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Investigating the Antidepressant Mechanism of 1 mg/kg Ketamine in Rats

Investigating the Antidepressant Mechanism of 1 mg/kg Ketamine in Rats

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Abstract

Unlike conventional antidepressive treatments ketamine exerts antidepressant (AD) efficacy within hours of administration. This rapid AD action is caused by a distinct pharmacological mechanism to conventional ADs and has renewed hope in a field with a once stagnant research effort. However, this effect is accompanied by psychotomimetic side effects, thus prompting the search for similar more tolerable ADs. To this end, animal research using rodents has been applied to understand the basic science behind ketamine's antidepressant mechanism.

Ketamine's AD effect manifests at low subanaesthetic doses, normally applied as a 0.5 mg/kg infusion in humans. In rodent research, doses of 10 – 25 mg/kg are predominantly applied on non-translational behavioural assays. With emerging evidence of an AD effect in rats at 1 mg/kg, we argue that the 10 – 25 mg/kg dose range is not optimally suited to investigate ketamine's AD mechanism in rodents, as increased non-AD side effects will result.

This work aimed to explore the influence of 1 mg/kg ketamine in rats and differentiate this from the 10 – 25 mg/kg dose range commonly applied in the literature, as well as compare this to the effects of ketamine's non-AD structural analogue PCP. Here, neural activation was assessed with immunohistochemistry whilst the object in place task was used to examine differences in memory impairment, a known side effect of ketamine. From this, we found evidence of differentiation between 1 mg/kg and 10 – 25 mg/kg ketamine. A novel rat behaviour assay using flavour preference was also explored, which could be applied to future AD research efforts.

Overall, whilst further work with larger n numbers is required, we propose that ketamine's AD efficacy lies with an excitatory "sweet spot", whereby ketamine's AD action is facilitated by a subtle stimulatory influence on the prefrontal cortex.

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Finally, I would like to thank my family, Catherine Tait and Rosie Higgins, who support me in everything I do and have helped me become the person I am today.

I dedicate this work to the memory of my father.

Keep on shinning Dad.

Authors declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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Abbreviations

ABT	Affective bias task
AD	Antidepressant
aMCC	Anterior midcingulate cortex
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
BDNF	Brain derived neurotrophic factor
BPRS	Brief Psychiatric Rating scale
CEN	Central executive network
CG	Cingulate
dACC	Dorsal anterior cingulate cortex
DAPI	4′,6-diamidino-2-phenylindole
DMN	Default mode network
DMSO	Dimethyl sulfoxide
FDA	Food and drug administration
fMRI	Functional magnetic resonance imaging
FST	Forced swim test
GABA	Gamma-aminobutyric acid
GAD67	Glutamate decarboxylase
HDRS	Hamilton depression rating scale
HNK	Hydroxynorketamine
HPA	Hypothalamic-Pituitary adrenal axis
i.p	Intraperitoneal
i.v.	Intravenous
IL	Infralimbic
JBT	Judgement bias task
LTP	Long term potentiation
mACh	Muscarinic acetylcholine
MADRS	Montgomery–Åsberg Depression Rating Scale
MAOI	Monoamine oxidase inhibitors
MDD	Major depressive disorder
mPFC	Medial prefrontal cortex

MRI	Magnetic resonance imaging
Mt	Motor
mTOR	Mammalian target of rapamycin
N,N-DMT	N, N-dimethyltryptamine
NAc	Nucleus accumbens
NMDAR	N-methyl-D-aspartate receptor
OIP	Object in place
PBS-T	Phosphate buffered saline, 0.2% Triton X
PCP	Phencyclidine
PFA	Paraformaldehyde
PFC	Prefrontal cortex
pgACC	Perigenual anterior cingulate cortex
PL	Prelimbic regions
QIDS	Quick Inventory of Depressive Symptomatology
RO	Receptor occupancy
RSFC	Resting state functional connectivity
s.c.	Subcutaneous
SN	Salience network
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressants
VAS	Visual analogue scale
VDCC	Voltage gated calcium channels
VTA	Ventral tegmental area

Chapter 1

Introduction

1.1 Depression

Depression is a prevalent psychiatric illness characterised by depressed mood, psychomotor impairment, sleeping disturbances and cognitive symptoms including indecisiveness and an inability to concentrate (Lux and Kendler, 2010). The disease affects 16% of the world population (Kessler *et al.*, 2003), and with limited treatment options the disease poses a severe strain on society.

Difficulties in treating depression arise from two main reasons. Firstly, its manifestation is more variable than is suggested by the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) criteria (commonly used to diagnose the disease), as the symptoms experienced between depressed patients can vary greatly, leading to subtypes (Schneck, 2009) with possibly distinct causes. Secondly, depression is prone to co-occur with other conditions, such as anxiety (Hirschfeld, 2001). This makes the condition more challenging to diagnose. Distinct possible subtypes, along with a complex and unknown aetiology has made the search for new treatments difficult and mostly unsuccessful.

1.1.1 Aetiology of depression

For decades, researchers have attempted to characterise the depressed brain in order to understand the root of the disease. However, there is no single unifying theory behind depression's manifestation. To understand the disorders complex neurobiology, we have often relied on neuroimaging and post-mortem studies. These have provided some insight into the structures involved although the underlying cause has remained elusive. Furthermore, imaging data and post-mortem studies should be interpreted with caution as findings may result from the disease and not represent its cause.

The prefrontal cortex (PFC) and hippocampus are regions implicated in the disease's cognitive manifestation from observations of reduced volumes detected from magnetic resonance imaging (MRI) and post mortem studies (Sheline, 2003). Additionally, neuroimaging studies have consistently reported increased activity in the amygdala, a region critical for emotional response and memory, in depressed patients (Drevets, 2001). Considering its role in emotional perception it has been suggested that amygdala overactivity can lead to negative perception to emotionally standard stimuli (Drevets, 2001). Indeed, stimulation of the amygdala can evoke emotional responses (Gloor *et al.*, 1982). With reciprocal connections between the PFC and amygdala, it's proposed that the regulation of negative emotional responses from the PFC of the amygdala is implicated in depression. Supporting the role of these circuits in depression, there is evidence of PFC based attenuation of fear responses resulting from modulation of the amygdala (Drevets, 2001).

Reward and motivation are also impacted in depression. Regions responsible include the nucleus accumbens (NAc) and ventral tegmental area (VTA), forming part of the mesolimbic system. Serving as a principle reward centre, the mesolimbic systems dopaminergic afferent neurons extend from the VTA towards the medial prefrontal cortex (mPFC) and amygdala, targets which have been shown to have depleted grey matter and glial cells in the depressed brain (Drevets, 2001). These circuits primarily rely on dopaminergic signalling, which has been suggested to be altered by tricyclic antidepressants (TCA) and monoamine oxidase inhibitors (MAOI) resulting in their effectiveness against atypical depression (Dunlop and Nemeroff, 2007).

The midbrain/brainstem nuclei possess large numbers of monoaminergic neurons, that is, serotonergic, noradrenergic and dopaminergic projections. These are involved in a range of functions including mood regulation. One of the principle origins of 5-ht projections in the brain, the raphe nuclei, has been suggested to modulate PFC transmission involved in depression due to the phenotype observed in a mouse gene knockout model (Albert, Vahid-Ansari and Luckhart, 2014). Additionally, serotonergic nuclei including the medial and dorsal raphe nuclei have heavy connections to the lateral habenula, a region of recent interest in the field of depression (Tchenio, Valentinova and Mameli, 2016). It is in fact the serotonergic system that the aetiology of depression was first attributed to (Delgado, 2000).

Whilst this research has pointed towards regions affected, the underlying cause of these disruptions is not understood. Of course, dysfunctional neurochemistry has been the subject of heavy investigation, with the first theories of the disease being based on monoaminergic depletion leading to disrupted synaptic function. Additionally, other neurotransmitters including the excitatory neurotransmitter glutamate, which targets the N-methyl-D-aspartate receptor (NMDAR), and the inhibitory transmitter gamma-aminobutyric acid (GABA) have been implicated. Evidence of reduced PFC GABA and glutamate in depressed patients (Hasler *et al.*, 2007) supports this.

However, our understanding of how glutamatergic dysfunction and its correction could cause/treat depression is not understood. Evidence of increased expression of NR2A (an NMDAR subunit) in the amygdala post mortem (Karolewicz *et al.*, 2009) could represent increased signalling of the region, leading to increased emotionally negative responses. Alternatively, magnetic resonance imaging has demonstrated reduced glutamate concentrations in the depressed brain (Mirza *et al.*, 2004). Interestingly, this has been shown to be reversed by ECT (Zhang *et al.*, 2013), a rapid antidepressant treatment, thus supporting glutamate as a causative factor of the disease. However, this does not address how monoaminergic antidepressants (ADs) relieve depression if depression is a glutamatergic

disease. It is possible that monoaminergic based antidepressants function by indirectly modulating the glutamatergic system. However, as glutamate is involved in almost every brain function targeting this system difficult.

Table 1. Summary of the possible aetiology of depression.

Theory	Mechanism	Evidence	Disputes
Monoamine theory of depression.	Reduced level of monoamines including 5-ht and NA are responsible for depressed mood.	Treatments which increase monoamine levels have AD efficacy.	Delayed onset of therapeutic effects despite acute increase in monoamines.
Chronic stress and the HPA.	Increased stress results in neurodegeneration.	Chronic stress can lead to neural atrophy in brain areas involved in mood regulation (Ghosal, Hare and Duman, 2017).	Not everyone that is stressed is depressed.
Neuropsychological	Disrupted processing of emotionally valenced information results in depressed mood.	Evidence of these cognitive bias in depressed patients and their amelioration with AD treatments (Harmer *et al*. 2009)	There is no conclusive biological mechanism proposed for this theory.
Genetic determinant.	Genetic predisposition determines an individual's susceptibility to environmental stressors and depression.	Genes linked to depression through genome wide association studies (Howard *et al.*, 2019).	Carriers of risk genes will not necessarily develop depression.

Chronic stress, a response used to model depression in rodents (Willner, 2017), has been attributed a causative factor for the depressed brain. With evidence of reduced cortical volumes in depressed patients (Bremner *et al.*, 2002), chronic stress has been associated with dendritic remodelling (Sanacora, Treccani and Popoli, 2012) and has been suggested to increase susceptibility to depressive symptoms (Gould, Zarate and Thompson, 2018). Interestingly, glucocorticoids influence glutamate neurotransmission by increasing glutamate levels, potentially causing excitotoxicity and neural atrophy (Sanacora, Treccani and Popoli, 2012) (Table 1).

In line with this, dysfunction between the hippocampus and hypothalamic-pituitary-adrenal axis has been associated with depressive episodes and the reduction of cortical volumes (Sheline, 2003). The Hypothalamic-Pituitary-Adrenal axis (HPA) is a primary system to regulate stress, where the paraventricular nucleus releases corticotrophin releasing factor, causing release of adrenocorticotophin thus causing cortisol release from the adrenal cortex. Whilst being a normal physiological response, prolonged activity can lead to the death of hippocampal neurons (Nestler *et al.*, 2002). However, whether stress is a definite cause of depression is in dispute. In fact, 50% of depressed patients do not exhibit elevated cortisol

(Nestler *et al.*, 2002) demonstrating that stress is not a universal cause of depression and is unlikely to elicit this effect alone. Thus, chronic stress is more likely a risk factor for the disease and not a definitive cause.

Therefore, varying susceptibility to environmental stressors will contribute to an individual's predisposition to depression which implies a role for genetics. For example, decreased expression of synaptic proteins such as SYN1 have been found in depressed patients (Kang *et al.*, 2012) which could contribute to increased susceptibility to stress induced neurodegeneration. Therefore, depression is likely a result of an interaction between the patient's genetic makeup and environment. However, the genetics of depression are complex, with 269 genes having been associated with depression (Howard *et al.*, 2019).

Evidently, the root cause of depression is complex, and its theories convoluted. The so far misunderstood interaction between the root cause of the disease and the environment has presented numerous hurdles to research efforts made to understand the disease. How to treat such a heterogenous disorder with an undetermined cause is the great challenge of psychiatric research, one which has previously been answered through serendipity. Despite this, one of the only ways to understand depression remains with the characterisation of antidepressant pharmacological mechanisms.

1.1.2 Current theories of depressions neurobiology

One of the first theories of depression was the monoamine hypothesis which developed from the discovery that clinically effective compounds (now known as the TCAs and MAOIs) possessed efficacy in major depressive disorder (MDD) and shared the characteristic of increasing synaptic monoamines (Racagni and Popoli, 2008). Originally, iproniazid, a treatment for tuberculosis was found to exert antidepressant effects through its ability to inhibit monoamine oxidase, the enzyme responsible for the breakdown of monoamines (Elhwuegi, 2004) leading to the idea that reduced monoamine levels caused depression. Similarly, characterisation of the pharmacology of the TCAs revealed they blocked reuptake of 5-HT and NA. Subsequently, more selective compounds were developed with reduced side effects and toxicity (Elhwuegi, 2004). This includes the selective serotonin reuptake inhibitors (SSRIs) such as paroxetine and fluoxetine, and autoreceptor antagonists such as mirtazapine (Elhwuegi, 2004). This long succession of antidepressant compounds which treat depression through their ability to increase synaptic monoamines supports the idea that depression results from cerebral monoamine deficiency (Schildkraut, 1995). This formed the backbone of the monoamine hypothesis.

Whilst appealing in its simplicity, inconsistencies have challenged this theory. This includes the inability of drugs such as cocaine or amphetamine, which increase monoaminergic activity, to induce antidepressant effects, although the drugs short half-life may also be responsible due to the transience in monoamine increase. Additionally, monoamine levels increase within hours after treatment of conventional antidepressants but are only effective after 2-4 weeks of treatment (Baldessarini, 1989). In attempt to address this irregularity, modifications of the monoamine hypothesis have been made. For example, the delay in therapeutic response has been attributed to an indirect mechanism of action. It is therefore posited that monoaminergic ADs do not directly remediate the symptoms of depression through increased monoamines, but rather they activate an ensuing signalling cascade resulting in adaptive changes.

One such hypothesis proposes that the delayed antidepressant effect of monoaminergic based antidepressants lies with the eventual desensitization of autoreceptors. These normally provide inhibitory feedback loops which temporarily prevent lasting monoamine increase. For example, the 5-HT1a receptor is responsible for an inhibitory feedback loop which reduces serotonergic neuronal activity (Gardier *et al.*, 1996). Thus, upon initial SSRI treatment, synaptic levels of 5-ht are elevated however subsequent stimulation of presynaptic 5-HT1a autoreceptors causes membrane hyperpolarisation therefore reducing serotonergic neuron firing. It is only upon chronic treatment that this negative feedback loop is diminished due to 5-HT1a desensitisation. This allows 5-HT levels to rise resulting in AD efficacy (Celada *et al.*, 2004). This mechanism has also been proposed for noradrenergic focused ADs, such as mirtazapine, which leads to desensitisation of the α2 adrenergic autoreceptor (Cottingham and Wang, 2012).

Alternatively, delayed antidepressant action has been attributed to the wait required for neurotrophic changes to develop, including synaptogenesis, as proposed by the neurotrophic hypothesis of depression. Evidence of neural atrophy in depressed patients such as reduced PFC and hippocampal volumes, reduced size of pyramidal neurons along with decreased GABAergic interneurons coupled with loss of glia, has led to the postulate that depression is a neurodegenerative disease (Duman and Li, 2012). With these events being synonymous to that of chronic stress induced atrophy, the neurotrophic hypothesis of depression posits the disease results from the degeneration of neurons due to insufficient trophic support from growth factors such as brain derived neurotrophic factor (BDNF) in regions responsible for mood regulation, and that this is reversed after antidepressant treatment (Duman and Monteggia, 2006).

BDNF is a widely expressed, small dimeric signalling protein essential for neuronal health, growth and survival. Its main action resides in activation of the TrkB receptor, ultimately

promoting neuron growth through increased gene expression and protein translation. This is achieved through binding to the TrkB receptor, resulting in its dimerization and tyrosine residue autophosphorylation. Subsequent effector molecule docking stimulates the PLCγ, PI3K and ERK cascades activating the CREB transcription factor and the mammalian target of rapamycin (mTOR) protein leading to increased gene expression and protein translation, respectively (Cunha, Brambilla and Thomas, 2010) (Figure 1). An immature form of the protein, BDNF-p75^{NTR} also effects cellular processes through the JNK and NF-kB signalling pathways (Cunha, Brambilla and Thomas, 2010).

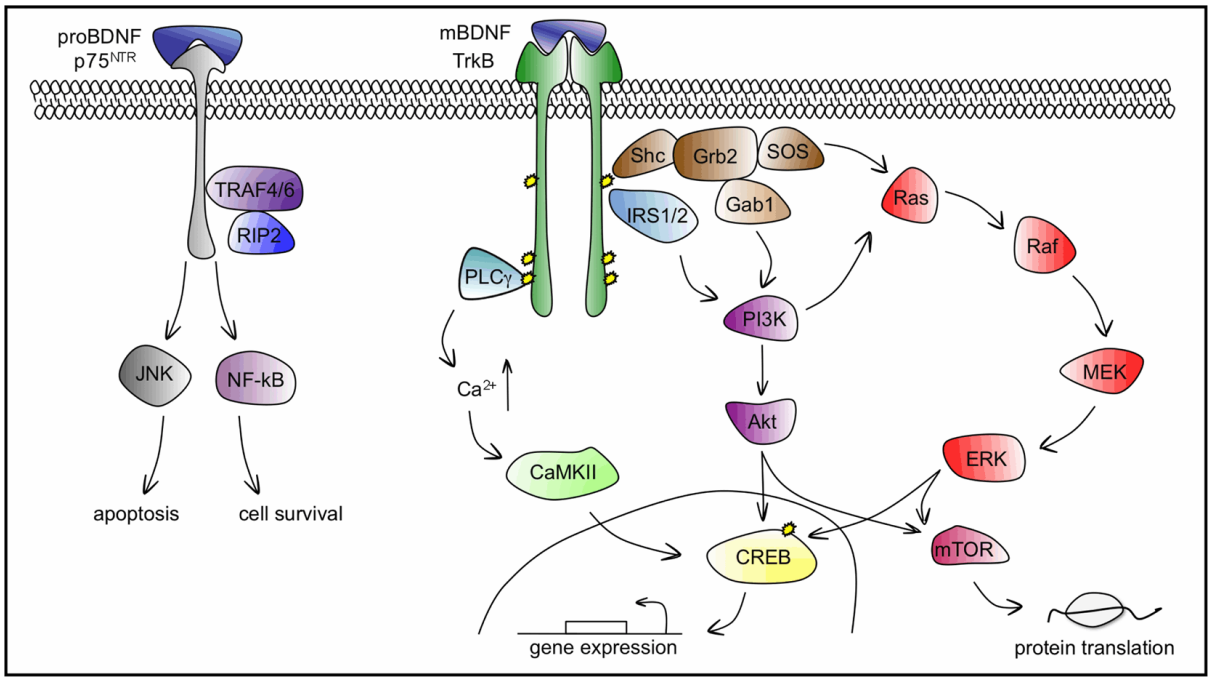

Figure 1. The mature BDNF (mBDNF) and proBDNF signalling pathways. Taken from Cunha, Brambilla and Thomas, 2010.

Findings of decreased BDNF in the PFC, amygdala and hippocampus in depressed patients in conjunction with elevated BDNF following chronic conventional AD therapy (SSRI's and noradrenaline selective reuptake inhibitors) (Duman and Monteggia, 2006) lends support to this hypothesis. Further corroboration for this originates from preclinical evidence of conventional antidepressant influence over the BDNF signalling pathway. For example, amytriptaline has been shown to increase ERK phosphorylation in rats (Galeotti and Ghelardini, 2012) and treatments such as sertraline, desipramine and tranylcypromine to inhibit stress induced BNDF downregulation in rats (Nibuya, Morinobu and Duman, 1995). Thus, it is proposed that increased 5-ht results in BDNF release subsequently leading to synaptogenesis and AD efficacy. Correspondingly, only chronic administration of

monoaminergic based antidepressants increases BNDF (Balu *et al.*, 2008), temporally consistent with the manifestation of remission.

1.1.3 The cognitive theory of depression

Whilst providing an explanation for AD efficacy, the aforementioned theories cannot fully account for the remission of depressive symptoms. To explain the manifestation of antidepressant efficacy, the drugs psychological action must be addressed. One theory, known as the cognitive theory of depression, proposes that depression results from disrupted cognitive processes (Beck, 1963). This relates to depressed patients having depressive thought processes consisting of illogical self-criticism and a strong tendency to negatively warp reality. Therefore, the cognitive theory of depression posits that depression results from biased perception of information, distorting the depressed patients interpretation of external stimuli and internal thoughts (Beck, 2005).

This can occur at memory retrieval, demonstrated by the tendency to remember negative words over positive, and can result in negative self-rumination (Harmer, Goodwin and Cowen, 2009). New and present information will also be affected and has been demonstrated in depressed patients where negative biases are observed during interpretation of neutral stimuli (Harmer *et al.*, 2009). These behaviours are not observed in healthy individuals. Thus, it is plausible that the amelioration of these negative cognitive bias is a necessity for AD efficacy, as proposed by the neuropsychological hypothesis of AD action (Harmer, Goodwin and Cowen, 2009).

Supporting the role of cognitive bias for AD action, these behaviours are ameliorated with conventional AD treatment. Interestingly, unlike their antidepressant efficacy, monoaminergic AD treatments attenuate negative bias with acute treatment. For example, Harmer *et al.*, 2003 observed how a single dose of reboxetine, duloxetine and citalopram increased recognition of happy facial expressions in healthy individuals. Additionally, Murphy *et al.*, 2009 found that a single dose of citalopram reduced activity of the amygdala in response to fearful faces. Therefore, conventional antidepressants have effects on emotional processing before the manifestation of their antidepressant effects, suggesting that the two are separate, albeit connected, effects.

Therefore, it is hypothesized that the delay in conventional antidepressant treatment results from these drugs not directly altering mood, but rather ameliorating these negative biases thus allowing the depressed patient to interpret events in positive way. This allows the patient to relearn positive, emotional experience. It is thus proposed that the relearning of positive

behaviour is the root of the delayed AD effect of antidepressant, despite attenuating negative biases hours after treatment (Harmer *et al.*, 2009).

1.2 Rapid acting antidepressants

Rapid acting antidepressants, which show AD efficacy within hours, show great improvement over conventional treatments and have posed major challenges to the receptor adaption and neurotrophic hypotheses of depression. Primary candidates include ketamine, now licensed by the Food and Drug Administration (FDA) as esketamine, and scopolamine. Scopolamine, an anticholinergic agent, has demonstrated rapid antidepressant effects lasting for 3 to 4 days post administration of the first infusion (Furey and Drevets, 2006). Whilst its precise mechanism is unknown its AD efficacy likely lies with the drugs inhibitory action on the cholinergic system. This is supported by the pro-depressive action of pharmacologically increased cholinergic activity in depressed individuals (Drevets, Zarate and Furey, 2013). Ketamine, on the other hand, has shed interest into the glutamatergic system. Ketamine is a non-competitive antagonist at the NMDAR, but also shows affinity for the D2 and sigma receptors. Similar to scopolamine, ketamine acts within hours (Figure 2) even in treatment resistant patients who do not respond to first line antidepressant drugs (Aleksandrova, Phillips and Wang, 2017).

Whilst the specific receptor targeted by scopolamine is unknown, it's thought that its selectivity for the M3 receptor subtype (30 times more than that of amitriptyline) may be vital for its AD action, as is suggested by its dose dependent effect (Furey and Drevets, 2006). Furthermore, it has been argued that muscarinic acetylcholine (mACh) receptor antagonism contributes to the AD action of TCAs, with amitriptyline being the most effective whilst displaying the highest mACh affinity thus supporting the role of mACh receptor in the AD action of scopolamine (Furey and Drevets, 2006).

Interestingly, this dose dependent characteristic is shared by ketamine, which is only known to exert AD activity at subanaesthetic doses, also potentially due to selective receptor subtype targeting. This could include receptors containing the GluN2B subunit, which has been associated with glutamatergic AD action (Miller *et al.*, 2014), or increasingly activated NMDARs which become more susceptible due to ketamine's use dependent mechanism. Furthermore, both drugs have demonstrated the requirement of BDNF signalling for eliciting an AD effect in rodents (Autry *et al.*, 2011; Ghosal *et al.*, 2018). Therefore, these rapid acting treatments possess similarities in their neurobiological mechanisms, although whether these relate to their AD effect remains to be determined.

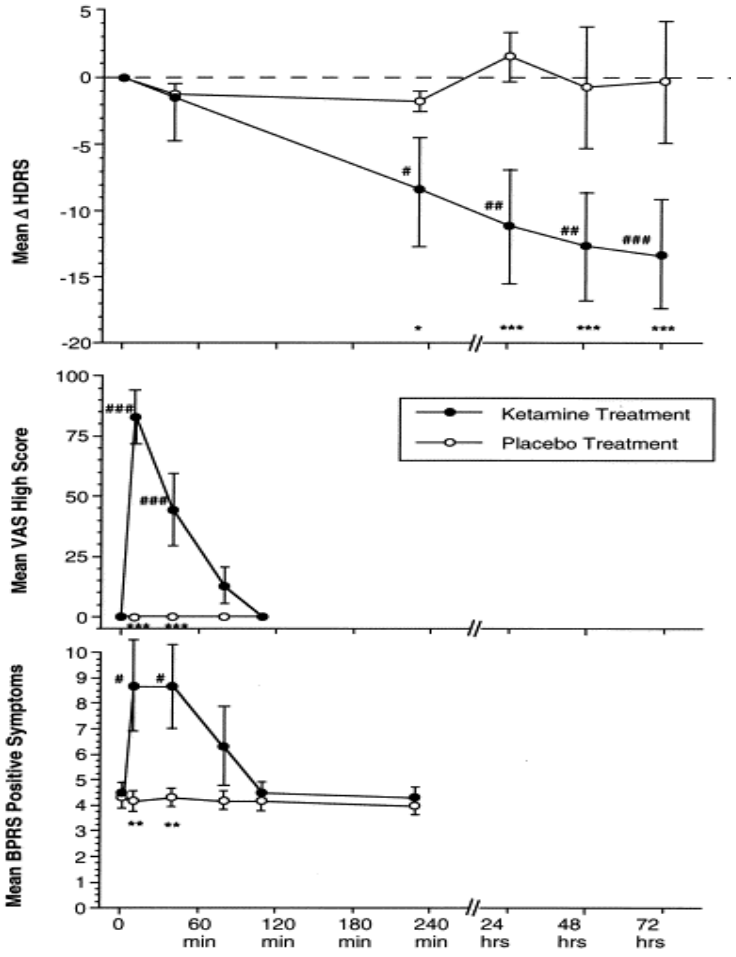

Figure 2. First evidence of ketamine's antidepressant effect in humans. Ketamine's antidepressant effect manifests within hours, characterised by reduction in the Hamilton Depression Rating Scale (HDRS) which does not temporally synchronize with its psychoactive properties or effect on cognition, as shown by the and Visual Analog Scales (VAS) and Brief Psychiatric Rating Scale (BPRS), respectfully. Data represents mean changes of scores, error bars represent SEM, * = p<0.05, ** = p<0.01 and *** = p<0.001 when compared against groups. # = p<0.05, ## = p<0.01 and ### = p<0.001 when compared to baseline. Graph taken from Berman *et al*., 2000.

Whilst promising, caution is required due to side effects and the unknown mechanism behind the rapid AD effect. Further, the long-term effects of these treatments have received little investigation despite the brief temporary nature of its antidepressant effect which lasts 1 week (Furey and Drevets, 2006; Newport *et al.*, 2015). Additionally, both treatments pose a real threat of memory loss as a side effect. Indeed, ketamine's main pharmacological action is NMDAR antagonism, a receptor central to Long Term Potentiation (LTP) posited to underlie learning and memory (Izquierdo *et al.*, 2008), whilst anticholinergic drugs have been known to facilitate cognitive decline (Papenberg *et al.*, 2017). However, there is evidence of six ketamine infusions actually improving cognitive performance (Zheng *et al.*, 2019), thus the true side effect of subanaesthetic ketamine remains to be determined.

Also, ketamine's psychotomimetic influence also poses a risk of abuse (Schatzberg, 2014), a threat demonstrated by the drugs popularity in the rave culture. Further, the hallucinatory properties of ketamine have been used to model schizophrenia (Farber, 2003) which aptly demonstrates the potential of distressing episodes, especially as robust screening methods used in clinical research (Murrough *et al.*, 2013) may not be employed in the clinic.

However, ketamine and scopolamine are not the only rapid acting antidepressants. Psychedelic drugs such psilocybin and ayahuasca have also demonstrated rapid antidepressant qualities. Although, research on these drugs has been challenging due to being listed as schedule 1 drugs. However, they have demonstrated interesting AD qualities. Ayahuasca is a mixture of two plants, *Psychotria viridis* and *Banisteriopsis caapi*, which possesses N, N-dimethyltryptamine (N,N-DMT) (Carbonaro and Gatch, 2016) and MAOI's such as harmine (Riba *et al.*, 2003). In a randomized placebo controlled trial, treatment resistant patients of depression experienced significant improvement in both HDRS and Montgomery–Åsberg Depression Rating Scale (MADRS) scores, an effect which manifested within one day and was maintained 7 days after receiving the ayahuasca treatment, containing 0.36 mg/kg of N,N-DMT (Palhano-Fontes *et al.*, 2019) which is believed to be the active compound in the treatment. Whilst an interesting finding, the rich mixture of compounds in ayahuasca makes it difficult to assert a mechanism behind its action.

Psilocybin, the active compound in magic mushrooms has also demonstrated a rapid antidepressant effect AD measured by the Quick Inventory of Depressive Symptomatology (QIDS) at 1 week (Carhart-Harris *et al.*, 2017). Interestingly, this effect may be due to psilocybin's influence on cognitive bias, evidenced by psilocybin's ability to reduce depressed patients sensitivity to negative emotion faces (Kometer *et al.*, 2012). Additionally, psilocybin possesses a mood elevating effect (Nicholas *et al.*, 2018) which has been associated with altered connectivity in the default mode network (Carhart-Harris *et al.*, 2017), although inconsistencies in the literature have made this difficult to determine.

1.2.1 Ketamine, a proof of concept

As mentioned, ketamine's main action is as an NMDAR receptor antagonist. The NMDAR is a tetrameric ligand gated ion channel which binds with its endogenous agonist glutamate and co-agonist glycine (Figure 3). Thus, the NMDAR belongs to the glutamatergic system, the primary excitatory drive of the brain. Being entirely distinct from the primary mechanism of current treatments, the NMDAR and glutamatergic system have been a focus of research to elucidate ketamine's rapid relief of depression for a novel source of AD treatments. Back translational research has largely supported the NMDARs role in this antidepressant effect, such as reduction of immobility in the widely used forced swim test (FST) for depression (Autry *et al.*, 2011). Furthermore, ketamine's antidepressant efficacy is shared with another NMDAR antagonist and GluN2B selective compound, CP-101,606 (Newport *et al.*, 2015).

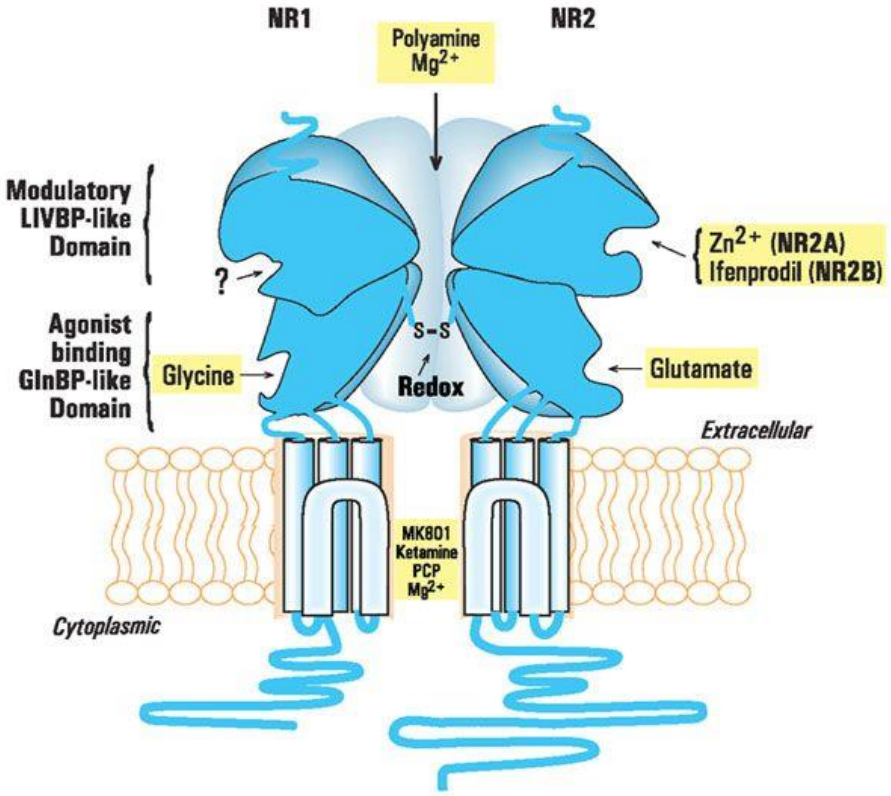

Figure 3. **Illustration of ketamine's binding within NMDAR ion channel pore.** Taken from (Kemp and McKernan, 2002). For simplicity, only two subunits are highlighted. Note, NR1 = GluN1 & NR2 = GluN2.

Through NMDAR antagonism, ketamine has been proposed to influence glutamatergic neurotransmission which results in downstream effects leading to AD efficacy. A hallmark of subanaesthetic ketamine administration is increase PFC glutamate, known as the glutamate surge (Abdallah *et al.*, 2018). An explanation for this effect, known as the disinhibition hypothesis, proposes that this results from disinhibition of tonically active inhibitory GABAergic interneurons extending to the PFC pyramidal neurons (Miller, Moran and Hall, 2016). This could then lead to synaptic plasticity which has been associated with its AD effect (Li *et al.*, 2010) as will be discussed below. Alternatively, a direct hypothesis proposes that ketamine promotes protein synthesis through directly inhibiting NMDARs which leads to a compensatory homeostatic increase in activity. This then results in protein synthesis supposedly responsible for ketamine's AD effect (Miller, Moran and Hall, 2016).

Both these hypotheses require the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR). The AMPAR is required for synapse formation and maintenance (McKinney, 2010), and is thus postulated to be essential for both the aforementioned direct and indirect hypotheses of ketamine (Miller, Moran and Hall, 2016). Indeed, the AMPAR receptor is widely present with the NMDAR, and is normally essential for the NMDARs activity due to removal of its magnesium block. Therefore, the AMPAR is highly likely to be required for an NMDAR mediated AD effect. In fact, some propose that the AD effect of ketamine solely lies with the AMPAR (Inta *et al.*, 2016).

A notion which has received attention is that it is not ketamine itself but its metabolite, hydroxynorketamine (HNK), which exerts therapeutic action (Zanos *et al.*, 2016) independent of NMDAR blockade whilst requiring AMPAR activation. However, whether the AMPAR can mediate AD efficacy independent of the NMDAR is in dispute. Further, Collingridge *et al.*, 2017 argue that the enantiomer of the metabolite to which Zanos *et al.*, 2016 attribute the antidepressant influence of to 2R, 6R-HNK, could not of been produced from the S-enatiomer of ketamine which has been shown to exert potent AD effects (Singh *et al.*, 2016; Daly *et al.*, 2018). Furthermore, evidence of AD effects of GluN2B selective NMDAR antagonists (Preskorn *et al.*, 2008; Miller *et al.*, 2014) supports NMDAR involvement. Additionally, AMPAR receptors are often required for NMDAR activation as they facilitate removal of the NMDARs Mg^{2+} block through membrane depolarisation (Bliss and Collingridge, 1993). Thus, ameliorating ketamine's AD effect through AMPAR antagonism does not prove an NMDAR independent effect.

It is important to consider that ketamine is widely administered as its racemic mixture, consisting of *R*-ketamine and *S*-ketamine which exists as a 1:1 mix. The posited implications this has for the NMDAR hypothesis is that contrasting reports have been made against the

antidepressant efficacy of each enantiomer (Zanos *et al.*, 2016; Collingridge *et al.*, 2017). Indeed, some posit that *R*-ketamine, which is 2 to 4 times less potent than esketamine at the NMDAR (Domino, 2010) is the more antidepressant compound such as in preclinical animal models (Zhang, Li and Hashimoto, 2014; Yang *et al.*, 2015). This would suggest that the NMDAR is not the principal mediator of ketamine's AD effect. In contrast, others believe that esketamine, which has shown promise over ketamine's racemic mixture (Singh *et al.*, 2016), is the more effective treatment. However, a direct comparison between the enantiomers at the same dose has not been made, although evidence of different fMRI response patterns (Masaki *et al.*, 2019) supports this investigation.

The fact that other NMDAR antagonists such as MK-801 lack AD influence (Newport *et al.*, 2015) has questioned what is behind ketamine's AD effect. This has triggered an important search to discern the characteristic of what makes ketamine AD compared to similar non-AD NMDAR antagonists such as MK-801 or AP-5. It is possible that ketamine's unique pharmacokinetic/pharmacodynamic profile may be responsible for its AD action against other NMDAR antagonists. For example, its short half-life and intermediary NMDAR binding affinity between memantine and MK-801 could facilitate AD action. On the other hand, ketamine's action on the opioid system, has been suggested to be responsible for its AD effect, as coadministration with the opioid system antagonist naltrexone has been shown to attenuate ketamine's AD effect (Williams *et al.*, 2018).

Despite some uncertainty, NMDAR antagonism remains a viable means of ketamine's antidepressant action. Furthermore, ketamine differs from its non-AD relatives such as MK-801 in NMDAR affinity and pharmacokinetic profile. The question of why some NMDAR receptor antagonists display antidepressant influence, whilst others do not, remains unanswered. Indeed, being able to differentiate these would undoubtedly contribute towards our understanding of ketamine's mechanism of action.

1.3 NMDAR antagonism and antidepressant action

1.3.1 Unselective NMDAR antagonists

Ketamine's binding site is found deep within the NMDAR channel (Figure 3) where it occludes the channel pore upon opening thus preventing ion flow. Furthermore, ketamine displays virtually no selectivity towards subtypes of the NMDAR (Zorumski, Izumi and Mennerick, 2016). This makes ketamine a use dependent, uncompetitive and unselective NMDAR antagonist, a mechanism shared by the similar antagonist's phencyclidine (PCP) and MK-801. However, these compounds dissociate much more slowly from the NMDAR than ketamine (Zorumski, Izumi and Mennerick, 2016), resulting in slower on/off kinetics and increasing drug induced channel opening, resulting in higher potency. Due to this, they cause more intense

psychotomimetic effects, as is well known for the once popular drug of abuse PCP, also known as ‘angel dust’. Consequently, these drugs have not been tested in human clinical trials for treating depression. Whilst being shown to be effective in rodent models of depression, such as the FST, MK-801 has differed from ketamine in that is does not last 24 hour after initial treatment in mice (Autry *et al.*, 2011; Yang *et al.*, 2016; Zanos *et al.*, 2016). PCP on the other hand has not been investigated as extensively as an antidepressant in human or animal assays, but has been shown to reduce immobility in the mouse FST (Solati, Ahmadi and Salari, 2011). However, due to the FST’s lack of translational validity, this is not a reliable indicator of AD efficacy.

Due to their similar pharmacology, any differentiation made between ketamine and its analogues could provide clues to ketamine’s AD mechanism. For example, memantine, a drug used to treat dementia and other neurological disorders such as Parkinson’s disease (Johnson and Kotermanski, 2006) has been investigated as an antidepressant. Despite possessing the desirable qualities of sharing similarities with ketamine’s mechanism of action and being clinically well tolerated (Gideons, Kavalali and Monteggia, 2014), memantine has failed to show antidepressant efficacy in humans (Zarate *et al.*, 2006b). Whilst binding to a similar site as ketamine, memantine exerts a weaker block of the NMDAR due to its low trapping effect, resulting from lower NMDAR affinity (Mealing *et al.*, 1999). This is likely the cause of its low psychotomimetic influence and potentially explains its lack of antidepressant action. This could suggest that a certain degree of NMDAR inhibition is required for AD response. However, this remains uncertain, although a connection between ketamine’s dissociative influence and AD effect has been made (Luckenbaugh *et al.*, 2014). Furthermore, its lack of effect on rodent models of depression have been attributed to its lack of effect on NMDARs at rest due to the influence of magnesium ions (Gideons, Kavalali and Monteggia, 2014).

1.3.2 GluN2B

The NMDAR is a tetrameric receptor, made up of two GluN1 and two GluN2 subunits (Figure 3). The GluN1 subunit binds glycine, whilst the GluN2 subunit binds glutamate and exists as four subtypes consisting of GluN2A, GluN2B, GluN2C and GluN2D. Some NMDAR antagonists such as CP-101,606 confer a selective preference over GluN2B containing receptors. CP-101,606 binds to a distinct region to that of ketamine, consisting of a cleft between the GluN1 and GluN2B ATD domains (Burger *et al.*, 2012). Despite this alternative mode of action, CP-101,606 has demonstrated antidepressant efficacy at both human trials (Preskorn *et al.*, 2008) and rodent models (Poleszak *et al.*, 2016). Though note that only one CP-101,606 trial has taken place and did not show as robust as an effect as ketamine

(Preskorn *et al.*, 2008). Despite little research, the ability of the GluN2B antagonist to induce antidepressant action could be used to dissect ketamine's mechanism.

A second GluN2B selective NMDAR antagonist, MK-0657, displayed hints of an antidepressant effect through oral administration, although this did not reach significance. However only 5 patients completed the study as the manufacturer discontinued the drugs production (Ibrahim *et al.*, 2012). Thus, whilst showing some promise, further investigation is required for GlunN2B selective antagonists to be employed in the clinic.

How the GluN2B containing NMDAR is involved in antidepressant efficacy is still in debate. One hypothesis is that inhibition of GluN2B NMDARs leads to the promotion of neurotrophic signalling as their activation has been shown to suppress mTOR protein synthesis (Miller *et al.*, 2014). However, this does not explain how a non-selective antagonist such as ketamine could achieve antidepressant efficacy which required GluN2B selectivity. In attempt to address this, it has been proposed that GluN2B receptors are predominantly located extrasynaptically, and are tonically activated by ambient glutamate thus increasing susceptibility to ketamine's open channel block (Miller *et al.*, 2014; Miller, Moran and Hall, 2016). However, the extrasynaptic location of GluN2B NMDARs is in dispute.

1.3.3 Other ways of targeting the NMDAR

To open, the NMDAR requires both the removal of the Mg^{2+} block via depolarisation and the binding of glutamate and its co-agonist glycine (Figure 3), which binds to a distinct site at the GluN1 subunit (Johnson and Ascher, 1987). It has therefore been attempted to use this as an opportunity for the development of another strategy to target the NMDAR to exert antidepressant effect. An example is D-cycloserine, a treatment for tuberculosis which has affinity for the glycine site at the NMDAR.

To date two trials have been conducted, one using a lower dose of 250 mg/day which did not improve depressive symptoms (Heresco-Levy *et al.*, 2006), whilst a second study using a higher dose of 1000 mg/day significantly improved HDRS scores (Heresco-Levy *et al.*, 2013). This suggests a dose dependent effect. Being a partial agonist D-cycloserine will begin to exert NMDAR antagonism only at higher concentrations, which corresponds with the results gained from the two aforementioned studies. However, these were preliminary studies with an n number of 22 (Heresco-Levy *et al.*, 2006) and 26 (Heresco-Levy *et al.*, 2013) and D-cycloserine was used as an adjunct therapy in each study, with patients already being on certified antidepressant treatments. Therefore, further investigation is required. A further attempt at developing an NMDAR co-agonist antidepressant has been attempted with GLXY-

13 also known as rapastinel, however its antidepressant efficacy has not been established (Newport *et al.*, 2015).

A potential use for D-cycloserine is as an adjunct therapy with ketamine has also been investigated. Here, Kantrowitz *et al.*, 2015 found that D-cylcoserine administration after an initial ketamine infusion caused a significant overall response over an 8-week period. However, this study was not blinded or placebo controlled as well as having a small sample size of 12. Therefore, further investigation is needed, particularly on the AD effect of D-cycloserine independent from other AD treatments.

1.4 Ketamine, a unique case

Whilst many attempts at replicating ketamine's AD action have been undertaken, none have achieved consistent efficacy (Newport *et al.*, 2015). This raises the question of what it is that gives ketamine this reliable therapeutic effect. A possibility is ketamine's rich pharmacology, which extends to the dopaminergic, serotonergic and opioid transmitter systems (Newport *et al.*, 2015). Particularly, ketamine's activity at the sigma receptor has been postulated to contribute to its antidepressant effect (Robson *et al.*, 2012). However, ketamine's affinity for the receptors behind its secondary actions are low and would likely require high doses to cause an effect which does not correspond to the low dose 0.5 mg/kg infusions at which it's antidepressant action manifests.

Alternatively, it could be specific properties which ketamine possesses at the NMDAR. This could be exemplified by memantine's inability to relieve depression (Zarate *et al.*, 2006b; Zarate *et al.*, 2013), which could be due to differential actions at the NMDAR such as influence of receptor desensitisation (Glasgow *et al.*, 2017) or memantines inability to block resting NMDAR currents in the presence of magnesium (Gideons, Kavalali and Monteggia, 2014). To further investigate, more comparisons between ketamine and similar antagonists such as PCP would benefit this search.

1.4.1 Dose dependent effect of ketamine

To achieve its AD effect ketamine must be administered in subanaesthetic doses. Typically, 0.5 mg/kg of ketamine is given as a single infusion across 40 minutes (Newport *et al.*, 2015; Zorumski, Izumi and Mennerick, 2016) which is associated with mild dissociative side effects rated by the Brief Psychiatric Rating Scale and Clinician Clinician-Administered Dissociation State Scales (Berman *et al.*, 2000).

In rats, a clear difference, both behaviourally and neurochemically exists between anaesthetic doses of ketamine (>80 mg/kg) and subanaesthetic doses (<30 mg/kg). Firstly, whilst doses

of 10 & 5 mg/kg have been shown to increased phosphorylated levels of mTOR, 4E-BP1 and p70S6K indicating synaptic strengthening, 80 mg/kg does not (Li *et al.*, 2010). Secondly, 3, 10 and 30 mg/kg ketamine increase both glutamate cycling and glucose metabolism in rat mPFC whilst 80 mg/kg does not (Chowdhury *et al.*, 2017). These differences provide evidence of distinct dose dependent biochemical effects in rodents.

Due to this the preclinical literature has focused on subanaesthetic doses ranging between 10 – 25 mg/kg when investigating ketamine's AD efficacy. However, evidence that 1 mg/kg ketamine modifies affective bias in rats (Stuart *et al.*, 2015) suggests that studying ketamine's AD effect at doses of 10 mg/kg may still be high enough cause secondary non-AD effects. In support, doses of 5 mg/kg and 4 mg/kg have been shown to impair responses to pre-pulse inhibition and have locomotor influence (Imre *et al.*, 2006; Radford *et al.*, 2017). Therefore, the behavioural aspect of the preclinical ketamine story could be fundamentally flawed as despite application of subanaesthetic doses within the 5 – 10 mg/kg range, which can still promote neurotrophic effects, may still be causing non-AD effects. Thus, the effects viewed at the behaviour level, particularly that of the FST, could be non-specific to ketamine's antidepressant action.

Logically, an effect more specific to the lower dose ranges could be due to ketamine preferably targeting a subpopulation of NMDA receptors. In support, a similar dose dependence may be seen with CP-101,606. As discussed, CP-101,606 has also been shown to display AD effects whilst maintaining dissociative effects. Indeed, dissociative effects have been observed from higher doses of CP-101,606 by Preskorn *et al.*, 2008, who reduced the dose from 0.75 mg/kg per hour for 1.5 hours, followed by 0.15 mg/kg 6.5 hours to 0.5 mg/kg per hour for 1.5 hours due to moderate/severe psychotomimetic effects. The more dissociative influence of the higher dose corresponds with the different pharmacological magnetic resonance imaging profiles between 15 mg/kg and 5 mg/kg CP-101,606 in rats observed by Tang *et al.*, 2018. They discovered that whilst a 5 mg/kg dose produced region specific activation at the mPFC and ventral orbital cortex and anterior cingulate cortex, 15 mg/kg resulted in more universal activity at the cortical/sub-cortical regions, an effect shared by 3 mg/kg ketamine. This discrepancy could suggest that even 3 mg/kg ketamine is high enough to exert nonspecific effects, increasing its dissociative influence.

Another issue with higher doses of ketamine is its effect on cognitive processes and memory. Indeed, 3 mg/kg ketamine can disrupt spatial memory in rats (Pitsikas, Boultadakis and Sakellaridis, 2008). Thus, doses within the 10 – 25 mg/kg range often used in the preclinical literature likely cause cognitive disturbances. This is exemplified by studies showing 8 mg/kg effects memory acquisition (Pallarés *et al.*, 1995), and the study by Dix *et al.*, 2010

demonstrating that 10 mg/kg ketamine consistently impaired performance in a cognitive battery test indicating reduced learning, memory and cognitive flexibility. However, as the 0.5 mg/kg infusion used in humans has also been linked to dissociative effects (Newport *et al.*, 2015), temporary cognitive impairments do not necessarily mean a lack of AD response. Furthermore, with evidence arguing against the AD efficacy of doses of 0.1 and 0.2 mg/kg ketamine (Fava *et al.*, 2018), the dissociative side effects of 0.5 mg/kg may be inseparable from ketamine's AD effect. Whether this is due to side effects from the necessary dosage, or a result of the same causative factor, remains to be determined.

Indeed, the neurobiology behind the AD action of subanaesthetic doses is not fully understood, although it likely results from the need of subtler neurocircuitry influences which anaesthetic doses cannot exert. This suggests that for antidepressant effects to manifest, a large proportion of NMDARs will remain unblocked (Zorumski, Izumi and Mennerick, 2016) facilitating subsequent recruitment for eliciting an antidepressant effect. Thus, global NMDAR suppression that accompanies anaesthetic doses would compromise this mechanism.

1.5 What is ketamine's antidepressant mechanism?

1.5.1 Neurotrophic signalling

The neurotrophic hypothesis has been used to describe ketamine's AD mechanism which has been suggested to act via neurotrophic signalling pathways, namely that of the mTOR pathway (Figure 4) (Duman and Voleti, 2012). It has been proposed that increased exocytosis of BDNF resulting from a ketamine triggered glutamate surge subsequently stimulates the TrkB receptor and therefore the synthesis of synaptic proteins including GluA1 and PSD95 (Duman *et al.*, 2012). Indeed, ketamine's AD effect in rodents has been ameliorated in BDNF knockout animals (Autry *et al.*, 2011), and by an infusion of a BDNF antibody (Lepack *et al.*, 2014). Additionally, intraperitoneal (i.p.) administration of the L-type calcium channel antagonist verapamil and nifedipine has been shown to inhibit ketamine's effect on immobility in the FST (Lepack *et al.*, 2014). Further downstream effectors have also been implicated, including mTOR and 4E-BP1 (Li *et al.*, 2010; Autry *et al.*, 2011).

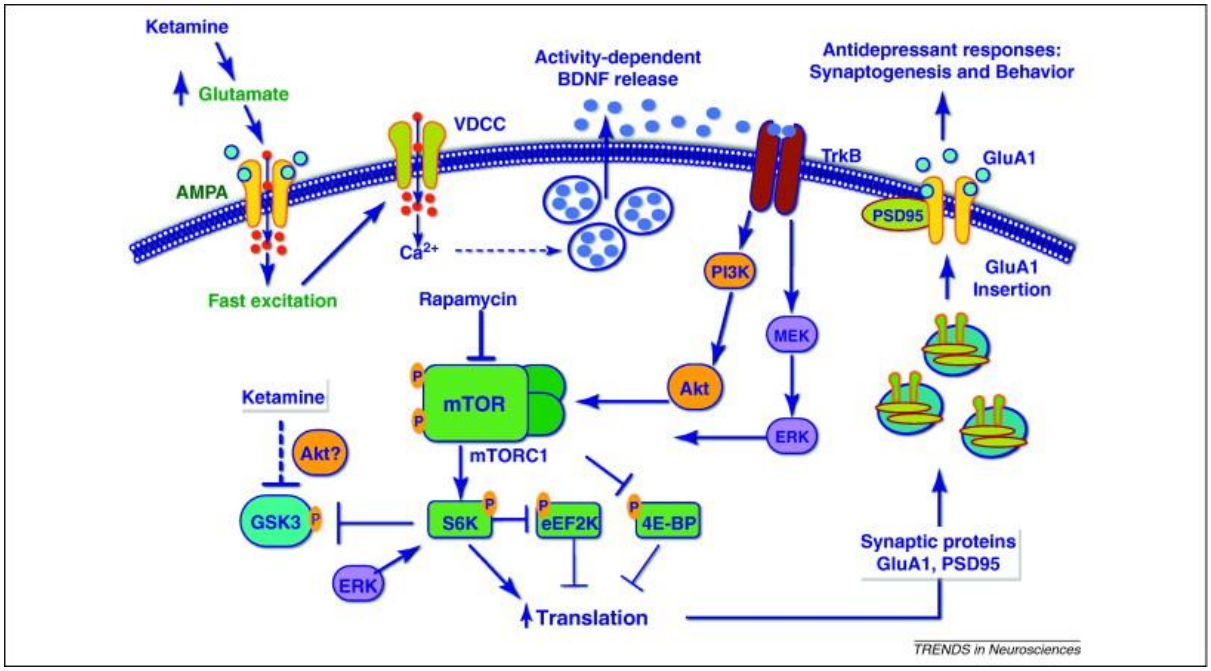

Figure 4. Signalling pathways attributed to ketamine's antidepressant mechanism in the neurotrophic hypothesis of depression. Disinhibition of GABAergic interneurons through ketamine NMDAR antagonism leads to increased synaptic glutamate. This in turn activates AMPAR facilitating calcium entry via voltage gated calcium channels (VDCC) which triggers BDNF exocytosis. Subsequently, the TrkB pathway is activated leading to increased expression of synaptic proteins thus promoting neurogenesis. Diagram is from Duman *et al.*, 2012.

Overall, a large proportion of preclinical research has supported this neurotrophic-based theory of ketamine's AD action. However, despite its appealing simplicity it would be rash to accept this as ketamine's AD action for several reasons. Firstly, understanding how intracellular cascades translate to the remission of depressive symptoms has been an age-old challenge of preclinical psychiatric research and the current literature fails to make such extrapolation beyond the use of despair related rodent models such as the FST. Secondly, inconsistencies exist in the timeline between the appearance of ketamine's therapeutic effect and neurogenesis. Specifically, it is unlikely that a seemingly chronic process such as dendritic spine remodelling could account for an AD effect which manifests within hours and fails to explain why ketamine's AD action is transient. Lastly, the behavioural element to this research is limited in that it is based on despair-based models of depression, which are based on the tautology of their capability to detect monoaminergic based AD efficacy and do not replicate the aetiology of depression. However, despite these irregularities, this research has identified a potential role for BDNF in ketamine's action, perhaps one that involves learning and memory.

1.5.2 Neuropsychological model

Acting within hours, ketamine's rapid onset has made it stand out from current therapies. This highlights the question of what causes the difference in the onset of action of current treatments and ketamine. Thus, the cognitive hypothesis has been applied in attempt to address this inconsistency.

As discussed, the amelioration of negative biases and subsequent effect on emotional relearning has been proposed to be responsible for the delayed action of conventional antidepressants (Harmer, Goodwin and Cowen, 2009). However, this does not correspond with the rapidity of ketamine's antidepressant effect, as its rapid onset would theoretically not be long enough for these same learning processes to take place. One postulate is that ketamine ameliorates negative biases based on recollection, and not new information, resulting in rapid but transient effect (Stuart *et al.*, 2015).

A possible mechanism is that ketamine acts to normalise neural processes which in the depressed person is leading to these negative biases. By monitoring depressed patients' brains using functional MRI (fMRI) whilst undertaking the dot probe task, which assesses attention and emotional processing and is used to assess cognitive bias through the interpretation emotional faces, Reed *et al.*, 2018 found that post ketamine administration the depressed group's activity at the right frontal cortex, dorsal anterior cingulate cortex (dACC), and left anterior gyrus was decreased to match that of placebo. Murrough *et al.*, 2015 demonstrated that ketamine reverses differences in striatal connectivity found in MDD patients compared to that of healthy controls using emotion perception tasks and fMRI. Furthermore, Abdallah *et al.*, 2017b, using resting state functional connectivity fMRI demonstrated that ketamine normalised reduced connectivity within the PFC in depressed patients.

Therefore, ketamine's ability to reverse these functional abnormalities in the depressed brain may result in its ameliorating effect on negative bias and thus AD effect. Indeed, evidence of the normalisation of altered brain activity in depressed patients in response to conventional ADs (Delaveau *et al.*, 2011) supports the possibility that new rapid acting ADs such as ketamine exert a similar normalising effect, albeit through different mechanisms.

1.5.3 Neurocircuitry

Three major neural networks including the default mode network (DMN) responsible for self-rumination, the central executive network (CEN) associated with cognitive function and the salience network (SN) which is involved in emotional regulation (Mulders *et al.*, 2015), have received noteworthy attention in the field. The DMN, centred around the medial prefrontal (anterior DMN) and anterior cingulate (posterior DMN) cortices has increased within network

connectivity at rest in depressed patients compared to healthy controls (Mulders *et al.*, 2015). This could potentially lead to maladaptive self-rumination. Other associations include altered connectivity between the SN and DMN, such as hypoconnectivity with the nucleus accumbens and increased connectivity with the amygdala, posited to influence emotional cognition (Kaiser *et al.*, 2015). Thus, ascertaining whether these depressive traits are reversed with ketamine is of prime interest due to the potential insight into its mechanism this could offer.

In an attempt to address this, extensive imaging studies have applied fMRI to investigate the depressed patient's brain to identify the networks responsible for the diseases affective and cognitive manifestation. A recent focus has resided on resting state functional connectivity (RSFC), which provides an insight into the brain which is not actively engaged in a task. This allows the search for key networks disrupted in the depressed brain at rest.

Ketamine has been shown to decrease functional connectivity between key regions such as the dorsal nexus, medial prefrontal cortex and pregenual anterior cingulate (Scheidegger *et al.*, 2012) suggesting ketamine's antidepressant effect lies with decreasing functional connectivity with the DMN. However, this study was undertaken in healthy controls and is not directly applicable to the depressed patient. Furthermore, a separate study by Mueller *et al*. 2018 failed to find a significant influence of subanaesthetic ketamine on the DMN, however increased connectivity between the ECN and anterior cingulum and frontal gyrus, along with decreased connectivity between the ECN and SN seeds were found.

Whilst these findings could suggest a mode of action for ketamine's antidepressant influence these are preliminary notions due to the inconsistency of findings. This is a common problem in the neuroimaging field due to the different methods of analysis used and the large number of heterogenous circuits studied. Furthermore, it is difficult to definitively separate ketamine's dissociative side effects from its therapeutic AD influence based solely on imaging data, and as Mueller *et al.*, 2018 propose, ketamine's reduction of ECN connectivity with SN regions including the occipital load could represent ketamine's dissociative effects. To resolve this, ketamine's pharmacodynamics should be compared with that of similar drugs to evaluate whether these effects can be isolated. However, in order to uncover ketamine's antidepressant mechanism, we cannot rely on biochemical observations or imaging data as it is impossible to determine how these will translate.

However, despite these difficulties this approach can elucidate on the neurocircuit dynamics involved in the cognitive aspects of the disorder which cannot currently be resolved at the molecular level. Furthermore, this area of research provides a link between ketamine's neuropharmacodynamics and the behavioural manifestation of its antidepressant effect. Indeed, altered connectivity within relevant circuits provides an additional platform for

understanding how functional changes in the brain present as symptoms of depression and its remission resulting from acute ketamine treatment. This includes those such as negative cognitive biases, which cannot be solely gained from *in vitro* research. Therefore, we must expand on these observations with novel methods of behavioural psychopharmacology using sophisticated animal research techniques.

1.6 Animal research and depression

Animal research has provided a vital method for the investigation of psychiatric illness and the means to understand the basic science behind these diseases. Using these approaches, the neurobiology of depression and discovery of therapeutic compounds has been aided greatly. Indeed, it was through animal research that the antidepressant properties of NMDAR antagonists were first encountered (Trullas and Skolnick, 1990), facilitating the discovery of ketamine's antidepressant activity.

Considering the difficulties of modelling higher cognitive functions in rodents, investigating depression using animal research is challenging and often resorts to stress induced states. Due to this, a set of behavioural tests sensitive to chronic stress have proven a popular screening method for antidepressant efficacy, such as the FST which relies on despair related behaviours. These animal assays are based on a tautology from an effect observed from monoaminergic antidepressants (Hendrie *et al.*, 2013), contributing to an unsuccessful search for new antidepressant medicines (Berton, Hahn and Thase, 2012). With most of the literature relying on either despair-based animal models including the FST, learned helplessness assay and novelty suppressed feeding test, the behavioural aspect of the ketamine story is lacking. This is because these assays were not designed to elucidate a drug's pharmacological mechanism, but to simply detect antidepressant effect (Nestler and Hyman, 2010).

However, recent developments in preclinical behavioural research has led to the development of novel behavioural assays based on depression's cognitive theory. The application of these more translational methods should enable developments of a more complete understanding of the mechanism of antidepressants such as ketamine.

1.6.1 Studying cognitive and affective biases in rodents

Sadness and anxiety are natural responses to negative events. However, in the case of the depressed individual these feelings are exacerbated and inappropriately regulated, leading to negative reactions to undeserving events. This stems from dysfunctional interpretation and regulation of information and responses, a postulate of the cognitive theory of depression first proposed by Beck, 1963. This theory has provided a good explanation for the remission of

depression and fits with evidence of cognitive bias in depressed individuals (Gotlib *et al.*, 2004; Peckham, McHugh and Otto, 2010).

The negative interpretation of ambiguous information is a serious impairment present in depressed patients, one which the widely used despair and hedonic based rodent models fail to elucidate. However, with increasing focus on cognitive bias, novel opportunities for preclinical behavioural studies have been presented. These include the affective bias task (ABT) and judgement bias task (JBT) (Hales *et al.*, 2014). As will be discussed, these assays provide a novel opportunity to study the cognitive influence of psychoactive drugs such as ketamine, providing further insight into their cognitive mechanism than the commonly used despair-based assays.

1.6.2 The judgement bias task

The rat judgement bias task comprises of learning to differentiate two distinct tones, one that corresponds to receiving an appetitive reward after the press of a lever, whilst the second tone represents a less positive outcome (low reward/no reward) or punishment. Once this is learnt, a third ambiguous tone is played, and the choice of the rat recorded. The animal's choice is then used to judge the animal's cognitive bias. A choice for the rewarding lever would indicate a positive bias, whilst the anticipation of punishment suggests a negative bias (Hales *et al.*, 2014). Supporting this test's validity, exposure to chronic stress results in a negative shift in cognitive bias, as more lever presses were made to avoid punishment (Papciak *et al.*, 2013).

As proposed by Harmer, Goodwin and Cowen, 2009, it is possible that the delayed action of current antidepressants resides with the eventual relearning of positive emotional experience which occurs after amelioration of negative affective biases. This temporal inconsistency has previously been impossible to investigate using behavioural animal research. Using the JBT, Hales *et al.* discovered differences between delayed onset and rapid onset antidepressants, where conventional monoaminergic treatments have no acute effect on cognitive bias whilst rapid acting antidepressants including ketamine, the GluN2B containing NMDAR selective antagonists CP-101,606 and the muscarinic antagonist scopolamine induced positive cognitive bias compared to vehicle baseline (Figure 5).

Interestingly, induction of positive bias was induced by chronic administration of the conventional antidepressant fluoxetine (Hales, Houghton and Robinson, 2017). These findings resemble the subjective reporting of remission in depressed patients, which occurs hours after ketamine administration but weeks after conventional monoaminergic antidepressants. In further corroboration with human AD trials, Hales *et al*. demonstrated that NMDAR antagonists similar to ketamine such as memantine which have not been shown to

possess antidepressant efficacy (Newport *et al.*, 2015), lack influence on cognitive bias in the JBT (Figure 5). This supports the role of cognitive bias in ketamine's AD effect.

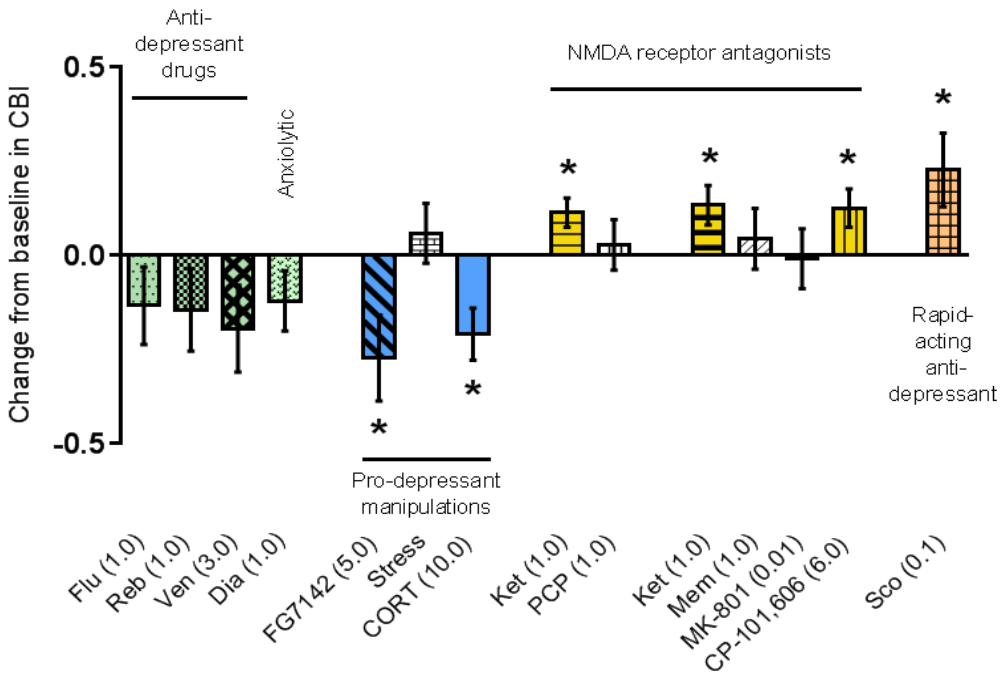

Figure 5. Effect of acute pharmacological manipulation of affective state in rats performing in the judgment bias task. Accumulated data from Hales, Robinson and Houghton, 2016; Hales, Houghton and Robinson, 2017; Bartlett *et al.* unpublished. Data represent choice made in reaction to ambiguous cue, error bars represent SEM, * = $p<0.05$.

1.6.3 The Affective Bias Task

Like the JBT, the ABT is based on the cognitive neuropsychological hypothesis but differs in its focal point being on the influence of affective state on memory, as in how affective state at the time of learning leads to emotional bias, or how drugs which modify affective state effect biases at the time of recall (Hales *et al.*, 2014).

Through learning to discriminate between two distinct digging substrates to attain a reward, the rats can be subjected to affective state manipulations associated with one of the specific digging substrates. Affective state manipulations may be pharmacological, such as an antidepressant compound, or environmental such as social enrichment. Thus, each of the rewarded digging substrates is encountered under different conditions, with one of these being under the affective state manipulation. The digging substrates are associated with rewards on alternating days against a blank, and then presented together on a final choice day. The rat's choice between the two reward paired substrates is then used to quantify their affective bias.

A ratio favouring the affective state manipulated digging substrate represents a positive bias, whilst a ratio favouring the unmanipulated substrate indicates a negative bias.

Having been reliably demonstrated to exhibit similar results to those attained in human studies described in section 1.1.3 (Figure 6), affirming its predictive validity, the ABT offers an additional source of research on ketamine's influence on cognitive biases. That is, to investigate ketamine's effect on the interpretation of emotionally valanced information.

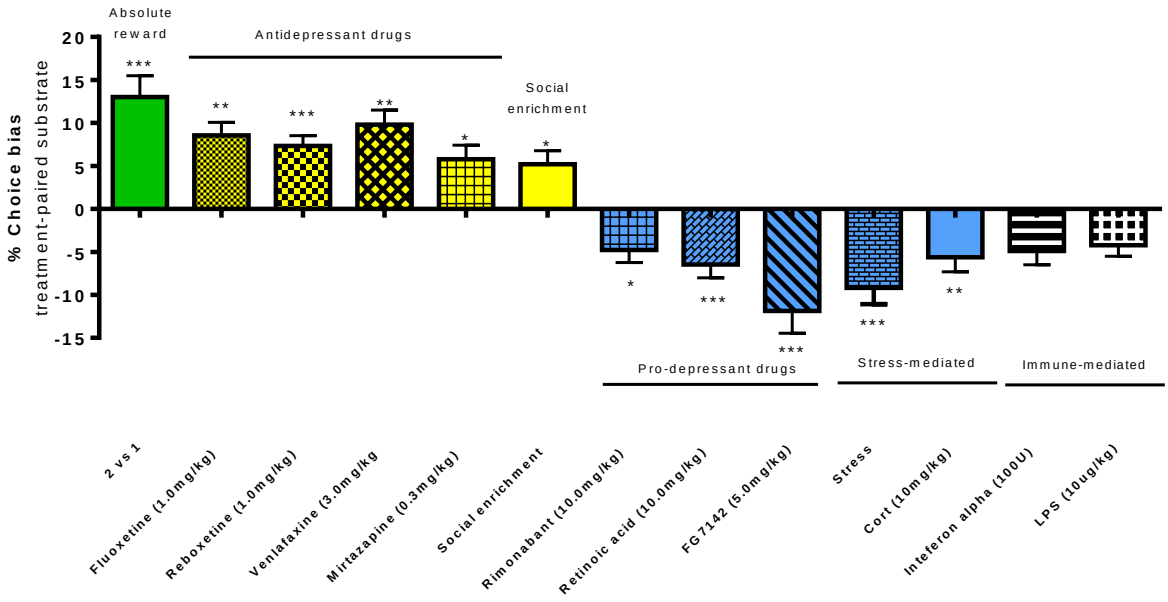

Figure 6. Validation of the ABT. Accumulated data for the bowl digging ABT (Stuart *et al.*, 2013; Stuart, Wood and Robinson, 2017). Data displayed as % choice bias, error bars represent SEM, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

Application of the ABT has distinguished ketamine from conventional antidepressant treatments. Whereas the conventional antidepressant fluoxetine is only able to induce a positive bias when administered before the pairing session, ketamine has this effect only when administered prior the choice session (Stuart *et al.*, 2015) (Figure 7). Stuart *et al.*, 2015 posit that whilst ketamine ameliorates previously acquired negative biases at recall, conventional antidepressants function by attenuating negative bias at the time of processing. This identifies a key difference in the influence of these antidepressant drugs on affective bias, which possibly explains the different temporal onsets of their therapeutic effect. However, ketamine's lack of effect on the processing of new information infers the drug will not work long term (Stuart *et al.*, 2015). Consistent with the different onset of these treatments, the ABT has provided a valuable insight into the cognitive mechanisms behind the antidepressant action of ketamine and how it differs from that of conventional treatments.

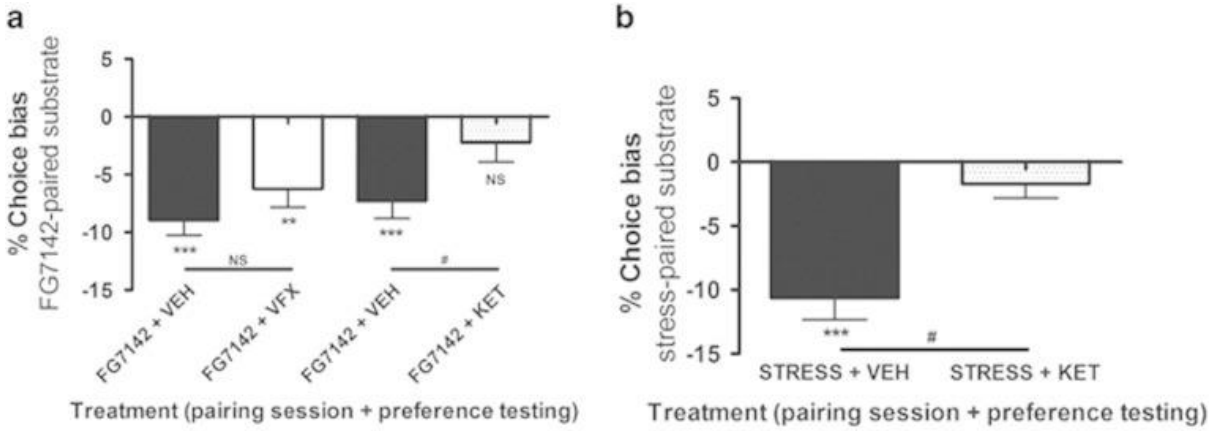

Figure 7. Ketamine ameliorates previously acquired negative biases. From Stuart et al., 2015. Stuart *et al*. demonstrate that 1 mg/kg ketamine (KET) is able to attenuates a previously acquired negative bias when administered before the choice session, whilst venlafaxine (VFX) did not improve over vehicle (VEH). Negative biases were induced through administration of FG7142 (a GABA receptor inverse agonist) (A) or restraint stress (B) before substrate pairing sessions. Data shown as mean±SEM, n = 16. ** = $p < 0.01$ and ** = $p<0.001$ when compared to theoretical 0% bias. # = $p<0.05$ and ## = $p<0.01$ when compared against vehicle control.

Taken together, the JBT and ABT have started to provide valuable insight into the role of cognitive bias in ketamine's antidepressant action. Whilst the JBT, consistent with the remission of symptoms, has shown to immediately effect the rat's judgement bias resembling the patients subjective reporting of remission (Hales, Houghton and Robinson, 2017), the ABT has shown that this effect could be achieved through attenuating negative bias at time of recall (Stuart *et al.*, 2015). This demonstrates the potential this line of research offers and warrants its continuation.

This is of significance because to truly understand ketamine's AD action we need to connect objective behavioural parameters to basic neuroscience. Application of the ABT allows this as it opens way for testing specific biological manipulations on a depressive behavioural trait whilst providing the advantages of *in vivo* research. By assessing the neurobiological effect of an efficacious treatment in this assay such as that of 1 mg/kg ketamine (Stuart *et al.*, 2015), deductions can be made as to what is behind this behavioural effect. This will allow more definitive research into ketamine's AD mechanism. Therefore, the current work aims to validate and optimise the ABT whilst characterising the influence of 1 mg/kg ketamine. The latter of which will provide insight to the basic science behind 1 mg/kg ketamine's effect in the ABT (Stuart *et al.*, 2015).

1.7 Aims and objectives

To investigate if an alternative approach to the ABT could provide a higher throughput and more reliable method to study the effects of ketamine on cognitive processes linked to MDD.

To try to understand the regions and circuits activated by low dose ketamine and compare these with higher doses and non-efficacious NMDAR antagonists.

To use the object in place version of the novel object recognition test to provide a different assay of cognition to determine the specificity of the effects seen with low dose ketamine.

Chapter 2

Investigating an alternative affective bias task using flavour preference

2.1 Introduction

Whilst the FST is known to be sensitive to monoaminergic ADs its predictive validity for non-monoaminergic ADs has been questioned (Berton, Hahn and Thase, 2012; Stuart *et al.*, 2013). Preclinical studies on ketamine often use the FST as a means to detect AD efficacy (Li *et al.*, 2010; Autry *et al.*, 2011; Zanos *et al.*, 2016) however these results may be confounded by ketamine's locomotor side effects. Additionally, non-AD antagonists such as MK-801 have also reduced immobility in the FST test (Autry *et al.*, 2011; Zanos *et al.*, 2016), although this only manifests at a 1-hour pre-treatment time this demonstrates that non antidepressant NMDAR antagonists can elicit a false positive in this test.

Furthermore, the test's primarily function is as a predictive validator and does not recreate depressions aetiology (Belzung and Lemoine, 2011). For this reason, the affective bias test provides an advantage over the sole predictive validity of the forced swim test due to its translational validity, as it recreates part of the depressive cognitive pattern known as affective bias (Stuart *et al.*, 2013). This offers a novel approach to understand how preclinical research may translate to remitting behaviour in humans.

However, despite exciting developments the ABT is highly sensitive to experimenter effects and requires a high level of skill in behavioural methods to successfully train animals to perform the task. To explore this, we undertook a repeat of the 2 vs 1 ABT and negative bias induction using cortisol injection undertaken by Hinchcliffe *et al.*, 2017. Having failed to achieve reliable reproducibility we conducted a trial of a modified version of the assay, replacing the experience of digging substrate with that of flavoured solutions, where the reward consisted of a sucrose solution and not reward pellets. The objective was to reduce the opportunity for the induction of bias from the experimenter to the animals by minimizing experimenter-animal interaction. By reducing opportunity of the experimenter influencing the animal's behaviour and therefore choice, the risk of compromising the affective bias should be minimized and increase the likelihood of reliable bias induction.

The ability to use flavours for associative learning has been well characterised in rats. First investigation into this was undertaken by Holman, 1975, who demonstrated rats exhibit a preference for a flavour paired with more concentrated saccharin solution than the flavour paired with a less concentrated saccharin solution. Furthermore, it is possible to maintain this association despite the removal of the reward associated as this type of learning can avoid extinction with properly designed experiments (Delamater, 2004). The possibility to facilitate associative learning using flavours provides the opportunity to apply the same principles used in the bowl digging ABT.

Like the ABT, the flavour preference ABT applies pavlovian conditioning in order to associate an unconditioned stimulus, in this case sucrose solutions, with a conditioned stimulus of flavour to form the unconditioned response of an affective bias, manifested by a preference to the more highly rewarded flavour. Thus, the formation of a positive association, such as a preference for a more highly rewarded flavoured solution, can be used to investigate the influence of drugs on cognitive bias, synonymous to the bowl digging ABT.

The design of a different experiment which aims to study affective bias in the same context not only offers more experimental options but will also support the test's theoretical validity. To achieve this, we assessed this variation of the affective bias task using the same experimental structure and concepts as the bowl digging task but replacing digging substrates with flavoured sucrose solutions. The ability to recreate an affective bias in this varied version of the ABT would also offer a less labour-intensive approach with greater opportunity for automation.

2.2 Methods

2.2.1 Animals

16 male lister hooded rats were used to conduct the ABT task (~500–550 g, Harlan, UK). Another 12 male lister hooded rats (300-350g) were then used to conduct the flavour preference ABT. Animals were housed in pairs and all experimental work was conducted within a 12:12 h light–dark cycle (lights off at 0700h), between 9 am – 5pm. Bowl digging ABT animals were food restricted to 100g of laboratory chow per day with *ad libitum* water whilst the flavour preference animals had *ad libitum* access to chow and water. All animals were provided with environmental enrichment (cardboard tube, plastic housing and chew block). Weight of the animals was monitored weekly.

All work was approved by the UK Home Office and was conducted in adherence to the regulations of the 1986 Animals (Scientific Procedures) Act and EU Directive 2010/63/EU.

2.2.2 Drug treatment

Corticosterone was purchased from Sigma Aldrich (UK). Cortisol was dissolved in 5% dimethyl sulfoxide (DMSO) and 95% sesame oil and a dose volume of 1 ml/kg was used. Treatments were administered through subcutaneous (s.c.) injection 30 minutes before pairing sessions.

2.2.3 Affective Bias test behavioural study

Apparatus: ABT experiments were carried out in a clear Perspex box arena with an area of 40 cm^2. A lining paper was laid down and replaced each day of testing. For substrate placement, two glazed bowls were placed at either side of the arena separated by approximately 2 inches.

A variety of materials were used for digging substrates, including hemp bedding, pompons, coconut fibres and shredded cardboard. After each ABT experiment, a fresh and new digging substrate combination was used.

Training: Animals had already been trained for the ABT prior these experiments. The training they received took place as explained in Table 2 (Stuart *et al.*, 2015).

Table 2. Training procedure for the rat affective bias test. Adapted from Stuart *et al.*, 2015.

Day 1: Arena habituation	Exploration of two bowls each containing 3 food pellets. The rat is allowed to approach both bowls and eat all the pellets. When all pellets are eaten the rat is removed, the bowls re-baited and the rat placed back into the arena. Criteria: total of 10 mins exploration.
Day 2: Digging training 1	Exploration of two bowls each containing 1cm sawdust and 3 food pellets (1 on surface and 2 buried). Each rat is allowed to dig in both bowls to find the pellets. When all pellets are eaten the rat is removed, the bowls re-baited and the rat placed back into the arena. Criteria: completion of 12 discrete trials (max. 2 min each trial)
Day 3: Digging training 2	Choice of two bowls each containing 2cm sawdust and 2 buried food pellets. Each rat is allowed to dig in one bowl to find the pellets. The other bowl is removed when the rat makes a choice (ie. Digs in a bowl). Criteria: completion of 12 discrete trials (max. 30 sec each trial).
Day 4: Digging training 3	Choice of two bowls each containing 2cm sawdust and 2 buried food pellets. Each rat is allowed to dig in one bowl to find the pellets. The other bowl is removed when the rat makes a choice (ie. Digs in a bowl). Criteria: completion of 12 discrete trials (max. 30 sec each trial).
Day 5: Novel substrate	Exploration of two bowls containing a novel digging substrate (eg. Mouse bedding) and 2 buried food pellets Each rat is allowed to dig in one bowl to find the pellets. The other bowl is removed when the rat makes a choice Criteria: completion of 12 discrete trials (max. 30 sec each trial).

Test protocol: Each ABT study took place over five days, consisting of four pairing days and a final test day. Throughout each run, three distinct substrates were used (Substrates A & B plus blank).

Rats experienced two distinct positive associations between two digging substrates, under neutral or affective state manipulation. To facilitate this, two distinct substrates are paired with the finding of reward. The rats formed this association during pairing days. At each pairing day, rats encountered one of the two reward paired substrates along with a “blank” substrate, which never hid a reward and was kept consistent during the test (Figure 8B). The reward paired substrates alternated across four days. The aim is for the rat to learn that to achieve the reward it must dig the reward paired substrates, and not the blank substrate. The reward is kept consistent (1 reward pellet) for both digging substrates (unless it is the 2 vs 1 ABT, see below) and across the pairing sessions.

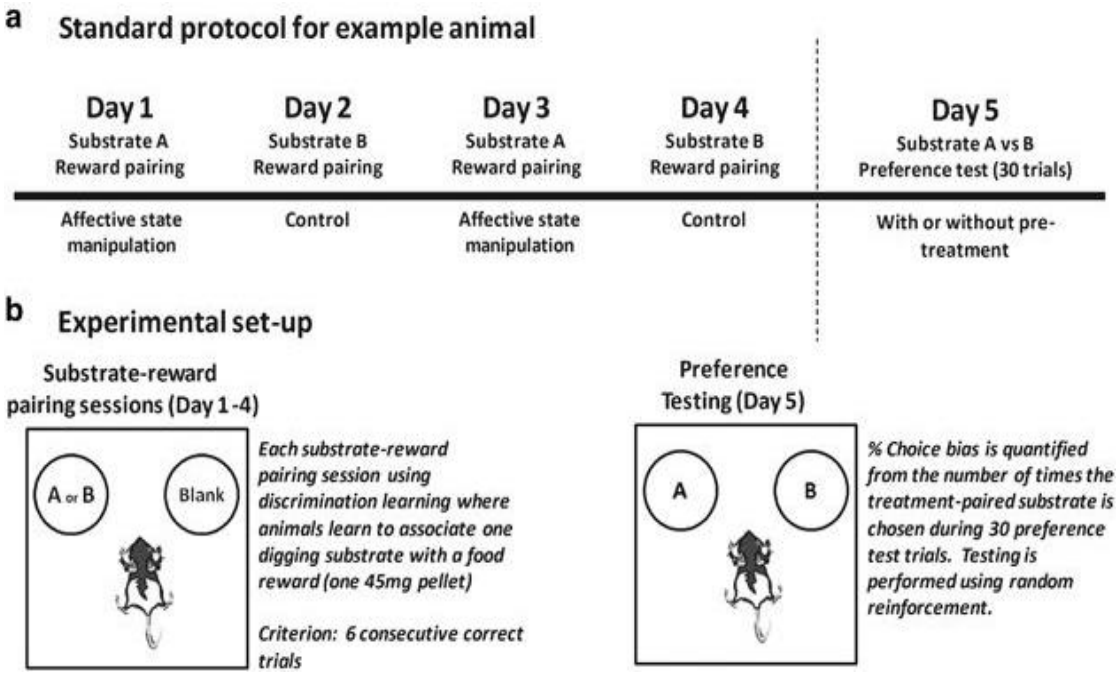

Figure 8. Affective Bias Test procedure. (A) Timeline of a single ABT study, consisting of four pairing days (two for each reward paired substrate) and a final test day (choice between the two reward-paired substrates). Treatment options here are either before pairing session with one of the rewarded substrates, shown here as substrate A, or before preference testing on choice day. (B) Set up for ABT for pairing sessions and choice day. Random reinforcement on choice day is achieved through pseudorandom pellet placement, where there is a 1 in 3 chance the rat will find the pellet. Taken from Stuart *et al.*, 2015.

After the animal had made their decision, judged by which bowl they began to dig first, the other bowl was removed and the latency to dig recorded. Once the animal had chosen the reward paired substrate six consecutive times their pairing day was complete. If an animal took longer than 20 seconds to decide this was counted as an omission, a maximum of twenty trials per animal was undertaken each pairing day.

The days on which the animals were paired with the affective state manipulated or control reward paired substrates would alternate over the four days. The position of the bowls was pseudorandom and counterbalanced to prevent the animals making associations based on direction/location and to ensure active participation of the task.

The choice day took place on day 5 when both previously reward-paired substrates were presented together for the first time and the rat was required to make a choice based on its prior memory of the substrate reward association (A or B). In this study the animals were not treated on choice day. The positions were again pseudorandomised. Unlike pairing days, the rat would undergo 30 consecutive trials. Bias was quantified relative to the affective state manipulated substrate with a positive bias seen when animals made more choices for the treatment-paired substrate versus control and vice versa for negative bias. As all other conditions except affective state were kept constant, any bias could be attributed to the affective state manipulation. Latency to dig was also recorded with a maximum of 10 seconds, anything longer was recorded as an omission. Random reinforcement using reward pellets was used to maintain the animal's interest in responding but limit any new learning.

Affective state manipulations: In the case of the 2 vs 1 ABT the manipulation consisted of an extra reward pellet during pairing sessions (not choice day) in order to induce a positive bias towards that substrate. To induce a negative bias, 10 mg/kg cortisol or vehicle was administered through s.c. injection 30 minutes before pairing sessions.

2.2.4 Flavour preference test

Apparatus: Flavour preference experiments took place in large clear plastic cages, distinct from those of residence. Sipper sacks were used to hold the liquid solutions in which metal sipping tubes were inserted. Once solutions needed to be changed the sacks were replaced and sipping tubes washed in distilled water. Due to the experimental procedure explained below, the bags would be changed after the four-day habituation period so that the subsequent sucrose free solutions on choice day would not be contaminated. Sipper sacks were replaced between experiments to avoid flavour contamination.

Approach

Habituation: Animals were place in the cages for 10 minutes with the two sipper sacks in place, one containing 0.1% saccharin and the other 0.1% saccharin with 10% sucrose. No flavours were added at this stage. Sipper sack position was fully counterbalanced. Over the habituation period a clear preference for the 10% sucrose solution developed (Figure 9). This resulted in a significant interaction between 10% saccharin solution consumption and trial (Greenhouse-Geisser corrected two-way repeated measures ANOVA, $F(1.89, 20.76) = 8.67$, $p = 0.002$).

Furthermore, Sidak corrected simple effects analysis statistically confirms the difference in consumption between the saccharin and saccharin/10% sucrose solutions appears from day 2 (mean increase 2.833 ml, 95% CI 1.790 to 3.877, $p<0.001$) and is maintained and increased from day 3 (mean increase 4.083 ml, 95% CI 2.197 to 5.969, $p = 0.001$) and day 4 (mean increase 5.333, 95% CI 3.572 to 7.096, $p<0.001$).

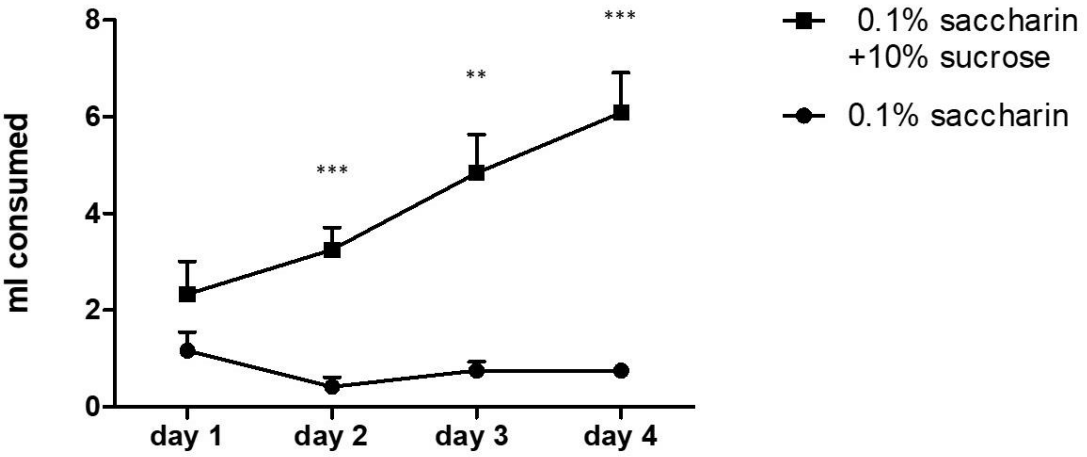

Figure 9. Flavour preference test habituation. Data shown as mean±SEM, n = 12. Two-way repeated measures ANOVA, Sidak corrected simple effects analysis, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

Test protocol:

To replicate the more highly rewarded substrate in the bowl digging 2 vs 1 ABT, one flavour was paired with a 15% sucrose solution whilst the less rewarded substrate contained 5% sucrose. The blank flavour was free of any sucrose. All flavours possessed 0.1% saccharin to maintain motivation on choice day where neither previously rewarded flavoured solutions included sucrose.

The rewarded flavours (A and B) were paired with rewarding sucrose solutions on alternating pairing days similar to the bowl digging ABT task. Here, the rat was exposed to the rewarded flavour along with the “blank” solution, which only contain 0.1% saccharin. Across the 4 days A or B would either be paired with 5% or 15% sucrose solution depending on the individual as flavour and reward allocation was fully counterbalanced. On the final choice day, the rat would choose between A or B, but this time the solutions only contained 0.1% saccharin in order to replicate the drive through random reinforcement also observed in the bowl digging ABT.

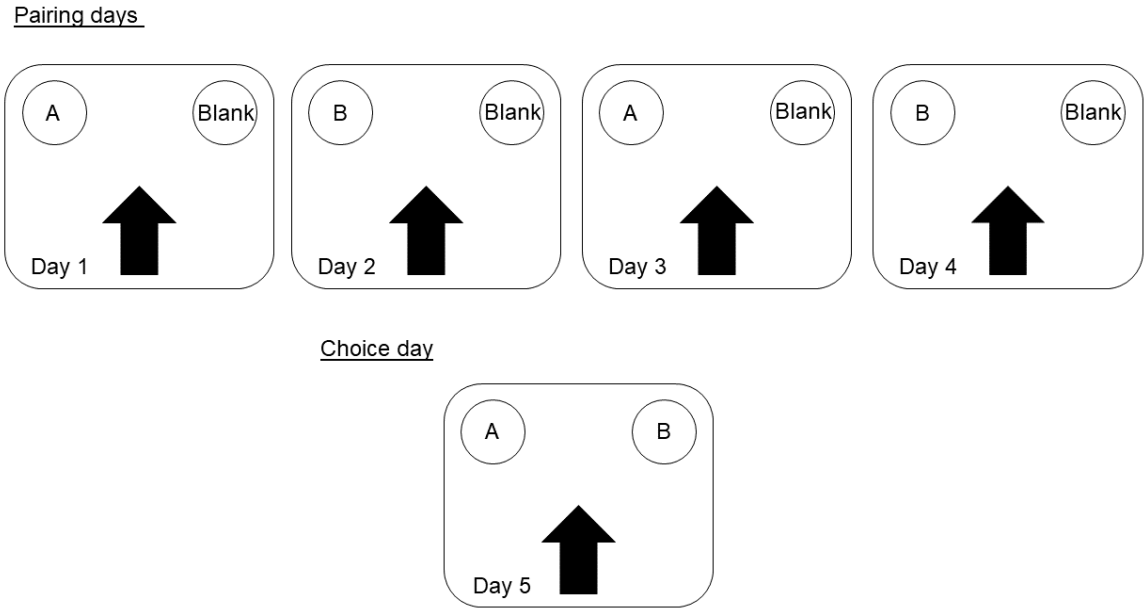

Figure 10. Environment layout of the flavour preference ABT. Synonymous with the bowl digging ABT, the flavour preference ABT utilises left and right positions of the associative memory cues. Note that position of flavours was fully counterbalanced.

2.2.5 Statistical analysis

For the bowel digging ABT, choice bias was calculated by subtracting 50 from the percentage of choices made for the affective bias manipulated substrate against the total number of trials (30). This gave a percentage choice bias. Similarly, for the flavour preference ABT, choice bias was assessed by calculating the percentage of the affective state manipulated flavour consumed against that of the total volume consumed and a value of 50 was then subtracted.

The experiments were assed using individual student *t*-test*s*. For the affective bias, directional bias and substrate bias results were tested against a theoretical mean of 0. To assess the number of pellets eaten results were tested against a theoretical mean of 10, to match the 1 in 3 probability of the rats finding the pellets by chance.

Unpaired student *t*-test*s* were also used for the flavour preference assay. During which, affective bias and flavour bias were compared against a theoretical mean of 0.

2.3 Results

2.3.1 Affective bias induction in ABT bowl digging task

The trials were assessed on an individual bases and individual *t*-tests were applied. A significant affective bias induction was only achieved during the third 2 vs 1 ABT attempt (one sample *t*-test, $t(15) = 3.656$, $p = 0.0023$, Figure 11A), which also displayed a significant substrate bias (one sample *t*-test, $t(15) = 3.656$, $p = 0.0023$, Figure 11B). A significant leftward bias was observed on choice day for trial 1 (one sample *t*-test, $t(11) = 4.729$, $p = 0.0006$) and trial 2 (one sample *t*-test, $t(15) = 2.145$, $p = 0.0487$). This was not observed in subsequent trials 3 & 4 (Figure 11C). The number of pellets eaten did not stray from expected value of 10 other than during ABT trial 2 (one sample *t*-test, $t(15) = 3.313$, $p = 0.0047$, Figure 11D).

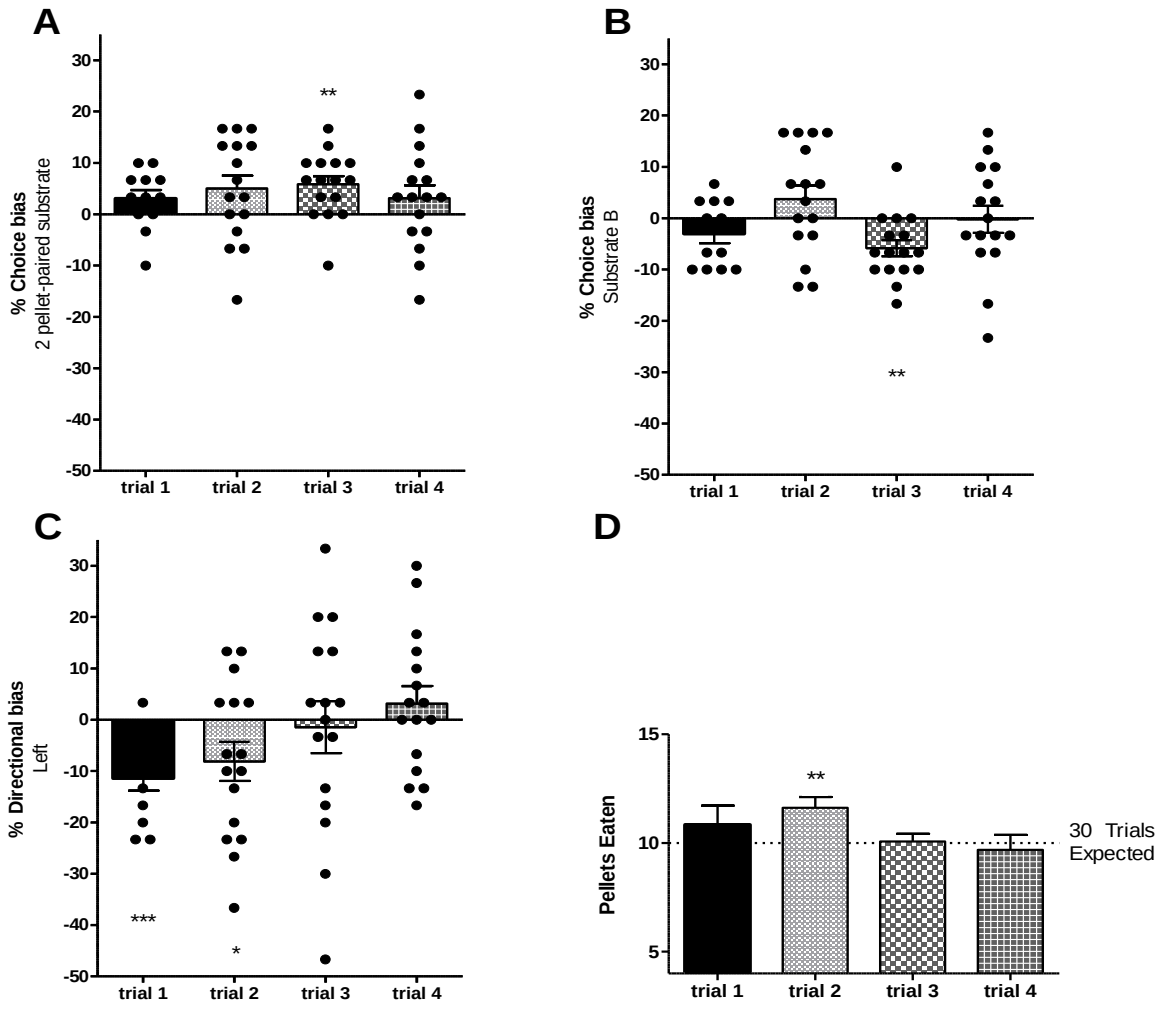

Figure 11. Results for the 2 vs 1 ABT. A) Affective bias index. B) Substrate bias. C) Directional bias. D) Number of pellets eaten. Data shown as mean±SEM, Trial 1 n = 12, Trials 2, 3 & 4 n = 16. Unpaired student *t*-test, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

No bias was observed after attempt to induce a negative bias through a cortisol paired substrate (Figure 12). Furthermore, a lack of substrate choice, directional or substrate bias was also recorded (Figure 12). The number of pellets eaten was not significantly different from that of the expected value.

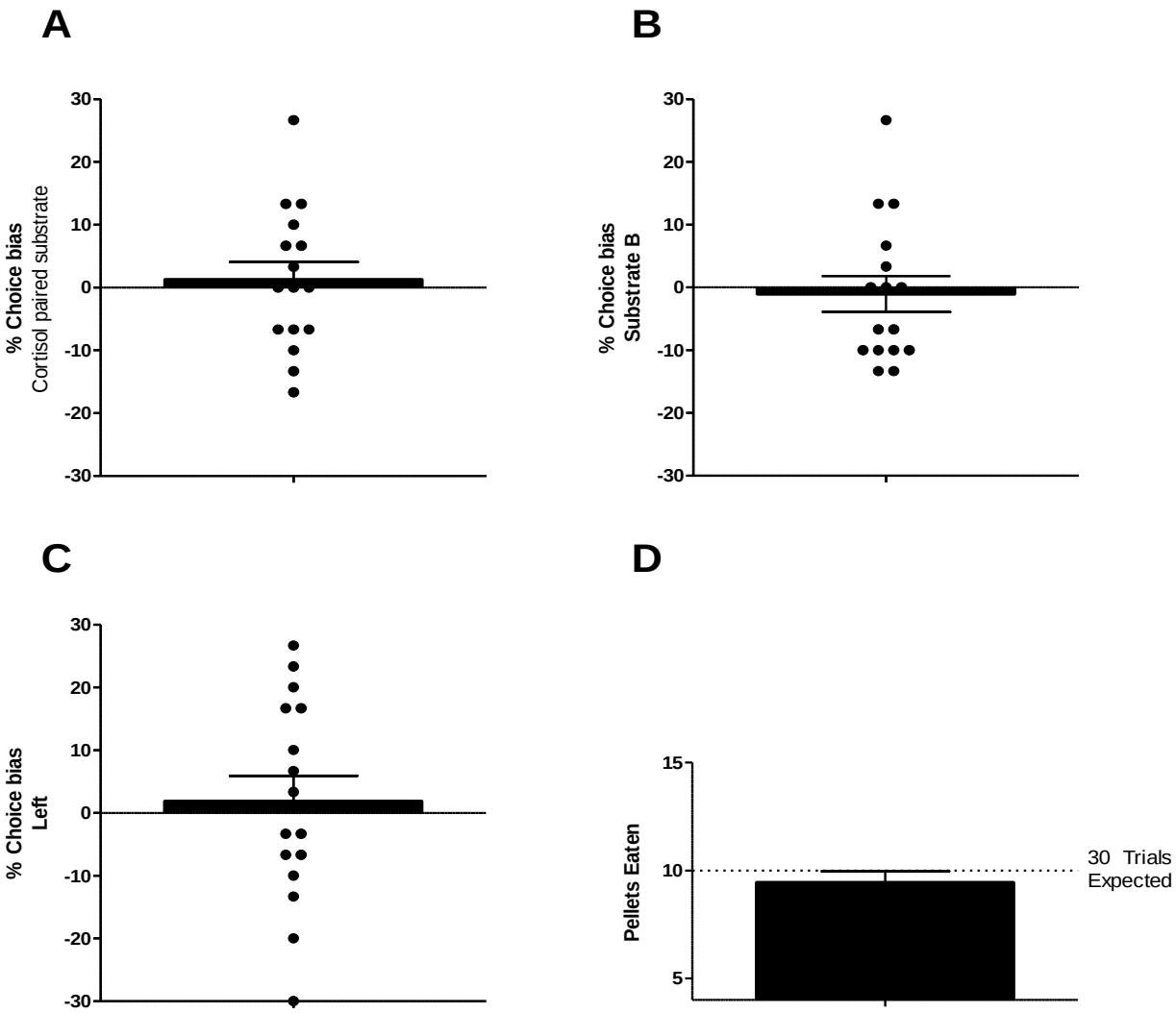

Figure 12. Lack of influence of cortisol on the bowl digging ABT. 10 mg/kg cortisol was administered 30 minutes prior pairing sessions with the cortisol-paired substrate through i.p. injection. Data shown as mean±SEM, n=8.

To review potential causes for the lack of a reliable affective bias, the trial to criterion data for the pairing sessions was reviewed. From this, it appears that by the second pairing session, the animals were able to locate the reward pellet within 6-7 consecutive trials (Figure 13).

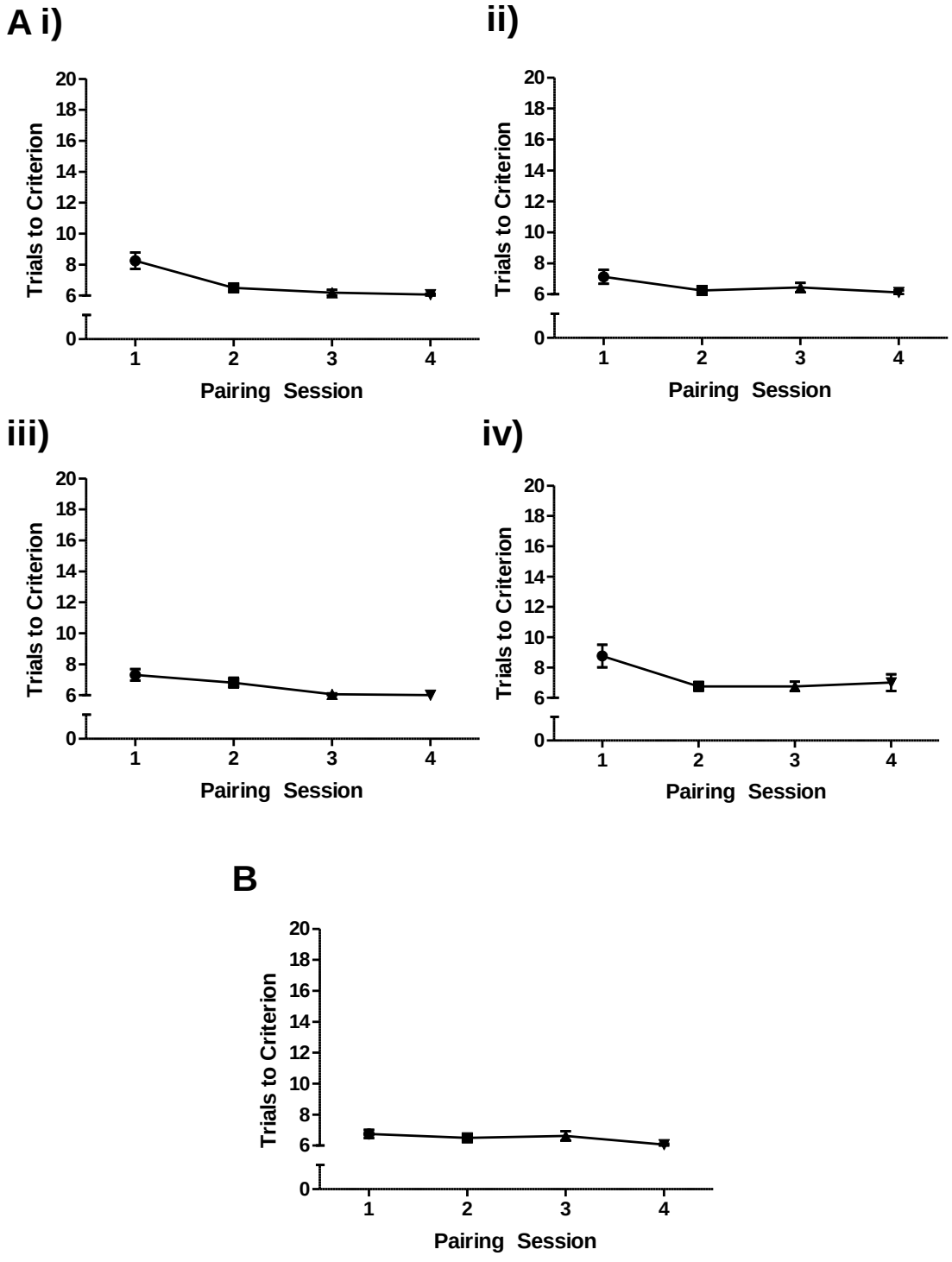

Figure 13. **Number of trials undertaken to reach criterion during bowl digging ABT.** A) 2 vs 1 ABT, i) trial 1, ii) trial 2, iii) trial 3, iv) trial 4. B) Cortisol ABT. Data shown as mean±SEM, Trial 1 n = 12, Trials 2, 3 & 4 n = 16.

2.3.2 Positive bias induction in the flavour preference ABT

Pairing of a 15% sucrose solution with a distinct flavour resulted in the induction of a positive bias as measured by solution consumption (Figure 14A & B). This was significant in both attempts, undertaken by the author (one sample *t*-test, $t(10) = 2.970$, $p = 0.0140$, Figure 14A) and by Bartlett J (one sample *t*-test, $t(10) = 2.948$, $p = 0.0146$, Figure 14B). No significant flavour bias was observed in either of the tests (Figure 14A & B).

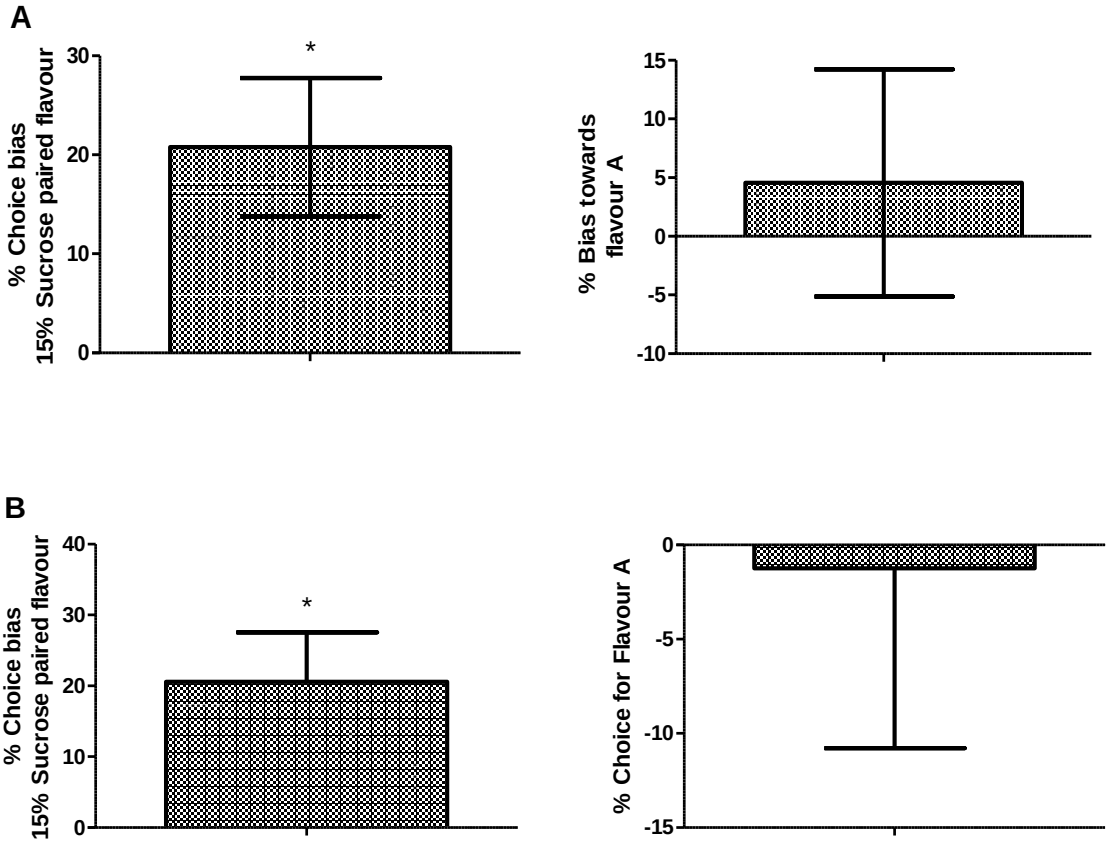

Figure 14. 2vs1 bias in flavour preference ABT. A) Conducted by the author B) Work of J. Bartlett. A positive bias towards the 15% sucrose paired solution induced in both trials, free of any flavour bias. Data shown as mean±SEM, n=12. Analysed using students *t*-test, *p* = *<0.05.

2.4 Discussion

2.4.1 The flavour preference ABT avoids technical difficulties encountered in the bowl digging ABT

We successfully induced a positive bias in the flavour preference ABT using flavour preference for associating the unconditioned and conditioned stimulus. We suggest the cause of the dissimilarity between the results gained from the bowl digging ABT and flavour preference ABT is due to reduced experimenter-animal interaction as the flavour preference ABT does not provide as much opportunity for the induction of unintentional biases in the task. For example, the sipper sacks are positioned prior to the placement of the rats into the test cages. This not only makes the task faster and easier to undertake but reduces the interaction between the experimenter and the animal therefore minimizing the opportunity for them to influence each other's behaviour and judgement. The flavour preference therefore offers an easier alternative to the bowl digging ABT which is more accessible to less experienced handlers.

Inducing a flavour preference using sugars is a well-established method to study animal behaviour (Dwyer and Quirk, 2008; Touzani, Bodnar and Sclafani, 2013). However, ours is the first to apply this to the study of affective bias. By removing the reward (sucrose) on choice day, the bias observed (Figure 14A & B) likely results from the animal's associative memory and is not influenced by satiety which can affect consumption (Sclafani, 2002). However, a reward induced bias cannot be directly compared to an affective bias, thus further tests on the flavour preference ABT with pharmacological affective bias manipulators are required.

Therefore, before further assumptions are made about the flavour preferences reliability, it should undergo similar pharmacological test as that performed in Stuart *et al.*, 2013 and Hinchcliffe *et al.*, 2017 to determine if its predictive validity matches that of the ABT. Despite these drawbacks, the flavour preference test has shown promise as another method for investigating affective biases in rodents. This therefore offers an additional approach to study cognitive bias in rodents, alongside the bowl digging ABT.

The flavour preference ABT may therefore prove an additional behavioural tool to investigate ketamine's mechanism of action. As flavour preference has been suggested to involve regions implicated in depression such the amygdala and PFC (Malkusz *et al.*, 2012), studying low subanaesthetic doses of ketamine at 1 mg/kg could consolidate the findings of ketamine's effect on the PFC in Stuart *et al*. 2015. However, considerations must be made when using the flavour preference test to investigate ketamine. For example, NMDAR neurotransmission in the amygdala has been shown to be necessary for flavour preference acquisition (Touzani, Bodnar and Sclafani, 2013), consistent with the NMDARs role in LTP and learning. Thus,

when testing ketamine's effect on affective bias at the time of information processing, a memory ameliorating effect must not be confused with attenuating affective bias.

2.4.2 Inability to induce affective bias in the ABT likely results from practical difficulties

As no positive bias with increased sugar pellet reward or negative bias with 10 mg/kg cortisol injection was induced (Figure 11 & 12) we did not replicate the results found by Hinchcliffe *et al.*, 2017. Review of the pairing day data across both the 2 vs 1 and cortisol experiments demonstrated that by the second pairing day the animals were able to achieve 6 consecutive trials with some success. However, no bias was observed on choice day. This suggests that the animals did not use associative memory to locate the pellets on pairing days. As the number of pellets eaten was not consistently above that of the expected value of 10 (Figure 11D & 12D), it is unlikely the animals used olfactory cues to locate the sugar pellets on choice days.

The animals used in the study had been previously trained and run in the ABT with reliable results and so the failure to replicate this was most likely attributed to the experimenter. One of the challenges of this experiment is the judgement of what constitutes a committed dig, and thus choice of the rat. To those new to the task this can present an opportunity for error as it can be difficult to judge whether the dig is due to exploratory behaviour which precedes their decision or is a result of the decision itself. Incorrect judgement of the rat's decision will therefore disrupt the formation of an affective bias and result in the rats using other strategies to locate the pellet.

2.4.3 Conclusions

Unlike the bowl digging ABT, a positive bias towards the higher value reward flavour was achieved using a flavour preference version of the ABT (Figure 14). This lends support to the notion that affective biases can be studied in rodents using this more efficient approach. Additionally, reduced experimenter-animal interactions decrease the risk of human induced error. However, as an appetitive bias is not synonymous with affective bias, this task must be validated with other affective state manipulations.

Overall, the flavour preference test offers a potential supplementary experiment with which to study affective bias in animals. The focus should now shift towards using these tasks to gain more insight into the cognitive effect of ketamine and how this relates to its AD mechanism. For example, the cognitive influence on affective bias needs to be tested the day after, not just straight after injection to determine whether the influence on cognitive bias lasts as long as its effect on the conventional FST (Autry *et al.*, 2011; Zanos *et al.*, 2016).

Chapter 3

Effect of ketamine on rat prefrontal cortex c-fos expression

3.1 Introduction

As discussed, Stuart *et al.*, 2015 found a cognitive effect of ketamine at an i.p. dose of 1 mg/kg, an effect attributed to actions at the PFC after replication of the result by direct infusion into the PFC. This effective dose of 1 mg/kg is the lowest dose used in an *in vivo* depression study as most publications use doses of 10-25 mg/kg in rodents (Autry *et al.*, 2011; Zanos *et al.*, 2016). As ketamine's AD effects manifest at low subanaesthetic doses (Berman *et al.*, 2000; Zarate *et al.*, 2006a) a 1 mg/kg dose may more closely resemble the doses used in clinical trials due to potential motor side effects which can occur between 10-25 mg/kg in rats (Qian, Brown and Carlton, 1996).

Low subanaesthetic doses of ketamine exert an excitatory influence at the PFC which has been associated with its AD effect (Li *et al.*, 2016). In support, subanaesthetic doses of ketamine have been demonstrated to elevate cortical glutamate in both humans (Stone *et al.*, 2012) and rats (Kim *et al.*, 2011), an effect that has been attributed to disinhibition of inhibitory GABAergic interneurons resulting in increased pyramidal activity (Homayoun and Moghaddam, 2007). Due to the association of PFC excitation and the AD effect of subanaesthetic ketamine the characterisation of the neurobiological effects of 1 mg/kg ketamine at the rat PFC is warranted.

The rat prefrontal cortex is a composite of sub regions including the cingulate (CG), infralimbic (IL) and prelimbic (PL) regions. These regions have been directly connected to ketamine's AD mechanism, as direct infusions into the rat IL cortex produce antidepressant effects also replicated by optogenetic stimulation (Covington *et al.*, 2010; Fuchikami *et al.*, 2015). Furthermore, the rat anterior cingulate cortex, a region involved with cognitive processes such as decision making (Kennerley *et al.*, 2006) and causes pro depressant effects in the FST upon lesioning (Bissiere *et al.*, 2006).

We therefore set out to investigate how the dose of 1 mg/kg ketamine influenced regional activation of the rat PFC. By monitoring activation of the IL, PL, and CG regions we sought to identify AD associated effects, whilst also monitoring activation of the motor cortex (Mt) to identify non-specific motor side effects. This was compared against its relative PCP at 0.3 mg/kg. The aim of this being to discern any differences between the two drugs, which might identify characteristics which give ketamine its AD properties. We also investigated a dose dependent influence at 1 mg/kg, 10mg/kg and 25 mg/kg to differentiate 1 mg/kg from these higher doses to again determine characteristics that provide 1 mg/kg ketamine's AD effects.

Our method of measuring neuronal activity was through analysing expression of c-fos protein, a product of a proto-oncogene whose expression is linked to neuronal action potentials

(Okuno, 2011). To investigate the role of GABAergic interneuron dishinbition in the effect of 1 mg/kg ketamine and 0.3 mg/kg PCP, we recorded glutamate decarboxylase 67 (GAD67)/c-fos colocalization in the PFC. GAD67 is an enzyme used to convert glutamate to GABA and is thus associated with the spatial location of GABAergic interneurons.

3.2 Methods

3.2.1 Animals and dosing methods

For the experiments comparing 1 mg/kg ketamine against 0.3 mg/kg PCP, 16 male lister hooded rats were used (~500–550 g, Harlan, UK). Another 16 male lister hooded rats (~300-350g, Harlan, UK) were used for the dose response experiment.

Animals were housed in pairs and all experimental work was conducted within a 12:12 h light–dark cycle (lights off at 0700h), between 9 am – 5pm. All animals had *ad libitum* access to laboratory chow and water and were provided with environmental enrichment (cardboard tube, plastic housing and chew block). Weight of the animals was monitored weekly.

All work was approved by the UK Home Office and was conducted in adherence to the regulations of the 1986 Animals (Scientific Procedures) Act and EU Directive 2010/63/EU.

3.2.2 Drug treatment

Both ketamine and PCP treatments were made with sterile 0.9% saline solution using a dose volume of 1 ml/kg. Ketamine used was purchased from Sigma Aldrich. PCP was purchased from Tocris, UK. Treatments were administered through i.p. injection 90 minutes before killing. Animals were killed through barbiturate overdose.

In the ketamine vs PCP experiment, 6 animals received 1 mg/kg ketamine, another 6 received 0.3 mg/kg PCP and the final 4 received saline control. During the ketamine dose experiment ketamine was administered at 1 mg/kg, 10 mg/kg and 25 mg/kg along with saline control. In this experiment 4 animals were allocated to each treatment group.

3.2.3 Immunohistochemistry

Tissue preparation: Animals were killed 90 minutes post drug/vehicle delivery using intraperitoneal barbiturate overdose. Once death was confirmed through cessation of breathing, and lack of response to paw/ankle pinch, brains were extracted and post-fixed in 4% paraformaldehyde (PFA) for 24 hours before being placed in 30% sucrose solution. Once brains had sunk in the sucrose solution the brains were cut using a freezing microtome into 40 µm sections. Using The Rat Brain in Stereotaxic Coordinates (Paxinos & Watson, 1982), neural landmarks such as the forceps minor were used to identify regions of interest, to ensure correct collection of the prefrontal cortex. Once sections were cut, they were placed in

cryoprotectant and stored at -20 degrees Celsius. Cryoprotectant was composed by adding 300g sucrose and 300ml ethylene glycol to 500ml of 0.1M phosphate buffer and making up to 1L with distilled water.

Immunohistochemistry protocol: The protocol used was a standard 2-day protocol with all steps taking place at room temperature.

- Day 1: Once removed from the freezer, sections underwent 3 consecutive five-minute washes with phosphate buffered saline, 0.2% Triton X (PBS-T). Subsequently, sections were incubated in blocking solution (3% donkey serum and 2% bovine serum albumin) for 30 minutes. Sections were then incubated with primary antibody in Immunobuffer I (2% Donkey serum in PBS-T) in the fridge overnight after 10 minutes on a rocker.

- Day 2: After incubation with primary antibody sections underwent three rounds of five-minute washes in PBS-T. From this point on the experiment was performed in darkness. After completion of washes, sections were incubated with secondary antibody in Immunobuffer I for 3 hours on a rocker at room temperature. Once incubated, sections were washed three times, each for five minutes, in phosphate buffer solution (pH of 7.4) and then incubated with 300 nM 4′,6-diamidino-2-phenylindole (DAPI) solution for five minutes. Sections were then washed a further three, five-minute washes in PBS solution before being floated in phosphate buffer to enable mounting on uncoated microscope slides. Sections were then left to dry for a couple of minutes before fluorescent mounting media was added, and coverslip placed. These were then sealed with nail varnish and placed on paper towels and carefully placed in the fridge dry.

Optimisation and antibody concentrations c-fos single stain:

- Optimisation of c-fos antibodies: For c-fos cell staining, a dilution factor of 1:2000 of the ABE457 rabbit anti c-fos antibody was used. This was determined from a pilot study where three concentrations were used in different wells (Figure 15). The optimum concentration for the primary antibody was determined to be a 1:2000 dilution as the amount of c-fos staining was not increased at a concentration of 1:1000 whilst being absent at the lower concentration after a 1:5000 dilution. Additionally, background fluorescence was more prominent at 1:1000 dilution of primary antibody.

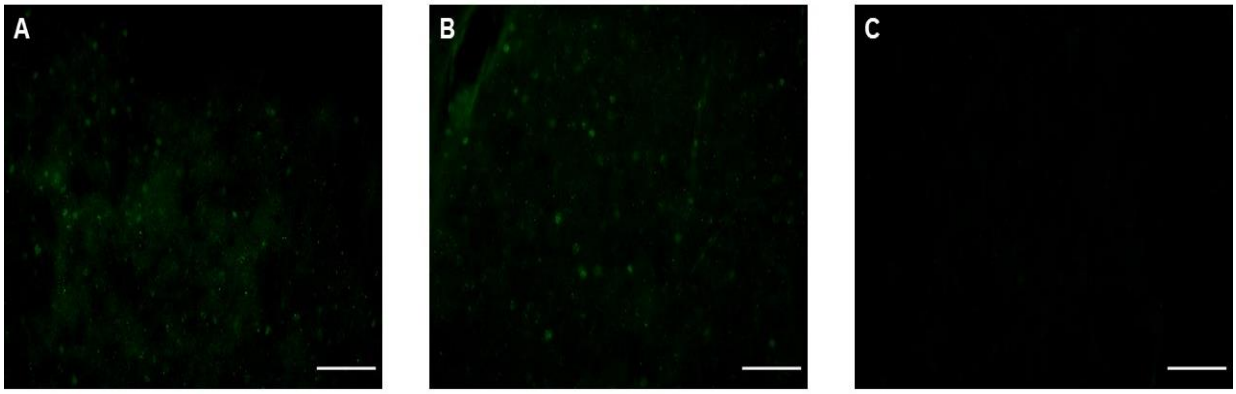

Figure 15. Optimisation of primary c-fos antibody (ABE457 rabbit anti c-fos). Images are of c-fos staining in rat prefrontal cortex in the prelimbic region, at x20 magnification. A) 1:1000 dilution of ABE457 rabbit anti c-fos. B) 1:2000 dilution of ABE457 rabbit anti c-fos antibody. C) 1:5000 dilution of ABE457 rabbit anti c-fos. All images above had a secondary antibody (donkey anti-rabbit Alexa Fluor 488) concentration of 1:500. Scale bars = 100 µm.

- Optimisation of secondary antibody (donkey anti-rabbit Alexa Fluor 488) concentration: Subsequently, a lower concentration of secondary antibody of 1:1000 dilution was found to be successful (Figure 16). Resulting in all subsequent experiments using this dilution factor as the same amount of fluorescent staining was found with reduced background fluorescence.

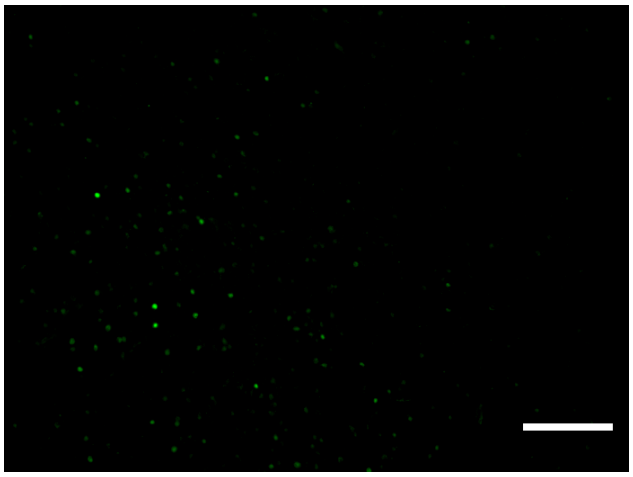

Figure 16. Optimisation of concentration for the donkey anti-rabbit Alexa Fluor 488 antibody (secondary antibody for c-fos staining), found at a dilution of 1:1000. Scale bar = 100 µm.

Antibody optimisation GAD67/c-fos stain:

- The double staining protocol followed the same process as the single staining protocol. During double staining, the respective antibodies were simply mixed together and then applied to the sections.
- Improved c-fos and GAD67 staining were achieved at dilutions of 1:1000 and 1:2500, respectively (Figure 17A & B) than at 1:2000 and 1:5000 (Figure 17D & E). Both secondary antibodies were applied at 1:1000.

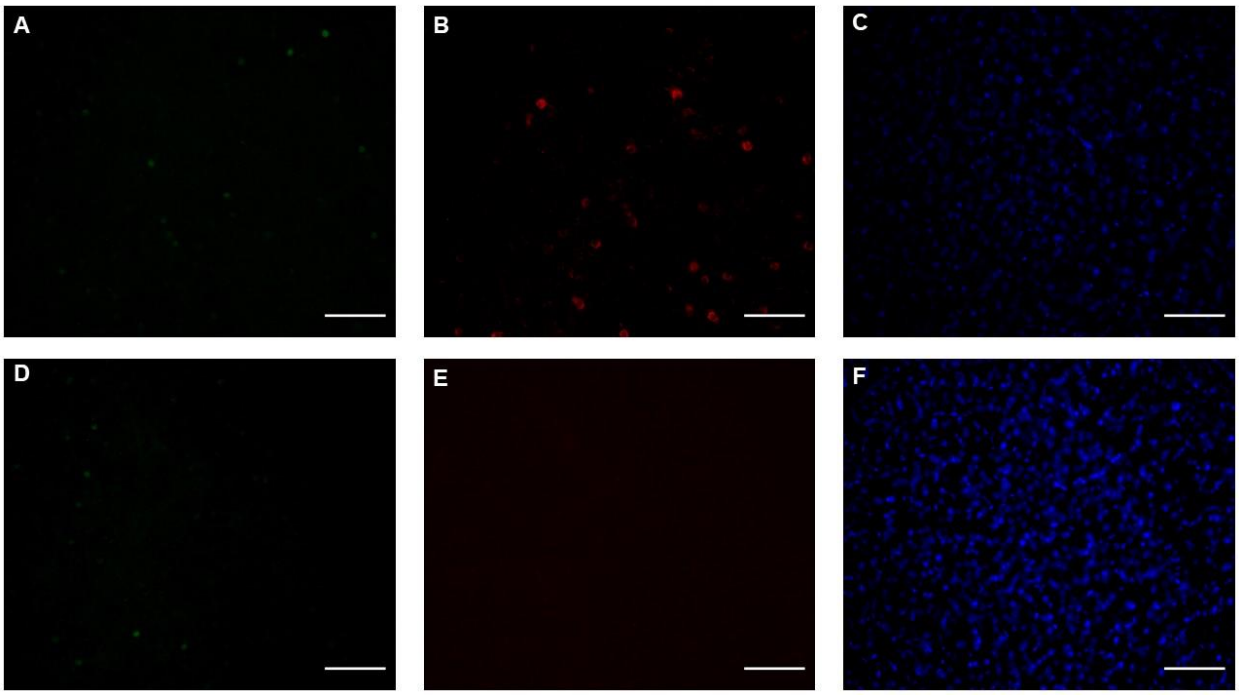

Figure 17. Optimisation of GAD67/c-fos double staining protocol. A) C-fos and B) GAD67 and were found at dilutions of 1:1000 for the c-fos primary and 1:2500 for GAD67. D) C-fos and E) GAD67 were found at 1:2000 for the c-fos antibody and 1:5000 for the GAD67 antibody. Both DAPI stains C) & F) were achieved at 300nM, as per the manufacturer's instructions. Secondary antibodies were applied at 1:1000. Scale bars = 100 µm.

Image analysis:

C-fos positive cell counting: C-fos count images were taken on the widefield 6 microscope, whereas GAD67 double staining photos were taken on a confocal microscope (University of Bristol Wolfson Bioimaging Facility). Pictures were taken at x10 magnification, with all settings consistent across all pictures. Photos were taken across 3 stereotaxic levels, on both left and right hemispheres, with an average being taken at the end. Position of images was kept consistent throughout, as shown in Figure 18.

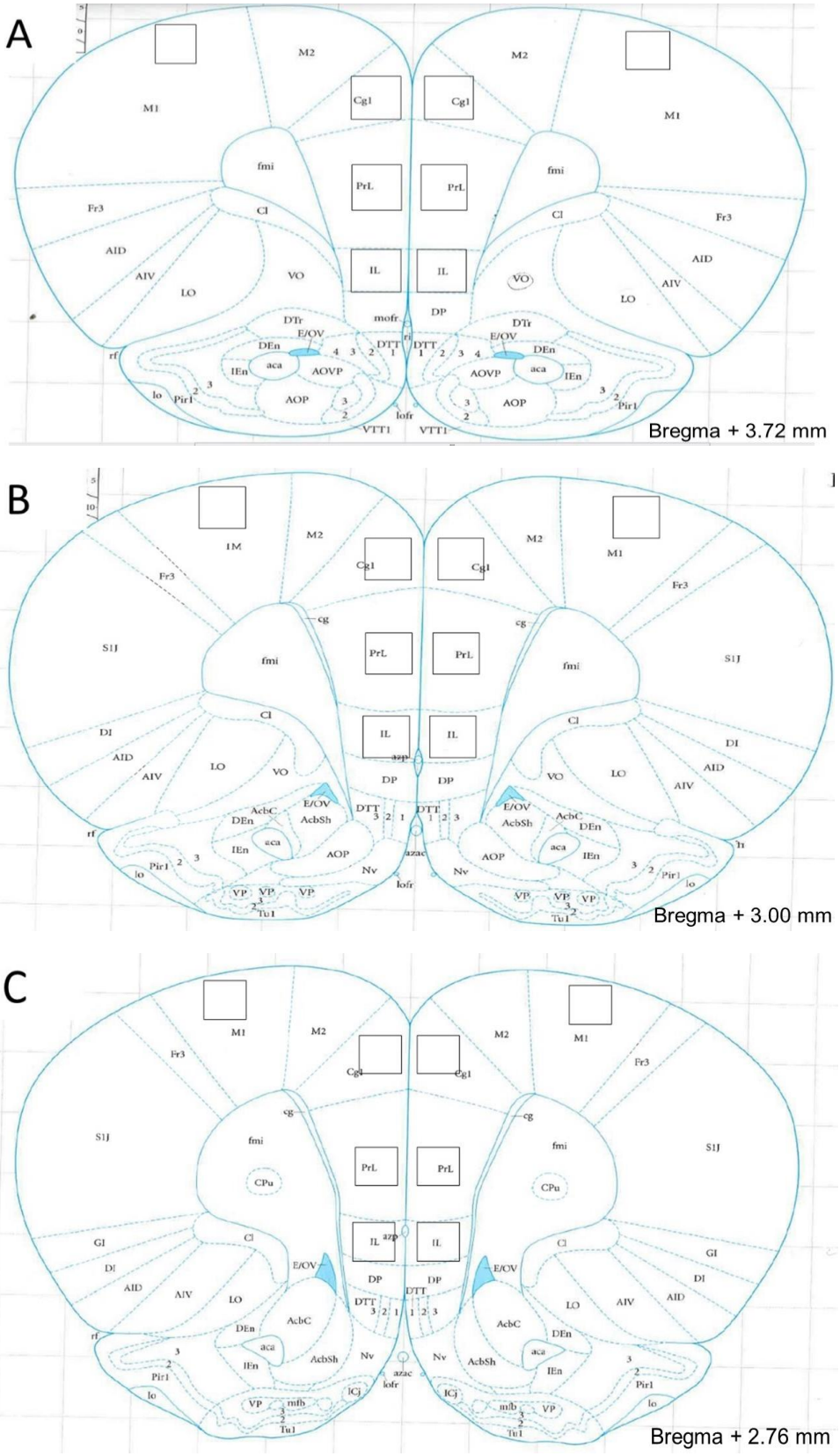

Figure 18. Location of microscope images taken from tissue sections. This was across designated stereotaxic level 1 (A), level 2 (B) and level 3 (C). Scanned from Paxinos & Watson, 1982.

Cellular counts were then undertaken using FIJI (FIJI Is Just ImageJ) software, using the cell counter plugging. DAPI staining was then used to help cellular identification through the merge channels function. Occasional adjustment of brightness and contrast were used to aid the manual counting of cells.

3.2.4 Statistical analysis

The primary statistical test applied was a mixed model ANOVA, with Sidak corrected *post hoc* analysis. Sidak simple effects analysis was used for specific comparisons between PFC sub regions and treatments.

3.3 Results

3.3.1 Effect of ketamine and PCP on PFC c-fos expression

Due to the number of slides lost (missing values shown in Table 3) from the first experiment no reliable statistical test could be applied and thus the data from this pilot are solely preliminary. Whilst marginal, our pilot suggested that 0.3 mg/kg PCP appears to increase c-fos expression more than 1 mg/kg ketamine (Figure 19). The first c-fos staining experiment conducted, on which no statistics were performed, shows a numerical increase in mean c-fos count of the drug treated animals compared to vehicle control. At the IL, ketamine caused a 23.30% increase in c-fos expression, whereas PCP caused a 71.66% increase. Increased c-fos expression at the PL appeared more pronounced, with ketamine and PCP leading to a 71.70% and 132.08% increase, respectively. Therefore, 0.3 mg/kg PCP potentially stimulates more c-fos expression at the IL and PL than 1 mg/kg ketamine. Due to number of missing values we undertook a repeat of this experiment.

Table 3. Raw data from c-fos immunostaining pilot experiment. Due to issues with slide mounting many slides were unable to be photographed. Due to this we were unable to apply a reliable statistical test.

	Counts per mm2															
	JP4-1	JP4-2	JP4-3	JP4-4	JP4-5	JP4-6	JP4-7	JP4-8	JP4-9	JP4-10	JP4-11	JP4-12	JP4-13	JP4-14	JP4-15	JP4-16
lvl1-IL-L	72	114	0	75	21	52	52	131	64			77	51		72	
lvl1-IL-R	83	137	0	145	34	0		127	95			114	96		57	
lvl1-PL-L	73	103	0	111	38	49	62	237	67		117	57	98		78	93
lvl1-PL-R	75	127	0	147	46			181	78		116	70	126		96	91
lvl2-IL-L	98	121	199	69			85		44	38	104	75				
lvl2-IL-R	93	116	227	93				129	36		93	69				
lvl2-PL-L	145	100	235	126	23		101	122	33	64	111	80				
lvl2-PL-R	134	88	214	113	8			145	47		65	65				
lvl3-IL-L	129	60	147	88	31				65		57	113				
lvl3-IL-R		131	144	54	8	59			57		51	132				
lvl3-PL-L	90	129	183	67	0						88	108				
lvl3-PL-R		135	201	86	10						82	104				

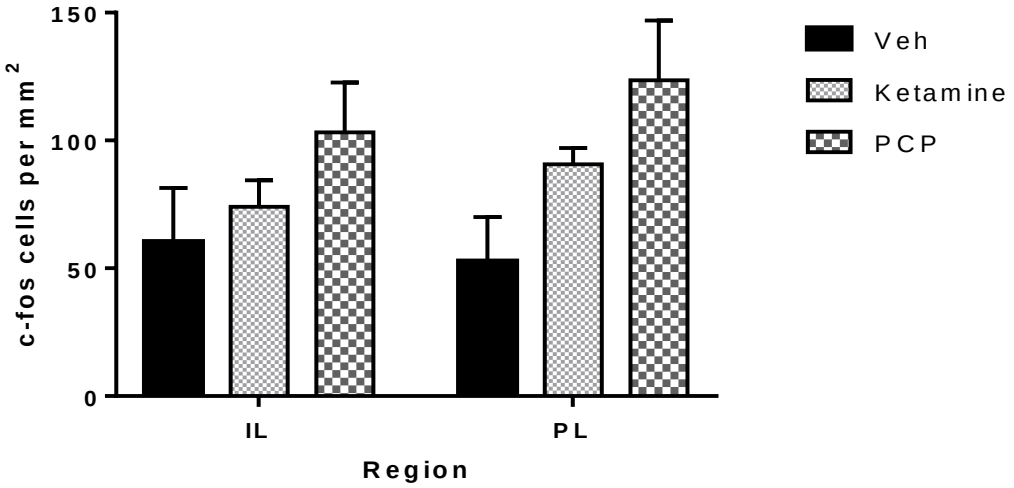

Figure 19. Pilot study for c-fos expression experiment. Expression was measured at the infralimbic (IL) and prelimbic (PL) cortices after i.p. administration of 1 mg/kg ketamine (n = 6), 0.3 mg/kg PCP (n = 6) or saline control (n = 3) at 90 minutes before sacrifice. Data shown is average c-fos count ±SEM.

The repeat of this experiment yielded a full and more reliable dataset. From this, a similar trend of a numerical increase in c-fos after treatment was observed (Table 4), although these were not significant, and therefore do not prove ketamine or PCP increased c-fos expression (Mixed Model ANOVA, F (2, 13) = 3.660, p = 0.055, Figure 20). There was therefore no interaction between drug and region (Mixed Model ANOVA, F (6, 39) = 1.688, p = 0.195, Figure 20).

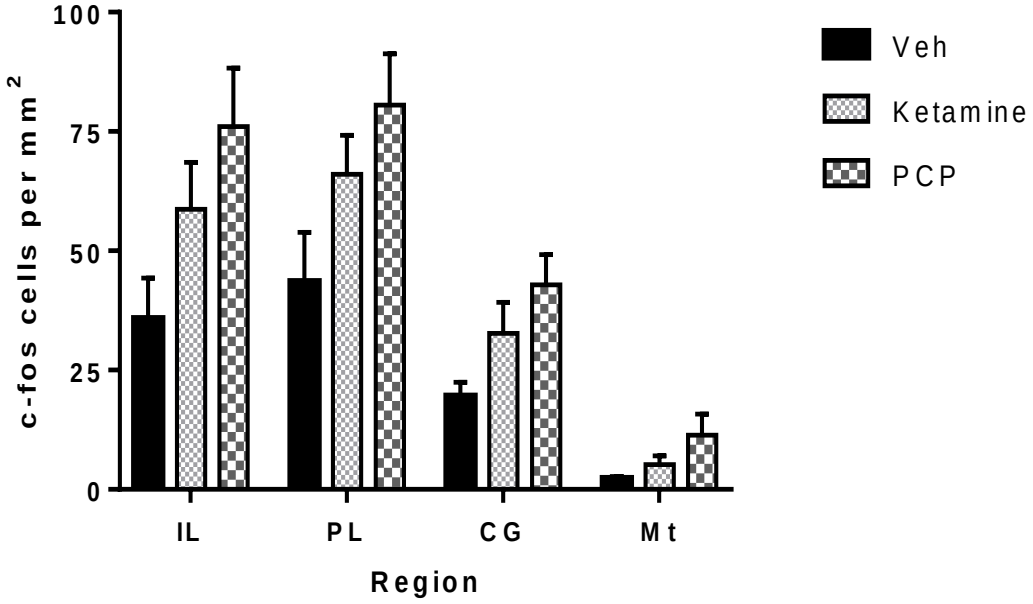

Figure 20. Effect of 0.3 mg/kg PCP and 1 mg/kg ketamine on c-fos expression at the rat PFC. Expression was measured at infralimbic (IL), prelimbic (PL), cingulate (CG) and motor (Mt) regions of the prefrontal cortex after i.p. administration of 1 mg/kg ketamine (n = 6), 0.3 mg/kg PCP (n = 6) or saline control (n = 4) at 90 minutes pre-termination. Data shown is average c-fos count ±SEM.

However, there is an arguably marginal effect, and if repeated with a larger n number significance may be reached. Indeed, statistical power was limited by the uneven group number due to the smaller control vehicle group (n = 4). Although, both 1 mg/kg ketamine and 0.3 mg/kg PCP lead to a numerical increase in c-fos expression at all four investigated regions.

Table 4. Comparison of the effect of 1 mg/kg ketamine and 3 mg/kg PCP on c-fos expression.

Comparison of effect of ketamine and PCP on PFC c-fos stimulation

Region	Comparison	Mean difference	Difference
IL	Ket vs Veh	22.917	64%
	PCP vs Veh	40.250	112%
	PCP vs Ket	17.333	29%
PL	Ket vs Veh	22.5	51%
	PCP vs Veh	37.0	84%
	PCP vs Ket	14.500	23%
CG	Ket vs Veh	13.167	69%
	PCP vs Veh	23.333	123%
	PCP vs Ket	10.167	31%
Mt	Ket vs Veh	2.917	146%
	PCP vs Veh	9.083	454%
	PCP vs Ket	6.167	123%

Out of the drug treatments, PCP appeared to stimulate more c-fos expression than ketamine as it caused a larger increase in mean c-fos expression across all regions, although this is a very preliminary result due to the lack of a main effect of treatment. Although, a significant difference when compared to vehicle was almost achieved (*posthoc* analysis, Sidak corrected, $p = 0.053$), more so than that of ketamine (*posthoc* analysis, SIDAK corrected, $p = 0.394$).

A consistent finding across both c-fos staining experiments was a difference in regional c-fos expression, regardless of treatment. This result was highly significant in the complete c-fos run (Mixed model ANOVA, $F(3, 39) = 71.714$, $p < 0.001$, Figure 20). Sidak corrected *posthoc* analysis revealed that all regions significantly differed at a significance of $p < 0.001$ apart from IL and PL regions (Sidak corrected *post-hoc* analysis, $p = 0.180$). However, regional c-fos expression was not affected by any of the treatments, as shown by the lack of drug/region interaction (Mixed Model ANOVA, $F(6, 39) = 1.688$, $p = 0.195$, Figure 20).

Although a significant increase in c-fos was achieved using separate independent samples *t*-tests at the IL ($p = 0.044$), PL ($p = 0.048$) and CG ($p = 0.023$) whilst no significant increase was achieved at the Mt ($p = 0.141$). Although these separate *t*-tests should not be considered as a final analysis due to the lack of control over type I errors.

3.3.2 Effect of different doses of ketamine on PFC c-fos expression

Overall, results gained from the dose response experiment showed a large amount of variance (Figure 21) and no significant effect of ketamine dose was found (Mixed model ANOVA, $F(3, 8) = 1.073$, $p = 0.413$). Whilst there appears to be a dose dependent increase in c-fos expression at the IL, PL and CG this should be interpreted with caution due to the large variance of the data, particularly that of the vehicle control and 1 mg/kg ketamine groups (Figure 21). Interestingly, an increase in c-fos expression at the Mt only seems to occur at the 25 mg/kg ketamine dose, although a repeat experiment would be required to confirm this due to the lack of any robust and reliable effect.

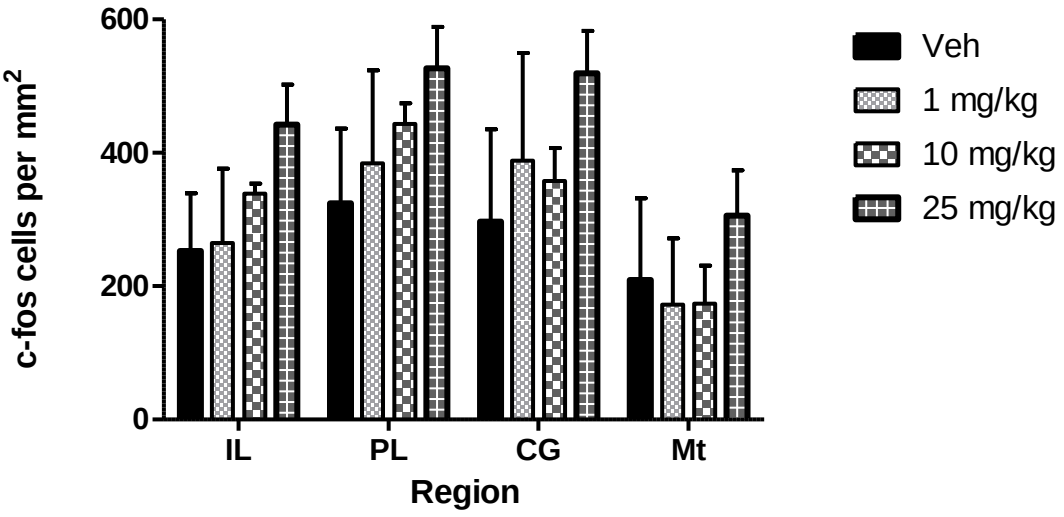

Figure 21. Ketamine dose experiment on PFC c-fos expression. C-fos expression was investigated at the infralimbic (IL), prelimbic (PL), cingulate (CG) and motor (Mt) regions of the rat prefrontal cortex after i.p. injection of 1 mg/kg, 10 mg/kg and 25 mg/kg ketamine with saline control (n = 4). Data shown is average c-fos count ±SEM.

Interestingly, there was an unforeseen effect of animal batch on c-fos expression which had a significant effect on c-fos expression between subjects (Mixed model ANOVA, F (1, 8) = 8.340, p = 0.020). There was also a significant interaction between batch and region (Mixed model ANOVA, F (3, 24) = 4.326, p = 0.014) and will have therefore influenced the c-fos experiment, and perhaps obscured an effect of ketamine dose. Simple effects analysis with SIDAK correction revealed a significant different between batches at the PL (mean difference 205.67 c-fos per mm^2, 95% CI 12.698 to 397, p = 0.039), CG (mean difference 281.992 c-fos per mm^2, 95% CI 86.389 to 477.594, p = 0.010) and Mt (mean difference 228.283 c-fos per mm^2, 95% CI 62.094 to 394.472, p = 0.013) whilst almost reaching significance at the IL (mean difference 150.096 c-fos per mm^2, 95% CI -15.817 to 316.009, p = 0.070). This provided us with cause to investigate the influence of batch on our results.

When separated by batch, the JB3 batch appear a clearer data set than that of the CH8 batch (Figure 22). The CH8 group displayed no main effect of dose between subjects (Mixed model ANOVA, F (3, 4) = 0.329), p = 0.806) whilst the JB3 batch did (Mixed model ANOVA, F (3, 4) = 10.100, P = 0.024). However, the small n number (n = 2) resulting from this batch separation reduces the reliability of this effect. Further, neither batch displayed any dose*region interaction (CH8 mixed model ANOVA, F (9, 12) = 1.648, p = 0.457 & JB3 mixed model ANOVA, F (9, 12) = 0.917, p = 0.599).

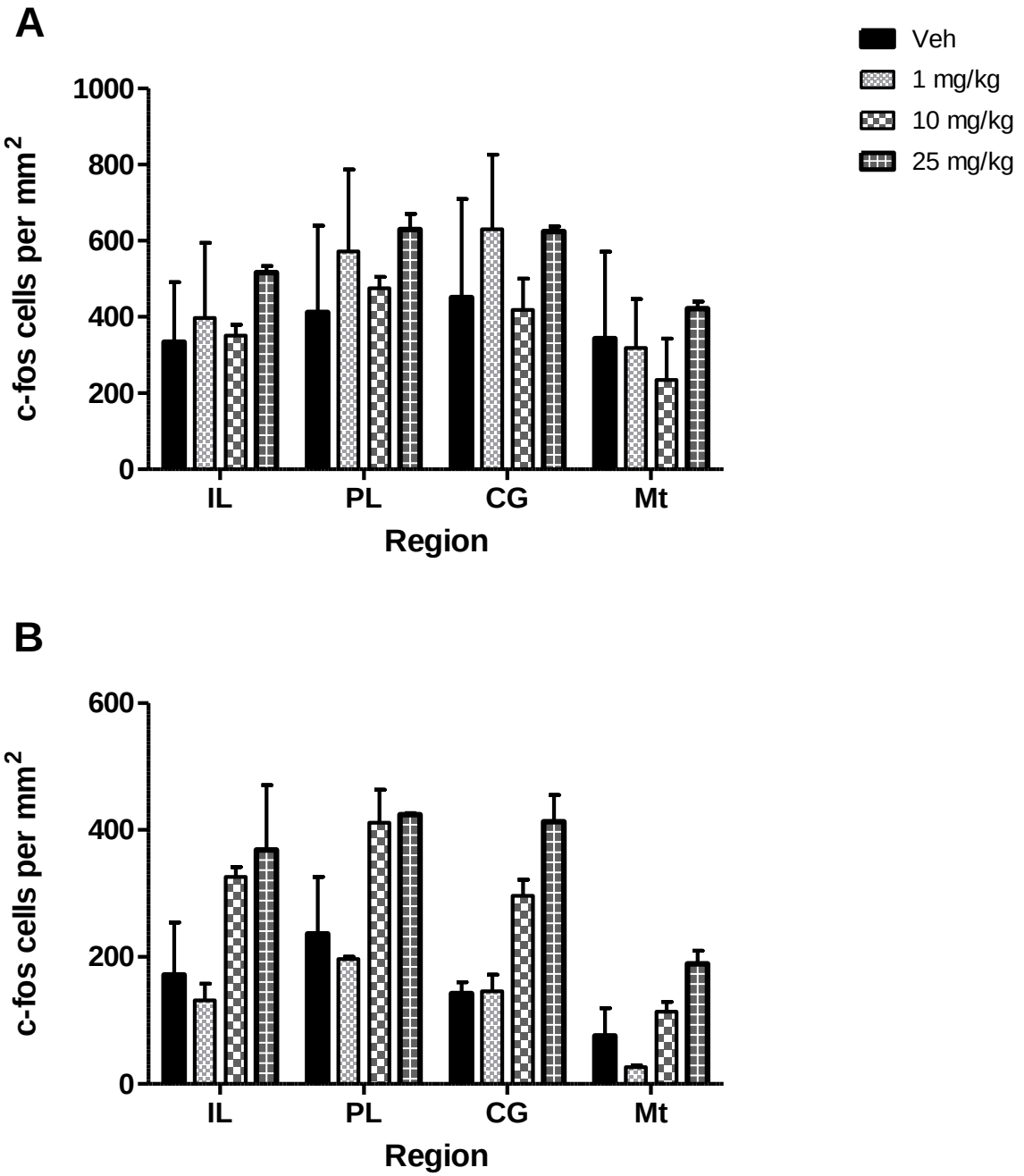

Figure 22. Ketamine dose experiment on PFC c-fos expression separated by batch. Data displayed for the infralimbic (IL), prelimbic (PL), cingulate (CG) and motor (Mt) regions of the rat pre-frontal cortex after i.p. injection of 1 mg/kg, 10 mg/kg and 25 mg/kg ketamine with saline control (n = 2) separated by batch (CH8 = A, JB3 = B). Data shown is average c-fos count ±SEM.

Considering the significant effect of batch, we identified two individuals which displayed very high c-fos levels and thus causing a large amount of variance in their respective groups of vehicle and 1 mg/kg ketamine (Figure 22). It appears that these individuals, CH8-7 and CH8-8, are possibly responsible for this difference in batch*region interaction as when they are excluded from the analysis the region*batch interaction is no longer significant (Mixed model ANOVA, F (3, 8) = 2.778, p = 0.071). Indeed, when excluded from the overall analysis a clear effect of dose is observed between subjects (Mixed model ANOVA, F (3, 6) = 19.021, p = 0.002) (Figure 23). There is also no dose*region interaction (Mixed model ANOVA, F (9, 18) = 1.715, p = 0.158), however, there is still a significant effect of batch (Mixed model ANOVA, F (1, 6) = 15.072, p = 0.008). Furthermore, the grounds to remove these two individuals is questionable. Indeed, although the counts of the PL and CG regions for CH8-7 approach a value of 2, overall the Z scores do not exceed 2 apart from the Mt count for CH8-8 (Table 5) and were therefore not officially counted as outliers. Thus, the data set with the excluded animals should be interpreted with caution.

Table 5. Z-scores of CH8 7 & 8.

Animal	z-scores			
	IL	PL	CG	Mt
CH8-7	1.70	1.95	1.98	1.35
CH8-8	1.05	1.17	1.45	2.08

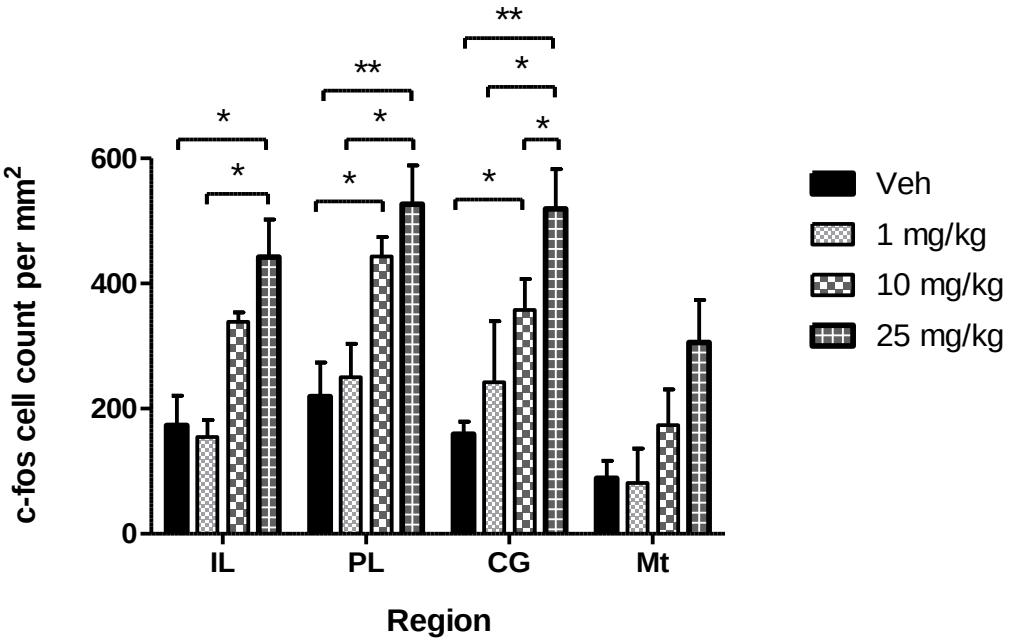

Figure 23. Ketamine dose experiment with CH8 – 7 (1 mg/kg) and CH8 – 8 (veh) removed. C-fos expression at the infralimbic (IL), prelimbic (PL), cingulate (CG) and motor (Mt) regions of the rat prefrontal cortex after i.p. injection of 1 mg/kg (n=3), 10 mg/kg (n=4) and 25 mg/kg (n=4) ketamine with saline control (n=3). Data shown is average c-fos count ±SEM. Mixed model ANOVA, Sidak corrected simple effects analysis, * = p<0.05, ** = p<0.01, *** = p<0.001.

Sidak corrected post hoc analyses of the CH8-7 and CH8-8 removed dataset revealed significant differences between vehicle and the 10 mg/kg (p = 0.037) and 25 mg/kg (p = 0.002) doses and between the 1 mg/kg and 25 mg/kg doses (p = 0.004). Simple effects analysis revealed significant differences between doses in each region. In the IL, the significant difference was between 25 mg/kg and both the vehicle (mean difference 268.167 c-fos per mm^2, 95% CI, 24.781 to 511.553, p = 0.032) or 1 mg/kg (mean difference 276.333 c-fos per mm^2, 95% CI 32.947 to 519.719, p = 0.028) treatments. At the PL region, vehicle differed from the 10 mg/kg (mean difference 232.883 c-fos per mm^2, 95% CI 29.752 to 436.014, p = 0.027) and 25 mg/kg (mean difference 316.375 c-fos per mm^2, 95% CI 113.244 to 519.506, p = 0.006) treatments. There was also a significant difference between the 1 mg/kg and 25 mg/kg treatments (mean difference 249.958 c-fos per mm^2, 95% CI 46.827 to 453.089, p = 0.019). At the CG region, vehicle differed from 10 mg/kg (mean difference 189.708 c-fos per mm^2, 95% CI 12.154 to 367.263, p = 0.037) and 25 mg/kg (mean difference 351.150 c-fos per mm^2, 95% CI 173.595 to 528.705, p = 0.002), 1 mg/kg differed from 25 mg/kg (mean difference 228.833 c-fos per mm^2, 95% CI 51.279 to 406.388, p = 0.015), and 10 mg/kg differed from 25 mg/kg (mean difference 161.442 c-fos per mm^2, 95% CI 2.632 to 320.252, p = 0.047). Finally, no significant differences were observed at the Mt, however significance was almost observed

between vehicle and the 25 mg/kg treatment (mean difference 210.375 c-fos per mm^2, 95% CI -2.108 to 422.858, $p = 0.052$).

3.3.3 GAD67 and c-fos colocalization

Overall no significant effect of drug treatment was seen on either c-fos expression or c-fos/GAD67 protein colocalization at the IL (Mixed model ANOVA, F (2, 13) = 0.173, $p = 0.843$, Figure 24) or PL (Mixed model ANOVA, F (2, 13) = 0.362, $p = 703$, Figure 24). There was a distinct difference in the populations of c-fos positive cells and the cells with colocalised c-fos/GAD67 at both the IL (Mixed model ANOVA, F (1, 13) = 81.337, $p<0.001$) and the PL (Mixed model ANOVA, F (1, 13) = 85.323, $p<0.001$). Although none of the treatments exerted significant influence on this, as there was no significant interaction between treatment and colocalization at either the IL (Mixed model ANOVA, F (2, 13) = 0.181, $p = 0.837$) or PL (Mixed model ANOVA, F (2, 13) = 0.517, $p = 0.608$).

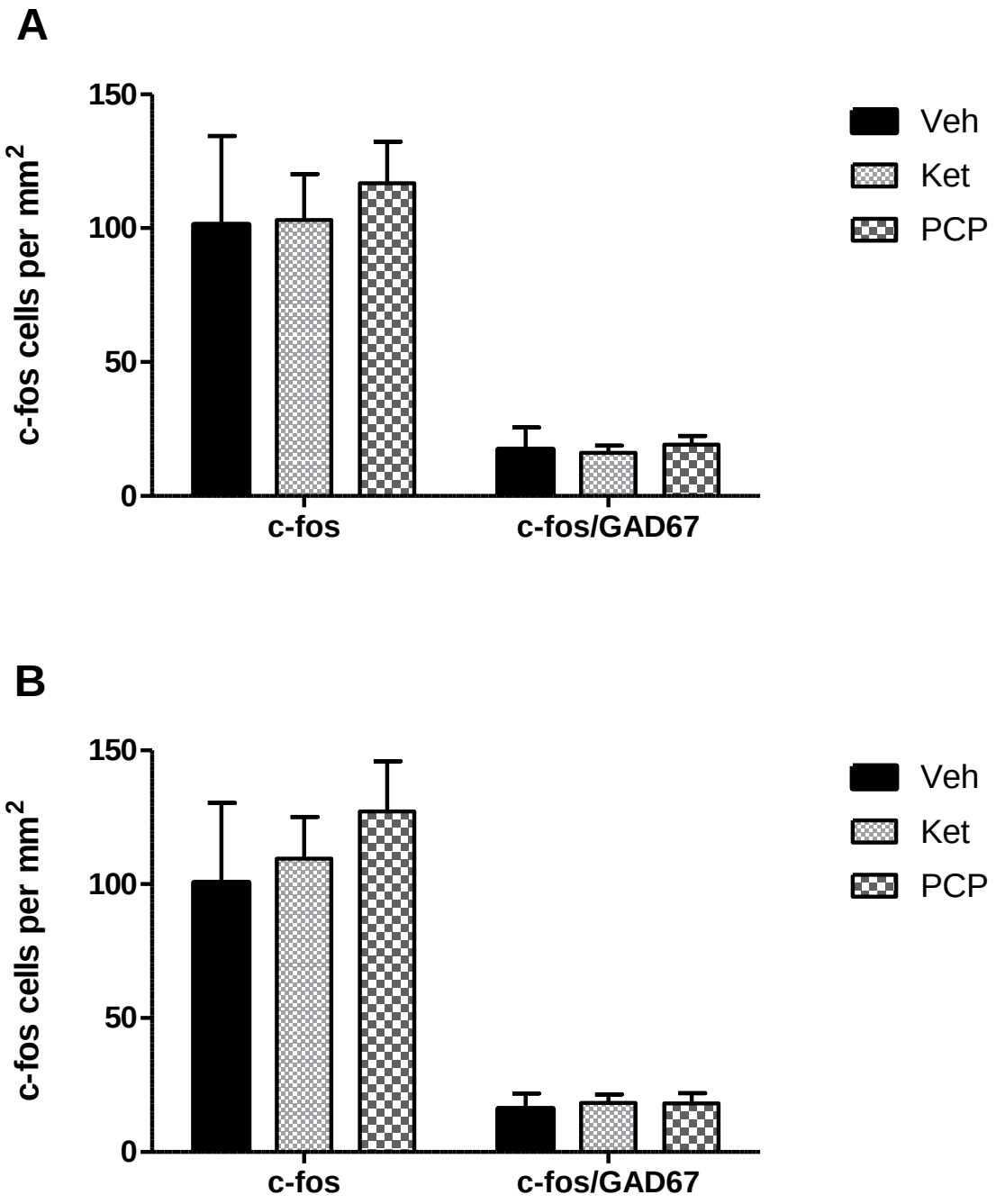

Figure 24. C-fos expression and c-fos/GAD67 colocalization at the infralimbic (A) and prelimbic (B) cortices after i.p. administration of 1 mg/kg ketamine (n=6), 0.3 mg/kg PCP (n=6) or saline control (n=4) 90 minutes before killing. Data shown is average c-fos count ±SEM.

3.4 Discussion

3.4.1 Influence of 1 mg/kg ketamine and 0.3 mg/kg ketamine on PFC c-fos expression

Overall, neither 0.3 mg/kg PCP or 1 mg/kg ketamine significantly increased c-fos expression. Whist it is well known that NMDAR antagonists increase PFC glutamate and activation (Homayoun and Moghaddam, 2007), we cannot confirm this at 1 mg/kg ketamine or 0.3 mg/kg PCP. However, whilst PFC stimulation is observed from ketamine in rats at doses between 10 mg/kg (Littlewood *et al.*, 2006) and 30 mg/kg (Kim *et al.*, 2011), it is questionable whether these doses correspond with the 0.5 mg/kg infusion of ketamine used in humans. Our finding of a numerical increase in PFC c-fos expression after a 1 mg/kg dose, demonstrated to be effective in the ABT test (Stuart *et al.*, 2015), may present evidence of a subtler excitatory effect, resembling more the 0.5 mg/kg infusion used in patients (Berman *et al.*, 2000) and different from that observed at higher doses ranging between 10 and 25 mg/kg used in rats.

Of course, interspecies extrapolation of pharmacological effects is difficult, especially in a pharmacokinetically sensitive response such as ketamine's AD effect. To address this, Shaffer *et al.*, 2014 applied a receptor occupancy (RO) normalization method using [^{3}H]MK-801 displacement studies combined with plasma concentration profiles of ketamine in rats after 10 mg/kg. Through this model, they predicted that the receptor occupancy profiles between 0.5 mg/kg infusion and 10 mg/kg s.c, are different on a temporal scale, with rat s.c reaching 65-33% at 0.085-0.5 hours, in contrast to the human 0.5 mg/kg infusion reaching 31% at 40 minutes. Interestingly, to mirror the RO profile, they predicted a 1.47 mg/kg infusion over 40 minutes in rats would reproduce this pattern. Therefore, it is reasonable to posit that 1 mg/kg i.p. dose more closely resembles the 0.5 mg/kg infusion used in human studies, and that this could mediate the AD effects, although further testing would be required.

Whilst no significant effect was discovered, PCP administration numerically increased c-fos expression at the IL, PL, CG and Mt more than ketamine (Table 4), on average of 30%. From this, we can speculate that 0.3 mg/kg PCP potentially elicited more PFC activation as 1 mg/kg ketamine or at least to a similar degree. As 0.3 mg/kg PCP has demonstrated no known AD effects and failed to induce positive bias in the JBT (Hales, Houghton and Robinson, 2017), whilst 1 mg/kg ketamine has induced positive bias in both the ABT and JBT (Stuart *et al.*, 2015; Hales, Houghton and Robinson, 2017), this could suggest that AD action is not solely NMDAR mediated. For example, ketamine's action at the opioid system has been postulated to be essential for its antidepressant effect (Williams *et al.*, 2018). Alternatively, the phamacodynamic differences between PCP and ketamine, such as NMDAR affinity, could be responsible for PCPs lack of AD efficacy.

However, NMDAR induced PFC stimulation has been strongly associated with ketamine's AD efficacy (Li *et al.*, 2016). Thus, it is unlikely that ketamine's glutamate surge fails to play a role in its therapeutic mechanism. Instead, 1 mg/kg ketamine may exert an excitatory 'sweet spot', which lies under the excitatory threshold required for c-fos expression. Indeed, 1 mg/kg ketamine could elicit sufficient excitatory influence to cause AD effects with reduced psychotomimetic influence, whilst 0.3 mg/kg PCP may start to exceed this optimum excitatory window and begin to exert more dissociative side effects. This effect could be attributed to differences in ketamine's and PCP's KI values at the NMDAR, as PCP's increased binding strength makes it the more potent drug (Lodge and Mercier, 2015). As PFC cortex stimulation is also associated with ketamine's hallucinatory influence (Breier *et al.*, 1997), the extent of excitation may be crux of distinguishing the two.

It is also possible that ketamine's unique pharmacology, including its action on the opioid system and subtle NMDAR induced stimulation at subanaesthetic doses, is responsible for its AD properties which other NMDAR antagonists such as PCP cannot recreate. As mentioned, PFC excitation and glutamate surge are associated with AD response, with ketamine's dissociative effects displaying a positive correlation with AD efficacy (Luckenbaugh *et al.*, 2014). Interestingly, Williams *et al.*, 2018 discovered that naltrexone, an opioid receptor antagonist, inhibited ketamine's AD efficacy but without influencing its dissociative side effects. Therefore, it is possible that ketamine's acute AD effect is mediated through the opioid system, whilst downstream effects resulting from NMDAR stimulation are necessary for downstream signalling resulting in longer lasting AD effects.

This could explain the lack of acute effect of 0.3 mg/kg PCP in the JBT (Hales, Houghton and Robinson, 2017). However, further research would be required, as ketamine's pharmacology extends to further transmitter systems such as the dopaminergic system. Additionally, it cannot be concluded that NMDAR receptor function is not required for ketamine's short-term AD effects, as other NMDAR antagonists such as CP-101,606 display rapid AD action (Miller *et al.*, 2014) but lack influence over the opioid system. Ultimately, the non-significant difference in PFC stimulation between 1 mg/kg ketamine and 0.3 mg/kg PCP suggests that ketamine's specific pharmacodynamic effects, which may not be related to stimulatory influence, contributes to their difference in AD effects (Hales, Houghton and Robinson, 2017). However, more sensitive methods and larger n numbers would be required to conclude this.

3.4.2 Dose dependent effect of ketamine on c-fos expression

The original dataset, shown in Figure 21, showed no statistical effect of dose. This was surprising as doses of 10 & 25 mg/kg have been demonstrated to stimulate c-fos expression (Duncan *et al.*, 1998; Imre *et al.*, 2006; Inta *et al.*, 2009). After further investigation, an effect of batch was discovered which could have obscured the influence of increasing dose on c-fos expression. When analysed separately, an effect of dose was observed in the JB3 group and not found in CH8. This effect of batch may have been due to two individuals, CH8-7 & CH8-8, which displayed unusually high levels of basal c-fos expression despite being treated with just vehicle control or the 1 mg/kg dose. As both animals were housed together, this could have been a caged specific effect, as stress is known to induce c-fos expression (Piao *et al.*, 2017). The exclusion of CH8-7 & CH8-8 results in a significant dose effect. However, the exclusion of these should be interpreted with care as there is no official basis on which to have the data removed. Furthermore, removal of these animals did not remove the significant effect of batch.

With CH8-7 & 8 removed, increases in c-fos expression occur at the IL, PL and CG at 10 mg/kg and 25 mg/kg. This corresponds with previous findings (Duncan *et al.*, 1998; Imre *et al.*, 2006; Inta *et al.*, 2009). With this, c-fos expression at 25 mg/kg significantly differed from 1 mg/kg at the IL, PL and CG. Differences between these doses gives cause to question AD effects observed at these doses with the FST (Yang *et al.*, 2018) when 1 mg/kg achieves an AD effect in the ABT (Stuart *et al.*, 2015) and JBT (Hales, Houghton and Robinson, 2017).

It is possible that 1 mg/kg ketamine provides more subtle stimulatory effect than the higher doses of 10 mg/kg & 25 mg/kg. This subtle effect may be the key to ketamine's AD efficacy, as higher doses of ketamine will begin to exert nonspecific effects. This relates to our previous notion of an excitatory 'sweet spot'. Interestingly, Miyamoto *et al.*, 2000 discovered that regional glucose uptake in the mouse brain was similar upon 30 mg/kg ketamine and 0.5 mg/kg MK-801 administration. Therefore, it is possible that at these higher doses, ketamine's neurobiological action begins to more closely resemble the action of its more potent NMDAR antagonist relatives. Thus, at these higher doses, ketamine's NMDAR antagonism may inhibit its stimulatory influence and overshadow secondary pharmacological actions.

Indeed, increased c-fos expression at the motor cortex at the 25 mg/kg dose, found in both the full data set (Figure 21) and data set with CH8-7 & 8 removed (Figure 23) was not significant but could suggest nonspecific locomotor effects of higher doses. This could suggest a dose dependent regional effect, in that higher ketamine doses of 25 mg/kg have less regionally specific stimulatory influence leading to motor side effects. Furthermore, whilst doses in the region of 10 mg/kg may lack influence over the Mt and more specifically target

more cognitive regions such as the IL and CG cortex, 8 mg/kg ketamine has still been reported to disrupt locomotion and cognitive function (Imre *et al.*, 2006).

Therefore, due to the subtlety of the influence of 1 mg/kg ketamine it is possible that, with our n numbers, c-fos staining is not sufficiently sensitive to detect this discrete effect, and that c-fos expression may represent ketamine's non-AD effects as the stimulation needed to induce significant expression is above the AD excitatory threshold. Thus, due to the small effect size a larger sample number would be required for future testing. From our CH8-7 & 8 removed data set, 10 mg/kg ketamine induces significant c-fos increase at the PL and CG cortex (Figure 23). Considering that doses around 10 mg/kg cause behavioural alterations associated with dissociation (Imre *et al.*, 2006), it is possible that this increase in c-fos is representative of ketamine's dissociative actions. Furthermore, c-fos expression at regions such as the cingulate cortex resulting from ketamine have been linked to its dissociative effects (Nishizawa *et al.*, 2000). Thus, at 10 mg/kg ketamine the excitatory AD threshold may have been exceeded.

Thus, it is possible that increased c-fos expression in rats is representative of ketamine's dissociative influence and not of its AD qualities. Indeed, 5 mg/kg ketamine has been shown to induce dissociative behaviours (Radford *et al.*, 2017), although this was administered as an intravenous (i.v.) bolus which will produce more severe effects due the rapid delivery and assumedly higher brain concentration. Furthermore, as a dose of 3 mg/kg has shown to be ineffective in the FST (Chowdhury *et al.*, 2017), despair based assays such as the FST may lack the sensitivity required to detect the AD effects of 1 mg/kg ketamine, which the ABT (Stuart *et al.*, 2015) and JBT (Hales, Houghton and Robinson, 2017) achieved.

3.4.3 Lack of effect of ketamine and PCP influence on c-fos/GAD67 colocalisation

To test whether any effect observed with ketamine or PCP was caused by the disinhibition of GABAergic interneurons, a popular notion behind the ketamine induced glutamate surge (Miller, Moran and Hall, 2016), we investigated whether any increased c-fos observed after 1 mg/kg ketamine or 0.3 mg/kg PCP colocalized with GAD67. During this double staining experiment no effect of either 0.3 mg/kg PCP or 1 mg/kg ketamine on c-fos expression was observed (Figure 24). Additionally, little colocalization was observed and this was not affected by either treatment.

The lack of findings here likely results from problems with staining. For example, when examined under the confocal microscope, a high level of autofluorescence was detected, and the signal from the primary antibody was dim. Thus, detection of c-fos expression and GAD67 colocalisation was challenging. This could also explain the lack of replication of results found in the previous single stain run (Figure 20). Although, c-fos expression in GABAergic neurons has been previously identified using c-fos and GAD67 colocalisation in rats (Doron and Rosenblum, 2010; Hossaini *et al.*, 2011) thus validating the experimental concept. However, this has not been performed specifically for GABAergic interneurons in the rat PFC.

We expected to find a lack of c-fos/GAD67 c-fos colocalization due to the disinhibition hypothesis of ketamine's action, which proposes subanaesthetic ketamine's excitatory action lies with selective inhibition of GABAergic interneurons, facilitating increased PFC pyramidal neuron activity (Miller, Moran and Hall, 2016). Therefore, due to ketamine's inhibitory action on GABAergic neurons, their activity and thus c-fos expression would be expected to be reduced.

3.4.4 Limitations & considerations

A caveat with the method of killing was the lack of perfusion. This may have obscured potential c-fos expressing neurons due to increased autofluorescence. Also, an inconsistency of different max values between experiments despite use of the same antibody was observed (Figure 25). A possible cause may be the age of the animals, with that of the PCP vs Ketamine experiment animals (Figure 25A) being older than those used in the ketamine dose experiment (Figure 25B).

Another significant limitation was not using a rocker over the incubation with the primary antibody. This was an oversight of the researcher, who only applied this during incubation with the secondary antibody.

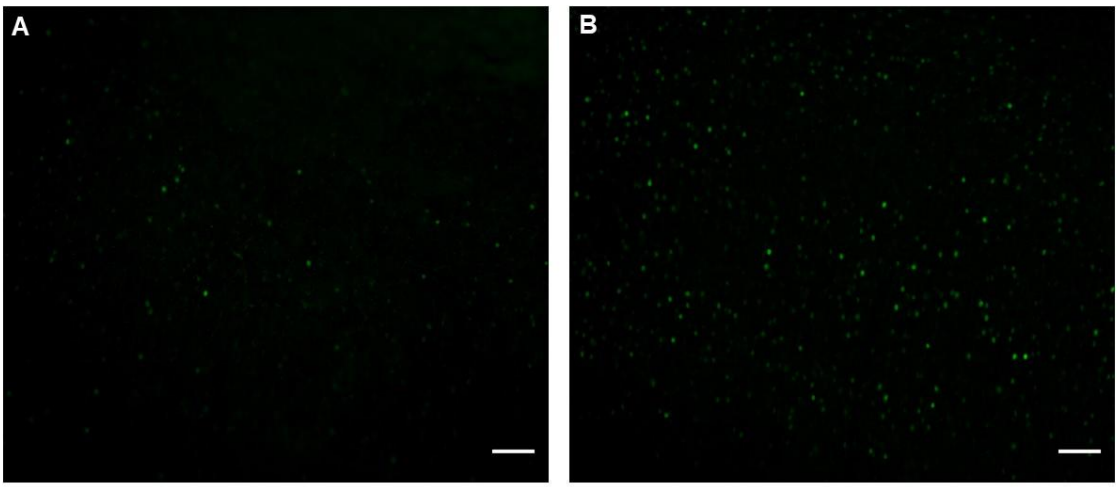

Figure 25. Comparison of c-fos expression observed between the ketamine vs. PCP experiment and ketamine dose experiment. Images are representative of the IL cortex of a 1 mg/kg ketamine treated animal from A) our PCP vs ketamine experiment and B) our ketamine dose experiment. Scale bars = 100 µm.

Another important consideration is the administration route used and how this may have influenced our results. With a half-life 0.28h in the rat brain (Shaffer *et al.*, 2014), ketamine is rapidly cleared. As it predominantly administered through an i.v. route, the drug is rapidly delivered. This leads to rapid increase and decline in plasma concentrations. Furthermore, whilst definite brain concentrations are unknown, this rapid plasma level elevation may be required for ketamine's excitatory and therapeutic effect. As we applied an intraperitoneal injection, this slower delivery rate method would have resulted in a lower peak plasma concentration. Differences in administration route along with difficult dose estimations between rats and humans can also affect NMDAR occupancy making translation more challenging (Shaffer *et al.*, 2014).

Lastly, we consider the implications of c-fos as a neural marker. Whilst c-fos protein is a well-established marker of neural activity, it is also a marker of stress and can even be induced with handling (Asanuma *et al.*, 1992). This sensitive nature of induction can therefore confound subtle pharmacological influences. Furthermore, c-fos protein is not universally expressed thus the effect of treatment on non-c-fos expressing neurons could not be accounted for.

3.4.5 Conclusions

Overall, the lack of a definite increase in PFC c-fos expression may suggest a subtle neurostimulatory effect lying under the c-fos expression inducing threshold. The numerical difference compared to PCP induced c-fos expression, which despite the use of a lower dose of 0.3 mg/kg appeared to exert more PFC stimulation than 1 mg/kg ketamine, may suggest that increased PFC stimulation is more associated with dissociative effects which PCP is more prone to induce. Alternatively, this may also rely on ketamine's specific pharmacology, as the effect of 1 mg/kg ketamine on cognitive bias versus that of 0.3 mg/kg PCP (Hales, Houghton and Robinson, 2017), combined with our observation on non-statistically significant different effects on c-fos expression between the two treatments, could suggest this difference does not lie with stimulatory action. However, c-fos is likely not a sensitive enough measure to conclude this.

1 mg/kg ketamine's effect on c-fos expression also distinguishes it from 10 and 25 mg/kg doses. Our findings of increased c-fos expression at 25 mg/kg compared to 1 mg/kg, and a stimulatory effect of 10 mg/kg at the PL and CG cortex compared to control, differentiate 1 mg/kg from these higher doses. As the literature supports the stimulatory influences of 10 & 25 mg/kg (Duncan *et al.*, 1998; Imre *et al.*, 2006; Inta *et al.*, 2009), it's reasonable to conclude that whilst these doses meet the stimulatory calcium influx and thus neuronal stimulation threshold capable of inducing c-fos expression, 1 mg/kg ketamine does not. Therefore, the stronger excitatory influence of 10 & 25 mg/kg may be unnecessary for an AD effect which can be observed at 1 mg/kg (Stuart *et al.*, 2015), and that these doses in fact differ more from the efficacious 0.5 mg/kg infusion used in humans than the 1 mg/kg dose in rats.

In summary, c-fos is likely not sensitive enough to detect the subtlety of ketamine's stimulatory influence or differential effects on GABAergic interneurons, resulting in non-statistically significant differences in 0.3 mg/kg PCP and our 1 mg/kg dose of ketamine. Future experiments could employ other methods of detecting neural activation and larger n numbers, to further characterize the neurobiological influence of 1 mg/kg ketamine. Despite this, our results lend support to the difference influence seen between 1 mg/kg and 10 and 25 mg/kg i.p. ketamine, which should be considered when investigating the drugs therapeutic mechanism.

Chapter 4

Influence of ketamine on object in place memory

4.1 Introduction

Given the influence of 1 mg/kg ketamine on affective bias (Stuart *et al.* 2015) and having demonstrated a lack of influence over reward-induced positive bias (unpublished), ketamine's influence appears to have a specific cognitive effect. For validation, we pursued ketamine's influence using another memory processing task. This would determine whether the effect observed is specific to affective bias, or whether non-specific effects such as memory disruption are confounding results from the affective bias task.

Acting as an NMDAR antagonist ketamine is liable to cause memory disruption due to the NMDARs role in memory formation. This consists of a 'coincidence detector', whereby the properties of the NMDAR allow it to trigger LTP, which occurs when membrane depolarisation removes Mg^{2+} block and glutamate binds to and opens the NMDAR channel. NMDAR activation then strengthens the synaptic connection through synaptic modifications such as increased AMPAR expression (Bliss and Collingridge, 1993). Thus, the NMDARs role in LTP, and evidence of ketamine's short term memory impairing influence (Adler *et al.*, 1998) warrants investigation of ketamine's potential memory disruption in the ABT.

Whilst the effect of NMDAR antagonists on recognition memory is well documented, the effects of the lower doses of ketamine on memory have received less attention. In rats, investigation of subanaesthetic doses typically ranges from 15 – 30 mg/kg (Imre *et al.*, 2006; Moosavi *et al.*, 2012; Wesierska, Maciasgonzalez and Bures, 1990). However, evidence of 1mg/kg ketamine induced memory impairment in the object recognition test at 20 minutes pre-treatment (Pitsikas, Boultadakis and Sakellaridis, 2008) suggests the 1 mg/kg dose in the ABT could lead to memory impairment. However, Stuart *et al*. 2015 administered ketamine 1 hour before treatment.

We thus aimed to elucidate a difference between 20 minute and 1-hour pre-treatment times, at the doses of 10 mg/kg and 1 mg/kg using the object in place task (OIP). This would allow us to determine the importance of dose and pre-treatment time for the memory disruptive effect of ketamine in rats, and thus uncover whether this could have confounded the results from Stuart *et al*. 2015. We focused on ketamine's effects on memory acquisition which is more sensitive to NMDAR blockade suggesting more NMDAR dependence (Barker and Warburton, 2015).

4.2 Methods

4.2.1 Animals

12 male lister hooded rats were used to conduct the object in place task (~300–350 g, Harlan, UK), the same strain used to undertake the affective bias test by Stuart *et al*. 2015. Animals were housed in pairs and all experimental work was conducted within a 12:12 h light–dark cycle (lights off at 0700h) between 9 am – 5pm. All animals had *ad libitum* access to laboratory chow and water and were provided with environmental enrichment (cardboard tube, plastic housing and chew block). Weight of the animals was monitored weekly.

All work was approved by the UK Home Office and was conducted in adherence to the regulations of the 1986 Animals (Scientific Procedures) Act.

4.2.2 Drug treatment

Each ketamine treatment was made with sterile 0.9% saline solution using a dose volume of 1 ml/kg. Ketamine used was purchased from Sigma Aldrich. Treatments were administered through i.p. injection either 20 minutes or 1 hour before memory acquisition stage.

4.2.3 Object in place task

Experimental set up

The experiment took place in a wooden arena (90x100 cm) with walls of 50 cm in height and no top. The left side wall was painted grey to provide the rats with orientation. The side to the experimenter was covered by a black curtain 1.5 m in height keeping them out of sight during the task. The floor of the arena was covered in saw dust. The objects used were constructed from Duplo and were different in size, colour and shape between each experiment. As the experiment progressed, the objects became more elaborate to retain the animal's interest. The position of objects was counterbalanced along with the pair that was swapped and object colour was randomized throughout the experiment. Video recordings of the rats was taken through a web cam which was positioned 1.5 m above the arena and inverted so that the arena was in full view.

Behaviour testing

Firstly, animals were handled for a week and subsequently habituated in the arena with no objects present for 4 days for 10-15 minutes each. Once habituated, the experiment consisted of a 5-minute sample phase followed by a 1-hour delay and a subsequent final 3-minute test phase for each animal. During the sample phase, the rat was placed in the centre of the arena facing forward and was left to encounter the 4 objects. Once the sample phase was complete, the rat was removed and returned to its cage. Following a one-hour delay, the rat was again

placed in the arena to begin the test phase. At the test phase, the position of either the 2 left or 2 right objects had swapped positions with each other. After each exposure the objects were wiped down with ethanol and saw dust removed to avoid the influence of any olfactory cues. Recognition memory was then assessed by measuring the exploration of each object pair. If OIP memory is intact the moved pair will receive more interest.

Due to the length of the delay, the experiment was undertaken with a staggered design. The schedule for the sample/test phases was kept consistent throughout the experiment, and the pre-treatments were scheduled around it.

4.2.4 Statistical analysis

Pilot: One sample *t*-test.

Ketamine experiment: Two-way repeated measures ANOVA, with Sidak corrected post-hoc analysis.

4.3 Results

To determine if the animals could perform the task a pilot study free of any treatment was conducted. Here the animals demonstrated significantly more interest in the moved objects (One sample students *t*-test: $t = 4.141$, $p = 0.002$, n = 12, Figure 26). We therefore established the rats were able to perform the OIP task and proceeded with the experiment.

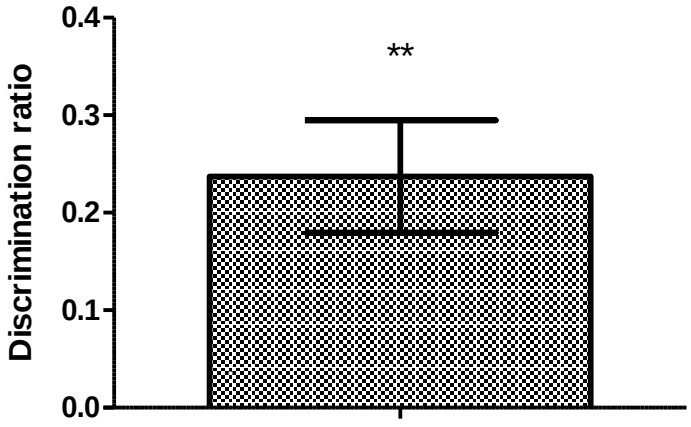

Figure 26. Pilot experiment for the object in place task. Data shown as mean±SEM, n = 12. Unpaired student *t*-test, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

Overall, we observed a significant effect of dose (Two-way repeated measures ANOVA, F (2, 22) = 5.678, p = 0.010). However, there was no overall effect of pre-treatment time (Two-way repeated measures ANOVA, F (1, 11) = 0.921, p = 0.358) and no interaction between time and dose (Two-way repeated measures ANOVA, F (2, 22) = 2.794, p = 0.083). Simple effects analysis with Sidak correction revealed that the significant effect of dose occurs within the 20-minute pre-treatment group, specifically between the vehicle and 10 mg/kg treatment (mean difference, 0.294, with 95% CI 0.017 to 0.571, p = 0.037). Simple effects analysis also revealed that a statistically significant difference was almost achieved between vehicle and 1 mg/kg, within the 20-minute pre-treatment group (mean difference, 0.324, with 95% CI -0.020 to 0.668, p = 0.066). Furthermore, no significant effect of dose was observed at the 1-hour pre-treatment, despite evident numerical difference between the 10 mg/kg dose compared to both the vehicle and 1 mg/kg treatment.

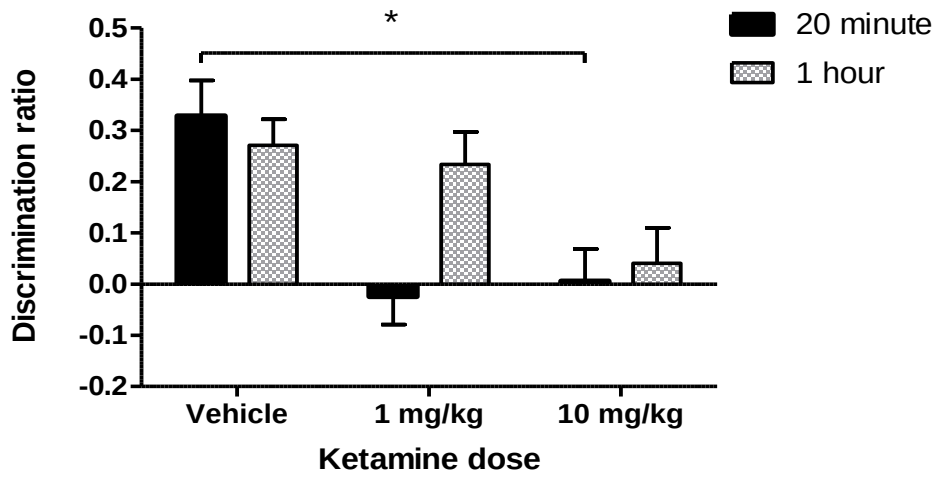

Figure 27. Effect of ketamine dose and pre-treatment time on the test phase of the OIP task. Data shown as mean±SEM, Trial 1 n = 12. Two-way repeated measures ANOVA, Sidak corrected simple effects analysis, * = p<0.05, ** = p<0.01, *** = p<0.001.

Sidak corrected simple effects analysis was also applied to assess the effect of pre-treatment at each dose. Differences between pre-treatment times at both vehicle (mean difference 0.072, with 95% CI -0.155 – 0.298, p = 0.501) and 10 mg/kg (mean difference 0.022, with 95% CI -0.200 – 0.244, p = 0.832) were not significant. However, the difference between pre-treatment time at 1 mg/kg was close to achieving significance (mean difference 0.252, with 95% CI -0.512 – 0.008, p = 0.056) and demonstrated a numerical difference between discrimination ratios.

With the object in place task requiring a sample phase for the acquisition of the objects and their location there is the opportunity to induce an object bias, that is a particular object pair is found more interesting than the other. To prevent this, counterbalance measures of both object position and colour were taken. To confirm the effectiveness of our counterbalance measures the sample phase data was analysed. No significant effect of dose (Two-way repeated measures ANOVA, F (2, 22) = 0.106, p = 0.900) or pre-treatment time (Two-way repeated measures ANOVA, F (1, 11) = 0.132, p = 0.723) was found. Thus, object bias was not an influence on any effect observed.

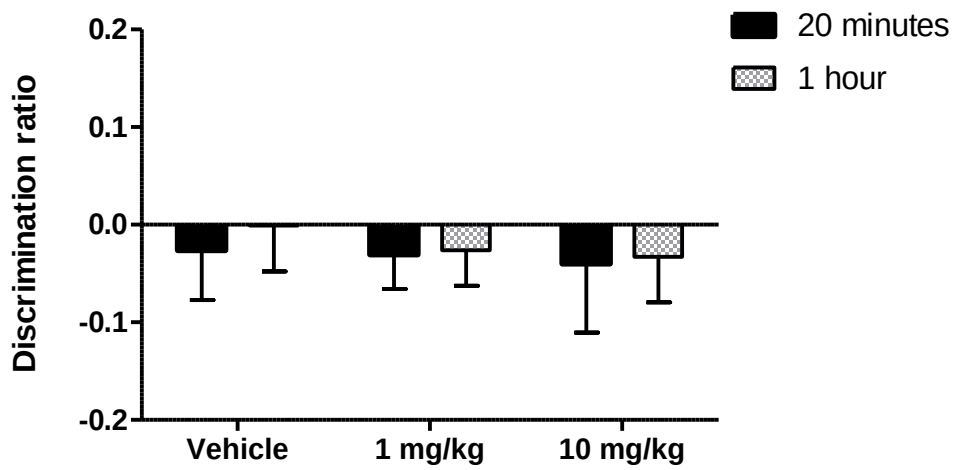

Figure 28. Sample phase of object in place assay. Data shown as mean±SEM, Trial 1 n = 12.

4.4 Discussion

4.4.1 10 mg/kg ketamine impairs OIP memory

Our finding of the memory impairing effect of 10 mg/kg demonstrates this dose, widely used in the FST, exerts cognitive deficits which could confound AD effects. However, this effect only reached statistical significance at the 20-minute pre-treatment time (Figure 27). Despite this, numerically, there is evidence of OIP memory attenuation at 10 mg/kg at the 1-hour pre-treatment time, as the animals show a lack of discrimination between the novel and sample object orientations (Figure 27). Although, a lack of statistical support does not allow a reliable conclusion. However, as object in place memory acquisition is dependent on NMDAR transmission (Barker and Warburton, 2015), memory disruption at the 1-hour pre-treatment is probable.

Support for a memory impairing effect of 10 mg/kg is provided by the current literature. For example, 15 mg/kg i.p, ketamine at 40 minutes prior training increases escape latency of the Morris water maze (Moosavi *et al.*, 2012) whilst Alessandri, Bättig and Welzl, 1989 demonstrated that at 20 minutes 12.5 mg/kg ketamine impairs water maze performance. Further, Chrobak, Hinman and Sabolek, 2008 demonstrated at 20-minute pre-treatment both 12.5 and 2.5 mg/kg impaired acquisition of the radial water maze task in rats. Thus, considering the previous works, 10 mg/kg ketamine is likely to impair OIP performance, demonstrating its unsuitability for studying specific cognitive processes such as the ABT.

4.4.2 Effect of pre-treatment time on 1 mg/kg ketamine induced memory impairment

Our findings at 1 mg/kg at 1-hour pre-treatment time support the validity of the results from Stuart *et al.*, 2015 as there was no evidence of memory impairment. At 20 minutes pre-treatment on the other hand, 1 mg/kg appears to have attenuated recognition memory. However, whilst there is a clear numerical difference between the discrimination index seen at the 20 minute and 1-hour pre-treatment with 1 mg/kg compared to vehicle treat groups, a statistically significant effect was not found. These results must therefore be interpreted with caution.

Considering ketamine's pharmacokinetic properties discussed in the previous section, 1-hour would theoretically provide enough time for sufficient plasma clearance and recovery from its cognitive impairing effect. Indeed, as demonstrated by Parwani *et al.*, 2005, ketamine's effect on memory is correlated with its plasma concentration and does not lead to lasting long-lasting effects. Furthermore, ketamine's memory impairing effect is transient and not present the next day despite repeated administrations in humans (Zhou *et al.*, 2018). Therefore, it is reasonable

to conclude that at subanaesthetic doses ketamine only impairs memory at a time of sufficient plasma concentration.

Consistent with this the animals tested here appeared to retain the ability to form OIP memory at 1 mg/kg at 1-hour pre-treatment. Thus, the animals receiving the dose and pre-treatment time used in Stuart *et al.*, 2015 would be free of memory impairment at the time of testing. This verifies that their results are due to the influence of affective bias, and not a memory impairment effect that simply disrupts the association between the digging substrate and affective state manipulation. Furthermore, the potential difference in OIP memory at the 1-hour and 20-minute time point suggests that the threshold for ketamine's affective bias specific effect is easily exceeded due to the fact that ketamine plasma levels at 20 minutes compared to 1-hour post treatment could be enough to cross it.

4.4.3 Conclusions

Our findings suggest that doses of 10 mg/kg, given 1-hour prior testing will exert memory impairing effects. This could affect behaviour when assessing of AD efficacy. Alternatively, 1 mg/kg at 1-hour pre-treatment appeared free of this memory disturbance. Whilst not statistically significant, the doses used in ABT/JBT (Stuart *et al.*, 2015; Hales, Houghton and Robinson, 2017) is supported by the numerical similarity between 1 mg/kg at 1 hour and vehicle control. Thus, we conclude the effect observed by Stuart *et al*. 2015 was specific to affective bias, and not the result of memory impairment.

We also demonstrated how 10 mg/kg ketamine exerts longer lasting effects to 1 mg/kg. Whilst there was evidence of a lack of memory impairment with 1 mg/kg at 1 hour, 10 mg/kg exerted robust memory disturbance at both pre-treatment times. This suggests that 10 mg/kg exerts longer lasting nonspecific effects including memory impairment and dissociation, likely due to increased plasma presence. This could confound ketamine's antidepressant action in rats.

Furthermore, 1 mg/kg could elicit mild disruption of the relevant networks due to its mild stimulatory effect on the PFC, with reduced nonspecific effects. This may more closely resemble the 0.5 mg/kg infusion given to human subjects (Berman *et al.*, 2000) than does the more disruptive 10 mg/kg. Thus, further application of 1 mg/kg ketamine in rats is warranted as it likely more closely resembles the treatment used in human studies.

Chapter 5

Final discussion

The principal aim of this project was to investigate the AD action of 1 mg/kg ketamine in rats to improve our understanding of its AD mechanism. Through differentiating the effects of 1 mg/kg with higher commonly used doses of 10-25 mg/kg in rats, and against the non-AD NMDAR antagonist PCP we aimed to uncover information on ketamine's AD mechanism. Additionally, we explored a novel method of assessing pharmacological affect bias manipulation. This would offer a valuable research tool for ketamine's preclinical research and support work by Stuart *et al.*, 2015.

We first endeavoured to assess the reproducibility of the ABT used to discover 1 mg/kg ketamine's ameliorating effect on previously acquired negative biases (Stuart *et al.*, 2015). Failure to replicate results achieved by Hinchcliffe *et al.*, 2017 through the lack of a 2 vs 1 positive bias or cortisol induced negative bias lead us to explore a modified version of the bowl digging ABT using flavoured solutions instead of digging substrates. Application of this modified methodology resulted in successful induction of a positive reward induced bias. From this, we attributed previous difficulties encountered in the bowl digging ABT to the extensive animal interaction required due to the practical aspect of the test which provided opportunity to compromise affective bias induction. Thus, future researchers applying these techniques should remain as objective as possible during these experiments. Additionally, successful induction of a positive bias through a distinct methodical approach supports the validity of the ABT, and thus its application to investigate ketamine's effect on affective bias.

An argument against ketamine's ameliorating effect on cognitive bias is that this may be the result of amnesia, which would disrupt the animals' associative memory. We therefore applied the object in place task to investigate whether the dose and pre-treatment time used by Stuart *et al.*, 2015 causes memory impairment. We found that the widely applied 10 mg/kg dose induced disruption of OIP memory. This was only significant at the 20-minute pre-treatment time but the lack of OIP discrimination formed at the 1-hour treatment suggests a memory impairment effect. At the 1 mg/kg dose, a potential effect of time was observed. Whilst lacking statistical backing, OIP memory at the 1-hour pre-treatment time appeared to be intact, which was not observed with the 20-minute pre-treatment. This suggests that the dose and pre-treatment time used in the ABT study by Stuart *et al.*, 2015 (1 mg/kg at 1 hour) does not influence memory and that there finding was representative of an effect on affective bias and not general memory impairment.

Another motive behind the application of the OIP test was the involvement of glutamatergic circuitries involving the PFC and Perirhinal cortex required in functional OIP memory (Barker and Warburton, 2008). As discussed, the PFC is heavily involved in depression and likely to be a principal region involved in ketamine's therapeutic effect. Therefore, application of the

OIP task provides scope to further elucidate ketamine's effect on PFC neurobiology. To this end, 10 mg/kg appears to exert a disruptive effect on this circuit, whilst 1 mg/kg at on hour may exert reduced if any disruption. Therefore, 1 mg/kg ketamine at one hour retains its specific influence whilst doses of 10 mg/kg exceed it and could lead to increased disturbance of the networks involved in ketamine's AD mechanism.

To characterize ketamine's effect on PFC cortex activation, we investigated changes in c-fos expression after ketamine treatment and how this compared to a low dose of 0.3 mg/kg PCP, a structural analogue of ketamine. Overall, our results did not provide a definitive conclusion on whether 1 mg/kg led to c-fos stimulation. In the first experiment, consisting of the comparison between ketamine and PCP, a numerical increase in c-fos expression was noted from ketamine and PCP. Speculatively, this could suggest a mild stimulatory effect of ketamine at 1 mg/kg, sufficient to produce an AD effect (Stuart *et al.*, 2015) and perhaps cause reduced memory impairing effects (Figure 27). Moreover, the larger stimulatory influence of PCP (Table 4) may represent a larger induced glutamate surge, likely resulting from its stronger NMDAR block and distinct pharmacodynamic properties. Additionally, 1 mg/kg ketamine's and 0.3 mg/kg PCPs distinct influence on cognitive bias, with ketamine successfully inducing a positive bias (Hales, Houghton and Robinson, 2017), despite no significant differences in PFC c-fos stimulation (Figure 20) could suggest secondary pharmacological actions are also responsible for ketamine's AD effect.

However, when investigating the effect of ketamine dose on c-fos expression, and c-fos/GAD-67 colocalization, a numerical increase in c-fos expression was not found at 1 mg/kg. This negative finding may suggest that 1 mg/kg ketamine elicits a subtle excitatory influence which fails to reach the threshold required for increasing c-fos expression, or that 1 mg/kg does not exert a stimulatory effect. Alternatively, c-fos expression and AD efficacy may be induced by independent mechanisms. Considering the evidence of 1 mg/kg ketamine possessing AD activity (Stuart *et al.*, 2015), and that this dose lacked a definite effect on c-fos expression the frequent use 10-25 mg/kg in rodent AD research (Zanos *et al.*, 2016; Yang *et al.*, 2018) should be interpreted with caution as secondary effects will increase at higher doses. Thus, it may be possible that increased c-fos expression observed at 10 and 25 mg/kg (Duncan *et al.*, 1998; Imre *et al.*, 2006; Inta *et al.*, 2009) is actually representative of more general effects, not specific to the affective bias pathway which is only achieved at lower doses of 1 mg/kg (Stuart *et al.*, 2015). This could be the crux of ketamine's AD pharmacology, in that higher doses, or other NMDAR antagonist lack 1 mg/kg ketamine's specific action at the PFC. Indeed, this threshold could lie within a thin margin which is difficult to replicate with other treatments as demonstrated by the difference we observed between a 20 minute and 1-hour pre-treatment time of 1 mg/kg on OIP memory (Figure 27).

The significance of dose in ketamine's AD action likely implicates its temporal pharmacokinetic properties. Having rapid pharmacokinetics, a brief plasma presence may be required for a transient glutamate surge critical for ketamine's AD effect. Thus, anything above this threshold may result in secondary and non-AD actions such as dissociative symptoms or memory impairment. Considering that our data show that OIP memory is intact at the AD dose of 1 mg/kg, but disrupted at 10 mg (Figure 27), it is possible that the more intense glutamate surges induced by higher dosage is unnecessary to elicit an AD effect. This difference in stimulatory effect is also demonstrated by the different c-fos expression observed at 1 & 10 mg/kg. Thus, to achieve and AD effect as seen by Stuart *et al.*, 2015 at 1 mg/kg, the level of stimulation seen at 10 mg/kg is excessive and could even occlude AD effects through increased nonspecific actions such as memory impairment.

Of course, when discussing dissimilitude between doses, and especially between species, the method of delivery is crucial. Therefore, the limitations of the compatibility of i.p. dosing in rats versus the 0.5 mg/kg infusion of ketamine in humans (Berman *et al.*, 2000) must be considered. As posited by Shaffer *et al.*, 2014 through their receptor occupancy model, 10 mg/kg i.p. injection is likely to result in different NMDAR occupancy than 0.5 mg/kg infusion. They posit that a 1.47 mg/kg infusion over 40 minutes in rats would most closely match the NMDAR occupancy profile of 0.5 mg/kg 40-minute infusion in humans. Therefore, it is possible that a 1 mg/kg i.p. dose in rodents results in NMDAR binding more similar to the human 40-minute infusion than the more commonly used doses of 10 and 25 mg/kg. Similar to the transient dissociative symptoms experienced during ketamine's clinical trial (Berman *et al.*, 2000), 1 mg/kg ketamine led to brief memory impairments in the OIP task assessed at 20 minutes which appeared to resolve after 1 hour (Figure 27). In contrast, 10 mg/kg disrupted OIP performance at both time points, resembling higher clinical doses leading to more cognitive disruption (Adler *et al.*, 1998).

Despite difficulties in interspecies extrapolation, we can be confident that a transient pharmacodynamic influence results in ketamine's lasting AD effects. This may be compromised at higher doses due to longer lived drug effects. When administered to rats, [^{3}H]MK-801 binding has been shown to be inhibited by 3 mg/kg (i.v.) ketamine in a time dependent manner, with [^{3}H]MK-801 receptor occupancy inhibition decreasing from 67% to 19% in 20 minutes (Fernandes *et al.*, 2015), demonstrating ketamine's brief action at the NMDAR. As a transient glutamate surge is associated with ketamine's AD effect (Milak *et al.*, 2016), higher doses may lead to increased secondary effects due to a prolonged glutamate surge. As NMDAR receptor occupancy is closely related to ketamine plasma concentration (Fernandes *et al.*, 2015), the increased clearance time necessary for higher ketamine doses will prolong any pharmacodynamic effect.

Evidence of prolonged glutamate increase by increasing ketamine dose is provided by Moghaddam *et al.*, 1997, who found a significant increase in extracellular PFC glutamate with i.p. 20 and 30 mg/kg at 80 minutes post injection, whilst 10 mg/kg only retained significance until 60 minutes post injection. The longer duration of this glutamate surge corresponds with the disrupted OIP performance observed at 10 mg/kg at 1 hour, and the potential increase in c-fos expression at 25 mg/kg. This could be representative of a prolonged glutamate surge accentuating psychotomimetic effects and memory impairment whilst reducing the specific targeting of the affective bias pathways.

Regional specificity may also be behind the difference between the AD efficacy of subanaesthetic doses. This regional specificity may arise due to different receptor densities. For example, Li *et al.*, 2017 found increased glutamate at the perigenual anterior cingulate cortex (pgACC) compared to the anterior midcingulate cortex (aMCC) and proposed this was due to AMPAR/NMDAR ratios. Low doses of ketamine may therefore possess increased therapeutic efficacy due to their ability to specifically target regions compared to more global inhibition elicited by higher doses. It may be that differences in NMDAR/AMPAR ratios facilitate targeting of specific subnetworks including regions such as the ACC. Our finding of a numerical increase in c-fos expression at the motor cortex at 25 mg/kg (Figure 21), a region not involved in ketamine's AD effect, supports this. This might also help explain the necessity of the AMPAR in ketamine's AD mechanism (Maeng *et al.*, 2008), as it may be that increased AMPAR receptor presence facilitates increased NMDAR channel opening, resulting in increased susceptibility to inhibition through ketamine's uncompetitive mechanism. This may be increasingly relevant for depressed patients due to differences in cerebral glutamine/glutamate levels (Yüksel and Öngür, 2010) and potential NMDAR/AMPAR expression (Treccani *et al.*, 2016).

Specific targeting of these regions may then result in correcting network dysfunction, amending abnormalities in core networks implicated in depression such as the DMN (Walter, Li and Demenescu, 2014). This acute effect of normalising abnormal connectivity (Walter, Li and Demenescu, 2014) could then also facilitate neuronal plasticity and the relearning of positive social behaviours associated with remission (Harmer, Goodwin and Cowen, 2009). This may first manifest as a change in cognitive bias, which facilitates remission through removing negative self-rumination and negative interpretation of information as proposed by the cognitive neuropsychological hypothesis of depression (Harmer, Goodwin and Cowen, 2009). Thus, this could explain the importance of the specific influence on affective bias observed at 1 mg/kg (Stuart *et al.*, 2015).

Whilst previous studies have researched ketamine's influence on cognitive bias their neurobiological observations are principally derived from neuroimaging studies such as MRI (Murrough *et al.*, 2015; Reed *et al.*, 2018). Whilst these provide parameters such as glucose metabolism or blood oxygenation, they fail to provide more intimate and direct variables. For this reason, the application of animal research in depression is essential, so that specific drug targets are identified.

The study of pharmacological influences on cognitive bias in rodents using the ABT remains a novel and reliable approach to investigate the AD mechanism of ketamine. The fact that we were able to identify the reason for the failure of the task in this current work supports the validity of this test and demonstrates its sensitivity. Furthermore, the positive bias induced in the modified flavour preference ABT provides further support towards the research on affective bias in rodents. Thus, our conclusions provide guidance for future researchers undertaking this test who must be aware of the importance of objectivity when undertaking the ABT. We also provided validation for a less labour-intensive alternative.

In order to fully elucidate ketamine's AD mechanism, it is essential to continue this line of research. Through it there is opportunity to dissect how ketamine effects cognitive bias at a basic level, which will ultimately allow design of new antidepressant treatments. Due to the potential importance of the affective bias influence in ketamine's AD effect, investigating this could result in a furthered understanding of its mechanism at both a cognitive and molecular level. Therefore, the importance of 1 mg/kg ketamine's neurobiological influences and their role in affective bias should be further characterised. For example, the importance of subanaesthetic ketamine's glutamate surge, which is associated with its AD action (Abdallah *et al.*, 2017a), should be investigated in the JBT and ABT. As we differentiated 1 mg/kg from higher doses of 10 and 25 mg/kg, it would be interesting to determine whether 1 mg/kg is sufficient to cause a glutamate surge in the rat PFC, and whether disruption of this would influence ketamine's efficacy in the JBT or ABT (Stuart *et al.*, 2015; Hales, Houghton and Robinson, 2017). This information could determine the importance of ketamine's stimulatory effect and whether it is required for the cognitive effects observed at 1 mg/kg in rats, and whether these are associated with a glutamate surge.

The role of ketamine's secondary pharmacological actions at 1 mg/kg in its cognitive influence observed in ABT and JBT should also be investigated. Due to the possible subtlety of ketamine's stimulatory influence at the PFC at 1 mg/kg, observed from a lack of significant effect on c-fos expression, and its potential similarity in its effects on c-fos expression as PCP (Figure 20), it is possible that ketamine's secondary pharmacological actions contribute to its AD effect. Indeed, as mentioned, naltrexone has shown to reduce ketamine's AD effects

(Williams *et al.*, 2018). Therefore, it is worth exploring the influence of, for example, opioid receptor antagonists on ketamine's neurobiological effects at 1 mg/kg in rats. This could involve *in vivo* electrophysiology readings at the PFC and fMRI. This could then be applied to results found from investigating how opioid receptor antagonism influences ketamine's effect on the JBT and ABT. From this, a further understanding of ketamine's AD action could be gained.

We conclude that dose plays a vital role in ketamine's AD mechanism, as it allows more specific and less disruptive action at relevant regions such as the mPFC. This potentially allows a subtler stimulatory influence facilitating AD action and reduced psychotomimetic side effects, as well as facilitating a more specific pharmacodynamic influence. Thus, the low dose of 1 mg/kg allows specific targeting of neural circuits leading to efficacy in the ABT (Stuart *et al.*, 2015) and JBT (Hales, Houghton and Robinson, 2017) and reduced nonspecific secondary actions such as memory impairment. However, limitations of our research including lack of significance and small n numbers warrants further research of 1 mg/kg ketamine. Furthermore, in order to gain the intimate knowledge required for designing new AD treatments, animal research using cognitive focused assays such as the ABT must be applied as they test a real depressive trait and go beyond a simple screening test. Through this, our understanding of depression will progress, and the discovery of effective new treatments free of ketamine's side effects could be made possible.

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