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FACULTY OF SOCIAL AND HUMAN SCIENCES

Psychology

7.5% CARBON DIOXIDE INHALATION: MODELLING ANXIETY AND ASSESSING PHARMACOLOGICAL EFFECTS

by

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Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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Generalised anxiety disorder (GAD) is a common and distressing mental health problem that is characterised by uncontrollable worry, inattention, and physiological arousal. A better understanding of anxiety disorders could be enabled with effective human models and may improve upon the poor predictive validity of animal models in the identification of treatments. The inhalation of 7.5% carbon dioxide (CO_2) produces robust increases in subjective and physiological symptoms of anxiety (Bailey, Argyropoulos, Kendrick, & Nutt, 2005) and impairs attentional control (Garner, Attwood, Baldwin, James, & Munafo, 2011a) in healthy volunteers. Research in animals (Ziemann et al., 2009) has provided evidence for the critical involvement of the amygdala in the detection and coordination of anxious behaviours to CO_2, however the role of the amygdala in the human response to CO_2 has not been identified. Study One used eye-blink startle magnitude, a well-validated, yet indirect measure of amygdala activity, to examine whether CO_2 inhalation in humans might provoke a fear response coordinated by the amygdala. Despite producing strong anxiogenic effects on subjective mood and autonomic arousal, 7.5% CO_2 had no effect on startle magnitudes. Instead, CO_2 inhalation slowed eye-blink startle latencies. This may be due to fewer available processing resources to respond to an external threat. Study Two investigated the extent to which duloxetine, an effective treatment for GAD, might reduce subjective anxiety and associated deficits in attentional control experienced during 7.5% CO_2 inhalation. Using the antisaccade task, CO_2 inhalation was found to increase the number of antisaccade errors (suggesting poor control of attention) in the placebo group; an effect that was not seen in the duloxetine group. No clear effect of duloxetine on CO_2-induced subjective anxiety or physiological arousal was revealed. Study Three extended these findings with the novel drug memantine, a licenced treatment for cognitive degeneration in Alzheimer's disease that has displayed some preclinical anxiolytic properties (Minkeviciene, Banerjee, & Tanila, 2008). Two week administration of memantine in healthy volunteers resulted in significantly fewer antisaccade errors than placebo, in the absence of any change in subjective mood. Collectively, these results suggest that modelled deficits in attentional control in healthy volunteers can be targeted by known and potential treatments of anxiety disorders, without a comparable decrease in subjective and autonomic symptoms. Examination of symptoms across the anxiety phenotype with human models could help identify better treatments, and understand the neural basis underlying pathological anxiety.

Keywords: Anxiety, 7.5% CO_2

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DECLARATION OF AUTHORSHIP

I, Verity Pinkney declare that this thesis entitled "7.5% CO_2 inhalation: modelling anxiety and assessing pharmacological effects" and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been submitted for publication as:

Pinkney, V., Bamford, S., Munafo, MR., Baldwin, DS., & Garner, M. (2014) Defensive eye-blink startle responses in a human experimental model of anxiety. *J Psychopharmacol, 18*(9): 874-880. Doi: 10.1177/0269881114532858.

A proportion of the data (N =10) for Study One was collected and submitted for an MSc in Research Methods (University of Southampton). This work was substantially extended for inclusion in this thesis.

Signed:

Date:

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Definitions and Abbreviations

5-HT	5-Hydroxytryptamine (Serotonin)
ABM	Attention Bias Modification
ACC	Anterior cingulate cortex
ADHD	Attention Deficit Hyperactivity Disorder
ANT	Attention Network Task
ASIC	Acid sensing ion channel
ATD	Acute tryptophan depletion
BMI	Body mass index
BNF	British National Formulary
BNST	Bed Nucleus of the Stria Terminalis
BZD	Benzodiazepine
CBT	Cognitive Behaviour Therapy
CCK4	Cholecystokinin tetrapeptide
CCTV	Closed Circuit Television
CO_2	Carbon dioxide
COPD	Chronic Obstructive Pulmonary Disease
CRF	Corticotropin-releasing hormone receptor
DBP	Diastolic blood pressure
DLPFC	Dorsolateral prefrontal cortex
DSM-V	Diagnostic and Statistical Manual of Mental Disorders (5^{th} Edition)
ECG	Electrocardiography
EMG	Electromyography
EPM	Elevated plus maze
fMRI	Functional magnetic resonance imaging
GABA	Gamma-Aminobutyric Acid
GAD	Generalised Anxiety Disorder
GAD-7	Generalised Anxiety Disorder 7-item scale
GAD-I	Generalised Anxiety Disorder Inventory
GAD-C	Generalised Anxiety Disorder Criteria Inventory
Glx	Glutamine and glutamate
HAM-A	Hamilton Anxiety Rating Scale
HPA	Hypothalamic-pituitary-adrenal axis

HR	Heart rate
HVPT	Hyperventilation Provocation Test
IAPS	International Affective Picture System
ICD-10	International Classification of Diseases and Related Health Problems
ITI	Inter-trial interval
LC	Locus Coeruleus
MADRS	Montgomery-Åsberg Depression Rating Scale
MDD	Major Depressive Disorder
NA	Noradrenaline
NICE	National Institute for Health and Care Excellence
NK	Neurokinin
NMDA	N-methyl-D-aspartate
NRI	Noradrenaline reuptake inhibitor
OCD	Obsessive Compulsive Disorder
PANAS	Positive and Negative Affect Schedule
PD	Panic Disorder
PFC	Prefrontal cortex
PMDD	Premenstrual Dysphoric Disorder
PSI	Panic Symptom Inventory
PSL-III-R	Panic Symptom List (3rd Edition, Revised)
PSWQ	Penn-State Worry Questionnaire
PTSD	Post-Traumatic Stress Disorder
SAD	Social Anxiety Disorder
SBP	Systolic blood pressure
SCR	Skin conductance response
SNRI	Serotonin-noradrenaline reuptake inhibitor
SSAI	State version of the Spielberger State-Trait Anxiety Inventory
SSRI	Selective serotonin reuptake inhibitor
SSRT	Stop-signal reaction time
STAI	Trait version of the Spielberger State-Trait Anxiety Inventory
TSST	Trier Social Stress Test
UWD	Urbach-Wiethe Disease
VAS	Visual analogue scale

Chapter 1: *Utilising 7.5% carbon dioxide challenge to model human anxiety and to assess pharmacological effects*

Anxiety is a commonly experienced, complex, emotional response that can be adaptive. It directs attention towards salient stimuli and promotes the use of coping behaviours when faced with increasingly demanding situations. However, prolonged and excessive anxiety can become problematic. Pathological anxiety is often early in onset and persistent over long periods which results in substantial impairments in wellbeing and occupational function and constitutes a significant economic burden on society. Anxiety disorders have been reported to account for more lost working days than many highly publicised physical illnesses (such as diabetes and heart disease; Wittchen & Jacobi, 2005) with an estimated annual cost of nearly €75 billion to the EU (Olesen et al., 2012).

Unfortunately, the treatments currently available to alleviate anxiety are not always effective and delivery and engagement with treatment is often suboptimal (Bruce et al., 2005). In addition, the search for new treatments is hindered by poor predictive validity of animal models to determine likely therapeutic success in humans (Nutt & Goodwin, 2011). This has resulted in considerable withdrawal of funding by pharmaceutical companies into the development of new anxiolytics (Van Gerven & Cohen, 2011) and has engendered concern about the future of research into the aetiology and treatment of anxiety (Nutt & Goodwin, 2011). This significantly limits the prospects that psychopharmacology will offer better treatment outcomes for the estimated 69.1 billion individuals in the EU (estimate 2010; Wittchen et al., 2011) who suffer from pathological anxiety.

Modelling anxiety is both complex and challenging. Many animal models exist to assess efficacy of antidepressant/anxiolytic drugs, and these largely rely on Pavlovian conditioning (pairing aversive events with a previously neutral stimulus, e.g. Vogel conflict tests) or the provocation of innate fears (e.g. defensive burying to predator specific stimuli) as behavioural measures of anxiety that can be compared pre- to post-treatment. However, although most animals are able to detect and respond to threat, these models are unlikely to produce the cognitive and behavioural features that are central to human anxiety (Nestler & Hyman, 2010), and it is often difficult to clearly

distinguish between measures of acute fear and chronic anxiety. This reduces the ability of animal models to differentiate between specific anxiety disorders, which vary in terms of higher order emotional characteristics rather than basic physiological reactivity. As such, animal models cannot easily replicate the broad range of clinical symptoms of anxiety (Nestler & Hyman, 2010; Nutt & Goodwin, 2011) and although these models are necessary for gaining an initial indication of safety and efficacy of new drugs, the degree to which they successfully translate across species remains unclear.

In order to address this issue, the development and subsequent validation of novel, experimental medicine models of anxiety in healthy human participants has emerged. Many human models of anxiety have been proposed (such as yohimibine administration, Charney, Heninger, & Redmond, 1983; and the Trier social stress test (TSST), Kirschbaum, Pirke, & Hellhammer, 1993), and these vary in their ability to provoke anxiety effectively. Some induce principal symptoms of specific disorders (such as CCK4-induced panic attacks; Jerabek, Boulenger, Bradwejn, Lavallee, & Jolicoeur, 1999), whilst others more broadly model anxiety, stress or fear (such as the TSST as a model of psychosocial stress, which has some specificity to social phobia/anxiety; Kirschbaum et al., 1993; see section 1.5.3). Robust and well-validated healthy human models could bridge the gap between preclinical assessments in animals and clinical trials in humans to determine the therapeutic and economic viability of potential anxiolytics earlier in drug development, and may also provide insight into the mechanisms that underlie human anxiety. With this in mind, 7.5% carbon dioxide (CO_2) inhalation is one model of anxiety in healthy volunteers that has shown promise. It is this model that is the central feature of the present review.

This thesis aims to address two questions:

1. Is 7.5% CO_2 inhalation a valid model of anxiety (rather than acute fear) in healthy volunteers, which shares similarities with generalised anxiety disorder (GAD)?
2. Can the 7.5% CO_2 model be used to evaluate potential efficacy of novel pharmacological treatments for pathological anxiety, such as GAD?

Consequently this review will provide a discussion and critique of both the 7.5% CO_2 model and GAD and will evaluate the similarities between the transient symptoms provoked by CO_2 and the clinical presentation of GAD. The 7.5% CO_2 model will then

be compared against analogous human models of anxiety. Finally, the effects of established anxiolytics on subjective and physiological responses to CO_2 will be examined.

1.1 Carbon dioxide (CO_2) inhalation

The inhalation of air enriched with CO_2 in healthy volunteers has been shown to safely and reliably provoke short-term, subjective and autonomic responses consistent with an anxious state (Bailey et al., 2005). The induction of anxiety through chemical means has largely been used as an exploratory tool for panic disorder, with a single, vital-capacity inhalation of air enriched with 35% CO_2 representing a well-established means of producing panic-like symptoms, such as dyspnoea, choking and dizziness (Van Den Hout & Griez, 1984). The administration of different concentrations of CO_2 allows for investigation across the broad spectrum of anxiety disorders, with higher doses (35% CO_2 most commonly) producing symptoms akin to panic disorder, and more recently, the suggestion that lower doses (5-8% CO_2) may induce some of the symptoms of generalised anxiety (Bailey, Dawson, Dourish, & Nutt, 2011a).

In recent years, inhalation of air enriched with 7.5% CO_2 has been identified as a useful experimental tool of anxiety. Inhalation of 7.5% CO_2 for 20 minutes produces measurable responses in healthy volunteers including changes in subjective state and autonomic arousal (Bailey et al., 2005). Specifically, 7.5% CO_2 temporarily raises heart rate and blood pressure and increases subjective anxiety and worry, whilst decreasing positive states such as feeling happy and relaxed (Bailey et al., 2005). These effects begin within the first few minutes of the inhalation and persist until its termination. The longer duration of lower CO_2 concentrations arguably induces a more sustained anxiety state rather than the short-lived, intense panic associated with 35% CO_2.

The transient experience of anxiety, worry and physical symptoms produced by 7.5% CO_2 shares some similarities with the chronic symptoms outlined in the DSM-5 criteria for GAD (see Table 1). This has led to the suggestion that 7.5% CO_2 inhalation may provide a useful tool with which to model this disorder, particularly with the search for new drug targets in mind. For example, restlessness, difficulty concentrating and elevated worry and anxiety, are commonly reported experiences of 7.5% CO_2 inhalation and also formulate part of the diagnosis for GAD (Bailey et al., 2005; see

Table 1). However, the degree to which the experiences of healthy volunteers breathing CO_2 encompass the full range of symptoms experienced by GAD patients is not known. Therefore, since 7.5% CO_2 is a state-based model, it is probable that it can only go so far in replicating the chronic symptoms of GAD. Despite this limitation, 7.5% CO_2 challenge could have value for the purposes of furthering anxiety research and drug development prior to costly clinical trials. This is discussed in more detail throughout section 1.4.

1.2 Generalised anxiety disorder (GAD)

Generalised anxiety disorder (GAD) is one of the most frequent anxiety disorders in primary care affecting an estimated 8.9 million people in the EU in 2011 (Wittchen et al., 2011). If not properly diagnosed or treated, GAD can develop into a chronic condition that lasts for months or years and often shares comorbidities with other debilitating forms of psychiatric illness, particularly major depression (American Psychiatric Association, 2013). The core cognitive feature of GAD is pervasive, uncontrollable worry and anxiety. These topics of worry are often indistinguishable from the concerns of healthy individuals, yet tend to be disproportionate to the situation. For diagnosis, worrisome thoughts must cause significant anxiety, distress and impairment to social and occupational functioning, and must occur more days than not. Physical symptoms, such as muscle tension and sleep disturbance accompany these cognitive symptoms and often drive initial utilisation and later overreliance on healthcare services (Wittchen, 2002) which can delay diagnosis (see Table 1 for full DSM-5 criteria). Finally, neurocognitive features, such as attentional threat-biases have also been observed in patients with GAD[1] (Mogg & Bradley, 2005).

[1] Some overlap between these domains is evident – for example worry is a subjective experience but is a likely consequence of poor cognitive control. For the purpose of this review, worry has been described as a subjective symptom of GAD since it is most often measured as such, although the intrinsic relationship between worry and neurocognitive processes should not be undervalued.

Table 1. DSM-5 Criteria for Generalised Anxiety Disorder.

DSM-5 Criteria for GAD
A. Excessive anxiety and worry about a number of events or activities, more days than not for 6 months or more
B. Difficulty controlling worry
C. 3 or more of the following symptoms, some of which are present more days than not:
1. Restlessness, feeling keyed up or on edge
2. Easily fatigued
3. Difficulty concentrating
4. Irritability
5. Muscle tension
6. Sleep disturbance, such as difficulty falling to sleep or restless sleep

Note. For diagnosis, A-C must cause significant distress or impairment to daily functioning, be independent to a physiological condition, and not be attributable to another mental disorder.

1.3 Anxiety and attention

Cognitive models regard maladaptive attentional processes to be central in the aetiology and maintenance of anxiety. Cognitive dysfunction in anxiety is characterised by general hypervigilance towards threat (Bar-Haim, Lamy, Pergamin, Bakermans-Kranenburg, & van IJzendoorn, 2007), difficulties disengaging from threat (Fox, Russo, & Dutton, 2002), and appraisal of ambiguous information as negative (or less positive; see Van Bockstaele et al., 2014 for a review on attentional biases in anxiety). Cognitive biases contribute to the emergence and continuation of a range of anxiety symptoms and disorders, including excessive uncontrollable worry, increased distractibility and apprehension.

The theoretical basis of attentional deficits in anxiety focuses largely on the concept of attentional control (Eysenck, Derakshan, Santos, & Calvo, 2007). Attentional control theory extends processing efficiency theory (Eysenck & Calvo, 1992), which assumes that anxiety impairs the efficiency of performance (such as reaction times) more so than the effectiveness of performance (such as response accuracy). Attentional control theory maintains this assumption, and also proposes that anxiety is experienced when a current goal is threatened and therefore increases attention to locating the source of the threat. This creates competition between task-relevant and stimulus-driven inputs, where focused attention on a goal is interrupted by the search for potential threat

(Eysenck et al., 2007). Thus according to attentional control theory, anxious individuals are more prone to distraction due to anxiety-driven hypervigilance. They struggle to ignore irrelevant information (whether threatening or not), which makes sustained attention increasingly challenging (e.g. Reinholdt-Dunne, Mogg, & Bradley, 2009).

1.3.1 Evidence from neuroimaging studies

In humans, current neural models suggest neurocognitive mechanisms involved in the response to threat are centrally coordinated by an amygdala-prefrontal circuitry. Disruption of this circuitry, characterised by hyper-responsive amygdala activity to threat and poor prefrontal control, is thought to be critical to the existence of threat-related attentional biases in anxious individuals (Bishop, 2008). This drives 'bottom-up' hypervigilance and insufficient 'top-down' control in the allocation of attention to, and interpretation of emotionally salient information. Evidence for deficient amygdala-prefrontal circuitry has observed hyper-responsivity of the amygdala to threat in state anxious individuals (Bishop, Duncan, & Lawrence, 2004b) and reduced recruitment of prefrontal regions to inhibit threat distractors (Bishop, Duncan, Brett, & Lawrence, 2004a). Poor connectivity between the amygdala and prefrontal regions has also been identified during threat processing (Monk et al., 2008). These findings are consistent with cognitive models of anxiety (e.g. attentional control theory; Eysenck et al., 2007), which suggest that poor recruitment of prefrontal mechanisms impairs attentional control over amygdala-driven hypervigilance to threat (Bishop, 2009).

1.3.2 Common measures of attentional biases in anxiety

Many behavioural tasks have been proposed to measure attentional biases in anxiety, of which the Stroop and dot-probe tasks are amongst the most well-known. The wealth of tasks developed has given insight into the type of stimuli (e.g. threat-related and/or distractor stimuli), the processing requirements (conscious versus non-conscious) and task requirements (such as inhibition, shifting functions) needed to elicit these biases. Taken together, these tasks provide strong evidence for the existence of attentional biases in subclinical and clinical anxiety.

1.3.2.1 Emotional variant of the Stroop task

The original colour-naming Stroop task has been modified to test attentional biases in anxiety (Williams, Mathews, & MacLeod, 1996). In the emotional variant of the Stroop task, participants are required to name the colour of emotionally salient (e.g. "fear") or emotionally neutral (e.g. "room") words. Reaction times indicate the ability to suppress the semantic context of a word in preference for its physical colour. The presence of threat-related biases is assumed if reaction times to name the colour of threat stimuli exceed that of neutral stimuli. In comparison to low anxious individuals, those high in anxiety display slower reaction times to threat, relative to neutral stimuli (Bar-Haim et al., 2007; Williams et al., 1996).

The emotional Stroop task has been criticised as it is difficult to pinpoint the cause of the interference induced by threat-related stimuli. For example, this might reflect deliberate avoidance of processing threatening information rather than greater attention towards these stimuli (De Ruiter & Brosschot, 1994). In addition, it is possible that delayed latencies to threat could be the result of processes aside to attention, such as increased negative mood (MacLeod, Mathews, & Tata, 1986). These limitations were instrumental in the development of the dot-probe task.

1.3.2.2 Dot-probe task

The dot-probe task (developed by MacLeod et al., 1986) has been widely used to examine attentional bias in anxiety. In this task, two words or images are presented simultaneously on either side of the visual field. One stimulus is threat-related (e.g. a fearful expression) and the other is neutral. A target probe is then presented in the location of the preceding threat stimulus (threat-congruent), or in the location of the neutral stimulus (threat-incongruent). The speed at which a participant responds to the probe is measured, and faster reaction times to probes following threatening stimuli relative to neutral stimuli indicate the presence of a threat-related attentional bias. High anxious individuals have been shown to respond faster to threat-congruent probes, relative to neutral probes (review by Bar-Haim et al., 2007). For example, patients with GAD show greater vigilance (faster reaction times) to probes following emotional, rather than neutral facial expressions (Bradley, Mogg, White, Groom, & De Bono, 1999).

1.3.2.3 Antisaccade Task

The antisaccade task requires participants to direct attention towards (prosaccade) or away (antisaccade) from images that vary in their content. Top-down control is needed on antisaccade trials to inhibit erroneous eye-movements towards an abruptly presented image, and to instead generate a saccade away. Error rates on antisaccade trials and latencies to initiate a correct saccade are the primary measures of the antisaccade task, and can indicate the degree to which processing efficiency (time taken to generate a correct antisaccade away from threat) and performance effectiveness (erroneous prosaccades towards threatening stimuli) are impaired.

Increased errors on antisaccade trials, and delayed latencies to generate a correct saccade away from a target are a common finding in subclinical anxiety (see Ainsworth & Garner, 2013 for a review; e.g. Derakshan, Ansari, Hansard, Shoker, & Eysenck, 2009). Initial use of a modified version of the task has also provided evidence to suggest similar deficits exist in GAD (Jazbec, McClure, Hardin, Pine, & Ernst, 2005). Electrophysiological measures of antisaccade performance in high anxious (vs. low anxious) individuals implicate activity in frontocentral sites in preparation of antisaccade trials (Ansari & Derakshan, 2011) which converges with neural models of anxiety (Bishop, 2007). This further supports evidence of poor recruitment of prefrontal regions in anxiety.

1.3.2.4 Attention Network Test

The Attention Network Test (ANT) assesses three core aspects of attention; alerting, orienting and executive control, within one behavioural task (Fan, McCandliss, Sommer, Raz, & Posner, 2002). These three networks play functionally different roles in attention, with the alerting system coordinating an active and sustained alert state, the orienting system instigating selection of salient information, and the executive control system monitoring and resolving conflict between competing inputs in a manner similar to attentional control (Posner & Petersen, 1990).

The ANT requires participants to respond to the direction of a central arrow (see Figure 4, Chapter 3). This arrow appears either above or below a central fixation cross, and is preceded by an alerting cue (a central or double asterisk), a spatial cue (an asterisk in the location of the following arrow), or no cue. The target arrow is also

flanked by four distractor arrows (two either side) that are either congruent (→→→→→) or incongruent (←←→←←) in direction to the target. The influence of these cues and flanker arrows can indicate how efficiently the three attention networks are performing. The alerting effect is calculated by subtracting the mean reaction time of double-cue trials from no cue-trials. The orienting effect is calculated by taking the mean reaction time of spatial cue trials from centre cue trials. Finally the executive control effect is calculated by subtracting the mean reaction time of congruent flanker trials from incongruent flanker trials.

Previous research suggests greater alerting and orienting in high state anxious individuals, consistent with a broad state of hypervigilance to potential threat (Pacheco-Unguetti, Acosta, Marques, & Lupianez, 2011). Furthermore, high trait anxious and clinically anxious individuals display deficiences in executive control, reflecting poor regulation of once adaptive prioritisation of bottom-up stimuli over task-relevant stimuli (Pacheco-Unguetti et al., 2011). These studies provide a distinction between the effects of state and trait anxiety on different mechanisms of attention in the absence of threat-related stimuli.

Parallels can be drawn between the executive control measure of the ANT and performance on antisaccade trials in the antisaccade task. Both constitute a measure of attentional control and particularly the inhibition function of the central executive (Miyake et al., 2000); the ability to suppress distracting information in preference for the task. However, the antisaccade task can utilise threat-relevant information, and is therefore able to examine the impact of threatening or disorder-specific stimuli on attentional control. This differs to the ANT which uses neutral distractor stimuli only. The ANT has faced some criticism due to evidence that repeated trials can diminish flanker interference and error rates. This would be a particular problem for repeated measures designs, although a recent assessment of the robustness of the ANT suggest consistent network scores overtime, despite evidence for practice effects (Ishigami & Klein, 2010). In addition, consistent correlations between cue condition and flanker condition have been seen in this task which suggest that the measures on the ANT may not be entirely independent of one another (MacLeod et al., 2010). In a review of the ANT, MacLeod et al. (2010) conclude that using more refined measures of the components of attention alongside the ANT would give greater confidence in the ability of this task to accurately measure each network.

1.3.3 Summary: attention and anxiety

The wealth of behavioural tasks examining attentional processes in anxiety provides strong evidence for the role of impaired attentional control and greater threat-related biases in the maintenance of an anxious state. This is further supported by theoretical models of anxiety, and converging evidence from imaging studies that together suggest overactive amygdala activation to threat in anxiety (which underlies threat-related attentional biases) is not adequately controlled by prefrontal regions.

1.4 Is 7.5% CO_2 inhalation a valid model of anxiety that produces symptoms akin to generalised anxiety disorder (GAD)?

In light of the broad spectrum of symptoms of GAD and the key role attentional processes play in anxiety disorders, for 7.5% CO_2 challenge to be considered a valid model of anxiety it would need to provoke symptoms across all domains (subjective, autonomic and neurocognitive). The evidence for each category will now be discussed in turn. Key evidence is summarised in Table 2.

1.4.1 Subjective and emotional response to 7.5% CO_2

1.4.1.1 Anxiety and worry

In the first published evaluation of the subjective effects of 20 minutes 7.5% CO_2 inhalation in healthy volunteers, Bailey et al. (2005) found changes in 10 of 11 visual analogue scales taken at peak effects including increased anxiety, worry and fear. This anxiety profile has since been replicated in a number of studies with a range of outcome measures, including simple visual analogue scales (e.g. de Oliveira, Chagas, Garcia, Crippa, & Zuardi, 2012; Poma et al., 2005) and established questionnaire measures for anxiety (using the state version of the state-trait anxiety inventory most notably (Bailey, Kendrick, Diaper, Potokar, & Nutt, 2007a; Garner et al., 2011a; Garner, Attwood, Baldwin, & Munafo, 2012) and the GAD criteria inventory (GAD-I; Argyropoulos, 2009; Bailey et al., 2011b)). Poma et al. (2005) for example, reported an average increase of 45.4 (± 32.1) points on a 100-point visual analogue scale for anxiety in responders; an effect that remained with repeated testing.

A core cognitive component of GAD is persistent, excessive worry that is difficult to control (American Psychiatric Association, 2013). Direct analysis of uncontrollable worry during 7.5% CO_2 has not been systematically conducted. Initial evidence has shown that visual analogue scales for worry increase at the peak effects of CO_2 inhalation when compared to air (e.g. Bailey et al., 2005), however whether CO_2 activates general worries, or worries that directly relate to the experience of the challenge remains unclear. Further research could assess worry during CO_2 inhalation by collecting broad qualitative reports of mood which would complement questionnaire measures. Periodic assessment of worrisome thoughts throughout CO_2 challenge (such as with a thought intrusions task; Ruscio & Borkovec, 2004) would also indicate if non-specific worry is a prominent feature of CO_2 inhalation. Since worry is a core clinical symptom of GAD, it is important to assess whether inhalation of 7.5% CO_2 can induce intrusive worry which can then be targeted by pharmacological and/or psychological therapies.

1.4.1.2 Panic

Significant increases in panic have also been found across a number of studies and measures (e.g. using the Panic Attack Questionnaire (PAQ), Bocola, Trecco, Paladini, Sollecito, & Martucci, 1998; and the Panic Symptoms List (PSL-III); Poma et al., 2005, both of which are based on DSM-III criteria for panic disorder). Bailey et al. (2005) observed increases in panic symptoms on the Panic Symptom Inventory (PSI) following 7.5% CO_2 inhalation, which differed from the symptoms following air inhalation (control). This scale focuses more so on physiological symptoms such as "heart racing", "apprehension" and "breathlessness" that are prominent features of panic disorder/attacks rather than changes in subjective mood. Although increases on the PSI after 7.5% CO_2 did not differ from a 35% CO_2 study (indicating similar arousal), there was variation in the type of symptoms reported, with greater ratings of "tension" and "tight muscles" at peak 7.5% CO_2. This illustrates quantitative differences between 7.5% and 35% CO_2 challenges, with lower concentrations eliciting symptoms more similar to the profile of GAD in DSM-5 rather than panic (see Table 1).

1.4.1.3 Fear and Anxiety

The distinction between fear and anxiety is important when characterising the effects of CO_2 challenge. The inhalation of lower concentrations of CO_2 is proposed to

more closely approximate anxiety towards a distal, often ambiguous threat, whereas single inhalation of 35% CO_2 robustly provokes panic and fear (Bailey et al., 2005; Van Den Hout & Griez, 1984). Measurable differences between stages of the defence cascade (see below) could be useful in the validation of 7.5% CO_2 challenge as a model of anxiety, rather than an acute fear response.

Evidence that fear and anxiety are distinct entities is largely supported by research examining fear-potentiated startle (Davis, Walker, Miles, & Grillon, 2010). The startle reflex is an automatic, defensive response (most often measured by eye-blink magnitude) to an intense, startling stimulus. Magnitude of eye-blinks to a startle can be amplified by contextual-fear, such as by threat of electric shock, or air-puff (e.g. Grillon, Baas, Lissek, Smith, & Milstein, 2004), and by presentation of unpleasant threat stimuli (e.g. viewing aversive images from standardized picture sets; Smith, Bradley, & Lang, 2005). In a recent review Davis et al. (2010) suggests that fear and anxiety can be dissociated with variants of the startle task, the findings of which lend support for differences in the neurology that underlies these two states. Since fear is operationalised as phasic, the pairing of discrete cues (startling noise) with an aversive event (e.g. electric shock) is used to provoke this state. Conversely, anxiety is operationalised by Davis et al. (2010) as "sustained fear", which can be elicited by the presentation of unpredictable, diffuse cues (e.g. threat of shock, context) that might result in an unpleasant event. Startle magnitudes under these scenarios appear to be mediated by independent (yet overlapping) neural systems, with the central nucleus of the amygdala particularly prominent in phasic fear, and bed nucleus of the stria terminalis in sustained fear (Davis et al., 2010; Walker & Davis, 1997). While inhalation of low concentrations of CO_2 has been seen to trigger anxious behaviour in small animals via the amygdala (Ziemann et al., 2009), its effects on defensive behaviours that are mediated by the extended amygdala in humans are not currently known (see Study One, Chapter 2).

Based on research in animals examining defence, fear and anxiety are considered functionally, behaviourally and pharmacologically distinct, despite regular co-occurrence and interaction (McNaughton & Corr, 2004). Defensive responses to threat can be considered across a defence cascade (Blanchard & Blanchard, 1989; Bradley & Lang, 2002; Faneslow, 1994) – see Figure 1. A three stage continuum of defensive behaviour is characterised by an anxious, preparatory state of vigilance (pre-

encounter), which is followed by freezing behaviours and orienting of attention to determine risk of threat (post-encounter). This culminates in direct defensive action (fight) or escape (flight) when danger is perceived as proximal and imminent (circa-strike). The post-encounter stage primes alertness and attentional vigilance to monitor the risk associated with an often ambiguous threat, and is related most strongly with anxiety. Conversely, the circa-strike stage is exemplified by a quick and intense state of fear and panic that encourages immediate action such as escape, avoidance or attack. These stages align with various cardiovascular changes that promote a behavioural response, for example heart rate deceleration has been observed as potential risk increases, prior to abrupt acceleration just before escape/capture (Low, Lang, Smith, & Bradley, 2008). Pharmacological assessments in rodents and humans suggest that anxiety behaviours (pre- and post-encounter) are sensitive to anxiolytics, whereas fear behaviours (circa-strike) are not (Blanchard, Griebel, Henrie, & Blanchard, 1997; McNaughton & Zangrossi, 2008 - see page 14 for a summary).

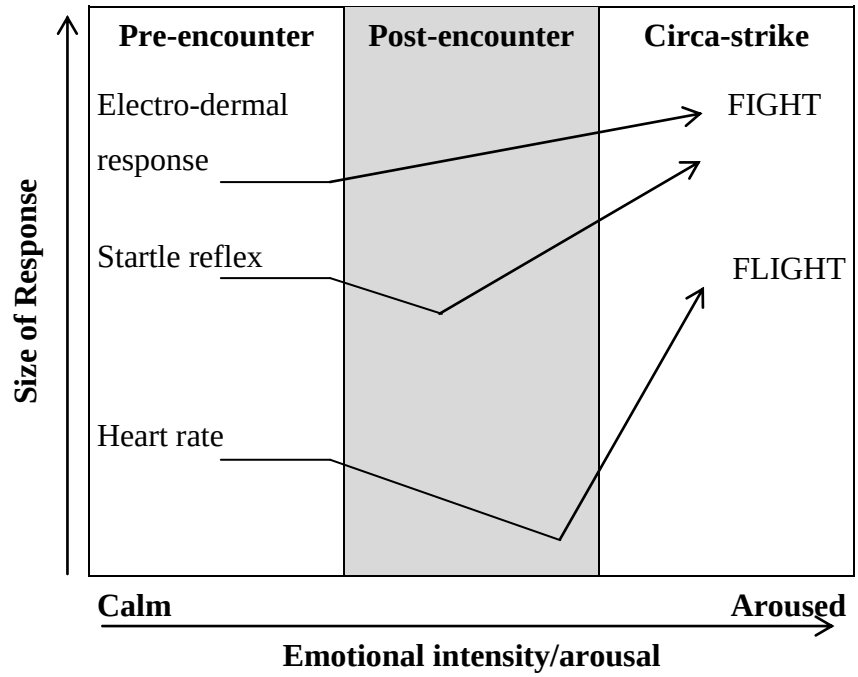

Figure 1. Defence Cascade Model. Adapted from Bradley, Codispoti, Cuthbert, and Lang (2001). The relationship between physiological responses (such as sweating, startle and heart rate) and defensive stage.

The behavioural and pharmacological differences between fear and anxiety are useful for comparison with the responses elicited by animal and human models. This distinction helps validate 7.5% CO_2 as a model of anxiety rather than acute fear.

1.4.1.4 Negative Affect

Broader effects of CO_2 on the symptoms of negative mood have also received attention due to the considerable comorbidity between anxiety and depression (Wittchen et al., 2002). For example, Hood et al. (2010) did not find any change in the depression/dejection scale of the Profile of Mood States (POMS; McNair, Lorr, & Droppleman, 1988) pre-to-post 7.5% CO_2 inhalation, yet Garner et al. (2011a) report consistent increases in negative mood immediately after CO_2 (using the positive and negative affect scale (PANAS); Watson, Clark, & Tellegen, 1988) that have since been replicated (Cooper et al., 2011; Cooper et al., 2013; Garner et al., 2012). This illustrates the influence of CO_2 inhalation on general mood states, particularly negativity. This would be expected by a model of anxiety when commonalities with depression are considered.

1.4.1.5 CO_2-induced anxiety in patients

In GAD patients, 7.5% CO_2 increases both the presence of anxiety (consistent with the symptoms of their diagnosis), and also panic (Seddon et al., 2011). This may reflect a limitation of the model to distinguish a general anxiety response from panic, although of those subjects, 2 of the 3 had experienced panic attacks previously. This is consistent with evidence reporting increased panic in those with a current diagnosis or past history of panic (Gorman et al., 2001), both of which are core exclusion criterion in healthy volunteer studies (Bailey et al., 2005). As such patients with comorbid psychiatric illness (a common occurrence in GAD) are more likely to show an unpredictable and varied response to CO_2 inhalation compared to patients with a single diagnosis.

1.4.2 Physiological responses to 7.5% CO_2

The ICD-10 criteria (World Health Organisation, 1992) subtypes the physiological symptoms reported in GAD. For example, autonomic arousal symptoms (e.g. accelerated heart rate, trembling, sweating); symptoms concerning chest and abdomen (e.g. difficulty breathing, nausea); and symptoms of tension (e.g. difficulty swallowing, aches and pains) reflect a range of somatic sensations often experienced by patients.

Table 2 summarises evidence of increased autonomic arousal as a result of 7.5% CO_2 inhalation. Elevated heart rate, blood pressure and sweating are reliable features of 7.5% CO_2 inhalation that are comparable to the autonomic arousal symptoms of GAD (Hoehn-Saric, McLeod, Funderburk, & Kowalski, 2004), further validating this model. Short durations of 7.5% CO_2 inhalation have also been shown to increase skin conductance levels suggesting elevated physiological and/or psychological arousal (Pappens, De Peuter, Vansteenwegen, Van den Bergh, & Van Diest, 2012). In addition, Garner et al. (2011a) found CO_2-induced increases in subjective anxiety were strongly associated with elevated heart rate but not blood pressure, consistent with possible use of interoceptive cues by participants when evaluating their current mood. Whilst continuous measurement of skin conductance (e.g. Poma et al., 2005), blood pressure and heart rate have been collected previously (e.g. Bailey et al., 2005; Cooper et al., 2011; Diaper et al., 2012a), temporal examination of this data would indicate if any ebb and flow in these symptoms exists during 7.5% CO_2 inhalation and give a more precise picture of change than single measures can provide.

Subjective reports on the panic symptom inventory (PSI; a scale that largely contains physiological items) have revealed greater numbers of panic symptoms immediately after 7.5% CO_2 inhalation (e.g. Bailey et al., 2005). Specific analysis of PSI data into psychic (such as “anxiety”, “feelings of unreality” and “fear of loss of control”) and somatic (such as “shakiness”, “churning of stomach” and “heavy headed”) categories would further clarify the kind of responses most commonly provoked by CO_2 inhalation and might indicate whether subjective and physiological reports are related or distinct. In conjunction with questionnaire data, recording qualitative descriptions would help to detail the full extent of physiological (and other) symptoms experienced during CO_2 inhalation.

Table 2. Subjective, autonomic and neurocognitive features of GAD in healthy human studies using 20 minutes of 7.5% CO_2 to model anxiety

Reference	Subjective		Cognitive			Autonomic		Explanatory notes
	Anxiety	Mood	Task		Performance	BP	HR	
Bailey et al. (2005)	✓	↑ feelings of worry, fear, tense and irritable. ↓ happiness.	-		-	✓	✓	-
Poma et al. (2005)	✓	-	-		-	-	-	↑ anxiety at test-retest indicates good repeatability of CO_2. ↑ respiratory rate, skin conductance, minute volume and end-tidal CO_2.
Diaper et al. (2011)	✓	↑ fear, feel like leaving, paralysed and ↓ happiness and relaxed.	Tracking and digit response task	**?**	No effect on tracking. Improved digit response, but slowed speed.	✓	✓	May reflect greater arousal or less attentional resources available.
	✓	↑ fear, feel like leaving, nervous, paralysed and ↓ alert, relaxed and happy.	Radar task	✗	No effects of CO_2 on task performance.	✓	✓	-
Cooper et al. (2011)	✓	↑ worry, fear, feel like leaving, stressed, tense, nervous and negative affect. ↓ positive affect, relaxed and happy.	Dot-probe	✗	Vigilant to emotional stimuli during CO_2 in Exp 1, and during air in Exp 2.	✓	✓	Inconsistent findings for bias for emotive faces

Is 7.5% CO_2 inhalation a valid model of anxiety?

Reference	Subjective		Cognitive			Autonomic		Explanatory notes
	Anxiety	Mood	Task		Performance	BP	HR	
Garner, Attwood, Baldwin, James, and Munafo (2011)	✓	↑ negative affect and ↓ positive affect	Antisaccade	✓	↑incorrect eye movements towards negative stimuli and delayed time to generate correct antisaccades	✓	✓	Suggests CO_2 induced hypervigilance and poor threat inhibition
Garner et al. (2012)	✓	↑ negative affect and ↓ positive affect	ANT	**?**	CO_2 improved alerting and orienting functions of attention	✓	✓	Might indicate hypervigilance but not changes in higher-order executive function
Attwood, Penton-Voak, Burton, and Munafo (2013)	✓	-	Face-matching task	**?**	↓ accuracy on face-matching task, specifically impairing hit rate but not false alarms.	✓	✓	Identification inaccuracy might be heightened by acute anxiety
Brambilla et al. (2013)	✓	-	-	-		-	-	-
Cooper et al. (2013)	✓	↑ negative affect. ↑ feeling worried, fearful, stressed, nervous, relaxed and tense.↓ happiness.	CCTV ratings	**?**	Trend (p = .082) for increased reports of suspicious behaviour	✗	✓	Suggests a possible bias toward negative evaluations

Is 7.5% CO_2 inhalation a valid model of anxiety?

Reference	Subjective		Cognitive			Autonomic		Explanatory notes
	Anxiety	Mood	Task		Performance	BP	HR	
Mattys, Seymour, Attwood, and Munafo (2013)	✓		Speech perception task	✓	↑ reliance on lexical status of a syllable and ↓ discrimination between phonemes	✓	✓	Suggests anxiety consumes resources away from important acoustic details and instead rely on lexical plausibility.
Attwood, Catling, Kwong, and Munafo (2015)	✓	↑ negative affect and ↓ positive affect	Face recall task	✓	↓ identification accuracy during CO_2	✓	✓	Anxiety at point of recognition can impair accurate facial recall

Note. BP = blood pressure. HR = heart rate. All studies reported used 20 minutes of 7.5% CO_2 except Brambilla et al. (2013); and Poma et al. (2005) where 7% was used. ✓indicates evidence for that feature of GAD was present. **X** indicates no support for that feature of GAD. **?** indicates the literature concerning this feature of GAD is unclear.

7.5% CO_2 challenge has a clear effect on cardiovascular symptoms, raising blood pressure and heart rate. Although anecdotal reports exist for a wider range of physiological responses to CO_2 inhalation (such as nausea, heavy-headedness), further analysis of these changes and how they interact with subjective responses is warranted.

1.4.3 Cognitive responses to 7.5% CO_2

An ability for 7.5% CO_2 to induce cognitive biases in healthy individuals would further validate experimental models of anxiety. Recent evidence suggests that 7.5% CO_2 inhalation in healthy individuals can induce biases in attention that are observed in unchallenged sub-clinical and clinical anxiety. Garner et al. (2011a) examined the effect of 7.5% CO_2 inhalation on attention to threat within an antisaccade task. 7.5% CO_2 inhalation was shown to increase erroneous eye movements towards negative images on antisaccade trials (Garner et al., 2011a). This is consistent with hypervigilance towards and poor control of attention away from threat and complements neural models of anxiety whereby the amygdala response to threat is poorly controlled by prefrontal regions (Bishop, 2009). This provides some evidence that 7.5% CO_2 inhalation can temporarily activate cognitive and behavioural mechanisms that characterise anxiety, including GAD.

However, in a series of experiments using an emotional variant of the dot-probe task Cooper, Rowe, Penton-Voak, and Ludwig (2009) found inconsistent effects of 7.5% CO_2 on allocation of attention to emotional faces, despite replication of the anxiogenic effects of CO_2. The first study identified an attentional bias towards emotional facial expressions (irrespective of valence) whilst subjects inhaled 7.5% CO_2, consistent with hypervigilance during a state of heightened anxiety. However, the second study found the same attentional bias to emotional faces but during inhalation of air. These findings question the ability of 7.5% CO_2 to reliably induce threat-related attentional biases seen in anxiety, but more likely reflect greater concern with the use of the dot-probe task as a reliable assessment of attentional bias in repeated measures designs (Schmukle, 2005).

Alterations in attentional networks during CO_2 challenge have also been examined. Garner et al. (2012) combined the attention network test (ANT; Fan et al., 2002) with 7.5% CO_2 inhalation to investigate effects of CO_2 on alerting, orienting and

executive control. 7.5% CO_2 inhalation selectively increased alerting and orienting functions of attention when compared to air, with no effect on executive control (Garner et al., 2012). This reflects a possible limitation of 7.5% CO_2 inhalation to replicate anxiety fully as, although stimulus-driven/bottom-up attentional processes were induced consistent with patterns observed in state anxiety, the model did not induce trait-anxious deficits in executive control. However, this study also reported associations between high trait anxiety and the orienting network function (but not alerting or executive control) which suggests trait anxiety has a more global effect on attention during CO_2 inhalation. This emphasises a limitation of a state-like model to induce chronic symptoms that are more akin to trait and clinically anxious individuals. Additional research is needed to disentangle the effects of trait and state anxiety on neurocognitive mechanisms to draw further claims about the validity of 7.5% CO_2 inhalation to temporarily replicate dispositional and/or situational elements of anxiety. Examination of high and low trait anxious groups (and possibly a clinically anxious group such as GAD patients) on response to 7.5% CO_2 challenge would provide this insight.

A predisposition to interpret ambiguous information as negative or threatening is central to neurocognitive models of anxiety (Eysenck, Mogg, May, Richards, & Mathews, 1991). This bias encourages greater perceptions of threat and danger than may truly be present, and so sustains an anxious state that is primed to spot potential harm. In a series of experiments Cooper et al. (2013) provide initial evidence to show that 7.5% CO_2 challenge can increase the number of interpretations of ambiguous information as negative. Participants viewed closed-circuit television (CCTV) clips whilst inhaling 7.5% CO_2 and normal room air. Increased ratings of negative and suspicious behaviour were identified during inhalation of 7.5% CO_2, with no differences in ratings of positive behaviour. This supports 7.5% CO_2 as a valid model of anxiety that can elicit cognitive biases consistent with an anxious state. To date this CCTV paradigm has not been used to reveal interpretative biases in trait anxious individuals. Thus future CO_2 studies should use established interpretative bias tasks (e.g. homophone and homograph paradigms) that have been validated in trait and clinical anxiety.

Together, these findings indicate 7.5% CO_2 challenge can successfully induce neurocognitive deficits seen in anxiety. Use of neurocognitive tasks within this model provide supplementary outcome measures that can be used to evaluate the therapeutic

potential of novel pharmacological compounds. Additional research is required to assess the reliability of these results and to probe whether inconsistent findings are due to restrictions of the model or reflect wider concerns with the employed tasks.

1.4.4 Biological similarities between anxiety and inhalation of 7.5% CO_2

A key question that remains unanswered is how inhalation of 7.5% CO_2 causes anxiety. Although 7.5% CO_2 inhalation has clear anxiogenic effects, the mechanisms through which these occur remain unclear. Animal research has provided compelling evidence to pinpoint neural structures that can both directly detect changing CO_2 levels and coordinate a fear response (Ziemann et al., 2009). However, whether these findings translate across species is not well investigated.

Various areas of the brain involved in fear and anxiety contain neurons that are sensitive to CO_2, such as the locus coeruleus, medullary raphe and the amygdala (Esquivel, Schruers, Maddock, Colasanti, & Griez, 2009). Recent research in rodents has identified the amygdala as an important chemosensor that directly detects extracellular hypercapnia and corresponding decreases in brain pH, via acid sensing ion channels1a (ASIC1a) (Ziemann et al., 2009). Inhalation of 10% CO_2 triggered fear behaviours in mice, such as increased freezing and reduced time spent in open field, and importantly, these behaviours were abolished or reduced in mice where ASIC1a expression was inhibited. Similarly, in humans CO_2 inhalation produces strong activation of limbic regions including the amygdala which was hypothesised to play an important role in respiratory control (Brannan et al., 2001). However, a recent study in patients with bilateral amygdala lesions revealed that 35% CO_2 inhalation could produce a robust fear response, despite pathological lesions that prevent any previous experience of fear (Feinstein et al., 2013).

Together, these findings indicate that whilst acid sensing ion channels within the amygdala are central in the identification and presentation of a fear response to increasing CO_2 (Price et al., 2014; Ziemann et al., 2009), other structures beside the amygdala must also play an important chemosensory role (Feinstein et al., 2013). For example, Taugher et al. (2014) identified that CO_2 inhalation causes acidosis in an area of the “extended amygdala” known as the bed nucleus of the stria terminalis (BNST), and this in turn induced freezing behaviours in mice. Later disruption of ASIC1a within

the BNST attenuated this defensive behaviour. In light of these complex and varied findings, further research is needed to provide cross-species support for the chemosensory properties of the amygdala, and to explore related neural areas that may also share this role.

These observations demonstrate that CO_2 challenge can increase the salience of threat via areas of an established neural fear network. However, the majority of evidence to indicate how CO_2 might evoke anxious responses comes from research examining the effects of various drugs developed to reduce these symptoms.

1.4.5 General properties of CO_2 inhalation

7.5% CO_2 inhalation has demonstrated good test-retest repeatability for both psychometric and autonomic measures in healthy subjects identified as "responders" (Poma et al., 2005). As this suggests, some volunteers do not show a discernible response, and so some studies have examined samples of pre-identified responders only (e.g. Brambilla et al., 2013; Poma et al., 2014). The reasons for nonresponse are unclear. This may reflect biological differences (such as altered sensitivity of central chemoreceptors), successful use of cognitive/behavioural techniques, or simple procedural issues in the way CO_2 is administered. Inhalation of higher concentrations of CO_2 in non-responders could identify whether these subjects differ physically, and if so, help identify potential receptor sites/drug targets. Alternatively, more potent concentrations of CO_2 may be needed to elicit an emotional response in subjects who do not respond at lower doses.

Further clarification of what constitutes a "response" would be beneficial to investigate whether true nonresponse exists. Previous investigations have used panic-specific measures such as the Panic Symptom Inventory (PSI) that may not be sensitive to lower CO_2 concentrations (Bailey et al., 2011b). Instead, responders could be identified via a predefined increase of anxiety on a visual analogue scale (VAS), or with a GAD-specific measure such as the Generalised Anxiety Disorder Inventory (GAD-I), a scale that has been shown to be sensitive to benzodiazepine administration at peak effects of 7.5% CO_2 (Argyropoulos, 2009). In spite of these concerns, the vast majority of participants demonstrate some response to 7.5% CO_2 inhalation.

1.4.6 Summary: Is 7.5% CO_2 inhalation a valid model of human anxiety and does it demonstrate specificity to GAD?

Based on the evidence discussed, 7.5% CO_2 inhalation produces robust increases in anxiety, autonomic arousal and neurocognitive deficits that share similarities with some, but not all of the symptoms reported by anxious patients. The model is therefore a useful experimental tool of anxiety. 7.5% CO_2 is safe, reliable, easy to implement and replicate and demonstrates good retest reliability in healthy (Poma et al., 2005) and clinical samples (Seddon et al., 2011). However, whether 7.5% CO_2 inhalation demonstrates a particular affinity to the symptoms of generalised anxiety still needs to be determined. At the present time, the relationship of this model to GAD is restricted by a lack of research examining whether 7.5% CO_2 can induce excessive, non-specific worrisome thoughts. Until this issue is addressed, 7.5% CO_2 inhalation is concluded to share strong characteristics with the physiological symptoms, and to a lesser degree, to the psychological symptoms of GAD.

1.5 Models of anxiety: how does 7.5% CO_2 inhalation compare?

The main purpose of models of anxiety is to gain an understanding of the mechanisms which underlie pathological anxiety, and to help identify treatments during the early stages of drug development.

In the past, animal models of human anxiety disorders were the first and most practical choice for assessing potential anxiolytics. These models were usually validated for use in drug discovery with benzodiazepines, since these were the most effective anxiolytic treatments of the time. However, whilst these models were adept at identifying anxiolytic properties of benzodiazepines (e.g. the elevated plus maze), they have since struggled to replicate the therapeutic effects of newer antidepressants (Borsini, Podhorna, & Marazziti, 2002). This has raised concern over the predictive validity of these early animal models (particularly the elevated plus maze and fear-potentiated startle) in the search for new anxiolytics that may have very different mechanisms of action to benzodiazepines (Haller, Aliczki, & Gyimesine Pelczer, 2012). In consequence, the search for new drugs with broader mechanisms of action is now being addressed with human models of anxiety, such as 7.5% CO_2 challenge (Nutt & Goodwin, 2011).

Is 7.5% CO_2 inhalation a valid model of anxiety?

In this section, human models that broadly induce a state of anxiety are discussed and comparisons are drawn with the 7.5% CO_2 model. Bailey et al. (2011a) categorised human models of anxiety based on how its symptoms are induced. These are:

a. through administration of a known anxiogenic pharmacological compound;
b. through physiological means such as utilising fear of pain, or through methods which challenge the respiratory system;
c. through psychological means, such as exposure to fearful or phobic stimuli.[2]

1.5.1 Pharmacological challenges

Pharmacological challenges involve the administration of substances known to elevate anxiety. For example, the α-adrenergic antagonist yohimbine (Charney et al., 1983), or injections of the neuropeptide CCK_4 (cholecystokinin tetrapeptide; e.g. Jerabek et al., 1999), induce anxiety and panic symptoms and increase autonomic arousal in healthy subjects; the effects of which closely compare with the symptoms of PD patients. Pharmacological challenges generally benefit from being translational across species, which allow for inferences to be made about how certain aspects of anxiety induced by these challenges might occur in humans. CO_2 inhalation also benefits in this respect, with increased fear behaviours apparent in rodents (e.g. Ziemann et al., 2009).

However, a major drawback of many pharmacological challenges is the need to use intravenous administration which is mildly painful and anxiety-provoking. This may emphasise individual differences in the level of tolerance to procedures of this kind. This also hinders the use of sensitive assessments of stress or anxiety (such as cortisol,

[2] The placement of anxiety models into these categories is open to a degree of individual interpretation. For example, in a discussion on human models of anxiety for drug development Siepmann and Joraschky (2007) divided human models of anxiety into two categories; chemical and psychological, with CO_2 inhalation placed in the former category. In this review, the distinction of models into three categories as Bailey et al. (2011a) proposed has been adopted, although models in the physiological category focuses more heavily on those which directly challenge the respiratory system, for example, hyperventilation and obstructive breathing methods. 7.5% CO_2 inhalation could be considered a physiological model as the most prominent response experienced is respiratory change, however it could also fall into the chemical/pharmacological subtype as although the respiratory system is challenged to some degree, this is not necessarily experienced by everyone at lower doses. CO_2-induced anxiety is also chemically induced. Where each model is placed in a category is not a primary concern as it is maintained that the majority (if not all) models of anxiety fall into these three categories, and some overlap is expected.

or skin conductance responses), as these procedures are likely to augment general levels of arousal which makes it difficult to differentiate the anxiogenic response of the procedure from the challenge.

Both CCK_4 and sodium lactate responses can be attenuated with pre-treatment with some anti-anxiety drugs (see Siepmann & Joraschky, 2007 for a review), however a major concern with pharmacological models of anxiety is the existence of possible interactions between the challenge agent and drug treatment. This makes identification of "pure" drug effects challenging and is particularly problematic with substances that do not have a clear effect on a transmitter system/s, such as sodium lactate. Instead, pharmacological challenges may be more useful to delineate potential mechanisms of different subtypes of anxiety, especially where the probable mechanism through which these models induce anxiety is clear. Models with known biological effects may be more sensitive to some drug classes and thus be valuable to examine the likely therapeutic viability of novel treatments known to work in a similar fashion. This works both ways, where the mechanisms by which a drug is anxiolytic are not clear, assessment with a battery of pharmacological challenges where the mechanisms of action are generally understood could provide a clearer picture of efficacious treatments and reduce the risk of both false positives and false negatives.

1.5.2 Physiological challenges

Models that induce anxiety through physiological means often make use of aversion of pain, such as the expectation of mild electric shock, or may challenge the respiratory system. Infusions of sodium lactate for example, produces increases in respiration that provoke similar levels of panic in PD patients to the pharmacological challenge CCK_4 infusion (Plag et al., 2012), but not in healthy volunteers. Respiratory challenges have largely stemmed from research into respiratory abnormalities in PD and from the noticeably high prevalence rates of anxiety in chronic breathing disorders (e.g. in COPD, Kunik et al., 2005).

Models that challenge the respiratory system tend to be used more as a diagnostic tool rather than for assessment of anxiolytic drugs. Voluntary hyperventilation tasks are unlikely to produce consistent panic-like responses in healthy volunteers so the usefulness of models of this kind is somewhat limited to patient

samples. For example, the hyperventilation provocation test (HVPT) requires subjects to voluntarily hyperventilate by over-breathing for few minutes. Subjects prone to panic disorder are expected to report symptoms more similar to panic attacks than controls, although even in patients the sensitivity of this test is low (Hornsveld & Garssen, 1996). As such, models with consistent anxiogenic effects in patient and healthy samples are preferential such as CO_2 inhalation, as these models can better bridge the gap between Phase I and II clinical trials to reduce the likelihood of poor therapeutic viability in patients at the later stages of drug development.

1.5.3 Psychological challenges

Psychological challenges often utilise common anxiogenic situations or phobic/fearful stimuli to evoke a state of anxiety. For example the Trier Social Stress Test (TSST; Kirschbaum et al., 1993) is a paradigm assessing psychosocial stress that uses the common fear of social embarrassment. Participants are asked to make a hurried oral presentation and perform a mental arithmetic task before a panel of judges. The judges refrain from providing positive feedback and the presentation is recorded. The TSST produces increases in anticipatory and state anxiety and also in biomarkers of stress, such as corticotropin (Schommer, Hellhammer, & Kirschbaum, 2003).

A common problem with psychological models is the influence of current affective state and individual differences, for example with some participants being more fearful of social embarrassment than others in the TSST. This also applies to the presentation of fearful or phobic stimuli (such as images of snakes and spiders) or threat of mild electric shocks in tasks such as the fear-potentiated startle. Thus, variation in the anxiety experienced by subjects in psychological challenges may make identification of true drug effects difficult, or may mask minor effects. 7.5% CO_2 inhalation is arguably less susceptible to this flaw as, although the physiological response to CO_2 varies between subjects, conscious attempts to alter this appears to have little effect (Ainsworth et al., 2015).

Whether dispositional or trait aspects of personality and prior experience modulates psychological interpretations of CO_2 inhalation has only recently begun to receive attention. Fluharty, Attwood and Munafo (2016) recently revealed that healthy volunteers high in “anxiety proneness” (high trait anxiety and anxiety sensitivity) tend

to have a greater subjective (but not autonomic) response to 7.5% CO_2 and air inhalation than those low in this trait. In addition, Garner et al. (2012) identified associations between self-reported trait anxiety and increased negative affect with autonomic arousal to 7.5% CO_2. The impact individual differences in stable personality traits such as anxiety sensitivity have on 7.5% CO_2 responses need to be examined in more detail. This will help inform future methodological decisions regarding the recruitment of healthy samples and in ensuring balanced groups.

A further issue with psychological challenges is that they sometimes struggle to provide a good control, "no-anxiety" comparator. Although a placebo version of the TSST has been developed (participants present a topic of their choice and perform a mental arithmetic task to an empty room), this does not reflect a true no-stress condition.7.5% CO_2 inhalation however, can be compared with inhalation of normal air, in a single-blind, counterbalanced design. Inclusion of an air condition allows for direct comparison between the change in peak subjective (i.e. immediately at the end of the 20 minutes), physiological, and neurocognitive performance during air and CO_2. Mild anxiety may occur during air trials due to anticipation of the effects of CO_2 or mask-anxiety, the impact of which could be reduced by allowing participants to acclimatise to the face mask. Analogous healthy human models do not always have the advantage of a comparator condition that shares such strong resemblance to the experimental condition.

However, in light of recent research by Fluharty et al. (2016) experimenters using the 7.5% CO_2 model should be aware that high trait anxiety and anxiety sensitivity is associated with a greater subjective response to air. This reflects a limitation of the control air condition, as it is differentially anxiogenic for subsets of individuals. Ensuring that groups are equally balanced in these traits may be necessary to counteract this, or participants could be pre-screened for high trait anxiety especially in studies where small effects may be expected (i.e. in drug evaluations).

Less research has been conducted with the TSST in terms of validation with known anxiolytics. One study assessed the effects of the BZD alprazolam on endocrine, autonomic and psychological stress in the TSST, and although a blunted endocrine response was seen, acute alprazolam administration did not affect increased state anxiety (Fries, Hellhammer, & Hellhammer, 2006). Individual differences in the

subjective response to the TSST may account for these inconsistencies, although further investigations with antidepressants are required.

Direct comparison between psychological challenges and 7.5% CO_2 has yet to be conducted. This would be beneficial to determine the advantages and disadvantages of different models across a variety of standardised measures, including autonomic arousal and subjective mood. Current variability in the way these tests are implemented and assessed makes it difficult to draw more substantial claims.

1.5.4 Summary: A systematic comparison of models of anxiety is needed

Many human models of anxiety have been proposed and these vary in their usefulness. No direct comparison of the most commonly employed models has been undertaken and so the degree to which anxiety is consistently induced by these provocation tests remains unknown. Variation in the way in which these models are implemented also makes evaluation between these models difficult. An experimental design which compares the effects of 7.5% CO_2 challenge to the effects of comparable models (such as the TSST or yohimbine infusion) with common assessment measures would demonstrate whether certain aspects of anxiety are displayed more robustly by some models more so than others. Direct assessment of a variety of anxiety models with diverse patient groups may also indicate whether the symptoms induced compare well to the symptoms of each disorder.

1.6 Neurotransmitter systems and anxiety: treatment effects on 7.5% CO_2 inhalation

For 7.5% CO_2 inhalation to be considered a valid model of anxiety, it must demonstrate sensitivity to drugs known to reduce anxiety. This section will introduce the main neurotransmitters targets and drug treatments that are available for anxiety before considering evidence that 7.5% CO_2 is sensitive to these treatments.

NICE guidelines recommend the use of selective serotonin reuptake inhibitors (SSRIs) as the first-line choice of pharmacological treatment for GAD, yet approximately 50% of patients do not make a full response, and only approximately one-third of patients achieve symptomatic remission (Buoli, Caldiroli, Caletti, Paoli, & Altamura, 2013; NICE, 2011b). Psychological therapies such as cognitive behavioural

therapy (CBT) which have known efficacy (e.g. Borkovec & Costello, 1993) are also recommended. Consequently, a multidisciplinary approach is needed (drawing on evidence from cognitive and behavioural psychology, genetics, psychiatry and neurology) to better understand the aetiology and maintenance of GAD, and thus the treatments that are most likely to be effective.

Several key neurotransmitters that are hypothesised to interact with the amygdala-prefrontal anxiety network have been the primary focus of pharmacological treatments for anxiety. Dysregulations of the noradrenaline, serotonin (Ressler & Nemeroff, 2000) and GABA (γ-aminobutyric acid; Nutt & Malizia, 2001) systems have been identified across many mood disorders. For example, serotonergic synapses are widely expressed in the amygdala, and areas of the prefrontal cortex have a direct inhibitory effect on serotonergic activity (Ressler & Nemeroff, 2000). fMRI research has also shown decreased amygdala activity after a 21 day course of the selective serotonin reuptake inhibitor escitalopram (Arce, Simmons, Lovero, Stein, & Paulus, 2008). Single (Murphy, Norbury, O'Sullivan, Cowen, & Harmer, 2009) or week-long (Harmer, Mackay, Reid, Cowen, & Goodwin, 2006) administration of the antidepressant citalopram in healthy volunteers also decreased amygdala activity to presentations of threat compared to those receiving placebo. As such, the existing drug treatments for anxiety disorders largely aim to target these systems and include selective serotonin reuptake inhibitors (SSRIs) serotonin-noradrenaline reuptake inhibitors (SNRIs), and benzodiazepines (BZDs, which enhance the inhibitory effects of GABA)[3].

Table 3 details the drugs that are currently licenced in the UK for treatment of GAD. Historically, BZDs were the drug of choice, although in recent years they have been criticised due to high risk of abuse, issues with tolerability and withdrawal. Current guidelines now recommend the use of SSRIs, SNRIs, or the anticonvulsant pregabalin (NICE, 2011b). Relatively few randomised comparator controlled studies have been conducted on acute treatments for GAD, however one analysis reports an overall mean effect size of 0.39 for acute pharmacological treatment (Hidalgo, Tupler,

[3] Since anxiety and depressive disorders share a number of symptoms and are highly comorbid, the treatments for these disorders overlap considerably. As such it is important to note that the majority of pharmacological treatments of anxiety (particularly BZDs, SSRIs and SNRIs) were originally developed for the treatment of depression. As a result they are often referred to as antidepressants, despite their widespread use for anxiety. Thus in this review, the terms “antidepressant”, and “anxiolytic/anti-anxiety” refer to the same drug treatments, and are used with particular reference to the disorder of discussion.

& Davidson, 2007). These drug therapies vary largely in their suitability, tolerability and efficacy, with greater effect sizes reported for pregabalin (0.50), the antihistamine hydrozyzine (0.45) and SNRIs (0.42), all of which are recommended for acute treatment (Baldwin et al., 2014). However, an estimated 30-50% of patients do not respond to anxiolytic treatment or are unable to tolerate unpleasant side effects. In light of this, there is room for improvement in the treatment of GAD with a definite need for further research into the efficacy of potential anti-anxiety treatments.

Table 3. UK licenced pharmacological treatments of Generalised Anxiety Disorder (NICE, 2011a).

Class	Drug	Dose (mg/day)
Anticonvulsants	Pregabalin	150-600
SSRIs	Escitalopram	10-20
	Paroxetine	20-50
SNRIs	Venlafaxine	75-375
	Duloxetine	60-120

Note. Sertraline is not currently licenced in the UK for treatment of GAD. Benzodiazepines are no longer recommended apart from as a short-term intervention for severe anxiety (< 2 weeks), due to high risk of abuse, dependence and tolerability and negative impacts on memory (Joint Formulary Committee., 2014).

Since the discovery of BZDs and later SSRIs and SNRIs, exploratory research has begun to probe the mechanisms of action behind these treatments. Abnormalities in the GABA, serotonin and noradrenaline neurotransmitter systems contribute to anxiety to varying degrees. Bailey and Nutt (2008) proposed that reduced $GABA_A$ transmission might modulate the relationship between CO_2 and anxiety, and altered levels of serotonin and noradrenaline might accompany this. Despite the focus on these particular neurotransmitters, it is important to note that there are likely to be many complex interactions between these (and other) transmitter systems in the expression of anxiety.

1.6.1 GABA

GABA is the primary inhibitory neurotransmitter, controlling excitability in the central nervous system and across the brain. Neural activity is maintained by a careful

balance between the inputs of glutamate, the most abundant excitatory neurotransmitter, and the inhibitory inputs of GABA (Nutt & Malizia, 2001). Once the mechanisms of action behind benzodiazepines were uncovered, investigation into the role of GABA increased.

Since then, a wealth of research has identified deficits in GABA receptor sites that may act as a precursor for anxiety disorders (see Mohler, 2012 for a review of GABAergic deficits). For example an imaging study in a small sample of GAD patients showed reduced BZD binding in left temporal areas (including the hippocampus) which is part of the distributed limbic system involved in emotion processing and presentation of fear and anxiety (Tiihonen et al., 1997). Furthermore, drugs that enhance the neurotransmission of GABA, such as BZDs, or that inhibit glutamate (e.g. riluzole; Mathew et al., 2005) are anxiolytic, whereas compounds that inhibit or disrupt the transmission of GABA are anxiogenic. For example, administration of flumazenil, a BZD antagonist, can produce anxiety and panic in PD patients but not in healthy controls (Nutt, Glue, Lawson, & Wilson, 1990). In rodents, mice heterozygous for the γ2-subunit gene display impaired $GABA_A$ receptor functioning, particularly in the hippocampus and exhibit neural and behavioural abnormalities that resemble deficits in GAD patients, such as decreased BZD binding and attentional biases towards threat. Importantly, these behavioural abnormalities are reversed with the administration of the BZD diazepam (Crestani et al., 1999).

Early approaches in the pharmacological treatment of GAD focussed on reducing the acute symptoms of anxiety (Reinhold, Mandos, Rickels, & Lohoff, 2011). BZDs provide rapid relief from the symptoms of GAD by presumably enhancing the inhibitory effects of GABA (γ-aminobutyric acid). The threshold of GABA required to open chloride channels is lowered, reducing excitability of a neuron by hyperpolarizing it with chloride ions. This results in anxiolytic and sedative effects (Nutt & Malizia, 2001). However the clinical use of BZDs has many limitations. This includes high risk of abuse and dependence, tolerability issues, and troublesome side-effects, with adverse effects on cognition (Baldwin et al., 2013; Lader, 2008). Although treatment with BZDs tends to effectively reduce acute symptoms, long-term use for the treatment of GAD is no longer recommended (NICE, 2011b).

Is 7.5% CO_2 inhalation a valid model of anxiety?

It has been suggested that $GABA_A$ may mediate the anxiogenic effects of CO_2 inhalation. Bailey and Nutt (2008) have provided an extensive discussion of translational research that speculates a relationship between $GABA_A$, anxiety and CO_2 inhalation. Key to their argument is a study by Cuccheddu et al. (1995). Cuccheddu et al. (1995) compared 1 minute inhalation of 35% CO_2 with FG7142, a BZD receptor inverse agonist that is anxiogenic, to assess whether heightened anxiety reduced licking behaviours during the Vogel conflict test (a water deprived rat is punished with electric shock when they attempt to drink, greater toleration of shocks as shown by increased licking behaviours is thought to be indicative of lower anxiety). Rats that inhaled 35% CO_2 displayed less licking periods to a similar extent as those treated with FG7142, indicating comparable levels of heightened anxiety. Furthermore, rats that were treated with the BZD alprazolam or FG7142 prior to CO_2 inhalation did not show reduced licking periods suggesting pre-treatment abolished avoidance behaviours. This suggests that CO_2-induced anxiety in rodents is sensitive to the actions of alprazolam. Although not conclusive, this indicates the possibility that CO_2 may interfere with GABAergic mechanisms and could offer a novel way to assess the efficacy of newly developed anti-anxiety drugs that target the GABA system.

1.6.2 The effect of benzodiazepine administration on CO_2-induced anxiety

Table 4 summarises the healthy human studies of pharmacological and psychological treatments that have been evaluated with 7.5% CO_2 challenge. Anxiolytic treatments that act on GABA/BZD receptor/binding sites have shown efficacy in the CO_2 challenge model. For example, single administration of the BZD lorazepam can reduce the subjective but not physiological effects of 7.5% CO_2 inhalation (Bailey et al., 2007a). This may be due to the subjective effects of BZDs occurring downstream of physiological effects (Bailey et al., 2011a). Furthermore, single administration of alprazolam, a full agonist of $GABA_A$, decreased anxiety and produced greater reductions in fear during 7.5% CO_2 inhalation when compared to the selective $GABA_A$ agonist zolpidem (which still reduced anxiety more so than placebo; Bailey et al., 2009). This extends findings that alprazolam can attenuate responses to CO_2 in rodents (Cuccheddu et al., 1995) and provides support for CO_2 challenge as a translational, cross-species model of anxiety that could sit between preclinical and clinical trials in drug development.

Table 4. Anxiolytic effects of pharmacological and psychological interventions in the 7.5% CO_2 model in healthy humans

Study reference	Intervention	Control	Details	Effects on subjective mood during CO_2
Bailey et al. (2007a)	N = 12	Within-subjects	Single dose 2mg lorazepam	↓ fear, tension, feel like leaving and worry
	N = 11		3 week 20mg paroxetine	↓ nervousness
Bailey, Papadopoulos, Lingford-Hughes, and Nutt (2007b)	N = 24	N = 24	Single dose 50mg D-cycloserine	No effect on subjective mood or autonomic arousal
Bailey, Papadopoulos, Seddon, and Nutt (2009)	N = 12	3-way cross-over	Single dose 1mg alprazolam	↓ fear, tense, stressed, alert, feel like leaving, total GAD-I scores and ↑ happy
			Single dose 5mg zolpidem	↓ feel like leaving, worried, stressed and total GAD-I scores
Papadopoulos, Rich, Nutt, and Bailey (2010)	N = 12	4-way cross over	Single dose 40mg propranolol Single dose 25mg hydroxyzine Single dose 0.5mg flupentixol	No effects of any drug on subjective mood
Bailey et al. (2011b)	N = 12	N= 11	1 week 40mg CRF_1 receptor antagonist R317573	↓ fear, feel like leaving, tense and total GAD-C, ↑ happy and relaxed
	N = 8		Single dose 2mg lorazepam	↓ fear, feel like leaving and tense, ↑ happy and relaxed
Diaper et al. (2012b)	N = 18	Within-subjects	Single subclinical dose 0.5mg lorazepam	No effects on subjective mood
			Single dose 2mg lorazepam	↓ feel like leaving. Trend for ↓ fear, anxiety, nervousness, irritability and worry compared to 0.5 mg lorazepam and placebo

Is 7.5% CO_2 inhalation a valid model of anxiety?

Study reference	Intervention	Control	Details	Effects on subjective mood during CO_2
de Oliveira et al. (2012)	N = 15	N = 15	Single dose 2mg lorazepam	Neither lorazepam nor oxytocin experienced ↑anxiety after CO_2 inhalation when compared to placebo. No effect on autonomic arousal
	N = 15		Single dose 24ml oxytocin intranasally	
Diaper et al. (2013)	N = 18	N = 18	21 days 150mg venlafaxine	No significant effects on subjective mood for either drug. Trend for ↓ feelings of tense and nervousness (*p's* <.072) for both treatments vs. placebo
	N = 18		21 days 200mg pregabalin	
Poma et al. (2014)	N = 19	Cross-over	Single dose 0.75mg alprazolam	↓ panic symptoms on PSL III-R (vs. placebo)
			Single dose 15mg vestipitant	↓ anxiety on visual analogue scale (vs. placebo)
			Single dose 25mg vofopitant	No anxiolytic effect
Ainsworth et al. (2015)	N = 11	N = 10 (asked to sit quietly)	10mins focussed attention training	Short intervention of mindfulness-based training ↓ subjective anxiety to 7.5% CO_2. No group differences on HR or BP nor antisaccade performance.
	N = 11		10mins open monitoring training	

Note. All studies reported are placebo-controlled in design apart from apart the 21 days paroxetine treatment by Bailey et al. (2007a). Poma et al. (2014) used 7% rather than 7.5% CO_2 for 20 minutes. GAD-I = Generalised Anxiety Disorder Inventory (Argyropoulos et al., 2007). GAD-C Generalised Anxiety Disorder Criteria Inventory. PSL III-R Panic Symptom List.

These studies found robust anxiolytic effects for BZDs such as alprazolam and lorazepam on responses to 7.5% CO_2 challenge. This suggests GABAergic mechanisms are involved in CO_2-induced anxiety.

1.6.3 Serotonin

The monoamine neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) is derived from the amino acid tryptophan and is abundant in the central nervous system. After release into the synaptic cleft, serotonin is transported back into the presynaptic neuron by a serotonin transporter pump, where it is broken down by the enzyme monoamine oxidase (Stahl, 1998). Findings that drugs that act on the serotonergic system demonstrate efficacy in mood disorders has fuelled interest in this system. The need to identify safer treatments in light of the risks of BZD treatment also encouraged further investigation.

SSRIs are recommended for the treatment of anxiety disorders. They are thought to increase the availability of serotonin in the synaptic cleft by inhibiting its reuptake back into the presynaptic neuron. Although remission rates are quite low for GAD, SSRIs demonstrate efficacy for anxiety disorders, including panic disorder (Bailey et al., 2011b). Therefore, it is likely that there are distinguishable differences between serotonergic function in GAD and panic disorder which could be exposed via challenge tests of each disorder.

Little is known about the precise mechanisms of serotonin in anxiety disorders due to an inability to directly measure serotonin levels in human participants (Durant, Christmas, & Nutt, 2009). For example, it is unclear whether anxiety disorders are characterised by serotonin deficiencies or excessive serotonin as different lines of evidence support elements of both theories (see Durant et al., 2009; Nutt & Bailey, 2003 for an overview). In spite of this, investigation of different compounds that influence serotonin levels is warranted to discover more suitable pharmacological treatments for anxiety, whilst researchers try to clarify this relationship through use of various challenge tests.

1.6.4 The effect of SSRIs on CO_2-induced anxiety

Evidence for a relationship between serotonin and 7.5% CO_2-induced anxiety is sparse. The research which does exist is not encouraging, with little to no effect on any responses to 7.5% CO_2 demonstrated. Bailey et al. (2007a) assessed whether a 3 week course of the SSRI paroxetine could reduce CO_2-induced anxiety (see Table 4). Chronic paroxetine administration significantly reduced nervousness and, although a trend for attenuated ratings of fear, anxiety and worry were reported, these did not reach statistical significance. This apparent insensitivity of 7.5% CO_2 to a serotonergic drug can be viewed in different ways. This may reflect an inability of 7.5% CO_2 to successfully replicate treatable anxiety via the same mechanisms that underlie these disorders, or may simply not be potent enough a model to show the benefits of treatment. This is possible as assessment of SSRIs with 35% CO_2 has shown anti-anxiety effects. For example, week long pre-treatment of citalopram in healthy volunteers (Bertani, Caldirola, Bussi, Bellodi, & Perna, 2001) and pre-treatment with paroxetine or sertraline in PD patients reduced reactivity to 35% CO_2 (Bertani, Perna, Arancio, Caldirola, & Bellodi, 1997), although this could reflect differences in response rates to SSRIs seen in panic disorder and evidence a further distinction between the two models.

Additional research is needed to examine the direct role of serotonin in hypercapnia. This is hindered by an inability to directly assess brain serotonin levels. Challenge tests such as acute tryptophan depletion (ATD), are used as an indirect assessment of the effects of alterations in brain serotonin levels and have been combined with 7.5% CO_2 inhalation to examine whether experimental reductions in 5-HT affect CO_2-induced anxiety. ATD reduces brain serotonin levels by depleting tryptophan, the precursor for 5-HT synthesis that is obtained through diet. Depletion of tryptophan can provoke the temporary return of symptoms for some disorders including depression and social anxiety, although whether this is the case for GAD is not known. As such, the combination of ATD and CO_2 inhalation was thought to worsen anxiety of patients more so than inhalation of CO_2 alone. Research using ATD with 7.5% CO_2 inhalation found no difference between tryptophan-depleted and control GAD patients on CO_2-induced anxiety (Hood et al., 2010). This parallels pharmacological research where pre-treatment with the SSRI paroxetine had no significant effect on CO_2-induced anxiety in healthy participants. Thus serotonin does not appear to modulate the effects of CO_2 challenge.

1.6.5 Noradrenaline

The noradrenaline system is largely centred on the locus coeruleus (LC), a neural structure which projects widely throughout the brain and spinal cord to areas implicated in fear and anxiety such as limbic and cortical structures. The LC is critically involved in the synthesis and release of noradrenaline, accounting for approximately 70% of noradrenaline innervation (Ressler & Nemeroff, 2000). This structure is thought to be involved in many physiological processes such as the modulation of attention (Sara, 2009) and autonomic control, of which abnormalities are key features of clinical anxiety. For example, noradrenergic function arising from the LC has been associated with the alerting network of attention (Coull, Nobre, & Frith, 2001; Fan et al., 2009; Witte & Marrocco, 1997). In GAD, excessive noradrenaline release and/or reduced inhibitory response of α2 receptors has been hypothesised, with some evidence for marginally elevated concentrations of noradrenaline in GAD versus controls (Kelly & Cooper, 1998; as cited in Nutt & Bailey, 2003).

Some attempts have been made to indirectly manipulate brain noradrenaline firing arising from the LC with challenge tests. The α_2-adrenergic receptor antagonist yohimbine increases noradrenaline firing from the LC and has been used as a challenge test to induce anxiety. Yohimbine challenge has been shown to differentiate between children with anxiety disorders from controls with higher ratings of anxiety in the former group (Sallee, Sethuraman, Sine, & Liu, 2000). This contrasts to the effects of clonidine, a α_2-receptor agonist, which has been shown to reduce anxiety brought about by yohimbine (Charney et al., 1983). This evidence provides strong support for the involvement of the noradrenergic system arising from the LC in anxiety, although precise examination of noradrenaline is required as its specific involvement is unclear.

Noradrenergic function of the LC is thought to be important in the anxiogenic response to CO_2 particularly hyper-arousal, autonomic changes and attentional abnormalities (Bailey, Argyropoulos, Lightman, & Nutt, 2003). Pineda and Aghajanian (1997) identified that exposure to low doses of CO_2 (5-10%) increased the firing of the LC neurons in the rat brain. This was dose-dependent with greater levels of CO_2 producing faster firing of the LC. Thus it is possible that noradrenergic neurones in LC are directly activated by central chemosensors. This parallels dose dependent effects in

healthy human studies utilising CO_2 challenge, with greater concentrations of CO_2 eliciting the most pronounced anxiety response (Leibold et al., 2013).

1.6.6 The effect of SNRIs and NRIs on CO_2-induced anxiety

Serotonin-noradrenaline reuptake inhibitors (SNRIs) are efficacious in the treatment of GAD, along with SSRIs (Baldwin, Woods, Lawson, & Taylor, 2011). Unlike SSRIs, SNRIs are inhibitors of both noradrenaline and serotonin reuptake. The SNRI duloxetine, a potent inhibitor of both 5-HT and NA, was ranked first for response in a meta-analysis of licenced UK treatments (compared to venlafaxine, paroxetine, escitalopram and pregabalin; Baldwin et al., 2011) and would be of interest to examine with challenge tests of anxiety.

Pre-treatment with SNRIs prior to CO_2 challenge is a novel way to assess whether CO_2-induced anxiety can be attenuated with drugs that act on the noradrenergic/serotonergic systems. Comparison of their effects (within the 7.5% CO_2 model) to SSRI pre-treatment could clarify the contribution of serotonin and noradrenaline to anxiety. However, to date research illustrates poor SNRI effects on the anxiety response to CO_2 and casts some doubt on the extent to which these systems are involved. Initial assessment of the SNRI venlafaxine has shown no anxiolytic effect during 20 minutes of 7.5% CO_2 inhalation (Diaper et al., 2013) although trends for lower ratings of nervousness and tension were recorded. Furthermore, administration of clonidine to healthy volunteers does not reduce subjective anxiety or autonomic arousal elicited by CO_2 inhalation, whereas administration of 0.75mg of the BZD alprazolam does (Woods, Krystal, Heninger, & Charney, 1989). As clonidine and venlafaxine did not alter CO_2-induced anxiety, this suggests CO_2 challenge may not principally induce anxiety via the noradrenergic system.

1.6.7 Summary

Evidence suggests that the acute administration of benzodiazepines but not chronic administration of some SSRIs and SNRIs can attenuate responses to 7.5% CO_2 challenge. This provides some validation of 7.5% CO_2 as a healthy human model of acute anxiety and in particular GAD, although these treatments are widely prescribed

across mood disorders. Further examination of the SSRI and SNRI class of drugs is warranted to understand the absence of effects on CO_2-induced anxiety.

1.7 A cognitive, neuropsychological theory of antidepressant drug action

A recent theory has been developed to explain why treatments of anxiety and depression often take a few days to weeks to produce improvements in mood. The suggested time at which to evaluate clinical efficacy of an antidepressant for the treatment of depression or anxiety has been widely debated, ranging from just one week to as much as eight weeks (Frazer & Benmansour, 2002; Lam, 2012). However, it is generally accepted that the therapeutic onset of antidepressants is not immediately apparent. Recent research indicates that gradual improvement in psychological symptoms can be detected within the first week (Lam, 2012; Taylor, Freemantle, Geddes, & Bhagwagar, 2006) which translates into more observable, and clinically significant improvements after a number of weeks.

Similarly to anxiety, cognitive theories of depression have highlighted the presence of negative biases in emotion processing as a critical feature of the disorder (such as negatively intepreting and recalling emotional information and trouble identifying positive emotions; Disner, Beevers, Haigh, & Beck, 2011; Mogg, Bradbury, & Bradley, 2006; Peckham, McHugh, & Otto, 2010) . The reversal of these biases is again thought to be important for successful treatment. Research in healthy volunteers suggests that the administration of some antidepressants can produce abrupt changes in the way the brain processes emotional material, whilst low mood and anxiety symptoms remain unaffected (e.g. Harmer et al., 2003a; Harmer et al., 2011; Harmer, Heinzen, O'Sullivan, Ayres, & Cowen, 2008; Harmer, Shelley, Cowen, & Goodwin, 2004; Murphy, Downham, Cowen, & Harmer, 2008; Norbury, Mackay, Cowen, Goodwin, & Harmer, 2008).

For example, in healthy volunteers prior administration with a single dose of some antidepressants can promote the processing of positive emotional information and/or downplay the salience of negative information. A single dose of the SNRI duloxetine has been shown to aid recognition of happy facial expressions and increase the number of incorrectly recalled positive characteristics compared to placebo (although improved recognition of disgusted faces was also reported; Harmer et al.,

2008). Similarly, a single clinical dose of the noradrenergic reuptake inhibitor (NRI) reboxetine facilitated recognition and recall of positive emotional information independent of a change in mood (Harmer, Hill, Taylor, Cowen, & Goodwin, 2003b). These findings are consistent with observed changes in neural activation and emotion processing that occurred after antidepressant treatment in regions that are associated with negative biases in depression (Norbury et al., 2008; see Phan, Wager, Taylor, & Liberzon, 2002 for a review).

In clinical populations, single administration of reboxetine in acutely depressed patients has been shown to abolish poor recognition of positive emotions commonly reported in major depression (Harmer et al., 2009b), whilst improvements in the recognition of happy facial expressions after two week antidepressant treatment has been related to a more favourable clinical response at 6 weeks (Tranter et al., 2009b). This highlights a possible causal link between early changes in emotion processing and later effects on mood. Whilst these clinical studies have largely considered cognitive biases in depressed patients, a similar approach may also be beneficial to our understanding of pathological anxiety.

Together, these observations prompted the formulation of a cognitive neuropsychological model of antidepressant drug action (Harmer & Cowen, 2013b; Harmer, Goodwin, & Cowen, 2009a). This theory hypothesises that antidepressants remedy negative biases early in the course of treatment to later change how ambiguous and negative sources of information are selected and interpreted. It is only after these changes in emotion processing are implemented and reinforced that improvements in mood are seen. Should this theory be proven correct, this may explain the observed delay between antidepressant administration and therapeutic relief from symptoms in depression and anxiety.

This theory prompts an interesting avenue for research that considers changes in attention and emotion processing before changes in mood. Potential drug effects on these processes should be acknowledged particularly if the administration duration is short as this may make it difficult to quantify subjective changes.

1.8 Is 7.5% CO_2 challenge amenable to novel anxiolytic drug treatments?

The 7.5% CO_2 model has value as an experimental and translational tool of anxiety in healthy human volunteers. This model is able to probe the effects of potent anti-anxiety treatments effectively, particularly drugs of the BZD class. Consequently recent studies have used the model to evaluate innovative treatments for chronic anxiety symptoms.

1.8.1 Pregabalin

The drug pregabalin has recently been licenced for the treatment of GAD. It binds to voltage-dependent calcium channels in the central nervous system which increases GABA, leading to greater inhibitory activity and thus having anxiolytic effects. A recent meta-analysis found pregabalin to be ranked among the most effective treatments for response (along with duloxetine and escitalopram) for the treatment of GAD (Baldwin et al., 2011).

Pregabalin has been assessed with the 7.5% CO_2 model in healthy volunteers (Diaper et al., 2013). Participants pre-treated for 3 weeks with pregabalin to a dose of 200mg underwent 20 minutes of 7.5% CO_2 inhalation and a single dose of 35% CO_2. Pregabalin had no significant effect on responses to either CO_2 challenge. This may not be unexpected as although pregabalin exerts similar clinical effects to the BZD class of drugs, its principal effects relate to diminution of glutamatergic activity (see section 1.6.2). However, a relatively low dose of pregabalin over the 21 days was administered and there were no baseline measures. Consequently the effects of pregabalin on CO_2-induced anxiety remain unclear.

1.8.2 Vestipitant

The drug vestipitant was investigated as a potential anxiolytic which could also be useful in the treatment of common comorbidities of anxiety including tinnitus and insomnia. Vestipitant is a selective neurokinin$_1$ (NK_1) receptor antagonist that in preclinical studies had been seen to increase punished responses in the Vogel conflict test, and reduce marble burying behaviour and ultrasonic vocalisations in rodents (Brocco et al., 2008). Clinically, the effects of vestipitant in humans is not well characterised in published reports.

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Poma et al. (2014) used 7% CO_2 inhalation to examine the anxiolytic effects of vestipitant in healthy humans, with the BZD alprazolam as an active control comparison. A single dose of vestipitant was revealed to produce significant reductions in anxiety (as measured with a visual analogue scale) but not in heart rate or skin conductance, whilst alprazolam reduced symptoms of panic. Thus vestipitant (and NK antagonists more broadly[4]) warrants further investigation in anxious populations to fully determine its anti-anxiety properties.

1.8.3 CRF-receptor targeted treatments

There is evidence to suggest that the corticotropin releasing factor (CRF) system is involved in stress responses and anxiety. CRF is a 41 amino acid peptide that is released in the brain in response to stress (Zorrilla, Valdez, Nozulak, Koob, & Markou, 2002). Release of CRF stimulates the secretion of adrenocorticotropic hormone (ACTH), which in turn stimulates production of cortisol. This is of interest as high levels of cortisol are related to abnormal serotonin and noradrenaline activity (see Stokes, 1995; as cited in Argyropoulos et al., 2002; Tafet et al., 2001). Elevated CRF has also been reported in major depression (e.g. Bissette, Klimek, Pan, Stockmeier, & Ordway, 2003; see Holsboer, 2000 for a review) and anxiety disorders (e.g. panic disorder; Erhardt et al., 2006).

CRF plays a key role in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis which is activated during stress. A main function of the HPA axis is the release of cortisol, synthesis of which is stimulated by a chain of events initiated by the secretion of CRF and vasopressin. Inhalation of 35% CO_2 has been shown to activate the HPA axis as illustrated by increased plasma cortisol levels in healthy volunteers. This suggests that 35% CO_2 inhalation is a significant stressor, with the potential to increase cortisol levels through release of CRF, or via the serotonergic or noradrenergic systems (Argyropoulos et al., 2002). Similar findings have not been replicated with 7.5% CO_2 inhalation (Bailey et al., 2007c), most likely because the 7.5% CO_2 model produces lower levels of stress and anxiety than the 35% CO_2. This provides further evidence for

[4] For example, saredutant (SR48968) is a neurokinin-2 receptor antagonist identified to have efficacy in animal models of anxiety and depression (Louis et al., 2008), although clinical applications in humans has produced disappointing results and the drug has now been dropped from further development (Griebel & Holsboer, 2012).

the specificity of CO_2 for subtypes of anxiety, due to the distinct anxiety profiles elicited by different concentrations.

In light of the potential role of CRF in anxiety, the development of novel therapeutic drugs that act on CRF_1 receptor sites has begun with some initially promising results (see Holsboer & Ising, 2008). For example, antalarmin (a CRF_1 receptor antagonist) reduced anxiety behaviours of rodents in the elevated plus maze (Zorrilla et al., 2002). A recent study in humans has investigated the effects of a CRF_1 receptor antagonist (R317573) on the outcomes of the CO_2 model (Bailey et al., 2011b). When compared to placebo, administration of R317573 was found to reduce subjective responses to 7.5% CO_2 inhalation, including generalised anxiety symptoms. This indicates that 7.5% CO_2 inhalation is sensitive to drugs other than BZDs. Expansion on these findings with other compounds that act on CRF_1 receptor sites (such as a human study with antalarmin) through use of the 7.5% CO_2 model may identify novel treatments of anxiety.

1.8.4 Summary

7.5% CO_2 challenge may be a useful model to predict if novel drug therapies are potentially anxiolytic in clinical samples. Further assessment with newly developed treatments (such as the CRF_1 receptor antagonist R317573) in anxious patients would demonstrate whether effective compounds in an experimental healthy human model can readily translate to clinical anxiety and therefore compliment preclinical models.

The evidence presented in this review has shown that the inhalation of 7.5% CO_2 is a viable model of anxiety in healthy humans that may be useful in drug development. However, whilst it is clear that 7.5% CO_2 inhalation is a useful experimental model of anxiety in healthy humans, further examination of critical features of GAD is needed to clarify whether 7.5% CO_2 inhalation is specific to this disorder. In addition, evidence that 7.5% CO_2 challenge is sensitive to anxiolytic medication provides further validation of this paradigm as an experimental model of anxiety. These findings indicate that 7.5% CO_2 may be a useful tool to differentiate between pharmacological treatments that have either acute (BZDs) or more gradual (SSRIs and SNRIs) effects. Further, examination of novel treatments would provide beneficial insight into the potential role of other neurotransmitter systems in anxiety disorders, such as the CRF system. The relationship

between these systems and the neural fear network indicate the complex nature of anxiety, and improved understanding of the precise mechanisms which underlie anxiety would aid the development of pharmacological/psychological interventions.

1.9 Future directions and thesis aims

This review highlights some key areas where additional evidence would help to validate the inhalation of 7.5% CO_2 as a model of anxiety, and in particular of GAD. While the evidence summarised in this review shows that the inhalation of low concentrations of CO_2 increases anxiety and autonomic arousal in humans (Bailey et al., 2005), and triggers anxious behaviour in small animals (Ziemann et al., 2009) and humans (Garner et al., 2011a; Garner et al., 2012), its effects on defensive behaviours that are mediated by the neural circuitry involved in anxiety are not known. Gaining an insight into the role of the amygdala in the human responses to CO_2 is important to understand the mechanisms through which this model induces anxious/fear behaviours.

Study One (Chapter 2) investigated the effect of 7.5% CO_2 inhalation on defensive behaviours. This work used the well-established human startle reflex to threat, the magnitude of which is widely acknowledged to be mediated by the amygdala across mammalian species. Previously, research in rodents has revealed that the amygdala is a direct sensor of increasing CO_2 concentrations, which coordinates subsequent defensive behaviours (such as freezing and reduced activity in the open-field test; Ziemann et al., 2009). Through examination of startle magnitudes and latencies to threat, this chapter aimed to gain insight into the level of involvement the amygdala has in CO_2-induced anxiety in humans. This extends current research that has found evidence for reduced startle magnitudes during brief inhalations of CO_2 (Ceunen, Vlaeyen, & Van Diest, 2013; Pappens et al., 2012), and further clarifies the role of neural regions implicated in this defensive response.

Study Two (Chapter 3) investigated whether the SNRI duloxetine could reduce anxiety responses to 7.5% CO_2 inhalation. Specifically, this work aimed to discover whether a known anxiolytic could reduce CO_2-induced anxiety and deficits in attention using a wide range of measures including two neurocognitive tasks. In light of the cognitive neuropsychological theory of antidepressant action (Harmer et al., 2009a), it is helpful to consider the potential for early effects of antidepressant/anxiolytic

administration on measures of attention within healthy human models of anxiety. Prior assessments of anxiolytics with the 7.5% CO_2 model may have overlooked important effects on attentional processes, particularly where single or short durations of administration were used (Bailey et al., 2007a; Diaper et al., 2013; Diaper et al., 2012b). Chapter 3 (and 4) of this thesis concentrated upon the possibility that anxiolytic administration could reverse deficits in attention (including hypervigilance to threat, and poor attentional control) measured with the antisaccade task and ANT. These tasks have been reported during CO_2 inhalation previously (Garner et al., 2011a; Garner et al., 2012) and extensively used in sub-clinical and clinically anxious populations (Ainsworth & Garner, 2013). This was examined alongside questionnaire and autonomic arousal measures of anxiety to provide a comprehensive assessment of anxiolytic action that fully spans the clinical presentation of anxiety.

A review of the literature also identified a lack of evidence concerning whether uncontrollable worry can be induced by 7.5% CO_2 inhalation. Since worry is a core clinical symptom of GAD that is directly related to cognitive control processes, it is important to assess whether inhalation of 7.5% CO_2 can induce intrusive worry, which is then amenable to pharmacological and/or psychological interventions. In order to accomplish this, Study Two (Chapter 3) included an objective and novel measure of spontaneously occurring thought intrusions, placed immediately after CO_2 and air inhalations[5]. The thought intrusions task (Borkovec & Costello, 1993; Hirsch, Hayes, & Mathews, 2009) measures the frequency of interrupting negative thoughts that occur before and after a period of instructed worry. In the past, the number of reported negative intrusions in this task has been shown to differentiate GAD patients from self-reported high worriers (Hirsch, Mathews, Lequertier, Perman, & Hayes, 2013). The simplicity and objectivity of this task makes it an appropriate tool to determine whether 7.5% CO_2 can provoke intrusive thoughts to a greater extent than air (and to a similar extent to instructed worry), the occurrence of which may be reduced by duloxetine. This extends previous research that has exclusively used subjective measures of worry (namely visual analogue scales; e.g. Bailey et al., 2005).

[5] Due to the prioritisation of the two neurocognitive tasks within the inhalations, the thought intrusions task was placed after CO_2 and air. It was acknowledged that it would be optimal for participants to complete the task during the inhalations.

Is 7.5% CO_2 inhalation a valid model of anxiety?

Careful consideration was given to the subjective measure/s of anxiety used in the latter stages of this research. Despite past attempts to enlist a GAD-specific measure (Argyropoulos, 2009), prior research has often focussed on measures of panic (e.g. using the panic symptoms list (PSL), or panic symptom inventory (PSI)) or state anxiety (such as with the state version of the Spielberger state-trait anxiety inventory: SSAI) to examine subjective responses to 7.5% CO_2 (e.g. Bailey et al., 2005; Poma et al., 2005). It was decided that a GAD-specific questionnaire would be preferential in this programme of work, as examining 7.5% CO_2 as a model of anxiety that replicates some key GAD symptoms was a prominent aim. The GAD-7 questionnaire (Spitzer, Kroenke, Williams, & Lowe, 2006) was chosen due to the relevance of questions to DSM-5 GAD criteria (e.g. item 2 - "not being able to stop or control worrying" – see Appendix A). In addition, the short length of the GAD-7 made this questionnaire a superior choice compared to longer, broader questionnaires (such as the 20-item SSAI, and 35-item PSI). As differences between placebo and active-drug might be small, the items on the GAD-7 were converted into visual analogue scales to enhance sensitivity to change (see Figure 11, Appendix A).

Study Three (Chapter 4) investigated the effects of the drug memantine on 7.5% CO_2-induced anxiety responses. Memantine is not currently licensed for the treatment of anxiety however there is evidence that memantine has some anxiolytic properties, which has led to 'off-label' administration by clinicians (Bertoglio & Carobrez, 2003; Minkeviciene et al., 2008). As memantine was primarily prescribed to alleviate symptoms of neurodegenerative diseases, positive effects on cognition were also of particular interest. This was again examined with the antisaccade task and ANT. This research should extend current evidence to suggest whether 7.5% CO_2 inhalation is able to identify key indicators of anxiolytic potential.

In summary, this thesis had two main goals; firstly to understand whether 7.5% CO_2 challenge is a model of anxiety (that shares some similarities with GAD) rather than fear by gaining insight into the role of the amygdala during CO_2 inhalation; and secondly, to identify whether the observed GAD-like responses to CO_2 can be reduced by current and novel anxiolytics. Through employing a range of assessments of anxiety including neuropsychological measures of attention, this thesis aims to add to the growing evidence that 7.5% CO_2 inhalation is a useful tool to model symptoms of pathological anxiety and evaluate novel therapeutics.

Chapter 2: *Defensive eye-blink startle responses in a human experimental model of anxiety*

2.1 Introduction

Inhalation of air enriched with 7.5% carbon dioxide (CO_2) produces reliable increases in subjective anxiety and autonomic arousal (e.g. blood pressure and heart rate, see section 1.4) in healthy humans (Bailey et al., 2005). The subjective effects of 7.5% CO_2 challenge are well characterised, and include increased anxiety, nervousness, worry, fearful apprehension and tension (Bailey et al., 2005; see section 1.4.1). These feelings are quantitatively and qualitatively less pronounced than the sudden acute feelings of panic (intense fear and discomfort) that accompany the single vital capacity inhalation of 35% CO_2 (see Colasanti et al., 2008). Accordingly, there is growing consensus that 7.5% CO_2 challenge provides an experimental model of anxiety that complements, but differs from the 35% CO_2 model of panic (refer to section 1.4.1.3).

Recent research has examined whether 7.5% CO_2 challenge can induce biases in cognition and emotion processing that promote the feelings of worry, nervous apprehension and perceptions of threat that characterise anxiety (section 1.4.3). For example, 7.5% CO_2 challenge increases attention (erroneous eye-movements) to aversive visual stimuli in an antisaccade task (Garner et al., 2011a), and increases hypervigilance through enhancing alerting (temporal) and orienting (spatial) attention network function (Garner et al., 2012). Similarly, studies in rodents show that exposure to 10% CO_2 increases behavioural inhibition, freezing and reduced activity in the open-field test (Ziemann et al., 2009). Thus across species, inhalation of low concentrations of CO_2 appears to trigger a range of behavioural responses typical of the anxiety phenotype.

Adaptive responses to threat can be considered across a defence cascade (see section 1.4.1.3). An anxious preparatory state is illustrated by vigilance, alertness, behavioural inhibition and appraisal, and enables the organism to monitor the risk associated with an anticipated, distal, often uncircumscribed threat. In contrast, active defence and avoidance (fight-flight) typify an acute fear state that is mobilized by identified, localized and proximal threat (for extended discussion of fear vs. anxiety in

humans and rodents see Blanchard, Hynd, Minke, Minemoto, & Blanchard, 2001; McNaughton, 2011; McNaughton & Corr, 2004).

One of the most reliable components of human defence is the eye-blink reflex, a rapid and intense contraction of the orbicularis muscle in response to a startling stimulus (typically a loud noise). This defensive reflex is greater in threatening contexts (e.g. when the delivery of an electric shock is unpredictable; Grillon, Baas, Cornwell, & Johnson, 2006a; Grillon et al., 2004), and when viewing threatening aversive pictures (e.g. Smith et al., 2005; Vrana, Spence, & Lang, 1988). Conversely, the startle response is reduced to positive/appetitive stimuli (e.g. Vrana et al., 1988). Startle responses are greater in fearful individuals (e.g. when phobic individuals view fear-provoking stimuli; Hamm, Cuthbert, Globisch, & Vaitl, 1997) and also in several anxious populations (see Vaidyanathan, Patrick, & Cuthbert, 2009 for a review) such as patients with PTSD (e.g. Morgan, Grillon, Southwick, Davis, & Charney, 1995), social anxiety (e.g. Garner, Clarke, Graystone, & Baldwin, 2011b), GAD (e.g. Ray et al., 2009), or panic disorder (e.g. Melzig, Weike, Zimmermann, & Hamm, 2007).

Comparatively few studies have examined the affective modulation of startle latency. There is evidence that startle responses are quicker to aversive relative to positive stimuli (Panayiotou, Witvliet, Robinson, & Vrana, 2011; Witvliet & Vrana, 1995), and to stimuli that elicit high relative to low levels of arousal (Cook, Hawk, Davis, & Stevenson, 1991; Hawk, Stevenson, & Cook, 1992; Witvliet & Vrana, 1995), although modulation of startle latency by affective valence (through pleasant – to neutral – to aversive) is less robust than for startle magnitude (Kumari et al., 1996; Corr et al., 1995). Startle latencies have been viewed as a measure of efficiency (i.e. time taken to produce the defensive startle response). Variation in latencies have been considered to indicate changes in neural processing speed (e.g. Pearce et al., 2013), and/or the degree to which emotional stimuli, mood, or psychopathology (via associated neural networks) might interfere with the startle pathway (e.g. Takahashi et al., 2014). Studies showing modulation of startle latency but not magnitude suggests that these parameters tap different processes, or are amenable to different factors (e.g. Panayiotou, Witvliet, Robinson & Vrana, 2011; Hutchinson, Niaura & Swift, 2000; Hackley & Graham, 1987). Indeed, latency has been considered to be more closely related to changing levels of arousal rather than emotional valence (Naudin, Canu & Costentin, 1999).

Startle reflexes are a common phenomenon across species, allowing for inferences to be drawn about human anxiety based on the neural structures identified in animal research (Grillon, 2002). Research in rodents (Hitchcock & Davis, 1986, 1991; Rosen, Hitchcock, Sananes, Miserendino, & Davis, 1991), human imaging (Pissiota et al., 2003), and lesion studies (Buchanan, Tranel, & Adolphs, 2004; Funayama, Grillon, Davis, & Phelps, 2001) implicates the extended amygdala, and in particular the central nucleus of the amygdala and the bed nucleus of the stria terminalis (BNST), in mediating startle potentiation. Lesion studies suggest that the central nucleus of the amygdala potentiates startle responses to brief, aversive stimuli of a short duration (i.e. mediates fear-potentiated startle). Conversely the BNST does not potentiate startle to discrete aversive cues, but does potentiate startle over sustained periods of anxiety (for example when nocturnal rodents are exposed to bright light, or when humans anticipate prolonged uncertain threat; see Grillon, 2008 for review). Likewise the central role of the extended amygdala in normal fear and pathological anxiety is well described (Davidson, 2002; Davis & Whalen, 2001). Furthermore, recent evidence in rodents suggests that the amygdala functions as an important chemosensor to directly detect increases in extracellular levels of carbon dioxide (via acid-sensing ion channels, ASIC1a) to increase behavioural inhibition and freezing (Ziemann et al., 2009).

While inhalation of low concentrations of CO_2 increases anxiety and autonomic arousal in humans (Bailey et al., 2005), and triggers anxious behaviour in small animals (Ziemann et al., 2009) and humans (Garner et al., 2011a; Garner et al., 2012), its effects on defensive behaviours that are mediated by the extended amygdala are not known. To date, only two studies have explored the effect of CO_2 challenge on the human eye-blink startle response (Ceunen et al., 2013; Pappens et al., 2012). Both studies examined the magnitude (but not latency) of three startle responses to acoustic probes delivered during a short (< 2 min) inhalation of 7.5% CO_2. Contrary to predictions, startle magnitudes were reduced (rather than potentiated) during CO_2 challenge relative to baseline. These findings contrast with evidence that 7.5% CO_2 challenge over longer durations (10-20 minutes) can *increase* anxious behaviour in response to threat in humans and animals.

This study investigated whether 7.5% CO_2 inhalation (versus air) can enhance the defensive eye-blink startle response in healthy human subjects. The anxiogenic effects of low-dose CO_2 inhalation are well characterised, however little is known about

the mechanisms through which CO_2 produces a state of anxiety in humans. Animal research has suggested that the amygdala is a critical chemosensor of increasing CO_2 which stimulates defensive behaviours (Ziemann et al., 2009). Consequently, the present research aimed to examine whether the amygdala might play a similar role in humans. By better understanding the way CO_2 inhalation produces fear and/or anxiety symptoms, clarification can be gained about whether this model best represents a state of sustained anxiety or an acute fear response. This may help address the suitability of 7.5% CO_2 inhalation as a model of anxiety rather than fear (see research question 1; Chapter 1:) and contributes to the limited literature that attempts to unpick how CO_2 produces fear/anxiety in humans.

Primary aims and predictions

We compared the effects of 7.5% CO_2 (versus air inhalation) on eye-blink startle reactivity to threatening (aversive) and non-threatening (neutral) picture stimuli. We also examined the effects of 7.5% CO_2 challenge on startle latency; a measure of efficiency that is often overlooked. Both parameters of eye-blink startle response were included to address whether CO_2 modulates the magnitude and efficiency of this defensive mechanism. An optimal adaptive startle response to threat should be both robust and quick. In line with previous findings, it was predicted that:

1. healthy volunteers would show increases in subjective anxiety and autonomic arousal following 7.5% CO_2 inhalation.
2. if 7.5% CO_2 inhalation triggers defensive behaviour coordinated by the amygdala (consistent with evidence for a central role of the amygdala in coordinating CO_2-induced fear behaviours in rodents; Ziemann et al., 2009) then eye-blink startles would be both larger and faster during CO_2, and this would be more apparent in the response to aversive images.

2.2 Method

Ethical considerations

The study protocol was approved by the University of Southampton Ethics and Research Governance Committee. All participants gave written informed consent prior to participation and were made explicitly aware that they could withdraw at any point.

Participants

Twenty-seven participants (16 female) aged 18-26 years old (*M* = 20.62, *SD* = 2.14) were recruited. Participants completed an initial health screen by telephone and a pre-test screening interview to confirm eligibility. Consistent with previous 7.5% CO_2 challenge studies (e.g. Garner et al., 2011a; Garner et al., 2012), exclusion criteria included current or history of psychiatric illness as assessed by the MINI International Neuropsychiatric Interview (based on DSM-IV; Sheehan et al., 1998), personal or family history of panic disorder or panic attacks, medication use within the last 8 weeks (apart from local treatment, occasional aspirin or paracetamol, and contraceptives), smoking, history of asthma/respiratory disease, diabetes, migraines or cardiovascular disease, excessive alcohol consumption (> 50 units/week for males, > 35 units/week for females; participants recruited in this study reported a mean of 9.38 units/week, *SD* = 6.86) or positive alcohol breath test, current or past alcohol or drug dependence, under- or overweight (body mass index < 18 or > 28kg/m^2), blood pressure exceeding 140/90 or heart rate of < 50bpm or > 90bpm at baseline, caffeine consumption of > 8 caffeinated drinks/day, or pregnancy/breastfeeding. Levels of trait anxiety (trait version of the State-Trait Anxiety Inventory (STAI), Spielberger, Gorsuch, Lushene, Vagg, & Jacobs, 1983; M = 32.10, SD = 6.67) and worry (Penn State Worry Questionnaire, Meyer, Miller, Metzger, & Borkovec, 1990; M = 45.41, SD = 12.05) were comparable with those observed in healthy control groups (Garner, Baldwin, Bradley, & Mogg, 2009)**.**

Procedure

Participants attended a single test session and completed an affective startle task twice, once during a 20 minute inhalation of air enriched with 7.5% CO_2 (a balance of 7.5% CO_2, 21% O_2 and 71.5% N_2) and once during a 20 minute inhalation of normal air. Inhalations were administered blind to participants and were separated by a 30 minute break to remove potential carry-over effects. Gas was administered through an oro-nasal face mask with inhalation order (i.e. CO_2 vs. air first) counterbalanced across participants in a within-subjects, single blind, cross-over design. Participants were contacted the following day to record any adverse events.

Measures of subjective state anxiety (Spielberger et al., 1983); positive and negative affect (PANAS; Watson et al., 1988), and blood pressure (Omron-M6 arm-

collar, Medisave-UK) were taken at pre-test baseline (10 minutes before the first inhalation) and immediately (within 1 minute) after each inhalation period (peak). Heart rate (HR) was measured at baseline (arm-collar). Electromyography (EMG) of the startle reflex, heart rate and skin conductance was continuously recorded throughout both inhalations.

Following the startle task participants completed a 7 minute behavioural measure of impulse-control; the stop-signal reaction time (SSRT) task. In this task each participant's SSRT is estimated from a staircase analysis of their reaction-time distribution. For several participants the algorithm was unable to converge on a reliable estimate of SSRT for both inhalations, perhaps reflecting an insufficient number of trials in our version of the task – thus reliable SSRT data is not available to report.

Startle task

Eye-blink electromyography (EMG) data were recorded using two 4mm Ag-AgCl electrodes placed under the centre and the outer canthus of the right eye. EMG was sampled at 1000Hz, amplified (x 10,000), rectified, filtered (30-500Hz) and integrated (20ms constant) using a Biopac MP150 data acquisition system and AcqKnowledge 4.1 software (Biopac Systems, CA, USA).

The startle task took 8 minutes to complete and was administered 2 minutes after the start of each twenty minute inhalation period. Participants were instructed that they would see a series of pictures and hear occasional noises. Participants viewed 32 images (16 aversive and 16 neutral) taken from the International Affective Picture Set (IAPS; Lang, Bradley, & Cuthbert, 2005). The images were selected on the basis of normative valence (scale -4 to +4) and arousal ratings[6] (0-8; aversive images: mean valence = -3.85 and mean arousal = 6.74; neutral images: mean valence = 2.30 and mean arousal = 4.24). Images subtended 22.2 x 15.1 visual degrees (viewed at 58cm) and were presented using Inquisit 2 (Millisecond.com, 2002) in a randomized order.

Startle reflexes were elicited with a 50ms, 96dB burst of white noise with near instantaneous rise/fall time delivered via headphones. A familiarisation block of 3

[6] Images selected from the IAPS database were 16 neutral and 16 negative images. Neutral: 1463, 1540, 1999, 5890, 5920, 7002, 7010, 7190, 7282, 7283, 7285, 7320, 7390, 7500, 7580, and 8510. Negative: 2053, 3000, 3010, 3030, 3053, 3060, 3071, 3100, 3102, 3120, 3130, 3150, 6350, 6510, 6560, and 6570.

habituation startle probes was followed by an experimental block comprising 32 randomly ordered trials (24 experimental picture startle trials, 4 inter-trial-interval (ITI) startle trials, and 4 no-startle trials). On experimental trials, aversive and neutral images were presented for 4000ms. The startle probe was presented 3000ms after image onset. Interspersed within the experimental trials were 4 no-startle trials and 4 trials where the startle probe was presented 7000ms after picture offset during a 14 second ITI. ITI- and no- startle trials were included to reduce the predictability of the startle probe. Picture valence was counterbalanced across trial type. Habituation and ITI startles were not included in analyses of startle magnitude or latency.

Data acquisition and preparation

All physiological responses (eye-blinks, heart rate, and skin conductance) were sampled at 1000Hz via a Biopac MP150 data acquisition system and were processed with AcqKnowledge 4.1 software. Data was processed blind to inhalation and trial type.

Startle magnitude and latency. Data from four participants were excluded from all startle analyses. Three participants were excluded due to a technical fault (recording failure) and a participant did not complete the task in full. Inspection of boxplots revealed startle responses that occurred < 50ms as extreme outliers – this equated to 3.3% of experimental trials and these data were removed from both magnitude and latency analyses. Startle magnitude was defined as the maximum response between 50-120ms after probe onset minus the mean EMG activity during the 50ms prior to probe onset. To correct for inter-subject variability, all blink magnitudes were standardised to T-scores (i.e. ((z x 10) +50) providing a distribution with a mean of 50 and SD of 10) within each participant using the condition mean and standard deviation which is a common procedure (see Blumenthal et al., 2005)[7]. Startle latencies are reported relative to probe onset.

[7]The score reflecting peak magnitude is calculated as the 50-120ms peak value after probe offset minus the mean value 0-50ms prior to probe onset. This is calculated in this way in order to remove any baseline noise **within** each trial and gives a peak magnitude score that more accurately reflects an increase from a stable baseline (where large variation can exist). However, as wide variation in this peak magnitude value has been observed within individuals without any clear link to an experimental manipulation, T scores are calculated to remove variation **across** trials for each individual. Thus the extent of change in magnitude can only be due to the experimental manipulation, as this varies freely of any variation within each individual.

Defensive eye-blink startle responses during 7.5% CO_2 inhalation

Heart rate. ECG was recorded with two electrodes placed on cleaned skin at the base of the thumb on both wrists at a sample rate of 1000Hz. The raw signal was cleaned using an FIR band pass filter (0.5 – 35Hz) and was matched to a representative ECG cycle template using AcqKnowledge 4.1 software. Raw tachograms were visually inspected for inconsistencies in R-R intervals, and were manually corrected where necessary.

Skin conductance. Skin conductance responses to pictures were also recorded with Ag-AgCl electrodes and conductive gel attached to the medial phalanges of the ring and middle fingers of the participant's non-dominant hand. Skin conductance responses (SCRs) to pictures were calculated by subtracting the mean skin conductance level 1000ms before picture onset (pre-trial baseline SCR) from the maximum skin conductance level between 900–4000ms window after picture onset (peak SCR) – this window excludes SCR responses to the acoustic startle probes.

Normality and statistical analyses

Comparisons between baseline and peak effects of air and CO_2 inhalation were made for subjective and physiological variables using repeated measures analysis of variance (ANOVA). All variables were normally distributed apart from negative affect scores on the PANAS. Non-parametric alternatives (Friedman's test) of the negative PANAS produced comparable results to parametric tests, so for ease of interpretation ANOVA are reported instead. Mean startle magnitude and latencies were analysed with separate repeated measures ANOVA with inhalation (7.5% CO_2 vs. air), and picture valence (negative vs. neutral) as within subjects factors[8]. For all analyses, Greenhouse-Geisser corrections were used where assumptions of sphericity were violated and Bonferroni corrections were used where appropriate to correct for multiple comparisons.

[8] Inclusion of order as a between subjects effect (mixed model ANOVA) did not qualitatively affect the pattern of results reported in the main text.

2.3 Results

7.5% CO_2 inhalation increased anxiety and cardiovascular function

Inhalation of 7.5% CO_2 significantly increased state anxiety and heart rate, and decreased positive affect (when compared with air; see Table 5). Systolic and diastolic blood pressure was elevated during both CO_2 and air inhalation, relative to baseline.

Effect of 7.5% CO_2 on startle and skin conductance responses

Within each dependent measure, repeated measures analyses of variance (ANOVA) examined the effects of inhalation (7.5% CO_2 vs. air), picture valence (aversive vs. neutral) and their interaction, on startle magnitude, startle latency and skin conductance response (see Table 6 for descriptive statistics). There were no significant effects on startle magnitude (*F's* < .274, *p*'s > .61, *ns*). A main effect of inhalation (*F* (1, 22) = 5.38, *p* = .030, η_p^2 = .196) revealed that startle latency was significantly slower during the inhalation of 7.5% CO_2 (*M* = 102.22, *SE* = 0.95) relative to air (*M* = 100.71, *SE* = 0.92; *t* (22) = 2.32, *p* = .030, d_{av} = 0.34). A main effect of inhalation was revealed for skin conductance (*F* (1, 25) = 8.85, *p* = .006, η_p^2 = .261) where skin conductance responses were significantly greater during inhalation of 7.5% CO_2 (*M* = .085, *SE* = .019) than air (*M* = .024, *SE* = .012, *t* (25) = 2.98, *p* = .006, d_{av} = 0.76) irrespective of picture content (*F* (1, 25) = 0.10, *p* = .752, *ns*). All other results were non-significant.

Defensive eye-blink startle responses during 7.5% CO_2 inhalation

Table 5. Effects of 20 minutes 7.5% CO_2 challenge on anxiety, mood and autonomic arousal.

	Baseline		Air		7.5% CO_2		ANOVA		
	M	*SD*	*M*	*SD*	*M*	*SD*	*F*	*p*	N_p^2
State anxiety	30.88[a]	(9.27)	33.75[a]	(8.72)	39.83[b]	(10.40)	15.87	.001	.379
Positive affect	30.00[a]	(7.91)	28.46	(7.80)	25.78[b]	(7.45)	7.69	.001	.228
Negative affect	12.26	(3.58)	12.48	(3.66)	13.63	(5.10)	2.36	.133	.079
Systolic BP	117.52[a]	(13.43)	124.60[b]	(17.95)	131.28[b]	(22.30)	14.00	.001	.368
Diastolic BP	70.60[a]	(7.42)	75.52[b]	(7.26)	75.76[b]	(12.12)	7.15	.005	.230
Heart Rate	71.92[a]	(11.09)	72.99[a]	(9.70)	78.43[b]	(12.65)	10.63	.001	.316

Note. Within each variable (row) values with different superscripts are significantly different from each other $p < .017$ (Bonferroni correction applied).

Table 6. Untransformed means (standard deviations) of startle indices, skin conductance and heart rate during air and 7.5% CO_2 inhalations.

	Air		7.5% CO_2	
	M	SD	M	SD
Startle magnitude (μV)	17.40	(17.70)	17.21	(16.16)
Negative	17.55	(17.56)	17.37	(17.05)
Neutral	17.25	(18.14)	17.04	(15.55)
Startle latency (ms)	100.71	(4.42)	102.22	(4.55)
Negative	100.58	(4.53)	101.75	(5.19)
Neutral	100.83	(4.70)	102.68	(4.16)
Skin conductance (μS)	.0242	(0.06)	.0848	(0.10)
Negative	.0343	(0.11)	.0941	(0.13)
Neutral	.0141	(0.06)	.0756	(0.09)

Associations between subjective and physiological responses to CO_2 inhalation

Difference scores (i.e. mean during CO_2 minus mean during air) were calculated to reflect the degree of CO_2-induced increases in i) subjective response, ii) autonomic response (heart rate, blood pressure), iii) magnitude and latency of startle response, and iv) skin conductance response. There were positive associations between CO_2-induced state anxiety, heart rate and blood pressure (see Table 7 and Figure 2). The effect of CO_2 on heart rate was strongly associated with reduced skin conductance responses during CO_2 relative to air and was further associated with slower startle latencies (see Figure 3). Those who experienced the greatest increases in HR during CO_2 inhalation tended to show the least pronounced change in SCR. Furthermore, CO_2-induced increases in negative affect correlated positively with increased anxiety and heart rate whereas CO_2-induced decreases in positive affect negatively correlated with increased anxiety and blood pressure. Finally greater negative affect during CO_2 was associated with larger startle responses during CO_2 relative to air.

Defensive eye-blink startle responses during 7.5% CO_2 inhalation

Table 7. Pearson's R correlations between CO_2-induced subjective and autonomic responses.

		1.	2.	3.	4.	5.	6.	7.	8.	9.
1.	State anxiety									
2.	Positive affect	-.644***								
3.	Negative affect	.771***	-.319							
4.	Systolic BP	.497*	-.536**	.267						
5.	Diastolic BP	.433*	-.370	.199	.575**					
6.	Heart rate	.461*	-.313	.424*	.499*	.535**				
7.	Startle magnitude	.040	.262	.414*	-.232	-.241	.179			
8.	Startle latency	.093	-.061	.153	-.082	-.283	.506*	.126		
9.	Skin conductance	-.079	-.050	-.116	-.206	-.159	-.452*	-.171	.022	

Note. * = significant < .05, ** = significant < .01 and *** = significant < .001.

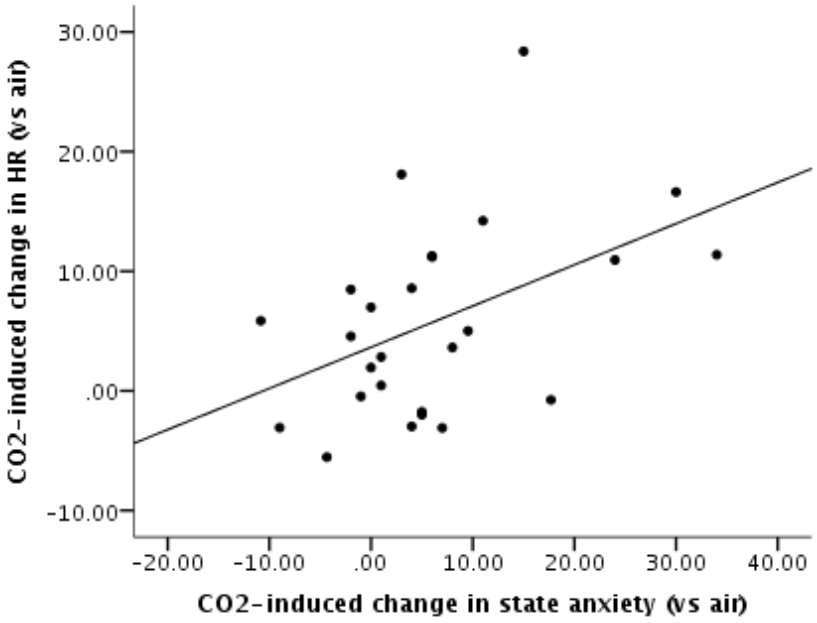

Figure 2. Associations between CO_2-induced increases in heart rate with increases in state anxiety.

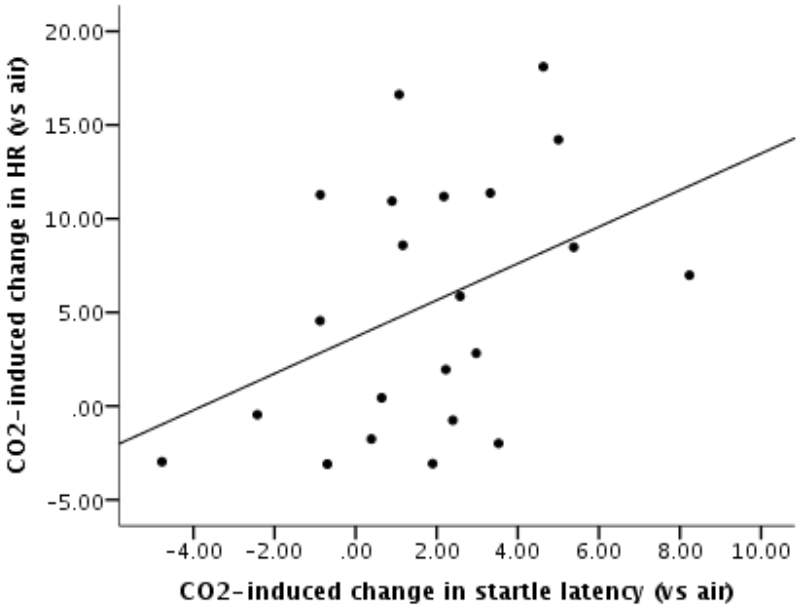

Figure 3. Associations between CO_2-induced increases in heart rate with increases in startle latency.

2.4 Discussion

The defensive startle response is characterised by a rapid and powerful eye-blink that is potentiated by the extended amygdala. This study examined the effects of 7.5% CO_2 challenge on both the magnitude and latency of startle responses. Contrary to predictions, 7.5% CO_2 inhalation did not modulate eye-blink magnitude despite robust effects on subjective mood and physiological arousal. Rather it slowed the latency of eye-blink responses to startle probes. These findings extend previous evidence that inhalation of 7.5% CO_2 for short periods (< 2 minutes) can reduce (rather than potentiate) the magnitude of startle responses to probes delivered in the absence of emotional stimuli (Ceunen et al., 2013; Pappens et al., 2012).

Why might 7.5% CO_2 challenge delay eye-blink startle latencies (present study), and/or reduce their magnitude (Ceunen et al., 2013; Pappens et al., 2012)? One possibility is that CO_2 challenge may limit processing resources required for defensive startle. Consistent with previous findings, CO_2 challenge produced large increases in subjective anxiety and autonomic arousal (including heart rate and skin conductance). Furthermore CO_2-induced increases in heart rate covaried with both subjective anxiety and longer startle latency during CO_2 challenge. Strong positive correlations between CO_2-induced increases in heart rate and subjective anxiety have been reported in previous studies (Garner et al., 2011a; Garner et al., 2012), and may reflect participants use of interoceptive 'threat' when rating their subjective anxiety. Notably, attenuated startle responses have been observed in paradigms that directly target interoceptive mechanisms (e.g. pain caused by cold pressor or mechanically resisted breathing; Ceunen et al., 2013, see also Pappens, Van den Bergh, Vansteenwegen, & Van Diest, 2011). Inhibited startle responses have also been reported when delivered during the post-encounter stage of the defence cascade (as characterised by decreasing distance towards confrontation), which was suggested to reflect inhibition and suppression of irrelevant material during task-focussed attention (Bradley, Codispoti, & Lang, 2006). Finally, startle responses are attenuated when cognitive load is high (e.g. through increased task demand - see Vytal, Cornwell, Arkin, & Grillon, 2012). Recent comparisons of 7.5% CO_2 challenge and cognitive load suggest that both manipulations might produce comparable deficits in behaviour through common effects on top-down attention/control mechanisms (Mattys, Seymour, Attwood, & Munafo, 2013). Thus CO_2-induced deficits in cognitive control, together with increased awareness of

competing interoceptive threat cues and corresponding increases in cognitive load, may limit the resources required to potentiate startle, thus slowing startle responses, and obscuring the typical effects of picture valence on startle magnitude (such as Vrana et al., 1988).

How do these findings fit with those from eye-blink startle studies in other forms of anxiety? Potentiated startle has been reliably demonstrated in PTSD (e.g. Morgan et al., 1995), specific phobias (e.g. Hamm et al., 1997), social anxiety (e.g. Cornwell, Johnson, Berardi, & Grillon, 2006; Garner et al., 2011b) and panic disorder (e.g. Grillon et al., 2008). In contrast, there is comparatively weak evidence of potentiated startle in generalized anxiety disorder (GAD; review by Vaidyanathan et al., 2009), and even evidence of reduced startle reactivity during anticipation of uncertain threat in GAD relative to other anxiety-related conditions (Grillon et al., 2009; see McTeague & Lang, 2012) which may be due to its high comorbidity with depression, where blunted startles are also a common feature (e.g. Taylor-Clift, Morris, Rottenberg, & Kovacs, 2011).

7.5% CO_2 challenge in healthy volunteers has been proposed as an experimental model of generalized anxiety disorder (Bailey et al., 2005; Bailey et al., 2011a). Drug treatments that are clinically effective for generalized anxiety can reduce some of the deleterious effects of 7.5% CO_2 challenge, and provide some support for the GAD model (e.g. Diaper et al., 2012b). Likewise 7.5% CO_2 challenge can mimic deficits in attentional control that are observed in (unchallenged) individuals with elevated generalized trait anxiety (Garner, Ainsworth, Munafo, & Baldwin, 2013). Consequently, the unexpected effects of 7.5% CO_2 on startle reported here, appear consistent with patterns of startle responding that are observed in conditions associated with broad negative affect, rather than acute periods of fear and panic (McTeague & Lang, 2012).

Converging evidence implicates the extended amygdala in potentiating startle responses (Pissiota et al., 2003) and mediating CO_2-induced behaviour in animals (Ziemann et al., 2009). However, the findings reported here and those of Pappens et al. (2012) and Ceunen et al. (2013) suggest subjective and autonomic response to CO_2 challenge can occur in the absence of defensive behaviour coordinated by the amygdala. New evidence that individuals with bilateral amygdala lesions can display strong subjective and autonomic responses to 35% CO_2 challenge suggests that mechanisms beyond the amygdala may mediate human response to CO_2 (Feinstein et al., 2013). The

responses of 3 patients with bilateral amygdala damage (caused by the rare genetic disorder Urbach-Wiethe disease; UWD) were assessed in a single inhalation of 35% CO_2. Previous research has shown that UWD patients do not experience fear to traumatic life events or to the presentation of fear-evoking stimuli (Feinstein, Adolphs, Damasio, & Tranel, 2011), and they struggle to identify faces as fearful (Adolphs, Tranel, Damasio, & Damasio, 1994; Tsuchiya, Moradi, Felsen, Yamazaki, & Adolphs, 2009). It was expected that UWD patients would not display signs of fear in response to CO_2. Contrary to this expectation, 35% CO_2 provoked panic and fear in all 3 UWD patients significantly more so than non-panickers in the control group. This indicates that an intact amygdala is not essential for CO_2 inhalation to cause fear and panic and suggests that other brain areas may mediate this response.

Esquivel et al. (2009) propose a distributed network of brain regions that underlie CO_2 challenge, including the locus coeruleus, hypothalamus, midbrain raphe and amygdala. Future research should clarify the neuro-pharmacological networks and peripheral chemoreceptor and mechanoreceptor systems that underlie the subjective, autonomic and behavioural response to CO_2 inhalation in humans, and factors that predict individual differences in response to challenge. To this end, research should examine whether anxiolytic drugs that modulate startle during anxious uncertainty but not phasic fear (review by Grillon, 2008; e.g. the benzodiazepine alprazolam, Grillon et al., 2006b) can also reduce the effects of CO_2 challenge on anxiety, autonomic arousal and startle reactivity. Furthermore, studies should try to take continuous measures of subjective mood, blood pressure, heart rate and respiration rate/volume (not measured here; see Poma et al., 2005), to help dissociate phasic and sustained responses throughout CO_2 challenge. This would extend current evidence of the cumulative effects of CO_2 on autonomic arousal, whereby heart rate increased throughout the inhalation period in an analysis of a randomly selected subgroup of the sample (mean HR between 5-10mins of CO_2 = 76.44 *SD* = 16.41, mean HR between 15-20mins of CO_2 = 81.09, *SD* = 19.75, t (9) = 2.79, p = .021). This suggests that the autonomic effects of 7.5% CO_2 rise early in the inhalation period and continue to increase gradually across the 20 minutes (see Bailey et al., 2005; Poma et al., 2005).

Our findings and those of Pappens et al. (2012) and Ceunen et al. (2013) suggest that 7.5% CO_2 challenge inhibits eye-blink startle. However these three studies differ markedly in design (within vs. between subjects), inhalation duration (ranging from <2

minutes to 20 minutes), number of startles, and affective-paradigm (contextual vs. emotional picture-potentiated). Our startle paradigm is based on those widely used in previous emotional picture-potentiated startle studies (e.g. Vrana et al., 1988), however it has not been widely used in within-subject designs, and it is possible that in our study habituation to aversive stimuli may increase the likelihood of type II error. Future research in this area would benefit from the recent development of standardized startle protocols that have already shown promise in validation studies, and that can differentiate startle responses during phasic cued fear vs. sustained contextual anxiety (e.g. NPU threat test, Schmitz & Grillon, 2012). For example, evidence that 7.5% CO_2 challenge mimics anxiety-potentiated rather than fear-potentiated startle in the NPU-threat test would further validate 7.5% CO_2 as a model of anxiety.

To summarise, despite strong effects of 7.5% CO_2 challenge on subjective anxiety and autonomic arousal, there was no evidence that CO_2 challenge potentiates defensive startle behaviour. Instead, the present research suggest that 7.5% CO_2 reduces the speed of startle responses, and extends previous findings where short durations of 7.5% CO_2 were shown to reduce the magnitude of startle eye-blinks (Ceunen et al., 2013; Pappens et al., 2012). Taken together, these findings are consistent with startle profiles observed during interoceptive threat (Ceunen et al., 2013; Pappens et al., 2011), increased cognitive load (Vytal et al., 2012), and in populations characterised by anxiety and depression rather than by acute fear and panic (McTeague & Lang, 2012).

Defensive eye-blink startle responses during 7.5% CO_2 inhalation

Chapter 3: *The effect of pre-treatment with duloxetine on CO_2 induced anxiety, autonomic arousal and attention in healthy volunteers*

3.1 Introduction

Preclinical animal models of anxiety (such as the elevated plus maze and open field test) often struggle to predict which compounds will be effective in humans (Haller et al., 2012; Nutt & Goodwin, 2011). Experimental models of anxiety in healthy volunteers could help bridge the gap between preliminary assessments of efficacy in animals and clinical trials in patients, and provide early insight into the likely success of new treatments for anxiety.

Inhalation of 7.5% carbon dioxide (CO_2) for 20 minutes produces subjective and physiological symptoms of anxiety in healthy humans (see sections 1.4: Chapter 1, and Chapter 2), raising heart rate and blood pressure and increasing feelings of tension, anxiety and worry. 7.5% CO_2 has also been shown to increase attention towards threat and enhance temporal and spatial attention network function, consistent with patterns in anxious individuals (Garner et al., 2011a; Garner et al., 2012). This model translates across species with low doses of CO_2 producing fear behaviours in rodents via the activation of neural (limbic) areas involved in fear and anxiety (Ziemann et al., 2009). The 7.5% CO_2 model differs from a single inhalation of air enriched with 35% CO_2 that models acute panic (Van Den Hout & Griez, 1984), and instead produces a weaker, yet more prolonged state of anxiety that shares features of the symptom profile of generalised anxiety disorder[9] (GAD; see Chapter 1; Bailey et al., 2011a). Consequently, 7.5% CO_2 inhalation has been advocated to be a new, translational, proof-of-concept model of GAD in healthy humans that can be used to evaluate the anxiolytic properties of potential treatments for anxiety (Bailey et al., 2011a).

Before 7.5% CO_2 inhalation can be used evaluate new treatments for anxiety, it must first be validated i) by determining the extent to which the model can induce core "symptoms" of anxiety and ii) by testing whether current interventions can also

[9] For example, subjective anxiety, edginess and uncontrollable worry, as well as physical symptoms of breathlessness, palpitations, nausea and poor concentration (as described in DSM-5 and the ICD-10; American Psychiatric Association, 2013; World Health Organisation, 1992).

attenuate the symptoms induced by the model. The broad anxiogenic effects of this model are well characterised (see section 1.4.6). Previous studies have found that CO_2-induced anxiety is reduced by drugs that act on the GABA/benzodiazepine receptor complex (such as alprazolam; Bailey et al., 2009 and lorazepam; Diaper et al., 2012b). However, the evidence is less clear for modern drug treatments within this model (see sections 1.6.4 and 1.6.6). For example, the anxiolytic properties of the SSRI paroxetine (Bailey et al., 2007a), SNRI venlafaxine, and the anticonvulsant pregabalin (Diaper et al., 2013) on CO_2-induced anxiety were relatively small. Furthermore, these studies have only examined drug effects on CO_2-induced subjective anxiety (using questionnaire measures) and autonomic arousal (heart rate and blood pressure). They have not examined whether these drugs might target CO_2-induced biases in cognitive-behavioural mechanisms (Garner et al., 2011a; Garner et al., 2012) involved in the aetiology and treatment of anxiety.

Duloxetine is an effective treatment for GAD (Koponen et al., 2007) and has recently been identified to possibly be "first for response" when compared to other licenced UK treatments (Baldwin et al., 2011). Duloxetine also improves attention and emotion processing. Eight week duloxetine treatment significantly enhanced attention, cognition and memory in elderly patients with major depression (Herrera-Guzman et al., 2009; Herrera-Guzman et al., 2010; Raskin et al., 2007) and reduced symptoms of inattention and hyperactivity in adolescents with ADHD (independent of an effect on anxiety/depressive symptoms; Mahmoudi-Gharaei, Dodangi, Tehrani-Doost, & Faghihi, 2011). In healthy volunteers, 2 week duloxetine administration decreased neural activation to emotional faces in affective processing regions including the amygdala (van Marle et al., 2011), and a single dose has been shown to aid recognition of happy facial expressions and increase (incorrect) recall of positive personality characteristics (although improved recognition of disgusted faces were also reported; Harmer et al., 2008).

The advent of the newer SNRI class of antidepressants such as duloxetine raises the interesting question whether drugs that act on 5-HT and NA might produce greater improvement in anxiety symptoms and corresponding deficits in attention than compounds that act on a single system (due to the strong links between NA transmission and attention; Aston-Jones, Rajkowski, & Cohen, 1999; Chamberlain & Robbins, 2013; Goddard et al., 2010; Sara, 2009). In particular, the potential role of NA

in coordinating attentional processes during CO_2 inhalation is supported by evidence finding that i) amygdala projections to the locus coeruleus (LC) modulate attention and autonomic processes (Davis & Whalen, 2001; Sara, 2009), ii) the noradrenergic α2 agonist clonidine impairs vigilance and alerting in humans and animals (Coull et al., 2001; Witte & Marrocco, 1997) iii) noradrenergic genotype predicts poor sustained attention (Greene, Bellgrove, Gill, & Robertson, 2009) and iv) dose-dependent increases of CO_2 produces firing of LC neurons in rodents (Pineda & Aghajanian, 1997) consistent with human models emphasising the role of NA and LC in CO_2 challenge (Bailey et al., 2003).

Recent theories of antidepressant drug action suggest early changes in attention and emotion processing may precede, and mediate subsequent clinical response (see Harmer & Cowen, 2013b; see Pringle, McCabe, Cowen, & Harmer, 2013 for a review, and see section 1.7 for a summary). Consequently, it would be prudent to extend the CO_2 model to include measures of attention and emotion processing that might reveal anxiolytic potential in the absence of subjective effects (which emerge following continued treatment in patients). Garner et al. (2011a) demonstrated that 7.5% CO_2 challenge increased eye-movement errors towards threatening distractor stimuli in an antisaccade task, and enhanced alerting and orienting attention network function in the ANT (Garner et al., 2012). In patients, anxiety increased the time taken to initiate correct antisaccades on trials punished with monetary loss for saccadic errors (Jazbec et al., 2005), and facilitated orienting to anger on prosaccade trials (Mueller et al., 2012). These findings align with current neurocognitive theories and neurological evidence for threat-related attentional bias in anxiety, which suggest that subcortical threat-detecting mechanisms (amygdala) are under-regulated by prefrontal regions implicated in functions of cognitive control (Bishop, 2007, 2008; Bishop et al., 2004a; Eysenck et al., 2007). Evidence of CO_2-induced hypervigilance to threat from the antisaccade task in particular mimics patterns of attention commonly shown in anxious groups (review by Ainsworth & Garner, 2013; Bar-Haim et al., 2007) and together with its good psychometric properties, is recommended for future use in CO_2-drug evaluations (see section 1.3.2.3). The ANT provides a secondary measure of attention that has been found to be sensitive to the effects of CO_2 challenge on alerting and orienting (but not executive control) attention networks (Garner et al., 2012).

The effect of duloxetine on 7.5% CO_2-induced anxiety

In addition, threat-related attentional biases and deficits in attentional control are likely to play a role in the uncontrollability of worry in GAD (Hirsch & Mathews, 2012). Typically, pathological worriers; shift attention to threat stimuli more readily (e.g. emotional faces; Bradley et al., 1999; e.g. threat words; MacLeod et al., 1986), negatively evaluate neutral associations of threat-words (Reinecke, Rinck, Becker, & Hoyer, 2013), report more disorder-related worry intrusions during a suppression task (Reinecke, Hoyer, Rinck, & Becker, 2013), and have less working memory available to perform simple tasks when worrying (Hayes, Hirsch, & Mathews, 2008). Importantly, these cognitive biases are receptive to treatment with CBT (Mogg, Bradley, Millar, & White, 1995), attention bias training (Hazen, Vasey, & Schmidt, 2009) and pharmacotherapy (Steiner, Petkus, Nguyen, & Loebach Wetherell, 2013). However, whether this defining feature of GAD is induced by 7.5% CO_2 inhalation has not been formally examined beyond a simple visual analogue "worry" scale (e.g. Bailey et al., 2005), and further assessment could validate 7.5% CO_2 challenge as a model of GAD.

Primary aims and predictions

By identifying whether a known anxiolytic such as duloxetine can reduce anxiety responses to 7.5% CO_2 inhalation, the overarching research questions described in Chapter 1 can begin to be addressed. If duloxetine is found to be effective at reducing CO_2-induced anxiety (and associated attentional deficits), this would suggest that 7.5% CO_2 can replicate some symptoms of pathological anxiety in healthy volunteers. Conversely, if duloxetine has no effect on CO_2-induced anxiety this may indicate that the 7.5% CO_2 model differentiates between treatments with acute anti-anxiety effects (e.g. benzodiazepines) and drugs which require more chronic dosing (e.g. SSRIs and SNRIs). This research would therefore encourage the use of translational healthy human models such as CO_2 inhalation for the purposes of future anxiety research and drug development.

In this experiment, the effects of duloxetine on CO_2-induced anxiety were evaluated. Subjects were randomised to receive a two week course of duloxetine (30mg titrated to 60mg after three days) or matched placebo. On day 14, participants completed measures of subjective mood, autonomic arousal and attentional control (ANT and antisaccade task) during a 20 minute inhalation of 7.5% CO_2 and air. Participants also completed a short thought intrusions task after each inhalation and

after a period of instructed worry (Ruscio & Borkovec, 2004). Previous research has found that prior treatment with an antidepressant can produce slight reductions in anxiety after CO_2 (e.g. Bailey et al., 2007a; Diaper et al., 2013). In addition, previous studies have also suggested that duloxetine can have positive effects on anxiety and attention in patient samples (Herrera-Guzman et al., 2009; Herrera-Guzman et al., 2010; Mahmoudi-Gharaei et al., 2011).

With respect to these findings, it was hypothesised that:

1. 14 day duloxetine administration will not alter general mood and anxiety in a healthy sample;
2. the duloxetine group (versus placebo) will experience less anxiety at peak CO_2 than the placebo group, with no change in autonomic arousal[10]. This will be demonstrated by significant interactions between drug (duloxetine vs. placebo) and time (baseline, peak-air vs. peak-CO_2) on subjective measures of mood and anxiety;
3. the duloxetine group (versus placebo) will make fewer antisaccade errors to threatening images and show improved executive control on the ANT during CO_2 challenge (again shown by a significant interaction between drug and peak-inhalation effects); duloxetine may also reduce the effects of 7.5% CO_2 inhalation on hypervigilance (alerting and orienting attention network function);
4. negative thought intrusions will be more frequent following CO_2 inhalation (than air); an effect that will be reduced in the duloxetine group.

3.2 Method

Participants

40 healthy volunteers (20 male) were randomised to receive either a two week course of duloxetine (30mg titrated to 60mg after 3 days) or matched placebo (drug groups balanced by gender, double-blind). Consistent with Study One (Chapter 2),

[10] Whilst no effect of duloxetine on autonomic arousal was anticipated during CO_2 inhalation, hypertension is an uncommon side effect of duloxetine (BNF; Joint Formulary Committee., 2014) and may lead to elevated blood pressure after administration.

participants completed a short phone screen and detailed screening interview to examine medical and psychological wellbeing prior to recruitment in this study. Exclusion criteria included current or history of psychiatric illness as assessed by the MINI International Neuropsychiatric Interview (based on DSM-IV; Sheehan et al., 1998), personal or family history of panic disorder or panic attacks, medication use within the last 8 weeks (apart from local treatment, occasional aspirin or paracetamol, and contraceptives), smoking, history of asthma/respiratory disease, diabetes, migraines or cardiovascular disease, excessive alcohol consumption (> 28 units/week for males, > 21 units/week for females) or positive alcohol breath test, current or past alcohol or drug dependence, under- or overweight (body mass index < 18 or > 28kg/m^2), blood pressure exceeding 140/90 or heart rate of < 50bpm or > 90bpm, caffeine consumption of > 8 caffeinated drinks/day, pregnancy/breastfeeding, or distress when viewing example images for the antisaccade task.

Additionally, potential contraindications to duloxetine were identified and also constituted exclusion criteria for the study. These included previous treatment with duloxetine or known hypersensitivity to duloxetine, renal or kidney problems, and known acute narrow glaucoma or increased intraocular pressure as detailed in the BNF (Joint Formulary Committee., 2014). All participants were contacted by phone every three days (days 3, 6, 9 and 12) during the two weeks to record any experienced side effects and guidance was provided whether to continue to the higher dose (2 x 30mg tablets of duloxetine, or 2 x matched placebo pills) on day 4. All participants completed the 2 week treatment phase of the study and were titrated to the 60mg dose of duloxetine (or equivalent placebo pills) on day 4. No serious adverse events were reported.

Procedure

In order to ensure that groups were equally matched, measures of anxiety (assessed with a modified version of the GAD-7 (see Appendix A), and the trait version of the Spielberger state-trait anxiety inventory (STAI); Spielberger et al., 1983; Spitzer et al., 2006), positive and negative affect (PANAS; Watson et al., 1988), worry (Penn-State Worry Questionnaire (PSWQ), Meyer et al., 1990), heart rate and blood pressure (both measured with a standard blood pressure monitor; Omron-M6 arm-collar,

Medisave-UK) were taken at screening. The GAD-7, PANAS and autonomic measures were repeated at the end of the drug administration period (day 14).

Participants attended a single 3-hour testing session on the last day of drug administration (day 14) to complete two 20-minute inhalations of 7.5% CO_2 and air (inhalation order counterbalanced across participants and drug group). Inhalations were administered blind to participants and were separated by a 30-minute break to remove potential carry-over effects. Gas was administered through an oro-nasal face mask with inhalation order (i.e. CO_2 vs. air first) counterbalanced across participants in a within-subjects, single blind, cross-over design. During both inhalations, the Attention Network Test (ANT) and the antisaccade task were completed (fixed-order). Subjective ratings of state anxiety (GAD-7), positive and negative affect (PANAS) and blood pressure were recorded after each inhalation. Continuous measures of autonomic arousal (heart rate and respiration rate) were assessed throughout both inhalations. The continuous measure of heart rate was the primary measure instead of the post-inhalation measures made with the monitor, as this was considered to be a more accurate assessment of HR change throughout the inhalation periods (see Chapter 2). Shortly after each inhalation participants completed a 5-minute thought intrusions task, and after the second inhalation, participants also completed a 5-minute worry induction followed by the thought intrusions task again (detailed below).

ANT

The ANT is illustrated in Figure 4. On each trial, a central fixation cross is presented for 400-1,600ms. This is followed by the presentation of a cue for 100ms (except on no-cue trials). After cue offset, the fixation cross is presented again for 400ms (or 500ms in no-cue trials), which is then followed by the target arrow (with flanker arrows). Participants are required to classify the direction of the central arrow with a manual button press response on a keyboard.

Centre cue and double cue trials alert participants to the onset of the target. On centre cue trials, a cue is displayed in the location of the fixation cross. On double cue trials, a cue is presented above and below the fixation cross. Spatial cues alert and orient participants to the location of the target. A spatial cue is presented either above or below the fixation cross, priming participants to the target location. These cues are always presented in the accurate location. Participants are then required to classify the direction

(left or right) of the target arrow as quickly and accurately as possible. The target arrow was flanked by pairs of distractor arrows that were either congruent (e.g. "←←←←←") or incongruent (e.g. "←←→←←") to the target arrow. Arrows subtended 0.55^{o} with 0.06^{o} between arrows. These were viewed at approximately 58cm. Flanker congruence, target direction, and target location were counterbalanced across centre, double, spatial and no-cue trials.

The ANT consists of 8 randomised practice trials (2 of each type of cue). This is followed by 64 randomly ordered experimental trials, with 16 trials per cue condition. Stimuli were presented using Inquisit 2 software. In total, the task took approximately 8-10 minutes to complete.

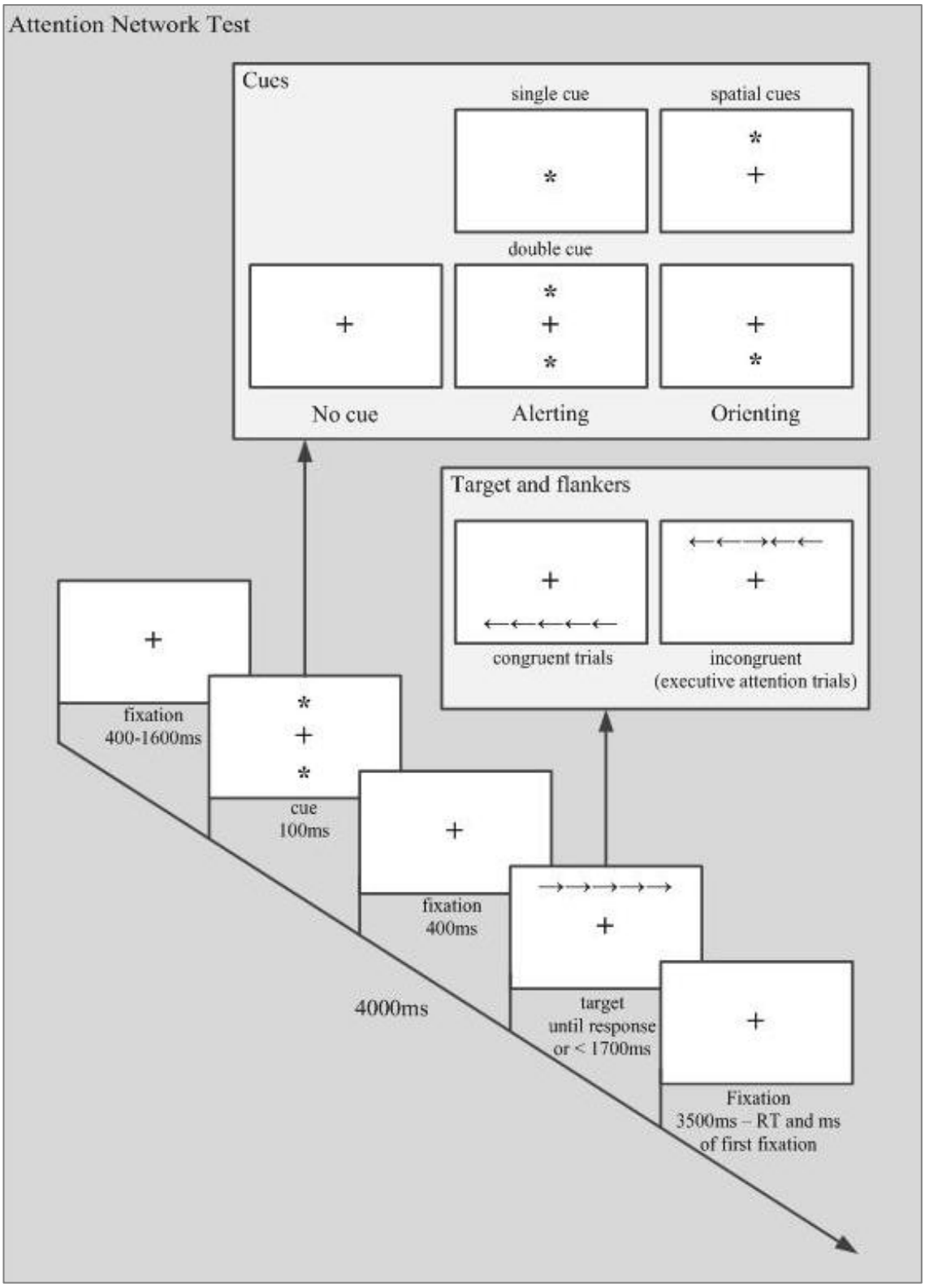

Figure 4. Temporal diagram of the Attention Network Task

Antisaccade

After completion of the ANT, participants completed the antisaccade task (Garner et al., 2011a). Participants were instructed to look towards or away from different types of images as quickly and accurately as possible. On each trial, participants were first presented with an instruction word (TOWARDS or AWAY) in the centre of the screen for 2000ms. 200ms after word offset, a neutral or negative colour image was presented on the left or the right side of the screen for 600ms. If the instruction was TOWARDS, participants were required to generate an eye-movement to look at the image (prosaccade trial). Conversely, if the instruction was AWAY, participants would instead generate an eye-movement to look in the opposite location to the image (antisaccade trial). To increase task demand, 50ms after image offset an arrow appeared on the left or right side of the screen (congruent with image location on 50% of trials). Participants were required to classify the direction of the arrow (up or down). A variable inter-trial interval (750-1250ms) was used with a mean duration of 1000ms. See Figure 5 for clarity.

Stimuli included 8 negative and 8 neutral images taken from the International Affective Picture Set (Lang et al., 2005). These images were selected on the basis of normative valence (scale -4 to +4) and arousal ratings (0-8; negative images: mean valence = 1.85 and mean arousal = 6.74; neutral images: mean valence = 6.30 and mean arousal = 4.24; see Figure 5 for example). Images subtended 8.98 x 6.07 visual degrees (viewed at approximately 58cm) and were presented using Inquisit 2 software (Millisecond.com, 2002).

Participants completed 8 practice pro- and antisaccade trials on which the cue was a yellow rectangle. This was followed by three experimental blocks of 32 trials, with each image presented twice per block in a randomised order (96 trials in total, each image presented 6 times). Pro- and antisaccade trials were also randomised in order and the images were balanced across these trials. In total the task took approximately 9-10 minutes to complete.

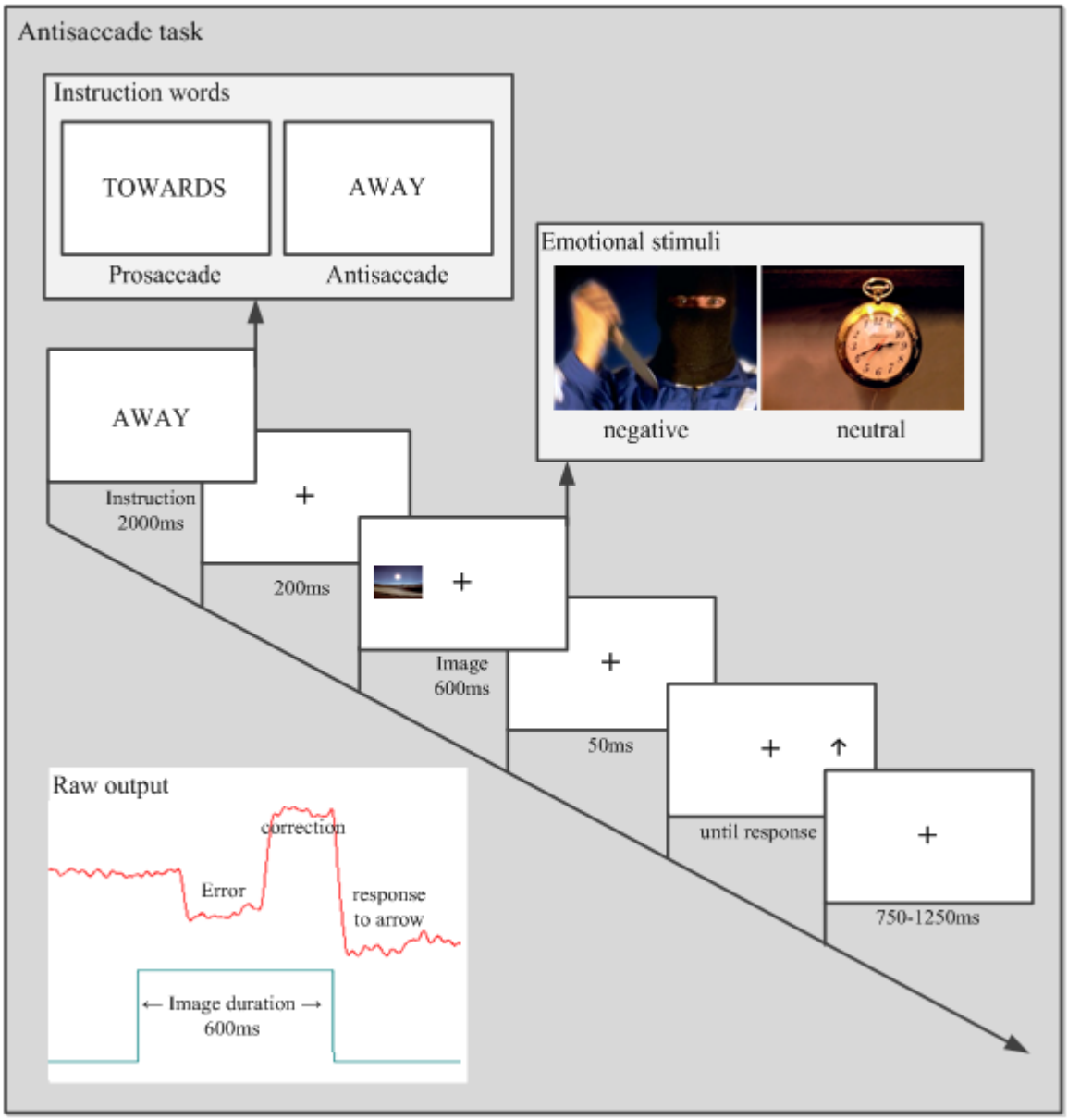

Figure 5. Example experimental trial and stimuli in the antisaccade task.

Thought intrusions task

The thought intrusions task was based on the method developed by Ruscio and Borkovec (2004) and was used to assess the frequency of thought intrusions throughout a five minute period of focussed attention. Participants were instructed to focus on their breathing for 5 minutes. During this time, 12 tones signalled participants to describe whether they were focussing on their breathing (as instructed), or whether a thought intrusion had occurred. If a thought intrusion was reported, participants briefly described the topic of the intrusion and categorised it as positive, negative or neutral in content (e.g. “friends – positive”).

The effect of duloxetine on 7.5% CO_2-induced anxiety

The thought intrusions task was completed three times; i) after air inhalation, ii) after CO_2 inhalation and iii) after a period of instructed worry. Once the two post-inhalation breathing focus phases were completed, participants also undertook a 5-minute worry induction phase. Participants were instructed to briefly describe a current worry to the experimenter and rated on visual analogue scales "how likely it was to happen", "how catastrophic it would be", and "how well they would cope with it". They then focussed on their chosen topic fully for five minutes before the thought intrusions task was completed for a third time.

Data acquisition and preparation

All physiological responses (heart rate, respiration rate and eye-movements) were sampled at 1000Hz via a Biopac MP150 data acquisition system and were processed with AcqKnowledge 4.1. Data were processed by experimenters blind to drug group and trial type.

Heart rate. During the two inhalations, heart rate was recorded through two electrodes placed on the inside of both wrists. The raw signal was band pass filtered (set between 0.5 and 35Hz) with 8000 coefficients and was matched to a representative QRS-template (see 2.2 Method, Chapter 2). After visual inspection of raw tachograms and manual artefact removal, an automated analysis of HR was performed to provide mean HR.

Respiration rate. Respiration rate was recorded with a respiration belt transducer. The raw signal was band pass filtered (low pass 0.05Hz, and high pass 1Hz) with 4000 coefficients. Breaths per minute (BPM) were detected in five minute intervals, resulting in four, five minute segments for both inhalations.

ANT. Reaction times from incorrect trials (3.2%) and where responses were greater than 1,000ms (1.4%) were removed from analyses. The removal of incorrect or slow trials did not vary by drug group or inhalation (F's < 1.00, p's > .323). Alerting, orienting and executive attention scores were calculated for each participant and inhalation. The alerting effect was calculated by subtracting the mean reaction time of double-cue trials from no cue-trials. The orienting effect was calculated by taking the mean reaction time of spatial cue trials from centre cue trials. Higher scores on the alerting and orienting effects of attention suggest greater efficiency of these networks.

Finally the executive control effect is calculated by subtracting the mean reaction time of congruent flanker trials from incongruent flanker trials (across all cue types). Higher scores on the executive control effect indicate less efficient resolution of conflict.

Antisaccade. For the antisaccade task, two single-use electrodes were placed on the outer canthus of each eye to record horizontal eye-movements via electrooculography. A low pass digital filter fixed at 20Hz was performed. Saccades were scored manually by two experimenters blind to trial type and drug group. Visual inspection ensured that the first eye-movement toward or away from the stimulus was captured correctly by the software. On occasions where participants made two eye-movements in quick succession, data was manually corrected to record only the first eye-movement made in response to the stimulus in order to accurately reflect the latency of the first eye movement. Consistent with previous studies, saccades with a latency < 100ms were removed from analyses as they were deemed to be anticipatory (Garner et al., 2011a).

Normality and statistical analyses

Assessments of normality with the Shapiro-Wilk test identified that the GAD-7 significantly deviated from a normal distribution, and as such a square root transformation was applied. All analyses of the GAD-7 were performed on the transformed data. To aid interpretation, raw means are reported in the tables. The negative scale of the PANAS was not normally distributed with a high proportion of participants scoring consistently low on negative items. Transformations did not consistently improve non-normality. As such non-parametric tests (Mann-Whitney U and Friedman tests) were carried out where possible on the negative scale of the PANAS. These tests produced comparable results to parametric alternatives and for clarity, parametric test statistics are reported.

For all analyses of variance (ANOVA), sphericity was assessed with Mauchly's test. Greenhouse-Geisser corrections were reported if the assumption of sphericity was violated. Post hoc analyses were conducted with simple main effects and pairwise comparisons. Unadjusted p-values are reported and compared against Bonferroni corrected p-values where appropriate.

3.3 Results

Group characteristics

Three participants were excluded from all analyses. Two participants did not complete the course of tablets (1 male from the duloxetine group and 1 female from the placebo group) and one male participant from the duloxetine group did not complete the CO_2 challenge (the gas ran out). Groups did not differ on demographics, mood, or measures of blood pressure and heart rate prior to treatment (see Table 8). Participants randomised to receive duloxetine had a slightly higher BMI (22.73kg/m^2) than those who received placebo (21.06kg/m^2).

Table 8. Participant demographics pre-treatment. Means (standard deviations).

	Placebo (N = 19)		Duloxetine (N = 18)		Independent t tests
Age	24.95	(8.98)	24.50	(6.20)	$t(35) = 0.18$, $p = .86$, *ns.*
BMI	21.06	(1.62)	22.73	(2.29)	$t(35) = 2.56$, $p = .015$, $d_s = 0.86$
GAD-7	9.57	(5.06)	9.26	(6.35)	$t(35) = 0.17$, $p = .87$, *ns.*
STAI	33.26	(6.20)	34.00	(8.81)	$t(35) = 0.30$, $p = .77$, *ns.*
PSWQ	39.26	(9.95)	39.61	(14.25)	$t(35) = 0.09$, $p = .93$, *ns.*
Positive affect	35.26	(4.07)	35.53	(5.32)	$t(34) = 0.17$, $p = .87$, *ns.*
Negative affect	13.37	(2.50)	13.22	(2.94)	$t(35) = 0.16$, $p = .87$, *ns.*
Heart rate	68.84	(11.16)	74.39	(10.04)	$t(35) = 1.59$, $p = .122$, *ns.*
SBP	125.05	(8.59)	124.17	(8.18)	$t(35) = 0.32.$, $p = .750$, *ns.*
DBP	69.47	(9.16)	71.72	(9.87)	$t(35) = 0.72$, $p = .477$, *ns.*

Side effect reporting and blinding

All participants (N = 37) completed the two week course of duloxetine or placebo without reported serious adverse event. Participants were asked to describe any symptoms they believed were the result of the drug at the end of the study and which group they believed they had been allocated to.

In total 36 potential side effects were reported by 20 participants. Eight participants who received placebo reported 11 side effects and 12 participants who

received duloxetine reported 25 side effects. The remaining 17 participants (11 of whom received placebo) did not report any side effects thought to be due to the drug. The most commonly reported side effects in the placebo group were headache (*N* = 3), nausea (*N* = 2) and changes in sleep (*N* = 2; difficulty falling asleep *N* = 1, lighter sleep *N* = 1). The most commonly reported side effects in the duloxetine group were changes in sleep (*N* = 5; longer to fall asleep *N* = 2, vivid dreaming *N* = 2, erratic sleep *N* = 1) and nausea (*N* = 5). A greater variety of side effects were described by those taking duloxetine than those taking placebo which included (but was not inclusive to) twitching and tingling fingers, dry mouth, decreased motivation, and fatigue.

Some evidence exists to suggest that participants were able to identify which drug they received (χ^2 (1) = 10.42, *p* = .001, φ = 0.51), however this appears to be driven by those taking placebo (17/20 correct) rather than duloxetine (13/20 correct) and this is presumably due to the lower incidence of side effects.

The effect of duloxetine on mood and autonomic arousal (day 0 vs. day 14)

To examine whether 2 week administration of duloxetine or placebo had an effect on mood or autonomic arousal, 2 x 2 mixed model ANOVAs were conducted with drug (placebo vs. duloxetine) as a between subjects factor and time (baseline vs. post-drug) as a within subjects factor (see Table 9).

The two groups did not differ significantly on levels of anxiety (GAD-7) or positive and negative affect over the two weeks (*p*'s > .123). Similarly, placebo and duloxetine did not differ on measures of heart rate (*p* = .372) and diastolic blood pressure (*p* = .114). For SBP, a significant interaction between drug group and time (pre- versus post-administration) was revealed (see Table 9, *p* = .043). Post hoc analyses found that SBP was lower for the placebo group post-administration compared to pre-administration (post hoc *p* = .004). No differences between duloxetine and placebo in SBP were found. Unexpectedly, a main effect of time was revealed for anxiety (*F*(1, 35) = 9.63, *p* =.004, η_p^2 = .216), with greater anxiety post-drug (*M* = 12.20, *SD* = 6.81) than pre-drug (*M* = 9.42, *SD* = 5.64). Likewise positive affect was lower post-drug (*M* = 33.63, *SD* = 4.95) than pre-drug (*M* = 35.37, *SD* = 4.70, *F*(1, 33) = 7.32, *p* = .011, η_p^2 = .181). This likely reflects anxious anticipation of the subsequent CO_2 challenge. All other main effects were non-significant (*F's* < 1.24, *p*'s > .272; main effects of duloxetine are shown in Table 32, Appendix A).

The effect of duloxetine on 7.5% CO_2-induced anxiety

Table 9. Mean (standard deviation) anxiety, mood and autonomic arousal at baseline and post-drug for the placebo and duloxetine groups.

	Placebo (*N* = 19)				Duloxetine (*N* = 18)				time*drug group
	Day 0		Day 14		Day 0		Day 14		
GAD-7	9.57	(5.06)	11.33	(6.29)	9.26	(6.35)	12.20	(6.81)	$F(1, 35) = 2.03$, $p = .163$ *ns*
PANAS positive	35.22	(4.18)	33.56	(4.37)	35.53	(5.32)	33.71	(5.64)	$F(1, 33) = 0.15$, $p = .904$ *ns*
PANAS negative	13.37	(2.50)	14.11	(3.59)	12.94	(2.77)	12.41	(1.87)	$F(1, 34) = 2.50$, $p = .123$ *ns*
Heart rate	68.84	(11.16)	70.26	(10.90)	74.39	(10.04)	73.17	(8.78)	$F(1, 35) = 0.82$, $p = .372$ *ns*
Systolic BP	125.05^{a}	(8.59)	119.16^{b}	(10.54)	124.17	(8.18)	124.06	(9.64)	$F(1, 35) = 4.43$, $p = .043$, $\eta_p^2 = .112$
Diastolic BP	69.47	(9.16)	66.16	(7.03)	71.72	(9.87)	72.33	(6.55)	$F(1, 35) = 2.62$, $p = .114$ *ns*

Note. Values with different superscripts were significantly different from each other.

Effects of duloxetine on subjective response to CO_2 challenge

To test whether duloxetine had an effect on anxiety or positive and negative mood, mixed model ANOVA were used with gas (baseline vs. peak air vs. peak CO_2) as a within-subjects factor and drug (placebo vs. duloxetine) and order (air first vs. CO_2 first) as between-subjects factors.

Mixed model ANOVA revealed strong effects of 7.5% CO_2 inhalation on subjective mood (see Table 10 for main effects of CO_2). Post- CO_2 levels of state anxiety and negative affect increased whilst positive affect decreased. Contrary to hypotheses, the effect of 7.5% CO_2 inhalation on anxiety and mood were not significantly attenuated following pre-treatment with duloxetine (i.e. no interaction between drug and gas were identified on subjective measures, *F'*s < 2.20, *p'*s >.130, *ns*)[11].No main effects or interactions with order were identified with measures of subjective mood.

Effect of duloxetine on autonomic response to CO_2 challenge

To test whether duloxetine had an effect on blood pressure, mixed model ANOVA was employed with gas (baseline vs. peak air vs. peak CO_2) as a within-subjects factor, and drug (placebo vs. duloxetine) and order (air first vs. CO_2 first) as between-subject factors. For heart rate and respiration rate, the within subjects factor of gas was conducted with two levels only (air vs. CO_2) as a baseline measure was not available.

Mixed model ANOVA found robust effects of 7.5% CO_2 on measures of autonomic arousal. 7.5% CO_2 raised respiration rate, heart rate and systolic blood pressure, but not diastolic blood pressure (see Table 10 for main effects of CO_2, and Table 33 for non-significant drug x time interactions), although some interactions with order were noted (to follow). The duloxetine group had elevated heart rate (M = 79.50, SE = 1.68; F (1, 32) = 5.21, p = .029, η_p^2 = .140) and diastolic blood pressure (M =

[11] Exploratory reassessment of the untransformed GAD-7 data does reveal a trend for a time*drug*order interaction: $F(2, 66) = 2.98$, $p = .080$, $\eta_p^2 = .083$, which represents a medium effect size (Richardson, 2011). Further examination of this indicates that when participants inhaled air first, those in the placebo group reported greater levels of anxiety (M = 46.23, SD = 24.53) than those in the duloxetine group (M = 26.52, SD = 24.02) at the peak effects of CO_2 (M_{diff} = 19.70, SE = 9.90, p = .055, d_s = 0.82, bonferroni critical p < .008). The raw mean GAD-7 scores at the peak effects of CO_2 were 37.85 (SD = 22.07) and 31.22 (SD = 20.92) for placebo and duloxetine respectively across both orders.

77.65, *SE* = 1.14; *F* (1, 33) = 16.22, *p* < .001, η_p^2 = .329) overall (across inhalations) compared to placebo (*M* = 71.85, *SE* = 1.63; *M* = 71.26, *SE* = 1.11 respectively).

Effects of order. Main effects and interactions with order were identified for autonomic measures. A significant main effect of order was revealed for diastolic blood pressure (*F* (1, 33) = 12.67, *p* < .001, η_p^2 = .277), where those who inhaled air first tended to have higher diastolic blood pressure than those who inhaled CO_2 first (M_{diff} = 5.65, *SE* = 1.59, *p* < .001). Furthermore a gas x order interaction for heart rate (*F* (1, 32) = 25.77, *p* < .001, η_p^2 = .446) was identified[12]. Simple main effects identified that participants who inhaled air first had a higher heart rate during the inhalation of air (*M* = 76.47, *SE* = 2.37) than those who inhaled CO_2 first (*M* = 65.92, *SE* = 2.24, *p* = .003, Bonferroni critical *p* < .0125). No effect of order was found in mean HR during inhalation of CO_2 (*p* = .491, *ns*). Mean HR during air and CO_2 were also examined for each order. Simple main effects revealed that those who received CO_2 first had a significantly greater HR during CO_2 (*M* = 80.67, *SE* = 2.31) than air (*M* = 65.92, *SE* = 2.24, *p* < .001), which was not seen in the air first group (*Mdiff*= 1.86, *p* = .322, *ns*). Thus it appears that CO_2-induced increases in HR were fuelled by the CO_2-first group.

The effect of duloxetine on attention network function during CO_2 challenge

Separate mixed model ANOVA were used to examine the effect of 7.5% CO_2 inhalation (versus air) on alerting, orienting and executive control network function. Drug group (placebo vs. duloxetine) and order (air first vs. CO_2 first) were between-subjects factors, and gas (air vs. 7.5% CO_2) was a within-subjects factor. The dependent variables were the function of the three attention networks. Table 34 and Table 35 (Appendix A) present mean attention network function (and reaction times) by drug group and by order.

Analysis of the alerting effect revealed a significant inhalation x order interaction (*F*(1, 33) = 6.40, *p* = .016, η_p^2 = .162). Post hoc comparisons suggest that

[12] Note that a single rather than a continuous measure (as provided with the blood pressure monitor) replicated these findings with a significant effect of gas (pre-inhalation vs. peak air vs. peak CO_2) *F* (1, 66) = 27.32, *p* < .001, η_p^2 = .453, an interaction between gas and order *F* (1.55, 51.17) = 7.66, *p* = .003, η_p^2 = .188, and a significant effect of drug group (placebo vs. duloxetine) *F* (1, 33) = 6.10, *p* =.019, η_p^2 = .156.

Table 10. Mean (SD) anxiety, mood and autonomic arousal for those receiving placebo and duloxetine at baseline, peak air and peak CO_2

	Baseline			Air			7.5% CO_2			ANOVA (time)		
	Placebo	Duloxetine	Total	Placebo	Duloxetine	Total	Placebo	Duloxetine	Total	F	p	η_p^2
GAD-7	9.00 (8.08)	10.86 (9.59)	9.91[a] (8.77)	11.55 (9.46)	11.04 (8.23)	11.30[a] (8.77)	37.85 (22.07)	31.22 (20.92)	34.62[b] (21.44)	63.35	< .001	.658
Positive affect	32.68 (5.51)	33.06 (7.77)	32.68[a] (6.61)	27.68 (6.47)	26.78 (9.18)	27.24[b] (7.80)	23.47 (8.55)	23.28 (9.31)	23.38[c] (8.80)	33.91	< .001	.507
Negative affect	12.32 (2.77)	11.72 (2.70)	12.03[a] (2.71)	11.42 (2.34)	11.00 (2.09)	11.22[a] (2.20)	20.84 (9.44)	18.11 (6.99)	19.51[b] (8.34)	36.16	< .001	.523
Systolic BP	117.47 (7.11)	121.78 (8.50)	119.57[a] (8.01)	116.89 (7.78)	122.06 (10.31)	119.41[a] (9.34)	129.42 (13.23)	134.00 (12.44)	131.65[b] (12.88)	29.33	< .001	.471
Diastolic BP	69.32 (6.20)	76.78 (5.42)	72.95 (6.88)	71.37 (6.38)	77.22 (7.95)	74.22 (7.68)	72.53 (8.86)	78.94 (5.59)	75.65 (8.04)	3.00	= .057, *ns*	.083
Continuous HR	-	-	-	67.31 (12.28)	74.57 (9.39)	70.94[a] (11.39)	75.83 (11.24)	83.11 (8.58)	79.47[b] (10.52)	42.75	< .001	.572
Respiration (breaths/minute)	-	-	-	16.07 (2.86)	16.52 (3.75)	16.29[a] (3.28)	19.07 (3.43)	20.18 (3.95)	19.61[b] (3.68)	25.57	< .001	.437

Note. Pairwise comparisons with Bonferroni correction applied ($p < .017$). Values with different superscripts were significantly different from each other.

performance during air inhalation varied depending on the order the gases were received. Those who received air first had a smaller alerting effect (M = 15.63, SD = 27.59, suggesting less efficient use of the alerting cue) during air than those who received CO_2 first (M = 32.88, SD = 19.69; p = .038). The raw means support this interpretation (see Table 11). However, these effects did not withstand after corrections for multiple tests were applied (Bonferroni p-value = .0125). No other main effects or interactions were significant (F's < 0.25, p's > .624).

Analysis of the orienting effect did not reveal any significant differences or interactions between the function of this network with inhalation, drug group, or order (F's < 0.88, p's > .356). Means are presented in Table 11.

Finally, analysis of the executive attention effect revealed a significant inhalation x order interaction ($F(1, 33) = 4.93$, p = .033, η_p^2 = .130). Post hoc comparisons identified a greater executive control effect (suggesting greater difficulty ignoring incongruent flankers) during the inhalation of air (M = 81.35, SD = 25.35) than 7.5% CO_2 (M = 66.49, SD = 25.14 – see Table 11). However, this effect was only apparent for participants who received air first (p = .008; CO_2 first p = .783, *ns*, Bonferroni p-value = .0125). Examination of the raw means suggests that the effect of CO_2 on executive attention was not driven by faster reaction times to incongruent trials (which would be indicative of improved executive control during CO_2) but was instead the result of slower reaction times on congruent trials during 7.5% CO_2 inhalation i.e. a performance deficit on congruent RTs (see Table 11 for all means; and see the Discussion for detailed interpretation of this effect). No other main effects or interactions were significant (F's <3.36, p's > .076; see Table 36 for means by drug group).

The effect of duloxetine on attentional bias during CO_2 challenge

Data from an additional two participants (both female taking placebo) were removed from the antisaccade analyses as these participants withdrew from the CO_2 inhalation after completing the ANT but before the antisaccade task, due to a

Table 11. Raw means (SDs) for the different cue/flanker types used to calculate alerting, orienting and executive control network function by order and gas

Order	Trial	Inhalation			
		Air		7.5% CO_2	
Air First (*N* = 18)	**Alerting**	**15.63**	**(27.59)**	**31.08**	**(19.69)**
	No cue	623.62	(83.99)	647.80	(74.05)
	Double cue	607.99	(92.95)	616.72	(79.07)
	Orienting	**35.00**	**(24.36)**	**30.06**	**(24.20)**
	Centre cue	612.32	(93.30)	615.15	(76.40)
	Spatial cue	577.32	(83.47)	585.10	(78.02)
	Executive Control	**81.35**	**(25.35)**	**66.49**	**(25.14)**
	Incongruent	645.99	(91.62)	649.44	(78.73)
	Congruent	564.64	(83.91)	582.95	(73.61)
CO_2 First (*N* = 19)	**Alerting**	**32.88**	**(19.69)**	**21.23**	**(34.23)**
	No cue	614.69	(66.01)	630.39	(70.18)
	Double cue	581.81	(64.40)	609.16	(64.16)
	Orienting	**36.71**	**(26.87)**	**35.75**	**(19.21)**
	Centre cue	592.86	(65.16)	610.67	(69.62)
	Spatial cue	556.15	(73.59)	574.93	(68.71)
	Executive Control	**69.79**	**(25.77)**	**71.28**	**(33.94)**
	Incongruent	621.28	(68.27)	641.93	(70.80)
	Congruent	551.48	(66.24)	570.65	(66.17)

pronounced response[13] (leaving a sample size of $N = 35$). Mixed model ANOVA examined the effects of duloxetine on antisaccade performance during CO_2 inhalation. In this instance, the within-subjects factors of gas (air vs. CO_2), trial type (prosaccade vs. antisaccade) and valence (neutral vs. negative) were included, and drug group (placebo vs. duloxetine) and order (air first vs. CO_2 first) were between-subjects factors (see Appendices, Table 37 for full descriptive statistics). The dependent variable was antisaccade performance (proportion of errors; range 0-1, values closer to 1 indicate poorer performance).

Mixed model ANOVA revealed a strong main effect of gas (F (1, 31) = 18.78, $p < .001$, $\eta_p^2 = .377$) and trial type (F (1, 31) = 79.43, $p < .001$, $\eta_p^2 = .719$) and a gas x trial type interaction (F (1, 31) = 8.72, $p = .006$, $\eta_p^2 = .219$). Participants made significantly more errors on antisaccade ($M = .41$, $SE = .04$) than prosaccade ($M = .05$, $SE = .01$) trials. Inhalation of CO_2 (versus air) impaired antisaccade performance ($M = .362$, $SE = .037$ for CO_2, $M = .461$, $SE = .042$ for air, $M_{diff} = .098$, $SE = .020$, $p = .001$, Bonferroni critical $p < .025$) but not prosaccade performance ($p = .237$, *ns*). No main effects or interactions with valence or order were found (F's < 2.74, p's > .108).

In addition, a trend for a gas x trial type x drug group interaction was revealed (F (1, 31) = 3.41, $p = .075$, $\eta_p^2 = .099$, *ns*), although this fell short of statistical significance. To examine this further, groups were split based on drug (placebo or duloxetine) and separate 2 (gas; air or CO_2) x 2 (trial type; prosaccade or antisaccade) repeated measures ANOVA tests were performed (Bonferroni critical $p < .025$). Responses to neutral and negative images were collapsed by trial type and the between-subjects factor of order was removed based on non-significant effects in the omnibus ANOVA.

In the placebo group, main effects of gas (F (1, 16) = 13.76, $p = .002$, $\eta_p^2 = .462$) and trial type (F (1, 16) = 34.64, $p < .001$, $\eta_p^2 = .684$) and a gas x trial type interaction (F (1, 16) = 8.17, $p = .011$, $\eta_p^2 = .338$) were revealed. However for the duloxetine group, main effects of gas (F (1, 17) = 6.29, $p = .023$, $\eta_p^2 = .270$) and trial type (F (1, 17) =

[13] The data from these participants were retained for the analyses of subjective mood and autonomic arousal despite not having completed a full 20 minute CO_2 inhalation (although all other measures were completed). The decision to retain this data was made due to the particularly strong responses noted for these individuals (to the extent that they chose to withdraw), even though a full inhalation was not completed. Conversely, a participant who did not complete the full 20 minute CO_2 inhalation due to a lack of gas was removed from all analyses. This was because we could not determine the level of response they would have experienced had the CO_2 inhalation continued for the full duration.

49.79, $p < .001$, $\eta_p^2 = .745$) were identified but no significant gas x trial type interaction was found ($p = .381$, *ns*, $\eta_p^2 = .046$). Post hoc analyses show that the placebo group continued to demonstrate poorer antisaccade performance during CO_2 inhalation ($M = .49$, $SE = .06$) than air ($M = .35$, $SE = .05$, $p < .001$, $d_{av} = 0.49$). There was no clear evidence of a similar effect in the duloxetine group ($p = .061$, $d_{av} = 0.29$, see Table 12). Prosaccade performance did not differ across inhalations for both drug groups (p's > .092). This indicates that whilst both drug groups made significantly more errors during 7.5% CO_2 than air, this CO_2-induced impairment on antisaccade trials was attenuated by pre-treatment with duloxetine.

Table 12. The proportion of errors (standard deviation) made on antisaccade and prosaccade trials during CO_2 and air inhalation by drug group

Errors	Placebo (N =17)				Duloxetine (N = 18)			
	Air		CO_2		Air		CO_2	
Prosaccade	0.05	(0.04)	0.07	(0.12)	0.03	(0.03)	0.06	(0.06)
Neutral	0.05	(0.04)	0.07	(0.11)	0.03	(0.04)	0.07	(0.07)
Negative	0.05	(0.04)	0.07	(0.14)	0.04	(0.04)	0.06	(0.07)
Antisaccade	0.35	(0.21)	0.49	(0.26)	0.37	(0.21)	0.43	(0.21)
Neutral	0.34	(0.21)	0.50	(0.28)	0.36	(0.22)	0.43	(0.22)
Negative	0.35	(0.22)	0.48	(0.25)	0.37	(0.22)	0.43	(0.22)

Note. Proportion of errors ranges between 0 and 1.

The effect of duloxetine on antisaccade latencies

Mixed model ANOVA was used to examine the effect of duloxetine on antisaccade latencies during CO_2 and air inhalations. Gas (air vs. CO_2), trial type (prosaccade vs. antisaccade) and valence (neutral vs. negative images) were within subject factors and drug group (placebo vs. duloxetine) and order (air first vs. CO_2 first) were between subjects factors (see Appendix A, Table 38 for full descriptive statistics). The dependent variable was mean latency (ms) to initiate a correct saccade.

A main effect of valence (F (1, 30) = 7.86, $p = .009$, $\eta_p^2 = 208$) was characterised by faster reaction times on trials containing negative images (M = 190.30ms, SE = 5.19) than neutral images M = 199.34ms, SE = 7.00) irrespective of trial

type. In addition, a gas x order interaction (F (1, 30) = 14.04, p = .001, η_p^2 = .319) was revealed, that was characterised by faster responses to generate correct eye-movements during CO_2 (M = 189.20, SE = 8.89) than air (M = 212.04, SE = 8.28, Bonferroni critical p = .013, p < .001) for those who received CO_2 inhalation first. This was not replicated in the air first group (p > .204). No other main effects or interactions were revealed (see Table 13 for descriptive statistics by inhalation, valence and trial type for each drug group).

Table 13. Latencies (standard deviation) on antisaccade and prosaccade trials during CO_2 and air inhalation by drug group

Latencies	Placebo (N =17)				Duloxetine (N = 18)			
	Air		CO_2		Air		CO_2	
Prosaccade	201.99	(34.75)	188.84	(34.05)	194.13	(40.71)	189.72	(36.42)
Neutral	207.18	(41.16)	188.92	(34.87)	196.80	(43.68)	192.14	(40.78)
Negative	196.76	(32.63)	183.62	(34.13)	191.47	(40.79)	187.30	(34.10)
Antisaccade	190.39	(35.67)	183.51	(45.36)	208.78	(59.14)	204.97	(52.69)
Neutral	202.60	(49.31)	187.64	(76.78)	211.16	(69.89)	206.50	(60.18)
Negative	179.10	(32.79)	176.72	(32.80)	206.42	(52.27)	203.45	(50.84)

The effect of duloxetine on frequency of thought intrusions post- air and 7.5% CO_2 inhalations

Two hypotheses were examined: first that 7.5% CO_2 inhalation will increase the frequency of negative thought intrusions to a similar degree as a period of instructed worry; and second, that duloxetine will be able to reduce the occurrence of intrusive negative thoughts. Mixed model ANOVA was used to assess these hypotheses, where time (post-air vs. post- CO_2 vs. post-worry) was a within subjects factor, and drug (placebo vs. duloxetine) and order (air-first vs. CO_2-first) were between subjects factors. The dependent variable was the number of negative intrusive thoughts.

Mixed model ANOVA identified a main effect of time (F (2, 66) = 5.28, p = .007, η_p^2 = .138), order (F (1, 33) = 5.42, p = .026, η_p^2 = .141), and also a significant time x drug group x order interaction (F (2, 66) = 5.17, p = .008, η_p^2 = .135). To

examine this interaction, two separate mixed model ANOVA tests were conducted when the data was split by order (bonferroni critical $p < .025$, see Table 14).

When air was inhaled first, a trend for a time x drug group interaction was revealed (F (2, 32) = 3.87, p = .031, η_p^2 = .195). Exploratory simple main effects identified that the placebo group showed the characteristic increase in negative thought intrusions after a period of instructed worry compared to post-air (M_{diff} = 1.56, p = .016; bonferroni critical $p < .017$). Similarly, the placebo group reported more negative thought intrusions post-CO_2 compared to post-air (M_{diff} = 1.78, p = .001), the frequency of which did not differ from the frequency post-worry (p = .704, *ns*). Importantly, in the duloxetine group there was no evidence that CO_2 challenge or instructed worry increased negative thought intrusions when compared to air (p's < .570, *ns*). For the CO_2-first group, a non-significant trend for a time x drug group interaction was identified (F (2, 34) = 3.396, p = .045, η_p^2 = .166). However, follow-up pairwise comparisons did not reveal any significant differences. This suggests that neither placebo nor duloxetine significantly increased negative thought intrusions after instructed worry or CO_2 inhalation for this order (see Table 14).

Table 14. Means (standard deviation) of negative thought intrusions for placebo and duloxetine when split by order

	Air first		CO_2 first	
	Placebo	Duloxetine	Placebo	Duloxetine
Post-air	0.22 (0.67)	0.67 (1.32)	0.20 (0.42)	0.00 (0.00)
Post- CO_2	2.00 (1.66)	0.67 (1.12)	0.20 (0.42)	1.00 (1.66)
Post-worry	1.78 (1.64)	0.33 (1.43)	0.60 (0.84)	0.47 (0.77)

Between-subjects ANOVA also examined whether the administration of duloxetine or placebo produced different responses on worry ratings of likelihood ("how likely it is to happen"), severity ("how catastrophic it would be"), and coping ("how well would you cope with it"). Order was included as a between subjects effect. No significant main effects or interactions between drug group or order were revealed (F's > 1.42, p's > .242).

Associations between CO_2-induced change in subjective mood, autonomic arousal and task performance

To capture CO_2-induced changes in mood, autonomic arousal and task performance, change scores were calculated by taking the peak air response from the peak CO_2 response (i.e. measures taken immediately at the end of each inhalation for subjective mood, blood pressure and thought intrusions, and measures taken throughout the CO_2 inhalation minus the air inhalation for heart rate, respiration rate, attention networks and antisaccade performance and latency). As expected, Pearson's R correlations revealed that CO_2-induced increases in anxiety were positively associated with increases in negative affect, systolic blood pressure and heart rate (see Table 15).

Table 15. Pearson's R correlations between CO_2-induced change in subjective mood and autonomic arousal (relative to peak air)

	GAD-7 (anxiety)	Positive affect	Negative affect	SBP	DBP	HR	Respiration Rate
GAD-7 (anxiety)	-	-	-	-	-	-	-
Positive affect	.152	-	-	-	-	-	-
Negative affect	.823***	.035	-	-	-	-	-
SBP	.391*	.060	.142	-	-	-	-
DBP	.074	.039	.018	.132	-	-	-
HR	.415*	-.045	.357*	.316	.108	-	-
Respiration rate	.129	-.021	.104	.184	.378*	.203	-

Note. * = significant < .05, ** = significant < .01 and *** = significant < .001

CO_2-induced anxiety was also moderately associated with increases in antisaccade errors during CO_2 inhalation (see Figure 6, Table 16), although this was irrespective of the valence of the image (negative vs. neutral on antisaccade trials). Similarly, increasing antisaccade errors during CO_2 inhalation were also associated with increasing heart rate (see Figure 7). An association of comparable magnitude was revealed between poorer executive attention in the ANT during CO_2 and greater heart rate, suggesting some consistency between measures of attention and changes in autonomic arousal (see Figure 8). This is supported further by a positive association between greater antisaccade errors during CO_2 and more frequent negative thought intrusions after CO_2 (relative to air). Finally an association between CO_2-induced increases in antisaccade errors with poorer use of alerting cues during CO_2 (better use of

cues during air) suggests that participants perform better on both tasks during air inhalation. Participants were able to ignore distracting images more easily and use the "alerting" double cue more appropriately during air.

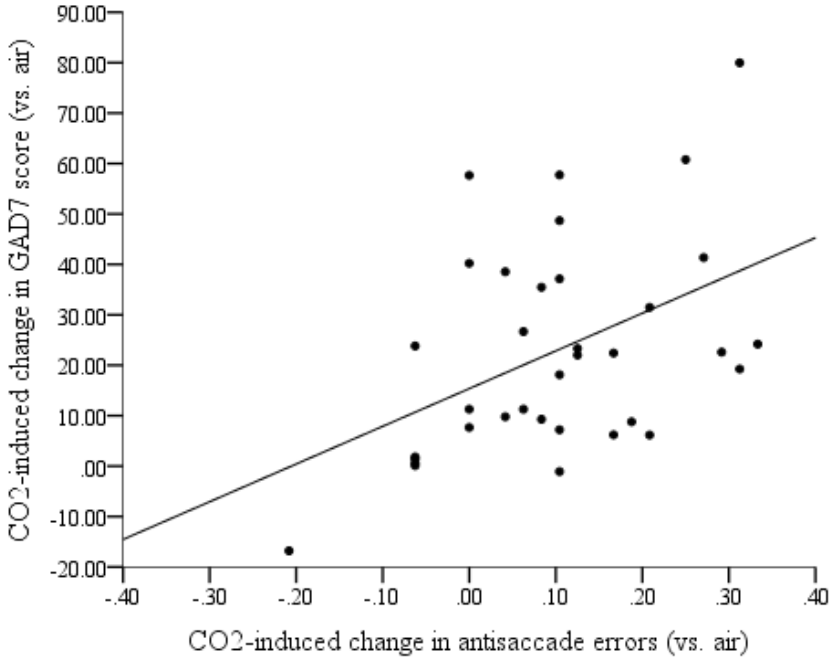

Figure 6. Relationship between anxiety response and antisaccade errors to 7.5% CO_2 inhalation, relative to air (r = .452)

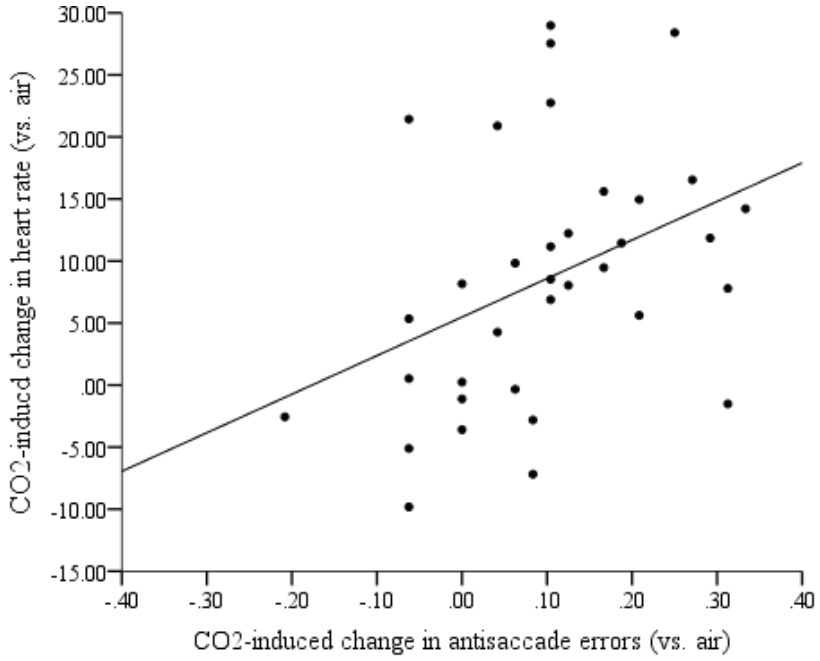

Figure 7. Relationship between heart rate and antisaccade errors to 7.5% CO_2 inhalation, relative to air (r = .388)

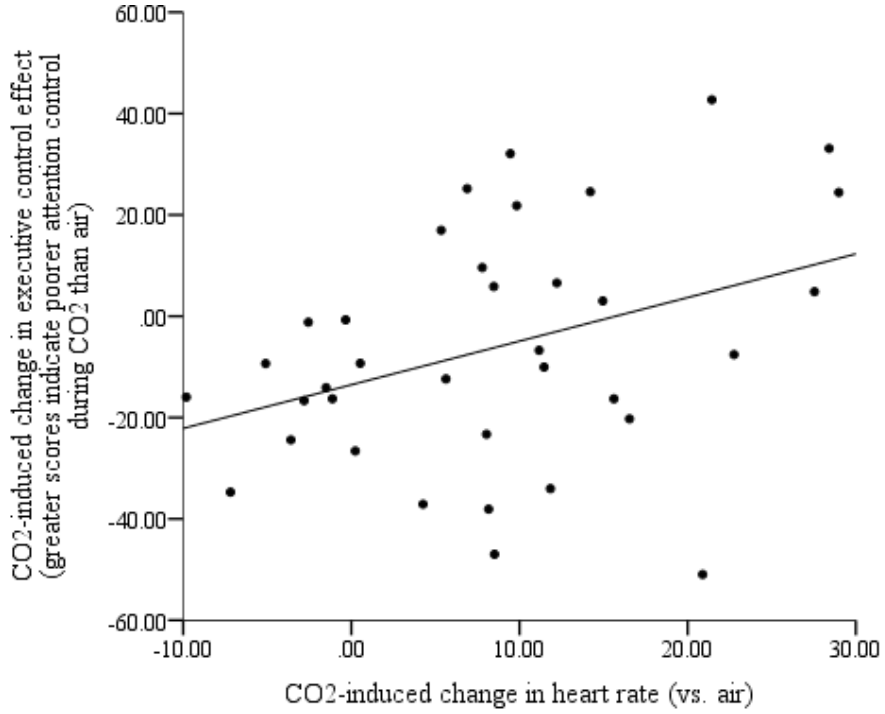

Figure 8. Relationship between increasing heart rate and poorer executive control during CO_2, relative to air (r = .368).

Table 16. Pearson's R correlations between attention network, antisaccade and thought intrusions task performance during CO_2 inhalation (relative to peak air) with CO_2-induced anxiety and heart rate

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
1.State anxiety (GAD-7)	-	-	-	-	-	-	-	-	-	-	-	-
2.Continuous heart rate	.415*	-	-	-	-	-	-	-	-	-	-	-
3.Alerting	-.154	-.468**	-	-	-	-	-	-	-	-	-	-
4.Orienting	-.365*	-.161	-.218	-	-	-	-	-	-	-	-	-
5.Executive control	-.009	.368*	-.224	.109	-	-	-	-	-	-	-	-
6.Antisaccade errors	.452**	.388*	-.419*	-.256	.098	-	-	-	-	-	-	-
7.Prosaccade errors	-.061	-.033	.065	-.184	-.174	.023	-	-	-	-	-	-
8.Proportion of antisaccade errors to negative vs. neutral	.173	-.159	.190	.057	-.062	-.158	-.097	-	-	-	-	-
9.Antisaccade latency	-.184	-.319	.171	.034	-.244	-.294	-.073	-.067	-	-	-	-
10.Prosaccade latency	-.230	-.492**	.121	.119	-.308	-.300	-.148	.123	.486**	-	-	-
11.Proportion of antisaccade latencies to negative vs. neutral	.057	.177	-.088	-.017	-.025	.059	.471**	.271	-.400*	-.083	-	-
12.Negative intrusions	.325*	-.160	-.038	-.198	-.027	.374*	-.045	.019	-.288	-.152	.001	-

Note. * = significant < .05, ** = significant < .01 and *** = significant < .001

3.4 Discussion

This study is the first to investigate the effects of a two week course of the SNRI duloxetine in a healthy volunteer, experimental medicine model of anxiety. The aim of this study was to examine whether duloxetine attenuates symptoms of anxiety elicited by a 20 minute inhalation of 7.5% CO_2. In particular, it focussed on CO_2-induced increases in anxiety and autonomic arousal, as well as deficits in attention on the ANT, antisaccade performance, and a thought sampling measure of negative intrusive thoughts.

In line with previous research (Bailey et al., 2005), 7.5% CO_2 inhalation produced robust increases in subjective and physical symptoms of anxiety, including elevated generalised anxiety, heart rate and blood pressure. Means at the peak effects of CO_2 suggest moderate GAD symptoms when compared to the criteria used for patient samples[14]. Strong associations between increased anxiety, negative mood and heart rate post-CO_2 were also revealed. However, duloxetine did not significantly affect subjective ratings of anxiety or autonomic arousal at the peak effects of CO_2 (although exploratory analyses of untransformed data hinted at reduced anxiety in the duloxetine group when air was inhaled first; $p = .055$). These findings mirror the marginal reductions in nervousness, anxiety and worry post-CO_2 that have been found in research with other psychotropic drugs (e.g. venlafaxine; for example Bailey et al., 2007a; Diaper et al., 2013) where few of these findings reached statistical significance.

Consistent with previous research (Garner et al., 2011a), 7.5% CO_2 inhalation was associated with impaired attentional control (as shown by greater numbers of antisaccade errors); a finding which adheres to the attentional deficits often reported in anxious populations (Bar-Haim et al., 2007). In addition, whilst all participants demonstrated a greater number of erroneous eye-movements on antisaccade trials during CO_2, this impairment was smaller for those receiving duloxetine than placebo. This suggests that duloxetine can reduce the negative impact of CO_2 on attentional control in the absence of a clear effect on subjective mood or autonomic arousal, however poorer attentional control was strongly associated with both CO_2-induced

[14] It has been suggested that a total score falling between 0-5 as mild anxiety, 6-10 as moderate anxiety and 11+ as severe anxiety on the GAD-7. This compares to scores of 0-33, 34-66 and 67+ respectively on the modified version used in this study.

increases in anxiety and heart rate. Although still moderate in size, the correlation between elevated heart rate and worsening antisaccade performance does not remain significant after controlling for anxiety (r = .246, p = .16, *ns*). This suggests that observed changes in mood may relate to attentional processes to some degree. The strong associations between these symptoms may reflect greater interoceptive awareness of heart rate which can provoke feelings of anxiety.

No clear effect of 7.5% CO_2 inhalation was found on alerting and orienting network function; a finding which differs from previous research (Garner et al., 2012). In addition, executive attention differed between the two inhalations for the air first order only. Inspection of the executive control bias score suggests that participants had greater difficulty ignoring the distractor arrows (incongruent vs. congruent) during air than CO_2, which appears to contradict the findings from the antisaccade task. However, further separate examination of reaction times on congruent and incongruent trials indicates that this effect was not driven by faster reaction times on incongruent trials (which would suggest superior executive control), but was instead due to slower reaction times on congruent trials. This highlights a problem with using difference scores to index attention network function in this task, as findings can be misleading without careful examination of the reactions times to each cue/flanker type.

Finally, 7.5% CO_2 inhalation was found to increase negative thought intrusions to a comparable level as a period of instructed worry, and there was some evidence that this was apparent for those receiving placebo but not duloxetine (although this occurred in the air first order only). This suggests that low-dose CO_2 inhalation can initiate maladaptive thought processes which are difficult to control, and lends support to 7.5% CO_2 inhalation as a model of generalized anxiety (including worry) rather than an acute fear/panic-like state. This also indicates that the ability to maintain attention on a simple breathing task can be impaired by CO_2 challenge – a pattern that covaries with the effects of CO_2 on antisaccade performance (see positive correlations; Table 16).

Human neurocognitive models of anxiety (Bishop, 2007) indicate that poor control of prefrontal regions to regulate amygdala activity may underlie the attention biases to threat that are common in anxious populations (Bar-Haim et al., 2007). Thus it is plausible that the effects of duloxetine on attention occur through this circuitry. This could be enabled by changes in NA and/or 5-HT transmission, or via complex knock-on

effects of these transmitters. NA in particular has been strongly linked to a range of attentional processes (De Martino, Strange, & Dolan, 2008) and dose-dependent activation of locus coeruleus neurones (the main site of NA synthesis, which has strong reciprocal connections with the amygdala) to rising CO_2 levels has been clearly demonstrated (Pineda & Aghajanian, 1997). In rodents, duloxetine increases extracellular levels of 5-HT and NA in the prefrontal cortex (Engelman et al. 1995, as cited in Norman & Olver, 2008), and in humans, prior administration of duloxetine has been shown to reduce the activity of the amygdala during an emotional face matching task (van Marle et al., 2011). Thus, hypercapnia may affect noradrenergic neurotransmission in the amygdala via LC chemoreceptors which in turn, may modulate attention and anxiety.

Current theories of antidepressant action (Harmer & Cowen, 2013b; Harmer et al., 2003b) have suggested that, similarly to psychological treatments, antidepressants produce early changes in attention that precede a later reduction in core subjective symptoms. This aligns with the current evidence to some degree, where duloxetine produced greater improvements in attentional control during CO_2 challenge than in anxiety (although the levels reported by the duloxetine group at peak- CO_2 do appear to be decreasing). However, since the present research administered duloxetine over a 2 week period, it is difficult to determine the temporal effects this antidepressant has on attentional biases elicited by CO_2.

These findings may indicate that 2 week administration of duloxetine in healthy volunteers is not sufficient to elicit changes in mood within this model. It is unclear whether this is the result of insufficient dose, duration, or inappropriate mode of action to modify CO_2-induced anxiety responses. The duration needed for an antidepressant to relieve anxiety/depressive symptoms in patient samples remains unclear (Harmer et al., 2009a; Mitchell, 2006), but noticeable improvements in depressed and anxious mood have been reported after 1-2 weeks in clinical populations (Hirschfeld, Mallinckrodt, Lee, & Detke, 2005; Raskin et al., 2007; Rynn et al., 2008). Further research is needed to examine a range of doses of duloxetine and administration periods in healthy volunteers and clinical samples, combined with periodic assessments of attention and mood to better track the temporal trajectory of these changes.

These results may instead reflect the limited potential for small changes in attentional control to reduce emergent anxiety in a one-off, novel, anxiogenic situation. This could explain the weak effects of similar antidepressants to produce meaningful decreases in anxiety symptoms during CO_2 even after longer administrations (Bailey et al., 2007a; Diaper et al., 2013). Future research should examine whether pre-existing deficits in attention in patient samples can be alleviated early on by duloxetine treatment i.e. before changes in subjective mood (in a similar design to Tranter et al., 2009a). This might help translate early improvements in attentional control into robust improvements in subjective anxiety. New studies should also revisit whether similar antidepressants can replicate the effects of duloxetine on CO_2-induced deficits in attentional control. Examination of single, acute doses using this healthy volunteer model may also help uncover whether CO_2-induced deficits in cognitive biases can be modified by acute antidepressant administration (as hypothesised by Harmer et al. 2013). Finally by re-examining serotonergic drugs (e.g. SSRIs) could reveal whether the effect on attention is a specific feature of the noradrenergic properties of duloxetine (considering the importance of noradrenaline in attentional processes), or a general property of antidepressant treatments.

Unlike previous research with the antisaccade task (Garner et al., 2011a), the detrimental effect of CO_2 on attentional control was not specific to threatening images. This may result from too small a sample size, particularly for the placebo group (N = 17), compared to the sample (N = 26) reported by Garner et al. (2011a). This may also reflect variability within our sample since further examination of mean antisaccade errors to negative and neutral stimuli in the placebo group did not indicate the presence of a threat bias during CO_2 (as would be expected with a direct replication of the antisaccade task in Garner et al., 2011a). This prevents conclusions being drawn about the effect of duloxetine on CO_2-induced threat biases specifically, constraining the interpretation to the broader effect of duloxetine on attention only. Replication of the antisaccade task within the CO_2 model may help to explain the reasons for this variability. Alternatively, within-subjects assessment of CO_2-induced deficits in attentional control at baseline would allow pre- to post- drug comparisons that may uncover whether a pre-existing bias to threat can be amended by duloxetine and would account for individual variation in task performance.

The effect of duloxetine on 7.5% CO_2-induced anxiety

Clinical anxiety has been found to strongly correlate with attentional and interpretational biases to threat (Bar-Haim et al., 2007), and more broadly with increased distractibility towards task-irrelevant information that is not necessarily threatening (- an important assumption of attentional control theory; Eysenck et al., 2007). Previous healthy volunteer studies have found specific effects of antidepressants including duloxetine on the processing of emotional material including threat. For example antidepressant administrations have been shown to enhance the recognition of happy faces (Harmer et al., 2003a; Harmer et al., 2003b) and produce variable effects on the recognition of fear and disgust (Browning, Reid, Cowen, Goodwin, & Harmer, 2007; Harmer et al., 2006; Harmer et al., 2004; Murphy et al., 2009; possibly reflecting variations in dosing schedules). This suggests that antidepressant effects on emotion are not restricted to patient samples. It should be noted that emotion recognition tasks require participants to make a conscious judgement about the emotion displayed in a face, and this differs to the processes involved in attentional tasks, where erroneous saccades are believed to reflect non-volitional slips in attention. Future research is needed to establish whether antidepressants have different effects on specific attentional biases to threat and broader interpretational biases to negative emotions in faces. This could be achieved with a placebo-controlled, between-subjects study using a single dose of duloxetine in healthy volunteers to examine error rates to threat in the antisaccade task.

In the present research, it was hypothesised that CO_2 inhalation would increase the number of negative intrusive thoughts to a similar degree as instructed worry, and this effect would be reduced by duloxetine. Whilst some support for this hypothesis was identified (although in the air first order only), it is entirely possible that duloxetine reduces negative intrusions by promoting the processing of neutral or positive thoughts, with no specific change in attentional control. This warrants investigation as previous research has shown that duloxetine can boost the recognition of happy and disgusted emotional faces (Harmer et al., 2008). Since the thought intrusions task records all intrusions, not just negative ones, this hypothesis could easily be examined. New research could look at the effects of antidepressants on intrusions by valence (positive, negative or neutral) to underpin if the effects observed are threat-specific, or relate to broader changes in attention processing of emotional material.

Limitations

One aim of this research was to examine the effects of duloxetine on 7.5% CO_2-induced deficits in attention. This was based on the theory that antidepressants can produce changes in cognitive processing which later translate to improvements in mood (Harmer et al., 2013). In this study a 2 week dosing schedule was used rather than a single, acute dose. By choosing this longer duration, conclusions concerning the time course of drug effects on CO_2-induced attention biases cannot be made. The two week administration used in this study was chosen for a number of reasons. Since duloxetine has a relatively poor side effect profile and a primary aim was to record drug effects on CO_2-induced anxiety, it was deemed important to limit the impact of unpleasant side effects on subjective measures. Previous research using a 60mg single dose of duloxetine has reported side effects, which may have affected the processing of emotional faces (Harmer et al., 2009). As such, a 2 week dosing schedule with titration from 30mg to 60mg on day 4 was considered to be the best way to manage side-effects prior to the testing session. Clinically, antidepressants such as duloxetine depend on chronic dosing to reveal efficacy on psychological measurement scales (e.g. HAM-A). Thus, longer durations of administration that closely mimic therapeutic treatment have been used in the majority of 7.5% CO_2 studies that examine drug effects on mood (see Table 4, section 1.7). Considering that this study is the first to examine potential effects of duloxetine within this model, the pros of using a longer dosing period to profile clinically relevant drug effects on a range of CO_2-induced anxiety were considered to outweigh the costs.

Although all participants in our study completed the 2 week course of duloxetine, a number of side effects were still reported. The frequency of side effects for the duloxetine group exceeded the placebo group. In light of the relatively poor side effect profile of duloxetine (when compared to other antidepressants) temporary unpleasantness arising from duloxetine may have negatively affected mood during the testing session. A study assessing a single dose of 60mg duloxetine in healthy volunteers reported increases in nausea, dizziness and impaired mood compared to a placebo group (Harmer et al., 2008). This could mask the presence of small, beneficial effects of duloxetine in the CO_2 model. Without formal assessment of side effects, it is difficult to determine whether the low starting dose and two week duration was successful at minimizing side-effects. Future studies should also consider observed

ingestion of study medication to improve confidence in compliance (based on the greater incidence of side effects from duloxetine).

In the present study, some effects with order were identified, despite counterbalancing the order in which the inhalations were delivered. These order effects tended to be stronger on autonomic measures than subjective mood. This makes it unlikely to be the direct consequence of anticipatory anxiety, where changes in subjective mood would also be expected.

Previous research has often used a fixed order, with air inhaled first (e.g. Attwood, Ataya, Bailey, Lightman, & Munafo, 2014; Bailey et al., 2007a; Bailey et al., 2011b; Bailey et al., 2009; Diaper et al., 2013). This is on the assumption that increased anxiety may be experienced in the first inhalation (regardless of gas inhaled) due to the experimental environment and/or anticipation of an aversive task. Using a fixed order with air first is presumed to prevent an overinflated anxiolytic effect of CO_2 and instead produce a more conservative comparison with a mildly anxiolytic air-first inhalation. This design is worth giving consideration in future research as it may preserve a degree of uncertainty regarding inhalation order. It would also be beneficial to assess how easily identifiable CO_2 inhalation is[15]. In the present study, counterbalancing the order was considered important to try to reduce potential practice effects in the neurocognitive tasks, and this appears to have been successful (as no order effects were revealed in antisaccade performance). However, it is unclear why instructed worry and/or CO_2 failed to induce an increase in negative thought intrusions when CO_2 was inhaled first. Future research needs to consider the pros and cons of using a fixed or counterbalanced order in within-subjects designs, and may instead compare the inhalations in a between-subjects manner or remove the air comparison entirely, since the anxiolytic effects of CO_2 have been well characterised.

Finally, it should be acknowledged that no measure of worry engagement was made in the breathing focus task. Individual variations in engagement during the worry period may explain the inconsistent pattern of results described here, and also account for the relatively low number of negative intrusions reported across both orders and

[15] At the end of the study, order was correctly determined by 37 out of 40 participants, although it is unclear if the completion of both inhalations was required to determine this.

groups. Few studies have described the thought intrusions task in a healthy sample (e.g. Baker, Baldwin & Garner, 2015), especially one pre-screened for high levels of anxiety. Research is needed to establish how robustly this task measures negative intrusive thoughts in healthy volunteers at baseline and during anxiety manipulations. Subsequent comparison of the number of negative intrusions of healthy volunteers during CO_2 inhalation with that of clinically anxious patients may help validate CO_2 challenge as a model of pathological anxiety.

Conclusion

This research revealed that prior, subchronic administration of duloxetine in healthy volunteers decreased the maladaptive effect of 7.5% CO_2 challenge on antisaccade accuracy, without a clear effect of drug on mood or autonomic arousal. The present study however, did not replicate the CO_2-induced attentional bias to threat that has been seen previously (Garner et al., 2012). Instead we found that 7.5% CO_2 inhalation worsened performance on antisaccade trials indiscriminately to image valence – an effect that appears to be amenable to duloxetine. Examining the impact of different antidepressants on well-established threat biases provoked by models of anxiety, and/or on biases consistently seen in clinical populations would help understand whether their effects on attention are emotion-specific or more general. In addition, by adopting a more systematic approach to the tests used to examine attentional biases, as well as manipulating the drug, dose and schedule of administration could help establish how specific neurotransmitter systems modulate attention. This research adds to the building literature that suggests antidepressants alter how we attend to cues in our environment in different ways (Harmer & Cowen, 2013), and aligns with neurocognitive theories of human anxiety and attention (Bishop, 2007).

The effect of duloxetine on 7.5% CO_2-induced anxiety

Chapter 4: *Memantine improves attention control on an antisaccade task during a state of heightened anxiety*

4.1 Introduction

In the preceding chapter, the antidepressant duloxetine was found to lessen the negative impact of 7.5% CO_2 on attentional control in the absence of a clear change in anxiety or autonomic arousal. This finding, along with a growing body of research (e.g. Bailey et al., 2007a; Bailey et al., 2009; Diaper et al., 2013; Diaper et al., 2012b), indicates that the 7.5% CO_2 model has some sensitivity to known anxiolytics. However, the ability of CO_2 challenge to predict likely therapeutic success of **novel** compounds has only recently received attention (Bailey et al., 2011b; de Oliveira et al., 2012; Poma et al., 2014) with no studies having yet examined beyond core subjective or autonomic features of anxiety.

The present chapter moves beyond evidence that 7.5% CO_2 inhalation can replicate treatable symptoms of anxiety (Chapters 2 and 3), and instead examined whether the model can be used to evaluate potential drug treatments for anxiety disorders. This chapter therefore aimed to identify whether the *N*-methyl-D-aspartate (NMDA) receptor antagonist memantine has any effect on CO_2-induced anxiety or associated attentional mechanisms. Memantine is currently licenced for moderate-to-severe Alzheimer's disease where it has been suggested to show some anti-anxiety properties (Cortese & Phan, 2005; Gauthier, Loft, & Cummings, 2008), and more recently it has been examined as a potential augmentation therapy for treating symptoms of OCD (Ghaleiha et al., 2013).

As described in Chapter 1 (section 1.3.1), the neural basis of anxiety focussed on the role of the extended amygdala. Current neural models suggest that the fear response is centrally coordinated by an amygdala-prefrontal circuitry and disruption of this circuitry, characterised by hyper-responsive amygdala activity to threat and poor prefrontal control, is thought to mediate threat-related attentional biases that promote and maintain an anxious state (Bishop, 2008). Furthermore, the processes of fear conditioning and fear extinction which are core to the development and maintenance of

anxiety have been strongly related to amygdala glutamatergic NMDA receptor function (Nandhra, Murphy, & Sule, 2013; Walker & Davis, 2002).

Glutamate is the primary excitatory neurotransmitter in the brain. Abnormal activity of the glutamate system has been reported in the neural areas involved in fear and anxiety. For example, elevated glutamate levels have been recorded from the central and basolateral nucleus of the amygdala and from the hippocampus of stressed rodents (Fontella et al., 2004; Reznikov et al., 2007), whilst in anxious humans, higher levels of glutamate in cerebrospinal fluid have been found in patients with OCD when compared to healthy controls (Chakrabarty, Bhattacharyya, Christopher, & Khanna, 2005). Since glutamate dysfunction has been shown in neural areas strongly involved in the presentation of fear, drugs that regulate glutamate levels and/or reduce glutamatergic neurotransmission in these areas may demonstrate anti-anxiety properties by disrupting processes such as fear conditioning and extinction.

Evidence exists to suggest that drugs which modulate the glutamatergic system may be useful in the treatment of anxiety disorders. For example an open-label trial of the drug riluzole, a presynaptic inhibitor or glutamate[16], found significant reductions in anxiety symptoms in a small sample of patients with GAD which persisted throughout the 8 week trial (Mathew et al., 2005). This is supported by studies in rodents, where riluzole produces anxiolytic effects in the elevated plus maze (EPM), light/dark, and open field tests (Sugiyama et al., 2012). Furthermore in rodents selected for high and low anxiety behaviours, pre-treatment with D-cycloserine (a partial agonist of glycine site of NMDA receptor) enhanced the extinction of a freezing response to a conditioned stimulus (aversive context) and this effect was accompanied by a reduction in glutamate concentrations in the basolateral amygdala (Lehner et al., 2010). Thus a growing body of research suggests that glutamatergic drugs demonstrate anxiety reducing properties in both mice models of anxiety, and in clinically anxious patients.

The present study investigated whether the drug memantine demonstrates efficacy in the 7.5% CO_2, healthy human model of anxiety. Memantine is a voltage-dependent, moderate affinity, uncompetitive antagonist on NMDA receptors, which when bound, blocks the activity of glutamate. Memantine is currently licensed for the

[16] However it should be noted that riluzole has a wide range of molecular mechanisms and it has not yet been determined which of these produces anti-anxiety effects (Pittenger et al., 2008).

treatment of moderate to severe Alzheimer's disease in Europe and the USA and has been shown to be safe, well tolerated (Gortelmeyer & Erbler, 1992), and effective at slowing the deterioration of cognitive, functional and behavioural symptoms of the disease (Peskind et al., 2006; Reisberg et al., 2003; Winblad, Jones, Writh, Stoffler, & Mobius, 2007). The positive effects of memantine in the treatment of neurodegenerative diseases are thought to occur by reducing the damage caused by excessive stimulation of glutamate which can cause excitotoxicity (nerve cell death). Glutamatergic medications also influence the neurotransmission of monoamines, for example, at high doses memantine has been shown to inhibit the reuptake of serotonin and dopamine (Onogi et al., 2009). NMDA antagonists (such as ketamine) have also been shown to increase brain serotonin levels in rodents (Tso, Blatchford, Callado, McLaughlin, & Stamford, 2004), and non-human primates (Yamanaka et al., 2014) and this is thought to play a part in their anxiolytic and antidepressant properties.

Preclinical studies of memantine have suggested possible anxiolytic effects. For example in rodents, chronic administration of memantine was found to decrease wall swimming latency in the Morris water maze, reduce aggressive attacks on intruder mice and, at higher doses (100mg/kg), memantine also increased time spent in the open arms of the EPM (Minkeviciene et al., 2008). The authors argue that higher doses of memantine than those needed to elicit cognitive improvements (such as improved spatial memory in the Morris water maze) may be required to produce an anxiolytic effect (such as greater exploration of open arms on the EPM; Minkeviciene et al., 2008), a theory that is supported by Takahashi et al. (2009) where high dose memantine strongly reduced distress vocalisations of pups but moderate doses increased ultrasonic vocalisations.

Clinically, memantine has been used 'off-label' for the treatment of a number of psychiatric disorders, including major depressive disorder (MDD), obsessive-compulsive disorder (OCD), schizophrenia and substance abuse (see Zdanys & Tampi, 2008 for a review). For example in MDD comorbid with alcohol dependence, treatment with memantine for 26 weeks was shown to have comparable efficacy to the SSRI escitalopram at reducing symptoms of depression (MADRS) and anxiety (HAM-A; Muhonen, Lonngvist, Juva, & Alho, 2008). However, in a placebo-controlled trial of memantine for MDD, no effects on depression or anxiety were found after 8 week

treatment (Zarate et al., 2006). Differences in dosing and outcome measures may explain the discrepancies between these findings.

There are relatively few studies examining the effects of memantine on clinical anxiety in humans, with most research focusing on OCD due to strong evidence for glutamate dysregulation in this disorder (Chakrabarty et al., 2005; Pittenger, Bloch, & Williams, 2011; Wu, Hanna, Rosenberg, & Arnold, 2012). For example, growing evidence supports the use of memantine as an augmentation therapy for severe, but not mild OCD, with clinically relevant decreases in symptoms reported after memantine augmentation in treatment resistant OCD patients (Haghighi et al., 2013; Stewart et al., 2010). In anxiety disorders, one small study has examined the effect of 12 weeks memantine treatment (20mg/day) in patients with GAD compared to OCD. Whilst small reductions in anxiety for the GAD group were revealed, better outcomes were identified for the OCD group with 7/10 patients experiencing > 45% reduction in OCD symptoms (Feusner, Kerwin, Saxena, & Bystritsky, 2009). This may reflect a stronger role for glutamate in OCD, yet the fact that memantine produced small reductions in GAD symptoms also suggests that drugs which modulate glutamate transmission may exert anti-anxiety effects across a range of disorders (whether as a downstream effect on other monoamine systems or as a direct effect via the blockade of glutamate). Similarly, Schwartz, Siddiqui, and Raza (2012) found clinically relevant reductions in anxiety symptoms in a small sample of partial responders to SSRIs for GAD (with or without comorbid social anxiety) when treatment was augmented with memantine, although these results should be taken cautiously due to the lack of a control comparison. As such there is preliminary evidence to suggest that glutamatergic drugs such as memantine may reduce anxiety in a range of disorders, and therefore further examination of memantine is warranted to determine its clinical potential for anxiety disorders.

Finally in a novel healthy volunteer model of antidepressant action, a single dose of memantine was found to produce a marginal reduction in biases to negative items in an emotion recognition memory task and further potentiate eye-blink startles to unpleasant (relative to neutral) images (a comparable finding to SSRI treatments that appear to initially increase emotion-potentiated startles; Pringle et al., 2012). However, no effect of memantine was found on cognitive assessments of working or declarative memory. This indicates that healthy volunteers are susceptible to the emotional effects of memantine which can be observed to some extent using neurocognitive tasks.

Together, these findings indicate that glutamatergic medications such as memantine can i) reduce symptoms of anxiety and depression in preclinical rodent models, ii) reduce false alarms for negative words in an emotional memory task in healthy humans, and iii) reduce some symptoms of anxiety in clinical cases (particularly OCD), however further research is warranted to clarify the therapeutic potential of memantine in treating anxiety disorders beyond OCD.

Primary aims and predictions

In this experiment, the effects of memantine on anxiety responses to 7.5% CO_2 inhalation were examined in healthy volunteers. Previous research has demonstrated some anxiolytic effects of memantine in both neurodegenerative and psychiatric diseases (Feusner et al., 2009; Schwartz et al., 2012; Stewart et al., 2010). In addition, since memantine is primarily used to lessen the decline in cognition associated with neurodegenerative diseases, whether memantine can reduce CO_2-induced impairments in attention (particularly inhibitory, attentional control processes) will be evaluated using the antisaccade task and ANT. In contrast to Study Two, the thought intrusions task was implemented as a separate measure from CO_2 challenge to fully examine whether memantine can reduce negative intrusive thoughts.

It was hypothesised that:

1. 14 day memantine administration will not alter general mood and anxiety in a healthy sample;
2. compared to placebo, memantine will reduce subjective anxiety experienced during CO_2 inhalation, with no change in autonomic arousal. This will be demonstrated by significant interactions between memantine (vs. placebo) and gas (baseline vs. peak-air vs. peak-CO_2);
3. memantine (versus placebo) will reduce antisaccade errors and may enhance executive attention (in the ANT) during CO_2 challenge, again illustrated by significant interactions between drug and gas;
4. pre-treatment with memantine (versus placebo) will inhibit negative intrusive thoughts during a thought intrusions task completed after a period of instructed worry.

The effect of memantine on 7.5% CO_2-induced anxiety

4.2 Method

Participants

Thirty-seven healthy volunteers (20 male) were randomised to receive either a two week course of memantine (5mg titrated to10mg after 7 days; N = 18, 11 males) or a placebo (N = 19, 9 male; drug groups balanced by gender, double-blind). Consistent with studies 1 and 2, participants initially completed a short phone screen and a subsequent screening interview to ensure eligibility criteria was met prior to recruitment in this study. All eligible participants were then contacted by phone on days 3, 7 and 11 to record the presence of any side effects and to discuss titration to a higher dose (10mg of memantine or matched placebo) on day 8. All participants achieved the maximum 10mg dose on day 8.

For the CO_2 session, the exclusion criteria included current or history of psychiatric illness as assessed by the MINI International Neuropsychiatric Interview (based on DSM-IV; Sheehan et al., 1998), personal or family history of panic disorder or panic attacks, medication use within the last 8 weeks (apart from local treatment, occasional aspirin or paracetamol, and contraceptives), smoking, history of asthma/respiratory disease, diabetes, migraines or cardiovascular disease, excessive alcohol consumption (> 28 units/week for males, > 21 units/week for females) or positive alcohol breath test, current or past alcohol or drug dependence, under- or overweight (body mass index < 18 or > 28kg/m^2), blood pressure exceeding 140/90 or heart rate of < 50bpm or > 90bpm, caffeine consumption of > 8 caffeinated drinks/day, pregnancy/breastfeeding, or distress when viewing example images for the antisaccade task. Additionally volunteers were excluded from participation if contraindications for memantine were identified. These included: a known hypersensitivity to memantine, previous treatment with memantine, epilepsy/history of convulsions, and a history or diagnosis of liver disease or impairment. This was based on the guidance provided by the BNF (Joint Formulary Committee., 2014).

Procedure

The procedure was largely a replication of that within Chapter 3. Briefly, this involved a 3 hour testing session on the final day of treatment (day 14), where participants completed two 20 minute inhalations of 7.5% CO_2 and air. Inhalations were

administered blind to participants and were separated by a 30 minute break to remove potential carry-over effects. Gas was administered through an oro-nasal face mask with inhalation order (CO_2 or air first) counterbalanced across participants in a within-subjects, single blind, cross-over design. Participants completed the Attention Network Test (ANT), followed by the antisaccade task during each inhalation. Measures of state anxiety (GAD-7), mood (PANAS) and blood pressure (SBP and DBP) were recorded before and after each inhalation. Continuous measures of heart rate and respiration rate were made throughout both 20 minute inhalations. After the two inhalations were completed, participants also undertook a thought intrusions task before and after a period of instructed worry (based on the protocol described in Hirsch et al., 2009).

Measures of anxiety (trait version of the Spielberger state-trait anxiety inventory (STAI), Spielberger et al., 1983; and a modified version of the GAD-7; Spitzer et al., 2006), mood (positive and negative scales of the PANAS; Watson et al., 1988), worry (Penn-State worry questionnaire (PSWQ); Meyer et al., 1990), heart rate and blood pressure (Omron heart rate monitor) were taken at screening to ensure that groups were well matched on these core measures, and these were again repeated on study day prior to the CO_2 session (day 14).

ANT

The ANT was identical to the procedure described in Chapter 3 (see Figure 4, Chapter 3 for ANT time course and trials). Briefly, participants view a central fixation cross (400-1,600ms) that is followed by a cue (100ms, except on no-cue trials). After cue offset (400ms, or 500ms in no-cue trials), a target arrow (with congruent or incongruent flankers) is displayed. Participants classify the direction of the central arrow as quickly and accurately as possible.

Centre cue and double cue trials alert participants to the onset of the target arrow. On centre cue trials, a cue is displayed in the location of the fixation cross. On double cue trials, a cue is presented above and below the fixation cross. Spatial cues alert and orient participants to the location of the target. On spatial cue trials, a cue is presented either above or below the fixation cross, priming participants to target location. The target arrow is flanked by two pairs of distractor arrows that are congruent (e.g. "←←←←←") or incongruent (e.g. "←←→←←") to the target. Flanker congruence,

target direction, and target location were counterbalanced across centre, double, spatial and no-cue trials.

Participants complete 8 randomised practice trials (2 of each type of cue) prior to 64 randomly ordered experimental trials (16 trials per cue condition).

Antisaccade

The antisaccade task was identical to the procedure described in Chapter 3. Participants are instructed to look towards (prosaccade) or away (antisaccade) from a negative or neutral peripheral stimulus as quickly and accurately as possible. Stimuli included 8 negative and 8 neutral images that were selected from the IAPs database (Lang et al., 2005) based on normative valence and arousal ratings. Task demand was increased by requiring participants to classify the direction of an arrow stimulus (up or down) shown shortly after each image. Participants completed 8 practice trials with a yellow rectangle, followed by 3 blocks of 32 experimental trials (96 trials in total, each image presented 6 times) where image order and trial type were randomised. Mean errors and latencies on antisaccade trials are reported as common indices of attentional control. Errors are presented as a proportion with values closer to 1 indicating worsening performance.

Thought intrusions task

The thought intrusions task was based on the protocol provided by Hirsch et al. (2009) and was completed twice; once before and once after a 5 minute period of instructed worry. Participants were instructed to focus their attention fully on their breathing for a period of 5 minutes. During this time, a tone was played by the computer every 25 seconds (providing a total of 12 trials). When the tone sounded, the participant indicated the focus of their attention at that moment. If they were on-task, the participant would report that they were focussed on their breathing. Alternatively if a thought intrusion had occurred, the participant would instead provide a brief description of their thought intrusion and state if they felt it was positive, negative or neutral in content (e.g. “friends – positive”).

At the end of the first thought intrusions task, participants summarised a current worry topic in the questionnaire booklet and briefly described this to the experimenter. They also rated on visual analogue scales “how likely it was to happen”, “how

catastrophic it would be" and "how well they felt they would cope with it". Participants then worried about their chosen topic for 5 minutes. At the end of the 5 minutes, the thought intrusions task was completed for the second time. The number of positive, negative and neutral intrusions was recorded.

Data acquisition and preparation

All physiological responses (heart rate, respiration rate and eye-movements) were sampled at 1000Hz via a Biopac MP150 data acquisition system and were processed with AcqKnowledge 4.1 software. Data was processed blind to drug group and trial type.

Heart rate. Throughout the inhalations, heart rate was recorded via two electrodes placed on the inside of both wrists. The raw signal was band pass filtered (set between 0.5 and 35Hz) with 8000 coefficients and was matched to a representative QRS-template. After visual inspection of raw tachograms and manual artefact removal, an automated analysis of HR was performed to provide mean HR.

Respiration rate. Respiration rate was recorded with a respiration belt transducer. The raw signal was band pass filtered (low pass 0.05Hz, and high pass 1Hz) with 4000 coefficients. Breaths per minute (BPM) were detected in five minute intervals and a mean was calculated across the full 20 minutes.

ANT. Reaction times from incorrect trials (3.5%) and greater than 1,000ms (1.8%) were removed from analyses. The removal of these trials did not vary by drug group or inhalation (F's < 3.21, p's > .084). Alerting, orienting and executive attention scores were calculated for each participant and inhalation using the following methods:

Alerting = Mean RT_*no-cue trials* – Mean RT_*double-cue trials*
Orienting = Mean RT_*centre-cue trials* – Mean RT_*spatial-cue trials*
Executive attention = Mean RT_*incongruent trials* – Mean RT_*congruent trials*

Higher scores on the alerting and orienting effects of attention suggest greater efficiency of these networks. Higher scores on the executive control effect indicate less efficient resolution of conflict.

Antisaccade. For the antisaccade task, two single-use electrodes were placed on the outer canthus of each eye to record horizontal eye-movements via

electrooculography. A low pass digital filter fixed at 20Hz was performed. Visual inspection ensured that the first eye-movement toward or away from the stimulus was captured correctly by the software. Where two eye-movements were made in quick succession, data was manually corrected to record only the first eye-movement to accurately reflect the latency. Consistent with previous studies, saccades with a latency < 100ms were removed from analyses as these were considered anticipatory.

Normality and statistical analyses

Normality of questionnaire and autonomic measures were assessed with Shapiro-Wilk tests. The GAD-7 was found to deviate from a normal distribution and a log transformation provided the best solution. All analyses with the GAD-7 were performed on log-transformed data, although raw means are reported in the tables for clarity. Similarly to the previous chapter, the negative scale of the PANAS was not normally distributed and transformations did not improve this. As such non-parametric tests (Mann-Whitney U and Friedman tests) were carried out where possible. The results of these tests produced comparable results to parametric alternatives and for clarity, parametric test statistics are reported.

For all analyses of variance (ANOVA), greenhouse-geisser corrections were used where assumptions of sphericity were violated. Post hoc analyses were conducted with simple main effects and pairwise comparisons. All reported *p* values are uncorrected. Bonferroni corrected critical *p* values are stated where appropriate for comparison.

4.3 Results

Group characteristics

Data from 4 participants were excluded from all analyses; 3 due to concerns about adherence to treatment (2 females taking placebo, 1 female taking memantine), and 1 who withdrew consent on study day due to circumstances unrelated to the study (1 male taking memantine). Therefore the final sample consisted of 33 healthy volunteers; 16 who received memantine (10 males) and 17 who received placebo (9 males). Groups did not differ on any measure of demographics, mood, or autonomic arousal prior to treatment (see Table 17).

Table 17. Mean (SD) demographics for all participants pre-treatment.

	Placebo (N = 17)		Memantine (N = 16)		Independent t tests
Age	21.00	(2.45)	23.00	(4.49)	$t(22.9) = -1.60$, $p = .119$, *ns*
BMI	22.72	(2.79)	22.13	(2.66)	$t(31) = 0.62$, $p = .539$, *ns*
GAD-7	10.01	(6.69)	8.77	(6.06)	$t(31) = 0.55$, $p = .585$, *ns*
Trait anxiety (STAI)	31.38	(5.56)	31.81	(3.82)	$t(30) = -0.26$, $p = .797$, *ns*
Worry (PSWQ)	40.41	(10.60)	37.60	(9.99)	$t(30) = 0.77$, $p = .448$, *ns*
Positive affect	35.47	(5.34)	35.93	(5.01)	$t(30) = -.025$, $p = .803$, *ns*
Negative affect	13.47	(2.29)	14.13	(4.16)	$t(31) = -0.56$, $p = .577$, *ns*
Heart rate	77.59	(7.36)	73.13	(13.28)	$t(23.1) = 1.18$, $p = .248$, *ns*
SBP	120.82	(14.79)	121.88	(10.84)	$t(31) = -0.23$, $p = .818$, *ns*
DBP	67.47	(8.57)	68.50	(7.69)	$t(31) = -0.36$, $p = .720$, *ns*

Side effect reporting and blinding

All participants completed the two week course of memantine (titrated to 10mg on day 8) or placebo without reported serious adverse event. At the end of the study, participants described any symptoms they had experienced which they believed were due to the tablets.

In total 29 potential side effects were reported by 17 participants. Six participants in the placebo group and 11 participants in the memantine group reported side effects. The most commonly reported side effects in the memantine group were lethargy (N = 4) and headaches (N = 2). The most common side effect reported by the placebo group were headaches (N = 3). A greater range of side effects were reported by those taking memantine than placebo and included (but not inclusive to) nausea, poor/improved concentration, a rash, and increasingly feeling anxious or upset.

At the end of the study, all participants were asked to identify which drug group they felt they were allocated to. In the placebo group, 10 of 17 participants accurately believed they received placebo, whilst only 5 of 16 participants in the memantine group accurately identified that they received memantine As such, no evidence was found to suggest that participants were accurate in their assessments ($\chi^2(1) = 0.35$, $p > .554$, *ns*), with the vast majority of participants believing that they received placebo (21/33). This

likely illustrates the good tolerability of memantine and an inability to differentiate true side effects from common ailments.

The effect of memantine on mood and autonomic arousal (day 0 – 14)

To examine whether 2 week administration of memantine or placebo had an effect on mood or autonomic arousal, 2 x 2 mixed model ANOVAs were conducted with drug (placebo vs. memantine) as a between subjects factor and time (baseline vs. post-drug) as a within subjects factor.

Two week treatment with memantine was found to have no effect on levels of anxiety or positive/negative mood. Similarly, no effect of memantine was found on measures of autonomic arousal including systolic and diastolic blood pressure and heart rate (see Table 18).

Similarly to Chapter 3, a main effect of time was revealed for anxiety ($F(1, 31) = 7.64$, $p = .010$, $\eta_p^2 = .198$), with greater anxiety post-drug ($M = 12.62$, $SD = 8.36$) than pre-drug ($M = 9.41$, $SD = 6.32$). This may reflect anxious anticipation of the CO_2 challenge that was to follow that day. However in contrast, a main effect of time was identified for systolic blood pressure ($F(1, 31) = 4.26$, $p = .048$, $\eta_p^2 = .121$), with lower SBP post-drug ($M = 117.55$, $SD = 12.30$) than pre-drug ($M = 121.33$, $SD = 12.83$). All other main effects were non-significant ($F's < 3.51$, $p's > .070$; main effects of memantine are shown in Table 39, Appendix B).

Effect of memantine on subjective and autonomic responses to CO_2 challenge

An additional 3 participants (2 males taking memantine; 1 female taking placebo) were excluded from the analyses of the subjective and autonomic effects of CO_2 challenge. These participants did not fulfil HR inclusion criteria (not less than 50bpm or exceeding 90bpm) for CO_2 challenge or did not complete both inhalations fully, despite completing all other measures. This leaves a final sample of 16 (9 M, 7 F) for the placebo group and 14 (8 M, 10 F) for the memantine group (a total of 30 participants).

Subjective response to CO_2. Comparisons of peak gas effects (vs. a pre-test baseline) were tested for anxiety (GAD-7) and positive and negative affect (PANAS) using separate mixed model analysis of variance (ANOVA). Gas (baseline vs. peak air vs. peak CO_2) was a within-subjects factor, and drug (placebo vs. memantine) and order

Table 18. Mean (standard deviation) anxiety, mood and autonomic arousal at baseline and post-drug for the placebo and memantine group.

	Placebo (*N* = 17)				Memantine (*N* = 16)				*time*drug group*
	Day 0		Day 14		Day 0		Day 14		
GAD-7	10.01	(6.69)	12.08	(7.09)	8.78	(6.06)	13.19	(9.73)	*F*(1, 31) = 0.73, *p* = .400, *ns*
PANAS positive	35.47	(5.34)	35.35	(5.85)	35.93	(5.01)	34.87	(4.05)	*F*(1, 30) = 0.45, *p* = .507, *ns*
PANAS negative	13.47	(2.29)	14.29	(3.70)	14.13	(4.16)	14.63	(5.34)	*F*(1, 31) = 0.80, *p* = .781, *ns*
Heart rate	77.59	(7.36)	77.88	(7.71)	73.13	(13.28)	74.63	(14.22)	*F*(1, 31) = 0.17, *p* = .681, *ns*
Systolic BP	120.82	(14.79)	117.24	(11.65)	121.88	(10.84)	117.88	(13.33)	*F*(1, 31) = 0.13, *p* = .912, *ns*
Diastolic BP	67.47	(8.57)	66.71	(7.39)	68.50	(7.69)	64.31	(7.89)	*F*(1, 31) = 1.68, *p* = .205, *ns*

(air first vs. CO_2 first) were between-subjects factors. In line with the hypotheses reported in section 4.1, significant interactions between drug group (placebo vs. memantine) and peak-inhalation effects (baseline vs. peak-air vs. peak-CO_2) were anticipated.

The strong effects of 7.5% CO_2 challenge seen in previous chapters (2 and 3) on subjective mood were replicated. 7.5% CO_2 inhalation significantly increased anxiety and negative affect with concurrent decreases in positive affect (see Table 19 for main effects). However, no effect of memantine was found on any subjective response to CO_2 (see Appendix B, Table 40 for drug group by gas interactions).

Autonomic response to CO_2. Mixed model ANOVA were used to test the effects of memantine on blood pressure, heart rate and respiration rate. For blood pressure, gas (baseline vs. peak air vs. peak CO_2) was a within-subjects factor, and drug (placebo vs. memantine) and order (air first vs. CO_2 first) were between-subjects factors. For heart rate and respiration rate, baseline measures were not recorded and so the within subjects factor of gas consisted of 2 levels only (peak air vs. peak CO_2).

7.5% CO_2 inhalation raised systolic blood pressure, heart rate and respiration rate consistent with previous findings (see Table 19 for main effect of time). However, no clear effect of memantine was found on any of these measures (see Appendix B, Table 40 for drug group by gas interactions). A significant gas by drug interaction was revealed for diastolic blood pressure, although simple main effects revealed that this was fuelled by changes in the placebo group. In the placebo group, diastolic BP was lower at the peak effects of air than at baseline (M_{diff} = 7.45, SE = 1.91, p = .002) and at peak CO_2 (M_{diff} = 7.71, SE = 2.52, p = .016). No differences in diastolic BP were revealed for the memantine group.

Effects of order. In addition to a main effect of time (F (1, 26) = 36.32, p < .001, η_p^2 = .583), a significant interaction between gas and order was identified for HR (F (1, 26) = 23.11, p < .001, η_p^2 = .471). Those who received CO_2 first experienced a significant increase in HR during CO_2 vs. air (M_{diff} = 10.87, p = .001), which was not revealed for the air first group (p = .396). No other meaningful main effects or interactions were identified.

Table 19. Mean (SD) anxiety, mood and autonomic arousal for those receiving placebo and memantine at baseline, peak air and peak CO_2

	Baseline			Air			7.5% CO_2			ANOVA (time)		
	Placebo	Memantine	Total	Placebo	Memantine	Total	Placebo	Memantine	Total	F	p	η_p^2
GAD-7	8.37 (5.14)	9.62 (10.94)	8.95[a] (8.23)	9.27 (10.94)	12.24 (11.04)	10.65[a] (10.90)	33.08 (17.32)	28.60 (16.28)	30.99[b] (16.71)	33.25	< .001	.561
Positive affect	34.88 (5.45)	32.43 (5.87)	33.73[a] (5.69)	27.75 (10.01)	26.57 (7.28)	27.20[b] (8.72)	25.69 (9.52)	20.71 (6.04)	23.37[c] (8.34)	29.96	< .001	.535
Negative affect	11.25 (1.61)	11.79 (2.04)	11.50[a] (1.81)	11.75 (2.86)	11.71 (2.23)	11.73[a] (2.55)	18.13 (7.85)	18.07 (7.87)	18.10[b] (7.72)	19.73	< .001	.431
Systolic BP	117.13 (13.26)	113.14 (10.68)	115.21[a] (12.04)	112.60 (9.72)	114.71 (10.61)	113.62[a] (10.03)	120.07 (13.08)	122.29 (10.26)	121.14[b] (11.65)	11.97	< .001	.324
Diastolic BP	73.47 (6.51)	69.14 (9.45)	71.38 (8.21)	66.27 (8.65)	69.64 (6.02)	67.90 (7.56)	73.67 (11.99)	69.64 (6.87)	71.72 (9.90)	2.90	= .064, *ns*	.075
Continuous HR	-	-	-	76.51 (8.86)	72.18 (8.25)	74.49[a] (8.71)	81.38 (9.87)	79.40 (10.03)	80.46[b] (9.82)	36.32	< .001	.583
Respiration	-	-	-	17.22 (4.11)	15.27 (3.36)	16.31[a] (3.85)	21.34 (5.51)	18.63 (2.87)	20.07[b] (4.61)	26.29	< .001	.503

Note. Pairwise comparisons with Bonferroni correction applied ($p < .017$). Values with different superscripts were significantly different from each other.

Effect of memantine on networks of attention during CO_2 inhalation

Separate mixed model ANOVA were used to examine the effect of memantine and 7.5% CO_2 inhalation (versus air) on alerting, orienting and executive control network function. Drug group (placebo vs. memantine) and order (air first vs. CO_2 first) were between-subjects factors, and gas (air vs. 7.5% CO_2) was a within-subjects factor. The alerting, orienting and executive control effects were the dependent variables. Table 20 summarises the function of each attentional network by drug group.

Analysis of the alerting effect did not reveal any significant main effects or interactions between this network function and inhalation, drug, or order (F's < 3.13, p's > .089). Similarly, analysis of the orienting did not reveal any significant main effects or interactions (F's >2.44, p's <.130).

Analysis of executive control revealed a significant inhalation x drug group interaction ($F(1, 26) = 5.43$, $p = .028$, $\eta_p^2 = .173$). Post hoc comparisons identified a greater executive control effect (suggesting impaired ability to ignore distractor arrows) during air ($M = 69.89$, $SD = 29.53$) than CO_2 ($M = 54.71$, $SD = 38.40$) for the placebo group only ($p = .023$). However this effect did not withhold after corrections for multiple comparisons were applied (Bonferroni p-value = .0125). Raw means (see Table 20) suggest that executive control performance was generally slower during CO_2 than air. This inhalation effect on executive attention was not significant for the memantine group ($p = .357$). No other main effects or interactions were significant (F's < 1.04, p's > .318).

Effect of memantine on antisaccade performance during CO_2 inhalation

Mixed model ANOVA was used to examine the effects of CO_2 inhalation on antisaccade performance. An additional two participants (1 male and 1 female; placebo) were removed from the analysis due to equipment failure and outlying poor number of valid eye movements (Z's > - 4.23); this leaves a sample of 28 participants (14 placebo, 14 memantine). In this instance, the within-subjects factors of trial type (prosaccade vs. antisaccade) and valence (neutral vs. negative) were included within the ANOVA,

Table 20. Means (SDs) of attention network function (ms) as measured by the ANT during 7.5% CO_2 and air inhalations, by drug group (placebo vs. memantine)

Networks	Placebo (*N* = 16)				Memantine (*N* = 14)				Total (*N* = 30)			
	Air		CO_2		Air		CO_2		Air		CO_2	
Alerting	**29.14**	**(38.37)**	**28.11**	**(32.87)**	**21.84**	**(26.99)**	**3.18**	**(33.43)**	**25.73**	**(33.19)**	**16.48**	**(34.92)**
No cue	610.82	(73.04)	629.40	(83.41)	586.48	(68.98)	604.55	(57.71)	599.46	(71.02)	617.80	(72.46)
Double cue	581.68	(75.90)	601.30	(86.58)	564.64	(61.82)	601.36	(77.58)	573.73	(69.05)	601.33	(81.09)
Orienting	**35.38**	**(21.29)**	**30.56**	**(19.23)**	**35.77**	**(16.68)**	**43.78**	**(29.82)**	**35.56**	**(18.95)**	**36.73**	**(25.20)**
Centre cue	586.55	(72.81)	607.15	(80.33)	565.00	(65.87)	596.73	(65.36)	576.49	(69.33)	602.29	(72.67)
Spatial cue	551.17	(66.11)	576.26	(87.22)	529.23	(67.62)	552.95	(69.31)	540.93	(66.63)	565.56	(78.94)
Executive control	**69.89**	**(29.53)**	**54.71**	**(38.40)**	**52.94**	**(19.47)**	**59.26**	**(23.65)**	**61.98**	**(26.37)**	**56.83**	**(31.92)**
Congruent	547.61	(74.53)	576.26	(86.73)	534.87	(64.98)	559.27	(66.03)	541.66	(69.34)	568.33	(76.94)
Incongruent	617.50	(68.01)	630.96	(83.65)	587.81	(65.54)	618.53	(66.58)	603.64	(67.42)	625.16	(75.14)

where gas compared antisaccade performance during air and CO_2 only[17]. The between subjects factors of drug (memantine vs. placebo) and order (air first vs. CO_2 first) were also included within the model. The dependent variable of antisaccade performance reflects the mean proportion between correct and incorrect trials and as such, values closer to 1 indicate greater numbers of errors.

Strong main effects of gas (F (1, 24) = 13.98, p = .001, η_p^2 = .368) and trial type (F (1, 24) = 60.69, p < .001, η_p^2 = .717) were identified. However, these main effects were superseded by a gas x trial type (F (1, 24) = 6.17, p = .020, η_p^2 = .204), a gas x order (F (1, 24) = 5.23, p = .031, η_p^2 = .179) and finally a gas x trial type x order interaction (F (1, 24) = 5.49, p = .028, η_p^2 = .186). In order to explore the three-way interaction further, data was split by trial type (prosaccade vs. antisaccade) and separate 2 (gas; air vs. 7.5% CO_2) x 2 (order; air first vs CO_2 first) mixed model ANOVA's were conducted (Bonferroni critical p < .025). Means were collapsed across negative and neutral images based on the lack of valence effects in the omnibus ANOVA.

For antisaccade trials, a main effect of gas (F (1, 26) = 12.72, p < .001, η_p^2 = .329) and an interaction between gas and order (F (1, 26) = 6.95, p = .014, η_p^2 = .211) were identified. Further examination of this interaction revealed that participants who received CO_2 first made a significantly larger proportion of antisaccade errors during CO_2 (M = .513, SE = .07) than air (M = .335, SE = .063, p < .001). No significant difference in antisaccade performance between the two inhalations was seen for those who received air first (p = 516, *ns*). No main effects or interactions were identified for prosaccade trials (p's > .116).

In addition to this interaction, a separate trial type by drug group (F (1, 24) = 5.15, p = .033, η_p^2 = .177) interaction was revealed. The means suggest that the memantine group made less antisaccade errors (M = .341, SE = .20) than the placebo group (M = .519, SE = .24), although this occurred irrespective of gas (p = .049, critical p < .025). No drug differences in prosaccade errors were found (p = .424, see Table 21). No other main effects or interactions were revealed.

[17] In addition, since images were presented on the left or the right, the location of the image was included in a subsequent analysis to ensure that this did not alter the interpretation of the present findings. No main effects or interactions with location were revealed (p's > .076).

Table 21. The proportion of errors (standard deviation) made on antisaccade and prosaccade trials during CO_2 and air inhalation by drug group

Errors	Placebo (N = 14)						Memantine (N = 14)					
	Air		CO_2		Total		Air		CO_2		Total	
Prosaccade	0.05	(0.05)	0.06	(0.06)	0.05	(0.05)	0.07	(0.13)	0.10	(0.15)	0.09	(0.13)
Neutral	0.04	(0.05)	0.07	(0.08)	0.06	(0.06)	0.09	(0.16)	0.11	(0.18)	0.10	(0.16)
Negative	0.05	(0.06)	0.06	(0.06)	0.05	(0.05)	0.05	(0.10)	0.09	(0.12)	0.07	(0.10)
Antisaccade	0.46	(0.27)	0.57	(0.25)	0.52	(0.24)	0.29	(0.23)	0.39	(0.19)	0.34	(0.20)
Neutral	0.49	(0.28)	0.57	(0.26)	0.53	(0.25)	0.29	(0.25)	0.38	(0.25)	0.33	(0.22)
Negative	0.44	(0.27)	0.58	(0.27)	0.51	(0.24)	0.30	(0.23)	0.40	(0.16)	0.35	(0.18)

Note. Proportion of errors ranges between 0 and 1.

The effect of memantine on antisaccade latencies during CO_2 challenge

Antisaccade latencies were analysed in the same way as antisaccade accuracy with mixed model ANOVA. Data from two participants (1 male, placebo group, 1 female, memantine group) were not included in this analysis due to a large number of anticipatory eye-movements on some trials. No main effects or interactions were found (all *F's* < 3.61, *p's* > .071, see Table 22).

Table 22. Latencies (standard deviation) on antisaccade and prosaccade trials during CO_2 and air inhalation by drug group

Latencies	Placebo (*N* = 13)				Memantine (*N* = 13)			
	Air		CO_2		Air		CO_2	
Prosaccade	190.68	(33.48)	184.10	(28.14)	201.13	(38.89)	199.70	(50.72)
Neutral	192.43	(35.61)	185.34	(31.39)	198.75	(40.00)	197.46	(48.18)
Negative	188.92	(33.74)	182.85	(27.34)	203.50	(40.81)	201.94	(56.37)
Antisaccade	193.50	(43.32)	178.46	(23.40)	202.27	(47.78)	193.87	(47.99)
Neutral	192.20	(46.64)	185.41	(32.38)	205.99	(55.61)	194.60	(59.65)
Negative	194.81	(43.37)	171.50	(22.41)	198.56	(48.39)	193.13	(60.10)

Associations between CO_2-induced change in subjective mood, autonomic arousal and antisaccade performance

To capture CO_2-induced changes in mood, autonomic arousal and antisaccade performance, difference scores were calculated (i.e. mean during CO_2 minus mean during air)[18]. Strong associations between CO_2-induced increases in anxiety, negative affect, heart rate and antisaccade errors were revealed (*r*'s > .401, *p*'s < .028; see Table 23 and Table 24, Figure 9 and Figure 10), indicating that those individuals who experienced the greatest increases in anxiety and heart rate during CO_2 also found it harder to look away from distracting images. CO_2-induced decreases in positive affect were also strongly associated with increased anxiety, negative affect and heart rate. Faster durations to initiate a correct antisaccade were found to be associated with

[18] i.e. measures taken immediately at the end of each inhalation for subjective mood and BP, and measures taken throughout the co2 minus air for heart and respiration rate and antisaccade performance.

greater anxiety and heart rate at peak CO_2 (vs. air). A strong association between delayed latencies to correctly look away during CO_2 and fewer antisaccade errors was also found. This may indicate a speed/accuracy trade-off when utilising attentional control during heightened anxiety, with better performance at the cost of slower eye-movements. Finally, poorer executive control in the ANT during CO_2 inhalation (i.e. slower durations to respond to the central arrow on incongruent than congruent trials) was associated with longer latencies to perform anti- and prosaccade trials correctly during CO_2 (versus air). This suggests some convergence between ANT and antisaccade performance measures of attentional control during CO_2 inhalation.

Table 23. Pearson's R correlations between CO_2-induced mood and autonomic arousal, relative to peak air.

	GAD-7 (anxiety)	Positive affect	Negative affect	SBP	DBP	HR	Respiration rate
GAD-7 (anxiety)	-	-	-	-	-	-	-
Positive affect	-.473**	-	-	-	-	-	-
Negative affect	.584***	-.528**	-	-	-	-	-
SBP	.144	-.122	.044	-	-	-	-
DBP	.182	-.319	.403*	-.106	-	-	-
HR	.507**	-.623***	.401*	.026	.228	-	-
Respiration rate	.041	.062	.159	.243	.031	.085	-

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Table 24. Pearson's R correlations between attention network function, errors and latencies in the antisaccade task during CO_2 inhalation (relative to peak air) with CO_2-induced state anxiety and heart rate

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
1.State anxiety (GAD-7)	-	-	-	-	-	-	-	-	-	-
2.Continuous heart rate	.507**	-	-	-	-	-	-	-	-	-
3.Alerting	.333	.194	-	-	-	-	-	-	-	-
4.Orienting	.076	-.025	.026	-	-	-	-	-	-	-
5.Executive control	-.205	-.115	.117	.282	-	-	-	-	-	-
6.Antisaccade errors	.426*	.533**	-.169	-.284	-.352	-	-	-	-	-
7.Prosaccade errors	-.016	-.057	.070	.249	.319	.071	-	-	-	-
8.Proportion of antisaccade errors to negative vs. neutral	-.143	.016	.047	.131	.172	-.143	.068	-	-	-
9.Antisaccade latency	-.394*	-.378*	-.026	.319	.438*	-.428*	.317	.315	-	-
10.Prosaccade latency	-.273	-.384*	-.215	.176	.520**	-.358	.258	.201	.573**	-
11.Proportion of antisaccade latencies to negative vs. neutral	-.127	-.189	-.116	-.179	-.037	.218	.096	.036	-.083	-.338

Note. * = significant < .05, ** = significant < .01 and *** = significant < .001.

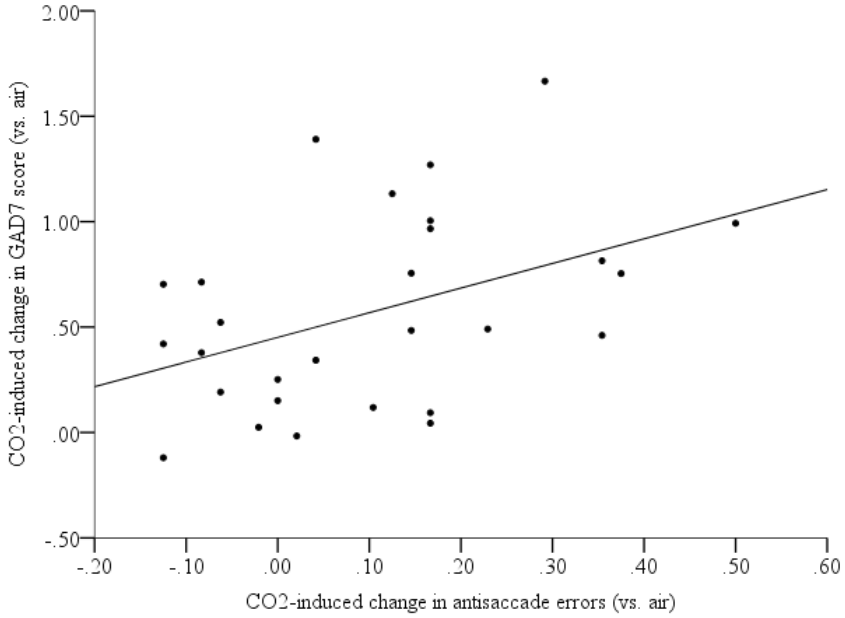

Figure 9. Relationship between CO_2-induced anxiety (log transformed GAD-7) and antisaccade errors (vs air)

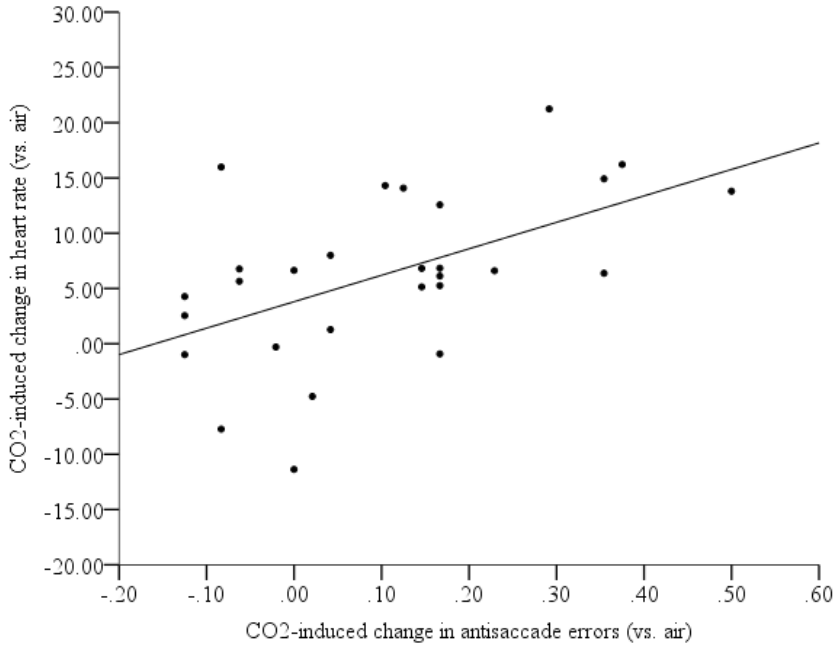

Figure 10. Relationship between CO_2-induced increases in heart rate and antisaccade errors (vs. air)

Instructed worry increases negative intrusions for placebo but not memantine

A mixed model ANOVA was conducted to examine the effect of memantine on frequency of negative thought intrusions before and after a period of instructed worry. Time (number of negative intrusions pre vs. post worry) was a within subjects factor and drug (placebo vs. memantine) was a between subjects factor.

Mixed model ANOVA did not reveal any significant main effects of drug group (F (1, 31) = 0.002, p = .963, *ns*) or time (F (1, 31) = 0.77, p = .387, *ns*). In addition, no significant interaction between drug group and time was revealed (F (1, 31) = 1.32, p = .260, *ns*). See Table 25 for mean number of negative thought intrusions by drug group.

Table 25. Mean number (SD) of negative thought intrusions reported by the memantine and placebo group before and after a period of instructed worry.

	Pre-worry	Post-worry
Placebo	0.53 (1.01)	1.00 (1.32)
Memantine	0.81 (1.52)	0.75 (0.93)

Independent samples t-tests also examined whether the memantine or placebo produced altered worry ratings of likelihood ("how likely it is to happen"), severity ("how catastrophic it would be"), and coping ("how well would you cope with it"). A marginal difference between the memantine and placebo group was revealed for ratings of ability to cope with a worry (t(31) = 2.01, p = .053, d_s = 0.70). The memantine group reported feeling better able to cope with their worry (M = 54.66, SD = 26.35) than the placebo group (M = 38.48, SD = 19.62). No differences were revealed for likelihood that the worry would occur, or how catastrophic that would be (t's < 1.26, p's > .219).

4.4 Discussion

In the present study, the effect of the NMDA-receptor antagonist memantine on subjective mood, autonomic arousal and attentional control was examined in a healthy human model of anxiety. The main finding of this study was that participants who received memantine made fewer antisaccade errors compared to those who received placebo. Moreover, no specific effect of memantine was revealed on CO_2-induced increases in antisaccade errors (compared to air – as shown by non-significant

interactions between drug and gas). This suggests memantine may have a global effect on attentional control rather than through the modulation of anxiety specifically. This is supported by the lack of evidence for an effect of memantine on the strong anxiety responses experienced during CO_2 inhalation, and the ambiguity over the potential anxiolytic properties of memantine in preclinical and clinical research (Feusner et al., 2009; Minkeviciene et al., 2008; Zarate et al., 2006).

This theory may also assist in explaining why memantine had no effect on the frequency of negative thought intrusions before and after a period of instructed worry. However, as the worry manipulation did not produce a significant increase in negative intrusions in either drug group, this hypothesis could not be directly tested. Future research should consider the main effect of memantine on intrusive thoughts and cognitive control, before partitioning by emotional content.

Attentional control theory suggests that in anxiety, attentional biases to threat reflect over-activation of stimulus-driven attentional processes at the cost of goal-directed attentional processes (Eysenck et al., 2007). Goal-directed processes employ top-down regulation of distractor stimuli in order to perform a task effectively. This converges with neurological evidence from anxious volunteers that focuses on a deficient amygdala-prefrontal circuitry. Hyperactive limbic areas (such as the amygdala) are thought to be poorly regulated by hypoactive cortical areas (such as the prefrontal cortex; Bishop, 2008). This behaviourally manifests as maladaptive attentional biases to threat, distractibility and poor control of attention (Bar-Haim et al., 2007). Given that these regions are densely populated with ionotropic glutamate receptors (Mcdonald, 1996), and modulation at these sites by glutamatergic compounds may disrupt fear conditioning and enhance extinction learning (Lee & Kim, 1998; Maren, Aharonov, Stote, & Fanselow, 1996; Walker & Davis, 2002; Walker, Ressler, Lu, & Davis, 2002), it is possible that the observed effects of memantine on attention occurred as a result of altered glutamate neurotransmission in limbic and/or cortical structures, boosting learning and cognitive control.

Other glutamatergic agents facilitate extinction learning (Norberg, Krystal & Tolin, 2008), interfere with memory consolidation, and activate areas involved in cognitive control in some patients (Aupperle et al., 2009). Previous research examining d-cycloserine (DCS), a partial agonist that enhances glutamate via the glycine-site of

NMDA receptors, did not affect CO_2-induced anxiety although did reduce self-reported task demand and improve task performance during a state of low anxiety (air inhalation – Bailey, Papadopoulos, Lingford-Hughes & Nutt, 2007). The manikin task used in this study requires learning a new strategy which may occur via altered glutamate transmission. This in turn could improve task performance when no emotional content is present (possibly by facilitating the speed of learning) and reduce self-reported experience of difficulty. This suggests that the actions of DCS - and possibly similar cognitive modulators that act on glutamate - may not be due to increased anxiety or arousal, but may instead increase learning by enhancing NMDA receptor activation leading to long-term potentiation (a necessity that underpins the biological basis for learning; Bliss & Collingridge, 1993).

Evidence exists to support this claim. Research in animals has shown that NMDA receptor activity (particularly in the amygdala) is important in extinction learning (Walker, Ressler, Lu & Davis, 2002). Specifically, increasing NMDA glutamate receptor activity can "overwrite" associated fear cues with an anxiety/fear response to facilitate extinction learning (Walker et al., 2002), and disrupt the reconsolidation of fear-related memories. In humans, considerable research has focussed on DCS as a possible augmentation therapy to aid the learning processes taught during CBT to reduce symptoms of anxiety/fear in a range of clinical disorders (see Norberg, Krystal & Tolin 2008, and Deveney 2009 for reviews). DCS has shown positive results for augmenting exposure-based CBT in social anxiety disorder (Guastella et al., 2008; Hofmann et al., 2006), specific phobias (Ressler et al., 2004), PTSD (Heresco-Levy et al., 2002; Difede et al., 2014) and OCD (Kushner et al., 2007 – see Bermudo-Soriano et al., 2012).

Whilst DCS is not thought to have direct anxiolytic properties, it may facilitate extinction and learning and override learned associations between negative cues and an aversive response – such as threat biases. For example, single dose DCS has been shown to augment attention bias training away from threat in trait anxious individuals, without any reduction in tolerance to stress or emotion reactivity to a mild stressor (Behar, McHugh, Peckham and Otto, 2010). Since the attentional bias to threat commonly seen in anxious populations was not reliably modelled in the present research by CO_2 inhalation, similar conclusions for memantine cannot extend beyond broader effects on attentional control. Similar glutamatergic agents to DCS, such as memantine

and riluzole should be systematically examined for beneficial effects on cognition and learning, and might be promising augmentation therapies for disorders characterised by maladaptive learnt associations (such as anxiety, phobias, OCD and addictions) alongside known anxiolytic treatments.

Interestingly, no effect of memantine was found on prosaccade performance, or on any of the attention networks measured by the ANT. This may be due to the relative ease of prosaccade trials during both CO_2 and air, where overall error rates are very low. This makes it difficult to establish whether the effect of memantine on task performance was specific to antisaccade trials only (and therefore on trials where conflict is introduced) or whether too few errors were made on prosaccade trials to robustly identify any improvement. This explanation may also be true of the alerting and orienting functions of the ANT where changes in performance (measured in ms) tend to be very small, but does not explain why no difference in executive network function was identified, considering the effect found on antisaccade trials. The reasons for this are unclear, but it is possible that the processes used to actively inhibit an eye-movement in the antisaccade task and focus on an arrow in the ANT vary, or differences exist in the validity of the tasks.

Few studies have directly examined the effects of memantine on attention in healthy volunteers and none have done so during experimentally induced anxiety. van Wageningen, Jorgensen, Specht, and Hugdahl (2009, 2010) used an auditory attention control task (thought to induce cognitive conflict) to examine the effect of memantine (titrated to 20mg over three weeks) on attentional control. Memantine attenuated activation in the ACC and PFC (among other areas), and this correlated with reduced glutamate-glutamine (Glx) concentrations in frontal regions. However, no change in performance on the task was identified, making it difficult to establish the extent to which these shifts in activation correspond to changes in attentional control or improved learning. Conversely, Schugens et al. (1997) found no effect of a single 30mg dose of memantine on mood, attention (using a letter cancellation task) or memory in healthy volunteers. A recent study by Chang et al. (2015) found that 12 week treatment with memantine improved cognitive performance and executive function in opiod dependent patients when used as an add on to methadone-maintenance therapy. Finally, a single dose of 10mg memantine in healthy volunteers did not affect mood, emotional memory, emotion recognition or performance on a visual-probe attention task (Pringle et al.,

2012). Why such variation exists across studies is unclear. This may reflect differences in task difficulty, sample choice, and methodology, such as a lack of knowledge on the optimal dose and duration required to elicit an effect.

Memantine had no impact on mood after two week administration, or on CO_2-induced anxiety and autonomic arousal. Given that the recruited sample were free from clinical symptoms, consistent changes in mood were not expected to be observed over the course of the two weeks. The absence of an effect of memantine on CO_2-induced anxiety may be due to a number of possibilities for example; insensitivity of the CO_2 model to detect changes in subjective mood (- although this is unlikely since reductions have been shown in assessments of known anxiolytics with this model; e.g. Bailey et al., 2007a); insufficient administration or dose; and/or weak efficacy of memantine as an anxiolytic. Anxiolytic effects of memantine are not well characterised in humans, with some research to suggest anti-anxiety properties (GAD and socially anxious patients, Schwartz et al., 2012; although note that no control comparison was used), while others report little or no change (GAD and OCD, Feusner et al., 2009; MDD, Zarate et al., 2006). It may follow that memantine like DCS, has no direct anxiolytic properties. Memantine may instead facilitate processes which, under the right conditions, produce or enhance subsequent changes in subjective mood. Further examination of memantine in clinically anxious subtypes with systematic randomised-controlled trials would be beneficial to clarify whether memantine has specific anxiolytic properties beyond the broader effects on attention and/or learning processes demonstrated here.

Limitations

Animal research has speculated that higher doses of memantine are required to produce noticeable and clinically relevant reductions in anxiety and these may need to exceed that of its use as a cognitive enhancer (Minkeviciene et al., 2008). In this research a final dose of 10mg was achieved for all participants, however, the BNF reports a maximal dose of 20mg can be reached. Whilst higher doses of memantine have been examined in humans, these are often single administration with a focus on schizophrenia rather than anxiety (e.g. Korostenskaja, Nikulinm, Kicic, Nikulina, & Kahkonen, 2007). Future research should consider replication of this work to achieve a final dose of 20mg. This may establish if memantine has anxiolytic properties in humans at higher doses.

Similarly to the previous chapter, no assessments of compliance were made in this study, although side effects consistent with memantine were reported. Participants who reported noncompliance were removed from all analyses. Partly as a result of noncompliance and due to withdrawal, the final sample size of this research was smaller than initially anticipated. Thus despite low power, the weak findings in this research are encouraging and it is hoped that a full replication with a larger sample size would provide further support for a global effect of memantine on attentional control.

It should be noted that in the worry task, no direct measure of worry engagement was made besides volunteers' descriptions of their chosen worry topic and ratings of likelihood and impact. The worries selected were therefore un-primed and unique to the individual. Poor engagement in the worry period is one possible reason for the low numbers of negative intrusions (and total intrusions) reported both pre- and post-worry in this research. Alternatively, this may be because the present sample were pre-screened for high levels of anxiety (including symptoms of generalised anxiety) and reported relatively low levels of worry (PSWQ; M = 39.09, SD = 10.25) when compared to similar samples (Behar, Alcaine, Zuellig, & Borkovec, 2003 report a college sample (exluding GAD) mean of 47.08). Little published research has clearly described the thought intrusions task in a sample free from psychiatric illness and this should be clarified with new research (e.g. Baker, Baldwin, & Garner, 2015 report a mean of 0.90 (SD = 1.1) negative intrusions pre-worry, and 1.58 (SD = 1.44) negative intrusions post-worry in their sample of undergraduate students). Finally, the failure of the worry induction phase to consistently increase negative intrusive thoughts across both groups is not unique to this study. No effect of time (pre-post worry) was revealed in a study examining this task in high worriers, GAD patients and PD patients (Hirsch et al., 2013).

Conclusion

In summary, the present research is the first report of the NMDA-receptor antagonist memantine in a healthy human model of anxiety. Whilst no effect of memantine was revealed on anxiety responses to CO_2, memantine did facilitate attentional control in an antisaccade task regardless of the inhalation received. Since similar glutamatergic agents have been suggested to have global effects on learning and memory, it is plausible that the effects of memantine are the result of similar processes. Future research is warranted to establish whether memantine (and compounds with

similar mechanisms of action such as DCS, riluzole and ketamine) exert global effects on attention in clinical samples, which may augment treatments that produce changes in subjective mood.

Chapter 5: *General Discussion*

5.1 Rationale:

There is a pressing need for new models of human anxiety due to the poor predictive validity of animal models in the identification of novel drug targets which successfully translate into clinical practice (Dawson, Dourish, & Goodwin, 2011; Haller et al., 2012; Markou, Chiamulera, Geyer, Tricklebank, & Steckler, 2009). The use of healthy human models to examine potential drug effects, not just on subjective mood, but also on the neurocognitive deficits that characterise anxiety disorders could aid the development of new treatments and repurpose existing drug treatments.

5.2 Thesis aims:

The programme of work described in this thesis had two main aims. First, to critically evaluate 7.5% CO_2 as a healthy human model of anxiety that may share some similarities with generalised anxiety disorder (GAD). This is with regard to the neural mechanisms that are instrumental in producing an anxious response (Chapters 1-2) and via the assessment of drugs known to reduce anxiety symptoms within this model (Chapter 3). Secondly, this research assessed whether the 7.5% CO_2 model can be used to evaluate potential efficacy of anxiolytic drug treatments. In particular, this focused on whether a current and/or novel drug treatment can decrease a range of anxiety symptoms (including neurocognitive deficits) induced by 7.5% CO_2 inhalation (Chapters 3-4).

This general discussion will summarise the findings from the three experiments conducted in this thesis. The combined weight of these findings will then be discussed, and the effects of duloxetine and memantine on responses to CO_2 will be compared. Whether these findings provide further support for 7.5% CO_2 inhalation as an experimental model of anxiety, and specifically of GAD, will then be considered. Finally, the limitations of this research will be acknowledged and suggestions for further work will be described.

5.3 Summary of experimental chapters

5.3.1 Study 1, Chapter 2: CO_2 inhalation does not potentiate eye-blink magnitude, and instead delays latencies to a startling stimulus

The defensive startle response is a rapid and powerful eye-blink that is potentiated by the extended amygdala in situations of possible or imminent threat. Rodent studies implicate the amygdala in the detection, and subsequent presentation of fear behaviours to increased concentrations of CO_2 (Price et al., 2014; Ziemann et al., 2009), however in humans, CO_2-induced fear remains unaffected in patients with extensive bilateral amygdala damage (Feinstein et al., 2013). This suggests that neural sites beyond the amygdala can sense changing CO_2 concentrations to stimulate defensive behaviours. In light of this, Study 1 sought to discover whether the amygdala might be similarly important in the detection of, and behavioural response to increased CO_2 in human subjects, as has been shown in rodents. Since the defensive startle is a well validated, yet indirect measure of amygdala potentiation in situations of high threat (Grillon, 2008), startle magnitude was examined during CO_2 inhalation.

In addition to startle magnitudes, startle latencies (i.e. the time it takes to generate an eye-blink to a startling stimulus) were also examined during CO_2 challenge for the first time. Extensive literature has described the phenomenon of hypervigilance towards threat in highly anxious populations (Bar-Haim et al., 2007), and this is most commonly shown by faster reaction times on threat-congruent trials in emotion processing paradigms (such as the emotional variant of the dot-probe). Despite this research, startle latencies to threatening and non-threatening stimuli are not often reported. As a result, Study 1 also investigated whether CO_2 inhalation produced speeded startle eye-blinks (of a greater magnitude) which would align with an anxious and hypervigilant phenotype.

Contrary to expectations, CO_2 inhalation did not modulate startle eye-blink magnitude, despite producing strong anxiogenic effects on mood and autonomic states (see Table 26 for summary). Rather, CO_2 inhalation slowed latencies of eye-blink responses to startle probes. This novel finding extends previous research which describes reduced startle magnitudes during short inhalations of 7.5% CO_2 (Ceunen et al., 2013; Pappens et al., 2012). One possible explanation for why CO_2 might delay eye-

blink latencies (Study 1) and/or reduce startle magnitudes (Ceunen et al., 2013; Pappens et al., 2012), is that the processing resources necessary to potentiate defensive startles may be reduced by CO_2 challenge. This theory aligns with evidence of attenuated startles when cognitive load is high, and when paradigms target interoceptive mechanisms that grab attention, such as during the cold pressor test.

Table 26. A summary of the main findings from Study 1.

7.5% CO_2 inhalation (versus peak air):		
Subjective	Increased state anxiety (d_{av} = 0.64)	No effect on negative affect
	Decreased positive affect (*d_{av} = 0.55)	
Autonomic	Increased heart rate (d_{av} = 0.52)	
	Increased systolic (*d_{av} = 0.77) and diastolic	
	BP(*d_{av} = 0.51)	
	Increased skin conductance (d_{av} = 0.76)	
Startle	Slowed startle latency (d_{av} = 0.34)	No effect on startle magnitude

Note. * indicates that the effect only occurred when compared to baseline, not air, and d_{av} effect sizes are reported as such.

5.3.2 Study 2, Chapter 3: Duloxetine modulates attentional bias of healthy volunteers during CO_2-induced anxiety

Study 2 investigated the effects of the SNRI duloxetine on subjective anxiety, autonomic arousal and neurocognitive responses to 7.5% CO_2. Previous evidence suggests that the CO_2 model can detect shifts in mood states as a result of single or sub-chronic administration of some anxiolytics (Bailey et al., 2007a; Bailey et al., 2011b; Bailey et al., 2009; Bertani et al., 1997; Bertani et al., 2001; Diaper et al., 2013; Diaper et al., 2012b; Papadopoulos et al., 2010; Schruers & Griez, 2004). However, the newer class of treatments that selectively modulate monoaminergic neurotransmission (SSRIs, SNRIs and NRIs) do not consistently attenuate CO_2-induced anxiety symptoms to a level considered clinically relevant (Bailey et al., 2007a; Diaper et al., 2013). Since duloxetine has been identified as "first for response" in the treatment of GAD, it is perhaps a better candidate with which to examine whether CO_2-induced anxiety can be targeted by monoaminergic drug administration.

A recent body of evidence has attempted to explain why the administration of monoaminergic drugs does not produce immediate relief from clinical symptoms of

anxiety/depression. Neuropsychological theories of drug action suggest that observable changes in clinical symptoms are mediated by changes in the way affective (particularly negative) information in our environment is processed (Harmer et al., 2009a). Unfortunately previous evaluations of drugs with CO_2 inhalation have exclusively focussed on measures of subjective mood states and autonomic arousal and have not included experimental measures of emotion/cognitive processing biases. Since attentional biases are thought to be critical to both the development and maintenance of pathological anxiety, drug effects on attentional deficits need to be examined to provide a comprehensive assessment of new treatments. Study 2 therefore examined the effect of duloxetine on CO_2-induced impairments in attention, using the ANT and the antisaccade tasks that have been used to profile attentional biases in clinical and subclinical anxiety previously.

In addition, a measure of thought intrusions (modified from Hirsch et al., 2009) was used immediately after each inhalation to examine whether focussed attention is impaired by CO_2 (vs. instructed worry). This was based on the proposal that 7.5% CO_2 inhalation is an appropriate model of GAD (Bailey et al., 2011a), the main diagnostic characteristic of which is uncontrollable, pervasive worry (American Psychiatric Association, 2013). Besides simple visual analogue scales of "worry" (e.g. Bailey et al., 2005), this is the first time that the frequency of negative intrusive thoughts have been recorded as a secondary measure of volitional control over worrisome thoughts.

Study 2 showed that whilst 7.5% CO_2 inhalation produced significant increases in subjective anxiety and systolic blood pressure, heart and respiration rate, these effects remained unchanged by prior administration of duloxetine (see Table 27). However, separate analyses of each drug group revealed a specific CO_2-induced impairment in antisaccade performance for the placebo group (as demonstrated by greater instances of erroneous eye-movements towards, rather than away from a stimulus on antisaccade trials), which was not present in the duloxetine group. Thus CO_2-induced deficits in attentional control appear to have been weakened by duloxetine, which occurred in the absence of a clear reduction in anxiety or autonomic arousal.

These findings were suggested to reflect possible changes in noradrenergic and/or serotonergic transmission after duloxetine treatment which improved attentional control over distractor stimuli during a state of heightened subjective anxiety. This

aligns to some extent with a cognitive neuropsychological theory of antidepressant action, where administration of an antidepressant is believed to produce improvements in the way emotional information is attended, processed and interpreted prior to changes in mood (Harmer et al., 2009a). However, since an attentional bias to threatening images during 7.5% CO_2 inhalation was not replicated in this research, assertions concerning the effect of duloxetine on anxiety-specific biases are speculative, and based on evidence from previous research (Harmer et al., 2008), rather than on the findings described here. Similarly, the two week dosing period used in this research prevents comment on the time course of drug effects on attention deficits produced by CO_2 inhalation.

Table 27. A summary of the main findings from Study 2.

	7.5% CO_2 inhalation (vs. air):	Effects of duloxetine:
Subjective	Increased state anxiety (d_{av} = 1.50) Increased negative affect (d_{av} = 1.57) Decreased positive affect (d_{av} = 0.47)	No effect of duloxetine*
Autonomic	Increased heart rate (d_{av} = 0.78) Increased systolic (but not diastolic) BP (d_{av} = 1.10) Increased respiration rate (d_{av} = 0.95)	No effect of duloxetine
ANT	Had no clear effect on the function of attention networks	No effect of duloxetine
Antisaccade	Increased errors on antisaccade (but not prosaccade) trials (d_{av} = 0.44). No interaction was revealed between CO_2 inhalation and image valence.	CO_2-induced impairment (vs. air) in antisaccade performance was only present for the placebo group (d_{av} = 0.49)
Thought intrusions	CO_2-induced increases (vs. air) in negative thought intrusions were only reported by the placebo group** (d_{av} = 1.53)	

Note. *A trend for an effect of duloxetine (vs. placebo) was revealed for untransformed GAD7 scores at the peak effects of CO_2, however this was for the air first order only (d_s = 0.81). **Only when air was inhaled first.

No effects of duloxetine or CO_2 inhalation were revealed on the function of the attention networks. Instead concern was raised over the use of difference scores to quantify these networks, as initial findings suggested poorer executive attention during air inhalation but not CO_2 inhalation (for air first order only). Subsequent examination of mean reaction times identified that this was not in fact the case, as apparent

reductions in distractor interference during CO_2 were attributable to slowed reaction times on congruent trials during CO_2 challenge as opposed to faster reaction times on incongruent trials (which would have suggested poorer executive control).

5.3.3 Study 3, Chapter 4: Memantine reduces the negative effect of CO_2 on attention control, whilst leaving CO_2-induced increases in anxiety unaffected

Study 3 investigated the potential anxiolytic effects of the drug memantine on subjective, autonomic and neurocognitive responses to 7.5% CO_2 inhalation. This study largely replicated the methodology of Study 2, and further emphasised the importance of including assessment of attention within models of anxiety to identify potential efficacy of new treatments. Memantine is currently licenced for the treatment of moderate-to-severe Alzheimer's disease, showing particular efficacy on cognitive symptoms. In addition, memantine has demonstrated some anxiolytic properties in preclinical models of anxiety (Morris water maze and elevated plus maze Minkeviciene et al., 2008) and may be beneficial in the treatment of psychiatric disorders that impair cognition including OCD, schizophrenia and substance abuse (Feusner et al., 2009; Haghighi et al., 2013; Zdanys & Tampi, 2008).

Similarly to Study 2, whilst memantine had no effect on CO_2-induced increases in anxiety or autonomic arousal, it did have a broad effect on attentional control across both inhalations (see Table 28). Those who received placebo experienced greater numbers of antisaccade errors compared to those who received memantine, irrespective of the inhalations received. No drug by inhalation interactions were revealed for any measure of CO_2-induced increases in anxiety, autonomic arousal, or pro- and antisaccade error rates. As a result, the global effect of memantine on attentional control (as measured by the antisaccade task) might reflect facilitated learning via modulation of NMDA glutamate receptors instead of a specific fear/anxiety effect. Further research should consider whether memantine might be used to modulate the attentional biases to threat commonly seen in anxious populations. This was not examined in the present research because CO_2 inhalation did not reliably model an attention bias to threat. Examination of memantine as a potential augmentation therapy alongside CBT or attention bias training would also be of considerable interest.

In contrast to Study 2, the standardised thought intrusions task (Hirsch et al., 2009) was used independently from 7.5% CO_2 challenge to provide a more powerful test of memantine on focussed attention and inhibition of negative thought intrusions following worry induction. No significant difference was revealed in the number of negative thought intrusions reported post-worry (compared to pre-worry) by the memantine or placebo group. This supports the theory that the effects of memantine are not emotion specific, and suggests that future research should consider whether drugs such as memantine (vs. placebo) might reduce frequency of thought intrusions more generally, alongside other standardised measures of attentional control.

Table 28. A summary of the main findings from Study 3

	7.5% CO_2 inhalation (vs. air):	Memantine (vs. placebo):
Subjective	Increased state anxiety (d_{av} = 1.59) Increased negative affect (d_{av} = 1.24) Decreased positive affect (d_{av} = 0.45)	No effect of memantine
Autonomic	Increased systolic blood pressure (d_{av} = 0.69) Increased heart rate (d_{av} = 0.64) Increased respiration rate (d_{av} = 0.89)	No effect of memantine
ANT	Did not affect alerting, orienting or executive control network function	No effect of memantine
Antisaccade	Increased errors on antisaccade (but not prosaccade) trials compared to air (d_{av} = 0.41). Did not increase errors or speed latencies towards negative (vs. neutral) images in pro- or antisaccade trials.	Memantine group made less antisaccade errors than the placebo group (d_s = 0.84)
Thought intrusions task	N/A	No effect of memantine on negative intrusions

5.4 Summary of experiments

In summary, the experiments presented in this thesis demonstrate three main findings; (1) 7.5% CO_2 inhalation in humans can robustly produce symptoms of anxiety including detrimental effects on the ability to control attention, (2) drugs with anti-anxiety properties can prevent some of the anxiety-related attentional deficits produced

by CO_2 inhalation or improve attentional control more broadly, whilst having weaker, or no effect on mood, and (3) CO_2 likely mimics anxiety through complex neurotransmitter interactions at structural sites beyond, but not necessarily excluding the amygdala. The main questions that arose from this series of studies will now be considered in turn.

5.5 Question 1: How might CO_2 reduce startle magnitudes, delay startle latencies and worsen performance on the antisaccade task: a role for interoceptive awareness?

An important question that needs to be considered is how does CO_2 inhalation produce smaller and slower startle magnitudes and worsen antisaccade performance? Recently, it has been suggested that interoceptive awareness shares, at least in part, the same neural pathways (and therefore processing resources) that are used by the visual system when attending to external cues. In particular, the neural pathways thought to underlie complex, top-down attentional processes (such as divided attention, inhibition etc.) seem to overlap with the neural regions used when focusing attention on visceral sensations (Critchley, Wiens, Rotshtein, Ohman, & Dolan, 2004). As a result, individuals high in interoceptive awareness may have a behavioural "advantage", performing better on tasks requiring complex/divided attention due to possible overlap between the processes used in evaluating bodily sensations and those used by the attentional system (Matthias, Schandry, Duschek, & Pollatos, 2009). However, it is plausible that in situations where bioregulatory sensations are more salient (such as during stress tests) and therefore more difficult to ignore, the processing resources needed to focus on an external attentional task (whether complex or not) are reduced, instead being consumed by interoceptive focus. This causes poor task performance, as resources are allocated more so on internal sensations than on primary task performance.

In Study 1 it was theorised that by inducing autonomic arousal, CO_2 inhalation increased the salience of bodily functions and heightened anxiety. Consequently, focus on internal sensations consumed attentional resources that would otherwise be directed on external stimuli (i.e. the task), resulting in smaller and slower startle eye-blinks (Study 1 and Ceunen et al., 2013; Pappens et al., 2012). The results from Study 2 also fit with this interpretation i.e. poor performance on the antisaccade task (particularly in

the placebo group) might reflect greater allocation of resources to interoceptive cues during CO_2 challenge.

This theory is further supported by the observed associations between (i) CO_2-induced elevations in anxiety and heart rate (Studies 1, 2 and 3), (ii) CO_2-induced increases in heart rate and slower startle latencies (Study 1), and (iii) CO_2-induce increases in heart rate and poorer antisaccade performance (Studies 2 and 3 – see Table 29 and Table 30). However, no direct measure of interoceptive awareness was included in this research. It should be acknowledged that whilst supportive to the theory that CO_2 may enhance the salience of physical symptoms and consequently increase anxiety, the associations presented here are not a direct examination of interoceptive awareness. Further research is needed to establish the truth of this claim.

Table 29. Associations between CO_2- induced increases in subjective anxiety and changes in subjective, autonomic and neurocognitive responses across Studies 1-3

Anxiety	Associations with CO_2-induced increases in subjective anxiety:		
	Study One	Study Two	Study Three
Subjective	↓ positive affect		↓ positive affect
	↑ negative affect	↑ negative affect	↑ negative affect
Autonomic	↑ heart rate	↑ heart rate	↑ heart rate
	↑ SBP	↑ SBP	
	↑ DBP		
Neurocognitive	-	↑ antisaccade errors	↑ antisaccade errors
		↓ use of spatial cues	
		↑ negative intrusions	

Table 30. Associations between CO_2-inducced increases in heart rate with changes in subjective, autonomic and neurocognitive responses across Studies 1to 3

Heart rate	Associations with CO_2-induced increases in heart rate:		
	Study One	Study Two	Study Three
Subjective	↑ anxiety	↑ anxiety	↑ anxiety
			↑ positive affect
	↑ negative affect	↑ negative affect	↑ negative affect
Autonomic	↑ SBP		
	↑ DBP		
Neurocognitive	↑ startle latency	-	-
	↓ skin conductance	-	-
	-	↑ antisaccade errors	↑ antisaccade errors
	-	↓ prosaccade latency	↓ prosaccade latency
	-	↓ executive control	↓ antisaccade latency

It must also be acknowledged that the causal relationship between autonomic arousal and anxiety induced by the model is unknown, although it seems probable that the biological response of increased heart rate and blood pressure precedes higher-order cognitive representations of subjective anxiety. This follows Klein's (1993) suffocation alarm theory of panic disorder. This theory hypothesised that in panic disorder the respiratory centres of the brain are overly sensitive to CO_2 concentrations, small changes to which can inappropriately signals a biological response (interpreted and experienced as panic). This may occur via central pH/ CO_2 sensitive neurons that are sensitised to respiratory markers of arousal and "air-hunger".

In all three studies, the presence of emotional stimuli had no effect on startle magnitude or antisaccade performance. This might indicate a general impairment of CO_2 on attentional processes such as distractibility to task-irrelevant information (threat and non-threat), and follows evidence for a hypervigilant state during CO_2 even when emotional material is absent (Garner et al., 2012). This also aligns with some of the assumptions of attentional control theory, where anxiety is believed to consume working memory resources, impairing the balance between stimulus-driven (distractor images, whether threatening or not, and startling stimuli) and goal-driven (correct antisaccade performance) processes. However, CO_2 did not impact on antisaccade latency (the time it takes to generate a correct saccade away from a stimuli), which is

considered an indicator of processing efficiency (see Chapter 1: 1.3). Why CO_2 failed to produce an effect on antisaccade latency is unclear and warrants further investigation as this contrasts with the findings from Garner et al. (2011a).

The lack of valence effects in the present research limits the conclusions that can be drawn, and may suggest a limitation of the 7.5% CO_2 model. As a model of anxiety, 7.5% CO_2 would be expected to replicate the strong attentional biases to threat that are characteristic of anxious populations. Valence effects have been identified in at least one 7.5% CO_2 study which used the antisaccade task (Garner et al., 2011a). However in a series of experiments using the visual dot-probe task, biases to emotional (vs. neutral) faces were found to be entirely inconsistent with the anxiogenic effects of CO_2 inhalation (Cooper et al., 2009). The reasons for this variation across studies is unclear, and whilst this may be explained by differences in methodology or samples, evidence is building to suggest that 7.5% CO_2 inhalation may induce a general state of hypervigilance that is not emotion-specific. Future research should attempt to replicate the findings of Garner et al. (2011a), alongside the examination of threat biases with a mix of paradigms (e.g. dot-probe with fearful and neutral expressions or words, antisaccade etc.). This will help establish whether 7.5% CO_2 inhalation is a suitable candidate to model the attentional biases to emotional stimuli seen in anxious groups.

5.6 Question 2: How do the effects of duloxetine and memantine on responses to 7.5% CO_2 challenge compare?

Studies 2 and 3 revealed some similarities and differences between duloxetine and memantine (see Table 31 for drug effects during 7.5% CO_2 inhalation). Though neither drug substantially altered self-reported anxiety and autonomic arousal across the two weeks, or during the inhalation of CO_2, they did have an effect on antisaccade performance. For example in Study 2, the placebo group demonstrated impaired antisaccade performance during CO_2 inhalation (compared to air, $d_{av} = 0.49$), which was not replicated to the same degree in the duloxetine group ($d_{av} = 0.29$). Whereas in Study 3, the memantine group made fewer antisaccade errors than the placebo group across both inhalations ($d_s = 0.84$).

Whilst memantine reduced antisaccade errors during CO_2, this was not specific to the poorer levels of antisaccade performance associated with the anxiogenic effects of

this inhalation, as improvements of a similar magnitude were also revealed during air. This differs to duloxetine, which appears to have a targeted effect on **anxiety-induced** deficits in attentional control (experienced during CO_2). These findings suggest that memantine may be a useful candidate to augment existing anxiety treatments, either through extinction learning or as a cognitive enhancer (e.g. CBT, attention bias training, exposure-based therapies), rather than as a direct treatment of these disorders. This follows the relatively weak evidence for improved subjective anxiety in humans following memantine treatment, and aligns with evidence for the cognitive enhancing properties of similar glutamatergic agents, without direct effects on mood (Bailey et al., 2007).

Table 31. Means (SDs) and effect sizes (Cohen's d_{av}) of the peak effects of air and 7.5% CO_2 on subjective mood, autonomic arousal and attentional tasks for duloxetine and memantine

Peak effects	Duloxetine			Memantine		
	Air	CO_2	Effect	Air	CO_2	Effect
GAD-7	11.04 (8.23)	31.22 (20.92)	1.38	12.24 (11.04)	28.60 (16.28)	1.20
Positive affect	26.78 (9.18)	23.28 (9.31)	0.38	26.57 (7.28)	20.71 (6.04)	0.88
Negative affect	11.00 (2.09)	18.11 (6.99)	1.57	11.71 (2.23)	18.07 (7.87)	1.26
Systolic BP	122.06 (10.31)	134.00 (12.44)	1.05	114.71 (10.61)	122.29 (10.26)	0.73
Diastolic BP	77.22 (7.95)	78.94 (5.59)	0.25	69.64 (6.02)	69.64 (6.87)	0.00
Heart Rate	74.57 (9.39)	83.11 (8.58)	0.95	72.18 (8.25)	79.40 (10.03)	0.79
Respiration	16.52 (3.75)	20.18 (3.95)	0.95	15.27 (3.36)	18.63 (2.87)	1.08
Alerting	25.68 (25.96)	27.67 (37.06)	0.06	21.84 (26.99)	3.18 (33.43)	0.62
Orienting	39.98 (29.20)	33.16 (21.01)	0.27	35.77 (16.68)	43.78 (29.82)	0.34
Executive control	82.30 (27.69)	71.90 (32.00)	0.35	52.94 (19.47)	59.26 (23.65)	0.29
Antisaccade errors	0.37 (0.21)	0.43 (0.21)	0.29	0.29 (0.23)	0.39 (0.19)	0.48
Prosaccade errors	0.03 (0.03)	0.06 (0.06)	0.67	0.07 (0.13)	0.10 (0.15)	0.21

It should also be noted that the effects of 7.5% CO_2 inhalation were similar in magnitude for the placebo groups across both studies (see Appendix C, Table 44). This further emphasises the robust effects of 7.5% CO_2 inhalation at provoking symptoms of

subjective anxiety, elevated autonomic arousal and poorer antisaccade performance with no obvious effect on alerting, orienting or executive attention network function.

Memantine appears to have had a stronger effect on attention with nearly 20% less errors made than the placebo group (d_s = 0.84, reflecting a large effect size), whereas CO_2-induced increases in antisaccade errors in the placebo group were absent in the duloxetine group. Since memantine is usually prescribed to slow the deterioration of cognitive function in patients with Alzheimer's disease, this is perhaps less surprising. In light of these findings, memantine warrants further examination in the treatment of psychiatric disorders characterised by impaired cognition and attention, such as GAD, with particular consideration paid to its ability to maintain or enhance attentional control. This would extend research that already suggests memantine as a potentially useful adjunct in the treatment of severe OCD to aid control of compulsive behaviour.

Since neither drug group exceeded the antisaccade performance of placebo during air inhalation, it appears that duloxetine has a protective effect on attentional control during anxiety and memantine enhances attention more globally (including during an anxious state). This is a novel finding which extends research supporting immediate changes in attention and emotion processing after single or subchronic administration of an antidepressant in healthy volunteers, where no anxiety manipulation was used (Bamford et al., 2015; Harmer et al., 2009a). This may reflect differences in methodology and outcome measures, where previous research has largely focused on drug induced changes in emotional memory and the processing of facial expressions rather than attentional control specifically. Further research may want to consider the effects of a single dose of an antidepressant on CO_2-induced anxiety to gain a clearer picture of the time course of attentional effects within a temporary anxious state.

The broader effects of memantine on attentional control than duloxetine (which was revealed during a period of heightened anxiety only) may be explained by the substantial differences in their mechanisms of action. Duloxetine is an SNRI that is a potent inhibitor of both serotonin and noradrenaline that has known anti-anxiety effects, whereas memantine is an antagonist of NMDA receptors which blocks the activity of glutamate and may be more involved in the modulation of learning processes. Despite

these differences, both drugs (memantine in particular) have direct and downstream effects on many neurotransmitter targets. For example, whilst memantine is best known for its effects on glutamate, it also has many CNS targets which include the modulation of serotonin (5-HT3 receptors in particular; Rammes, Rupprecht, Ferrari, Zieglgansberger, & Parsons, 2001) and dopamine (Seeman, Caruso, & Lasaga, 2008; Spanagel, Eilbacher, & Wilke, 1994) uptake. In addition, although duloxetine lacks significant affinity for many other receptor sites, it has been shown to indirectly increase dopamine neurotransmission in the frontal cortex of rodents by preventing the function of the noradrenaline transporter in the reuptake of noradrenaline and dually, extracellular dopamine (Kihara & Ikeda, 1995; Tanda, Carboni, Fran, & Di Chiara, 1994). In light of this, the present findings on attention by duloxetine and memantine may reflect complex and varied interactions between neurotransmitter targets, some of which may be shared features of the two drugs.

5.7 Question 3: Why are there no clear effects of duloxetine or memantine on CO_2-induced subjective anxiety?

The lack of a clear effect of duloxetine and memantine on CO_2-induced anxiety follows previous research where CO_2 assessments with similar antidepressants (such as venlafaxine) have failed to produce consistent reductions in experimentally induced anxiety. There are several possible explanations for this apparent insensitivity of CO_2 to drugs of this kind.

Firstly, CO_2 may not elicit anxiety that is comparable to clinical disorders. Whilst the neural areas involved in anxiety (largely focus on the amygdala and associated structures) are well described, how these regions relate to specific disorders, which transmitter systems are disrupted and where this occurs, has not been clarified. Research in animals suggests the involvement of ASIC1a located in the amygdala as direct sensors of increasing CO_2 that stimulate defensive behaviours. However, the picture in humans is much less clear. Patients with extensive bilateral amygdala damage display an intact fear response to 35% CO_2 inhalation (Feinstein et al., 2013), and this, coupled with evidence for slower, smaller startle magnitudes (Study 1; Ceunen et al., 2013; Pappens et al., 2012) points to the importance of structures beyond the amygdala in the human response to CO_2.

One potential structure of interest is the bed nucleus of the stria terminalis (BNST). Whilst this forebrain structure receives direct input from the amygdala, it also projects to regions involved in autonomic and behavioural aspects of anxiety, such as the lateral hypothalamus (involved in respiratory control; Song et al., 2012) and the noradrenergic neurons of the locus coeruleus (suggested to be involved in CO_2-induced anxiety; Bailey et al., 2003). The BNST has also been implicated in sustained fear/anxiety responses, as measured with tasks such as light/dark potentiated startle or situations of unpredictability, such as threat of shock (see Davis et al., 2010 for a review). Recent research in rodents has identified distinct subregions of the BNST which have opposite effects on anxiety. The activation of the oval BNST is anxiogenic, promoting fear behaviour (reducing time in open-field and exploration of open arms of elevated-plus maze) and increasing respiration rate, whilst stimulation of the anterodorsal BNST is anxiolytic, reducing avoidance of open spaces (Kim et al., 2013). Since previous research has shown that the BNST is more important in the generation of sustained, rather than acute fear (despite interconnections with the amygdala), and that this structure expresses ASIC1a, the role of the BNST in CO_2-induced anxiety has received recent attention (Taugher et al., 2014). New research has revealed that ASIC1a in the BNST can directly detect acidosis arising from CO_2 inhalation (which lowers pH), that in turn evokes defensive behaviours in mice such as freezing. This is the first direct evidence to show that structures beyond the amygdala can detect changing concentrations of CO_2 and helps to explain inconsistencies between preclinical research and human lesion studies.

In addition, recent evidence shows that genetic variation in the amiloride-sensitive cation channel 2 (ACCN2) gene - the human ortholog of the ASIC1a gene - is associated with panic disorder, particularly when the disorder is early in onset and where respiratory symptoms are more prominent (Smoller et al., 2014). The same variation in ACCN2 was additionally related to increased amygdala reactivity to emotional faces and amygdala volume. Smoller et al. (2014) suggested that this genetic variation in the ACCN2 gene may increase risk of PD by lowering the threshold for acidosis needed to stimulate the amygdala/associated structures – and thus increase the ease of which to generate an anxiety response. This indicates that overexpression of the ASIC1a/ACCN2 gene in the fear circuit might predispose some individuals to be overly

reactive to CO_2. Thus it may be prudent to examine antagonists of ASIC1a within the CO_2 model as these may demonstrate therapeutic potential.

It is possible that the lack of a clear effect on mood by duloxetine within the CO_2 model might be a true reflection of the variable response rate to antidepressant drug treatment in anxious patients. Similar drug assessments within the CO_2 model have yielded varied results, with traditional benzodiazepines producing strong and replicable reductions in CO_2-induced anxiety (see Bailey & Nutt, 2008 for a review), whilst modern SSRI and SNRI's produce smaller to negligible effects (Bailey et al., 2007a; Diaper et al., 2013). SSRI and SNRI antidepressants are first-line for the pharmacological treatment of anxiety disorders, although response rates vary markedly. This is due to many factors such as individual patient characteristics (comorbidity for example), duration of treatment, severity of anxiety, presence of side effects, and specific treatment choice. As a result, it is difficult to predict which drugs will be most effective for each patient. Whilst many possible predictors of response have been suggested, currently a "best guess" strategy based on the clinical profile, secondary indicators of health and preference of the patient is used to decide on a treatment method, with switching between treatments a common occurrence.

It has been proposed that antidepressant treatments might first reverse emotion processing biases (specifically attention, memory and perception of emotionally salient information) prior to changes in subjective mood (Harmer & Cowen, 2013a). The cognitive neuropsychological model suggests that by interacting with the environment and relearning, changes in emotion processing can produce later improvements in clinical symptoms. This theory is supported by evidence for changes in emotion processing and memory after single dose administration of some SSRIs, SNRIs and benzodiazepines in healthy volunteers (Bamford et al., 2015; Harmer et al., 2011; Harmer et al., 2008; Pringle et al., 2013; Pringle et al., 2012). In Study 2, the ability to control attention was impaired by CO_2 to a greater extent in the placebo group than duloxetine group despite no effect on subjective anxiety. This finding aligns to some degree with the cognitive neuropsychological theory, although comment cannot be passed on the immediacy of attentional effects due to the two week drug administration period used. This dosing schedule was chosen to ensure that any impact of side effects was reduced (particularly considering the unpleasantness of CO_2 inhalation), and allowed staggered titration to a clinically relevant dose in both studies. This was

important to cleanly assess potential drug effects on CO_2-induced anxiety, with changes in attention posing an additional avenue of interest. In a healthy sample, substantial cognitive biases are not anticipated. If this theory is to be believed, day-to-day relearning is unlikely to occur for the majority of healthy subjects which may not produce a change in subjective mood. Therefore assessment of clinical populations or high trait anxious groups may instead reveal whether improvements in attentional control during CO_2 also translate to improved mood when a longer dosing period is used (so as to allow time for biases to be modified by treatment). In addition, whether single or acute administration of a drug in healthy volunteers can alter the attentional biases temporarily induced by this model would be an area worth exploring, especially with regard to the changes seen in healthy populations during situations of low stress (e.g. Harmer et al., 2011; 2008).

5.8 Question 4: Taken together, do these studies lend further support to 7.5% CO_2 inhalation as a model of generalised anxiety disorder?

Finally, one of the key aims of this thesis was to assess whether the 7.5% CO_2 model has particular specificity to GAD. Whilst Chapter 1 revealed that there is a wealth of evidence available to support 7.5% CO_2 inhalation as a valid model of anxiety, it also identified a lack of research examining whether CO_2 can activate the neural regions implicated in anxiety and provoke intrusive and uncontrollable worry – a key characteristic of GAD.

Study 1 considered whether CO_2 inhalation could produce changes in startle eye-blink magnitude which might suggest amygdala activation. The imaging research on neural pathways and behavioural assessments of startle magnitude in patients with GAD is both lacking and inconclusive (Etkin, Prater, Schatzberg, Menon, & Greicius, 2009). Increased activity of the bilateral dorsal amygdala in anticipation of aversive and neutral images has been shown to differentiate GAD patients from controls (Nitschke et al., 2009), yet attenuated amygdala activation to fearful faces (relative to neutral faces) has also been reported (Blair et al., 2008). Deficits in amygdala activation in GAD have been suggested to only emerge when associated deficits are present in frontal areas that are often implicated in regulatory processes (Bishop, 2007; Blair et al., 2008). Consistent with this, Monk et al. (2008) identified increased amygdala activity in adolescents with GAD that was inversely associated with prefrontal activity in response

to threat. Thus, in light of the core cognitive component of worry in GAD, this disorder may be better characterised by abnormalities in prefrontal regions; a theory which aligns with the neural pathways thought to underlie rumination and attentional control (Paulesu et al., 2010).

Study 2 also considered the validity of CO_2 inhalation as a model of GAD with particular emphasis on the cognitive symptoms of the disorder. It was hypothesised that duloxetine - an effective treatment of GAD - would reduce CO_2-induced anxiety across a broad spectrum of symptoms. This theory received some support, since duloxetine lessened the negative impact of CO_2 on attentional control. However as previously discussed, duloxetine administration did not prevent CO_2-induced increases in subjective anxiety, despite using a scale specific to the symptoms of GAD (the GAD-7) as an outcome measure. It is unclear whether this indicates that the model is unable to identify subjective effect of drugs used to treat generalised anxiety; whether CO_2 does not produce anxiety via the same mechanisms as pathological anxiety; or whether the choice of measurement scale was too specific to detect small changes in mood. However, Study 2 did reveal that CO_2 inhalation could exacerbate the frequency of negative intrusive thoughts to a similar extent as a period of instructed worry which were attenuated by duloxetine (albeit in the air first condition only), although to a small degree. As such, a marginal reduction in negative intrusions may not translate well to measures of subjective mood where it might be more difficult to quantify actual change in worry.

Based on the findings in this thesis and previous research, 7.5% CO_2 inhalation produces a wide array of anxiety symptoms some of which characterise GAD. Research examining the neurobiology that underlies GAD is lacking. A better understanding of the structures and neurotransmitter pathways associated with GAD would help to clarify whether low-dose CO_2 inhalation utilises similar pathways, and would reveal if the findings from Study 1 are consistent with a GAD profile, or a unique feature of CO_2-induced anxiety. In addition, further insight into why drugs known to reduce subjective symptoms of clinical anxiety do not produce reductions in experimentally induced anxiety is required.

5.9 Limitations, conclusions and future directions

In all three studies, occasions arose where some participants were unable to differentiate between CO_2 and air trials. Variations in individual responses to 7.5% CO_2 may be due to differences in trait anxiety and anxiety sensitivity, with a recent study identifying a positive association between initial anxiety proneness and subjective and physiological responses to 7.5% CO_2 and air inhalation (Fluharty, Attwood & Munafo, 2016). Understanding these variations in response to CO_2 through the examination of "non-responders", "partial responders" and "extreme responders" would be valuable to identify the causal factors that underlie CO_2 sensitivity and may indicate ways to predict susceptibility to pathological anxiety in the future.

A particular advantage of the 7.5% CO_2 model is the ability to make direct comparisons between a high anxiety (7.5% CO_2) and low anxiety (air) condition, which are identical besides the gas inhaled. This is superior to other healthy human models of anxiety, such as the Trier Social Stress Test, which either have a control condition that is quite distinct from the anxiety manipulation or no clear control condition at all. However, it should be acknowledged that the air inhalation is not perfect, with greater mean state anxiety reported during air than at baseline, and differences in response due to the order that the inhalations are received (seen in study 2). This could be due to a number of possibilities such as individual differences in anxiety sensitivity, trait anxiety and/or depression or may reflect variations in the experimental condition such as increased anticipatory or mask anxiety for the first inhalation, and researcher bias.

A recent study examining individual differences in trait anxiety and anxiety sensitivity on responses to 7.5% CO_2 inhalation revealed a stronger relationship between anxiety proneness and subjective responses during air inhalation than CO_2 (Fluharty, Attwood & Munafo, 2016). It was suggested that whilst high trait anxious and anxiety sensitive individuals may experience a greater subjective response to CO_2, the difference between those low and high in anxiety proneness may be more similar during CO_2 than air. Thus, a smaller difference between the two conditions in anxiety prone individuals makes it more difficult to discern a clear 7.5% CO_2 response (from air) in highly anxiety-prone samples and may mask potentially important effects when testing pharmacological agents. It is also plausible that variations in stable aspects of anxiety, such as anxiety sensitivity may impact on cognitive performance during CO_2,

as per the strong relationship between trait anxiety and attentional bias to threat (e.g. Broadbent & Broadbent, 1988; Koster, Crombez, Verschuere, Van Damme, & Wiersema, 2006; Bar-Haim et al., 2007 for review). With regards to these findings, future research should consider anxiety sensitivity and trait anxiety when recruiting volunteers or interpreting results. High trait anxiety and anxiety sensitivity could feature as additional exclusion criteria, or at the very least should be examined at baseline to ensure groups are balanced and samples are well-characterised.

In addition, the order in which gases are delivered should be considered in future research. Despite counterbalancing by order, order effects were identified on cognitive measures in study 2 (frequency of negative thoughts), and physiological indices (particularly heart rate) in studies 2 and 3. This may be indicative of participants becoming more at ease with the testing environment over time, or reflect variations in anticipatory anxiety prior to the initial inhalation. Some previous research has used a fixed order with participants receiving air first (e.g. Diaper et al., 2012b). This aims to ensure that any anticipatory anxiety experienced during the first inhalation always occurs during air, and not CO_2. Whilst this makes it more difficult to find an effect between air (plus anticipatory anxiety) and CO_2, when an anxiety response post-CO_2 (vs. air) is revealed, it is believed that this is more likely to be the result of the gas rather than anticipation – although this hypothesis has not been formally examined. Researchers should weigh up the pros and cons of using a fixed, versus counterbalanced order. Studies which examine the interaction between individual differences in trait anxiety and anxiety sensitivity on order are warranted, and would provide useful insight to inform future methodological decisions when using the 7.5% CO_2 model.

Inconsistencies between the findings from the antisaccade task and the ANT were revealed in both Studies 2 and 3. It was hypothesised that if increased error rates and speeded latencies were observed in the antisaccade task, then these would co-occur with deficiencies in attention network function such as hypervigilance and facilitated orienting (alerting and orienting networks) and distractor interference (executive control). This hypothesis was not supported by either study. Study Two in particular demonstrated some interpretative difficulties with ANT bias scores, whereby initial examination of the difference scores suggested 7.5% CO_2 inhalation might promote, rather than impair executive attention network function; a finding that was subsequently refuted on examination of the raw reaction times. This issue stems from the use of

difference scores to profile attention network function, which can be incorrectly considered to demonstrate improved/impaired network function when considered in the absence of reaction times for each cue. Meaningful assessment of difference scores is however a necessity with this task, since no one trial is an isolated or direct measure of a specific network. As a result, direct measures of an attention network (such as antisaccade error rates and latencies for attentional/executive control) might provide a superior assessment with which to examine specific aspects of attention. In future, it is therefore recommended that mean reaction times should be examined alongside difference scores when analysing the ANT in order to avoid costly interpretative errors, and/or consideration should be given to comparable tasks that might be more suitable to examine the hypothesis in question.

The final samples in studies 2 and 3 that were used to examine the primary effect of drug on CO_2-induced attentional deficits were relatively small (Study 2 $N_{total} = 35$ ($N_{placebo} = 19$, $N_{duloxetine} = 18$), Study 3 $N_{total} = 28$ ($N_{placebo} = 14$, $N_{memantine} = 14$)). Whilst similar research has used samples of a comparable size (e.g. $N_{placebo} = 18$, $N_{drug} = 18$; Diaper et al., 2013), to date no studies have searched for a possible interaction between drug and attention during CO_2-induced anxiety. Since the effect size for threat-related attention biases in anxiety is expected to be reasonably small (approximated at $d = 0.45$ in a large meta-analysis; Bar-Haim et al., 2007), the sample required to achieve adequate power (minimum acceptable level is often considered to be 80%) and detect the presence of a small interaction effect may exceed that of the present studies. By being underpowered, the conclusions that can be drawn from Studies 2 and 3 are somewhat limited. Since low power reduces the chance that an observed effect is a true effect (low positive predictive value), overinflates the magnitude of a true effect when one is detected, and increases the likelihood of false negatives (see Button et al., 2013), the results of the present studies should be interpreted with this in mind. Any replication or extension of this work should consider the impact this may have on determining sample size, as well as the expected magnitude of the effect of interest.

Future research should therefore aim to be better powered. Studies with comparable mixed methods designs that examine drug effects on attention should be used to guide power analyses and to estimate appropriate sample sizes for the research question. This will help improve the chance of detecting a true effect when one exists (reducing the likelihood of type 2 error), whilst decreasing the risk of accepting a

significant result as meaningful when it is not (type 1 error; Button et al., 2013). Power analyses should also be conducted early in the design of the research using established methods such as the statistical package G*Power (Faul, Erdfelder, Lang & Buchner, 2007). This will require a prior consideration of the statistical methodology needed to appropriately address the hypothesis, and provides greater confidence in the observed results.

It must also be acknowledged that the effect of duloxetine on antisaccade performance fell short of statistical significance ($p = .075$). This could be due to a variety of reasons including; an insensitivity of 7.5% CO_2 inhalation to accurately model pathological anxiety; weaker efficacy of more modern treatments on an enduring rather than intense episode of anxiety; and/or insufficient power and sample size. However, the fact that drug effects on attention (specific to CO_2 in study 2, and more globally in study 3) were revealed in both drug studies suggests some confidence in these findings.

At a time where the search for new mental health drug treatments is slowing, ways with which to reinvigorate this pursuit are incredibly important, especially if they enable greater accuracy in predicting those interventions which will be effective, and those which will not. This thesis provides a timely examination of a promising, translational model of anxiety (if not GAD) in a healthy population, which can bridge preclinical and clinical trials and provide researchers an opportunity to evaluate new drugs in a valid and cost-effective way.

The main findings from this thesis are that (1) 7.5% CO_2 inhalation in humans can robustly produce symptoms of anxiety including detrimental effects on the ability to control attention, (2) duloxetine and memantine can prevent some of the anxiety-related attentional deficits produced by CO_2 inhalation, whilst having weaker/no effect on mood, and (3) the way in which CO_2 is likely to mimic anxiety occurs via structural sites beyond, but not necessarily excluding the amygdala. As such, this thesis provides evidence that the CO_2 model is a useful tool with which to examine both the mechanisms of anxiety and the effectiveness of new pharmacological treatments. It is hoped that this model will be instrumental in future to provide better treatment outcomes for this common and debilitating condition.

Appendix A (Study Two)

Figure 11. Example of modified GAD-7 questionnaire for CO_2 session.

During the last **20 minutes** how often have you been bothered by the following problems? Rate each word by drawing a **vertical line** on the scale below to indicate the extent you feel this way.

FEELING NERVOUS, ANXIOUS OR ON EDGE

Not at all Some of the time Most of the time All of the time

……

NOT BEING ABLE TO STOP OR CONTROL WORRYING

Not at all Some of the time Most of the time All of the time

……

WORRYING TOO MUCH ABOUT DIFFERENT THINGS

Not at all Some of the time Most of the time All of the time

……

TROUBLE RELAXING

Not at all Some of the time Most of the time All of the time

……

BEING SO RESTLESS THAT IT IS HARD TO SIT STILL

Not at all Some of the time Most of the time All of the time

……

BECOMING EASILY ANNOYED OR IRRITABLE

Not at all Some of the time Most of the time All of the time

……

FEELING AFRAID AS IF SOMETHING AWFUL MIGHT HAPPEN

Not at all Some of the time Most of the time All of the time

……

Appendix A (Study Two)

Table 32. Means (SD) and main effects of duloxetine on mood and autonomic arousal prior to 7.5% CO_2 challenge

	Placebo (*N* = 19)				Duloxetine (*N* = 18)				*Main effect of drug*
	Day 0		Day 14		Day 0		Day 14		
GAD-7	9.57	(5.06)	11.33	(6.29)	9.26	(6.35)	12.20	(6.81)	$F(1, 35) = 0.03$, $p = .858$ *ns*
PANAS positive	35.22	(4.18)	33.56	(4.37)	35.53	(5.32)	33.71	(5.64)	$F(1, 33) = 0.02$, $p = .882$ *ns*
PANAS negative	13.37	(2.50)	14.11	(3.59)	12.94	(2.77)	12.41	(1.87)	$F(1, 34) = 1.61$, $p = .213$ *ns*
Heart rate	68.84	(11.16)	70.26	(10.90)	74.39	(10.04)	73.17	(8.78)	$F(1, 35) = 1.92$, $p = .175$ *ns*
Systolic BP	125.05	(8.59)	119.16	(10.54)	124.17	(8.18)	124.06	(9.64)	$F(1, 35) = 0.54$, $p = .467$ *ns*
Diastolic BP	69.47	(9.16)	66.16	(7.03)	71.72	(9.87)	72.33	(6.55)	$F(1, 35) = 2.99$, $p = .092$ *ns*

Table 33. Means (*SD*s) and time*drug group interactions assessing the effect of duloxetine on 7.5% CO_2-induced anxiety and autonomic arousal.

	Baseline		Air		7.5% CO_2		ANOVA (time)		
	Placebo	Duloxetine	Placebo	Duloxetine	Placebo	Duloxetine	F	p	η_p^2
GAD-7	9.00 (8.08)	10.86 (9.59)	11.55 (9.46)	11.04 (8.23)	37.85 (22.07)	31.22 (20.92)	1.46	= .240, *ns*	.042
Positive affect	32.68 (5.51)	33.06 (7.77)	27.68 (6.47)	26.78 (9.18)	23.47 (8.55)	23.28 (9.31)	0.13	= .877, *ns*	.004
Negative affect	12.32 (2.77)	11.72 (2.70)	11.42 (2.34)	11.00 (2.09)	20.84 (9.44)	18.11 (6.99)	0.77	= .469, *ns*	.023
Systolic BP	117.47 (7.11)	121.78 (8.50)	116.89 (7.78)	122.06 (10.31)	129.42 (13.23)	134.00 (12.44)	0.03	= .975, *ns*	.001
Diastolic BP	69.32 (6.20)	76.78 (5.42)	71.37 (6.38)	77.22 (7.95)	72.53 (8.86)	78.94 (5.59)	0.25	= .779, *ns*	.008
Continuous HR	-	-	67.31 (12.28)	74.57 (9.39)	75.83 (11.24)	83.11 (8.58)	0.04	= .853, *ns*	.001
Respiration (breaths/minute)	-	-	16.07 (2.86)	16.52 (3.75)	19.07 (3.43)	20.18 (3.95)	0.18	= .676, *ns*	.005

Appendix A (Study Two)

Table 34. Means (SDs) of attention network function (RTs) as measured by the ANT during 7.5% CO_2 and air inhalations, by drug group (placebo vs. duloxetine)

Networks	Placebo (N =19)				Duloxetine (N = 18)			
	Air		CO_2		Air		CO_2	
Alerting	23.36	(24.90)	24.46	(25.74)	25.68	(25.96)	27.67	(37.06)
Orienting	35.88	(25.34)	32.80	(22.84)	39.98	(29.20)	33.16	(21.01)
Executive control	68.89	(22.87)	66.16	(27.87)	82.30	(27.69)	71.90	(32.00)

Table 35. Means (SDs) of attention network function (reaction times) as measured with the ANT during CO_2 and air by drug group, by order

Order	Attentional network	Placebo (N =19)				Duloxetine (N = 18)			
		Air		CO_2		Air		CO_2	
Air first (N = 18)	Alerting	16.23	(31.84)	30.02	(23.59)	15.03	(27.59)	32.15	(33.39)
	Orienting	28.68	(20.09)	26.38	(27.63)	41.31	(27.70)	33.73	(21.23)
	Executive control	75.50	(25.27)	66.75	(30.07)	87.20	(25.51)	66.24	(20.94)
CO_2 first (N = 19)	Alerting	29.78	(15.38)	19.46	(27.79)	36.33	(24.10)	23.19	(41.94)
	Orienting	34.96	(22.62)	38.58	(16.93)	38.66	(32.25)	32.59	(22.05)
	Executive control	62.94	(19.90)	65.63	(27.36)	69.79	(25.77)	77.56	(40.82)

Table 36. Mean RTs (SDs) to the four cue types and two flanker types on the Attention Network Test during 7.5% CO_2 and air for placebo and duloxetine, by order (air first vs. CO_2 first)

Order	Trial	Placebo (N =19)				Duloxetine (N = 18)			
		Air		CO_2		Air		CO_2	
Air First (N = 18)	No cue	618.28	(56.81)	655.86	(66.65)	628.96	(108.16)	639.75	(84.04)
	Double cue	602.04	(70.47)	625.84	(74.80)	613.93	(115.39)	607.60	(86.62)
	Centre cue	605.23	(80.24)	630.73	(70.07)	619.41	(109.30)	599.58	(83.35)
	Spatial cue	576.54	(77.30)	604.35	(61.66)	578.10	(93.96)	565.84	(91.10)
	Congruent	562.77	(63.15)	595.82	(62.39)	566.50	(104.71)	570.07	(85.14)
	Incongruent	638.27	(77.52)	662.57	(73.34)	653.70	(108.14)	636.31	(86.06)
CO_2 First (N = 19)	No cue	599.90	(71.10)	605.64	(66.65)	631.14	(59.49)	657.90	(66.84)
	Double cue	570.12	(68.61)	586.17	(48.27)	594.81	(60.63)	634.71	(72.43)
	Centre cue	580.24	(72.20)	588.60	(58.35)	606.88	(57.19)	635.20	(76.10)
	Spatial cue	545.28	(69.91)	550.01	(52.02)	568.22	(79.83)	602.61	(77.07)
	Congruent	542.41	(72.95)	549.79	(61.12)	561.56	(60.57)	593.83	(67.08)
	Incongruent	605.36	(67.78)	615.42	(52.15)	638.96	(68.17)	671.38	(79.78)

Appendix A (Study Two)

Table 37. The proportion of errors (standard deviation) made on antisaccade and prosaccade trials during CO_2 and air inhalation by drug group (placebo vs. duloxetine) and by order

Order	Trial	Placebo (N =17)				Duloxetine (N = 18)			
		Air		CO_2		Air		CO_2	
Air First (N = 16)	Prosaccade	0.06	(0.05)	0.04	(0.03)	0.02	(0.02)	0.06	(0.04)
	Neutral	0.06	(0.06)	0.03	(0.03)	0.01	(0.02)	0.06	(0.05)
	Negative	0.06	(0.05)	0.04	(0.04)	0.02	(0.03)	0.05	(0.06)
	Antisaccade	0.38	(0.24)	0.51	(0.37)	0.43	(0.25)	0.44	(0.28)
	Neutral	0.38	(0.23)	0.51	(0.39)	0.43	(0.25)	0.43	(0.28)
	Negative	0.39	(0.27)	0.50	(0.34)	0.44	(0.25)	0.44	(0.28)
CO_2 First (N = 19)	Prosaccade	0.04	(0.03)	0.09	(0.15)	0.04	(0.04)	0.06	(0.07)
	Neutral	0.04	(0.03)	0.09	(0.13)	0.04	(0.05)	0.07	(0.08)
	Negative	0.05	(0.03)	0.09	(0.18)	0.05	(0.05)	0.06	(0.08)
	Antisaccade	0.32	(0.20)	0.47	(0.18)	0.31	(0.17)	0.43	(0.13)
	Neutral	0.32	(0.21)	0.49	(0.19)	0.30	(0.17)	0.44	(0.16)
	Negative	0.33	(0.20)	0.46	(0.18)	0.33	(0.18)	0.42	(0.16)

Table 38. Latencies (standard deviation) on antisaccade and prosaccade trials during CO_2 and air inhalation by drug group and by order (ms)

Order	Trial	Placebo (N =17)				Duloxetine (N = 18)			
		Air		CO_2		Air		CO_2	
Air First (N = 16)	Prosaccade	192.43	(46.13)	189.83	(47.72)	176.26	(24.49)	186.96	(36.71)
	Neutral	200.37	(55.61)	188.48	(47.28)	171.89	(24.83)	190.91	(42.53)
	Negative	184.48	(38.82)	191.18	(48.84)	180.62	(26.32)	183.02	(34.23)
	Antisaccade	188.20	(53.68)	187.94	(68.54)	183.74	(44.34)	206.82	(67.78)
	Neutral	201.95	(71.35)	211.63	(114.91)	184.18	(47.08)	203.61	(77.37)
	Negative	174.44	(40.80)	164.25	(26.67)	183.30	(42.00)	210.03	(59.96)
CO_2 First (N = 19)	Prosaccade	208.69	(24.66)	188.14	(23.27)	212.01	(46.94)	192.48	(38.13)
	Neutral	212.48	(28.02)	189.26	(24.56)	221.71	(45.18)	193.36	(41.49)
	Negative	206.31	(25.12)	177.73	(17.51)	202.31	(50.77)	191.59	(35.48)
	Antisaccade	191.93	(18.29)	180.41	(22.37)	233.83	(63.67)	203.13	(36.01)
	Neutral	203.11	(27.21)	168.98	(16.49)	238.14	(80.78)	209.39	(41.14)
	Negative	182.73	(27.10)	186.42	(35.24)	229.53	(53.28)	196.87	(42.70)

Appendix B (Study Three)

Table 39. Means (SD) and main effect of memantine on mood and autonomic arousal prior to 7.5% CO_2 challenge

	Placebo (N = 17)				Memantine (N = 16)				*Main effect of drug*
	Day 0		Day 14		Day 0		Day 14		
GAD-7	9.57	(5.06)	11.33	(6.29)	9.26	(6.35)	12.20	(6.81)	$F(1, 31) = 0.04$, $p = .849$, *ns*
PANAS positive	35.22	(4.18)	33.56	(4.37)	35.53	(5.32)	33.71	(5.64)	$F(1, 30) = 0.00$, $p = .994$, *ns*
PANAS negative	13.37	(2.50)	14.11	(3.59)	12.94	(2.77)	12.41	(1.87)	$F(1, 31) = 0.15$, $p = .700$, *ns*
Heart rate	68.84	(11.16)	70.26	(10.90)	74.39	(10.04)	73.17	(8.78)	$F(1, 31) = 1.19$, $p = .284$, *ns*
Systolic BP	125.05	(8.59)	119.16	(10.54)	124.17	(8.18)	124.06	(9.64)	$F(1, 31) = 0.04$, $p = .836$, *ns*
Diastolic BP	69.47	(9.16)	66.16	(7.03)	71.72	(9.87)	72.33	(6.55)	$F(1, 31) = 0.08$, $p = .779$, *ns*

Table 40. Means (SD) and time*drug group interactions assessing the effect of memantine on 7.5% CO_2-induced anxiety and autonomic arousal.

	Baseline		Air		7.5% CO_2		ANOVA (time*drug group)		
	Placebo	Memantine	Placebo	Memantine	Placebo	Memantine	F	p	η_p^2
GAD-7	8.37 (5.14)	9.62 (10.94)	9.27 (10.94)	12.24 (11.04)	33.08 (17.32)	28.60 (16.28)	1.40	= .255, *ns*	.051
Positive affect	34.88 (5.45)	32.43 (5.87)	27.75 (10.01)	26.57 (7.28)	25.69 (9.52)	20.71 (6.04)	1.00	= .373, *ns*	.037
Negative affect	11.25 (1.61)	11.79 (2.04)	11.75 (2.86)	11.71 (2.23)	18.13 (7.85)	18.07 (7.87)	0.04	= .961, *ns*	.002
Systolic BP	117.13 (13.26)	113.14 (10.68)	112.60 (9.72)	114.71 (10.61)	120.07 (13.08)	122.29 (10.26)	2.39	= .102, *ns*	.087
Diastolic BP	73.47^a (6.51)	69.14 (9.45)	66.27^b (8.65)	69.64 (6.02)	73.67^a (11.99)	69.64 (6.87)	3.29	< .046	.116
Continuous HR	-	-	76.51 (8.86)	72.18 (8.25)	81.38 (9.87)	79.40 (10.03)	1.36	= .254, *ns*	.050
Respiration	-	-	17.22 (4.11)	15.27 (3.36)	21.34 (5.51)	18.63 (2.87)	0.27	=.607, *ns*	.010

Note. Pairwise comparisons with Bonferroni correction applied ($p < .017$). Values with different superscripts were significantly different from each other.

Appendix B (Study Three)

Table 41 The proportion of errors (standard deviation) made on antisaccade and prosaccade trials during CO_2 and air inhalation by drug group (placebo vs. memantine) and by order (air first vs. CO_2 first).

Order	Trial	Placebo (N =14)				Memantine (N = 14)			
		Air		CO_2		Air		CO_2	
Air First	Prosaccade	0.04	(0.06)	0.06	(0.09)	0.09	(0.18)	0.12	(0.17)
	Neutral	0.03	(0.06)	0.06	(0.11)	0.11	(0.23)	0.13	(0.21)
	Negative	0.06	(0.07)	0.06	(0.07)	0.07	(0.14)	0.11	(0.15)
	Antisaccade	0.53	(0.27)	0.53	(0.21)	0.31	(0.22)	0.37	(0.18)
	Neutral	0.57	(0.29)	0.54	(0.26)	0.29	(0.25)	0.35	(0.27)
	Negative	0.49	(0.25)	0.53	(0.21)	0.33	(0.21)	0.38	(0.13)
CO_2 First	Prosaccade	0.05	(0.03)	0.07	(0.03)	0.05	(0.04)	0.08	(0.13)
	Neutral	0.05	(0.02)	0.08	(0.04)	0.06	(0.05)	0.10	(0.17)
	Negative	0.04	(0.05)	0.05	(0.04)	0.04	(0.04)	0.07	(0.10)
	Antisaccade	0.40	(0.28)	0.62	(0.29)	0.27	(0.25)	0.41	(0.21)
	Neutral	0.40	(0.27)	0.60	(0.28)	0.29	(0.26)	0.41	(0.24)
	Negative	0.39	(0.29)	0.64	(0.32)	0.26	(0.26)	0.41	(0.20)

Table 42. Latencies (standard deviation) on antisaccade and prosaccade trials during CO_2 and air inhalation by drug group and by order (ms)

Order	Trial	Placebo (N =13)				Memantine (N = 13)			
		Air		CO_2		Air		CO_2	
Air First	Prosaccade	191.81	(40.69)	198.68	(28.78)	188.62	(21.03)	184.43	(7.53)
	Neutral	192.27	(42.36)	201.18	(33.37)	185.22	(21.09)	182.99	(8.15)
	Negative	191.34	(39.20)	196.18	(26.57)	192.01	(30.34)	185.87	(18.42)
	Antisaccade	196.04	(47.84)	187.79	(20.72)	177.15	(14.69)	184.46	(35.85)
	Neutral	198.89	(47.12)	198.44	(37.16)	175.22	(15.24)	175.84	(24.65)
	Negative	193.19	(48.88)	177.14	(15.30)	179.08	(17.41)	193.08	(62.17)
CO_2 First	Prosaccade	189.36	(26.47)	167.08	(16.17)	211.85	(48.63)	212.79	(68.30)
	Neutral	192.62	(29.82)	166.85	(16.31)	210.35	(49.89)	209.86	(64.79)
	Negative	186.09	(29.50)	167.31	(20.22)	213.35	(48.15)	215.72	(74.78)
	Antisaccade	190.54	(41.69)	167.56	(23.12)	223.81	(56.69)	201.93	(58.05)
	Neutral	184.40	(49.19)	170.20	(18.55)	232.37	(65.06)	210.68	(77.18)
	Negative	194.81	(43.37)	164.93	(28.78)	215.25	(61.05)	193.18	(63.28)

Appendix B (Study Three)

Subsequent analysis of the effect of memantine on the frequency of positive, negative and neutral intrusions in the thought intrusions task

A mixed model ANOVA was conducted to examine the effect of memantine on positive, negative and neutral thought intrusions before and after instructed worry. Time (pre vs. post worry) and valence (positive vs. negative vs. neutral) were within subjects factors and drug (placebo vs. memantine) was included as a between subjects factor.

Mixed model ANOVA did not reveal any significant main effects of drug, time or valence. However, a significant 3-way interaction was identified between time, valence and drug ($F(2, 62) = 3.27$, $p = .045$, $\eta_p^2 = .095$). To understand this interaction further, the data was split by drug group (placebo or memantine) and separate repeated measures ANOVA tests examining time (pre and post worry) and valence (positive, negative and neutral) were performed (Bonferroni correction results in a critical $p = .025$). For the placebo group, a trend for a time x valence interaction was revealed ($F(2, 32) = 3.66$, $p = .037$, $\eta_p^2 = .186$) although this was not statistically significant after corrections for multiple comparisons were applied. Pairwise comparisons suggest that the frequency of negative intrusive thoughts increased ($M_{diff} = 0.47$, $SE = 0.21$) after instructed worry for participants in the placebo group (unadjusted $p = .014$). This pattern was not seen for the memantine group as no significant main effects or interactions were revealed. Overall frequency of intrusive thoughts irrespective of valence does not appear to differ between groups (see Table 43).

Table 43. Mean number (*SD*) of positive, negative and neutral thought intrusions reported by the memantine and placebo group before and after instructed worry.

	Pre-worry		Post-worry	
	Placebo	Memantine	Placebo	Memantine
Neutral	0.82 (1.07)	0.94 (1.44)	0.65 (0.86)	0.50 (0.73)
Positive	1.12 (1.05)	0.50 (0.73)	0.88 (0.99)	1.06 (1.00)
Negative	0.53 (1.01)	0.81 (1.52)	1.00 (1.32)	0.75 (0.93)
Total	2.47 (1.46)	2.25 (2.52)	2.53 (1.70)	2.31 (1.45)

Appendix C (General Discussion)

Table 44. Means (SDs) and effect sizes (Cohen's d_{av}) for measures of subjective anxiety, autonomic arousal, and attention at the peak effects of 7.5% CO_2 (versus air) for the placebo groups in Study 2 and 3.

Peak effects	Study 2			Study 3		
	Air	CO_2	Effect	Air	CO_2	Effect
GAD-7	11.55 (9.46)	37.85 (22.07)	1.67	9.27 (10.94)	33.08 (17.32)	1.69
Positive affect	27.68 (6.47)	23.47 (8.55)	0.56	27.75 (10.01)	25.69 (9.52)	0.21
Negative affect	11.42 (2.34)	20.84 (9.44)	1.60	11.75 (2.86)	18.13 (7.85)	1.19
Systolic BP	116.89 (7.78)	129.42 (13.23)	1.19	112.60 (9.72)	120.07 (13.08)	0.66
Diastolic BP	71.37 (6.38)	72.53 (8.86)	0.15	66.27 (8.65)	73.67 (11.99)	0.72
Heart Rate	67.31 (12.28)	75.83 (11.24)	0.72	76.51 (8.86)	81.38 (9.87)	0.52
Respiration	16.07 (2.86)	19.07 (3.43)	0.95	17.22 (4.11)	21.34 (5.51)	0.86
Alerting	23.36 (24.90)	24.46 (25.74)	0.04	29.14 (38.37)	28.11 (32.87)	0.03
Orienting	35.88 (25.34)	32.80 (22.84)	0.13	35.38 (21.29)	30.56 (19.23)	0.24
Executive control	68.89 (22.87)	66.16 (27.87)	0.11	69.89 (29.53)	54.71 (38.40)	0.46
Antisaccade errors	0.35 (0.21)	0.49 (0.26)	0.60	0.46 (0.27)	0.57 (0.25)	0.42
Prosaccade errors	0.05 (0.04)	0.07 (0.12)	0.25	0.05 (0.05)	0.06 (0.06)	0.18

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