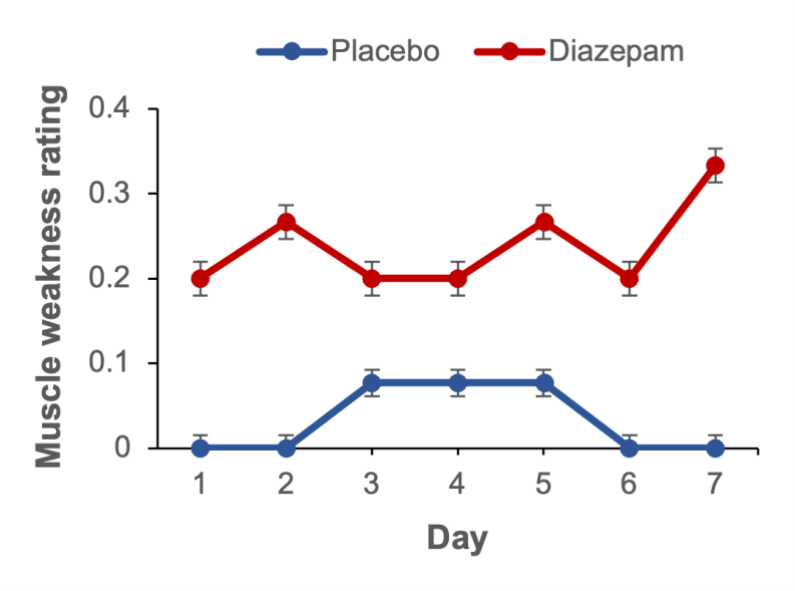

Figure 6.4 – *Muscle weakness side effect ratings*

The diazepam group (red) experienced significantly more muscle weakness throughout the intervention week compared to the placebo group (blue). Ratings are expressed as mean ± SEM.

There was no significant main effect of treatment group on any of the VAS (all *ps* > .129), except on the "well-coordinated – clumsy" scale. Participants in the diazepam group were significantly clumsier throughout the intervention week compared to the placebo group ($F(1, 26) = 8.206$, $p = .008$, $\eta_p^2 = .240$), as shown in Figure 6.5, which is in accordance with the observations from the side effect ratings of lack of coordination.

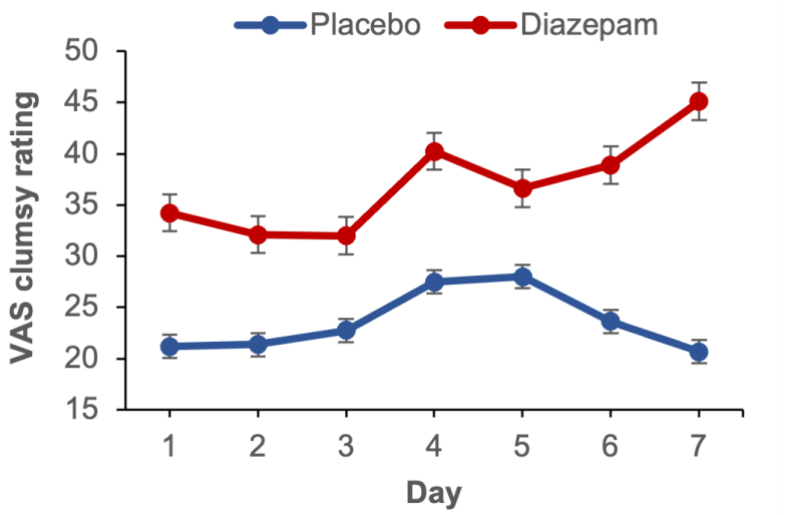

Figure 6.5 – *Clumsiness visual analogue scale ratings*

The diazepam group (red) was significantly clumsier throughout the intervention week compared to the placebo group (blue). Ratings are expressed as mean ± SEM.

6.3.3 *Behavioural performance*

There were no group differences in everyday emotion regulation strategies (ERQ), nor in negative affect ratings during the task (all *ps* > .102). There was however a main effect of condition (reappraise vs. maintain; $F(1, 26) = 82.255$, $p = <.001$, $\eta_p^2 = .760$), where both groups had significantly lower negative affect ratings after reappraise blocks compared to maintain blocks, which suggests that cognitive reappraisal techniques were applied successfully (see Figure 6.6).

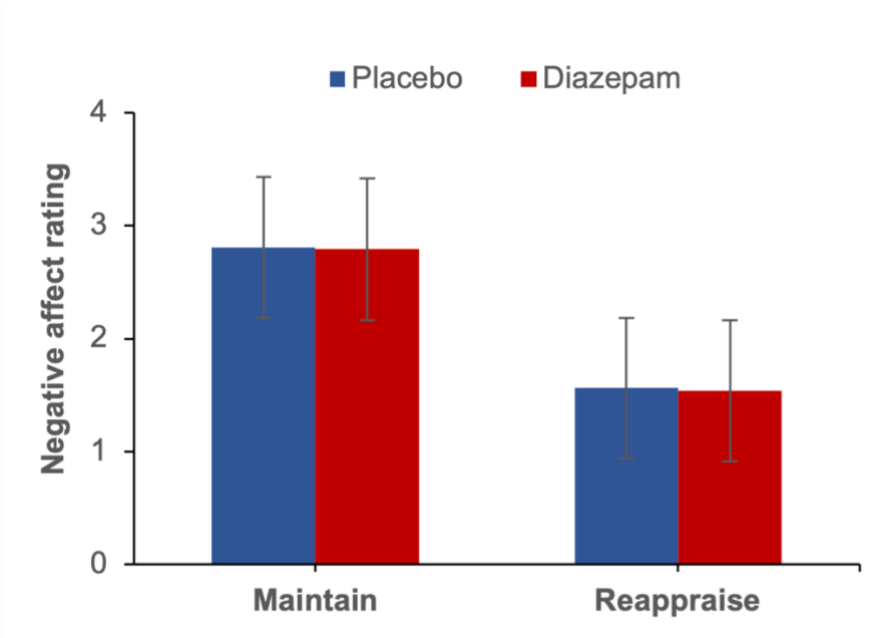

Figure 6.6 – *Negative affect ratings during the emotion regulation task*

Both the placebo group (blue) and the diazepam group (red) had significantly lower negative affect ratings after reappraise blocks compared to maintain blocks. Ratings are expressed as mean ± SEM.

Furthermore, there was a main effect of regulation (expressive suppression vs. reappraisal; $F(1, 26) = 31.180$, $p = <.001$, $\eta_p^2 = .545$), where both groups used reappraisal strategies more often than suppression of emotional expression in their everyday lives according to the ERQ (see Figure 6.7).

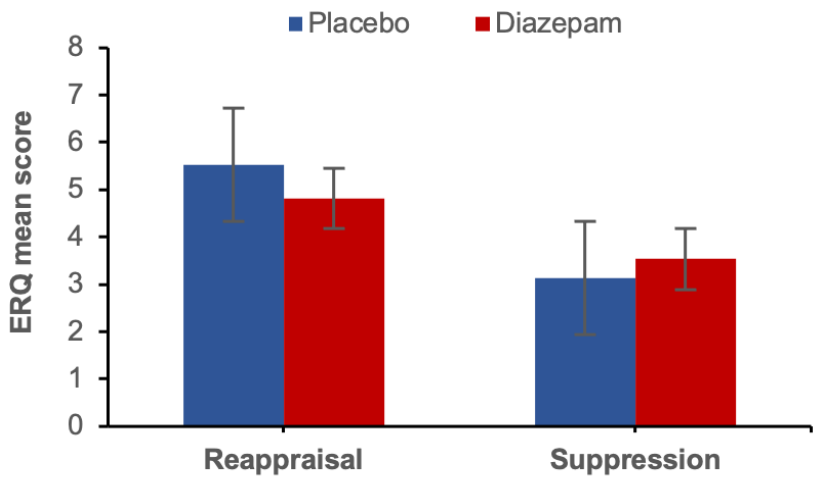

Figure 6.7 – *Emotion Regulation Questionnaire mean scores*

Both the placebo group (blue) and the diazepam group (red) had significantly lower scores for using suppression of emotional expression in their everyday lives compared to using reappraisal strategies. Ratings are expressed as mean ± SEM.

6.3.4 *Main effect of task*

Significant activity was observed in relation to viewing the aversive pictures (reappraise and maintain combined) across both groups in clusters that included the bilateral thalamus extending to the bilateral temporal occipital fusiform cortex, frontal pole, paracingulate gyrus, superior and inferior frontal gyrus, and middle frontal gyrus, juxtapositional lobule cortex, hippocampus, insular cortex, amygdala, and pre- and postcentral gyrus (see Figure 6.8 and Table 6.2). These areas are associated with attention and memory, motor control and movement, and visual and emotional processing, and are consistent with previous research (Phan et al, 2005).

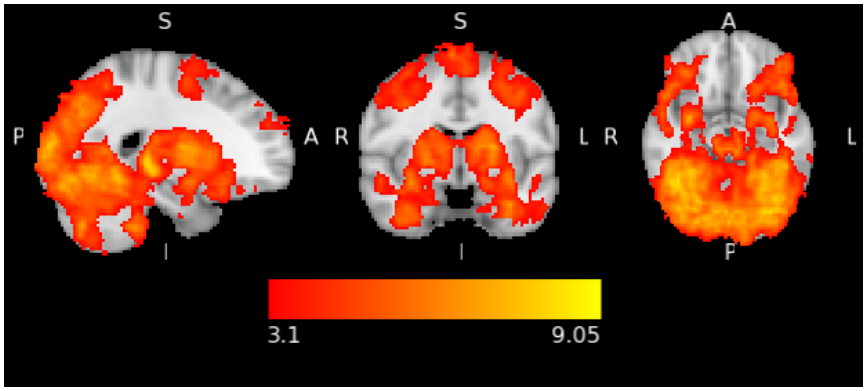

Figure 6.8 – *Whole-brain activation in response to aversive pictures across groups*

Sagittal, coronal, and axial images depicting neural activation in response to the aversive pictures (reappraise and maintain combined) across groups (diazepam and placebo) during the emotion regulation task. Images are thresholded at $Z > 3.1$ and $p < 0.05$ corrected.

Table 6.2 – Main effect of the emotion regulation task across groups ($Z > 3.1$, $p < 0.05$)

Cluster	Brain area	Side	Cluster size (voxels)	MNI max (x, y, z)	*z*-score	*p*-value
Task activation (mean of reappraise & maintain)						
1	Bilateral thalamus extending into bilateral temporal occipital fusiform cortex, paracingulate gyrus, frontal pole, superior & inferior frontal gyrus, middle frontal gyrus, juxtapositional lobule cortex, hippocampus, insular cortex, & amygdala	R	108238	22, -28, -2	8.96	<.001
2	Pre- & postcentral gyrus	L	112	-2, -34, 74	4.49	.014
Task activation (reappraise>maintain)						
1	Bilateral frontal pole extending into superior & inferior frontal gyrus, middle frontal gyrus, & paracingulate gyrus	R	5211	30, 60, 8	5.54	<.001
2	Middle frontal gyrus & frontal pole	R	167	48, 34, 24	4.34	.001
3	Middle frontal gyrus	L	155	-40, 14, 42	4.19	.002

Furthermore, increased activation of frontal areas, which included the bilateral frontal pole extending into the bilateral superior and inferior frontal gyrus, middle frontal gyrus, and paracingulate gyrus, was observed when cognitive strategies of reappraisal were implemented across both groups, compared to passively viewing the pictures (reappraise>maintain, overall mean; see Figure 6.9

and Table 6.2). This increased activity in frontal areas is consistent with previous research (Phan et al, 2005; Reinecke et al, 2015), thus confirming that participants were successful in completing the task.

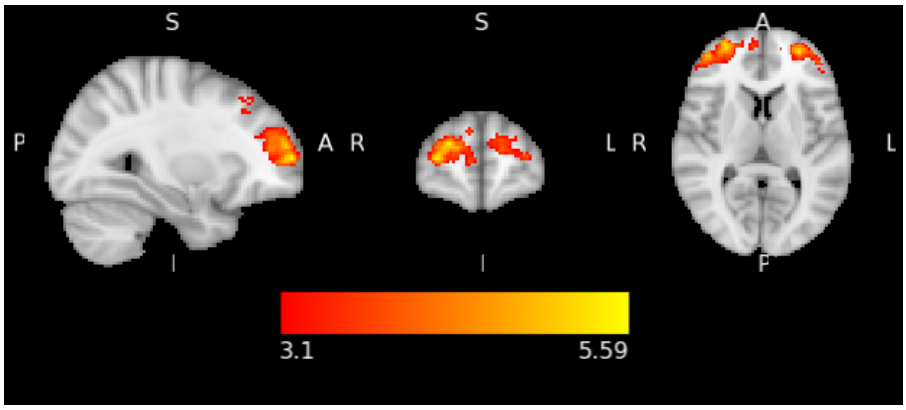

Figure 6.9 – *Whole-brain activation in response to the reappraise condition across groups*

Sagittal, coronal, and axial images depicting neural activation in response to the reappraise condition, compared to the maintain condition, across groups (diazepam and placebo) during the emotion regulation task. Images are thresholded at $Z > 3.1$ and $p < 0.05$ corrected.

6.3.5 *Effect of treatment*

A whole-brain analysis ($Z > 3.1$ thresholded, $p < 0.05$ corrected) revealed decreased BOLD activation in the diazepam group relative to the placebo group when cognitive strategies of reappraisal were implemented (reappraise condition), compared to passively viewing the pictures (maintain condition), in the right ventrolateral prefrontal cortex (vlPFC; x = 54, y = 36, z = 0; $Z = 4.44$; voxel cluster size: 147; see Figure 6.10). This difference was not affected by adding GM maps as a covariate in the model. The difference was also not affected by adding the side effect of drowsiness as a covariate in a further sensitivity analysis. No group differences were

seen in any of the other contrasts. Furthermore, there were no group differences in the amygdala and the insular cortex in the small volume correction analysis.

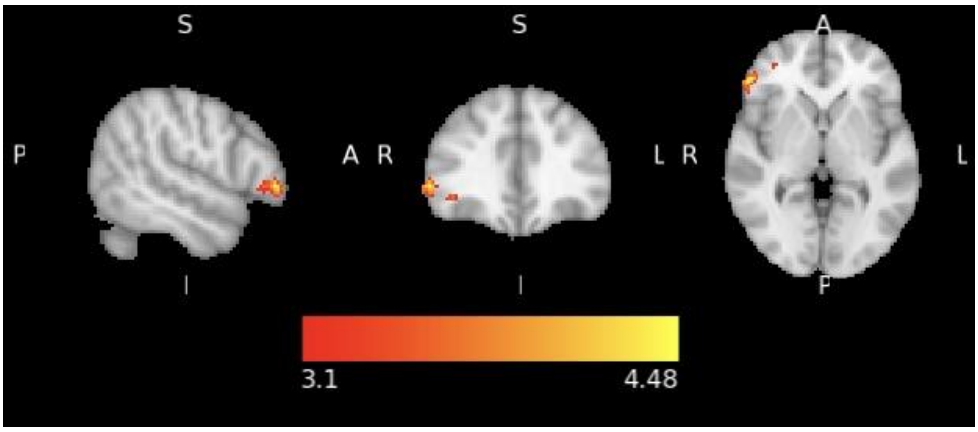

Figure 6.10 – *Whole-brain activation in response to the reappraise condition in the placebo group*

Sagittal, coronal, and axial images depicting significantly increased activation in the placebo group compared to the diazepam group for the reappraise vs. maintain contrast in the right vlPFC (peak voxels: x = 54, y = 36, z = 0; $Z = 4.44$; voxel cluster size: 147), thus suggesting a downregulation in this area in the diazepam group. Images are thresholded at $Z > 3.1$ and $p < 0.05$ corrected.

6.3.6 *Connectivity analyses*

In the psychophysiological interaction analysis, the diazepam group showed lower left amygdala–precuneous cortex connectivity than the placebo group during the maintain and reappraise blocks. The diazepam group also showed lower left and right amygdala–pre- and post-central gyrus connectivity than the placebo group during reappraise blocks (see Figure 6.11 and Table 6.3).

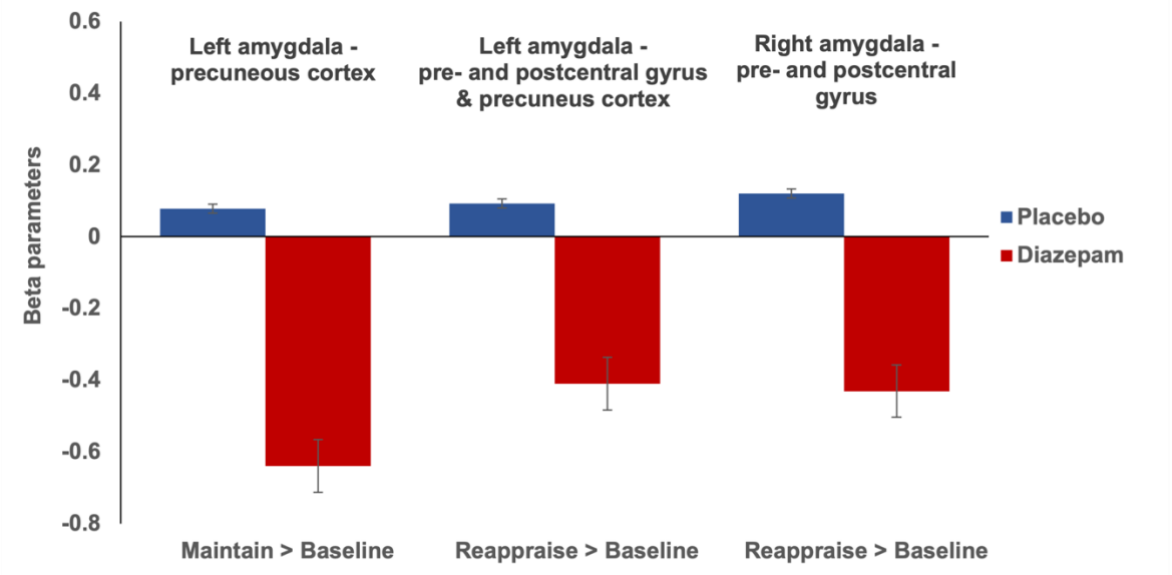

Figure 6.11 – *Parameter estimates extracted from the PPI analysis of the left and right amygdala*

Parameter estimates extracted from the significant psychophysiological interaction (PPI) results depicting decreased coupling between the left amygdala and the precuneous cortex during maintain and reappraise blocks, and between the left and right amygdala and the pre- and postcentral gyrus during reappraise blocks, in the diazepam group (red) compared to the placebo group (blue). Results are thresholded at $Z > 3.1$ and $p < 0.05$ corrected. Error bars represent ± SEM.

Table 6.3 – Psychophysiological interactions (PPI) with the amygdala

	Brain area	Side	Cluster size (voxels)	MNI max (x, y, z)	*z*-score	*p*-value
Group differences in left amygdala coupling (placebo>diazepam)						
maintain>baseline	Precuneous cortex, lateral occipital cortex, & cuneal cortex	R	76	12, -72, 44	4.02	.035
reappraise>baseline	Pre- and postcentral gyrus	L	204	-6, -34, 66	4.13	<.001
	Precuneous cortex & postcentral gyrus	L	78	-14, -46, 52	3.71	.025
mean	Pre- and postcentral gyrus	L	431	-36, -26, 60	4.37	<.001
	Cuneal cortex & precuneous cortex	R	201	10, -74, 30	4.27	<.001
	Precentral gyrus	L	170	-4, -26, 56	3.80	<.001
	Lateral occipital cortex	R	156	30, -70, 34	4.20	<.001
	Precuneous cortex & supracalcarine cortex	R	71	20, -56, 16	3.64	.043
Group differences in right amygdala coupling (placebo>diazepam)						
reappraise>baseline	Pre- and postcentral gyrus	L	349	-18, -30, 68	4.64	<.001

Furthermore, the diazepam group showed lower right insular cortex–precuneous cortex connectivity than the placebo group during both the maintain and reappraise blocks (see Figure 6.12 and Table 6.4). There were however no significant interactions with the left insular cortex.

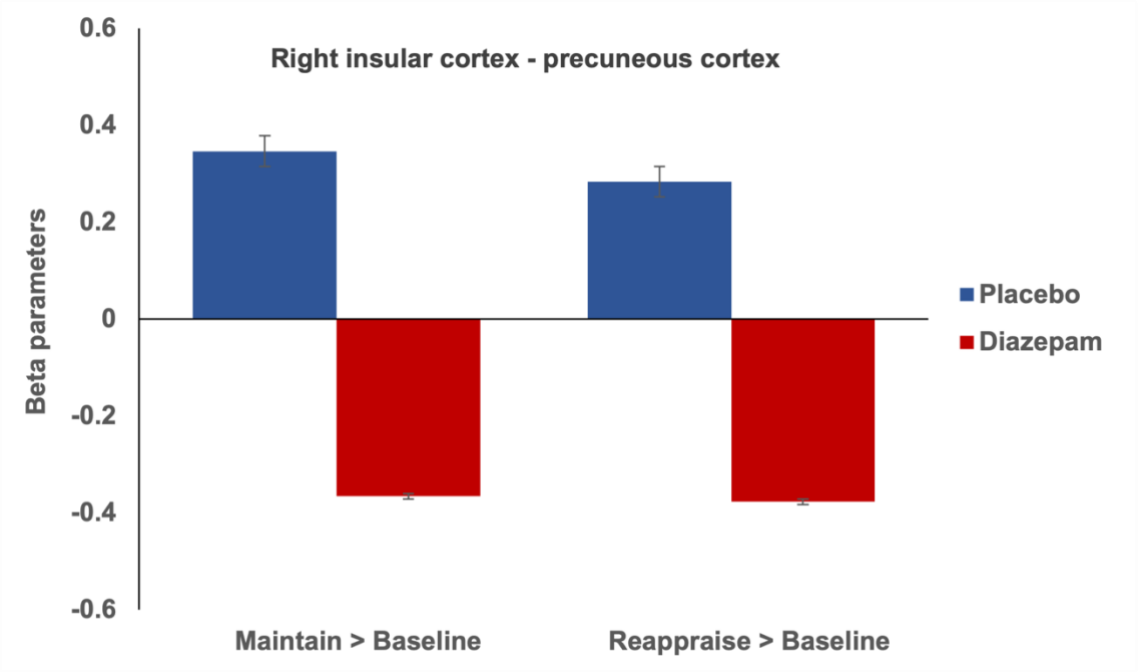

Figure 6.12 – *Parameter estimates extracted from the PPI analysis of the right insular cortex*

Parameter estimates extracted from the significant psychophysiological interaction (PPI) results depicting decreased coupling between the right insular cortex and the precuneous cortex during both the maintain and reappraise blocks in the diazepam group (red) compared to the placebo group (blue). Results are thresholded at $Z > 3.1$ and $p < 0.05$ corrected. Error bars represent ± SEM.

Table 6.4 – Psychophysiological interactions (PPI) with the insular cortex

	Brain area	Side	Cluster size (voxels)	MNI max (x, y, z)	*z*-score	*p*-value
Group differences in right insular cortex coupling (placebo>diazepam)						
maintain>baseline	Precuneous cortex	R	652	2, -60, 56	4.48	<.001
	Superior parietal lobule & postcentral gyrus	L	80	-32, -40, 66	3.97	.017
	Lateral occipital cortex, superior parietal lobule, & angular gyrus	L	71	-34, -60, 48	4.41	.031
reappraise>baseline	Precuneous cortex	R	335	4, -48, 50	4.31	<.001
	Precuneous cortex	L	72	-8, -62, 46	4.15	.028
mean	Precuneous cortex	L	2112	-8, -62, 46	4.69	<.001
	Inferior temporal & middle temporal gyrus	R	87	60, -46, -14	3.78	.010
	Supramarginal gyrus & angular gyrus	L	73	-48, -48, 42	3.86	.025
	Lateral occipital cortex & angular gyrus	L	68	-42, -60, 46	4.09	.035

6.4 Discussion

This study sought to investigate whether diazepam modulates activity within the limbic and prefrontal areas in relation to adaptive emotion regulation. In light of prior research on acute benzodiazepine administration (Del-Ben et al, 2012; Paulus et al, 2005) and $GABA_A$ receptor α_2 and α_3 subunits being located in high concentrations in the amygdala and frontal regions (D'Hulst et al, 2009), it was predicted that a 7-day treatment of diazepam would attenuate activation in the amygdala and insular cortex and possibly also modulate activity within the prefrontal areas in response to cognitive reappraisal. Contrary to predictions, there was no evidence for an effect of a 7-day diazepam treatment on amygdala or insular cortex activity during cognitive reappraisal. A further investigation however revealed that the diazepam group did show lower left and right amygdala–pre- and post-central gyrus connectivity during cognitive reappraisal. The diazepam group also showed lower left amygdala–precuneous cortex connectivity and lower right insular cortex–precuneous cortex connectivity when viewing aversive pictures in general. Moreover, the diazepam group showed a downregulation in the right vlPFC during cognitive reappraisal, compared to passively viewing the pictures, without any subjective changes in mood and state anxiety. The diazepam group also experienced more drowsiness, more clumsiness, and more muscle weakness throughout the study week than the placebo group. The vlPFC downregulation was however not affected by adding drowsiness as a covariate, nor by adding GM maps, which suggests that the analysis was not confounded by differences in drowsiness or brain structure between the groups.

There were no group differences in everyday emotion regulation strategies (ERQ) and negative affect ratings during the task. Both groups had lower ratings

after reappraisal compared to passively viewing the pictures, which suggests that cognitive reappraisal techniques were applied successfully. Both groups also used reappraisal strategies more often than suppression of emotional expression in their everyday lives, which suggests they were comparable in terms of everyday use of reappraisal techniques and thus any differences observed should be due to the intervention. Furthermore, the aversive pictures increased activity within visual, frontal, and limbic areas, and an increase in activity in frontal areas was observed when cognitive strategies of reappraisal were implemented in both groups, which is consistent with previous research (Phan et al, 2005; Reinecke et al, 2015) and confirms that participants were successful in completing the task.

Although the short-term treatment of diazepam did not seem to modulate activity within the amygdala and insular cortex during reappraisal, it did affect functional coupling during it. A lower left and right amygdala–pre- and post-central gyrus connectivity was observed in the diazepam group compared to the placebo group during cognitive reappraisal. The pre- and postcentral gyrus are motor and somatosensory regions that have also been shown to be reliably engaged during emotional processing (Saarimäki et al, 2016; Nummenmaa, Hirvonen, Parkkola, & Hietanen, 2008; De Gelder, Snyder, Greve, Gerard, & Hadjikhani, 2004; Pichon, de Gelder, & Grezes, 2008; Kropf, Syan, Minuzzi, & Frey, 2018). Furthermore, it has been established that there is clear connectivity between the amygdala and the pre- and postcentral gyrus (Rizzo et al, 2018; Toschi, Duggento, & Passamonti, 2017; Grezes, Valabregue, Gholipour, & Chevallier, 2014). The pre- and postcentral gyrus may thus potentially be receiving inputs from the amygdala during affective appraisal to facilitate sensory and motor reactions to affective signals. Furthermore, a meta-analysis of functional neuroimaging studies assessing emotion regulation in

mood and anxiety disorders concluded that patients consistently presented higher functional activations in the pre- and postcentral gyrus during cognitive reappraisal compared to healthy controls, which according to the meta-analysis might contribute to amplified physiological and motor responses, and increased physiological feedback in anxious individuals (Picó-Pérez, Radua, Steward, Menchón, & Soriano-Mas, 2017). Diazepam might thus potentially be exerting its anxiolytic effect by lowering the connectivity between the amygdala and the pre- and postcentral gyrus in anxious individuals during adaptive emotion regulation, which in turn possibly helps reduce sensory and motor reactions to aversive signals during it.

A lower left amygdala–precuneous cortex connectivity was also observed in the diazepam group compared to the placebo group, as well as lower right insular cortex–precuneous cortex connectivity, when they viewed the aversive pictures in general. The precuneous cortex has been implicated in both the mental representation of the self and attribution of emotions to the self, which involves reflection upon one's own feelings (Ochsner et al, 2004; Cavanna & Trimble, 2006), while the amygdala and insular cortex are known to be involved in affective appraisal (Silvers et al, 2014). The amygdala and insular cortex may thus potentially be receiving input from the precuneous in regard to emotional self-attribution during affective appraisal. It might be hypothesized that less connectivity between these areas might lead people to relate aversive emotional material less to their own personal feelings. An increased left amygdala–precuneous cortex connectivity has been observed in generalized anxiety disorder in relation to emotionally unpleasant pictures (Strawn et al, 2012), and subjects with anxiety have also been shown to have higher connectivity between the left amygdala and the precuneous during resting state fMRI compared to healthy controls (Toazza et al, 2016). Diazepam

might thus potentially be exerting its anxiolytic effect by lowering the connectivity between the limbic system and the precuneous cortex, thus possibly helping anxious people take aversive stimuli less personally. A future study could test this hypothesis using a task involving judgements of how participants feel about emotional photos (pleasant, unpleasant, or neutral) vs. where they were taken (inside, outside, unsure), similar to Ochsner et al. (2004), to further investigate whether diazepam does affect emotional self-attribution during affective appraisal.

Finally, a downregulation in the right vlPFC was observed in the diazepam group when cognitive strategies of reappraisal were implemented compared to the placebo group. Hypo-activation in prefrontal areas has generally been observed in anxiety disorders during emotion regulation (Ball et al, 2013) and hyper-activation in deep limbic structures during emotional processing (Etkin & Wager, 2007). More recent neurobiological frameworks have however suggested that some prefrontal areas, such as the vlPFC, may also be hyperactive during emotional processing (Moon et al, 2015; Reinecke et al, 2015), which possibly reflects an increased utilisation of dysfunctional emotion regulation strategies like cognitive avoidance (Hofmann et al, 2012; Reinecke et al, 2015). These dysfunctional strategies are thought to play a big part in maintaining anxiety disorders and are thus targeted during CBT. It has also been shown that amygdala attenuation during threat processing in anxious subjects is associated with decreased activity in the right vlPFC after CBT (Månsson et al, 2013). Diazepam could thus potentially be exerting its anxiolytic effect by downregulating hyperactivity within this area in relation to the characteristic emotion dysregulation seen in anxiety disorders. Future studies should focus on a clinical sample to further investigate this.

In summary, a 7-day diazepam treatment did not have an effect on amygdala or insular cortex activity during reappraisal. It did however affect the functional coupling of these regions with other regions that have been implicated in emotional processing. A lower connectivity between the left and right amygdala and the pre- and post-central gyrus was observed in the diazepam group, compared to the placebo group, during reappraisal. This lower connectivity might reflect a reduction in sensory and motor reactions to aversive signals during emotion regulation, which could be one of the mechanisms through which diazepam may exert its anxiolytic effects. Furthermore, a lower connectivity between limbic areas (left amygdala and right insular cortex) and the precuneous cortex was observed in the diazepam group while viewing aversive pictures in general. It could be hypothesized that this lower connectivity might reflect emotional material being less related to one's own personal feelings, which in turn reduces its emotional affect. Diazepam might thus potentially be exerting its anxiolytic effect by lowering this connectivity. Finally, a downregulation in the right vlPFC was observed in the diazepam group during reappraisal. Diazepam could thus potentially be exerting its anxiolytic effect by downregulating hyperactivity within this area in relation to the characteristic emotion dysregulation seen in anxiety disorders. Taken together, these results provide valuable insights into potential mechanisms through which diazepam may exert its anxiolytic effects.

Chapter 7: The effects of diazepam on emotional processing during a face classification task

7.1 Introduction

As outlined in Chapter 1, the majority of people with anxiety disorders experiences serious impairments in their daily lives by their symptoms. These symptoms often include increased attentional biases (Campbell-Sills et al, 2014) and heightened processing of threat-relevant stimuli (Mogg & Bradley, 2002; MacLeod, Mathews, & Tata, 1986), which are normalised after psychological and pharmacological treatments (Mathews, Mogg, Kentish, & Eysenck, 1995; Mogg, Baldwin, Brodrick, & Bradley, 2004). Decreased threat processing has also been observed in healthy participants after administration of anxiolytic pharmacological agents. A short-term (7-day) administration of the SSRI citalopram for example made participants more likely to misclassify fearful, angry, and disgusted facial expressions as happy, which suggests a positive bias in facial expression recognition, and increased the relative recall of positive compared to negative emotional material (Harmer, Shelley, Cowen, & Goodwin, 2004). Additionally, these threat-related biases have been associated with certain patterns of neural activity, particularly increased amygdala activation (Monk et al, 2008; Blair et al, 2008), and it has been shown that SSRIs cause a reduction in amygdala activity amongst anxious patients (Faria et al, 2012). Similar amygdala activity changes have also been observed in relation to threat in healthy participants receiving an acute dose (Murphy et al, 2009) and after repeated administration for 7 days (Harmer et al, 2006). A hyperactive amygdala response might thus contribute to these biases towards threatening information and anxiolytic pharmacological agents may be exerting their effects by normalising these neural responses. Given that these effects happened in the absence of any

pathophysiology and without any subjective changes in mood and anxiety, it strongly suggests that modifying the processing of emotionally relevant material may play an important role in SSRIs' anxiolytic effects, as opposed to being just a by-product of anxiety relief. However, as SSRIs are effective for both anxiety and depression, it can be difficult to distinguish between their anxiolytic and antidepressant effects. Benzodiazepines on the other hand are not efficacious in treating depression, so any effects they exert on cognition and neural activation may be more directly related to their anxiolytic action. A better understanding of the mechanisms behind benzodiazepines anxiolytic effects could potentially help researchers tease apart the anxiolytic and antidepressant effects of SSRIs and help inform the development of even better anxiolytics.

A number of studies have investigated acute effects of the long-acting benzodiazepine diazepam on emotional processing in healthy volunteers. A single dose of diazepam has been shown to reduce accuracy in detecting threat-related facial expressions (Blair & Curran, 1999; Zangara et al, 2002; Del-Ben et al, 2012), and to reduce startle responses and increase attentional vigilance to masked happy faces compared to threatening or ambiguous ones at a non-sedating dose (Murphy et al, 2008). Moreover, a non-sedating dose of diazepam has been shown to attenuate activation in the amygdala, insula, and orbitofrontal cortex in response to fearful faces, while also increasing activation in the anterior cingulate cortex (ACC), which could possibly be due to its top-down regulation of these structures (Del-Ben et al, 2012). Another study showed that a single dose of the benzodiazepine lorazepam attenuated activation in the amygdala and insula in response to emotional stimuli in a dose-dependent manner in healthy participants without any subjective changes in anxiety (Paulus et al, 2005). Furthermore, a repeated

administration of diazepam for 7 days has been shown to reduce vigilant–avoidant patterns of emotional attention (Pringle et al, 2016), and to increase functional connectivity in brain areas of emotional processing independent of task selection and clinical status (Pflanz et al, 2015). Benzodiazepines do thus appear to affect emotional processing in healthy participants without any subjective changes in anxiety, similar to SSRIs (Murphy et al, 2009; Harmer et al, 2006). Furthermore, $GABA_A$ receptor α_2 and α_3 subunits, which are thought to be responsible for benzodiazepines' anxiolytic effects (McKernan et al, 2000), are located in high concentrations in the amygdala and frontal regions (D'Hulst et al, 2009). Based on these findings, benzodiazepines' anxiolytic effects may be due to them modulating activity within the limbic and prefrontal areas related to emotional processing. Most of the current research has focused on their acute effects; it may however be more clinically relevant to investigate if the effects are the same after short-term administration, as benzodiazepines are usually prescribed over a period of two to four weeks and there may be differences between acute and short-term treatments, as has been shown for SSRIs (Murphy et al, 2009). Furthermore, a short-term administration may elicit more reliable changes on emotional processing as plasma drug levels may be more stable.

This study therefore sought to replicate and extend previous research on threat-related neural responses to anxiolytic medications. The objective was to investigate whether diazepam modulates activity within the limbic and prefrontal areas related to the processing of emotional facial expressions. In light of prior research on acute benzodiazepine administration (Del-Ben et al, 2012; Paulus et al, 2005) and $GABA_A$ receptor α_2 and α_3 subunits being located in high concentrations in the amygdala and frontal regions (D'Hulst et al, 2009), it was

predicted that a 7-day treatment of diazepam would attenuate activation in the amygdala and insular cortex in response to fearful facial expressions and possibly also modulate activity within the prefrontal areas in relation to emotional facial expressions.

7.2 Methods

7.2.1 *Participants & procedure*

Information about the participants and the procedure is presented in Chapter 5. In brief, this was a double-blind, randomised, placebo-controlled study done in 2012 that investigated the effects of a 7-day treatment of diazepam (15mg) on the neural correlates of emotional processing. 34 healthy volunteers were recruited, but 2 participants had to be excluded from the analysis of this task due to response capture and anatomical problems. The analysis thus included 32 participants, with 14 in the placebo group and 18 in the diazepam group.

7.2.2 *Experimental task*

In the face classification task, participants were asked to classify faces as either male or female as quickly and as accurately as possible via a button press during an fMRI scan. The stimuli were photographs from the NimStim database (Tottenham et al, 2009) of faces with fearful, happy, and sad facial expressions (see Figure 7.1). E-prime (version 2.0; Psychology Software Tools Inc., Pittsburgh, PA, USA) was used to run the task and record responses. Each trial began with the presentation of a fixation cross for 2900ms, followed by a presentation of a face for 100ms. Each block consisted of 10 trials of the same emotional valence and lasted 30s. A fixation cross was presented between each block for 30s. Fearful facial expressions were presented in the first block, happy in the second, and sad in the third; this sequence was then repeated 4 times, which resulted in 12 blocks in total.

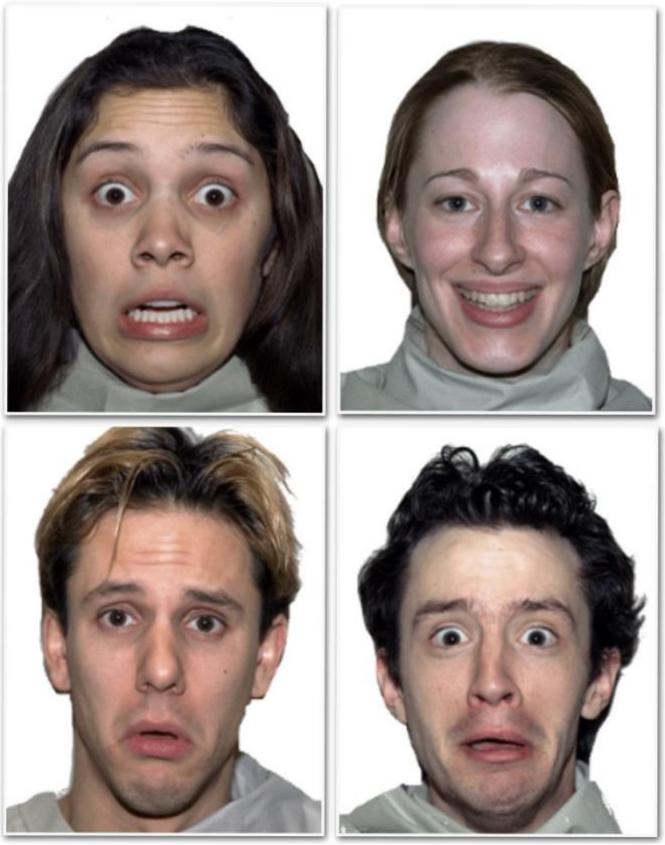

Figure 7.1 – *Examples of pictures used during the face classification task*

7.2.3 *Behavioural analysis*

The behavioural analysis was carried out using IBM SPSS Statistics for Windows, version 27 (IBM Corp., Armonk, N.Y., USA). To determine if there were any significant effects of treatment group on accuracy or reaction times, a 3×2 mixed ANOVA was conducted. Treatment condition (diazepam vs. placebo) was entered as the between-group factor and valence (fearful, happy, sad) as the within-subjects factor. For information about the analysis of the subjective ratings please see section 5.2.4 of Chapter 5.

7.2.4 *Image acquisition*

A 3T Siemens Magnetom Tim-Trio whole-body scanner system (Siemens, Erlangen, Germany) with a 32-channel head-coil, located at the OCMR, was used to acquire T1-weighted structural images and T2-weighted transverse echo planar images (EPI). Functional imaging consisted of 45 T2-weighted EPI slices (TR = 3000ms, TE = 30ms, flip angle = 87°, matrix size = 64 × 64, voxel dimension = 3mm isotropic, slice thickness = 3mm, FOV = 192 × 192mm^2, 251 volumes, echo spacing = 0.49ms). Gradient-echo fieldmap images were acquired for distortion correction (44 slices, slice thickness = 3.5mm, voxel dimension = 3.5mm isotropic, FOV = 192 × 192mm^2, TR = 488ms, TE1 = 5.19ms, TE2 = 7.65ms, flip angle = 60°), and T1-weighted structural images were acquired using an MP-RAGE sequence for anatomical alignment (192 slices, slice thickness = 1mm, TR = 2040ms, TE = 4.7ms, flip angle = 8°, voxel dimension = 1mm isotropic, FOV = 192 × 192mm^2).

7.2.5 *Image analysis*

MRI data was analysed using FSL (FMRIB Software Library v6.6) tools (https://fsl.fmrib.ox.ac.uk/fsl). Structural anatomical scans were brain extracted using FSL's Brain Extraction Tool (BET; Smith, 2002). To denoise (removal of noise due to movement, scanner, or cardiovascular artifacts) the single subject functional data of this task, it was first pre-processed using Multivariate Exploratory Linear Decomposition into Independent Components (MELODIC version 3.15, part of FSL). Pre-processing consisted of brain extraction using BET (Smith, 2002), motion correction using FMRIB's Linear Image Registration Tool (MCFLIRT; Jenkinson, Bannister, Brady, & Smith, 2002), distortion correction using gradient-echo fieldmaps, spatial smoothing using a Gaussian kernel of FWHM 5mm, grand-mean

intensity normalisation of the entire 4D dataset by a single multiplicative factor, registration of the functional space template to the anatomical space and the Montreal Neurological Institute (MNI) 152 space using the FMRIB's Linear Image Registration Tool (FLIRT; Jenkinson & Smith, 2001; Jenkinson, Bannister, Brady, & Smith, 2002), high-pass temporal filtering equivalent to 90s (Gaussian-weighted least-squares straight line fitting, with sigma = 45.0s), masking of non-brain voxels, voxel-wise de-meaning of the data, and normalisation of the voxel-wise variance.

A probabilistic independent component analysis (ICA; Beckmann and Smith, 2004) was then done using FSL's MELODIC, which involved splitting the pre-processed data into independent components. First, the pre-processed data was whitened and projected into a subject dependent dimensional subspace using probabilistic principal component analysis (PCA), where the number of dimensions was estimated using the Laplace approximation to the Bayesian evidence of the model order (Minka, 2000; Beckmann and Smith, 2004). The whitened observations were decomposed into sets of vectors which describe signal variation across the temporal domain (time-courses) and across the spatial domain (maps) by optimising for non-Gaussian spatial source distributions using a fixed-point iteration technique (Hyvärinen, 1999). Estimated component maps were divided by the standard deviation of the residual noise and thresholded by fitting a mixture model to the histogram of intensity values (Beckmann and Smith, 2004). FMRIB's ICA-based Xnoiseifier (FIX; Salimi-Khorshidi et al, 2014; Griffanti et al, 2014) was then manually trained to denoise the functional data for this task by removing movement, scanner, or cardiovascular artifacts. This was done by manually creating a training dataset for FIX from five subjects in each group (N=10 in total). The manual labelling of components was done by me and another independent researcher (Dr Marieke

Martens) and then compared. A conservative approach was used in that if not agreed the component was included.

Time-series statistical analysis was then carried out on the denoised data using FMRIB's Improved Linear Model (FILM) with local autocorrelation correction (Woolrich, Ripley, Brady, & Smith, 2001), and a custom 3 column format convolved with a gamma hemodynamic response function, and its temporal derivative, was used to model the data. The main contrasts of interest were fear vs. happy, fear vs. sad, happy vs. sad, but estimates of happy vs. fear, sad vs. fear, sad vs. happy, fear vs. baseline, happy vs. baseline, and sad vs. baseline were also obtained for reference, where fixation blocks were the baseline reference. The group level analysis was carried out using FMRIB's Local Analysis of Mixed Effects (FLAME; Woolrich, Behrens, Beckmann, Jenkinson, & Smith, 2004). The general linear model (GLM) included the 2 groups (diazepam & placebo), where group averages, average across both groups, and differences between groups for each contrast of interest were tested. Significant activations were determined by cluster-based thresholding of $Z > 3.1$ and a family-wise error-corrected cluster significance threshold of $p < 0.05$ (Worsley, 2001).

Grey matter (GM) images of each participant were extracted using FMRIB's Automated Segmentation tool (FAST; Zhang et al, 2001). These were then registered to standard space, smoothed to match the intrinsic smoothness of the fMRI data (2.63mm), voxel-wise demeaned across all subjects, and added to the general linear model to remove any potential structural differences explaining the BOLD contrast differences.

In addition to the whole-brain analysis, a small volume correction (SVC) was preformed using predefined anatomical regions of interest (ROIs), which included

the left and right amygdala and the left and right insular cortex. These regions were defined using the Harvard-Oxford Cortical and Subcortical Structural Atlases and a 50% threshold.

7.3 Results

7.3.1 *Sociodemographic, clinical, and personality characteristics*

Sociodemographic, clinical, and personality characteristics of participants included in the face classification task analysis are presented in Table 7.1. The analysis of this task consisted of 32 participants, as one participant did not complete the task successfully and another had anatomical anomalies. Both of the excluded participants were in the placebo group, so the analysis included 14 participants in the placebo group and 18 in the diazepam group. The two groups were well matched on sociodemographic, clinical, and personality parameters.

Table 7.1 – Sociodemographic, clinical, and personality characteristics

	Placebo (n=14)	*Diazepam (n=18)*
Gender		
Male	n=8	n=8
Female	n=6	n=10
Age	22.21 (1.14)	22.85 (1.03)
Verbal IQ (NART)	113.25 (1.50)	115.50 (1.68)
Beck Depression Inventory	1.64 (0.53)	1.54 (0.48)
Spielberger State and Trait Anxiety Inventories		
State-Anxiety	25.50 (1.05)	26.46 (1.62)
Trait-Anxiety	28.14 (1.42)	28.08 (1.77)
Eysenck Personality Questionnaire		
Neuroticism	5.00 (0.71)	5.54 (0.91)
Psychoticism	3.00 (0.70)	2.69 (0.61)
Extraversion	17.36 (0.83)	15.77 (1.02)
Lie/Social Desirability	7.00 (1.18)	7.08 (1.09)

Values are means (SEM)

7.3.2 *Subjective rating results*

Similar to what was found in Chapter 6, diazepam did not have a significant effect on subjective mood or state anxiety during the intervention week (BFS, PANAS, STAI-S, BDI; all *ps* > .072). Furthermore, there were no significant main effects of treatment group on the drowsiness, lightheadedness, confusion, forgetfulness, agitation, and muscle weakness side effect ratings (all *ps* > .064). However, similar to what was found in Chapter 6, there was a significant main effect of group on the lack of coordination ratings ($F(1, 30) = 9.333$, $p = .005$, $\eta_p^2 = .237$), where participants in the diazepam group were significantly less coordinated throughout the study week compared to the placebo group. Similarly, there was no significant main effect of treatment group on any of the VAS (all *ps* > .204), except on the "well-coordinated – clumsy" scale, where participants in the diazepam group were significantly clumsier throughout the intervention week compared to the placebo group ($F(1, 30) = 6.558$, $p = .016$, $\eta_p^2 = .179$).

7.3.3 *Behavioural performance*

Overall accuracy in identifying the gender was high (>90%), thus confirming that participants were successful in completing the task. Furthermore, there were no group differences in accuracy or reaction times (all *p*s > .770).

7.3.4 *Main effect of task*

Significant activity was observed for each facial expression across the two combined groups in clusters that included the bilateral temporal occipital fusiform cortex extending to the paracingulate gyrus, superior and inferior frontal gyrus,

middle frontal gyrus, lateral occipital cortex, cerebellum, putamen, frontal pole, precentral and postcentral gyrus, central opercular cortex, frontal operculum cortex, angular gyrus, superior parietal lobule, supramarginal gyrus, juxtapositional lobule cortex, and insular cortex (see Figure 7.2 and Table 7.2). These areas are associated with attention and memory, motor control and movement, and visual and emotional processing, and are consistent with previous research (Capitão et al, 2019; Kesler et al, 2001). There was however no activation observed for any of the facial expression across the two combined groups in the amygdala.

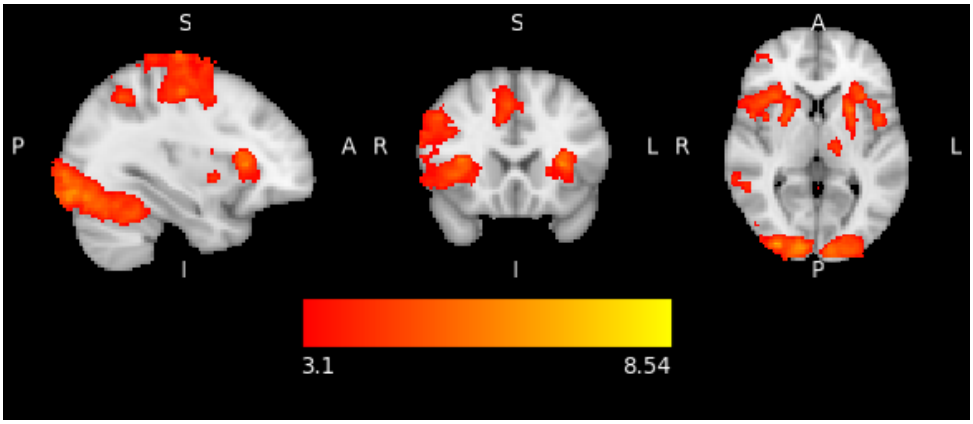

Figure 7.2 – *Whole-brain activation in response to all facial expressions across groups*

Sagittal, coronal, and axial images depicting neural activation in response all facial expressions (fearful, happy, sad) across groups (diazepam and placebo) during the face classification task. Images are thresholded at $Z > 3.1$ and $p < 0.05$ corrected.

Table 7.2 – Main effect of the face classification task across groups ($Z > 3.1$, $p < 0.05$)

Cluster	Brain area	Side	Cluster size (voxels)	MNI max (x, y, z)	*z*-score	*p*-value
Task activation (fear>baseline)						
1	Temporal occipital fusiform cortex extending to bilateral paracingulate gyrus, superior & inferior frontal gyrus, & middle frontal gyrus	R	10954	40, -48, -20	7.43	<.001
2	Juxtapositional lobule cortex	R	6038	0, -2, 58	6.45	<.001
3	Central opercular cortex	L	1629	-44, -2, 14	5.86	<.001
4	Frontal operculum cortex & insular cortex	R	592	34, 20, 10	4.75	<.001
5	Precentral gyrus, & middle & inferior frontal gyrus	R	474	52, 12, 34	4.61	<.001
6	Cerebellum	R	411	18, -60, -52	6.21	<.001
7	Supramarginal gyrus, middle temporal gyrus, & angular gyrus	R	381	58, -42, 10	4.78	<.001
8	Precentral gyrus	R	349	42, -6, 66	4.69	<.001
9	Putamen	R	256	22, 12, 0	4.20	<.001
10	Lateral occipital cortex, angular gyrus, & superior parietal lobule	R	251	26, 33, 63	4.15	<.001
11	Parietal operculum cortex & central opercular cortex	L	210	-48, -24, 20	4.94	<.001
12	Precentral & middle frontal gyrus	L	192	-56, 10, 38	4.68	<.001
13	Frontal pole	R	132	44, 48, -4	4.21	.008
Task activation (happy>baseline)						
1	Temporal occipital fusiform cortex extending to bilateral paracingulate gyrus, superior & inferior frontal gyrus, & middle frontal gyrus	R	10634	38, -56, -20	7.97	<.001
2	Precentral gyrus	L	3898	-46, -18, 68	5.71	<.001
3	Frontal operculum cortex & insular cortex	R	2557	34, 20, 8	5.55	<.001
4	Paracingulate gyrus, juxtapositional lobule cortex, & superior frontal gyrus	R	1918	2, 8, 52	6.79	<.001
5	Insular cortex & frontal operculum cortex	L	938	-30, 20, 8	5.27	<.001
6	Angular gyrus, superior parietal lobule, & lateral occipital cortex	R	784	38, -54, 50	5.22	<.001
7	Precentral gyrus & middle frontal gyrus	L	296	-56, 10, 38	5.29	<.001
8	Cerebellum	R	285	20, -58, -50	5.64	<.001
9	Frontal pole	R	279	32, 50, -12	4.05	<.001
10	Central opercular cortex	L	267	-42, -4, 14	5.65	<.001
11	Central opercular cortex & postcentral gyrus	L	230	-58, -18, 20	5.66	<.001

Task activation (sad>baseline)						
1	Temporal occipital fusiform cortex extending to bilateral paracingulate gyrus, superior & inferior frontal gyrus, & middle frontal gyrus	R	9948	38, -46, -16	6.60	<.001
2	Precentral gyrus	L	2428	-34, -20, 74	5.24	<.001
3	Inferior frontal gyrus, precentral gyrus, & middle frontal gyrus	R	1767	50, 14, 24	5.17	<.001
4	Angular gyrus, superior parietal lobule, & lateral occipital cortex	R	1272	36, -54, 42	5.53	<.001
5	Juxtapositional lobule cortex	L	1081	0, 0, 56	6.64	<.001
6	Frontal pole	R	498	44, 46, -4	5.14	<.001
7	Insular cortex & frontal operculum cortex	R	415	34, 20, 4	4.99	<.001
8	Putamen	L	184	-24, 2, -8	4.34	.002
9	Central opercular cortex	L	146	-42, -4, 16	4.40	.009
10	Superior parietal lobule, supramarginal gyrus, & lateral occipital cortex	L	137	-30, -54, 48	4.53	.012
11	Frontal operculum cortex & insular cortex	L	137	-32, 22, 10	4.82	.012
12	Cerebellum	R	109	18, -64, -46	4.57	.036
Task activation (mean across facial expressions)						
1	Temporal occipital fusiform cortex extending to bilateral paracingulate gyrus, superior & inferior frontal gyrus, & middle frontal gyrus	R	13676	40, -44, -20	8.45	<.001
2	Inferior frontal gyrus, precentral gyrus, & middle frontal gyrus	R	4408	50, 12, 24	5.89	<.001
3	Precentral gyrus & postcentral gyrus	L	4261	-34, -20, 54	5.90	<.001
4	Juxtapositional lobule cortex	L	2177	0, 0, 56	6.88	<.001
5	Insular cortex & frontal operculum cortex	L	1956	-30, 22, 8	6.14	<.001
6	Lateral occipital cortex, angular gyrus, & superior parietal lobule	R	1149	40, -58, 50	5.43	<.001
7	Middle frontal gyrus & precentral gyrus	R	504	36, -2, 58	4.63	<.001
8	Precentral gyrus & middle frontal gyrus	L	256	-56, 10, 38	5.15	<.001
9	Central opercular cortex & postcentral gyrus	L	206	-58, -18, 20	4.91	<.001

7.3.5 *Effect of treatment*

A whole-brain analysis ($Z > 3.1$ thresholded, $p < 0.05$ corrected) revealed increased BOLD activation in the diazepam group relative to placebo group in response to happy facial expressions compared to baseline in the left ventrolateral prefrontal cortex (vlPFC; x = −54, y = 30, z = 8; $Z = 4.20$; voxel cluster size: 117; see Figure 7.3). This difference was not affected by adding GM maps as a covariate in the model. No group differences were seen in any of the other contrasts. Furthermore, there were no group differences in the amygdala and the insular cortex in the small volume correction analysis.

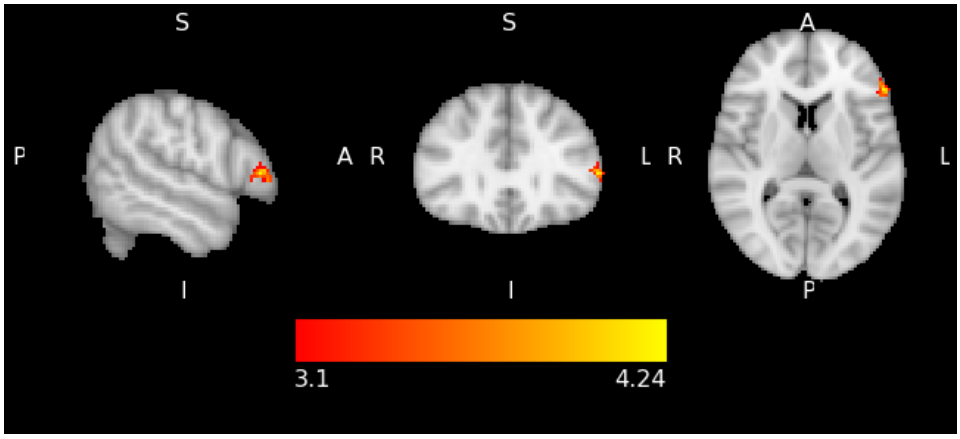

Figure 7.3 – *Whole-brain activation in response to happy facial expressions compared to baseline*

Sagittal, coronal, and axial images depicting significantly increased activation in the diazepam group compared to the placebo group for the happy vs. baseline contrast in the left vlPFC (peak voxels: x = −54, y = 30, z = 8; $Z = 4.20$; voxel cluster size: 117). Images are thresholded at $Z > 3.1$ and $p < 0.05$ corrected.

7.4 Discussion

This study's objective was to replicate and extend previous research on threat-related neural responses to anxiolytic medications by investigating whether diazepam modulates activity within the limbic and prefrontal areas in relation to the processing of emotional facial expressions. In light of prior research on acute benzodiazepine administration (Del-Ben et al, 2012; Paulus et al, 2005) and $GABA_A$ receptor α_2 and α_3 subunits being located in high concentrations in the amygdala and frontal regions (D'Hulst et al, 2009), it was predicted that a 7-day treatment of diazepam would attenuate activation in the amygdala and insular cortex in response to fearful facial expressions, and possibly also modulate activity within the prefrontal areas in relation to emotional facial expressions in healthy participants. Contrary to predictions, there was no evidence for an effect of a 7-day diazepam treatment on neural responses in the amygdala or insular cortex to threat-related facial expressions. The diazepam group did on the other hand show an increase in activation in the left vlPFC during the gender classification of happy faces compared to baseline, without any subjective changes in mood and state anxiety, although they did experience a lack of coordination throughout the study week. The increased activation was not affected by adding GM maps as a covariate, suggesting that the analysis was not confounded by structural differences between the two groups. Furthermore, there were no group differences in the accuracy of the gender classification or reaction times to any of the facial expressions.

The lack of an effect of treatment condition on amygdala activation does not come as a surprise given that there was no activation observed for any of the facial expression across the two combined groups in the amygdala. This may have been motion-related, as participants in both groups moved significantly in the scanner

during this task. A single-subject independent component analysis was done to try to mitigate this problem, but the activity in the amygdala may not have been salvageable as the amygdala is a relatively small structure. Typical patterns of activation were however observed in other regions involved in vision and emotional face perception, such as the temporal occipital fusiform cortex, lateral occipital cortex, paracingulate gyrus, superior and inferior frontal gyrus, and middle frontal gyrus, cerebellum, putamen, frontal pole, precentral and postcentral gyrus, and insular cortex (Capitão et al., 2019; Kesler et al, 2001).

The lack of an effect of diazepam on insular cortex may possibly be due to the development of neuronal tolerance. Benzodiazepines exert their therapeutic effects by producing allosteric changes that enhance the effect of the neurotransmitter GABA at the $GABA_A$ receptors. It has however been shown that neurons receiving GABAergic input show tolerance to benzodiazepines' effects after a period of time, where for example after 3 days on a diazepam treatment, sensitivity of dorsal raphe neurons to GABA was facilitated in rats, but after 7 days the GABAergic sensitivity had returned to baseline levels (Gonsalves & Gallager, 1987). Similar effects have also been observed in other brain regions, such as the pars reticulata of the substantia nigra and hippocampus (Wilson & Gallager, 1987; Xie & Tietz, 1991). The effects of repeated benzodiazepine treatment on GABAergic transmission in the insular cortex have not been studied specifically, but it remains a possibility that similar compensatory effects could have happened in the participants of this study. The insular cortex activity changes observed in studies administering acute doses of diazepam might thus reflect an initial increase in GABA binding, which may then decrease again when tolerance develops after 7 days, thus possibly explaining why no differences were observed in this study. A future study

might want to focus on testing participants at multiple time points to investigate whether neural tolerance does develop in the limbic system, as well as to investigate whether there are regional differences in tolerance between areas, as adaptations could occur on different time scales depending on receptor subtypes and brain regions involved. Furthermore, future studies might also want to include angry facial expressions, as diazepam has also been shown to reduce participants' ability to recognise angry faces (Blair & Curran, 1999; Zangara et al, 2002).

Diazepam has been shown to increase attentional vigilance to masked happy faces and it has been suggested that such a change in attentional orienting may represent an important mechanism through which diazepam exerts its therapeutic effects on clinical anxiety (Murphy et al, 2008). Furthermore, a short-term administration of diazepam has been shown to increase accuracy in categorising positive compared to negative personality characteristic words, when using self-referential judgements, as well as shorten the time needed to classify the positive compared to the negative words (Pringle et al, 2016), which potentially reflects a positive bias. The diazepam group in this current study showed an increase in activation in the left vlPFC during the gender classification of happy faces compared to baseline. A possible reason for it not showing a significant increase compared to fearful or sad facial expressions is that positive and negative valence systems have been suggested to share a common regulatory control implemented by the vlPFC (Kim, Weisenbach, & Zald, 2019; Ochsner, Silvers, & Buhle, 2012), meaning that the vlPFC is activated by both negative and positive stimuli, so the difference in activation may be more pronounced and thus more detectable when the happy expressions are compared to baseline. Furthermore, transcranial magnetic stimulation (TMS) disruption of vlPFC activity has been shown to decrease

sensitivity to happy facial expressions in healthy participants, without any effects on reaction times (Chick, Rolle, Trivedi, Monuszko, & Etkin, 2020). Enhanced activity in the vlPFC in relation to positively valanced stimuli could thus potentially underlie the kind of increased positive attentional vigilance and positive bias observed in other diazepam studies (Murphy et al, 2008; Pringle et al, 2016). This may give an important insight into a potential mechanism through which diazepam may exert its anxiolytic effects. These effects are reminiscent of the effects of SSRIs, where a 7-day administration of the SSRI citalopram for example made participants more likely to misclassify negative facial expressions as happy, suggesting a positive bias in facial expression recognition, and increased the relative recall of positive compared to negative emotional material (Harmer et al, 2004). This raises the possibility that there may be common pathways involved, and that modulation of neural activity related to emotional processing may be an important part of benzodiazepines' and SSRIs' anxiolytic effects. It is however worth noting that diazepam has not been shown to significantly affect attentional vigilance to unmasked happy faces (Murphy et al, 2008; Pringle et al, 2016), which should be addressed in future studies. A future study might want to focus on vlPFC responses during an attentional dot probe task, with both masked and unmasked emotional faces, to further investigate whether diazepam increases attentional vigilance to positive stimuli by enhancing vlPFC activity and whether the effects are limited to stimuli presented subliminally.

In summary, there was no evidence for an effect of a 7-day diazepam treatment on neural responses in the amygdala or insular cortex to threat-related facial expressions, which was contrary to predictions. The lack of an effect on amygdala activation may have been due to excessive motion during the task, as no amygdala activation was observed for any of the facial expression across the two

combined groups, so no conclusions can be drawn regarding the amygdala from this task. However, the lack of an effect of diazepam on insular cortex activation may possibly be due to the development of neuronal tolerance, as it has been shown that neurons receiving GABAergic input can show tolerance to benzodiazepines' effects after a period of time (Gonsalves & Gallager, 1987; Wilson & Gallager, 1987; Xie & Tietz, 1991). The diazepam group did on the other hand show an increase in activation in the left vlPFC during the gender classification of happy faces compared to baseline, without any subjective changes in mood and state anxiety. Diazepam may thus possibly be inducing a positive bias in the processing of emotion-related stimuli, which has also been observed in other studies (Murphy et al, 2008; Pringle et al, 2016), by enhancing activity in the vlPFC during processing of positive stimuli. This may give an important insight into a potential mechanism through which diazepam may exert its anxiolytic effects.

Chapter 8: The effects of diazepam on emotional processing during an emotional counting Stroop task

8.1 Introduction

As mentioned in Chapter 1, Silvers, Buhle, & Ochsner (2014) developed a model of the processes and neural systems that support emotion generation and regulation, which proposes that prefrontal, cingulate, and parietal control regions modulate activity in affective appraisal regions, such as the amygdala and insula, as well as occipito-temporal regions involved in semantic and perceptual representations. Furthermore, they have suggested that people with anxiety disorders may have an inability to accurately appraise threat, or an inability to reappraise threat, or even both, which has been linked to abnormal activity in frontal control regions and affective appraisal regions (Silvers et al, 2014). It has been a long-held view that fear and anxiety are linked to hyper-activation in deep limbic structures, such as the amygdala and insula (Etkin & Wager, 2007), where amygdalar hyperactivity has for example been observed during negative emotional processing in people with social anxiety disorder (Stein, Goldin, Sareen, Zorrilla, & Brown, 2002; Phan, Fitzgerald, Nathan, & Tancer, 2006), generalized anxiety disorder (Monk et al, 2008), and PTSD (Rauch et al, 2000; Shin et al, 2005). Furthermore, hypo-activation in prefrontal areas has been observed in anxiety disorders during emotion regulation (Ball, Ramsawh, Campbell-Sills, Paulus, & Stein, 2013), which involves the modification of emotional responses via the engagement of top-down control processes (Silvers et al, 2014). This hypo-activation is thought to contribute to the characteristic emotion dysregulation seen in anxiety disorders, thus possibly reflecting insufficient top-down control (Ball et al, 2013).

A key frontal control region involved in emotion modulation is the anterior cingulate cortex (ACC). The ACC has extensive connections with both limbic and prefrontal regions and is thought to be primarily involved in assessing the salience of emotional information and the regulation of emotional responses (for a review, see Bush, Luu, & Posner, 2000). Furthermore, ACC activation in healthy individuals has been shown to occur in the absence of behavioural interference, such as response time increases, which has been suggested to be due to its regulatory response (Whalen et al, 1998). As the ACC is considered a regulatory area that is activated during normal emotional processing, it has led studies to focus on whether activation within the ACC is affected in people with anxiety disorders. Studies have indeed found that anxiety is linked to hypo-activity in the ACC during emotional processing. This hypo-activity has for example been reported in social anxiety disorder (Klumpp, Post, Angstadt, Fitzgerald, & Phan, 2013a), PTSD (Offringa et al, 2013), and generalized anxiety disorder (Palm, Elliott, McKie, Deakin, & Anderson, 2011; Schlund, Verduzco, Cataldo, & Hoehn-Saric, 2012; Etkin, Prater, Hoeft, Menon, & Schatzberg, 2010).

It has been suggested that SSRIs may potentially exert their anxiolytic effects by enhancing ACC response during threat-related processing, where for example activation in this region increased in anxious youth after treatment with the SSRI sertraline, and the increase was related to greater reductions in anxiety and avoidance symptoms following treatment (Burkhouse et al, 2018). However, as mentioned in the previous two chapters, it can be difficult to distinguish between SSRIs anxiolytic and antidepressant effects. Benzodiazepines on the other hand are not efficacious in treating depression, which makes them good candidates to help tease apart the anxiolytic and antidepressant effects of SSRIs and to help

inform the development of future anxiolytic treatments. Benzodiazepines have been shown to affect the ACC and limbic regions. A non-sedating dose of the benzodiazepine diazepam was for example shown to attenuate activation in the amygdala, insula, and orbitofrontal cortex in response to fearful faces, while also increasing activation in the ACC, which was suggested to be due to its top-down regulation of these structures. Diazepam was also shown to decrease activation in the bilateral ACC in response to angry faces in the same study (Del-Ben et al, 2012). Furthermore, $GABA_A$ receptor α_2 and α_3 subunits, which are thought to be responsible for benzodiazepines' anxiolytic effects (McKernan et al, 2000), are located in high concentrations in the amygdala and frontal regions (D'Hulst et al, 2009). Based on these findings, benzodiazepines' anxiolytic effects may involve modulation in the processing of threat within frontal control regions, such as the ACC, during emotional processing.

This study therefore sought to investigate whether diazepam has an effect on activity related to emotional processing within the ACC. This was investigated using an emotional counting Stroop (ecStroop) task, which is an fMRI adaptation of the Stroop paradigm that has been shown to reliably activate the ACC (Whalen et al, 1998). Current research has focused on diazepam's acute effects, but as mentioned in previous chapters it may be more clinically relevant to investigate if the effects are the same after short-term administration. There may also be differences between acute and short-term treatments, as has been shown for SSRIs (Murphy et al, 2009). Furthermore, a short-term administration may elicit more reliable changes on emotional processing as plasma drug levels may be more stable. In light of prior research showing an effect of acute diazepam administration on ACC activation (Del-Ben et al, 2012), it was predicted that a 7-day treatment of

diazepam would modulate activation in the ACC in response to emotional words during the ecStroop task in healthy participants.

8.2 Methods

8.2.1 *Participants & procedure*

For information about the participants and the procedure please see Chapter 5. In brief, this was a double-blind, randomised, placebo-controlled study done in 2012 that investigated the effects of a 7-day treatment of diazepam (15mg) on the neural correlates of emotional processing. 34 healthy volunteers were recruited, but 6 participants had to be excluded from the analysis of this task due to response capture, motion, and anatomical problems. The analysis thus included 28 participants, with 14 in the placebo group and 14 in the diazepam group.

8.2.2 *Experimental task*

In the emotional counting Stroop (ecStroop) task, participants were instructed to report the number of presented words, regardless of word meaning, while undergoing an fMRI scan. The task was adapted from Whalen et al.'s (1998) task and included a subset of words from previous research on anxiety (Mathews, Mogg, May, & Eysenck, 1989; MacLeod & Mathews, 1988). The task included the following word categories: positive, neutral, socially threatening, and physically threatening, where the last two categories were combined to generate a threat word category. All categories were matched in respect to word length, imageability, and frequency (see Table 8.1). E-prime (version 2.0; Psychology Software Tools Inc., Pittsburgh, PA, USA) was used to run the task and record responses. The task included 16 picture blocks, with a total of 160 words, where each block consisted of 10 trials of the same emotional word valence and lasted 20s. In each trial, participants were presented with 1 to 4 identical words and were instructed to report the number of

words on the screen using a button box. The words were presented for 1500ms in each trial, followed by an intertrial interval of 500ms, and were offset from the centre to minimise visual blurring of the words. A fixation cross was presented between each block for 20 seconds. 4 blocks of each category (physical threat, social threat, positive, neutral) were presented in a pseudo-randomised order and the order was counterbalanced. A diagram of the task is presented in Figure 8.1.

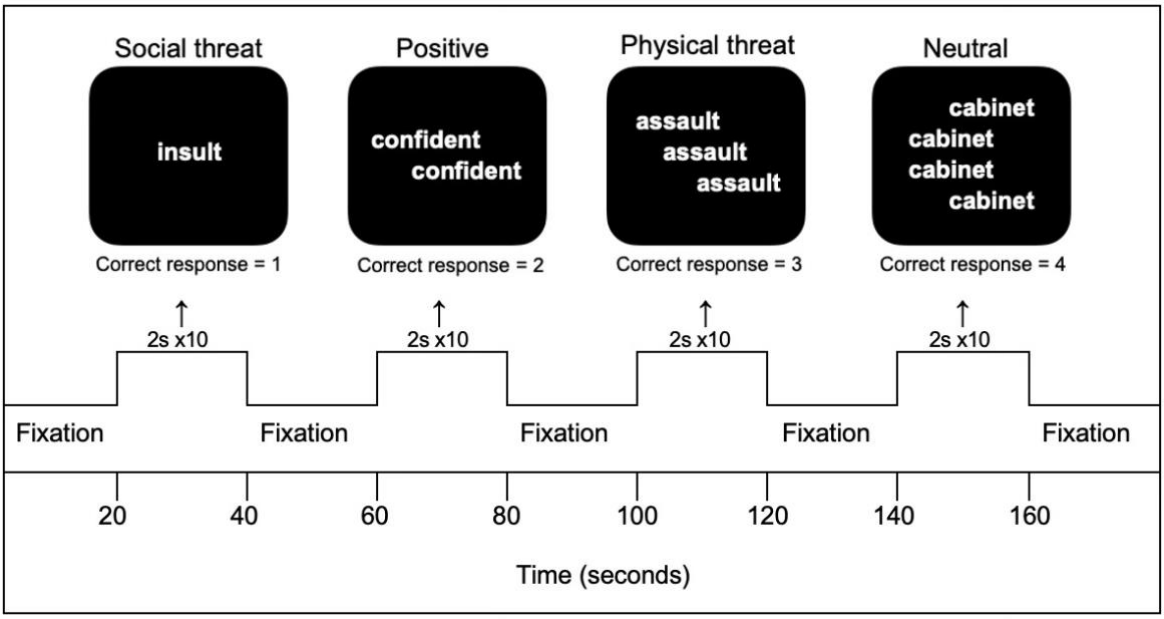

Figure 8.1 – *A diagram of the block design and individual trials of the ecStroop task*

Table 8.1 – Words used in the emotional counting Stroop task

Social Threat	Physical Threat	Positive	Neutral
Sin	Disease	Nice	Mileage
Sad	Panic	Embrace	Cartilage
Insult	Sickness	Confident	Plate
Pathetic	Assault	Brave	Surface
Despair	Catastrophe	Merry	Cabinet
Terrible	Sick	Calm	Upholstery
Gloomy	Shoot	Fun	Towel
Bad	Pain	Pious	Wait
Bitter	Harm	Generous	Peck
Pessimistic	Attack	Wealth	Surplus
Hostile	Rape	Pleasant	Heir
Clumsy	Suffocate	Friendship	Trunk
Alone	Brutal	Prosper	Term
Grief	Emergency	Gracious	Prime
Stern	Punishment	Romance	Clean
Misery	Hurt	Tranquil	Bleach
Immature	Destruction	Trust	Deal
Mad	Weakness	Joy	Ginger
Quarrel	Accident	Laugh	Wear
Poor	Infection	Love	Molecule
Evil	Kill	Humour	Graphic
Shame	Gore	Hope	Guess
Rude	Afraid	Safe	Grow
Upset	Agony	Bright	Sweep
Liar	Groan	Clever	Display
Spite	Death	Miracle	Grate
Meek	Vicious	Pure	Couch
Dumb	Disaster	Superior	Electricity
Failure	Fear	Holiday	Brass
Bore	Moan	Witty	Majestic
Punish	Tragedy	Kiss	Join
Angry	Bury	Keen	Decorate
Ashamed	Enemy	Splendid	Thrifty
Sneer	Die	Rejoice	Vase
Awkward	Danger	Dignified	Cellar
Ridicule	Nasty	Noble	Unit
Lonely	Ache	Polite	Predict
Error	Poison	Talented	Forth
Stubborn	Hazard	Smile	Closet
Blunder	Evil	Secure	Item

8.2.3 *Behavioural analysis*

The behavioural analysis was carried out using IBM SPSS Statistics for Windows, version 27 (IBM Corp., Armonk, N.Y., USA). To determine if there were any significant effects of treatment group on accuracy or reaction times, a 4×2 mixed ANOVA was conducted. Treatment condition (diazepam vs. placebo) was entered as the between-group factor and word valence (socially threatening, physically threatening, positive, neutral) as the within-subjects factor. For information about the analysis of the subjective ratings please see section 5.2.4 of Chapter 5.

8.2.4 *Image acquisition*

A 3T Siemens Magnetom Tim-Trio whole-body scanner system (Siemens, Erlangen, Germany) with a 32-channel head-coil, located at the OCMR, was used to acquire T1-weighted structural images and T2-weighted transverse echo planar images (EPI). Functional imaging consisted of 45 T2-weighted EPI slices (TR = 3000ms, TE = 30ms, flip angle = 87°, matrix size = 64 × 64, voxel dimension = 3mm isotropic, slice thickness = 3mm, FOV = 192 × 192mm^2, 203 volumes, echo spacing = 0.49ms). Gradient-echo fieldmap images were acquired for distortion correction (44 slices, slice thickness = 3.5mm, voxel dimension = 3.5mm isotropic, FOV = 192 × 192mm^2, TR = 488ms, TE1 = 5.19ms, TE2 = 7.65ms, flip angle = 60°), and T1-weighted structural images were acquired using an MP-RAGE sequence for anatomical alignment (192 slices, slice thickness = 1mm, TR = 2040ms, TE = 4.7ms, flip angle = 8°, voxel dimension = 1mm isotropic, FOV = 192 × 192mm^2).

8.2.5 *Image analysis*

MRI data were analysed using FSL (FMRIB Software Library v6.6) tools (https://fsl.fmrib.ox.ac.uk/fsl). Structural anatomical scans were brain extracted using FSL's Brain Extraction Tool (BET; Smith, 2002). Pre-processing consisted of brain extraction using BET (Smith, 2002), motion correction using FMRIB's Linear Image Registration Tool (MCFLIRT; Jenkinson, Bannister, Brady, & Smith, 2002), distortion correction using gradient-echo fieldmaps, spatial smoothing using a Gaussian kernel of FWHM 5mm, grand-mean intensity normalisation of the entire 4D dataset by a single multiplicative factor, registration of the functional space template to the anatomical space and the Montreal Neurological Institute (MNI) 152 space using the FMRIB's Linear Image Registration Tool (FLIRT; Jenkinson & Smith, 2001; Jenkinson, Bannister, Brady, & Smith, 2002), and high-pass temporal filtering equivalent to 90s (Gaussian-weighted least-squares straight line fitting, with sigma = 45.0s). Time-series statistical analysis was then carried out using FMRIB's Improved Linear Model (FILM) with local autocorrelation correction (Woolrich, Ripley, Brady, & Smith, 2001), and a custom 3-column format convolved with a gamma hemodynamic response function, and its temporal derivative, was used to model the data. The main contrasts of interest were threat vs. neutral and positive vs. neutral, but estimates of neutral vs. threat, neutral vs. positive, threat vs. positive, positive vs. threat, social threat vs. baseline, positive vs. baseline, physical threat vs. baseline, neutral vs. baseline were also obtained for reference, where fixation blocks were the baseline reference. The *fsl_motion_outliers* tool was used to identify and remove timepoints in the data that had been corrupted by excessive motion.

The group level analysis was carried out using FMRIB's Local Analysis of Mixed Effects (FLAME; Woolrich, Behrens, Beckmann, Jenkinson, & Smith, 2004). The general linear model (GLM) included the 2 groups (diazepam & placebo), where group averages, average across both groups, and differences between groups for each contrast of interest were tested. Significant activations were determined by cluster-based thresholding of $Z > 3.1$ and a family-wise error-corrected cluster significance threshold of $p < 0.05$ (Worsley, 2001).

In addition to the whole-brain analysis, a small volume correction (SVC) was preformed using predefined anatomical regions of interest (ROIs), which included the left and right ACC. These regions were defined using the Harvard-Oxford Cortical Structural Atlas and were set to a liberal threshold of 10% to allow for the inclusion of all 3 peak activation ACC coordinates found in Del-Ben and colleagues' study on diazepam (2012) in the analysis.

8.3 Results

8.3.1 *Sociodemographic, clinical, and personality characteristics*

Sociodemographic, clinical, and personality characteristics of participants included in the ecStroop task analysis are presented in Table 8.2. The analysis of this task consisted of 28 participants, as 6 participants had to be excluded due to response capture (placebo=1, diazepam=2), motion (diazepam=2), and anatomical (placebo=1) problems. The analysis thus included 14 participants in the placebo group and 14 in the diazepam group. The two groups were well matched on sociodemographic, clinical, and personality parameters.

Table 8.2 – Sociodemographic, clinical, and personality characteristics

	Placebo (n=14)	*Diazepam (n=14)*
Gender		
Male	n=8	n=6
Female	n=6	n=8
Age	22.43 (1.10)	23.20 (1.28)
Verbal IQ (NART)	113.57 (1.43)	116.87 (1.65)
Beck Depression Inventory	1.64 (0.53)	1.20 (0.47)
Spielberger State and Trait Anxiety Inventories		
State-Anxiety	25.43 (1.05)	26.50 (2.05)
Trait-Anxiety	28.00 (1.46)	28.90 (2.13)
Eysenck Personality Questionnaire		
Neuroticism	5.36 (0.64)	5.70 (1.03)
Psychoticism	2.93 (0.72)	2.90 (0.75)
Extraversion	16.71 (1.07)	15.50 (1.29)
Lie/Social Desirability	6.79 (1.10)	8.40 (1.09)

Values are means (SEM)

8.3.2 *Subjective rating results*

Similar to what was found in Chapters 6 and 7, diazepam did not have a significant effect on subjective mood or state anxiety during the intervention week (BFS, PANAS, STAI-S, BDI; all *ps* > .053). Furthermore, there were no significant main effects of treatment group on the lightheadedness, confusion, forgetfulness, and agitation side effect ratings (all *ps* > .058). However, similar to what was found in Chapter 6, participants in the diazepam group were significantly drowsier ($F(1, 26) = 4.265$, $p = .049$, $\eta_p^2 = .141$) and less coordinated ($F(1, 26) = 11.695$, $p = .002$, $\eta_p^2 = .310$), and experienced more muscle weakness ($F(1, 26) = 4.710$, $p = .039$, $\eta_p^2 = .153$) throughout the study week compared to the placebo group. Similarly, there was no significant main effect of treatment group on any of the VAS (all *ps* > .139), except on the "well-coordinated – clumsy" scale, where participants in the diazepam group were significantly clumsier throughout the intervention week compared to the placebo group ($F(1, 26) = 6.011$, $p = .021$, $\eta_p^2 = .188$).

8.3.3 *Behavioural performance*

Overall accuracy in reporting the number of words was high (>90%), thus confirming that participants were successful in completing the task. There was no significant effect of word valence on reaction times ($F(1.437, 37.363) = .879$, $p = .392$, $\eta_p^2 = .033$), which is consistent with Whalen et al. (1998). Furthermore, there were no group differences in accuracy or reaction times, and no significant interactions between treatment group and accuracy or reaction times (all *p*s > .095).

8.3.4 *Main effect of task*

Significant activity was observed for each word category across the two combined groups in clusters that including the lingual gyrus extending to bilateral ACC, superior & inferior frontal gyrus, and middle frontal gyrus, frontal pole, putamen, cerebellum, thalamus, insular cortex, and juxtapositional lobule cortex (see Figure 8.2 and Table 8.3). These areas are associated with attention and memory, motor control and movement, and emotional and visual word processing.

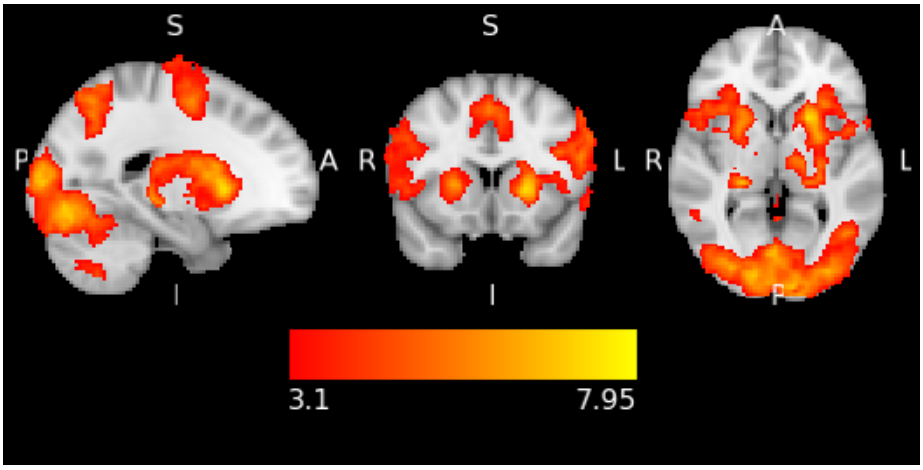

Figure 8.2 – *Whole-brain activation in response to the word categories across groups*

Sagittal, coronal, and axial images depicting neural activation in response to the four word categories (social threat, physical threat, positive, neutral) across groups (diazepam and placebo) during the ecStroop task. Images are thresholded at $Z > 3.1$ and $p < 0.05$ corrected.

Table 8.3 – Main effect of the emotional counting Stroop task across groups ($Z > 3.1$, $p < 0.05$)

Cluster	Brain area	Side	Cluster size (voxels)	MNI max (x, y, z)	*z*-score	*p*-value
Task activation (mean of the four word categories)						
1	Lingual gyrus extending to bilateral ACC, superior & inferior frontal gyrus, and middle frontal gyrus, frontal pole, putamen, cerebellum, thalamus, insular cortex, and juxtapositional lobule cortex	L	41486	-2, -88, -10	7.87	<.001
2	Putamen	L	5779	-20, 14, 2	7.32	<.001
3	Thalamus	R	286	24, -28, 0	6.90	<.001
4	Frontal pole	R	115	18, 58, -2	4.75	.014
Task activation (threat>neutral)						
1	Middle temporal gyrus	L	99	-58, -52, 6	4.11	.024
Task activation (positive>neutral)						
1	Occipital pole	L	108	-10, -94, 14	4.05	.010

Furthermore, increased activity in the left middle temporal gyrus (MTG) was observed in response to threat-related words across both groups, compared to neutral words (threat>neutral, overall mean; see Table 8.3 and Figure 8.3), which is consistent with previous research (Shin, et al., 2001). Increased activity was also observed in the left occipital pole in response to positive words across both groups, compared to neutral words (positive>neutral, overall mean; see Table 8.3 and Figure 8.4).

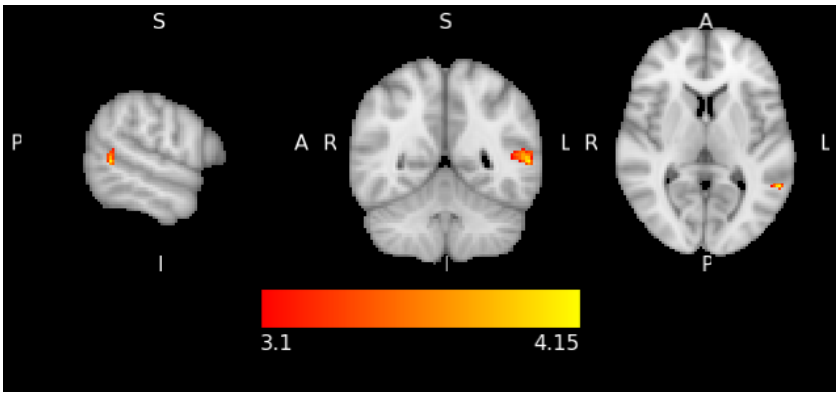

Figure 8.3 – *Whole-brain activation in response to threat-related words across groups*

Sagittal, coronal, and axial images depicting neural activation in the left middle temporal gyrus in response to threat-related words compared to neutral words across groups (diazepam and placebo) during the ecStroop task. Images are thresholded at $Z > 3.1$ and $p < 0.05$ corrected.

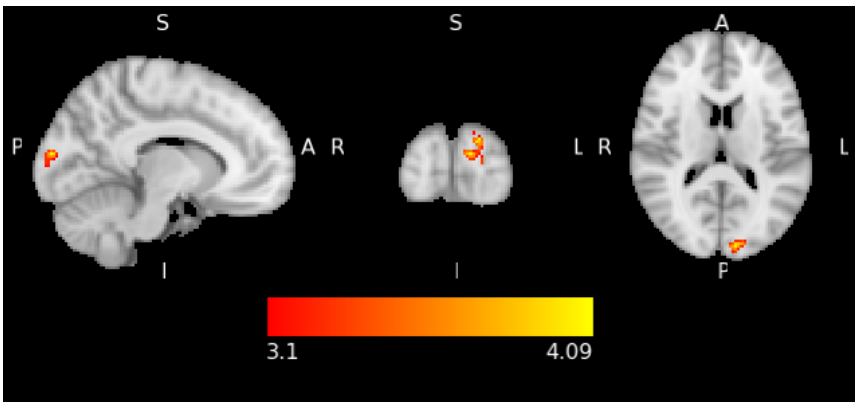

Figure 8.4 – *Whole-brain activation in response to positive words across groups*

Sagittal, coronal, and axial images depicting neural activation in the left occipital pole in response to positive words compared to neutral words across groups (diazepam and placebo) during the ecStroop task. Images are thresholded at $Z > 3.1$ and $p < 0.05$ corrected.

8.3.5 *Effect of treatment*

No group differences were found in the whole-brain analysis. The small volume correction analysis ($Z > 3.1$ thresholded, $p < 0.05$ corrected) revealed increased

BOLD activation in both the left and right ACC across both groups for all word categories (threat-related, positive, and neutral). Furthermore, it revealed increased BOLD activation in the right ACC in the diazepam group, compared to the placebo group, in response to positive words relative to baseline (see Table 8.4 and Figure 8.5).

Table 8.4 – Small volume correction (SVC) in the anterior cingulate cortex ($Z > 3.1$, $p < 0.05$)

	Side	Cluster size (voxels)	MNI max (x, y, z)	*z*-score	*p*-value
Mean across groups					
social threat>baseline	R	105	2, 20, 42	4.31	<.001
social threat>baseline	R	22	10, 26, 28	4.13	.030
physical threat>baseline	R	102	4, 12, 46	4.32	<.001
positive>baseline	R	167	8, 20, 38	5.00	<.001
neutral>baseline	R	99	10, 20, 38	4.33	<.001
mean	R	187	10, 20, 36	4.56	<.001
social threat>baseline	L	201	-4, 6, 48	5.45	<.001
physical threat>baseline	L	211	-6, 6, 48	5.45	<.001
positive>baseline	L	219	-6, 6, 48	5.42	<.001
neutral>baseline	L	201	-6, 6, 48	6.20	<.001
mean	L	262	-6, 6, 48	5.78	<.001
Group differences (diazepam>placebo)					
positive>baseline	R	20	12, 28, 26	3.96	.032

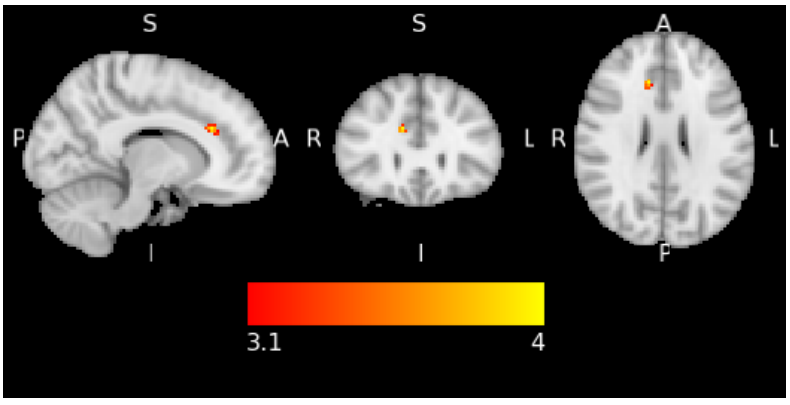

Figure 8.5 – *Right ACC activation in response to positive words in the diazepam group*

Sagittal, coronal, and axial images depicting significantly increased activation in the right ACC in the diazepam group relative to the placebo group for positive words compared to baseline. Images are thresholded at $Z > 3.1$ and $p < 0.05$ corrected.

8.4 Discussion

This study sought to investigate whether diazepam affects activity related to emotional processing within the ACC during an emotional counting Stroop (ecStroop) task. In light of prior research showing an effect of acute diazepam administration on ACC activation (Del-Ben et al, 2012), it was predicted that a 7-day treatment of diazepam would modulate activation in the ACC in response to emotional words during the ecStroop task in healthy participants. To investigate this, a small volume correction analysis was done using left and right ACC masks, defined by the Harvard-Oxford Cortical Structural Atlas, with a threshold of 10% to make sure the masks covered the 3 peak activation ACC coordinates found in Del-Ben and colleagues‘ study on diazepam (2012). The diazepam group in the current study showed increased activation in the right ACC while reporting the number of positive words, compared to baseline, without any subjective changes in mood and state anxiety, although the group did experience more drowsiness and muscle weakness, and reported being less coordinated throughout the study week than the placebo group.

The ecStroop task caused typical increases in activity within areas associated with attention and memory, motor control and movement, and emotional and visual word processing. Furthermore, increased activity in the left occipital pole in response to positive words and in the left MTG in response to threat-related words was observed across both groups, compared to neutral words, which is consistent with previous research (Shin, et al., 2001). Overall accuracy in reporting the number of words was also high, thus confirming that participants were successful in completing the task. Moreover, there were no group differences in the accuracy or

reaction times to any of the word categories, and no significant effect of word valence on reaction times, which is consistent with Whalen et al. (1998).

The diazepam group in the current study showed increased activity in the right ACC while reporting the number of positive words compared to baseline. A possible reason for it not showing a significant increase compared to neutral or threat-related words is that the right ACC was similarly activated for all categories of words (social threat, physical threat, positive, neutral), so the difference in activation may be more pronounced and thus more detectable when the positive words are compared to baseline. Del-Ben and colleagues (2012) found that an acute dose of diazepam increased activation in the right ACC in response to fearful faces and decreased activation in the right and left ACC in response to angry faces. Interestingly the right ACC peak activation coordinates for positive words in this current study (peak voxels: x = 12, y = 28, z = 26) were very close to the ACC peak activation coordinates in Del-Ben and colleagues' (2012) study for fearful faces (peak voxels: x = 11, y = 25, z = 23). This may represent a difference between acute and short-term diazepam treatments. An acute dose of benzodiazepines may thus potentially induce an initial ACC activation increase to threat-related stimuli due to its top-down regulation of the amygdala and insula during threat-processing (Del-Ben et al, 2012), while a subchronic treatment may potentially induce an activation increase to positive stimuli in the same area in the longer run. These results are in line with previous research on short-term administration of diazepam and the results from Chapter 7. Pringle and colleagues (2016) for example found that a short-term treatment of diazepam increased accuracy in categorising positive compared to negative personality characteristic words, when using self-referential judgements, and shortened the time needed to classify the positive compared to the negative

words. Moreover, diazepam was shown to increase activation in the left vlPFC in response to happy faces compared to baseline in Chapter 7. Both the ACC and vlPFC have been implicated in the assessment of the salience of emotional information and the regulation of emotional responses (Bush et al, 2000; Kim et al, 2019). Diazepam may thus potentially be exerting its short-term effects by modulating activity within these areas to positive stimuli, thus producing a positive bias in the processing of emotion-related stimuli. A future study might want to focus on testing participants at multiple time points to further investigate whether there are differences between acute and subchronic administrations by using a task that engages both the vlPFC and the ACC. It is worth noting that as activation in control areas is usually thought to reduce subcortical activity, thus leading to a reduced emotional response (Silvers et al, 2014), it raises the possibility that this increase in activation to positive stimuli may be leading to inhibition of subcortical areas and thus a reduced positive experience, but given the absence of any subcortical activation differences in the current study, it is unlikely. Future studies may however want to focus on a clinical sample to further investigate this.

In summary, a 7-day diazepam treatment increased activation in the right ACC in response to positive words, compared to baseline, without causing any subjective changes in mood and state anxiety. These results are in line with previous research on short-term administration of diazepam (Pringle et al, 2016) and the results from Chapter 7, where diazepam was shown to increase activation in the left vlPFC in response to happy faces. Both the ACC and vlPFC have been implicated in the assessment of the salience of emotional information and the regulation of emotional responses (Bush et al, 2000; Kim et al, 2019). Diazepam may thus potentially be exerting its short-term effects by modulating activity within

these areas to positive stimuli, thus producing a positive bias in the processing of emotion-related stimuli. Taken together, these results provide valuable insights into potential mechanisms through which diazepam may exert its short-term anxiolytic effects.

Chapter 9: General discussion

9.1 Summary of findings

Given the prevalence (Kessler et al, 2005) and substantial economic costs (Kessler & Greenberg, 2002) of anxiety disorders, and the shortcomings of current treatments, there is dire need for research that helps inform the development of new treatments and medications. The aim of this thesis was to further our understanding of two medications relevant to anxiety, losartan and diazepam, to help inform the use of existing treatments and lead to more effective ones.

9.1.1 *Losartan study*

Exposure-based cognitive behavioural therapy (CBT) has been the most effective and widely employed treatment of anxiety disorders (Deacon & Abramowitz, 2004), but overall treatment response rates are still only estimated to be around 50% across them (Loerinc et al, 2015). Researchers have thus begun looking for strategies to improve the treatment, which has involved identifying the mechanisms underlying its efficacy. Mechanisms such as attentional biases and hippocampal functioning have been shown to be important for exposure success (Price et al, 2011; Maren, 2011), which means that augmenting them may be a promising way to increase clinical effects of the treatment.

Research has indicated that the brain renin-angiotensin system (RAS) may play an important role in the pathophysiology and extinction of anxiety (Braszko et al, 2003; Ciobica et al, 2011; Gard, 2002). The angiotensin receptor antagonist losartan may potentially be a promising pharmacological candidate to enhance the efficacy of exposure-based therapies, as it has been shown to facilitate extinction

learning (Marvar et al, 2014; Zhou et al, 2019) and to enhance early threat discrimination and threat processing (Reinecke et al, 2018). Increasing evidence is emerging that it also enhances cognition (Fogari et al, 2003; Tedesco et al,1999), with even a single dose of 50mg being shown to improve memory function (Mechaeil et al, 2011; Raghavendra et al, 1998) and to enhance learning from positive relative to negative events (Pulcu et al, 2019). It however remains to be fully clarified how losartan affects some of the mechanisms relevant to exposure success in humans, such as attentional biases (Price et al, 2011) and hippocampal functioning (Maren, 2011). Chapters 2 to 4 in this thesis thus focused on a double-blind, randomised, placebo-controlled study, where the objective was to investigate the key effects of a single dose of losartan on neural correlates of memory encoding and on attentional biases in healthy, high trait-anxious volunteers.

Losartan has been shown to improve memory function in animals (Raghavendra et al, 1998) and healthy humans (Mechaeil et al, 2011), but it remains to be clarified how losartan affects hippocampal functioning and memory encoding. The effects of a single dose of losartan on hippocampal functioning, measured using a memory encoding task, were thus investigated in Chapter 3. As losartan has been shown to improve memory function (Raghavendra et al, 1998; Mechaeil et al, 2011), it was predicted that losartan would increase hippocampal activation during memory encoding. There was however no evidence found for an effect of losartan on neural responses in the hippocampus during memory encoding, nor on memory recognition outside of the scanner. Power to detect smaller effects was limited given the relatively small sample size used in the analysis due to the COVID-19 pandemic disruption, which could potentially explain why no effect was found. Another possible reason for the lack of an effect on memory recognition performance may

be that losartan might have more of an effect after a longer time of memory consolidation. Longer time intervals might thus possibly be needed between encoding and recognition testing for an effect, as has been shown in previous researach (Xu et al, 2021). Furthermore, given that losartan has been associated with enhanced extinction memory in rodents (Marvar et al, 2014) and given that the magnitude of extinction memory has been shown to be positively correlated with hippocampal activation during extinction recall in humans (Milad et al, 2007), losartan may have more of an effect on hippocampal activity during extinction recall rather than during encoding. Losartan's effects on the hippocampus may also potentially be more pronounced in response to threat signals, as has been shown with vmPFC activation (Zhou et al, 2019) and during encoding of negative stimuli (Xu et al, 2021). A less stringent group analysis ($Z > 2.3$ thresholded, $p < 0.05$ corrected) revealed an increase in the paracingulate gyrus, insular cortex, lingual gyrus, and fusiform gyrus in response to both familiar and novel photos in the losartan group compared to the placebo group. These regions have been shown to be activated during a similar encoding paradigm (Machielsen et al, 2000). However, as they were activated in response to both novel and familiar photos, the activity may be more related to higher-order visual processing of stimuli rather than encoding. Losartan might thus possibly be modulating activity within regions related to higher-order visual processing.

Losartan has been shown to enhance both early threat discrimination (Reinecke et al, 2018) and learning from positive relative to negative events (Pulcu et al, 2019). It however remains to be clarified how losartan affects attentional biases, which have been shown to be relevant to exposure success in humans (Price et al, 2011). The effects of a single dose of losartan on attentional biases,

measured using a dot probe task, were thus investigated in Chapter 4. As losartan has been shown to improve early discrimination of negative versus positive stimuli (Reinecke et al, 2018) and to induce a positive learning bias (Pulcu et al, 2019), it was predicted that losartan would reduce negative attentional biases and increase positive ones. The findings showed that the losartan group had higher attentional bias index scores than the placebo group on the Positive Disengagement Bias Index, meaning that the losartan group's attention was more firmly held by positive stimuli compared to neutral stimuli. Losartan has been shown to initiate a shift from aversive to appetitive learning (Pulcu et al, 2019), as well as to shift motivational and emotional salience away from social punishment towards social reward (Zhou et al, 2021). An increase in positive attentional bias, as seen in the present study, may potentially represent a mechanism by which losartan can induce this shift. No other group differences were found on any of the other attentional bias indices, which may have been due to lack of statistical power.

Power to detect smaller effects was limited in this study given the relatively small sample size used in this analysis due to the COVID-19 pandemic disruption. Small sample sizes are usually thought to contribute to false negatives, which can conceal the presence of real effects, so definite conclusions cannot be drawn until more participants have been recruited in this study.

9.1.1.1 *Implications of the current findings for the pharmacological augmentation of psychological treatments*

The goal of the current thesis was to improve our current knowledge of how losartan affects attentional biases and hippocampal functioning, which have been shown to be relevant to exposure success in humans (Price et al, 2011; Maren, 2011), to

potentially provide further support that losartan may be a promising pharmacological candidate to enhance the efficacy of exposure-based therapies.

Although no evidence was found for an effect of losartan on neural responses in the hippocampus during memory encoding, the findings showed that a single dose of losartan, in the absence of overall effects on heart rate, blood pressure, and mood, increased activation in the paracingulate gyrus, insular cortex, lingual gyrus, and fusiform gyrus in response to both novel and familiar photos, which possibly reflects modulation of higher-order visual processing. This modulation may be relevant for cognitive behavioural therapy (CBT), as greater reactivity in higher-order visual regions to threat has been shown to predict CBT success (Klumpp et al, 2013b).

The findings also showed that a single dose of losartan increased positive attentional bias, which was reflected in attention being more firmly held by positive stimuli compared to neutral in the losartan group. An increase in positive bias may potentially represent a mechanism by which losartan can induce a shift from negative to positive learning, which has been described in previous research (Pulcu et al, 2019; Zhou et al, 2021). It has been suggested that losartan may have the potential to also facilitate positive relative to negative learning during exposure therapy in humans (Pulcu et al, 2019) and that adjunct positive valence training, which involves depicting a feared situation in a more positive light, may enhance the longevity of exposure treatment (Dour, Brown, & Craske, 2016). By enhancing positive attentional bias, losartan may thus possibly have the potential to more firmly focus people's attention to positive aspects of a feared situation, which could be beneficial during exposure therapy, but this of course requires further testing.

Given that both greater reactivity in higher-order visual regions and positive valence training have been shown to be relevant for therapy success (Klumpp et al, 2013b; Dour et al, 2016), the current results may provide further support that losartan might potentially have synergistic effects with exposure therapy, but this remains to be tested directly.

9.1.1.2 *Strengths and limitations*

The findings of the current study should be interpreted in light of several strengths and limitations pertaining to the study.

As healthy participants were assessed in the current study, as opposed to patients, potential effects of the treatment could be tested unconfounded by psychopathology or changes in symptoms, which allows for a clearer assessment of potential effects. It however limits the generalisability of the results to clinical populations, but the selection of participants on the basis of trait anxiety did allow some cognitive aspects of anxiety to be probed, which gave further clinical relevance without the need to recruit patients. Furthermore, to the best of my knowledge this is the first investigation of the effects of a single dose of losartan on attentional biases and neural responses in the hippocampus during memory encoding in highly anxious individuals. Moreover, the current study was a randomized double-blind placebo control study, which is considered the "gold standard" of intervention-based studies. However, in addition to the strengths mentioned above, there are also some limitations that need to be addressed.

As mentioned above, the generalisability of the results to clinical populations is limited. Moreover, the limitations associated with fMRI also need to be considered. As the BOLD signal is an indirect measure of neural activity that is

based on physiological changes associated with neural activity, it raises the possibility that it reflects global modulations of signalling and/or vascular reactivity rather than a specific modulation on neural activity induced by the drug. Additionally, the current study made use of power calculations based on previous research into the effects of a single dose of losartan on memory in healthy volunteers (Mechaeil et al, 2011) in order to determine desired sample sizes, which suggested sample sizes of 18 per drug group. Recruitment in the study was unfortunately more difficult than anticipated due to the COVID-19 pandemic disruption and therefore may have been underpowered. Small sample sizes are usually thought to contribute to false negatives, which can conceal the presence of real effects, so definite conclusions cannot be drawn until more participants have been recruited in this study.

9.1.1.3 *Future research*

No evidence was found for an effect of losartan on neural responses in the hippocampus during memory encoding in the current study. However, given that losartan has been associated with enhanced extinction memory in rodents (Marvar et al, 2014) and that the magnitude of extinction memory has been shown to be positively correlated with hippocampal activation during extinction recall in humans (Milad et al, 2007), a future study should focus on hippocampal activity in relation to extinction recall, as losartan's effects on the hippocampus may potentially be more pronounced during recall and in response to threat signals, as has been shown with vmPFC activation (Zhou et al, 2019). A future study could investigate this by using a fear conditioning and extinction protocol, similar to Milad et al. (2007), where hippocampal activation to an extinguished conditioned stimulus can be compared to activation to an unextinguished one during extinction recall. Further investigation

of this is clearly warranted, given the association between enhanced extinction memory and better CBT outcomes in anxiety disorders (Berry et al, 2009; Forcadell et al, 2017). Furthermore, as findings from the current study suggest that losartan might modulate activity within regions related to higher-order visual processing, future studies may want to include a visual processing task to further investigate this; especially as modulation of activity within higher-order visual regions might have synergistic effects with exposure therapy. Moreover, a future study could also focus on investigating how losartan affects brain regions related to attentional biases during a similar dot probe task in an fMRI scanner, to replicate and further expand the positive attentional bias finding of this study.

To control for the possible confounding effects of global drug-related modulation of the BOLD signal mentioned in section 9.1.1.2, it would be a useful addition in future studies to include a control task that explores the BOLD response within a region that is not expected to be modulated by the drug in question. If the selected region is not affected by the drug, it suggests a global confound is not present and helps to support the hypothesis that the drug does have a specific localised brain activity effect (Iannetti & Wise, 2007). Including an arterial spin labeling (ASL) scan in the MRI protocol, which directly measures cerebral perfusion, would also be a useful addition. Furthermore, as healthy, high trait-anxious participants were assessed in the current study, future studies should focus on assessing if these findings can be replicated in patients with anxiety disorders. Replications in healthy participants are also necessary to increase confidence in the findings and to reduce the likelihood of false positives, as are replications in larger and more diverse samples.

9.1.2 *Diazepam study*

Apart from CBT, the most common therapies for anxiety disorders include selective serotonin reuptake inhibitors (SSRIs) and benzodiazepines, as they have been proven to have anxiolytic efficacy (Baldwin et al, 2005), but both groups of medications have limitations. A better understanding of how existing medications exert their anxiolytic effects may help guide development of new medications. Benzodiazepines appear to affect emotional processing without any subjective changes in anxiety in healthy participants (Del-Ben et al, 2012; Paulus et al, 2005), similar to SSRIs (Murphy et al, 2009; Harmer et al, 2006). As benzodiazepines are not effective in treating depression, researching their effects provide a means of teasing apart antidepressant and anxiolytic effects, since any effects observed can directly be related to their anxiolytic action, as opposed to SSRIs. Although the pharmacological mechanisms of benzodiazepines are relatively well understood, a comprehensive understanding of the cognitive neuropsychological mechanisms behind their anxiolytic effects is still lacking. Furthermore, most of the current research has focused on benzodiazepines' acute effects; it may however be more clinically relevant to investigate if the effects are the same after short-term administration, as they are usually prescribed over a period of two to four weeks and there may be differences between acute and short-term treatments, as has been shown for SSRIs (Murphy et al, 2009). A short-term administration may also elicit more reliable changes on emotional processing, as plasma drug levels may be more stable. Chapters 5 to 8 in this thesis thus focused on a double-blind, randomised, placebo-controlled study from 2012, where the objective was to investigate the effects of a 7-day treatment of the benzodiazepine diazepam on the neural correlates of emotional processing in healthy volunteers.

The effects of a 7-day diazepam treatment on the neural correlates of adaptive emotion regulation during an emotion regulation task were investigated in Chapter 6. In light of prior research on acute benzodiazepine administration (Del-Ben et al, 2012; Paulus et al, 2005) and $GABA_A$ receptor α_2 and α_3 subunits being located in high concentrations in the amygdala and frontal regions (D'Hulst et al, 2009), it was predicted that the treatment would attenuate activation in the amygdala and insular cortex and possibly also modulate activity within the prefrontal areas in response to cognitive reappraisal. The treatment did not have an effect on amygdala or insular cortex activity during reappraisal. It did however affect the functional coupling of these regions with other regions that have been implicated in emotional processing. A lower connectivity between limbic areas and the precuneous cortex was observed in the diazepam group, compared to the placebo group, in response to aversive pictures. Furthermore, a lower connectivity between the left and right amygdala and the pre- and post-central gyrus was observed in the diazepam group during reappraisal, as well as a downregulation in the right vlPFC during it.

The effects of a 7-day diazepam treatment on the neural correlates of emotional facial expression processing during a face classification task were investigated in Chapter 7. In light of prior research on acute benzodiazepine administration (Del-Ben et al, 2012; Paulus et al, 2005) and $GABA_A$ receptor α_2 and α_3 subunits being located in high concentrations in the amygdala and frontal regions (D'Hulst et al, 2009), it was predicted that the treatment would attenuate activation in the amygdala and insular cortex in response to fearful facial expressions and possibly also modulate activity within the prefrontal areas in relation to emotional facial expressions. No effects were found on neural responses in the amygdala or

insular cortex to threat-related facial expressions, which was contrary to predictions. The lack of an effect on amygdala activation may have been due to excessive motion during the task, as no amygdala activation was observed for any of the facial expression across the two combined groups, so no conclusions can be drawn regarding the amygdala from this task. However, the lack of an effect of diazepam on insular cortex activation may possibly be due to the development of neuronal tolerance, as it has been shown that neurons receiving GABAergic input can show tolerance to benzodiazepines' effects after a period of time (Gonsalves & Gallager, 1987; Wilson & Gallager, 1987; Xie & Tietz, 1991). The diazepam group did on the other hand show an increase in activation in the left vlPFC in response to happy faces.

The effects of a 7-day diazepam treatment on the neural correlates of emotional processing within the ACC during an emotional counting Stroop task were investigated in Chapter 8. In light of prior research showing an effect of acute diazepam administration on ACC activation (Del-Ben et al, 2012), it was predicted that the treatment would modulate activation in the ACC in response to emotional words. The results showed that the treatment increased activation in the right ACC in response to positive words, which is in line with previous research on short-term administration of diazepam (Pringle et al, 2016) and the results from Chapter 7.

Taken together, diazepam may potentially be exerting its short-term effects by modulating neural activity within prefrontal and cingulate regions, as well as connectivity between parietal and limbic regions, related to emotional processing.

9.1.2.1 *Implications of the current findings for the identification of anxiety-specific mechanisms of action*

The goal of the current thesis was to improve our current knowledge of the cognitive neuropsychological mechanisms behind diazepam's effects, to potentially help tease apart the anxiolytic and antidepressant effects of SSRIs and help inform the development of future anxiolytic treatments. The neurocognitive model of emotional processing and anxiety illustrated in Chapter 1 proposes that prefrontal, cingulate, and parietal control regions modulate activity in affective appraisal regions and occipito-temporal regions during generation and regulation of emotions. It also proposes that anxiety is linked to both hypo-activation (e.g., dlPFC & dmPFC) and hyper-activation (e.g., vlPFC) in prefrontal control regions, as well as hyper-activation in affective appraisal regions, such as the amygdala and insular cortex, during it. The current study sought to investigate whether a 7-day diazepam treatment would modulate activity within these frontal control regions and affective appraisal regions during emotional processing, which would indicate anxiety-specific mechanisms of action, as benzodiazepines are efficacious in treating anxiety, but not depression. The current findings showed that the treatment modulated neural activity within prefrontal and cingulate control regions, as well as connectivity between parietal and affective appraisal regions, during emotional processing. The treatment lowered connectivity between affective appraisal regions and the precuneous cortex in response to aversive pictures, which could be hypothesised to reflect an increased "distancing" from the emotional experience and its affect. Moreover, the treatment lowered connectivity between the left and right amygdala and the pre- and post-central gyrus during cognitive reappraisal, which may reflect a reduction in sensory and motor reactions to aversive signals during

emotion regulation. The treatment also reduced activity in the right vlPFC during reappraisal. It has been proposed that people with anxiety disorders may have an inability to accurately appraise threat, or an inability to reappraise threat, or even both, which has been linked to abnormal activity in control regions and affective appraisal regions (Silvers et al, 2014). It has also been suggested that SSRIs may exert their effects by improving emotion regulation, as SSRI treatments have been associated with an increased use of cognitive reappraisal and a decreased use of suppression of emotional expression in both depressed and anxious patients (McRae, et al, 2014; Feurer et al, 2021). Modulation of this neural activity and connectivity during cognitive reappraisal could potentially underlie the improved emotion regulation observed in studies on SSRIs and may give an important insight into potential mechanisms through which benzodiazepines and SSRIs may exert their anxiolytic effects. Furthermore, the 7-day diazepam treatment also increased activation in the left vlPFC and right ACC in response to positive stimuli, which could potentially underlie the increased positive attentional vigilance and positive bias that has been observed in previous diazepam studies (Murphy et al, 2008; Pringle et al, 2016) and may give another important insight into potential mechanisms through which diazepam may exert its anxiolytic effects. These effects are reminiscent of the effects of SSRIs, where a 7-day administration of the SSRI citalopram for example made participants more likely to misclassify negative facial expressions as happy, suggesting a positive bias in facial expression recognition, and increased the relative recall of positive compared to negative emotional material (Harmer et al, 2004). Taken together, these findings raise the possibility that there may be common pathways involved, and that modulation of neural activity related to

emotional processing may be an important part of benzodiazepines' and SSRIs' anxiolytic effects.

9.1.2.2 *Strengths and limitations*

The findings of this study should also be interpreted in light of several strengths and limitations.

As healthy participants were assessed in the current study, it allowed for a clearer assessment of potential effects of the treatment, as the results were unconfounded by psychopathology or changes in symptoms. Also, to the best of my knowledge this is the first investigation of the potential effects of a diazepam treatment on emotional regulation processing. Furthermore, in contrast to previous brain imaging studies on various effects of benzodiazepines, which have focused on a single-dose intervention, the current study investigated a short-term intervention, which has higher clinical ecological validity. The current study was also a randomized double-blind placebo control study, which, as mentioned before, is considered the "gold standard" of intervention-based studies. However, in addition to the strengths mentioned above, there are also some limitations that need to be addressed.

Similar to the losartan study, the generalisability of the current results to clinical populations is limited. Furthermore, the relatively small sample size may have impacted the power of the study to detect broader effects of diazepam. Limitations associated with fMRI also need to be considered, such as the possible confounding effects of global drug-related modulation of the BOLD signal mentioned in section 9.1.1.2. The lack of an objective measure to confirm drug schedule adherence also needs to be addressed. None of the subjects reported missing a

dose or not taking it at the correct time on the test day, but it was not directly tested. With the potential risk of being excluded from the study, there is a slight chance participants may not have been fully honest about it. However, given the group differences in the side effects of lack of coordination, drowsiness, and muscle weakness, it suggests that most of the participants adhered to the drug schedule. Finally, limitations associated with excessive motion during the face classification task also need to be considered, although a single-subject independent component analysis was done to try to mitigate this problem.

9.1.2.3 *Future research*

As findings from the current study suggest that diazepam might modulate activity in the vlPFC and ACC, a future study could investigate this further by using a dot probe task in a scanner. This task could include both masked and unmasked emotional faces, to further investigate whether diazepam increases attentional vigilance to positive stimuli by enhancing vlPFC and ACC activity and whether the effects are limited to stimuli presented subliminally. Furthermore, in addition to fearful and happy facial expressions, the task could include angry facial expressions, as diazepam has also been shown to reduce participants' ability to recognise angry faces (Blair & Curran, 1999; Zangara et al, 2002). A future study might also want to focus on testing participants at multiple time points, to investigate whether neural tolerance does develop between acute and subchronic administrations, as well as to investigate whether there are regional differences in tolerance between areas, as adaptations could occur on different time scales depending on receptor subtypes and brain regions involved.

To control for the possible confounding effects of global drug-related modulation of the BOLD signal, it would be a useful addition in future studies to include a control task that explores the BOLD response within a region that is not expected to be modulated by the drug in question, or to include an ASL scan (see section 9.1.1.3). Furthermore, measuring diazepam plasma concentrations, especially on the test day, might be a useful addition in future studies to ascertain compliance with drug intake. As healthy participants were assessed in the current study in a relatively small sample, future studies should also focus on assessing if these findings can be replicated in a larger and a more diverse cohort of participants (i.e., in highly anxious participants and clinical populations).

9.2 Conclusions

To conclude, this thesis investigated the effects of the two medications losartan and diazepam on memory and emotional processing in two different studies. The results showed that a single dose of losartan, in the absence of overall effects on heart rate, blood pressure, and mood, increased activation in the paracingulate gyrus, insular cortex, lingual gyrus, and fusiform gyrus, which possibly reflects modulation of higher-order visual processing. There was however no evidence found for an effect of losartan on neural responses in the hippocampus during non-emotional memory encoding. The results also showed that losartan increased positive attentional bias, which was reflected in attention being more firmly held by positive stimuli compared to neutral stimuli. Given that both greater reactivity in higher-order visual regions and positive valence training have been shown to be relevant for therapy success (Klumpp et al, 2013b; Dour et al, 2016), the current results may provide further support that losartan might potentially have synergistic effects with exposure therapy. Definite conclusion can however not be drawn until more participants have been recruited in the losartan study. The results also showed that a 7-day diazepam treatment lowered connectivity between the amygdala and the pre- and post-central gyrus during cognitive reappraisal, and between limbic regions and the precuneous cortex in response to aversive pictures. The treatment also led to a decrease in activation in the right vlPFC during reappraisal, and to an increase in activation in the left vlPFC and right ACC in response to positive stimuli, without any subjective changes in mood and state anxiety. These results may provide valuable insights into potential mechanisms through which diazepam may exert its anxiolytic effects. The observations from both studies deserve closer scientific

investigation in future studies and, if replicated, can help inform the use of existing treatments of anxiety disorders and hopefully lead to more effective ones.

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