A frightening view on schizophrenia

Combining fear conditioning and ketamine administration to investigate emotional blunting in an animal model of schizophrenia

Charmaine Y Pietersen

Paranimfen:

Tony Vladusich

Irma Maria Lucia Clara Meyners

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Promotor: Prof. dr. J.A. den Boer

Copromotor: Dr. F.J. Bosker

Beoordelingscommissie:

Prof. P. Luiten

Prof. J. Korf

Prof. B. Olivier

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Table of contents

List of abbreviations

5HT	serotonin
5HIAA	5-Hydroxyindoleacetic Acid
abla	anterior basolateral amygdala
ACC, antcing	anterior cingulate
ACPD	(+/-)-trans-1-aminocyclopentane-1, 3-dicarboxylic acid
AMPA	amino-3-hydroxy-5-methyl-4-isoxazole
AP5	2-amino-5-phosphonopentanoic acid;
APV	2-amino-5 phosphonovalerate
BLA, bla	Basolateral amygdala
BOLD	Blood oxygen level dependent
CEA, cea	central amygdala nucleus
CI	conditioned inhibition
CLOZ, Cloz, cloz	clozapine
CPP	(6)-3-(2-carboxypiperazin-4-yl) propyl-1-phosphoric acid
CS	conditioned stimulus
$D_{1,2,3,4,5}$	dopamine receptor
DAB	di-aminobenzidine
dB	decibels
DA	dopamine
DEC	Dierenexperimentele commissie
Df	degrees of freedom
DG, dg	dentate gyrus
DR, dr	dorsal raphe
FC	fear conditioned
fMRI	functional magnetic resonance imaging
freq	frequency
GABA	γ-aminobutyric acid
HALO, Halo	haloperidol
HVA	homovanillic acid
i.p.	intra-peritoneal

KET, Ket	ketamine
la	lateral amygdala
LC, lc	locus coeruleus
LTP	long-term potentiation
LY354740, LY 379268	Eli Lilly compounds
LY 379268	(-)-2-Oxa-4-aminobicyclo [3.1.0.] hexane-4,6-dicarboxylate
LY	LY379268
MK-801	5-methyl-10, 11-dihydro-5H-dibenzocyclohepten-5, 10-imine
NaAc	sodium acetate;
NAAG	N-acetylaspartylglutamate
Nacc, nacc	nucleus accumbens
nacc_core	nucleus accumbens core
nacc_shell	nucleus accumbens shell
NAS	nickel ammonium sulphate
NFC	no fear conditioning (control)
NMDA	N-methyl-d-aspartate
mGlu	metabotropic glutamate receptor
PBS	potassium phosphate buffered saline
pbla	posterior basolateral amygdala
PCP	phencyclidine
PAG	peri-aqueductal grey
PET	positron emission tomography
PFC	prefrontal cortex
PPI	prepulse inhibition
PVN, pvn	paraventricular nucleus
SAL	saline
s.c.	sub-cutaneous
TBS	tris buffered saline
td	total duration
US	unconditioned stimulus
VTA	ventral tegmental area

List of tables and figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 1

Introduction

1 Schizophrenia

Schizophrenia is a common disorder, occurring in all races and cultures, afflicting men and woman alike. It has been described as a chronic, severe and disabling disorder and can persist throughout ones life (Tsai and Coyle, 2002). It has therefore been ranked as one of the world's top ten causes of disability (Mueser and McGurk, 2004). Schizophrenia is a relatively old disorder, having being described by Shakespeare, where some of his characters exhibit behaviour typical of schizoaffective disorder e.g. Ophelia in Hamlet (Andreasen, 1976). Falvet first described schizophrenia in 1851 as a cyclical madness. Twenty years later, Hecker discovered the same group of symptoms and named it after the goddess of frivolity, Hebe i.e. Hebephrenia. The first comprehensive description was, however, only provided by Kraeplin around the end of the 19th century. He called it dementia praecox. Bleuler (1911) coined the term schizophrenia, which literally means split mind. Schizophrenia is not, however, a multiple personality disorder, as so many would think, but rather a fragmentation of thought or a disassociation between subjective feeling and thought. Modern day psychiatry classifies schizophrenic symptoms into 3 main categories: positive symptoms, negative symptoms and cognitive symptoms (Tsai and Coyle, 2002). Positive symptoms refer to delusions, hallucinations, and thought disorder. Negative symptoms include apathy, social incompetence and emotional blunting. General cognitive functions are also distorted, resulting in impairments in attention, memory and executive function.

One finds both structural and functional brain abnormalities in schizophrenia. Studies have shown that there is significant atrophy in the parahippocampal region (Sim et al., 2005) often associated with schizophrenic symptomology (Prasad et al., 2004), while other studies associate schizophrenia with significant atrophy of the cerebral cortex (Tsai and Coyle, 2002). Most data suggest, however, that abnormalities are mainly distributed throughout the thalamo-cortico-limbic brain regions (Tsai and Coyle, 2002; Snitz et al., 2005), areas typically involved in the processing of emotions and motivation. Among these, perhaps the most important are the amygdala, prefrontal cortex (anterior cingulate cortex) and hippocampus (Fig. 1).

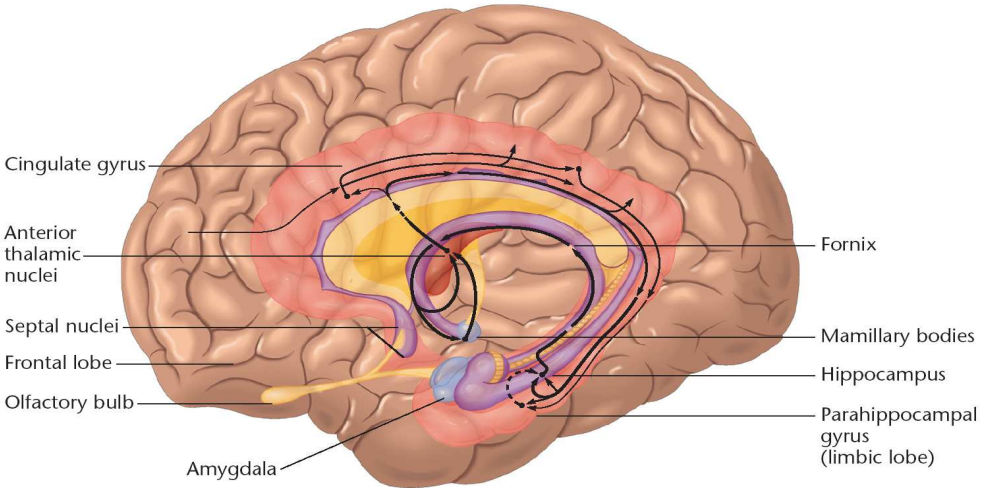


Figure 1: *Human brain circuits of the limbic system. The limbic system plays a pivotal role in emotional processing. Disruption of limbic processing networks has been centrally implicated in the pathophysiology of negative schizophrenic symptoms. From http://cti.itc.virginia.edu/~psyc220.*

2 Structural and functional abnormalities in schizophrenia

2.1 Amygdala

A key component of the limbic system is the amygdala (Fig. 2). The amygdala is a group of interconnected nuclei located in the temporal lobe of mammals (Walker and Davis, 2002) and plays an important role in the acquisition and expression of conditioned fear (Maren and Fanselow, 1996; LeDoux, 1998; LeDoux, 2000; Maren, 2001). Results of imaging studies suggest that the amygdala might be the link between the visual representation of fear (fearful faces) and the concept of fear (Adolphs et al., 2005). In line with this theory, it has been shown that bilateral amygdala damage in humans impairs the processing of fearful facial expressions (Adolphs et al., 1995). Schizophrenics themselves showed reduced activation of the left amygdala and bilateral hippocampal areas in a task requiring discrimination of positive from negative facial affect valence (Gur et al., 2002). These patients failed to activate limbic areas involved in valence discrimination, even though they performed just as well as healthy subjects verbally (Gur et al., 2002). Dysfunction in the amygdala could therefore underlie one of the negative symptoms characterising schizophrenia: emotional blunting. There is still, however, considerable controversy as to whether deficits in recognising affective states in faces are due to generalised problems in face processing, dysfunctional emotional processing, or to the general cognitive dysfunction seen in schizophrenia (see Fullam and Dolan, 2006 and references therein).

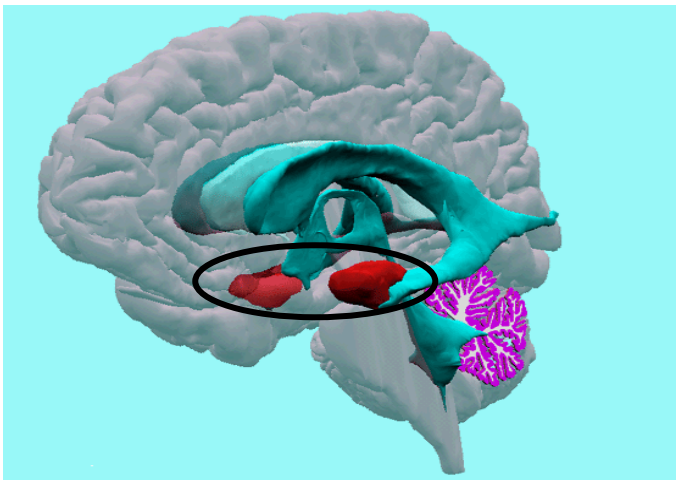


Figure 2: *Location of the left and right amygdala in the human brain. Lesions to the amygdala may underlie disturbances in attributing emotional significance to sensory stimuli and cognitive states. Abnormalities in amygdala functioning have also been implicated in schizophrenia. One of the major goals of the current thesis will be to examine the role of amygdala in a putative animal model of negative schizophrenic symptoms. Taken from http://www.liebermanparkinsonclinic.com.*

2.2 Prefrontal cortex

The prefrontal cortex (PFC) consists of the prelimbic, dorsal and anterior cingulate cortex (ACC) and medial pre-central cortices (Ananth et al., 2001). These areas form part of the broader prefrontal-limbic circuit, which includes the amygdala and orbitofrontal cortex. This entire system plays an important role in anticipating aversive stimuli and seems to mediate anticipatory planning and emotional regulation, particularly within social contexts (Veit et al., 2002). Emotional expression and perception are therefore considered to be a subcategory of social cognition (Pinkham et al., 2003). Dysregulation of this system could be the source of the social incompetence displayed by schizophrenics.

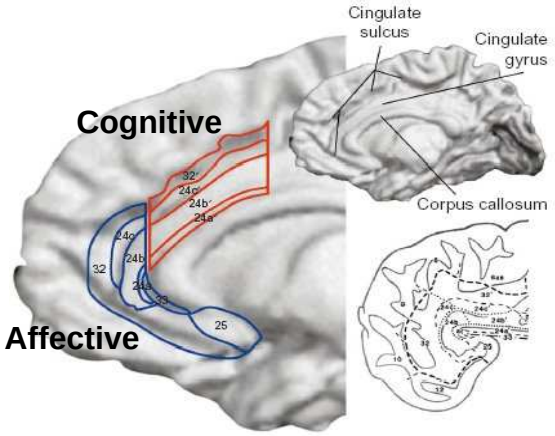


Figure 3: *Cognitive and affective subdivisions of the anterior cingulate cortex (ACC) in the human brain. Recent research suggests that abnormalities in ACC function may underlie several behavioural and cognitive disorders, such as schizophrenia. From Bush et al. (2000).*

The ACC (Fig. 3), a subsection of the PFC, has been shown to play a crucial role in

motivation (Devinsky et al., 1995) and acts primarily by influencing activity in other brain regions involved in cognitive, motor, endocrine and visceral responses (Bush et al., 2000). Consistent with this notion, lesions of the ACC produce symptoms including apathy, inattention, dysregulation of autonomic function, akinetic mutism and emotional instability (Bush et al., 2000), symptoms similar to those seen in the schizophrenic patient. The ACC includes specific modules for the processing of sensory, cognitive, and emotional information (Bush et al., 2000). The cognitive subdivision is part of a distributed attentional network and is connected to lateral PFC and motor areas, while the affective subdivision is connected to the amygdala, PAG, nucleus accumbens, hypothalamus, anterior insula, hippocampus and orbitofrontal cortex (Bush et al., 2000). These two subdivisions demonstrate reciprocal suppression during cognitively demanding tasks and intense emotional states (Bush et al., 2000). On the other hand, some studies have reported that recognition of emotional states correlates with cognitive functioning, especially memory processes and executive functioning (Kee et al., 1998; Sachs et al., 2004), implicating interactions between affective and cognitive subdivisions on some tasks. A recent report has also demonstrated an association between facial affect recognition and cognitive tasks, such as memory, executive functioning and psychomotor speed (Bozikas et al., 2004). Reduced activation of the ACC was also noted during an affect discrimination task with faces in schizophrenics (Hempel et al., 2003). The ACC therefore integrates input from various sources including motivation, evaluation of error, and representations from cognitive and emotional networks.

2.3 Hippocampus

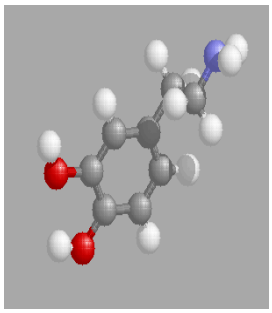
Together with the amygdala, ACC, and orbitofrontal cortex, the hippocampus (Fig. 1) is thought to be part of a circuit involved in cognitive-emotional information processing (Poldrack and Gabrieli, 1997; Shu et al., 2003), where it is primarily involved in the formation of declarative memory and memory consolidation (Eichenbaum, 1999, 2000). The hippocampus plays a role in relational and complex conditional learning in both animals and humans (Phillips and LeDoux, 1992; Peper et al., 2001; Sanders et al., 2003). Huff et al. (2006), for example, found increased levels of cFos and Arc mRNA (immediate early genes, markers of neuronal activity; see Section 10.1) in the hippocampus after context exposure and/or shock. Inactivation of the basolateral amygdala (BLA) in this experiment attenuated this

increase during the context plus shock condition (contextual fear conditioning) suggesting that the BLA plays an important role in regulating gene expression induced in the hippocampus by contextual fear conditioning (Huff et al., 2006). As shock alone has little impact on the expression of immediate early genes, (Hall et al., 2000), the context exposure itself must be the main trigger for immediate early gene expression in the hippocampus (Huff et al., 2006). Another study by Bechara et al. (1995) showed that a patient with damage to the amygdala failed to acquire conditioned autonomic responses to visual or auditory fear-inducing stimuli, but could still recall the factual content (which stimulus was paired with the unconditioned stimulus). Patients with damage to the hippocampus, in contrast, acquired conditioning, but could not recall the factual content (Bechara et al., 1995). A clinical study also showed that patients with amygdala damage could still report which US was associated with a CS, indicating that the declarative (hippocampal) knowledge of the US-CS association was intact and that these two memory systems are able to operate independently (Phelps and Anderson, 1997). More specifically, it seems that the CA3 (specific area of the hippocampus involved in learning) NMDA receptors (see Section 3.2.1 for full description) are critical for learning a novel paired-associates problem, especially for rapid concurrent acquisition of multiple, novel stimuli (Rajji et al., 2006). This suggests that antagonism of the NMDA receptor in the hippocampus could cause a deficit in contextual information processing by disrupting CA3-dependent acquisition of meaningful cues (Rajji et al., 2006).

3 Abnormalities associated with neurotransmitter systems

Functional neurotransmitter abnormalities have long been known to be present in the schizophrenic brain. Several neurotransmitter theories have been postulated in order to describe the symptoms present in patients. These neurotransmitter systems include dopamine, glutamate, serotonin and noradrenalin. The two main hypotheses that continue to attract most research, and therefore will be discussed here at length, are the dopamine and glutamate hypotheses.

3.1 Dopamine



The dominant theory of schizophrenia is that of a dysregulated dopaminergic system (Tsai and Coyle, 2002). There are three main dopaminergic pathways in the brain. The first originates from the ventral tegmental area and projects to the nucleus accumbens and the medial PFC and several other parts of the mesolimbic system. The second arises from within the substantia nigra and projects to the dorsal striatum, and is primarily involved in movement. A third runs from the hypothalamus to the pituitary and most likely has an endocrine function (Fig. 4). There are also two main dopamine receptor groups, D_1 and D_2, out of five in total (up to D_5). D_1 receptors tend to be distributed in cortical regions, while D_2 are subcortical (Williamson, 2006).

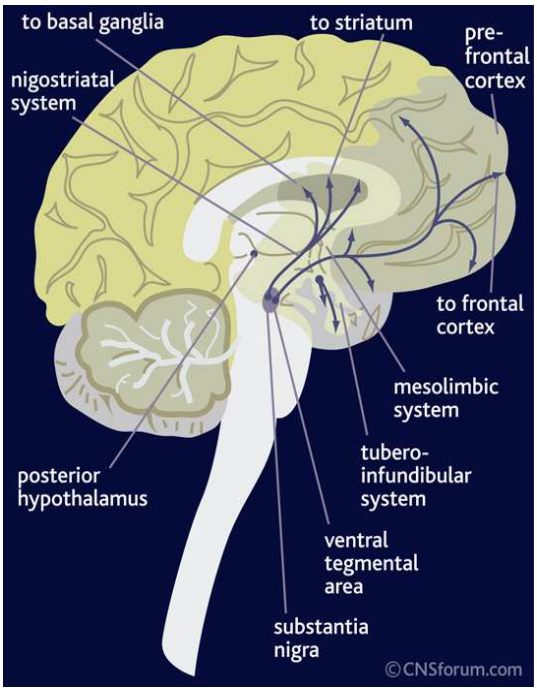
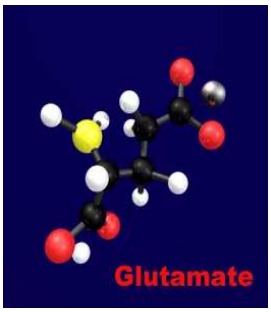


Figure 4: *Projections of the major dopamine pathways in the human brain. Dopamine has long been implicated in the pathophysiology of schizophrenia. Particular attention has been paid to dopamine midbrain projections (ventral tegmental area) to prefrontal cortex. From www.CNSforum.com.*

The dopamine hypothesis has its origins in the observation that typical antipsychotics (dopamine D_2 receptor antagonists) tend to ameliorate positive symptoms (Peroutka and Snyder, 1980; Jones and Pilowsky, 2002), whereas dopamine receptor agonists, such as amphetamine, augment the central dopaminergic system, inducing a schizophrenic-type psychosis (Robinson and Becker, 1986; Laruelle et al., 1999). An emerging theory involving dopamine is that its system is hypoactive in the cortex, while in subcortical areas it is hyperactive (Deutch, 1992). In particular, Weatherspoon et al. (1996) propose that dopaminergic hyperactivity in the nucleus

accumbens mediates positive symptoms, while dopaminergic hypoactivity in the PFC underlies negative symptomology. Dopaminergic dysfunction has also been implicated in abnormal cognitive functioning (Jentsch et al., 2000). By modulating dopamine receptors in the PFC, one can influence working memory (Castner et al., 2004). A study by Verma and Moghaddam (1996) showed that both acute and chronic deficiencies in dopamine neurotransmission disrupt the associative functions of the PFC. In a review by Moore et al. (1999), however, the authors state that dopamine transmission is not altered in schizophrenia as a result of a primary defect in the dopamine neurons, but rather as a result of abnormalities in their regulation by prefrontal and limbic cortical regions, where other neurotransmitters, such as glutamate, are also involved. Thus the dopamine hypothesis alone cannot fully explain all the functional abnormalities leading to the various symptoms of schizophrenia.

3.2 Glutamate



Glutamate is the main excitatory neurotransmitter and is used in more than 40% of all synapses. It is also the main neurotransmitter of the pyramidal cells that connect the cerebral cortex and limbic system (Tsai and Coyle, 2002), although it is found almost everywhere in the brain. Glutamate receptors can be either metabotropic or ionotropic (Fig. 5) (Goff and Coyle, 2001). Ionotropic receptors open calcium channels, and overactivity in these receptors can lead to excitotoxicity (Williamson, 2006). NMDA glutamate receptors are an example of a voltage-gated ionotropic receptor group (Tsai and Coyle, 2002). They are concentrated in the hippocampus, ACC and other parts of the limbic system (Williamson, 2006). Other ionotropic receptors include the AMPA/kainate subtype. Metabotropic receptors are G-protein mediated and found mostly in the forebrain areas (Moghaddam, 2004). It has been suggested that a pathological disruption of the glutamatergic input from afferent cortical systems could be responsible for the increase in dopamine responsivity in schizophrenic patients (Grace, 2000).

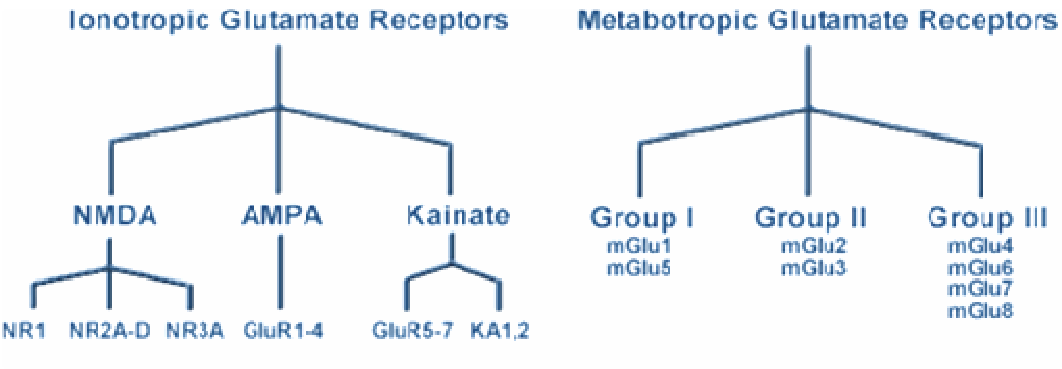


Figure 5: *Ionotropic and metabotropic glutamate receptor families. Antagonists of NMDA ionotropic receptors, such as ketamine, have proven particularly useful in studying both positive and negative symptoms of schizophrenia.*

3.2.1 NMDA (N-methyl-d-aspartate) receptors

The glutamate hypothesis originates from investigations showing that glutamate NMDA receptor antagonists induce psychosis in healthy volunteers, or elicits psychotic symptoms in refractory schizophrenic patients (Tsai and Coyle, 2002). Some studies (Moghaddam et al., 1997; Krystal et al., 2000; Abel et al., 2003) suggest that a hypofunctioning glutamatergic system could be specifically related to both the cognitive and emotional deficits displayed by schizophrenic patients (see Riedel et al., 2003, for a review of cognitive deficits). For example, a study investigating NAAG (N-acetylaspartylglutamate), a neuropeptide found in the NMDA receptors (Tsai et al., 1995), showed that levels of NAAG were increased in the schizophrenic brain, whereas its peptidase (enzyme) activity and glutamate levels were decreased (Tsai et al., 1995). Ibrahim et al. (2000) also found NMDA receptor hypoactivity in the limbic thalamus of schizophrenic patients, consistent with the attenuated glutamate activity hypothesis. An inverse correlation between negative symptoms and glutamate concentration has also been noted (van der Heijden et al., 2004). One proposed mechanism for how glutamate hypofunction might occur is via an excitotoxic process in early life that destroys postsynaptic cells that house the glutamate receptor system, thereby rendering the glutamate neural network defective (Heresco-Levy, 2003; Aleman and Kahn, 2005).

3.2.2 AMPA (amino-3-hydroxy-5-methyl-4-isoxazole)/Kainate receptors

NMDA receptors usually coexist with AMPA or kainate receptors and may be involved in augmentation of the glutamate signal (Bergink et al., 2004). Both these receptors mediate fast excitatory synaptic transmission (Cotman et al., 1995) and

promote the activation of the NMDA receptor. As they are co-localised, the distributions of the AMPA/kainate receptors are similar to the NMDA receptor (Bergink et al., 2004). These receptors are therefore typically located in the cortex and limbic system and exhibit effects on cognition, perception and mood (Krystal et al., 1999).

3.2.3 Metabotropic mGlu 2/3: LY 379268

G-protein-coupled glutamate receptors were discovered in the 1980s (Pin and Duvoisin, 1995) and to date 8 subtypes have been cloned (Bergink et al., 2004). It was found that these receptor subtypes were often located together on the same neurons and interacted within complex neural networks (Bergink et al., 2004). Metabotropic glutamate receptors are divided into 3 subgroups. Group one is connected to phospholipase C-related cellular cascades, while groups 2 and 3 are negatively coupled to adenylate cyclase (Nakanishi, 1992; Conn and Pin, 1997). Glutamate receptors 2/3 belong to the second subgroup and are highly expressed in the forebrain regions, although agonists of these receptors have been shown to work in the hippocampus, locus coeruleus, amygdala and PFC (Ohishi et al., 1993; Cartmell et al., 1999). Unlike the fast-acting ionotropic glutamate receptors, mGluR subtypes exert long-lasting effects through intracellular signals (Bergink et al., 2004).

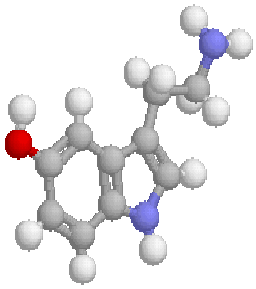
3.3 Interactions between dopamine and glutamate

Interactions between these neurotransmitter systems are known to occur. For example, MK-801, a glutamate NMDA receptor antagonist, exerts an effect on dopamine metabolism in the medial PFC and striatum (Dai et al., 1995), while NMDA receptor antagonists in general increase the firing rate of dopamine neurons in the ventral tegmental area (French and Ceci, 1990). Stimulation of metabotropic glutamate receptors by ACPD ((+/-)-trans-1-aminocyclopentane-1,3-dicarboxylic acid) dose-dependently increases the release of dopamine in the striatum (Verma and Moghaddam, 1998). Stimulation of AMPA or kainate glutamate receptors also leads to the increased release of dopamine in the PFC (Jedema and Moghaddam, 1996). Conversely, treatment with typical antipsychotics, which act primarily on dopamine receptors, results in a significant increase in glutamate levels in schizophrenic patients (van der Heijden et al., 2004). Such findings suggest that the dopamine and glutamate systems function in an antagonistic fashion, and that an

imbalance in the normal interactions between these systems may give rise to schizophrenic symptoms (de Bartolomeis et al., 2005).

In order to investigate the interaction between the two main neurotransmitter hypotheses of schizophrenia, Krystal et al. (2005) administered both amphetamine and ketamine (see Section 4.2) to healthy volunteers in a randomised double-blind psychopharmacological trial. While both drugs alone led to positive symptoms, combined administration was less severe than the sum of the effects of each drug individually. Their combination did, however, produce additive effects on euphoria and thought disorder (Krystal et al., 2005). Amphetamine attenuated the disruption of working memory induced by ketamine. In addition to positive symptoms, ketamine also led to negative symptoms and impairments in attention, working memory and declarative memory. Conversely, amphetamine mostly led to positive symptoms and psychomotor activation, rather than negative and cognitive symptoms (Krystal et al., 2005). This study therefore provides evidence of interactions between the dopaminergic and glutamatergic neurotransmitter systems, while also elucidating their own unique profiles.

3.4 Serotonin



Another neurochemical model of schizophrenia is that of the LSD/serotonin $5HT_2$ receptor hypothesis. It is interesting to note that the amygdala possesses moderate to high levels of 5 subtypes of serotonin receptors, with the $5HT_2$ receptors located in the basolateral nucleus, and the $5HT_{1A}$ receptors in the central nucleus (Pralong et al., 2002). An intimate relationship between the serotonergic (Fig. 6) and glutamatergic systems has also been established (Aghajanian and Marek, 2000). Evidence for this lies in the fact that $5HT_2$ antagonists are able to block both the behavioural effects of NMDA antagonists and psychedelic hallucinogens that make use of the serotonin system (Aghajanian and Marek, 2000). These hallucinogens, acting via $5HT_2$ receptors, also appear to enhance glutamatergic transmission in the locus coeruleus and cerebral cortex (Aghajanian and Marek, 2000).

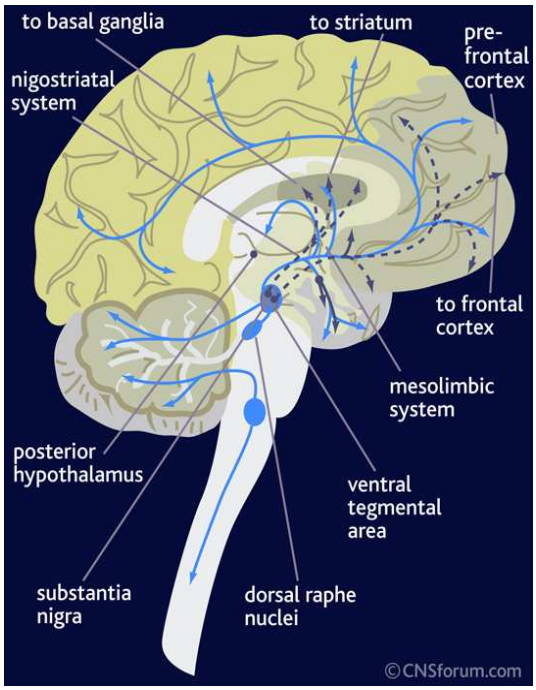
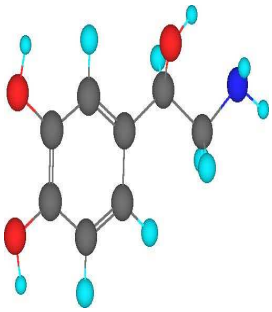


Figure 6: *Serotonin pathways in the human brain. There is some evidence to implicate pathophysiology of serotonin and its receptors in schizophrenia. From www.CNSforum.com.*

Post-mortem schizophrenic brains show increased $5HT_{1A}$ receptor density in the PFC (Bantick et al., 2001). Atypical antipsychotics also affect the serotonin $5HT_{2A}$ receptor, lending support to the serotonin hypothesis. Clozapine, an example of an atypical antipsychotic, combines D_2 receptor antagonism and $5HT_{1A}$ agonism (Bantick et al., 2001). Olanzapine, another atypical antipsychotic, also significantly increases the HVA/5HIAA (dopamine/serotonin metabolites) ratio in the cerebral spinal fluid of schizophrenic patients (Scheepers et al., 2001).

3.5 Noradrenalin



Noradrenalin innervates the human neocortex and limbic forebrain substantially (Fig. 7) (Yamamoto and Hornykiewicz, 2004) and has been proposed to play a role in the neurobiology of schizophrenia as early as 1971 (Stein and Wise, 1971).

Modulation of noradrenergic activity leads to similar symptoms as those seen in schizophrenia, including attention impairments, stress sensitivity and social avoidance (see references in Yamamoto and Hornykiewicz, 2004). Higher than normal concentrations of noradrenalin have been found in the cerebral spinal fluid of schizophrenic patients (Gomes et al., 1980; Sternberg et al., 1981; Kemali et al., 1990), mainly associated with paranoid symptoms.

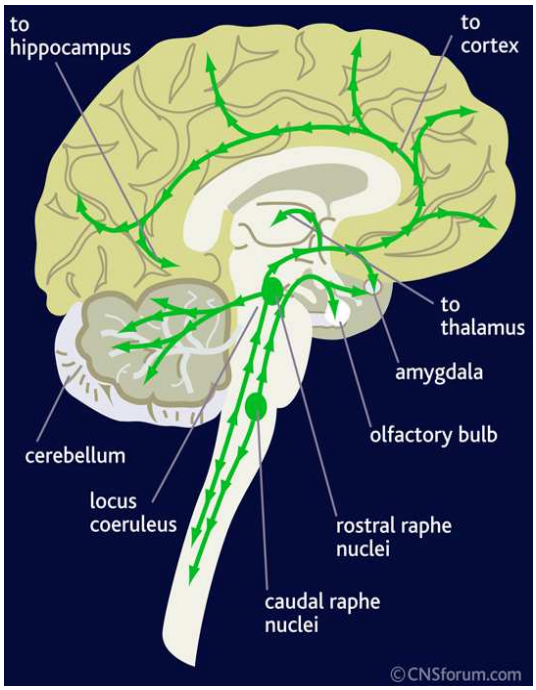


Figure 7: *Noradrenergic pathways in the human brain. Although noradrenaline has been implicated in schizophrenic pathology, its role has not been studied as extensively as dopamine and glutamate. From www.CNSforum.com.*

Noradrenalin also has effects on emotional learning. Within the BLA noradrenalin enhances glutamatergic synaptic plasticity (Ferry et al., 1997), which is thought to underlie learning and memory functions (Huang and Kandel, 1996). Alone, it exerts both inhibitory and excitatory effects via the α_2 and β adrenoreceptors respectively (Pralong et al., 2002), both of which are found in the BLA. It has been shown that noradrenalin was released in the amygdala after foot shock, and the concentration increased as the intensity of the footshock increased (Galvez et al., 1996), indicating a role for noradrenalin in fear learning. Noradrenergic projections from the locus coeruleus to the amygdala have also been shown to influence memory storage, as noradrenalin infused directly into the amygdala attenuated memory impairment (Liang et al., 1995).

4 Drugs acting on the glutamate system that mimic schizophrenic symptoms

4.1 PCP

Amongst the NMDA receptor antagonists, phencyclidine (PCP) and ketamine have been used in several studies investigating the hypothesis of glutamate hypofunction in schizophrenia. PCP has been extensively validated as a model of schizophrenia in animals and elicits both positive and negative symptomology (Javitt and Zukin, 1991). It has been found to dose-dependently induce stereotyped behaviour and

social withdrawal in rats (Sams-Dodd, 1999). It also produces deficits in working memory (spatial and object), which is reversed by clozapine (Jentsch et al., 1997; Castner et al., 2004). As well as being a glutamate receptor antagonist, PCP also acts on the dopaminergic system. It acts to reduce dopamine metabolism in the prefrontal, orbital and limbic frontal cortical regions in monkeys, thus mimicking the hypofrontality seen in schizophrenics (Jentsch et al., 1999).

4.2 Ketamine

Ketamine has been described as a phencyclidine hydrochloride derivative, dissociative anaesthetic and a non-competitive antagonist of the glutamate NMDA receptor (Krystal et al., 1994). Dissociative anaesthetics typically replicate the negative symptoms and cognitive impairments of schizophrenia, unlike the amphetamine model (which involves dopamine receptors), which recreates only the positive symptoms (Coyle and Tsai, 2004). Sub-anaesthetic doses of ketamine in healthy individuals lead to paranoia, perceptual alterations and memory loss, as well as positive and negative symptoms of schizophrenia (Krystal et al., 1994; Malhotra et al., 1997a; Abi-Saab et al., 1998). In the rat, ketamine administration is generally associated with behavioural symptoms, such as hyperlocomotion and stereotypy (Irifune et al., 1991; Uchihashi et al., 1992), and leads to PPI disruption, which is also seen in schizophrenic patients (Braff et al. 2001). In considering ketamine as an appropriate model for schizophrenia, Becker et al. (2003) noted alterations in social behaviour and a long-lasting disruption of latent inhibition (see below) up to 4 weeks after sub-chronic ketamine treatment. Ketamine also affects with learning and memory. In a study by Krystal et al. (2000), the authors showed that ketamine appeared to interfere with the acquisition, but not the expression, of functions related to abstract procedural learning in healthy volunteers. These impairments were found on tasks associated with frontal and cortical activities. In another study, ketamine administered to healthy volunteers significantly reduced changes in fMRI BOLD response in the posterior cingulate, preuncus, and ACC during retrieval of episodic memories (Northoff et al., 2005).

With regards to its effects on neurotransmitter systems, ketamine tends to exert a biphasic influence on the outflow of glutamate in the PFC: low sub-anaesthetic doses

increase these levels, whereas an anaesthetic dose decreases these levels (Moghaddam et al., 1997). It also has a stimulatory effect on the PFC dopamine release, which can be reduced by local application of an AMPA/kainate receptor antagonist (Moghaddam et al., 1997). Other studies showed that ketamine administration resulted in increased dopamine and serotonin secretion, as well as glutamate release, (perhaps through non-NMDA receptors, e.g. AMPA receptors) in the rat PFC (Lindefors et al., 1997; Lorrain et al., 2003). A recent study has shown that these effects of ketamine are in fact due to a *direct* stimulation of the D_2 and $5HT_2$ receptors (Kapur and Seeman, 2002). It could therefore be said that ketamine affects most of the neurotransmitter systems involved in the pathophysiology of schizophrenia.

5 Antipsychotics

Antipsychotic treatment began in the 1950s with the discovery of chlorpromazine (Willamson, 2006). Many more antipsychotics have been developed since then. Fewer than half of the patients receiving these medications however, reach full remission, and only 70 to 80% would be characterised as responding well to neuroleptics (Kane, 1989). As chlorpromazine is a D_2 receptor antagonist, this led to the development of other drugs known as typical antipsychotics such as haloperidol, which also act on the D_2 receptor (Jones and Pilowsky, 2002). It also supported the dopamine hypothesis. These drugs, however, have severe (extra-pyramidal) side effects, with patients often displaying parkinsonian-type symptoms. These antipsychotic treatments also have negative effects on cognition (Tsai and Coyle, 2002). When clozapine was developed in 1961, it was found that it did not fit in with other antipsychotics in terms of its receptor binding profile, and therefore other mechanisms of action were investigated (Pilowsky et al., 1992), signifying the discovery of atypicals. Both typical and atypical antipsychotics have been found to preferentially act on D_2 receptors in the cortex (Xiberas et al., 2001). Atypical antipsychotics, however, differ from typical psychotics in their limbic-specific affinity to dopamine D_2 receptors and in their high ratio of serotonin $5HT_2$ receptor to dopamine D_2 receptor binding (Meltzer et al., 1989; Worrel et al., 2000). Examples of atypical antipsychotics are clozapine, risperidone, quetiapine, and olanzapine. Atypical antipsychotics have subsequently been shown to be more effective in the treatment of schizophrenic symptoms than typical neuroleptics, and provide a greater beneficial

effect on cognition and negative symptoms (Worrel et al., 2000). Today, clozapine is a standard treatment for forms of schizophrenia that have not responded to other forms of treatment (Worrel et al., 2000).

5.1 Clozapine and haloperidol

In addition to being an NMDA and D_2 antagonist, clozapine also has an affinity for $5HT_{2A}$ as well as muscarinic cholinergic and adrenergic receptors (Duncan et al., 1998; Johnson et al., 2005; Ma et al., 2006). In relation to the dopaminergic system, clozapine has the greatest affinity for the D_4 receptor and haloperidol, the D_2 receptor (Meltzer, 1996; Duncan et al., 1998). Taken together with the fact that clozapine is a potent $5HT_2$ antagonist, this could contribute to the differences noted in the actions of these drugs (Duncan et al., 1998). Clozapine also differs from conventional neuroleptics in its cardinal effects on the glutamate system (Heresco-Levy, 2003). An increase in glutamate concentrations has been found after administration of clozapine in several studies (Goff et al., 1996; Evins et al., 1997). Animal studies have also shown this increase in the medial prefrontal cortical glutamate concentrations, an effect not associated with haloperidol (Daly and Moghaddam, 1993; Yamamoto and Cooperman, 1994). Clozapine has also been shown to be the most potent of the antipsychotic agents in blocking NMDA receptor antagonist-induced neurotoxicity (Farber et al., 1993; Olney and Farber, 1994).

The effects of haloperidol and clozapine on the glutamate NMDA receptor also differ. A study by Sams-Dodd (1996) showed that haloperidol did not selectively antagonise the effects produced by PCP in rats, while chronic clozapine treatment inhibited PCP-induced stereotypical behaviour and social isolation. In another study, clozapine (5 or 10 mg/kg) abolished the increased metabolism (2-deoxyglucose uptake) in the prelimbic cortex and nucleus accumbens, anterior ventral thalamic nucleus and hippocampal formation induced by ketamine administration (Duncan et al., 1998). This finding is consistent with the notion that clozapine's blocking/reversal of glutamate receptor antagonists is primarily via the NMDA receptor (Duncan et al., 1998). In the same study, haloperidol was administered 45 minutes prior to ketamine administration (0.5 mg/kg), but failed to alter the behavioural response or metabolic activation induced by ketamine (Duncan et al., 1998), consistent with its putative D_2

mechanism of action. An *in vitro* study has also shown clozapine to displace MK-801 from striatal tissue samples (Lidsky et al., 1993). Clinically, ketamine increases positive and negative symptoms in schizophrenics, an effect that is reduced with clozapine treatment (Malhotra et al., 1997a,b). There is still, however, the need for novel antipsychotics that counteract negative symptoms and cognitive deficits associated with chronic schizophrenia, particularly emotional blunting. These results of clozapine (atypicals) working on the NMDA receptor has led to a shift in research from modulating dopaminergic to glutamatergic systems (Heresco-Levy, 2003).

5.2 mGLu2/3 receptor agonists: LY 354740 and LY 379268

In an attempt to develop antipsychotics acting at the metabotropic glutamate receptor, it was found that novel glutamate receptor (2/3) agonists, LY354740 and LY379268, effectively reversed PCP-evoked motor activations, without impairment to the animals' motor capabilities (Cartmell et al., 1999). LY354740 was able to block ketamine-induced cognitive impairment in normal human volunteers (Swanson et al., 2005). In another study investigating the Glu2/3 agonist, LY379268, this compound increased dopamine levels in the PFC (Cartmell et al., 2000). This effect was, however, less than that of clozapine, and was evoked only after a longer time period.

6 Cognition and emotion

6.1 Cognitive and negative symptoms of schizophrenia

Memory functioning is the largest cognitive deficit seen in schizophrenia (Aleman, et al., 1999). As prefrontal regions are implicated as the origin of cognitive disorders and are involved in emotional regulation, memory functioning may be relevant for some negative symptoms. In a study by Sanfilipo et al. (2002), the authors found that cognitive deficits were strongly related to negative symptoms and/or disorganised behaviour. This study illustrates that schizophrenic patients show marked impairment in age-adjusted cognitive performance (Sanfilipo et al., 2002), relative to control subjects, of which memory and verbal processing are most affected. Patients also had significantly smaller bilateral volumes in grey, but not white matter, in the PFC (Sanfilipo et al., 2002). The authors therefore hypothesised that negative symptoms may involve the disruption of frontal-subcortical connections (Sanfilipo et al., 2002). The schizophrenic group also showed relationships between cognitive performance

and negative symptoms (Sanfilipo et al., 2002) with global negative symptoms (and particularly affective blunting) inversely related to cognitive flexibility.

6.2 Emotional learning

Emotional arousal is thought to have immediate effects during encoding that are interpreted to reflect attentional influences on memory (Labar and Cabeza, 2006). Such concepts of emotional modulation on learning and memory have rarely been explored, despite its well-known importance in human memory (Lang et al., 2000). Affective space is theoretically divided into two dimensions, arousal and valence. Arousal and valence affect the two different forms of memory - declarative and non-declarative (Labar and Cabeza, 2006). Declarative memory includes memory for events, or episodic memory, while non-declarative memory includes fear conditioning, an associative learning paradigm (Eysenck, 1988; Labar and Cabeza, 2006).

6.3 Association

Bleuler (1911) believed that a general “loosening of associations” represented the core deficit in schizophrenia. Indeed, it has been shown that some forms of associative learning (e.g. eyelid conditional discrimination) are disrupted in schizophrenic patients (O’Carroll, 1995; Rushe et al., 1999; Hofer et al., 2001). Furthermore, in an animal study mimicking schizophrenic symptoms with NMDA antagonists, Enomoto et al. (2005) treated mice for 14 days with PCP, which impaired pavlovian fear conditioning up to 8 days after cessation of PCP treatment (Enomoto et al., 2005). Repeated olanzapine for 7 days, but not haloperidol, reversed the associative learning impairment caused by PCP (Enomoto et al., 2005). Such findings suggest that a breakdown in simple associative processes could underlie some of the negative symptoms, such as emotional blunting, seen in schizophrenia. One way to combine examining emotional blunting and associative learning in an animal model is through fear conditioning, as fear is at present one of the most documented emotions in laboratory animals. The association network involved in emotional modulation of memory differs from declarative memory (hippocampus) as it has direct connections to primary motivation-related brain areas (Lang et al., 2000). Brain areas involved in emotional regulation are therefore typically activated by appetitive or aversive stimuli, resulting either in approach or

withdrawal behaviours respectively (Lang et al., 2000).

7 Fear conditioning

Classical fear conditioning is used throughout the literature to study fear circuits in the brain (Maren, 2001; Gerrits et al., 2003; Li et al, 2004). It involves the pairing of a neutral conditioned stimulus with an aversive unconditioned stimulus (Fig. 8). After a few trials, the conditioned stimulus elicits the same response as the aversive stimulus (Walker and Davis, 2002). The aversive stimulus can be visual, auditory, tactile, gustatory or olfactory. In animal models, such as the mouse or rat, measuring the freezing behaviour of animals in response to the conditioned stimulus can allow one to determine whether fear conditioning was acquired or not. Fear conditioning is also considered to be stress inducing (Sotty et al., 1996; Suzuki et al., 2002). Accordingly, the stress the animal experiences may be a good indicator of arousal, with the aversive stimulus eliciting the negatively-valenced emotional response. This response can then be measured after conditioning, in the absence of physical stressors (e.g. in the 5 minutes following a conditioning session).

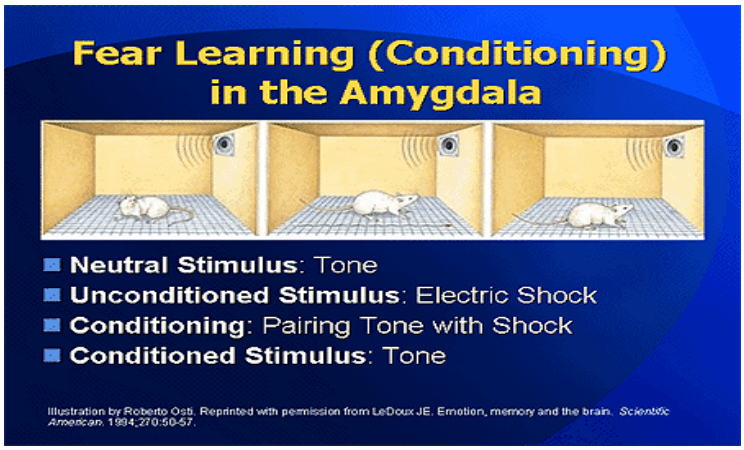


Figure 8: *Fear conditioning takes place in the amygdala. An emotionally-neutral stimulus (e.g. tone) is repeatedly paired with an emotionally-valent stimulus (e.g. shock). The tone then acquires some of the emotional valence associated with the shock, as expressed in various behavioural and neural assays. The fear conditioning paradigm is utilized extensively throughout the current thesis to assess an animal model of negative schizophrenic symptoms. After authors LeDoux et al. (1994), taken from www.medscape.com.*

7.1 Fear conditioning and schizophrenia

There has also been some evidence that fear conditioning is deficient in the schizophrenic patient. In a simple conditioning task combined with aversive emotional stimuli, schizophrenic patients failed to develop an increase in response

frequency to aversively reinforced trials, whereas healthy volunteers acquired a differential response to reinforced vs. unreinforced trials (Hofer et al., 2001). In another study, schizophrenics displayed a continuous deficit in emotional learning tasks after remission of symptoms, while performance on the non-emotional learning tasks was improved and no longer differed from controls (Exner et al., 2004). In the same study, schizophrenic patients also manifested diminished right amygdala volume (Exner et al., 2004). The authors therefore proposed that deficits of schizophrenics in emotional processing could be related to defective amygdala function. Conditioned inhibition (CI), another form of pavlovian conditioning, occurs when the CI stimulus in fact inhibits the prediction of the US by the CS. In a study investigating the relationship between CI and schizotypy scores (a prediction for the development of schizophrenia), Migo et al. (2006) found a negative correlation between these two elements, suggesting impaired learning capabilities in those susceptible to developing schizophrenia.

Taken together, one could suggest that a deficit in associative learning, such as fear conditioning, is in fact present in schizophrenia. Investigating the neural mechanisms thereof in an animal model could therefore elicit new information with regards to the mechanisms underlying the cognitive deficits seen in schizophrenia.

7.2 Brain circuits of fear conditioning

In Pavlovian conditioning, the neural mechanisms are highly conserved across species (Labar and Cabeza, 2006). Fear conditioning induces long-term potentiation, a form of synaptic plasticity that is thought to underlie learning, along both subcortical and cortical routes of information processing to the amygdala (Rogan et al., 1997; Tsvetkov et al., 2002). Brain regions that mediate the acquisition of fear in humans include the anterior cingulate cortex, insula, thalamus, sensory neocortex and amygdala (Buchel and Dolan, 2000; Birbaumer et al., 2005; Labar and Cabeza,

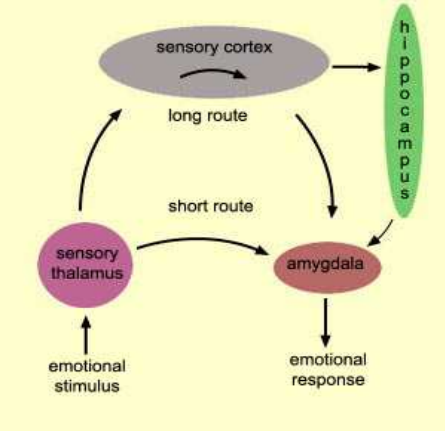


Figure 9: *Circuit underlying fear conditioning in the rat brain. The present study will examine neural activity in several regions of the rat brain following fear conditioning. Taken from* http://www.thebrain.mcgill.ca.

2006). The hippocampus also plays a role in conditioning (Maren, 2001; Sanders et al., 2003), especially in trace conditioning studies (Buchel and Dolan, 2000). The hippocampal formation provides inputs to the basolateral nuclei of the amygdala (Maren, 2001) and also has projections that extend to the PFC (Grace, 2000). According to Thiels and Klann (2002), the hippocampus, although important in contextual fear conditioned memory, does not play a part in tone-dependent fear conditioning, whereas the amygdala does (Phillips and LeDoux, 1992). LeDoux (1998) has previously illustrated a general overview of this fear circuitry in the rat brain in his review (Fig. 9).

According to this scheme, a conditioned stimulus, such as a tone (noise) is first processed by the auditory system, whereafter the information can take two routes to the amygdala (LeDoux, 1998). In rats, it has been shown that the lateral nucleus of the amygdala receives auditory input via 2 mechanisms: rapid, but impoverished input from the auditory thalamus and slow, rich input from the auditory cortex. The amygdala then integrates the 2 pathways during the acquisition and expression of conditioned fear responses (Li et al., 1996).

7.2.1 Amygdala, learning and memory in humans

The contributions of the amygdala, PFC, and the medial temporal lobe in memory are well characterised. This system participates both in the initial period of memory consolidation and the later retrieval of emotional memories, including those from the personal past (Labar and Cabeza, 2006). The amygdala is also involved in the ability to attribute mental states to others (Shaw et al., 2004) and in the processing of social cues (Adolphs et al., 1998).

A rare disease, called Urbach-Wiethe syndrome, involves selective amygdala pathology. These patients provide an important information source in trying to understand the workings and influences of the amygdala. They typically show impairments in long-term recall or recognition of emotional words, pictures and stories (Markowitsch et al., 1994; Adolphs et al., 1997). A later study by Adolphs et al. (1999) showed that bilateral amygdala damage also impaired fearful face recognition (as seen in schizophrenia) and attributed this deficit to the dysfunction of a general fear circuit in which the amygdala plays a central role. In addition, these

patients were inconsistent in recognising emotions. The authors suggested that this impairment is due to an inability to retrieve emotional knowledge, especially with negative emotions (Adolphs et al., 1999). Patients with amygdala lesions (unilateral temporal lobectomy) do, however, remember words that are affectively valent, but low in arousal (unlike fearful stimuli) relative to neutral ones (Phelps et al., 1997). Emotional arousal may therefore play an important role in amygdala-related memory formation (Phelps and Anderson, 1997). Conversely, patients with amygdala damage also exhibited intact responses to arousing events. The authors claim that these deficits in emotional processing are perhaps not specifically related to perception, but rather are memory-based (Phelps and Anderson, 1997).

Evidence for this proposal is found in PET studies, which established that the amount of amygdala activation during encoding of memory correlates positively with delayed recall of aversive, but not neutral, film clips, as well as delayed recognition of emotionally arousing pictures that are both positive and negative in valence (Cahill et al., 1996). One PET study has found a positive correlation between brain glucose metabolic rate in the right amygdala during memory encoding and the number of emotional films recalled three weeks later (Hamann et al., 1999). This finding was not significant for neutral films. Left and right amygdala activity was also related to episodic memory for aversive (and pleasant) stimuli (Hamann et al., 1999).

7.2.2 Amygdala and fear conditioning

Damage to the amygdala consistently impairs fear conditioning and fear-potentiated startle responses (paradigms used to investigate associative emotion-based learning) in non-human animals (Peper et al., 2001; Buchanan et al., 2004). In humans, it has been shown that the amygdala's strongest response to conditioned fear is during the acquisition phase, when emotional associations are initially formed (Buchel et al., 1998; LaBar et al., 1998). This finding is similar to the electrophysiological response profiles of some lateral amygdala neurons during fear conditioning in rats (Quirk et al., 1995). In animals, bilateral excitotoxic amygdala lesions in rats result in a blockade of mesocortical monoaminergic responses to stress induced by re-exposure to stimuli previously paired with an unconditioned stressor (Goldstein et al., 1996). These lesions also attenuated associated

adrenocortical activation, freezing, ultrasonic vocalisation, and defecation (Goldstein et al., 1996). The authors suggest that the amygdala is critical in linking aversive stimuli to the normally contingent behavioural, neuroendocrine, and cortical monoamine responses to stress (Fig. 10) (Goldstein et al., 1996).

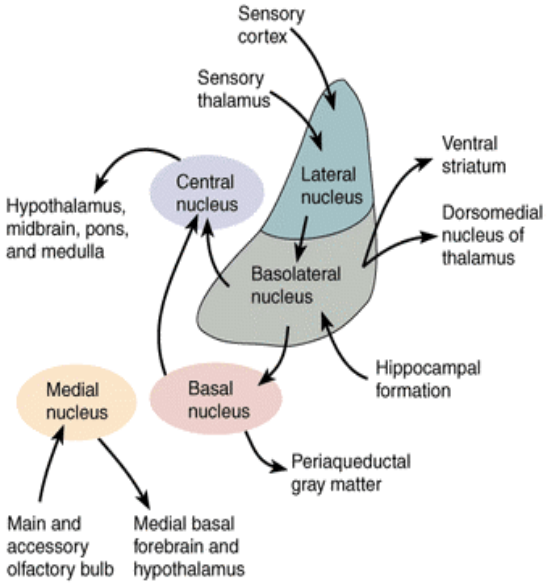


Figure 10: *The amygdala and its major input and output projections. The amygdala receives projections from sensory cortex and provides outputs to brain areas involved in autonomic, endocrine, motor, memory and cognitive functions. The amygdala several major nuclei, perhaps the most important (from a fear conditioning perspective) being the basolateral and lateral nuclei and the central nucleus. The respective function of the regions is a topic of ongoing research and will be an important consideration in the present study. From* [*http://homepage.psy.utexas.edu*](http://homepage.psy.utexas.edu)*.*

A study investigating patients with unilateral amygdala lesions demonstrated impairments in the conditioned startle potentiation (see below) by aversive and threatening stimulation, revealing an important role for the amygdaloid complex in this response (Weike et al., 2005). This response was also lateralised, with right hemisphere lesioned-patients showing accurate startle reflex during an instructed fear paradigm, but an impaired one when no instructions were given regarding the pairings of aversive stimuli and CS. The opposite pattern was noted in patients with left hemisphere lesions (Weike et al., 2005). Morris et al. (1998) also noted lateralisation effects when they investigated the amygdala's response to fear conditioning in humans by making use of a masking paradigm. Activity in the right amygdala was enhanced significantly during presentation of masked conditioned faces, whereas activity in the left amygdala was relatively enhanced when the conditioned angry face was clearly seen (unmasked). In this context, Markowitsch (1998) suggests that the left amygdala be more closely related to the encoding and

extraction of detailed stimulus features, whereas the right amygdala is involved in retrieval, with a special affinity for pictorial emotional material. Meta-analysis has also revealed that left amygdala (more than right) activation is related to cognitive processing of emotional stimuli (Wager et al., 2003). Further research needs to be undertaken to fully explore lateralisation effects of the amygdala.

The amygdala, being composed of several nuclei (Fig. 10), has various functions depending on the nucleus. According to an fMRI study, which involved the pairing of a red light with an electrical shock, the central amygdala (CEA) is primarily involved in the execution of autonomic responses to fearful events, whereas the basolateral nuclei are involved in the connection of the event to the fearful stimulus. Data from several animal studies agree with this statement. For example, Fanselow and Kim (1994) found that bilateral administration of AP5, a glutamate antagonist, into the BLA prior to fear conditioning prevented fear acquisition, but infusions into the central nucleus did not. Similar results were found in a study by Shors and Matthew (1998). In the study by Killcross et al. (1997), lesions made within the basolateral nucleus of the amygdala prevented the animals from being able to learn to avoid the shock, while the lesions in the central nucleus did not do so. The study by Koo et al. (2004) showed that the fibres that run through the central nucleus from the basolateral nucleus, and not the neurons within the central nucleus itself, are responsible for fear conditioning, thus implicating the basolateral nucleus alone in the fear conditioning process. It has also been shown that lesions in the BLA that are given 28 days after training produce deficits in expression of conditioned fear, indicating storage of the association between CS and US in the BLA (Lee et al., 1996; Maren et al., 1996).

The convergence of the sensory pathways within the basolateral nucleus of the amygdala (Fig. 10) has in the past made it an interesting site to investigate fear conditioning (Walker and Davis, 2002). According to Huff and Rudy (2004), the basolateral region of the amygdala modulates memory formation in other regions of the brain and is a storage site for CS-US association (Schauz and Koch, 2000, Gale et al., 2004). Gale et al. (2004) investigated the role of the BLA in expression of fear memories varying from 1 day to 16 months. Lesions of the basolateral nuclei, made

shortly before or after training, produced profound deficits in fear conditioning to auditory, visual and contextual stimuli. Lesioned rats also showed robust deficits during all recent and remote memory tests. In particular, post-training lesions produced robust freezing deficits to contextual and auditory stimuli, independently of training-to-lesion interval (Gale et al., 2004), indicating a role for the BLA in memory storage of both auditory and contextual fear (Gale et al., 2004). BLA lesions, however, did not interfere with the general ability to freeze (Gale et al., 2004). This evidence suggests that the BLA plays a specialized role in encoding the emotional aspects of fear conditioning, perhaps coordinating the consolidation of declarative memory in extra-amygdala regions (Grace, 2000; Gale et al., 2004). Over-training can, however, allow contextual training to occur independent of the BLA (Gale et al., 2004), indicating that other regions can compensate for loss of BLA activity. While direct fear responses to specific threats are primarily mediated by the amygdala, sustained anxiety responses that persist beyond the immediate threat include structures other than the amygdala for mediation (Walker and Davis, 2002).

7.2.3 Glutamate NMDA receptors, fear learning and the amygdala

It has already been established in this Introduction that the amygdala, in particular the BLA, is critical for fear conditioning. In this process, the glutamate receptors, especially those located on the BLA, play an important role. In fact, NMDA receptors are more highly concentrated in the basolateral nucleus than in the central nucleus of the amygdala (Monaghan and Cotman, 1985) reinforcing the BLA's role in fear conditioning. Amygdala NMDA receptors have been shown to participate in the initial acquisition of Pavlovian fear memories, and may also participate in post-training consolidation processes important for avoidance learning (Walker and Davis, 2002). One study showed that pre-training infusion of the NMDA antagonist, APV (2-amino-5 phosphonovalerate), into the BLA, impairs the acquisition of the two-way active avoidance reaction: animals failed to acquire the directionality of the escape reaction and showed deficits in attention to conditioned stimuli (Savonenko et al., 2003). APV did not retard the acquisition of freezing to contextual cues, however, but did dramatically deteriorate the retention of contextual fear (Savonenko et al., 2003). This deficit coincided with a significant attenuation of cFos activation in the amygdala (Savonenko et al., 2003): cFos is associated with the acquisition of new memories in this particular paradigm (Radwanska et al., 2002). cFos expression in the amygdala

has also been correlated with measures of emotional learning, but not with sensory stimulation (e.g. during foot shock) (Savonenko et al., 1999). Savonenko et al. (2003) conclude that the blockade of NMDA receptors in the BLA during their paradigm represents a disrupted CS-US association in pavlovian fear conditioning. Another study also indicated that when the NMDA antagonist, AP5, is infused into the BLA prior to fear conditioning (light-shock), fear learning is disrupted, as assessed 1 week later (Miserendino et al., 1990). Infusions 5 days after training, and 1 week before testing, had no effect on the fear-potentiated startle reflex. These findings were replicated using auditory and olfactory cues as CS (Walker and Davis, 2002).

7.2.4 Prefrontal cortex

7.2.4.1 Learning and conditioning

A variety of cognitive abnormalities have been described in schizophrenia, including disturbances in selective attention and working memory. Hypofrontality in the PFC is also a robust finding in schizophrenia research (Williamson, 1987; Lewis et al., 2004), and likely provides a neural basis for many of the cognitive deficits (Castner et al., 2004; Lewis et al., 2004). One fMRI study showed learning-related changes in activation within the ACC (Knight et al., 1999). Within the ACC itself, the amount of active tissue increased as a function of repeated CS-US trials, but did not change with unpaired light and shock presentations (Knight et al., 1999), providing evidence for a role in associative learning. The authors did, however, suggest that the ACC may facilitate, but not necessarily be critical, in learning affective behaviour, as determined by lesion and physiological studies (Knight et al., 1999). It has recently been suggested that one possible mechanism of how this facilitation is achieved is through connections between the ACC and the amygdala (Tang et al., 2005). In fact, many ACC neurons have been found to project directly to the amygdala (Aggleton et al., 1980). It has also been suggested that the amygdala relays signals to the ACC that could be important for adjusting motivational levels or for forming reward expectations in the ACC (Sugase-Miyamoto and Richmond, 2005). In line with this idea, an animal study showed that auditory fear memory produced by pairing ACC stimulation with a tone was blocked by an NMDA receptor antagonist, AP5, administered locally into the amygdala (Tang et al., 2005). This was not the case for contextual fear memory, which may be mediated by other structures, such as the

hippocampus (Tang et al., 2005).

7.2.4.2 Pain

As well as being involved in associative learning, the ACC is also involved in the processing of both pain sensation and pain emotion (Gao et al., 2004). Pain could therefore be a confounding variable in a shock-related fear conditioning paradigm, as it also involves the ACC. Historically, it has been shown that surgical ablation of the ACC and surrounding cortical tissue decreased pain-related unpleasantness without affecting the patient's ability to discriminate the intensity or localization of the noxious stimuli (Foltz and White, 1962). An animal study investigating the magnitude of formalin-induced conditioned place avoidance showed that this behaviour was reduced in ACC and amygdala lesioned rats, indicating that different neural substrates underlie pain affect and pain sensation (Gao et al., 2004). The authors suggested that lesions in the ACC cause a decrease in aversion or perceived unpleasantness to the noxious stimulus (Gao et al., 2004). They also proposed that the ACC may be specifically involved in pain-related negative emotion, rather than aversive associative learning, as ACC lesions did not affect the foot shock induced avoidance, but did block pain related avoidance (Gao et al., 2004).

7.2.4.3 NMDA receptors, fear learning and the ACC

Glutamate receptors in the ACC also contribute to emotional learning. Activation of metabotropic glutamate receptors in the ACC facilitates behavioural responses in both the tail-flick reflex and hot-plate tests, providing direct evidence for the involvement of glutamate in pain mediation in the ACC (Tang et al., 2005). mGlu receptors have also been shown to be involved in learning in the ACC, as demonstrated by the enhanced the escape response due to chemical activation of these receptors (Tang et al., 2005)

7.2.5 Use of NMDA antagonists in the disruption of fear conditioning

As some NMDA receptor antagonists induce sensory distortions in humans, the use of these antagonists in a fear conditioning (emotional learning) paradigm has been questioned. Walker and Davis (2002) claim that the effects of NMDA receptor blockade cannot be attributed to a general disruption of amygdala activity, or to a specific inability of the rat to process the CS. Observations have been made that

NMDA receptor-mediated currents contribute minimally to synaptic transmission, but in fact play a more active role in triggering intracellular cascades, such as those involved in neural plasticity (Walker and Davis, 2002). Could the NMDA antagonist (AP-5)-induced learning impairments then be attributed to a disruption of the US processing? Miserendino (1990) has reported that reactions to foot shocks between controls and AP5 rats (injected into the amygdala) were indistinguishable, even at a dose four times that required to impair learning. Subsequent studies have confirmed this result (Campeau et al., 1992). Therefore there is no evidence for the analgesic influence of this treatment. Walker and Davis (2002) also claim that the ability of AP5 to disrupt learning could not be attributed to the disruption of neural transmission in pathways that convey footshock information to the amygdala. The disruption must therefore involve the impaired association of a US and a CS (Walker and Davis, 2002).

8 Animal models of schizophrenia

8.1 Latent inhibition and blocking

Animal models of schizophrenia typically combine either pharmacological treatments, adult lesions or neonatal lesions, with some form of conditioning paradigm (Marcotte et al., 2001). Attentional (cognitive) deficits in schizophrenia are often investigated by a type of conditioned fear response, known as latent inhibition (e.g. Sotty et al., 1996). This phenomenon is elicited when the conditioned stimulus is presented in the absence of the unconditioned aversive stimulus prior to conditioning, which then delays the learning process (Sotty et al., 1996; Escobar et al., 2002). Radulovic et al. (1998) described latent inhibition as a decrease in attentional processing during the encounter of a stimulus. These tests are reputed to reflect the normal functioning of attention, which has repeatedly been shown to be disrupted in acute schizophrenics (Escobar et al., 2002). Kamin blocking is defined as a procedure involving a pre-exposure stage, where an association between a conditioned stimulus CS and an unconditioned stimulus US is made. A second series of pairings are then presented with the same US as before. Kamin blocking is then indicated by a decreased rate of learning of the second pairing exhibited by the subjects exposed to the original CS-US relationship (Jones et al., 1997).

Schizophrenic patients and their schizotypal and non-schizotypal relatives display disrupted latent inhibition and Kamin-blocking effects (Martins Serra et al., 2001). In another study, latent inhibition was also not observed in acute unmedicated patients, while it was observed in chronic, medicated patients and controls (Vaitl et al., 2002). Animal models have also shown disrupted latent inhibition with drugs known to induce schizophrenic-type psychotic states, such as NMDA antagonists. In a conditioned emotional response model in rats for example, MK-801 led to abnormally persistent latent inhibition during the conditioning stage (Gaisler-Salomon and Weiner, 2003). This effect was subsequently reversed by clozapine, but not haloperidol, administered during pre-exposure (Gaisler-Salomon and Weiner, 2003). One shortcoming of the attentional model prevalent in studies of latent inhibition and blocking is that it only explains changes occurring in response to the conditioned stimulus (Escobar et al., 2002) and not the association of the US and CS.

8.2 PPI

The startle reflex consists of a collection of physiological responses in response to a sudden intense stimulus. The major advantage of the startle reflex paradigm is that the resulting behavioural responses can be studied in a variety of species including both humans and rodents (Braff and Geyer, 1990; Koch, 1996). It therefore serves as a valuable tool to assess different forms of information processing, such as fear-potentiation and prepulse inhibition.

Schizophrenia patients are deficient in the normal inhibition of the startle reflex that occurs when the startling stimulus is preceded by a weak pre-stimulus (for a review, see Braff et al. 2001). This loss of normal prepulse inhibition (PPI) is thought to be a measure of the deficient sensorimotor gating (Braff and Geyer, 1990) that underlies sensory flooding and cognitive fragmentation in these patients (McGhie and Chapman, 1961). Similar deficits in PPI can be produced in rodents by pharmacological or developmental manipulation, which provide models of sensorimotor gating deficits in schizophrenic patients with face, predictive and construct validity (Geyer et al., 2001; Swerdlow et al., 2001). Yet, PPI remains primarily a model of attentional processing, and does not explore the emotional modulation of cognitive processes.

9 Fear processing and anxiety disorders

9.1 Basic features

Fear/stress is considered to be a normal response to threatening or potentially threatening stimuli, as seen in the fear conditioning paradigm. Fear is defined as a reaction to an aversive or threatening stimulus leading towards behaviour directed at escape or avoidance (Lang et al., 2000). This reaction typically utilises the fear conditioning circuit mentioned above. Fear responses include not only the classic three “cannonical” options of fight, flight, or freeze (Cannon, 1929) but also anticipatory fear and increased levels of arousal (Yerkes, 1921), which can be measured in the absence of the aversive stimulus in order to gauge the emotional arousal. Anxiety, however, is defined as a more general and longer lasting state of distress, involving physiological arousal, but sometimes without functional behaviour (Lang et al., 2000). It has been suggested that this emotional state makes more use of the bed nucleus stria terminalis than the CEA, although these two areas project onto the same end substrates (Lang et al., 2000). If these responses become maladaptive i.e. if the response is incongruent to the situation, they may constitute a disorder. Anxiety disorders incorporate panic disorder, social phobia, obsessive-compulsive disorder, post-traumatic stress disorder and generalized anxiety disorder.

9.2 Brain circuits

The brain circuit involved in anxiety disorders is the equivalent of the fear conditioning circuit (Davidson, 2002; see section 7.2). It was suggested in Davidson (2002) that disinhibition of the PFC’s control of the amygdala could lead to maintenance of a learned aversive response, leading to anxiety disorder.

9.3 mGlu receptors and anxiety

As glutamate is the main excitatory neurotransmitter in the brain, and since anxiety disorders may involve overexcitation of the fear circuit, it is logical that new therapeutic approaches towards anxiety disorders include drugs that modulate glutamate functioning. Disruption of the glutamate system is therefore not only associated with schizophrenia, but also with anxiety disorders. In particular, both NMDA and AMPA/kainate receptor antagonists have shown anxiolytic properties (Jardim et al., 2005; Alt et al., 2006; Boyce-Rustay and Holmes, 2006). In the clinical

setting, however, these drugs have not been convenient in treating anxiety disorders, due to memory impairment and central nervous system depression (Danysz and Parsons, 1998). The direction of the search for anxiolytics has therefore turned to the metabotropic glutamate receptors, as mGlu receptors do not have the profound negative effects of the ionotropic receptors on the nervous system (Fig. 11). The group II mGlu (2/3) receptor agonists, in particular, have been shown to be potential anxiolytics through inhibition of glutamate release. Of particular interest, these agonists suppress excitatory neurotransmission in the amygdala (Swanson et al., 2005).

One study has shown that pre-treatment with LY354740, an mGlu 2/3 agonist, prevents stress-induced cFos induction in the hippocampal regions, but does not modify the elevated-plus-maze-induced cFos induction seen in the CEA nucleus (Linden et al., 2004). As indicated previously, it is well known that the hippocampus is primarily involved in contextual conditioning (Labar and Disterhoft, 1998; Holland and

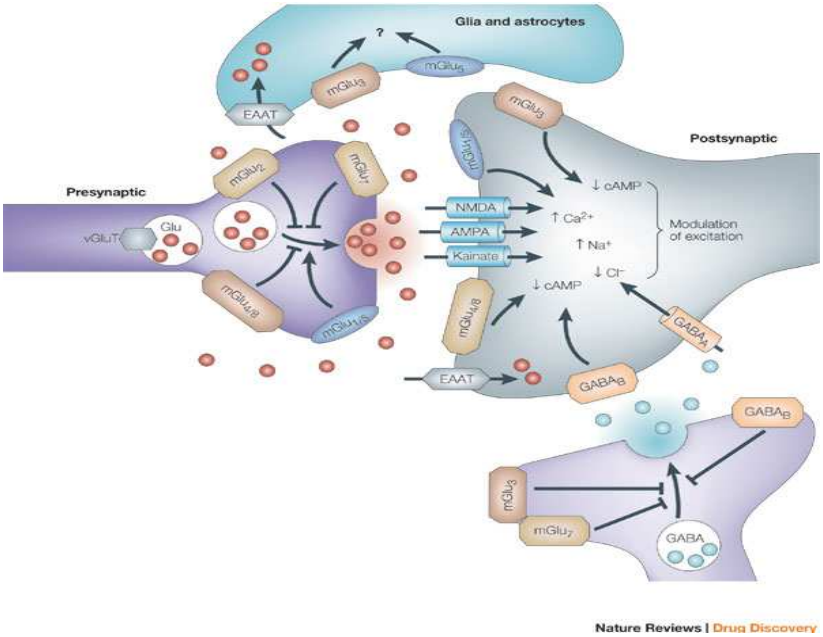


Figure 11: *Localisation and function of glutamate receptors on a hypothetical synapse according to Swanson et al. (2005). Ionotropic glutamate receptors have not been successful in the clinic, and therefore focus has switched to the mGlu receptors. mGlu (2/3) receptor agonists, in particular, have been shown to be potential anxiolytics through inhibition of glutamate release*

Bouton, 1999; Gewirtz et al., 2000; Maren and Holt, 2000; Anagnostaras et al., 2001). In anxiety disorders, contextual factors contribute more to fear generalisation, traumatic memory retrieval and relapse after exposure therapy (Mineka et al., 1999) than possibly sensory conditioning, the latter involving primarily the amygdala (Thiels and Klann, 2002). Psychological stress, dysregulation of central monoamine systems, and dysfunction of the amygdala have, however, been proposed to play a role in the development of post-traumatic stress disorder (Charney et al., 1993; Goldstein et al., 1994).

10 Schizophrenia, fear conditioning and ketamine: a novel approach to negative symptoms

In patients suffering from negative symptoms, we often find the opposite phenomenon to anxiety disorder, with an absence of emotional response to fear-inducing stimuli (emotional blunting), including diminished functional and structural integrity of amygdala and other important fear and motivation areas. As summarized above, the functional integrity of the amygdala and related brain regions is critically dependent on normal glutamate NMDA functioning.

In the present study, we wish to develop an animal model to study emotional blunting, a key negative symptom in schizophrenia. We therefore make use of a fear conditioning paradigm. Although this paradigm is usually used to investigate anxiety disorders, as pointed out by Aleman and Kahn (2005), anxiety and schizophrenia are interlinked. Studies have shown that anxiety often precedes the onset of hallucinations (Delespaul et al., 2002) and higher levels of anxiety are related to a predisposition for hallucinations (Allen et al., 2005). Neuro-imaging studies suggest that positive symptoms are associated with increased amygdala activity, whereas negative symptoms are associated with hypoactivation (Taylor et al., 2002; Fahim et al., 2005). Anxiety is present in the onset stages of schizophrenia, yet largely absent in the longer-term stages of the disorder (Cutting, 2003). Aleman and Kahn (2005) propose a two-hit model of amygdala abnormalities in schizophrenia. They speculate that prolonged activation of the amygdala during psychotic states in the onset stages of schizophrenia could lead to glutamate excitoxicity resulting in amygdala lesions and long-term hypofunctioning (see also Heresco-Levy, 2003). A decrease in amygdala grey matter density is also noted in schizophrenics over the course of the disorder (Hulshoff Pol et al., 2001). Here we simulate glutamate excitotoxicity through glutamate antagonism, namely through ketamine administration, leading to hypofunctioning of the amygdala and other brain areas involved in fear conditioning. Accordingly, we combine a conditioning paradigm (Chapter 2) with ketamine (Chapter 3), and examine conventional measures of fearful behaviour (e.g. freezing) and neural activity (see below) in a putative rat model of emotional blunting (Chapters 3-5).

10.1 cFos expression as a measure of functional integrity

cFos is an immediate-early gene that can be used as an index of neuronal activity, since it is speculated to occur as a consequence of synaptic activity (Sagar et al., 1988). Immediate-early genes are so-called because of their direct transcriptional activation due to neurotransmitters or drugs (Sagar et al., 1988; Ananth et al., 2001). At rest, Fos is produced in small quantities in the neuron. In response to a stimulus, cFos mRNA is produced *en masse* and translocated into the cytoplasm to be translated into protein (Ananth et al., 2001). If cFos is induced, the time period when the protein product is maximal is between 1 and 4 hours post-experimentation (Sharp, 1997).

As mentioned earlier, glutamate NMDA receptor antagonists are reputed to induce several symptoms characterising schizophrenia. cFos studies would therefore be helpful in pinpointing their locus of action in the rat brain. For example, induction of cFos mRNA was noted 1 hour after injection of PCP (0.86 mg/kg or 8.6 mg/kg i.p.) in the anterior and posterior cingulate areas and in the thalamus (Gao et al., 1998). This activation was sustained up to 3 hours after injection. In the same experiment, MK-801 (0.1 mg/kg; 1 mg/kg i.p.) also induced cFos mRNA expression (at both dosages) in limbic and cortical areas, including the medial prefrontal, parietal and cingulate cortices (Gao et al., 1998). If cFos induction can be measured with respect to drugs that induce negative schizophrenic symptoms (e.g. ketamine), then logically the capacity of antipsychotics to relieve or block these symptoms can also be measured in terms of cFos expression (e.g. Nguyen et al., 1992; Chapter 4).

10.2 Central hypotheses of the thesis and their significance

The present model is aimed at elucidating the putative glutamate-regulated breakdown in fear processing, and related cognitive-emotional processes, observed in patients suffering from schizophrenia, and similarly, in healthy controls following ketamine administration (Krystal et al., 2000; Abel et al., 2003). As fear conditioning involves basic associative learning and memory processes, we wish to investigate whether emotional blunting (a negative symptom) is caused by an interruption of basic emotional processing in the amygdala and other brain areas in the fear circuit. We therefore combine the paradigm of fear conditioning with ketamine administration

in order to develop an animal model of the negative symptoms of schizophrenia.

Based on the above, we therefore propose the following logical sequence of hypotheses:

1) Fear conditioning will lead to:
    a. Increase in behaviour associated with fear (e.g. freezing).
    b. Increase in cFos expression in those brain areas involved in fear conditioning, including the ACC and BLA.
    c. Increased glutamate release and dopamine modulation in those same brain areas.
2) Ketamine administration will abolish all the above-mentioned effects of fear conditioning to baseline levels of behaviour, cFos expression and neurotransmitter levels.
3) Administration of clozapine, but not haloperidol or LY 379268 (an anxiolytic), will block the effects of ketamine in all measured output parameters, either fully or partially.

Evidence in favour of all three hypotheses would support the notion that glutamatergic hypofunctioning in the amygdala and related brain areas underlies negative schizophrenic symptoms, thereby paving the way for future studies to explore novel drug treatments of these notoriously drug-resistant symptoms.

10.3 The emotional-cognitive perspective

The putative model may shed light on the emergence of cognitive symptoms through the breakdown of normal interactions between emotional and cognitive processing, as proposed in the theory of Grossberg (2000) (Fig. 12). According to this theory, brain regions involved in emotion and motivational learning, such as the amygdala, interact with cognitive brain regions that selectively focus attention on sensory features relevant to a conditioning task. A deficit in motivational learning can lead to problems in attentional focusing by means of the bi-directional linkage between brain areas subserving these functional roles. Cardinal et al. (2002) have proposed that the rodent BLA performs the role of motivational learning, while the ACC selects specific

sensory features associated with a conditioned stimulus. Based on these hypotheses, we speculate that ketamine will lead to the abolition of fear-related activity in the BLA and the ACC, which in the present study may represent the cognitive-emotional breakdown predicted in Grossberg's (2000) theory of schizophrenia.

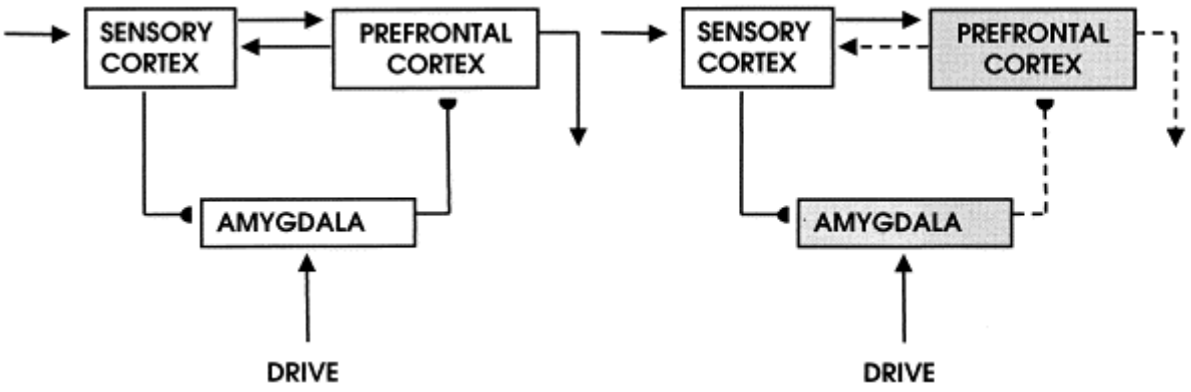


Figure 12: *Grossberg's (2000) emotional-cognitive model of schizophrenia. In the normal case, the amygdala is hypothesised to facilitate motivated behaviour through interactions with the prefrontal cortex and the sensory cortex (left). When the activities of drive representations in the amygdala are compromised (right), say through excitotoxic lesions, processing of emotionally-valent sensory and cognitive (in prefrontal cortex) information is compromised, leading to problems in associative linking of sensory, emotional and cognitive states. This breakdown may correspond to the negative schizophrenic syndrome.*

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Chapter 2

Fear conditioning and shock intensity: The choice between minimising the stress induced and reducing the number of animals used

CY Pietersen, FJ Bosker, F Postema, JA den Boer

Department of Psychiatry, Medical Faculty, UMCG, The Netherlands

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Abstract

Many fear conditioning studies use electric shock as the aversive stimulus. The intensity of shocks varies throughout the literature. In this study, shock intensities ranging from 0mA to 1.5mA were used, and the effects on the rats assessed by both behavioural and biochemical stress parameters.

Results indicated a significant difference with respect to defecation and freezing behaviour between controls and those animals that received a shock. Significant differences in corticosterone levels were also noted between controls and those groups that received a shock.

No significant differences were found between the shock groups with regards to the stress parameters measured in our fear-conditioning paradigm, indicating that the two shock groups were similarly stressed. Increased significance levels were noted for freezing behaviour as well as a lower standard error of means was found in the highest shock intensity group.

We therefore recommend using the higher shock intensity (1.5mA) as the rats in the higher shock intensity group were more homogenously fear-conditioned and therefore the results should be more reproducible and robust than in the lower shock intensity group. This would allow for fewer rats to be used in order to gain an accurate impression of the conditioning paradigm employed.

1 Introduction

Classic fear conditioning is used throughout the literature to study fear and reward circuits in the brain (Maren, 2001; Gerrits et al., 2003; Li et al, 2004). It typically involves pairing a neutral conditioned stimulus with an aversive unconditioned stimulus. After a few trials, the conditioned stimulus then elicits the same response as the aversive stimulus (Walker and Davis, 2002), namely the conditioned response. By measuring the freezing behaviour of the animals in response to the conditioned stimulus, one can then determine whether fear conditioning was acquired or not. However, many discrepancies occur in the various studies concerned. This extends from the type and intensity of the aversive stimuli employed to the number of times an animal is conditioned (1 day to 21 days) (Kaehler et al., 2000; Pezze et al., 2002; Gerrits et al., 2003; Kikusui et al., 2003).

Fear conditioning is also considered to be stress inducing (Sotty et al., 1996; Suzuki et al., 2002). Accordingly, the stress the animal experiences, may be a good indicator of fear response. This is especially true when it is measured after conditioning and in the absence of physical stressors. Essentially the emotional stress reaction in response to the conditioning can then be obtained.

We were interested in measuring the stress that the animal experiences, not only to determine whether fear conditioning was acquired, but also to determine quantitatively what the animal endures. The Hypothalamic-Pituitary-Adrenal (HPA) axis is the pathway through which activation by a stressor ultimately leads to the release of cortisol (in humans) or corticosterone (in animals) (Nash and Maickel, 1988; Udelsman and Chrousos, 1988). Therefore, in order to determine the stress effect of the different shock intensities induced in our paradigm, plasma corticosterone levels were measured.

The behavioural parameters, freezing and defecation, are also commonly used as indicators of stress (Goldstein et al., 1996; Suzuki et al., 2002) and were taken as such in our study. They are also indicative of fear or anxiety (Babar et al., 2001; Goldstein et al., 1996). Freezing in particular is the key behaviour to determine

whether fear conditioning has been obtained or not (Inoue et al., 1993; Holahan et al., 2002; Levita et al., 2002).

The aim of this study was to investigate the effects of classic pavlovian fear conditioning on various stress parameters in order to quantify the stress experienced by the rat as well as the relation between the variance of the behaviour shown to the shock intensity delivered. We therefore wanted to determine which shock intensity causes the least amount of stress to the animal undergoing an acute fear conditioning paradigm, as well as optimising the results obtained.

2 Materials and methods

2.1 Animals

Male Sprague-Dawley rats (outbred strain, Harlan, The Netherlands) (n=15) weighing between 225-250g were obtained from the central animal facility (Groningen, The Netherlands). The animals were tested for viruses, bacteria, mycoplasma, fungi, parasites and pathological lesions and were found to be healthy and specified pathogen free. After arrival from the animal breeding facility, they were housed individually and conventionally in cages (38x22x18cm, lxbxh) with enriched environments (wooden stick, tissue paper) and allowed to acclimatise for two to three days. They were then handled daily for five days for five minutes per day in order to eliminate handling stress as a confounding variable. The treatment schedule and protocol were approved by the local animal experimental commission (DEC: Dierenexperimentele commissie, Groningen, The Netherlands), under the law for the care of experimental laboratory animals (Experiment number: DEC 2823).

2.2 Husbandry during experiment

Animals were housed individually in perspex cages lined with sawdust bedding (LTE E-001, ABEDD) in a temperature (± 23℃) and humidit y controlled (40 to 60 %) conventional environment. They were kept on a 12-hour light/dark cycle, with the light cycle beginning at 7am, ending at the beginning of the dark cycle at 7pm. Ventilation in combination with air conditioning was provided by means of an outlet filtration system. Methods to refine experimental techniques were applied in accordance with

the three R's (replacement, reduction, refinement). In order to determine the effects on the whole organism, the animals could not be replaced with other techniques.

2.3 Feeding

Food (pellets) obtained from Hope farms (RMH-B, Woerden, The Netherlands) was given to the animals and consisted of a normal grain diet enriched with dietary vitamins, the composition of which is given in Table 1. Water was obtained from normal taps suitable for human consumption. Both food and water were available *ad lib.*

Table 1 The chemical analysis and vitamin content of rat pellet food

RMH-B rodent chunks 2181	
Analysis (%)	
24.0	Raw protein
5.5	Raw fat
4.0	Cellulose
5.6	Raw ash
0.65	Calcium
0.49	Total phosphor
10.0	Fluid
0.4	Sodium
0.88	Potassium
0.12	Magnesium
Vitamins	
20600 IE/kg	A
1900 IE/kg	D_3
95 IE/kg	C
60 IE/kg	E
11 mg/kg	Copper

2.4 Experimental Procedure

A total of 15 rats were used in this experiment. The animals were divided into 3 groups: controls (n=5), fear conditioned with 1,0mA (n=5) and fear conditioned with 1,5mA (n=5) as several behavioural studies indicate that five animals are adequate in order to obtain significant results (Wilensky et al, 2000; Majak and Pitkanen, 2003). The rats were taken out of their home cage and placed individually in the shock box. This was a specially constructed wooden container (25x25x25cm) with a floor made of a metal grid. A central computer controlled the current and noise emission making use of a programme that was specially developed for this study (N594 ver. 2.00,

Rijksuniversteit Groningen, The Netherlands, 2002). The DC current was scrambled and randomised across the individual bars. Rats (n=10) were then subjected to fear conditioning on two consecutive days by means of pairing a noise (60dB, pure tone) with a shock (1.0 or 1.5mA). One shock session consisted of a 1-minute period (Fig. 1) that was repeated consecutively ten times per day. All sessions took place in the morning (± 10am) and lasted 10 minutes in total.

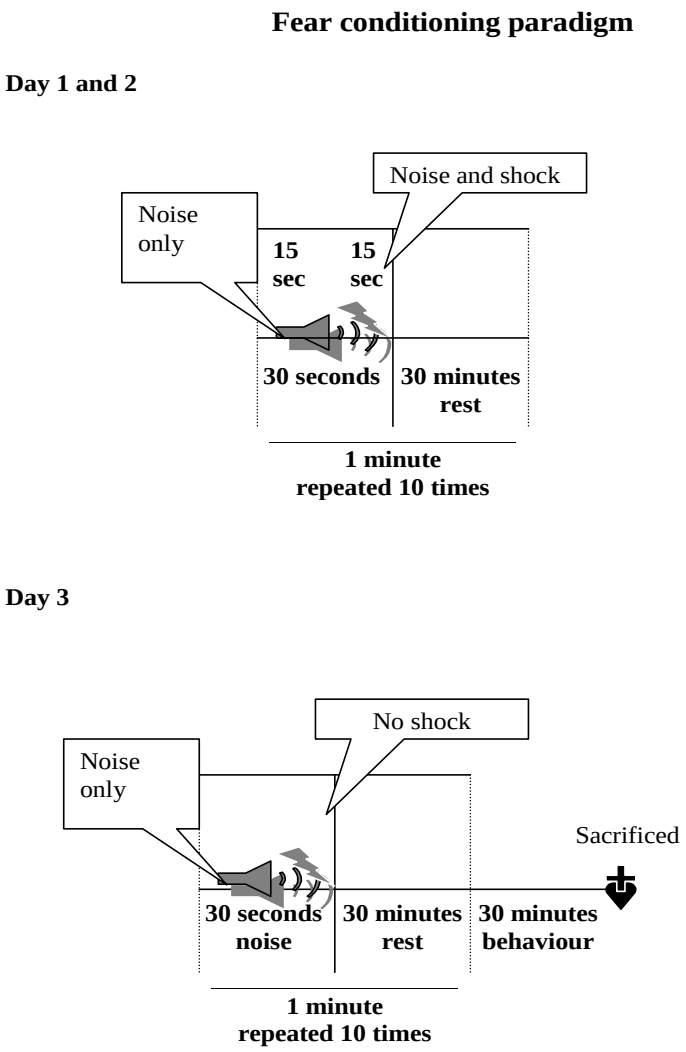

Figure 1: *Fear conditioning paradigm. This diagram is representative of the 1-minute shock session that is repeated 10 times per day on day 1 and day 2. On day 3, the same process is followed, but without administering the shocks, where after the behaviour is noted and the rats are sacrificed via decapitation.*

On the third day, the same procedure was followed, but without administering the shocks. The behaviour was then noted for 30 minutes after the last noise session. Control animals received the same treatment without receiving the shocks (n=5).

2.5 Behavioural measurements

Behaviours were recorded for each rat by means of a video camera and recorder (Philips Explorer Camcorder) directly after the last noise session for 30 minutes. They were then subsequently analysed with the aid of the computer programme, The Observer (Noldus version 3.0, The Netherlands). Freezing as well as defecation (total number of faecal boli) was noted. The rats were then sacrificed by means of decapitation (after a brief exposure to 70% CO_2 (in oxygen or air) for a quick loss of consciousness without hypoxia) and trunk blood was taken for the measurement of corticosterone levels.

2.6 Corticosterone levels

Plasma corticosterone levels were measured by HPLC with UV detection (Trentani et al., 2002). Dexamethasone was used as an internal standard during corticosterone quantification. Plasma was extracted with 3ml of diethylether, vortexed for 5 minutes and then centrifuged for five minutes at 3000g at 4℃. The organic phase was carefully removed and then evaporated to dryness in a 50 ℃ waterbath. The detection limit of corticosterone was 10nM/ 0,35µg/dl.

2.7 Statistics

After establishing homogeneity of variances and a normal distribution, the main effect of fear conditioning as a group factor was assessed between the three groups (0, 1.0, 1.5mA) with a univariate analysis of variance, one-way ANOVA (SPSS package, version 10). When a significance of $p < 0.05$ was found, Tukey's posthoc was applied to the data. Here too significance was taken at $p < 0.05$. Defecation data did not show a normal distribution and therefore a Kruskal-Wallis non-parametric test was performed to determine main effects of fear conditioning with a Mann-Whitney test as post hoc. In both cases significance was determined at $p<0.05$.

3 Results

3.1 Behavioural Measurements

The behavioural data indicated that those animals that received a shock exhibited more stress-related behaviours, including an increase in the total amount of time spent freezing and in defecation. Statistically significant main effects of fear

conditioning were noted between the groups with respect to defecation (χ^2 =9,015; Df = 2; p<0.011). Mann-Whitney post-hoc tests revealed significant differences between controls (n=5) and those animals that received a shock intensity of 1.0mA (n=5; p=0.008) (fig. 2) or 1.5mA (n=4; p=0.016). However no differences were noted between the two shock groups (p=0.73).

Differences between the groups were also noted with respect to total duration of freezing behaviour displayed ($F_{2,12}$=7.09, p=0.009) with controls being significantly lower than the 1.0mA (p=0.032) and 1.5mA (p=0.011) shock intensity groups (Fig. 2). No differences were found between the two shock intensity groups in this parameter (p=0.832).

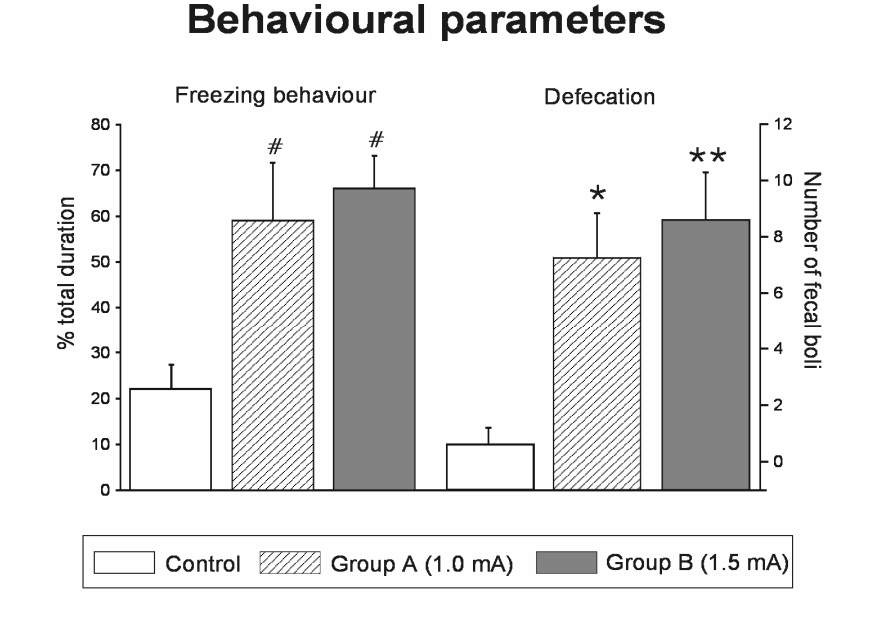

***Figure 2**: Behavioural parameters. Behavioural parameters are depicted here as mean ± SEM for total freezing duration and the median with interquartile boxes and full range whiskers for defecation. Group B (1.5mA, n = 5) and group A (1.0mA, n = 5) displayed increased freezing behaviour as compared to controls (0mA, n = 5) ($^{\#}p<0.05$). No differences were noted between shock groups (p=0.83). With regards to defecation, both groups again (A: n = 5, $^{\#\#}p<0.01$, B: n = 4, $^{\#}p<0.05$) showed an increased amount of faecal boli excreted as compared to controls (n = 5). The shock groups did not significantly differ from each other (p=0.73). The asterisk* represents an outlier.*

3.2 Corticosterone levels

Statistical differences ($F_{2,11}$=14.46, p=0.001) were noted in corticosterone levels between controls and those groups that received a shock (A: n=5, p=0.002; B: n=4, p=0.002), but again no differences were found between the two different shock intensity groups (p=0.950, Fig. 3). A lower standard error of the mean was however noted in the 1.5mA group. One sample in the 1.5mA group was disregarded due to analytical failure.

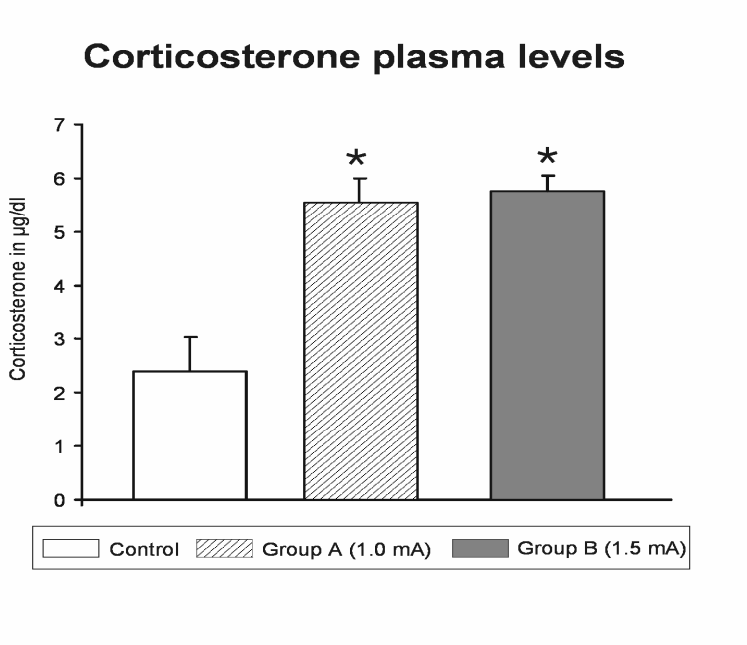

***Figure 3**: Corticosterone plasma levels. Corticosterone levels are depicted here as the mean ± SEM. The two shock groups (A: n = 5, B: n = 4) have significantly higher corticosterone levels (*p=0.002) when compared to controls (n = 5). Again, no significant differences between the two shock groups were found (p=0.95).*

4 Discussion

The aim of this study was to investigate the effects of two different shock intensities in the context of classical pavlovian fear conditioning on various stress parameters in order to determine its effects quantitatively on the animal. We approached this aim by aversively conditioning rats to a sound for 2 days, with no shock being given on the third day in order to evaluate only the psychological effects of the conditioning on freezing, defecation behaviour and corticosterone levels.

The main finding of our study was that those rats that were subjected to fear conditioning with our acute shock paradigm, were indeed stressed and thereby fear conditioned (Goldstein et al., 1996; Suzuki et al., 2002). Our behavioural results show increased freezing and defecation behaviour (Fig. 2). However, as the rats did not receive any shocks on the day of measurement, we can deduce that the behaviour displayed was emotional and in anticipation of a stressful event associated with the tone emitted and therefore not based on the painful stimulus itself, thus indicating that they were indeed fear conditioned.

In both shock intensity groups, a significant increase in corticosterone levels was noted (Fig. 3), indicating that these two groups were more stressed than the control

group (Paris et al., 1987; Pitman et al., 1995; Codero et al., 2002). The fact that there was no difference between the two shock intensity groups, suggests that the rats in these two groups were equally stressed, or that the maximum stress threshold (ceiling) had been reached, and therefore an increase of 0.5mA in shock intensity did not have a significant effect on corticosterone levels between the two groups. Either shock intensity, based on this result, could therefore be employed without imposing severe consequences on the amount of corticosterone released or stress endured.

Looking at defecation, another stress marker (Goldstein et al., 1996, Suzuki et al., 2002), both shock groups were again significantly increased as compared to controls (Fig.2) with the two shock groups not differing from each other. We therefore conclude that the rats subjected to fear conditioning with electric shocks of varying intensity (1.0mA or 1.5mA) experienced the same amount of stress.

Freezing behaviour is considered to be the most reliable output measure of fear conditioning (Inoue et al., 1993; Holahan et al., 2002; Levita et al., 2002). In our paradigm, no differences were noted between shock groups with respect to freezing behaviour, indicating that our rats were similarly stressed and conditioned. The standard error of the mean however, appears to be smaller in the higher shock intensity group compared to the lower shock intensity group (fig. 2). The higher shock intensity group also displayed an increased significance level compared to controls than the lower shock intensity group ($p=0.032$ in the 1.0mA vs. $p=0.011$ in the 1.5mA group).

Although this study does limit itself to changing only one parameter, shock intensity, and several other parameters would also have an effect on the robustness of the fear conditioning, our results suggest that the rats in the higher shock intensity group were more homogenously fear-conditioned and therefore the results should be more reproducible and robust than in the lower shock intensity group. This would allow for fewer rats to be used in order to gain an accurate impression of the conditioning paradigm employed.

5 Acknowledgements

We would like to make special mention of Thomas Cremers and Minke Jongsma for their help with corticosterone analysis and general guidance.

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Chapter 3

Ketamine administration disturbs behavioural and distributed neural correlates of fear conditioning in the rat

CY Pietersen, FJ Bosker, F Postema, DS Fokkema, J Korf, JA den Boer

Department of Psychiatry, Medical Faculty, UMCG, The Netherlands

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Abstract

The neurotransmitter glutamate and its associated receptors perform an important role in the brain circuitry underlying normal fear processing. The glutamate NMDA receptor, in particular, is necessary for the acquisition and recollection of conditioned-fear responses. Here the authors examine how acute blockage of the NMDA receptor with sub-aesthetic doses of ketamine affect behavioural assays of fear-conditioned stress (e.g. freezing) and cFos expression in a network of brain areas that have previously been implicated in fear processing.

Fear-conditioned rats displayed significantly more freezing behaviour than non-conditioned controls. In fear-conditioned rats that also received ketamine, this conditioning effect was largely neutralised. Fear conditioning also led to increased cFos expression in various areas central to fear processing, including the basolateral nucleus of the amygdala, the paraventricular nucleus of the hypothalamus and the anterior cingulate. Ketamine abolished such increases in cFos expression in most brain areas investigated.

The present study therefore demonstrates that systemic ketamine administration in rats interferes with fear conditioning on a behavioural level and in a network of brain regions associated with fear and anxiety. The combination of ketamine and fear conditioning may therefore provide a useful model of abnormal fear processing, as observed in certain psychiatric conditions.

1 Introduction

Classical fear conditioning is a technique generally used to explore fear circuits in the brain. It involves learning an association between a neutral conditioned stimulus and an aversive unconditioned stimulus (Walker and Davis, 2002). The neural pathways underlying fear conditioning, and more generally, fear processing, have been thoroughly investigated in the rat, with the primary focus being the amygdala (Davis et al., 1994; Maren and Fanselow, 1996; LeDoux, 1998; LeDoux, 2000; Maren, 2001).

Previous studies have shown that NMDA receptors in the amygdala are essential for long-term potentiation (LTP), a process that underlies fear learning (Li et al., 1995; Lee et al., 2001). Amygdalar NMDA receptors are necessary for the convergence and association of the unconditioned and conditioned stimuli (Walker and Davis, 2002). Kim and McGaugh (1992) for example, examined how injecting various NMDA antagonists (AP5, MK-801, CPP) into the rat amygdala altered conditioned-fear behaviour in an inhibitory avoidance task. While acquisition of conditioned responses remained intact, deficits in inhibitory avoidance were noted 48 hours later. It has been hypothesized that temporally precise fear responses to specific threats are mediated by the amygdala, whereas sustained anxiety responses that persist beyond the immediate threat are mediated, at least in part, by structures such as the anterior cingulate, nucleus accumbens, orbitofrontal cortex and insula (Davidson and Irwin, 1999; Cardinal et al., 2002; Walker and Davis, 2002). In one study, for example, the pairing of anterior-cingulate stimulation with an auditory tone produced conditioned fear responses in the rat (Tang et al., 2005). These responses were then blocked by the infusion of an NMDA-receptor antagonist into the amygdala. Such studies indicate a functional relationship between the anterior cingulate and the amygdala during fear processing.

To further investigate the functional basis of normal and altered fear processing, we induced a state of fear-conditioned stress (Suzuki et al., 2002), a state dependent on the environmental context in which classical conditioning occurs in the rat. We then examined behavioural (e.g. freezing) and neural (cFos expression) assays of fear conditioning following the (systemic) administration of ketamine, an NMDA

antagonist, and in control animals. Based on available knowledge of the processing regions likely to be involved in fear and stress responses (LeDoux, 1998; Davidson and Irwin, 1999; Cardinal et al., 2002; Walker and Davis, 2002), we identified several candidate brain regions for investigation, including the amygdala and anterior cingulate. We hypothesized that ketamine would abolish neural and behavioural responses associated with fear conditioning, thereby inducing a state of abnormal fear processing which may prove useful in developing animal models of certain psychiatric conditions, such as schizophrenia.

2 Materials and methods

2.1 Animals

All animals were cared for in accordance with the principles laid down by the European Communities Council Directive (1986) for the Protection of Vertebrate Animals used for Experimental or Other Scientific Purposes (86/EEC). Sprague-Dawley rats (n=24) weighing between 225-250 g were obtained from the central animal facility (Groningen, The Netherlands) and were housed individually in a temperature (± 23℃) and humidity controlled (40 to 60%) environment. Food and water were delivered *ad libitum*. After arrival from the animal breeding facility, rats were allowed to acclimatize for two to three days. They were then handled daily for five days in order to eliminate handling stress as a confounding variable.
The rats were divided into four groups: Fear conditioned and ketamine, Fear conditioned and saline (fear conditioning), no fear conditioning and ketamine (ketamine), and no fear conditioning and saline (control).

2.2 Drugs and injection paradigm

Ketamine Hydrochloride was obtained from Sigma (Germany). Ketamine injections (16 mg/kg, s.c.) and saline shams were administered half an hour before fear conditioning for the first 2 days (i.e. only during the actual conditioning phase of the experiment). Previous observations in our lab (Imre et al., 2006), as well as other studies (Pallarés et al., 1995), show that half an hour is sufficient for the ketamine-induced alterations in locomotor activity to normalise. All injections were omitted on the third day of conditioning testing to avoid unnecessary drug interaction with

behavioural measurements. The ketamine dose was determined empirically (i.e. it was the highest dose possible that did not affect locomotor behaviour).

2.3 Shock paradigm

The paradigm according to Suzuki et al. (2002) was followed with some minor modifications. We chose this more-stressful protocol compared to traditional fear conditioning studies because we wanted to induce persistent changes at both the biochemical and behavioural level.

The rats were taken out of their home cage and placed individually in the shock box. This was a specially constructed wooden container with a floor made of a metal grid. A central computer controlled the current and noise emission, making use of a program that was specially developed for this study (N594 version 2.00, Rijksuniversteit Groningen, The Netherlands, 2002). Rats destined to undergo fear conditioning were then subjected to a shock (1.5 mA) that was paired with a noise (60 dB tone) for two days (Fig.1). This shock intensity was based on a pilot study (Pietersen et al., 2006) indicating that 1.0 and 1.5mA shocks induced comparable stress levels (corticosterone and behaviour), but also showing that the latter shock intensity was clearly superior in terms of variability of all incurred stress parameters.

One shock session consisted of a 1-minute period. During the first 30 seconds the noise was emitted. Within the second half (15 seconds) of this 30-second period, the shock was delivered. The last 30 seconds served as a rest period. All sessions took place in the morning and were repeated consecutively ten times per day, resulting in one session lasting 10 minutes in total. Control rats followed the same routine with noise emission, but without experiencing any shocks.
On the third day, the same procedure was followed, but without administering shocks. The behaviour following fear-conditioned stress was then noted for 30 minutes after the last noise session. This time point was chosen as it has been shown that freezing behaviour persisted long after the last noise session (Suzuki et al., 2002).

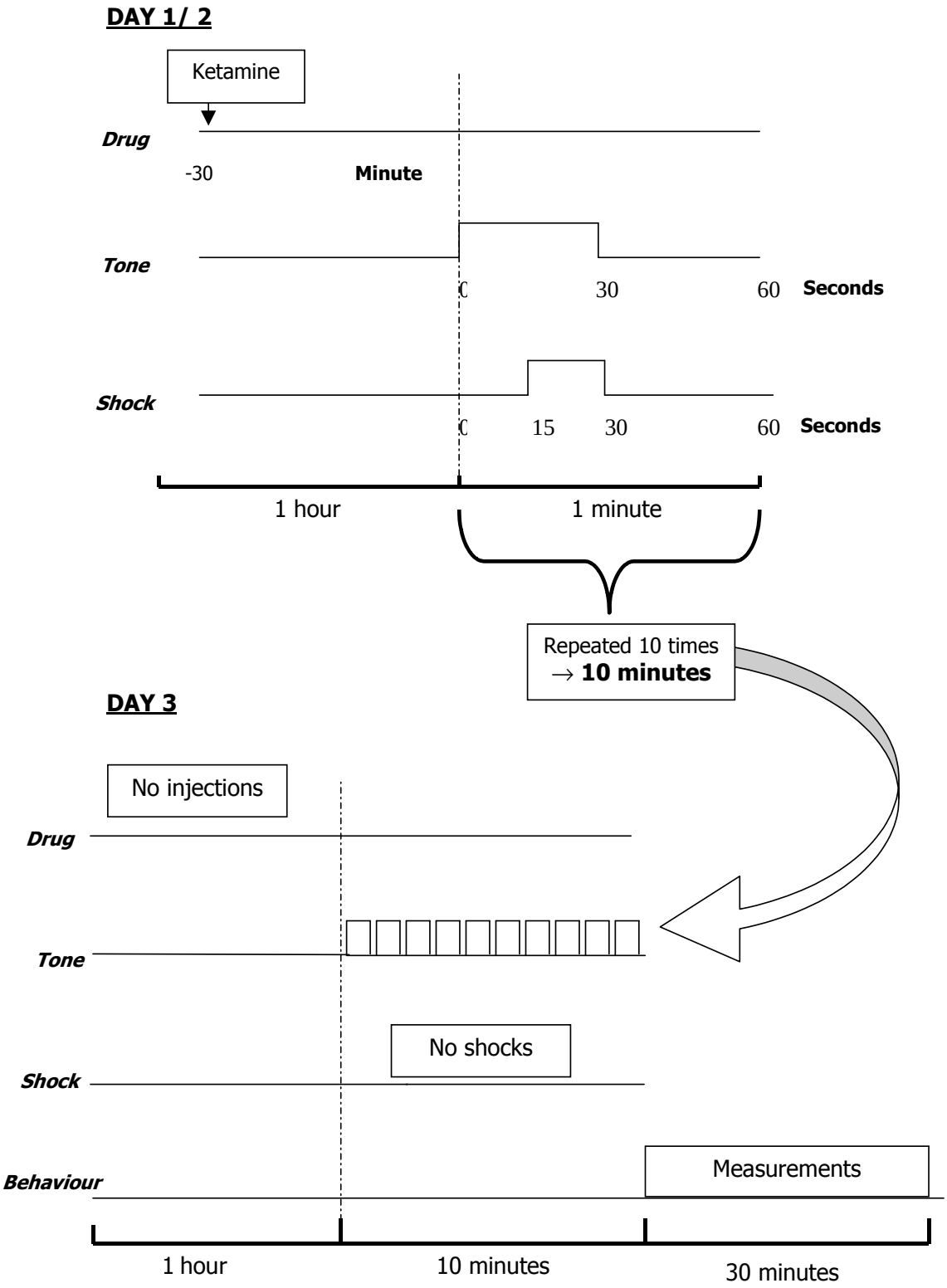

Figure 1: *Injection and shock paradigm. This diagram represents the methodology employed during fear conditioning. On day one and two, the same protocol is followed with only a sound given during the first 15 seconds. In the next 15 seconds, the sound is presented in combination with a shock (1.5mA). The last 30 seconds serves as a rest period. On day 3, no shocks or injections are given, although the animal follows the same protocol. This 1-minute session is repeated 10 times per day in succession. The behaviour is noted for 30 minutes after the last test session on the third day*.

2.4 Behavioural observation

Behaviours were recorded for each rat by means of a video camera (Philips Explorer Camcorder) directly after the last noise session. They were then subsequently analysed with the aid of the computer program, The Observer (Noldus version 3.0, The Netherlands). An independent observer unaware of experimental conditions noted freezing, grooming, rearing and resting. Freezing was denoted as an absence of any movement except that needed for respiration and whisker twitching. Rearing was defined as the raising of the body onto the hind legs, while resting served as a default state whereby none of the other behaviours were being displayed. The freezing behaviour, as well as being a behavioural expression of stress, is also the main determinant of successful fear conditioning. Rearing and grooming were denoted as anxiolytic behaviours (Morrow et al., 2002; Sharp et al., 2002; Daniels et al., 2004).

2.5 cFos expression

2.5.1 Perfusion and preparation

Half an hour after the end of the behavioural observation, the rats were perfused trans-cardially with 4% paraformaldehyde (Merck, Germany) for 20 minutes. The brains were then removed and placed into 4% paraformaldehyde, and kept at 6℃ for 2 days. Thereafter, they were transferred into 0.02 M potassium phosphate buffered saline (PBS; pH 7.4) with 1% sodium azide (Boom, Meppel, The Netherlands) to prevent bacterial growth and were stored at 6°C. I n preparation for cFos staining, whole brains were dehydrated in a 30% sucrose solution overnight and subsequently frozen with gaseous CO_2 at –80°C. The brains were cut using the Leica CM 3050 cryostat machine at 40 micrometers thin slices and stored at 6°C in 0.02 M PBS buffer (pH 7.4).

2.5.2 cFos staining: Immunocytochemistry

Coronal cryostat sections of 40 mm were collected in 0.01 M Tris buffered saline (TBS, pH 7.4) and rinsed 3x5 min. After pre-incubation with 0.3 % H_2O_2 (10 min, in 0.01 M TBS, pH 7.4), the sections were washed with 0.01 M TBS (4x5 min, pH 7.4) and incubated with an rabbit polyclonal antibody raised against cFos (Ab-5 Oncogene Research Products, Calbiochem, 1:10.000 in 0.01 M TBS-Triton 0.01%,

4% normal goat serum) for 24–30 h at room temperature. Subsequently, the sections were washed in 0.01 M TBS (8x5 min, pH 7.4) and incubated for 2h at room temperature with biotinylated goat anti-Rabbit IgG (Vector, 1:1000 in 0.01 M TBS). After rinsing with 0.01 M TBS (6x5 min, pH 7.4), the immunoreactivity was visualized with a standard ABC method (Vectastain ABC kit, Vector, (1 drop A+1 drop B)/20 ml TBS for 2h). After washing with TBS 0.01 M (6x5 min, pH 7.4) the peroxidase reaction was developed with a di-aminobenzidine (DAB)-nickel solution and 0.3% H_2O_2 (0.5mg DAB/ml Distilled water; 1.0% nickel ammonium sulphate (NAS) in 0.1 M sodium acetate (NaAc, pH 6.0). To stop the reaction, the sections were washed with 0.1M NaAc, pH 6.0 (3x5minutes), then 0.01 M TBS (3x5 min, pH 7.4), and were subsequently mounted on gelatin-coated slides, air dried, dehydrated, and coverslipped with DePeX (Gurr) (Boom, Meppel, The Netherlands).

The area of the region of interest was measured and, after background correction, the number of immunopositive nuclei was quantified using a computerized image analysis system (Leica Qwin version 2.3, Leica Microsystems Imaging Solutions). The average number of cFos immunoreactive cells was calculated and expressed as number of positive nuclei (counts)/ 0.1 mm^2.

Areas included in the cFos analysis were: the paraventricular nucleus, central and basolateral amygdala nuclei, nucleus accumbens, dorsal raphe, locus coeruleus, anterior cingulate and dentate gyrus. The Swanson (1992) co-ordinates are given in Table 1 as millimetres from Bregma.

Table 1*:* Brain areas: Swanson (1992) rostral-caudal co-ordinates

Area	Mm from Bregma
Anterior cingulate	+2.80 to +2.15
Basolateral nucleus of amygdala	-2.45 to –2.85
Central nucleus of amygdala	-2.45 to –2.85
Dentate gyrus	-2.45 to –2.85
Dorsal raphe	-7.10 to –8.60
Locus coeruleus	-9.60 to –10.10
Nucleus accumbens	+2.80 to +2.15
Paraventricular nucleus	-1.53 to –2.00

2.6 Statistics

A two-way ANOVA was utilized in the program JMP 4.0.4 (SAS institute). When the ANOVA indicated a "fit" with a p value < 0.05, a determination for the dependent variables fear conditioning vs. no fear conditioning and saline vs. ketamine treatments was performed. In addition, an interaction factor was included. When appropriate (i.e. a significant interaction factor of $p<0.05$), individual group comparisons were made using a Tukey's HSD. All behavioural data analysis, as well as the paraventricular nucleus and dorsal raphe data, were performed on log (base 10) transformed data via JMP 5.1 statistical package in order to normalise the distribution.

3 Results

3.1 Behaviour

The results of the ANOVA on frequency, percentage total duration of the half-hour session and the mean time that the animal displayed certain behaviour are analysed and are stated here. Results of the post-hoc test for individual comparisons are represented in Fig. 2.

3.1.1 Frequencies

Fear-conditioned stress increased freeze frequency in the presence of saline, but had no effect in combination with ketamine ($F_{1, 20} = 11.55$; $p<0.01$). Ketamine also decreased rest frequency in the presence of fear conditioning ($F_{1, 20} = 11.54$; $p<0.01$).

3.1.2 Percentage total duration

In terms of percentage total duration, fear-conditioned stress again increased freeze duration, whereas in combination with ketamine this effect was reduced to control levels ($F_{1, 20} = 24.53$; $p<0.0001$). Ketamine itself decreased freezing duration with fear conditioning, but increased freeze duration without fear conditioning. A significant interaction effect was noted in resting duration ($F_{1, 20} = 6.56$; $p<0.05$), but no individual differences were noted between groups. Fear-conditioned stress also decreased total grooming duration ($F_{1, 20} = 10.07$; $p<0.01$), while ketamine in

combination with fear conditioning reversed this effect ($F_{1, 20} = 4.88$; $p<0.05$). No interaction effect was noted here.

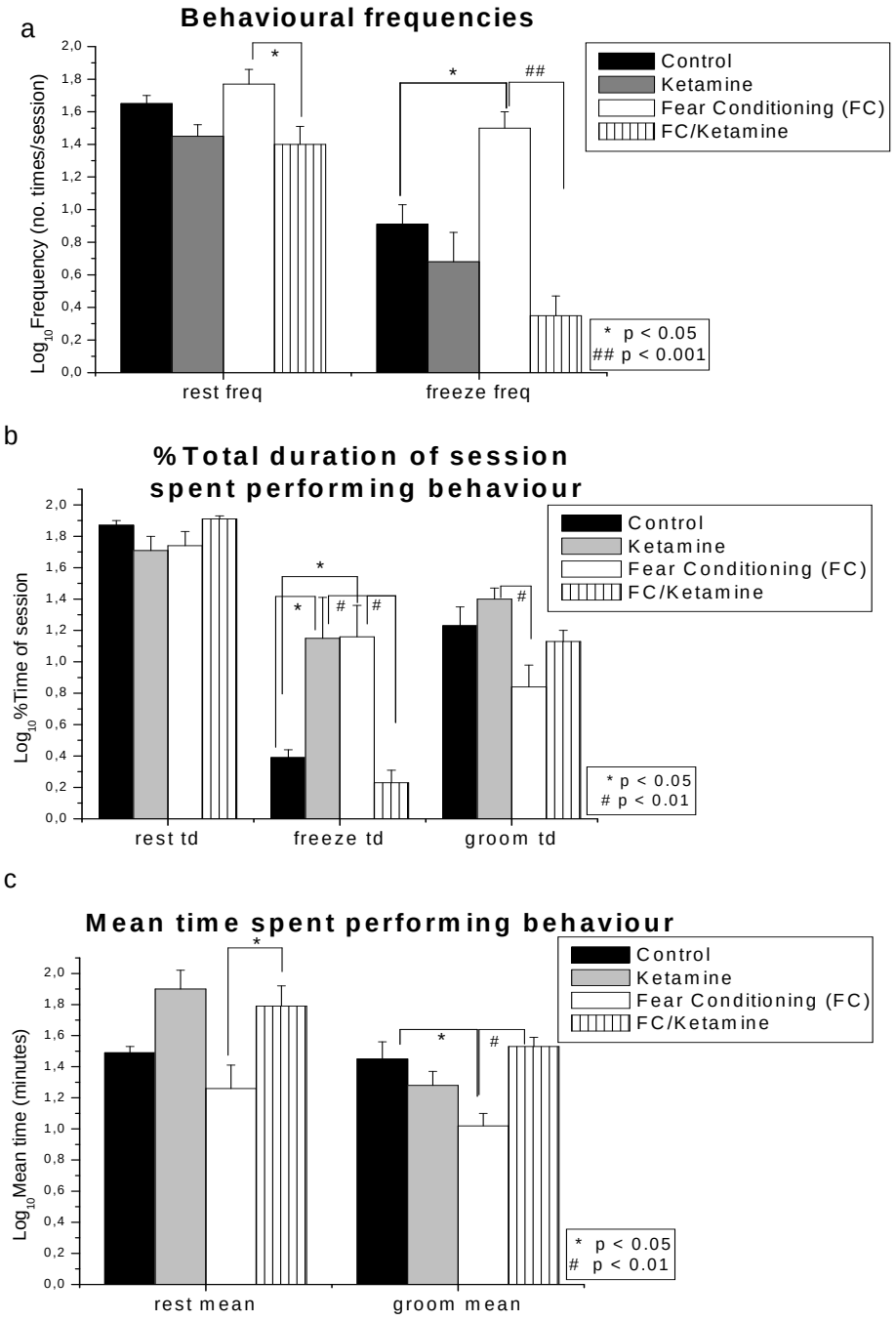

***Figure 2**: Behavioural observations. Significant differences shown on the graphs are according to Tukey's post hoc test. Significant differences between non-related groups (i.e. no fear conditioning and saline vs. fear conditioned and ketamine) are not shown. Fear conditioning (a) increased freeze frequency (p = 0.028), (b) increased total duration of freezing (p = 0.023), and (c) decreased the mean time spent grooming (p = 0.012). Ketamine reversed (a) the effect of fear conditioning on rest (p = 0.027) and freeze frequency (p < 0.001), (b) total duration of freezing (p = 0.005), and (c) mean time spent resting (p = 0.025) and grooming (p = 0.003). Ketamine also (b) increased the total duration of freezing in the presence of saline (p = 0.024), but not in the presence of fear conditioning (p = 0.005). (freq = frequency, td = total duration)*

3.1.3 Means

Ketamine had a main effect on the mean resting period ($F_{1, 20}$ = 15.55; p<0.001), but no effects were noted in terms of freezing behaviour. Grooming had significant interaction effects ($F_{1, 20}$ = 15.16; p<0.001), with fear-conditioned stress decreasing mean grooming time in the presence of saline, but not in the presence of ketamine. Ketamine increased grooming under fear conditioning, but had no effect without fear conditioning.

3.2 cFos expression

Results of Tukey's post-hoc test for individual comparisons are represented in Fig. 3. The two-way ANOVA revealed a fear conditioning main effect in the dorsal raphe ($F_{1, 16}$ = 10.30; p<0.01), central amygdala ($F_{1, 19}$ = 5.77; p<0.05) and basolateral amygdala ($F_{1, 19}$ = 23.43; p<0.0001), while ketamine had a significant main effect on cFos in the basolateral amygdala ($F_{1, 19}$ = 19.15; p<0.001). A significant interaction between fear conditioning and ketamine treatment was found in the anterior cingulate ($F_{1, 19}$ = 8.41; p<0.01), locus coeruleus ($F_{1, 17}$ = 4.36; p=0.05), nucleus accumbens ($F_{1, 19}$ = 9.53; p<0.01) and paraventricular nucleus ($F_{1, 19}$ = 23.46; p<0.001). In the dentate gyrus, ketamine decreased cFos expression ($F_{3, 20}$ = 17.44; p<0.001). Typical areas analysed and cFos staining are shown in Fig. 4.

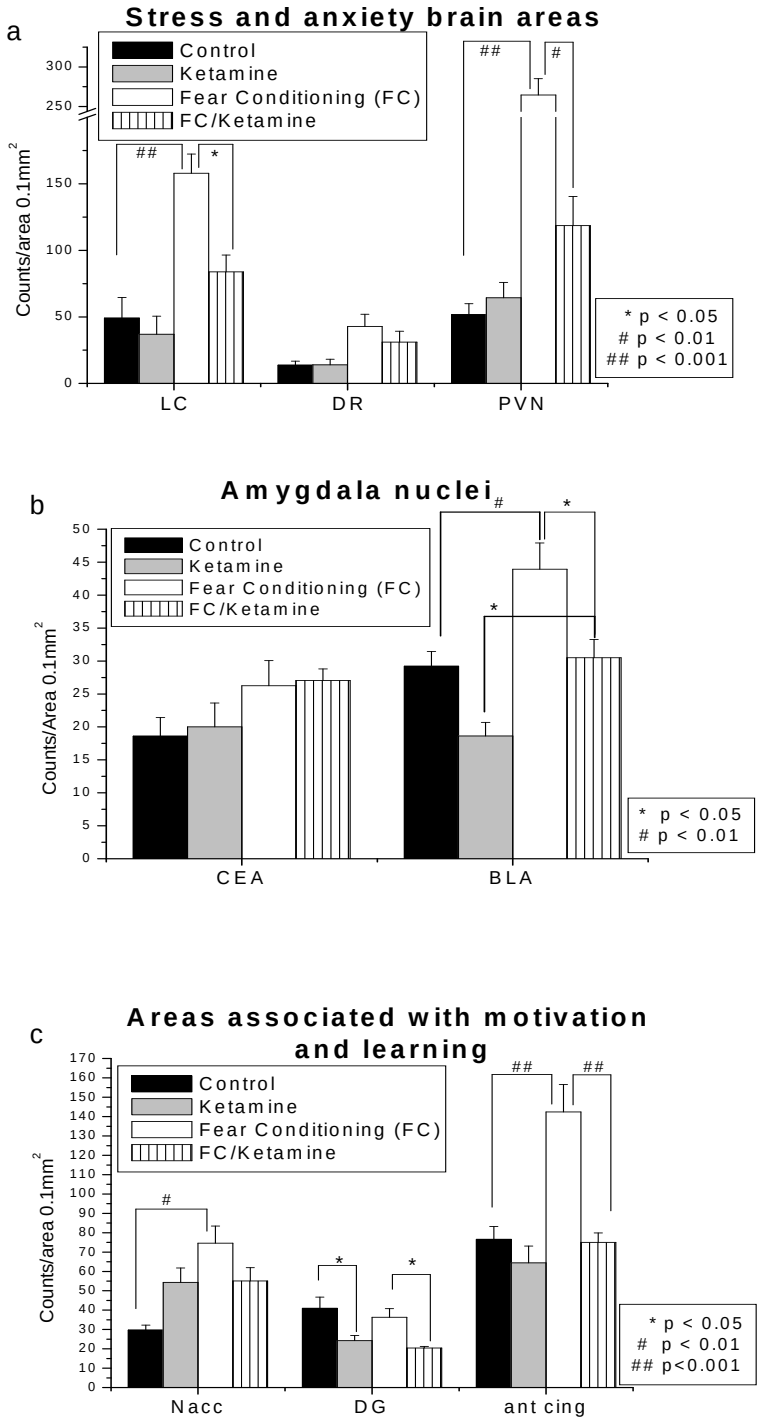

Figure 3*: cFos expression in brain areas. Significance differences shown on the graphs are according to Tukey's post hoc test. Significance differences between non-related groups (i.e. no fear conditioning and saline vs. fear conditioned and ketamine) are not shown. Fear conditioning increased cFos expression in (a) the locus coeruleus ($p < 0.001$), paraventricular nucleus ($p < 0.001$), (b) basolateral amygdala ($p = 0.008$), (c) nucleus accumbens ($p = 0.002$) and anterior cingulate ($p < 0.001$). Ketamine itself decreased cFos expression in (c) the dentate gyrus in the presence of saline ($p = 0.031$) and fear conditioning ($p = 0.043$). A similar trend was seen in (b) the basolateral nucleus of the amygdala ($p = 0.051$), which disappeared in the presence of fear conditioning ($p = 0.026$). Ketamine significantly reversed the effect of fear conditioning in (a) the locus coeruleus ($p = 0.016$), paraventricular nucleus ($p = 0.009$), (b) basolateral amygdala ($p = 0.015$), and (c) anterior cingulate ($p < 0.001$). (DR = dorsal raphe, LC = locus coeruleus, PVN = paraventricular nucleus, CEA = central nucleus amygdala, BLA = basolateral nucleus amygdala, antcing = anterior cingulate, DG = dentate gyrus, Nacc = nucleus accumbens)*

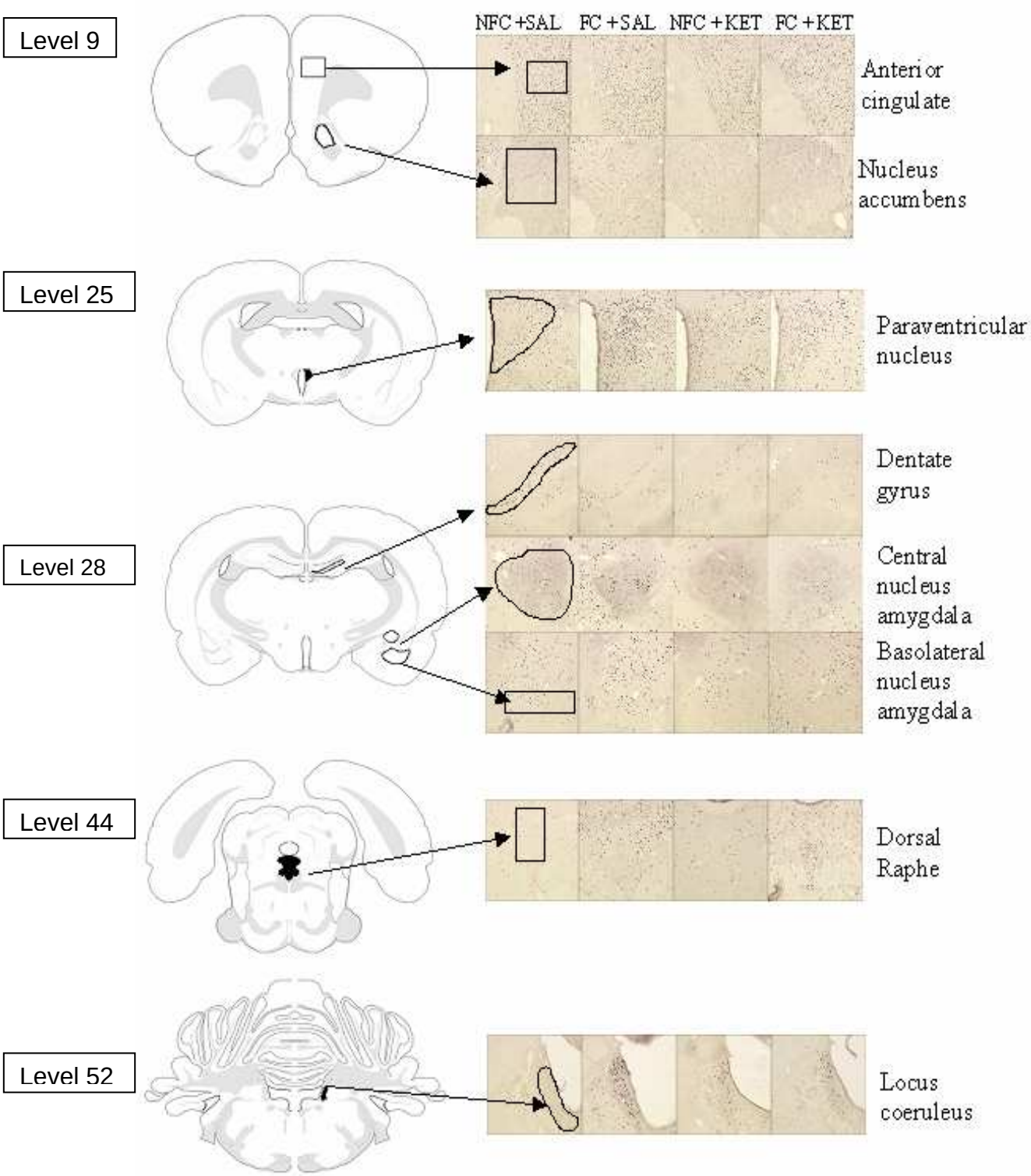

***Figure 4**: cFos expression. Typical examples of the brain areas stained for cFos expression, visually showing the effects of the various treatments. Delineated areas depict actual areas measured. Brain slice levels were taken from the Swanson rat brain atlas (1992), with appropriate co-ordinates listed in Table 1.*

4 Discussion

4.1 Behavioural and neural correlates of stress

The main aim of this study was to examine how the influence of systemic ketamine administration manifests itself in neural and behavioural assays of fear-conditioned stress in rats. Fear conditioning was successful in eliciting stress, as witnessed by the increased (decreased) freezing (grooming) behaviour (Fig. 2) up to 30 minutes after the last conditioned tone. Consistent with our behavioural observations, the locus coeruleus, the dorsal raphe and the paraventricular nucleus, all areas involved in either stress regulation or associated with anxiety (Nash and Maickel, 1988; Chauoloff, 2000; Dunn et al., 2004) showed increased levels of cFos expression induced by fear-conditioned stress (Fig. 3). In relation to our central hypothesis, administration of ketamine to fear-conditioned rats normalised stress-related behaviours (Fig. 2) and cFos levels in the above-mentioned brain areas, except for the dorsal raphe (Fig. 3). Thus, both the neural and behavioural data suggest that our fear-conditioning paradigm was effective in eliciting a stressful state and that ketamine was successful in normalising this state.

4.2 Differential activation in amygdala nuclei

As indicated in the Introduction, the amygdala performs a key role in the learning and expression of fear (LeDoux, 1992; Phillips and LeDoux, 1992; LeDoux, 1998; Walker and Davis, 2002). To this end, we found considerable fear-elicited cFos expression in the basolateral amygdala, but found only a small increase in cFos expression in the central nucleus (the increase was significant only after combining data from ketamine and non-ketamine groups). Whereas administration of ketamine normalised cFos expression in the basolateral nucleus, we found no such effect in the central nucleus. Interestingly, ketamine administration in the absence of fear conditioning decreased cFos expression slightly in the basolateral nucleus (although the effect was not significant) but not in the central nucleus.

The differential activation of the basolateral and central nuclei in response to fear conditioning and ketamine administration likely reflects the different functional roles performed by these areas (Cardinal et al., 2002). Goosens and Maren (2003), for example, have shown that that infusion of the NMDA antagonist D, L-2-amino-5-

phosphonovalerate (APV) into either the basolateral or central nuclei blocks the acquisition of conditional fear. Residual fear memory is, however, retained following APV administration into the central nucleus, but not the basolateral, indicating that the latter nucleus is more critical to fear learning and memory. Koo et al. (2004) showed that the fibres that run through the central nucleus from the basolateral nucleus, and not the neurons within the central nucleus itself, are involved in fear conditioning. Our data also suggest that the basolateral amygdala plays the more important role in fear conditioning, since we found no effect of conditioning or ketamine in the central nucleus. Although our paradigm does not permit us to say whether ketamine blocked the acquisition or the expression of fear, previous experiments involving focal infusion of NMDA receptor antagonists (AP5 (2-amino-5-phosphonopentanoic acid) and APV) into the amygdala are consistent with the impairment of fear learning rather than expression (Fanselow and Kim, 1994; Shauz and Koch, 2000; Goosens and Maren, 2003). In summary, our data is consistent with the idea that ketamine-induced glutamatergic hypofunction impairs processes related to fear conditioning in the basolateral amygdala (Fanselow and Kim, 1994).

4.3 Other brain areas implicated in fear processing- the anterior cingulate and nucleus accumbens

The rat anterior cingulate has previously been shown to be involved in associative learning (Cardinal et al., 2002), particularly fear conditioning (Frankland et al., 2004; Gao et al., 2004). In our study, fear conditioning was associated with a marked increase in cFos expression in this area, with the increase being more than double the amount of cFos elicited in the control condition (Fig. 3). Administration of ketamine completely abolished the fear-related cFos expression, consistent with the tight bi-directional anatomical and functional linkage between the anterior cingulate and basolateral nucleus of the amygdala (Cardinal et al., 2002). The nucleus accumbens, an area primarily involved in motivation (Reynolds and Berridge, 2003; Salamone et al., 2005), also forms an integral part of this circuit (Levita et al., 2002), as it receives projections from both the anterior cingulate and the basolateral nucleus (Cardinal et al., 2002). We also observed an increase in fear-related cFos expression in the nucleus accumbens (Fig.3). Ketamine administration did not, however, drive cFos expression back down to the level observed in the control group. Interestingly, ketamine alone elicited a slight, but non-significant, increase in cFos expression.

4.4 Failure to detect fear-induced activation in the dentate gyrus

We failed to find fear-related cFos expression in the dentate gyrus of the hippocampal formation. Indeed, ketamine administration was associated with slight decreases in cFos expression, below the level of the control group, in both the presence and absence of fear conditioning. The absence of fear-related cFos activity might be explained by the notion that the hippocampus plays a role in contextual fear memory but not in the type of tone-dependent fear conditioning (Thiels and Klann, 2002) used in our study. Perhaps other hippocampal regions, such as CA3, may have manifested fear-dependent cFos activity, although the main focus of our study was not the hippocampus.

4.5 Ketamine's confounding properties

Could the effects we observed be due to the anaesthetic properties of ketamine? Importantly, we used a sub-anaesthetic dose of ketamine that has previously been tested for its soporific actions upon rats' locomotor activity (Imre et al., 2006): locomotor activity normalised by the 30 minute mark at which we began fear conditioning in the present study. A study by Pallarés et al. (1995) also indicated that a 12mg/kg ketamine dose injected half an hour before testing did not interfere with locomotor activity. The authors concluded that the deficits encountered with the operant behavioural learning paradigm in their study could therefore not be due to the anaesthetic properties of the drug.

As ketamine is known to cause perceptual distortions, is it also possible that the drug prevented proper sensory encoding of the conditioned stimulus rather than blocking fear processing? de Bruin et al. (1999) showed that a 10mg dose of ketamine did not alter auditory evoked potentials in a double-click paradigm in rats. This result suggests that conditioned-stimulus encoding was likely to be intact at the 16mg dose used in our study, although it does not completely rule out the possibility of an analgesic effect. A study using an NMDA antagonist, AP5, infused into the amygdala, reported that reactions to footshocks between controls and AP5 rats were indistinguishable, even at a dose 4-fold higher than that required to impair learning (Miserendino et al., 1990; see also Campeau et al., 1992). These authors concluded that AP5 treatment did not have an analgesic effect but rather disrupted the

association of the unconditioned and conditioned stimuli (Walker and Davis, 2002). In our study, we administered ketamine systemically, rather than locally. Yet our qualitative behavioural observations during conditioning suggest that ketamine-treated and control rats responded in the same fashion (e.g. jumping height during the shock appeared the same). The putative analgesic effect of ketamine is therefore unlikely to have played a significant role in our study.

4.6 Relevance for psychiatric disorders

In related work in our lab, we have demonstrated that administration of atypical, but not typical, neuroleptics partly restores the impaired neural and behavioural conditioned-fear responses introduced by ketamine (Pietersen et al., submitted). We therefore speculate that the combination of ketamine administration and fear conditioning may in future provide an animal model for the emotional-processing deficits seen in schizophrenia (Paradiso et al., 2003; Sachs et al., 2004; Takahashi et al., 2004). It may, however, also prove valuable in the modelling of other psychiatric disorders featuring abnormal fear processing such as anxiety disorders (Doronbekov et al., 2005; Swanson et al., 2005).

5 Conclusion

The authors conclude that the administration of the glutamate antagonist ketamine blocks the expression of fear-conditioned stress in rats at multiple neural sites and at the behavioural level. We further suggest that the combination of fear conditioning and ketamine may provide an effective model in linking the breakdown of fear processing to hypoglutamatergic states. In particular, our work could have implications for disorders in which fear processing is abnormal, such as schizophrenia (Tsai and Coyle, 2002; Coyle and Tsai, 2004).

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Chapter 4

The effects of clozapine, haloperidol and LY379268 in a putative animal model of cognitive-emotional disturbances in schizophrenia

CY Pietersen[1], FJ Bosker[1], J Doorduin[1], F Postema[1], JV Haas[2], MP Johnson[2], T Koch[1], T Vladusich[1], JA den Boer[1]

[1]Department of Psychiatry, Medical Faculty, UMCG, The Netherlands

[2]Eli Lilly and Company, Indianapolis, Indiana 46285, USA

In preparation

Abstract

Fear processing is especially relevant to schizophrenia, as many patients manifest deficits in the processing and attribution of negative emotional states. Many studies suggest that a hypofunctioning glutamatergic system could be related to both the cognitive and emotional deficits, including fear processing, displayed by schizophrenic patients.

We investigated behavioural and neural (cFos) correlates of a ketamine-induced hypoglutamatergic state on fear conditioning in the rat, emphasizing brain areas implicated in fear processing and schizophrenia. We sought to evaluate the validity of the fear-conditioning/ketamine animal model by examining the effects of antipsychotics typically used in the clinical setting (clozapine and haloperidol) and a putative atypical antipsychotic (LY 379268). We hypothesized that ketamine administration would interfere with fear conditioning, and that clozapine and perhaps LY 379268, but not haloperidol (which ameliorates mainly positive symptoms), would renormalize behavioural and neural assays of fear conditioning.

Fear conditioning led to changes in behaviour, including increases in freezing duration and frequency, the main determinant of fear conditioning. Fear conditioning also led to increases in cFos expression in the anterior cingulate, nucleus accumbens shell, paraventricular nucleus, and anterior portion of the basolateral amygdala and lateral amygdala. Ketamine, in turn, abolished the increase in cFos expression associated with fear conditioning in these areas. Behavioural assays of conditioning were consistent with these findings. Although clozapine administration renormalized conditioning-induced cFos expression after ketamine administration, behavioural evidence did not correlate with these findings. Haloperidol and LY 379268 did, however, partially renormalize freezing frequency, but with no neural correlates, with the exception of the anterior cingulate.

1 Introduction

A need for novel antipsychotics that counteract negative symptoms and cognitive deficits associated with chronic schizophrenia has led to a shift in research from modulating dopaminergic to glutamatergic systems (Heresco-Levy, 2003). The two main neurotransmitter hypotheses of schizophrenia differ in origin. The dopamine hypothesis has its origins in the observation that typical antipsychotics (dopamine receptor antagonists) tend to ameliorate positive symptoms (Peroutka and Snyder, 1980; Heinz, 2000; Jones and Pilowsky, 2002). In comparison, glutamate has been implicated by virtue of the observation that administration of non-competitive NMDA (N-methyl-D-aspartate) receptor antagonists such as PCP and ketamine to healthy volunteers reproduces many of the negative symptoms and cognitive impairments seen in schizophrenia (Tsai and Coyle, 2002; Coyle and Tsai, 2004). Studies have suggested that a hypofunctioning glutamatergic system could be related to both the cognitive and emotional deficits displayed by schizophrenic patients (Moghaddam et al, 1997; Krystal et al, 2000; Abel et al, 2003; see Riedel et al, 2003, for a review of cognitive deficits).

Deficits in emotional processing include the inability to process fear adequately. Many patients manifest deficits in the recognition of fearful faces (Edwards et al, 2001; Johnston et al, 2001; Gur et al, 2002) in addition to general abnormalities in the processing and attribution of negative emotional states (Paradiso et al, 2003; Takahashi et al, 2004). In a simple conditioning task using aversive emotional stimuli, for example, schizophrenic patients failed to develop an increase in response frequency to aversively-reinforced trials, whereas healthy volunteers acquired a differential response to reinforced versus non-reinforced trials (Hofer et al, 2001; see also O'Carroll, 1995; Rushe et al., 1999).

We investigated fear conditioning in the rat—an animal that has provided the basis for several extant models of schizophrenia (Grace 2000; Schauz and Koch, 2000; Marcotte et al, 2001; Schmajuk et al, 2001; Castner et al, 2003). Our model combines fear conditioning with ketamine administration, as previous studies have shown that the NMDA receptor is involved in fear conditioning. For example, Goosens and Maren (2003) have shown that that infusion of the NMDA antagonist D,

L-2-amino-5-phosphonovalerate (APV) into either the basolateral or central nuclei of the amygdala, an area centrally involved in the processing of fear (Maren and Fanselow, 1996; LeDoux, 1998), blocks the acquisition of conditional fear. Importantly, bilateral damage to the amygdala has also been shown to impair the processing of fearful facial expressions in healthy human subjects (Adolphs et al, 1995) and reduced amygdala volumes have been found in schizophrenic patients (Sachdev et al, 2000; Joyal et al, 2003; Exner et al, 2004; Niu et al, 2004).

Although the fear conditioning paradigm is usually used to investigate anxiety disorders, some studies indicate that anxiety and schizophrenia are interlinked (Delespaul et al., 2002; Cutting, 2003; Allen et al., 2005). Neuro-imaging studies suggest that positive symptoms are associated with increased amygdala activity, whereas negative symptoms are associated with hypoactivation (Taylor et al., 2002; Fahim et al., 2005). Anxiety is present in the onset stages of schizophrenia, yet largely absent in the longer-term stages of the disorder (Cutting, 2003). Aleman and Kahn (2005) propose a two-hit model of amygdala abnormalities in schizophrenia. They speculate that prolonged activation of the amygdala during psychotic states in the onset stages of schizophrenia could lead to glutamate excitoxicity, resulting in amygdala lesions and long-term hypofunctioning (see also Heresco-Levy, 2003). A decrease in amygdala grey matter density is also noted in schizophrenics over the course of the disorder (Hulshoff Pol et al., 2001). Here we simulate glutamate excitotoxicity through glutamate antagonism, namely through ketamine administration, leading to hypofunctioning of the amygdala and other brain areas involved in fear conditioning. We hypothesize that the hypoglutamatergic state induced by ketamine administration will interfere with normal fear processing and acquisition, due to abnormalities in basic association of fear cues in the amygdala and related areas (see also Yagi et al, 1998; Johnson et al, 2000).

We examine neural (cFos) and behavioural (freezing) assays of fear conditioning in the presence and absence of administered ketamine. We further administer two antipsychotics used in the clinical setting, haloperidol and clozapine, in order to validate the etiological aspects of this model. Haloperidol, a typical antipsychotic, is used for treating positive symptoms of schizophrenia (Peroutka and Snyder, 1980; Levinson, 1991; Heinz, 2000; Jones and Pilowsky, 2002). Clozapine, in contrast, is

an atypical antipsychotic that has been found to alleviate negative and cognitive symptoms of schizophrenia (Heresco-Levy, 2003). Clozapine also differs from conventional neuroleptics, such as haloperidol, in the way it affects the glutamate system (Yamamoto and Cooperman, 1994; Heresco-Levy, 2003). For example, animal studies have shown an increase in medial prefrontal cortical glutamate concentrations after clozapine administration, while haloperidol did not elicit this increase (Daly and Moghaddam, 1993). Another animal study, comparing the effects of haloperidol and clozapine on ketamine-induced alterations in metabolism, found that clozapine completely blocked the effects of ketamine in several brain areas, whereas haloperidol did not (Duncan et al., 1998). We therefore hypothesize that clozapine, but not haloperidol, will renormalize the behaviour changes induced by fear conditioning following ketamine administration. We also administer a new compound (LY 379268; (-)-2-Oxa-4-aminobicyclo [3.1.0.] hexane-4,6-dicarboxylate), a metabotropic glutamate 2/3-receptor agonist, which is currently being tested for its involvement in fear learning (Walker and Davis, 2002). It is presently unclear whether LY 379268 can affect conditional fear processing in the rat. A recent paper, however, does suggest that agonists of this receptor possess anxiolytic properties (Swanson et al., 2005). The metabotropic glutamate 2/3-receptor is located primarily in forebrain regions, and LY 379268 has been shown to decrease glutamate release in these areas (Moghaddam and Adams, 1998). We therefore postulate that LY 379268 will elicit an effect on ketamine's actions in forebrain areas.

To measure the effects of these treatments, we investigate cFos expression (an assay of neural activity) in the central amygdala and the two subdivisions of the basolateral nucleus. As previously mentioned, the amygdala is centrally involved in the processing of fear (LeDoux, 1992; Phillips et al, 1992; Maren and Fanselow, 1996; LeDoux, 1998) and structural aberrations in the amygdala have been found in schizophrenic patients (Sachdev et al, 2000; Joyal et al, 2003; Exner et al, 2004; Niu et al, 2004). We also investigate cFos expression in the anterior cingulate and the nucleus accumbens (core and shell). The prefrontal cortex plays a central role in working memory (Castner et al, 2004), the disruption of which may contribute to cognitive deficits in schizophrenia. It also has direct connections with limbic structures and can therefore influence the expression of emotions, especially fear and anxiety (Lacroix et al, 2000). The rat anterior cingulate, a sub-area of the

prefrontal cortex, has previously been shown to be involved in associative learning, particularly fear conditioning (Frankland et al, 2004; Gao et al, 2004) and in cognitive processes, such as attention (Cardinal et al, 2002; Han et al, 2003). Lesions of this area in humans produce symptoms including apathy, inattention, dysregulation of autonomic function and emotional instability (Bush et al, 2000), all symptoms present in schizophrenic patients.

The nucleus accumbens has also been implicated in the neurobiology of schizophrenia (Grace, 2000) and is an area primarily involved in motivation (Reynolds and Berridge, 2003; Salamone et al, 2005). Studies show that this nucleus has two distinct subdivisions, each with its own function: the core is mainly associated with motor function, while the shell is connected with the limbic system and is primarily involved with emotional regulation (Heimer et al., 1997). It is also intimately linked with the anterior cingulate (Grace, 2000; Cardinal et al, 2002) and basolateral amygdala (Johnson et al., 1994) and receives glutamatergic projections from these areas.

To summarize, we hypothesize that the influence of ketamine on fear conditioning will manifest itself as a decrease in cFos expression, relative to fear-conditioned saline controls, in brain regions associated with fear processing. Further, we expect that clozapine will restore normal freezing behaviour and cFos activity abolished by ketamine. We also hypothesize that haloperidol, as it mainly affects positive symptoms, will not normalize these assays. We also tentatively postulate that LY 379268 will elicit an effect on ketamine's actions on cFos expression, particularly in forebrain areas.

2 Materials and methods

2.1 Animals

All animals were cared for in accordance with the principles laid down by the European Communities Council Directive (1986) for the Protection of Vertebrate Animals used for Experimental or Other Scientific Purposes (86/EEC), which is comparable to the guidelines laid down in the "Principles of laboratory and animal care". Sprague-Dawley rats (n=48) weighing between 225-250 g were obtained from

the central animal facility (Groningen, The Netherlands) and were housed individually in a temperature (± 23℃) and humidity controlled (40 to 60%) environment. Food and water were delivered *ad libitum*. After arrival from the animal breeding facility, they were allowed to acclimatize for two to three days. They were then handled daily for five days in order to eliminate handling stress as a confounding variable.

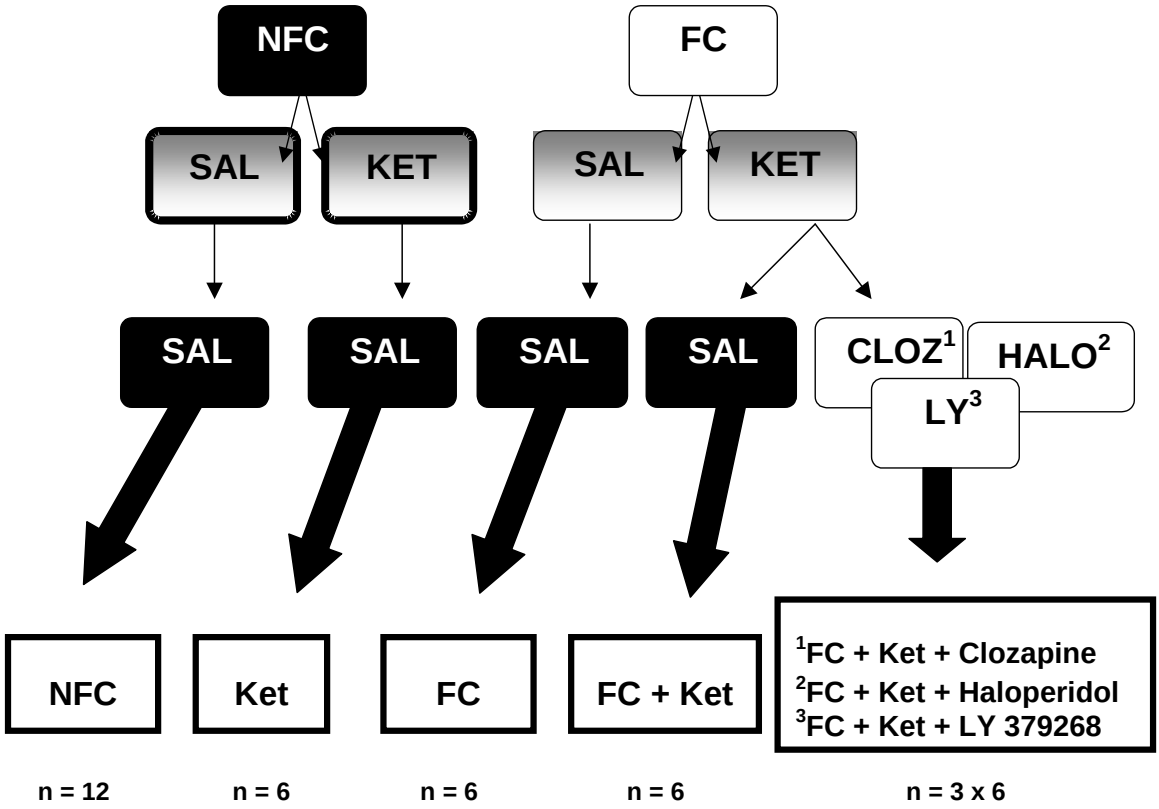

Figure 1*: Experimental group divisions. Diagram portraying the rat group divisions. At the top of the hierarchy, we divided rats into two main groups: those receiving fear conditioning (n = 30), and those not (n = 18). Those animals receiving fear conditioning, were then further divided into rats receiving ketamine administration (n = 24) and rats receiving saline shams (n = 6). The latter group would form the fear conditioning only group (FC). The rats not receiving fear conditioning were also divided into two groups depending on whether they would receive a ketamine (n = 6) or saline injection (n = 12); the former group making up the ketamine only group (Ket), and the latter being the control group (NFC). The remaining fear conditioned rats also receiving ketamine were then further divided into those receiving either a saline injection (FC + Ket) or those receiving an additional antipsychotic injection consisting of clozapine (FC + Ket + CLOZ; n = 6), haloperidol (FC + Ket + HALO; n = 6), or LY 379268 (FC + Ket + LY; n = 6). CLOZ, clozapine; FC, Fear conditioning; HALO, haloperidol; KET, ketamine; LY, LY379268; NFC, no fear conditioning; SAL, saline.*

The rats were divided into seven groups as illustrated in Fig. 1. At the top of the hierarchy, we had a fear conditioned (FC, n = 6) group, a fear conditioned with ketamine administration (FC + Ket, n = 6) group, and a non-fear-conditioned (NFC, n = 12) group. An additional four groups receiving the FC + Ket treatment also received antipsychotics; each group received either a clozapine (FC + Ket + CLOZ; n = 6),

haloperidol (FC + Ket + HALO; n = 6), or LY 379268 (FC + Ket + LY; n = 6) injection, in addition to ketamine and fear conditioning. We also included a ketamine control group, which did not receive fear conditioning or antipsychotic treatment (Ket; n = 6).

2.2 Drugs and injection paradigm

Haloperidol was diluted from 5mg/1ml Haldol® injection capsules. Both clozapine and ketamine were dissolved in physiological saline (0.9%), with hydrochloric acid (HCL) added to clozapine to aid dissolving. One µl/ml of 5N sodium hydroxide (NaOH) was added to the LY 379268 in saline solution before sonication for dissolving purposes. Haloperidol (0.25mg/kg, i.p.), clozapine (5mg/kg, i.p.) and LY 379268 (3mg/kg, s.c.) were administered half an hour before ketamine injections (16 mg/kg, s.c.). Ketamine injections as well as saline shams were administered half an hour before fear conditioning. All injections only took place on the first two days, i.e. only during the actual conditioning phase of the experiment. Injections were omitted on the third day of conditioning testing to avoid unnecessary drug interaction with behavioural measurements. Previous observations in our lab showed that half an hour was sufficient for ketamine-induced increases in locomotor activity to subside (Imre et al., 2006). All other drug doses were determined empirically, i.e. it was the highest dose possible that did not affect locomotor behaviour or induce catalepsy. Clozapine was obtained from Sandoz Pharma AG, Switzerland; Haloperidal from Janssen-Cilag, The Netherlands; LY 379268 from Eli Lilly, USA; and ketamine hydrochloride from Sigma, Germany.

2.3 Shock paradigm

The rats were taken out of their home cage and placed individually in the shock box. This was a specially constructed wooden container with a floor made of a metal grid. A central computer controlled the current and tone emission making use of a program that was specially developed for this study (N594 version 2.00, Rijksuniversteit Groningen, The Netherlands, 2002). Rats destined to undergo fear conditioning were then subjected to a shock (1.5 mA) that was paired with a tone (60 dB tone) during conditioning trials on the two days (Fig. 2). This shock intensity was based on a pilot study indicating that 1.0 and 1.5mA shocks induced comparable stress levels (corticosterone and behaviour), but that the latter shock intensity was superior in terms of variability of all incurred stress parameters (Pietersen et al., 2006a).

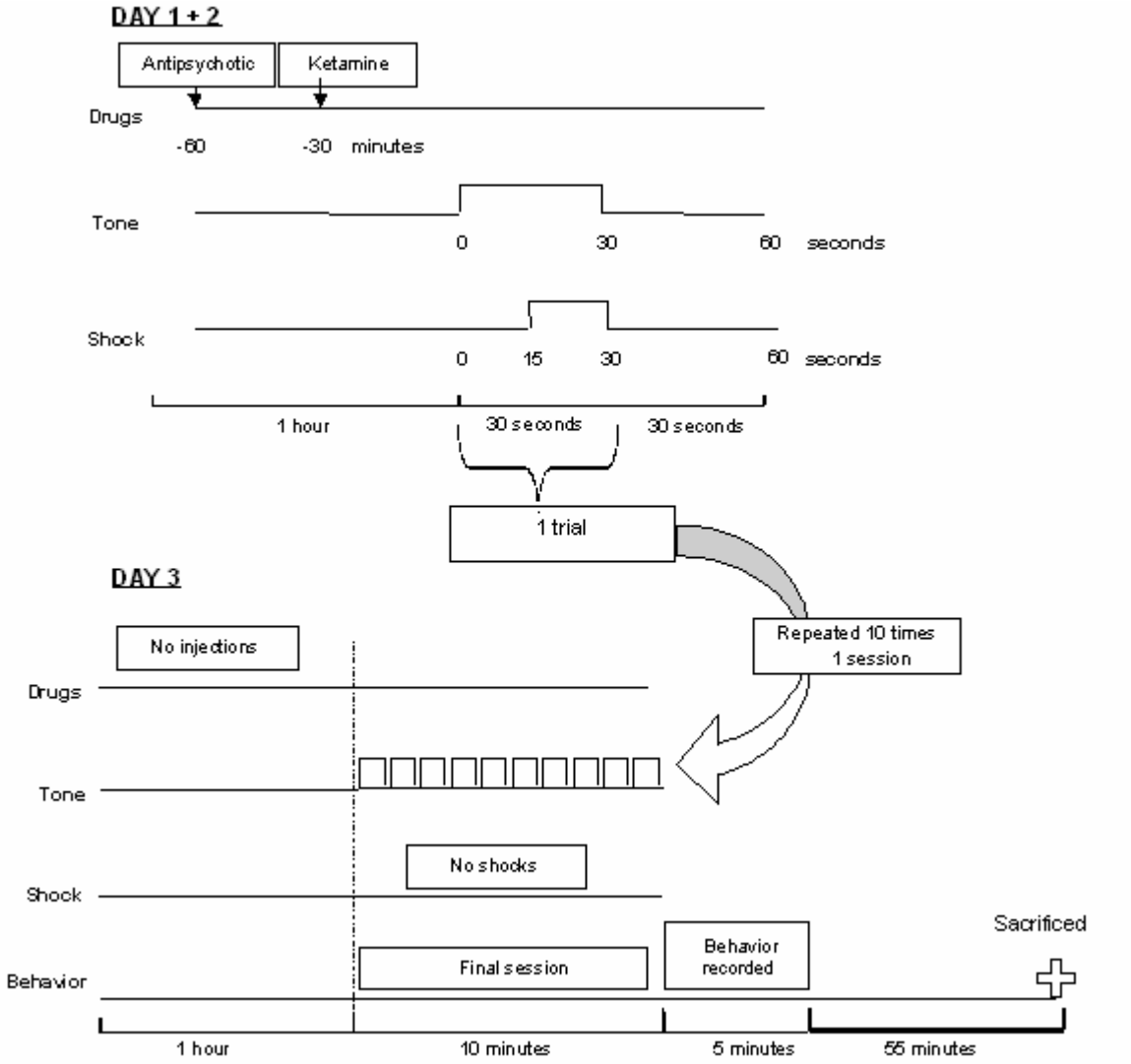

***Figure 2**: Injection and shock schedule. One trial consisted of a 30 second period. During the 30 seconds, a tone was emitted. Within the second half (15 seconds) of this 30-second period, the shock was delivered. Thirty seconds following the trial served as a rest period. All trials took place in the morning and were repeated consecutively ten times per day, resulting in one session lasting 10 minutes in total. Control rats followed the same routine with tone emission, but without experiencing any shocks. On the third day, the same procedure was followed, but without administering shocks. This was done to avoid measuring behavioural outputs due to direct drug interference or pain stimuli. The behaviour was noted for 5 minutes after the test session on the third day and was sacrificed 1 hour after the end of the test session.*

One shock session consisted of a 1-minute period. All shock sessions took place in the mornings. We presented rats with a tone during the first 15 seconds. Thereafter, in the next 15 seconds the tone in combination with a shock is emitted. Thirty seconds thereafter, the process is repeated. This 1-minute session is repeated 10 times per day in succession, resulting in one trial of 10 minutes. This protocol is repeated on day 2. Control rats followed the same routine with tone emission, but without experiencing any shocks. On day 3, neither groups receive shocks nor injections; otherwise the animals follow the same protocol. This was done to avoid

measuring behavioural outputs due to direct drug interference or pain stimuli. Following this conditioned fear stress, the behaviour was noted for 5 minutes after the test trial on the third day in order to determine if a fear response was acquired in reaction to the whole stress procedure (tone and context). Previous studies in our lab (unpublished data) have shown that minimal extinction occurs during the first 5 minutes of the last test session and that fear-conditioned freezing behaviour was still evident.

2.4 Behavioural observation

Behaviours were recorded for each rat by means of a video camera (Philips Explorer Camcorder) directly after the last test session on the third day. They were then subsequently analyzed with the aid of the computer program, The Observer (Noldus version 3.0, The Netherlands). An independent observer unaware of experimental conditions noted both the frequency and total duration of freezing, grooming, rearing and resting behaviour. Freezing was denoted as a conscious action absent of any movement, except that needed for respiration and whisker twitching. Rearing was defined as the raising of the body onto the hind legs, while resting served as a default state when none of the other behaviours were being displayed. Freezing behaviour, as well as being a behavioural expression of stress, is also the main determinant of fear conditioning having being achieved (Bolles and Collier, 1976; Holahan and White, 2002).

2.5 cFos expression

2.5.1 Perfusion and preparation

One hour after the end of the final test session, the rats were perfused trans-cardially with 4% paraformaldehyde (Merck, Germany) for 20 minutes. This time point was chosen so as to incorporate all events happening in the brain during the tone signals in the last testing session. The brains were then removed and placed into 4% paraformaldehyde, and kept at 6 ℃ for two days. Th ereafter, they were transferred into 0.02 M potassium phosphate buffered saline (PBS; pH 7.4) with 1% sodium azide (Boom, Meppel, The Netherlands) to prevent bacterial growth and were stored at 6℃. In preparation for cFos staining, whole bra ins were dehydrated in a 30% sucrose solution overnight and subsequently frozen with gaseous CO_2 at –80 ℃. The

brains were cut using the Leica CM 3050 cryostat machine at 40 micrometers thin slices and stored at 6 ℃ in 0.02 M PBS buffer (pH 7.4).

2.5.2 cFos staining: Immunocytochemistry

Coronal cryostat sections of 40 mm were collected in 0.01 M Tris buffered saline (TBS, pH 7.4) and rinsed 3×5 min. After pre-incubation with 0.3 % H_2O_2 (10 min, in 0.01 M TBS, pH 7.4), the sections were washed with 0.01 M TBS (4×5 min, pH 7.4) and incubated with an rabbit polyclonal antibody raised against cFos (Ab-5 Oncogene Research Products, Calbiochem, 1:10.000 in 0.01 M TBS-Triton 0.01%, 4% normal goat serum) for 48–60 hours at room temperature. Subsequently, the sections were washed in 0.01 M TBS (8×5 min, pH 7.4) and incubated for 2hours at room temperature with biotinylated goat anti-Rabbit IgG (Vector, 1:1000 in 0.01 M TBS). After rinsing with 0.01 M TBS (6×5 min, pH 7.4), the immunoreactivity was visualized with a standard ABC method (Vectastain ABC kit, Vector, (1 drop A+1 drop B)/20 ml TBS for 2hours). After washing with TBS 0.01 M (6×5 min, pH 7.4) the peroxidase reaction was developed with a di-aminobenzidine (DAB)-nickel solution and 0.3% H_2O_2 (0.5mg DAB/ml Distilled water; 1.0% nickel ammonium sulphate (NAS)) in 0.1 M sodium acetate (NaAc, pH 6.0). To stop the reaction, the sections were washed with 0.1M NaAc, pH 6.0 (3x5minutes) and then 0.01 M TBS (3×5 min, pH 7.4) and were subsequently mounted on gelatin-coated slides, air dried, dehydrated, and coverslipped with DePeX (Gurr) (Boom, Meppel, The Netherlands).

The area of the region of interest was measured and, after background correction, the number of immunopositive nuclei was quantified using a computerized image analysis system (Leica Qwin version 2.3, Leica Microsystems Imaging Solutions). The average number of cFos immunoreactive cells was calculated and expressed as number of positive nuclei or Counts/Area (0.1 mm^2). Areas included in the cFos analysis were: the paraventricular nucleus, central, basolateral amygdala nuclei (subdivided into anterior and posterior nuclei) and lateral nucleus of the amygdala, nucleus accumbens (core and shell), and anterior cingulate. The Swanson (1992) co-ordinates (rostral-caudal) are given in Table 1 as millimetres from Bregma.

Table 1: Brain areas: Swanson (1992) rostral-caudal stereotaxic co-ordinates

Area	Mm from Bregma
Anterior cingulate	+2.80 to +2.15
Nucleus accumbens: core and shell	+2.80 to +0.45
Paraventricular nucleus	-1.53 to –2.00
Central nucleus of amygdala	-2.45 to –2.85
Anterior basolateral nucleus amygdala	-2.45 to –2.85
Posterior basolateral nucleus amygdala	-2.45 to –2.85
Lateral nucleus amygdala	-2.45 to –2.85

2.6 Statistics

Due to the presence of occasional outliers, the behavioural data were analyzed by one-way analysis of variance (ANOVA) on rank-transformed data, which is equivalent to the Kruskal-Wallis test (Montgomery, 1984). When the overall F test of treatment group equality was significant at the 5% level ($p<0.05$), planned comparisons among treatment groups were made with the least significant difference (LSD) method (pairwise comparisons). When the overall F test was not significant at the 5% level, planned comparisons were made with the Bonferroni method (Milliken and Johnson, 1992).

An independent Student's t-test was first applied to the cFos data with regards to the FC and NFC groups to determine if there was an effect of fear conditioning. This was done in order to determine which brain areas were to be further analyzed for data collection and which could be discarded. If a fear conditioning effect was found ($p<0.05$), all groups were then counted in appropriate brain areas revealed by the t-test and subsequently analyzed by means of a one-way analysis of variance (ANOVA), followed by post-hoc pairwise comparisons (LSD). Logged equivalents were used in order to eliminate skew distributions where necessary.

The set of planned comparisons were as follows: FC vs. NFC; FC vs. FC + Ket; NFC vs. Ket; FC + Ket vs. FC + Ket + Cloz; FC + Ket vs. FC + Ket + Halo; and FC + Ket vs. FC + Ket + LY. Statistical analyses were performed with JMP Release 5.1.1 software or SPSS v.12.

3 Results

3.1 Behaviour

The total duration and frequency of behaviours 5 minutes after the test trial were analyzed, and are represented in Fig. 3. The behaviours of 3 rats in the control group were not included due to technical difficulties with the video recording. The one-way ANOVA revealed significant overall differences for the following behaviours: resting duration ($F_{6, 38} = 3.32$; $p = 0.0099$) and frequency ($F_{6, 38} = 15.23$; $p < 0.0001$), freezing duration ($F_{6, 38} = 6.51$; $p < 0.0001$) and frequency ($F_{6, 38} = 20.42$; $p < 0.0001$), and rearing duration ($F_{6, 38} = 6.79$; $p < 0.0001$) and frequency ($F_{6, 38} = 5.35$; $p = 0.0004$).

3.1.1 Effects of fear conditioning

The least significant differences (LSD) post hoc showed fear-conditioning effects in most of the behaviours investigated (FC vs. NFC). These include a decrease in resting duration ($p = 0.0064$; Fig. 3a), and increases in rearing duration ($p = 0.0262$; Fig. 3a) and resting frequency ($p < 0.0001$; Fig. 3b). More importantly, increases in freezing duration ($p = 0.0001$; Fig. 3a, c) and frequency ($p < 0.0001$; Fig. 3b, d) were noted.

3.1.2 Effects of ketamine alone and on fear conditioning

According to the LSD post hoc, ketamine alone did not influence any of the behaviours measured. It augmented the effect of fear conditioning with respect to rearing duration ($p = 0.0023$). In agreement with our hypothesis, however, ketamine blocked the effects of fear conditioning with respect to both freezing duration ($p = 0.0213$; Fig. 3c) and frequency ($p = 0.0002$; Fig. 3d).

3.1.3 Antipsychotic effects on fear conditioning + ketamine combination

Comparing the effect of antipsychotics on rats undergoing fear conditioning with ketamine administration (FC + Ket vs. FC + Ket + Cloz/Halo/LY), we find significant

differences with respect to rearing duration. Decreases in rearing duration were noted due to clozapine (p = 0.0123) and haloperidol (p = 0.0043) administration, both blocking the effect of ketamine. While antipsychotics did not reverse the effect of ketamine on fear conditioning with respect to freezing duration (Fig. 3c), haloperidol (p = 0.0040) and LY 379268 (p = 0.0026), but not clozapine (p = 0.1033), did reverse the effect of ketamine with respect to freezing frequency (Fig. 3d).

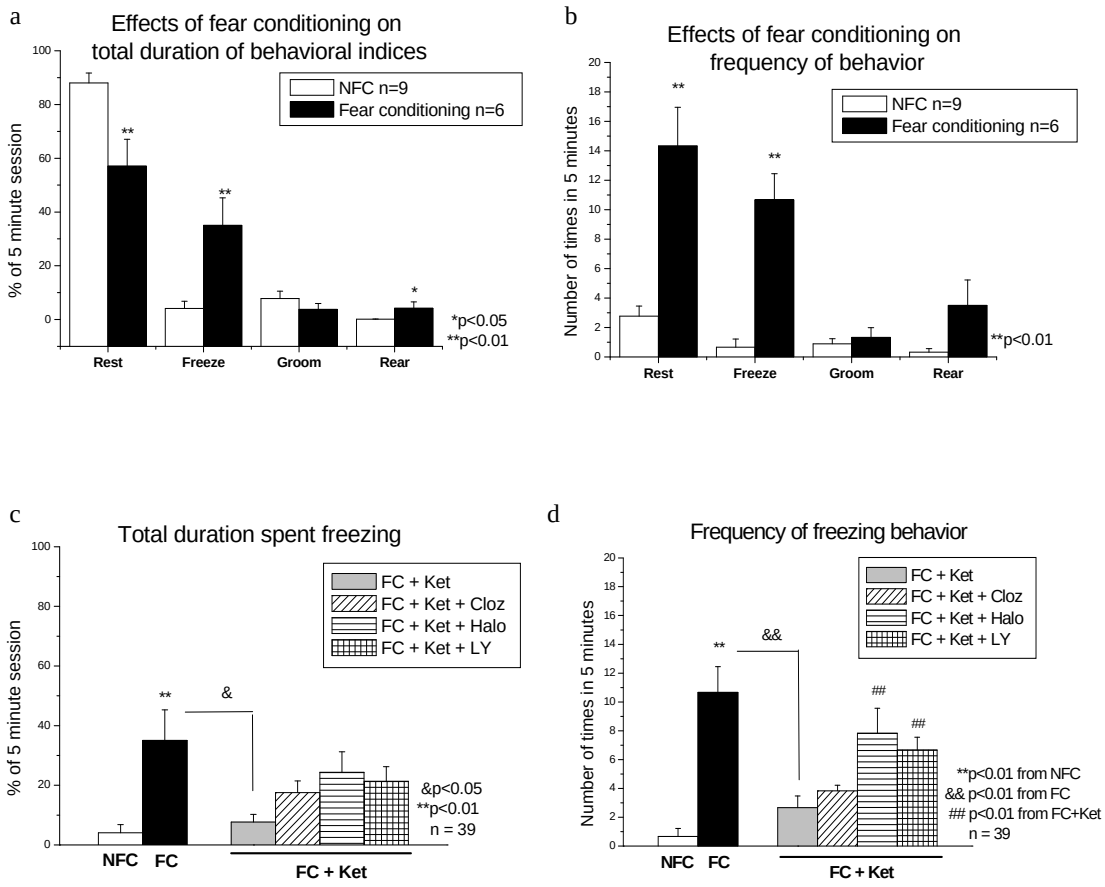

Figure 3*: Behavioural data. All significances were determined using the LSD post hoc test after a significant (p<0.05) one-way ANOVA. Fear conditioning affects almost all of the behaviours including a decrease in resting duration (p = 0.0064; a), and increases in rearing duration (p = 0.0262; a), resting frequency (p < 0.0001; b), and freezing duration (p = 0.0001; a) and frequency (p < 0.0001; b). As hypothesized, ketamine blocked the effect of fear conditioning (FC vs. FC + Ket), reducing freezing duration p = 0.0213; c) and frequency (p = 0.0002; d). Haloperidol (p = 0.0040) and LY 379268 (p = 0.0026) were able to partially restore this blockade (FC + Ket + Halo/LY vs. FC + Ket), but only in terms of freezing frequency (d). Cloz, clozapine; FC, Fear conditioning; Halo, Haloperidol; Ket, Ketamine; LY, LY 379268; NFC, no fear conditioning.*

3.2 cFos expression

Results of the cFos data are represented in Figs. 4 and 5, with typical examples of cFos stainings and the delineations of the areas represented in Fig. 6. An independent Student's t-test revealed fear-conditioning effects in the anterior

cingulate ($p = 0.016$), nucleus accumbens shell ($p = 0.001$), and the paraventricular nucleus ($p < 0.0001$). No fear conditioning effects were noted in the nucleus accumbens core ($p = 0.649$) and therefore this area was not included in further analyses. With regards to the amygdala, significant fear conditioning effects were found in the anterior portion of the basolateral amygdala ($p = 0.008$) and lateral amygdala ($p = 0.008$), with no effects of fear conditioning in the (medial) central amygdala ($p = 0.654$) or the posterior portion of the basolateral amygdala ($p = 0.483$). The latter two areas were not included in further analyses. After all groups were subsequently analysed, the one-way ANOVA revealed significant overall F-tests for the following brain areas: anterior cingulate ($F_{6, 39} = 5.96$; $p < 0.001$), nucleus accumbens shell ($F_{6, 40} = 8.96$; $p < 0.001$), paraventricular nucleus ($F_{6, 40} = 25.89$; $p < 0.001$), anterior basolateral amygdala ($F_{6, 39} = 9.49$; $p < 0.001$) and lateral amygdala ($F_{6, 39} = 11.68$; $p < 0.001$).

3.2.1 Effects of fear conditioning

The least significant differences (LSD) post hoc showed increases in cFos expression due to fear conditioning in all the brain areas included after elimination of those with negative T-test results. These include the anterior cingulate ($p = 0.003$; Fig. 4a), nucleus accumbens shell ($p < 0.0001$; Fig. 4a), and paraventricular nucleus ($p < 0.0001$; Fig. 4a). More importantly, increases due to fear conditioning were noted in the anterior portion of the basolateral amygdala ($p = 0.002$) and lateral amygdala ($p < 0.0001$) (Fig. 5a).

3.2.2 Effects of ketamine alone and on fear conditioning

Decreases of cFos expression due to ketamine alone, as revealed by the LSD post hoc, were also noted in most areas except the anterior cingulate ($p = 0.087$) and nucleus accumbens shell ($p = 0.09$) (Fig. 4b), i.e. paraventricular nucleus ($p = 0.047$; Fig. 4b), anterior basolateral amygdala ($p = 0.003$; Fig. 5b) and lateral amygdala ($p = 0.01$; Fig. 5b). Ketamine also led to the hypothesized blocking of cFos expression due to fear conditioning in all the brain areas investigated, i.e. the anterior cingulate ($p < 0.0001$), nucleus accumbens shell ($p = 0.002$), paraventricular nucleus ($p < 0.0001$) (Fig. 4b), anterior basolateral amygdala ($p = 0.001$) and lateral amygdala ($p = 0.004$) (Fig. 5b).

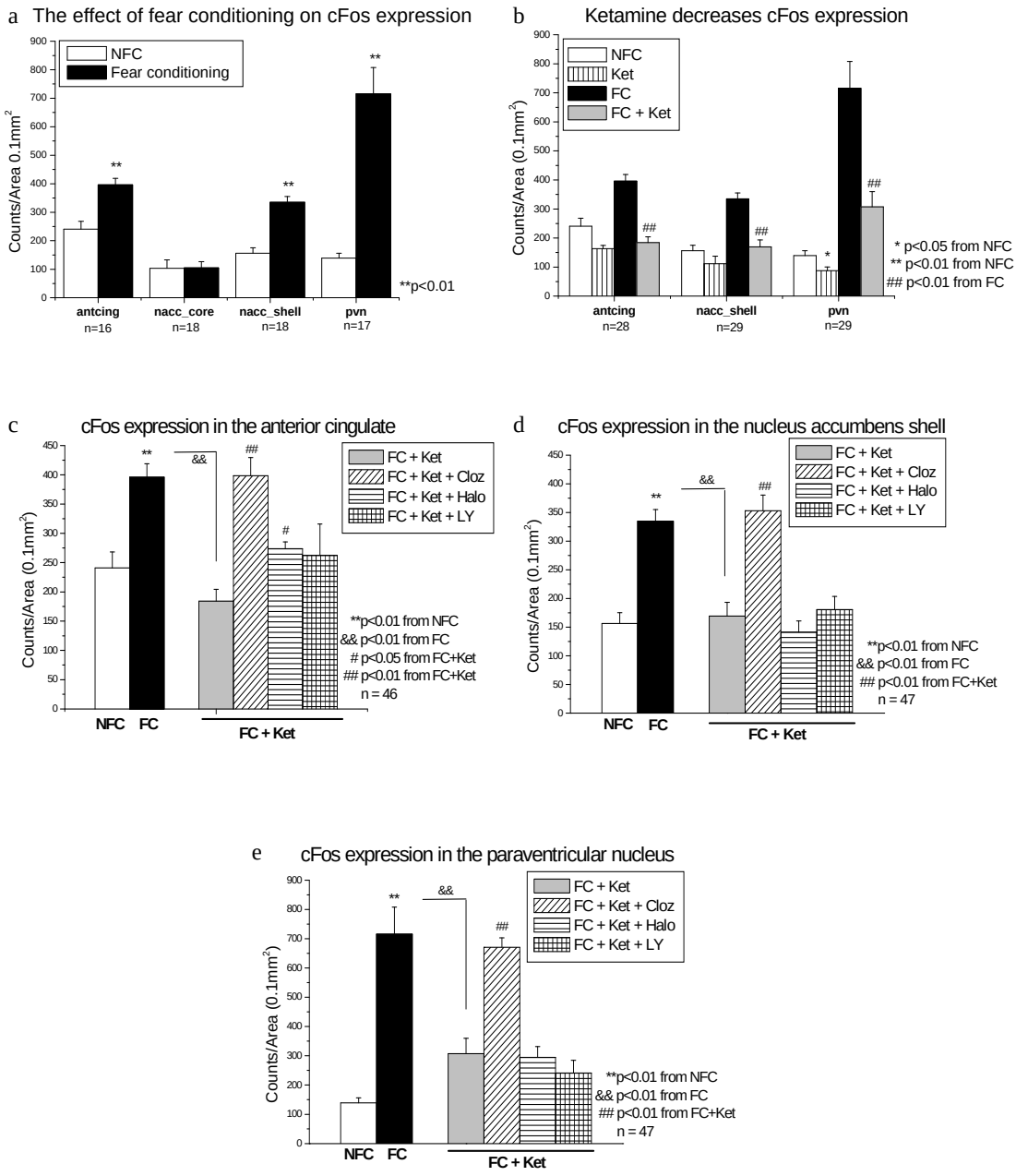

Figure 4*: cFos expression in other brain areas. All significances were determined using the LSD post hoc test after a significant (p<0.05) one-way ANOVA. Fear conditioning (FC) increased cFos expression as compared to the NFC group (a) in the anterior cingulate (p = 0.003), nucleus accumbens shell (p < 0.0001), and paraventricular nucleus (p < 0.0001). Ketamine (b) decreased cFos expression alone (NFC vs. Ket) in the paraventricular nucleus only (p = 0.047); while also blocking the effect of fear conditioning (FC vs. FC + Ket) in the anterior cingulate (p < 0.0001), nucleus accumbens shell (p = 0.002), and paraventricular nucleus (p < 0.0001). These results are also depicted in graphs c, d, and e. As hypothesized, clozapine was able to counteract the blockade of ketamine on fear conditioning (FC + Ket vs. FC + Ket + Cloz) in the anterior cingulate (p < 0.0001; c), nucleus accumbens shell (p = 0.001; d), paraventricular nucleus (p = 0.001; e). A slight restoration by haloperidol was noted in the anterior cingulate (p = 0.042; c). antcing, anterior cingulate; Cloz, clozapine; FC, fear conditioning; Halo, haloperidol; Ket, Ketamine; LY, LY 379278; nacc_core, nucleus accumbens core; nacc_shell, nucleus accumbens shell; NFC, no fear conditioning; pvn, paraventricular nucleus.*

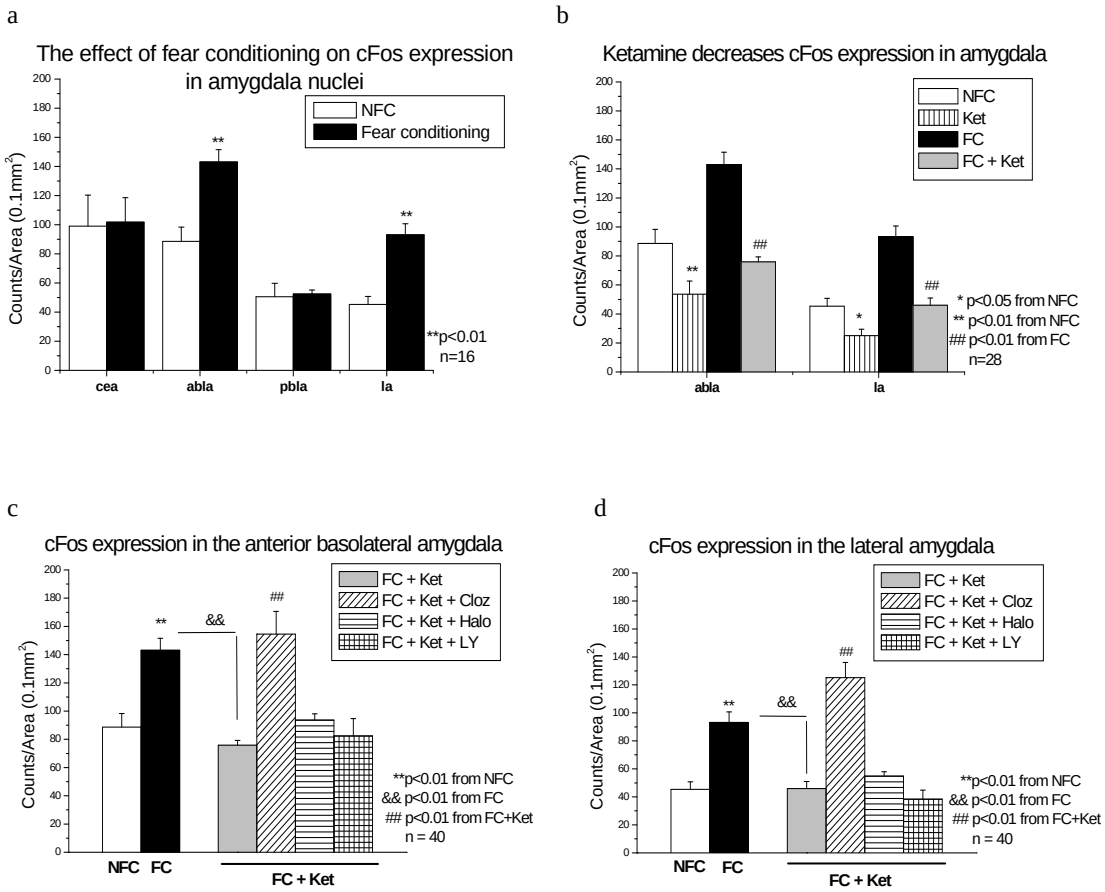

Figure 5*: cFos expression in amygdala nuclei. The number of rats indicated on the graph refers to the number of rats per brain area. All significances were determined using the LSD post hoc test after a significant (p<0.05) one-way ANOVA. Fear conditioning (FC) increased cFos expression as compared to the NFC group (a) in the anterior basolateral amygdala (p = 0.002) and lateral amygdala (p < 0.0001). Ketamine (b) decreased cFos expression alone (NFC vs. Ket) in the same areas: anterior basolateral amygdala (p = 0.003) and lateral amygdala (p = 0.01); while also blocking the effect of fear conditioning (FC vs. FC + Ket) in the same areas: anterior basolateral amygdala (p = 0.001) and lateral amygdala (p = 0.004). These results are also depicted in graphs c, d. As hypothesized, clozapine, and not haloperidol or LY379268, was able to counteract the blockade of ketamine on fear conditioning (FC + Ket vs. FC + Ket + Cloz) in the anterior basolateral amygdala (p < 0.0001; c) and lateral amygdala (p < 0.0001; d). abla, anterior basolateral amygdala; cea, central amygdala (medial); FC, fear conditioning; Ket, Ketamine; la, lateral amygdala; NFC, no fear conditioning; pbla, posterior basolateral amygdala.*

3.2.3 Antipsychotics vs. fear conditioning + ketamine combination

It was hypothesized that clozapine (FC + Ket + Cloz), and not haloperidol or LY 379268 (FC + Ket + Halo/LY) would reverse the block the effect of ketamine on fear conditioning (FC + Ket) with regards to cFos expression. These hypotheses were supported by the data, which showed normal fear conditioning effects on cFos expression after clozapine administration. Significant differences between groups due to clozapine administration (FC + Ket vs. FC + Ket + Cloz) were noted in the anterior cingulate (p < 0.0001; Fig. 4c), nucleus accumbens shell (p = 0.001; Fig. 4d),

paraventricular nucleus (p = 0.001; Fig. 4e), anterior basolateral amygdala (p < 0.0001; Fig. 5c) and lateral amygdala (p < 0.0001; Fig. 5d). Haloperidol did show some effect in the anterior cingulate, although not as significant as clozapine (p = 0.042; Fig. 4c). No other effects of haloperidol or LY 379268 drugs were found in any of the other areas investigated.

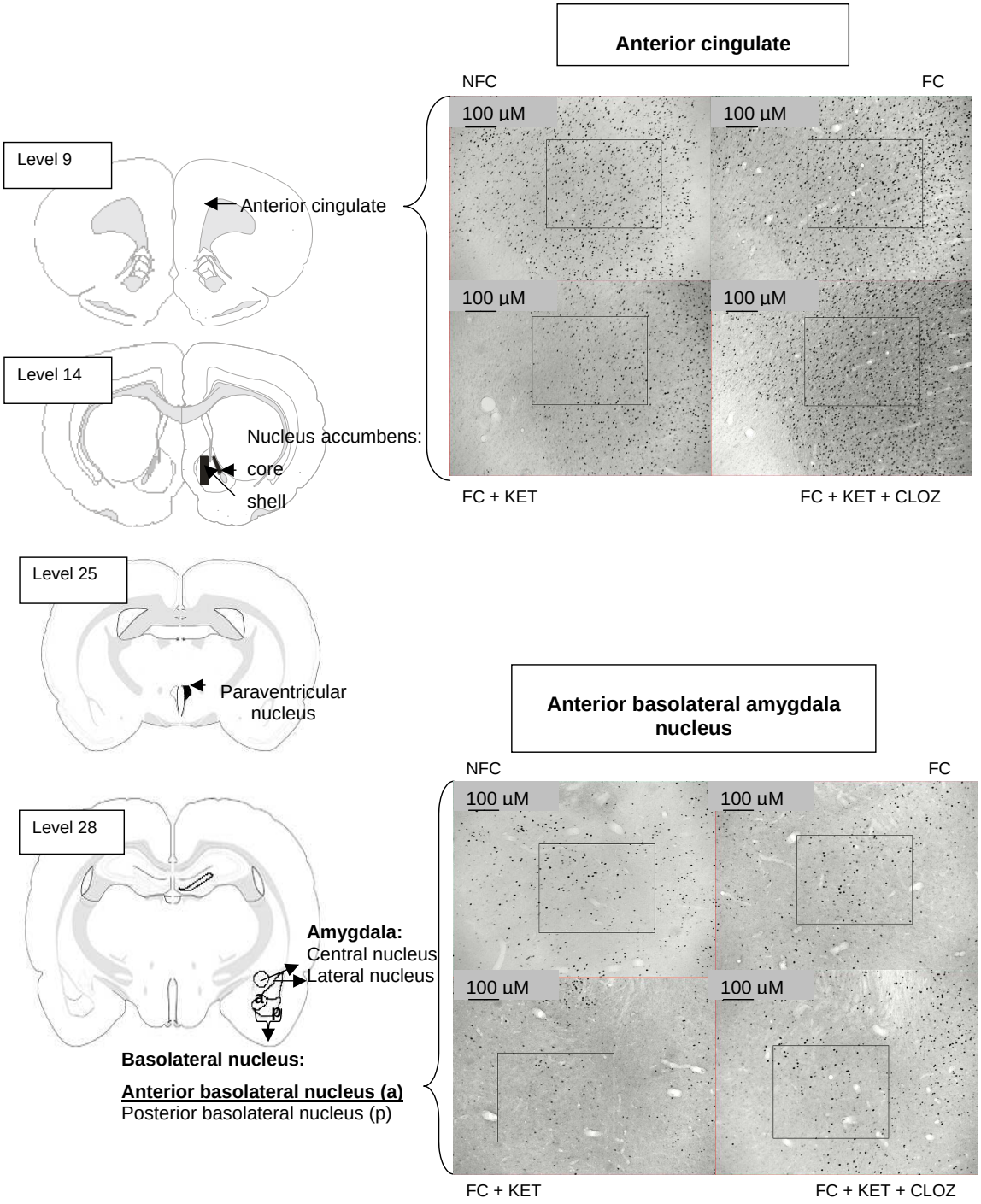

***Figure 6**: cFos immunocytochemical labelling. Typical examples of the brain areas stained for cFos expression, visually showing the effects of some of the treatments. Delineated areas depict areas measured. Brain slice levels were taken from the Swanson rat brain atlas (1992), with appropriate co-ordinates listed in Table 1. CLOZ, clozapine; FC, Fear conditioning; KET, ketamine; NFC, no fear conditioning.*

4 Discussion

Fear processing is especially relevant to schizophrenia, as many patients manifest deficits in the recognition of fearful faces (Edwards et al., 2001; Johnston et al., 2001; Gur et al., 2002), in addition to general deficits in the processing and attribution of negative emotional states (Paradiso et al., 2003; Takahashi et al., 2004). Studies suggest that a hypofunctioning glutamatergic system could be related to both the cognitive and emotional deficits, including fear processing, displayed by schizophrenic patients (Moghaddam et al., 1997; Krystal et al., 2000; Abel et al., 2003; see Riedel et al., 2003 for a review of cognitive deficits). In a previous study (Pietersen et al., 2006b), we investigated the effects of a hypoglutamatergic state on fear conditioning, as measured through the paradigm of fear-conditioned stress. In that study, we found profound effects of conditioning on freezing behaviour, consistent with observed increases in cFos expression in areas related to stress (locus coeruleus, paraventricular nucleus), fear (basolateral nucleus of the amygdala), and motivation and learning (nucleus accumbens and anterior cingulate). Ketamine, the glutamate antagonist, reversed these changes in behaviour and cFos expression in all above-mentioned brain areas, except the nucleus accumbens. This previous study, however, focused on the role of glutamate in anxiety disorders.

In the present study, we attempted to replicate the results of the previous study, in addition to evaluating the effects of antipsychotics typically used in the clinical setting (clozapine and haloperidol) in an attempt to validate our paradigm as a model of negative schizophrenia. We also tested the effects of a potentially new antipsychotic, LY 379268, a 2/3 metabotropic glutamate agonist, which has been shown to alter glutamate release in the forebrain area (Moghaddam and Adams, 1998). Our hypothesis was that fear conditioning would increase behavioural assays of fear (in particular, freezing) in addition to increasing cFos expression, particularly in the lateral amygdala, basolateral amygdala and nucleus accumbens shell and core. Additionally, we expected that ketamine would block all fear-conditioning effects. We also hypothesized that clozapine would restore the fear-conditioning effects on behaviour and cFos expression abolished by ketamine, thereby restoring normal cognitive-emotional processing. We expected that haloperidol, however, would not

normalize these neural and behavioural assays, as it mainly affects positive symptoms.

Fear conditioning led to increases in freezing behaviour (both duration and frequency) as well as decreases in resting duration (Fig. 3a, b). The increased freezing behaviour shows increased anxiety and more importantly that fear conditioning was achieved. The decreased resting duration also suggests increased anxiety, even though an increase in resting frequency was noted. This could be due to the fact that resting was the default state to which all behaviours returned upon completion, and therefore as the frequency of any one behaviour increases, so does the resting frequency. Increases in rearing behaviour were also noted, which traditionally suggests a non-anxious rat. In this scenario, however, it could be an avoidance response in anticipation of the shock.

The expression of cFos (an immediate early gene) is usually associated with neural activity (Sagar et al., 1988; Ananth et al., 2001). In our study, cFos expression was increased in brain areas relevant to fear learning after conditioning, such as the anterior cingulate, nucleus accumbens shell, paraventricular nucleus and amygdala nuclei, particularly the anterior basolateral and lateral amygdala (Fig. 4a, 5a). Other studies (Sananes and Davis, 1992; Goosens and Maren, 2001) have also selectively implicated the anterior portion of the basolateral nucleus in fear conditioning. Consistent with our results, Scicli et al. (2004) found that the anterior portion of the basolateral nucleus, in addition the lateral nucleus (although also a subsection of the central nucleus of the amygdala), showed increased levels of cFos expression following fear conditioning. Fear conditioning in our study did not evoke cFos expression in the central amygdala or posterior basolateral amygdala (nor the nucleus accumbens core), implying that these areas are not central to fear learning. These findings are in agreement with our previous study (Pietersen et al., 2006b), and with other studies in the literature (Fanselow and Kim, 1994; Shors and Matthews, 1998; Pezze et al., 2002; Reynolds and Berridge, 2003; Koo et al., 2004).

Ketamine, in the absence of fear conditioning, did not influence any of the behaviours measured. As in the previous study (Pietersen et al., 2006b), however, ketamine did block fear conditioning, as seen by the decrease in freezing duration (Fig. 3c) and

frequency (Fig. 3d). This was also reflected in the cFos data, as ketamine blocked fear conditioning-induced increases in cFos expression (Fig. 4b, 5b).

Clozapine did not have any influence on freezing behaviour but powerfully blocked the effects of ketamine on cFos expression, restoring cFos back to fear conditioning levels in the anterior basolateral amygdala and lateral amygdala (Fig. 5c, d). This pattern of results was also seen in other areas associated with fear learning, including the anterior cingulate, nucleus accumbens shell and paraventricular nucleus (Fig. 4c, d, e), areas intimately linked with the amygdala. Interestingly, along with the amygdala, the former two areas have also been implicated in the pathophysiology of schizophrenia (Weatherspoon et al., 1996; Hempel et al., 2003). With the exception of the anterior cingulate, neither haloperidol nor LY 379268 had any effect on cFos expression. The behavioural data, however, paints a slightly different picture, since haloperidol and LY 379268 significantly restored freezing frequency. Although the number of freezing events increased with haloperidol and LY 379268 administration, however, the total duration of time spent freezing did not change. This implies that rats froze more often but for shorter periods, making it difficult to interpret the effects of haloperidol and LY 379268 in clear cut terms. The discrepancy between freezing frequency and duration found here parallels the behavioural effects of clozapine administered alone (Pietersen et al., submitted), and clearly requires additional study to resolve. The correlation between freezing frequency and cFos expression in the anterior cingulate may relate to the function of this brain area in generating anticipation of pain (Gao et al., 2004; Sugase-Miyamoto and Richmond, 2005; Tang et al., 2005). That is, changes in freezing frequency may be due to an anticipation effect generated in the anterior cingulate, whereas changes in total freezing duration appear to be related to amygdala function.

The discrepancy between the effects of clozapine on behavioural and neural correlates requires some explanation. It must be stated from the outset, however, that clozapine's efficacy in the clinical situation is not absolute (Bender et al., 2006; Thornton et al., 2006), a fact which should be taken into consideration when evaluating the model. Other studies making use of clozapine and haloperidol indicate that clozapine is superior in reversing the neural effects of NMDA antagonists. For example, clozapine (5 or 10 mg/kg) abolished the increased metabolism (2-

deoxyglucose uptake) in the rat prelimbic cortex, nucleus accumbens, anterior ventral thalamic nucleus and hippocampal formation induced by ketamine administration (Duncan et al., 1998). In the same study, haloperidol administered 45 minutes prior to ketamine administration (0.5 mg/kg) did not alter the behavioural response or metabolic activation induced by ketamine (Duncan et al., 1998), consistent with its putative D_2 mechanism of action. This would account for clozapine's success and haloperidol's failure to reverse the effects of ketamine in the key brain areas studied here, most importantly in the amygdala. It does not, however, explain why clozapine failed to restore normal fear behaviour.

Pietersen et al. (submitted, Chapter 5) provide a conceptual model of amygdala fear processing to explain why clozapine does not reverse the fear-blocking effects of ketamine. This model is supported by analyses of glutamate and dopamine tissue concentrations obtained with a protocol similar to that used here. The key findings from this study were: (a) fear conditioning induced elevated glutamate levels in the basolateral amygdala, but did not affect dopamine levels in either the basolateral or central amygdala; (b) ketamine blocked the rise in glutamate levels in the basolateral amygdala and simultaneously elevated dopamine levels in the central amygdala; (c) clozapine reversed the effects of ketamine on glutamate levels in the basolateral amygdala, but did not act to reverse the elevation of dopamine in the central amygdala. Pietersen et al. (submitted, Chapter 5) speculated that elevated dopamine levels in the central amygdala might have acted to block the behavioural expression of activity in the basolateral amygdala, perhaps through GABA inhibition within the central amygdala itself.

We predict that a chronic study is needed in order to observe the remedial effects of clozapine on fear behaviour. While it is possible that the dose used in our study was not strong enough to elicit behavioural effects, we used the lowest dose possible without deleteriously affecting locomotor activity, a critical imperative for our behavioural measurements. The dosage used here is also in line with the dose a patient would receive in a clinical setting, as determined by D_2 receptor occupancy (Kapur et al, 2003). As indicated above, it is well known in the clinical situation that antipsychotics take a considerable period of time (up to 6 weeks) to start resolving symptoms. Enomoto et al. (2005) treated mice for 14 days with phencyclidine (PCP),

an NMDA antagonist similar to ketamine, leading to impaired fear conditioning. Repeated chronic administration of olanzapine, but not haloperidol, for seven days reversed the impairment caused by PCP (Enomoto et al., 2005). A study by Sams-Dodd (1996) also showed that chronic clozapine treatment inhibited PCP-induced stereotypical behaviour and social isolation. We speculate that chronic clozapine administration may work to remediate fear conditioning by decreasing elevated dopamine levels in the central amygdala. This idea predicts that one would see a gradual decrease in central-amygdala dopamine levels in our animal model over time. Coupled with the rapid action of clozapine in reversing the actions of ketamine on glutamate, this would renormalize behavioural assays of fear conditioning. Should this prediction turn out to be correct, it would have important implications for the modus operandi of clozapine in treating negative schizophrenic symptoms. Indeed, the very remedial effectiveness of clozapine may be a key reason why deficits in associative learning, particularly fear conditioning, are not widely considered symptomatic of the schizophrenic condition (but see Hofer et al., 2001; see also O'Carroll, 1995; Rushe et al., 1999).

5 Conclusion

In summary, our results support our main hypothesis that glutamate NMDA antagonism interferes with fear conditioning. cFos assays suggest that, in addition to anxiety disorders, this paradigm could also be used to investigate cognitive-emotional dysfunctions seen in schizophrenia, such as emotional blunting, as clozapine (but not haloperidol) blocked the effect of ketamine on fear conditioning. Lack of behavioural evidence, however, forces us to conclude that this model is still in its infancy and needs to be refined before behavioural changes can be induced. Future studies include neurotransmitter and genetic analysis, in order to further investigate the model's potential in predicting therapeutic outcomes for treatments of the cognitive-emotional deficits observed in schizophrenia.

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Chapter 5

Interactions between dopamine and glutamate in the amygdala in a putative rodent model of the negative symptoms of schizophrenia

CY Pietersen, FJ Bosker, T Vladusich, J Doorduin, ME Jongsma, F Postema, JA den Boer

Department of Psychiatry, Medical Faculty, UMCG, The Netherlands

In preparation

Abstract

Psychiatric disorders, such as schizophrenia, involve dysfunctional emotional processing. Some of these disturbances may stem from amygdala dysfunction, a brain region involved in fear conditioning. The present study forms part of the validation of a putative animal model for the negative (emotional) symptoms of schizophrenia. The model involves the blockage of normal fear conditioning—a putative assay of emotional blunting in schizophrenia—by means of ketamine administration. We examine glutamate and dopamine tissue concentrations in rats treated with ketamine before fear conditioning. Ketamine blocked freezing behaviour and reversed elevated glutamate due to fear conditioning in key regions of the fear circuit, particularly the basolateral amygdala, and increased dopamine levels in the nucleus accumbens. Clozapine, an atypical antipsychotic moderately effective in treating emotional dysfunction in schizophrenia, restored normal neurotransmitter levels but did not restore normal freezing behaviour. We found no effects of fear conditioning in terms of dopamine tissue content in the central amygdala, though ketamine and clozapine, administered alone or in combination, elevated dopamine levels. We reconcile the neurochemical and behavioural data in terms of a model of amygdala function in which glutamate and dopamine neurons perform antagonistic roles.

1 Introduction

Schizophrenia is a chronic and disabling disease. Elucidating its neurochemical origins could provide important information regarding its aetiology and may also provide some clue for future therapeutical interventions. Currently, there are two predominant neurochemical hypotheses of schizophrenia: the glutamate hypothesis and the dopamine hypothesis. The glutamate hypothesis originates from investigations showing that glutamate N-methyl-D-aspartate (NMDA) receptor antagonists, such as ketamine, induce psychosis in healthy volunteers, or elicit psychotic symptoms in refractory schizophrenic patients (Krystal et al., 1994; Malhotra et al., 1997; Abi-Saab et al., 1998). Some studies (Moghaddam et al., 1997; Krystal et al., 2000; Abel et al., 2003) suggest that a hypofunctioning glutamatergic system could be specifically related to the cognitive and emotional deficits displayed by schizophrenic patients (see Riedel et al., 2003, for a review of cognitive deficits). The dopamine hypothesis has its origins in the observation that typical antipsychotics (dopamine D_2 receptor antagonists) tend to ameliorate positive symptoms (Peroutka and Snyder, 1980; Jones and Pilowsky, 2002), whereas indirect dopamine D_2 receptor agonists such as amphetamine, induce a schizophrenic-type psychosis (Robinson and Becker, 1986; Breier et al., 1997; Laruelle et al., 1999). A single-neurotransmitter hypothesis is therefore probably not sufficient to fully describe the deficits reflected in the schizophrenic brain.

Currently it is unknown whether the schizophrenic-like symptoms induced by NMDA receptor antagonists arise from direct effects on the glutamate system, the dopamine system, or from interactions between the glutamate and dopamine systems. It has been reported, for example, that 5-methyl-10, 11-dihydro-5H-dibenzocyclohepten-5, 10-imine (MK-801), a glutamate NMDA receptor antagonist, exerts an effect on dopamine metabolism in the rat medial prefrontal cortex and striatum (Dai et al., 1995), while NMDA receptor antagonists in general increase the firing rates of dopamine neurons in the rat midbrain (French and Ceci, 1990). Ketamine administration results in increased dopamine and serotonin release (Lindefors et al., 1997; Lorrain et al., 2003), an effect that is likely to arise through direct stimulation of the dopamine D_2 and serotonin $5HT_2$ receptors (Kapur and Seeman, 2002) respectively. Dopamine D_2 receptor antagonists, in turn, have been found to

ameliorate ketamine-induced impairment of some prefrontal cortex-dependent cognitive functions in rodents (Verma and Moghaddam, 1996). Such findings suggest that the dopamine and glutamate systems function in an antagonistic fashion, and that an imbalance in the normal interactions between these systems may give rise to schizophrenic symptoms (de Bartolomeis et al., 2005).

Here we investigate the relationship between glutamate and dopamine in a putative animal model of negative schizophrenic symptoms. The present work forms part of a larger study (Pietersen et al., 2006) which involves combining ketamine administration with fear conditioning in the rat in order to model negative symptoms. Although not widely recognized as a core deficit in schizophrenia (Aleman and Kahn, 2005), the use of fear conditioning to investigate negative symptoms has some face validity. A recent study has shown that schizophrenic patients participating in a discriminative aversive conditioning task failed to develop conditioned responses to aversive stimuli, whereas healthy volunteers acquired a differential response to reinforced versus non-reinforced trials (Hofer et al., 2001; see also Kosmidis et al., 1999). Schizophrenic patients also manifest deficits in the recognition of fearful faces (Edwards et al., 2001; Johnston et al., 2001; Gur et al., 2002), and it is likely that disturbances in emotional processing stem from dysfunction of the amygdala (Phelps and Anderson, 1997; Adolphs et al., 1999; Peper et al., 2001; Aleman and Kahn, 2005). This is particularly relevant in light of evidence that bilateral amygdala damage in humans interferes with fear conditioning and with emotional learning of negatively arousing stimuli per se (Adolphs et al., 1999; Peper et al., 2001).

Translating human emotions into an animal model is difficult, particularly given the complexity of human emotion and the subjective nature of emotional states. The biological fear circuit, however, has been thoroughly investigated in the rat (Davis et al., 1994; LeDoux, 1998; Maren, 2001) and is highly conserved across species (Labar and Cabeza, 2006). Furthermore, fear conditioning is primarily mediated by NMDA glutamate receptors located in the amygdala (Fanselow and Kim, 1994; Goosens and Maren, 2003; Johnson et al., 2000) and leads to alterations in extracellular concentrations of both glutamate and dopamine (Yokoyama et al., 2005). The combination of fear conditioning with ketamine administration—recalling that ketamine alters glutamate and dopamine functioning and mimics some negative

schizophrenic symptoms—therefore provides an opportunity to study negative symptoms in an animal model. Previous work showed that ketamine administration blocks the acquisition of fear conditioning in the rat, both behaviourally and in terms of cFos expression in fear circuitry (Pietersen et al., 2006), but did not reveal whether such changes were due to glutamate or dopamine malfunctioning.

In the present study, we measure the influence of fear conditioning on tissue glutamate and dopamine levels in multiple regions of the rat fear circuit following ketamine administration. A key aim is to examine whether the atypical antipsychotic clozapine is able to reverse the blocking effect of ketamine, in order to provide further support of the putative animal model. We hypothesise that ketamine will interfere with the neurochemical alterations in brain areas associated with fear conditioning, primarily through action on NMDA receptors in the amygdala. As dopamine neurotransmission within the amygdala-nucleus accumbens circuit also plays an important role in associative processes underlying conditioned emotional responses (Young et al., 2005), we expect that alterations in this circuit due to fear conditioning will also be blocked by ketamine - perhaps in an antagonistic manner compared to glutamate. We also hypothesise that clozapine, an atypical antipsychotic known to be moderately effective in treating emotional dysfunction in schizophrenia, will partially restore these neurochemical alterations.

2 Materials and methods

2.1 Animals

All animals were cared for in accordance with the principles laid down by the European Communities Council Directive (1986) for the Protection of Vertebrate Animals used for Experimental or Other Scientific Purposes (86/609/EEC), which is comparable to the guidelines laid down in the “Principles of laboratory and animal care”. Sprague-Dawley rats (n = 29) weighing between 225-250 g were obtained from the central animal facility (Groningen, The Netherlands) and were housed individually in a temperature (±23℃) and humidity controlled (40 to 60%) environment. Food and water were delivered *ad libitum*. After arrival from the animal breeding facility, rats were allowed to acclimatize for two to three days. They were

then handled daily for five days in order to eliminate handling stress as a confounding variable.

The rats were divided into five groups: sham control (NFC), fear conditioned (FC), FC + ketamine (Ket), FC + clozapine (Cloz) and FC + Ket + Cloz. Clozapine was obtained from Sandoz Pharma AG, Switzerland and ketamine hydrochloride from Sigma, Germany.

2.2 Drugs and injection paradigm

Both clozapine and ketamine were dissolved in saline (0.9%), with hydrochloric acid added to the clozapine/saline solution to aid dissolving. Clozapine (5mg/kg, i.p.) was administered 30 minutes before ketamine injections (16 mg/kg, s.c.). Ketamine injections and saline shams were administered 30 minutes before fear conditioning. All injections took place on the first 2 days (i.e. only during the actual conditioning phase of the experiment). Drug administrations were omitted on the third day of testing to avoid unnecessary drug interaction effects with behavioural measurements. Previous observations in our lab showed that 30 minutes was sufficient for the ketamine-induced increases in locomotor activity to subside (Imre et al., 2006). The clozapine dose was determined empirically i.e. it was the highest dose possible that did not affect locomotor behaviour or induce catalepsy. It is also in line with the dose a patient would receive in a clinical setting, as determined by D_2 receptor occupancy (Kapur et al., 2003).

2.3 Shock paradigm

Rats were taken out of their home cage and placed individually in the shock box. This was a specially constructed wooden container with a floor made of a metal grid. A central computer controlled the current and tone emission, making use of a program that was specially developed for this study (N594 version 2.00, Rijksuniversiteit Groningen, The Netherlands, 2002). Rats destined to undergo fear conditioning were then subjected to a shock (1.5 mA) that was paired with a tone (60 dB tone) during 10 minute training sessions on the first two days of the experiment (Fig.1). This shock intensity was based on a pilot study indicating that 1.5mA shocks induced less variability on all measured stress parameters than 1mA shocks.

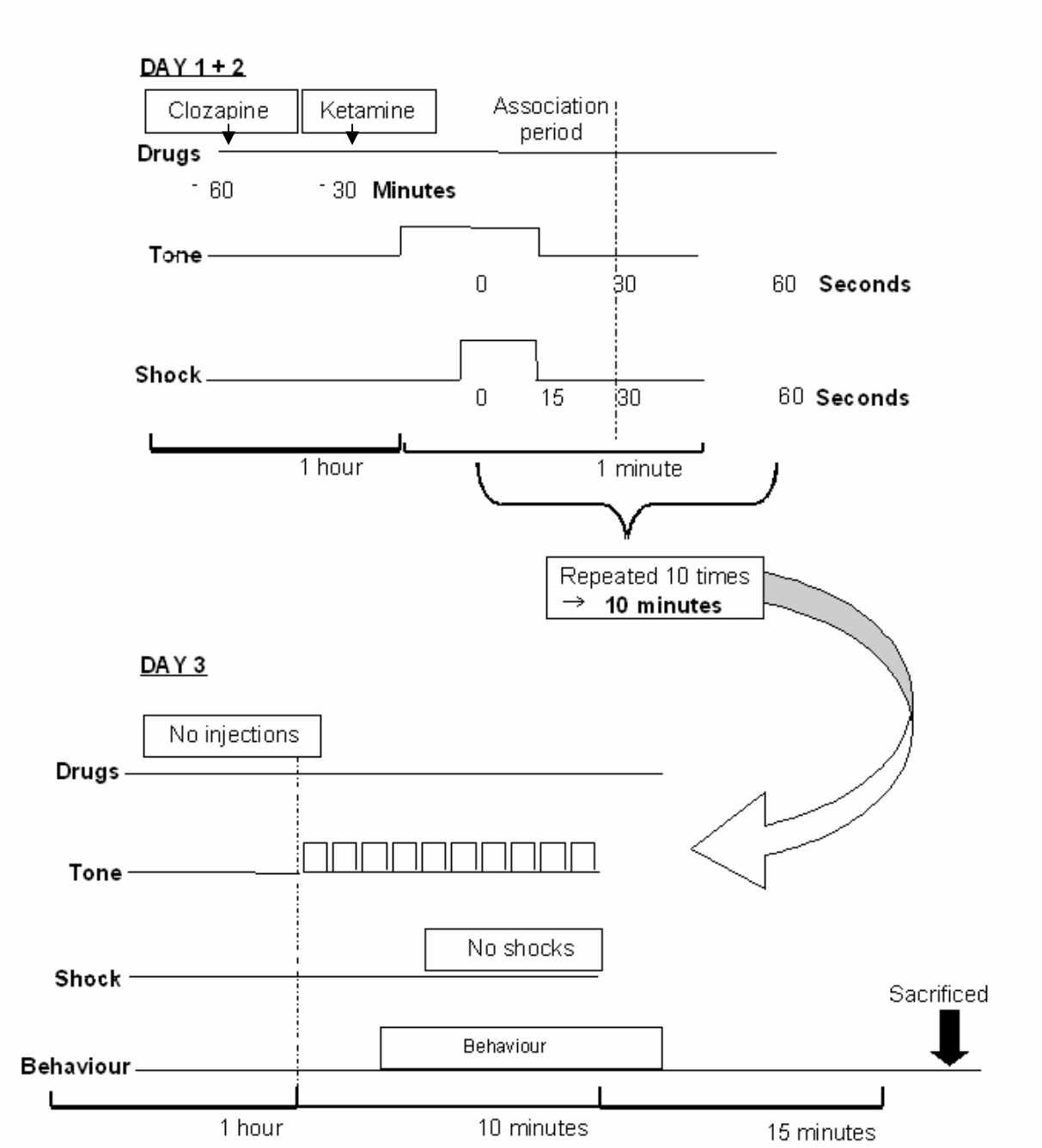

Figure 1*: Injection and shock schedule. One shock trial consisted of a 1-minute period. We presented rats with a tone during the first 15 seconds. In the next 15 seconds, the tone was combined with a shock. The next 30 seconds served as a rest period. This 1-minute trial was repeated 10 times in succession, resulting in one session of 10 minutes. The same protocol was repeated on day 1 and 2. Control rats followed the same routine with tone emission, but without experiencing any shocks. On day 3, neither group received shocks or injections; otherwise the animals follow the same protocol. Freezing behaviour was observed and recorded for 10 minutes during the testing session on day 3.*

All shock sessions took place in the mornings. One shock trial consisted of a 30 second period, which was followed by 30 seconds without tone or shock. One shock session, consisting of 10 trials, lasted for 10 minutes. We presented rats with a tone during the first 15 seconds of each trial. During the next 15 seconds of each trial, the tone was paired with the shock. Control (NFC) rats followed the same routine, but without experiencing any shocks. On day 3, no groups received shocks or injections. This was done to avoid measuring behavioural outputs due to direct drug interference or pain stimuli. Behavioural measures were noted during the test session (10 minutes) on day 3. This was done in order to establish fear-conditioned freezing behaviour associated with the tone.

2.4 Behavioural observation

Behaviours were recorded for each rat by means of a video camera (Philips Explorer Camcorder) directly after the last test session. They were then subsequently analyzed with the aid of the computer program, The Observer (Noldus version 3.0, The Netherlands). An independent observer unaware of experimental conditions noted freezing frequency (the number of freezing events in one session) and total duration of time spent freezing expressed as a percentage. Freezing was denoted as an action absent of any movement, except that needed for respiration and whisker twitching. Freezing behaviour, as well as being a behavioural expression of stress, is also the main determinant of fear conditioning having being achieved (Bolles and Collier, 1976; Holahan and White, 2002).

2.5 Tissue collection and punching technique

Fifteen minutes after the test session (day 3), rats were anaesthetised with 5% isoflurane and decapitated; brains were quickly removed and frozen at -80℃. Serial 300 µm coronal sections were made with a cryostat microtome (-15℃) and frozen on dry ice. Tissue samples were dissected from the anterior cingulate, nucleus accumbens, paraventricular nucleus, central and basolateral amygdala nuclei, dentate gyrus, dorsal raphe and locus coeruleus (Fig. 2). The Swanson (1992) co-ordinates are given in Table 1.

Table 1: Brain areas: Swanson (1992) rostral-caudal stereotaxic co-ordinates

Area	Mm from Bregma
Anterior cingulate	+2.80 to +2.15
Basolateral nucleus of amygdala	-2.45 to –2.85
Central nucleus of amygdala	-2.45 to –2.85
Dentate gyrus	-2.45 to –2.85
Dorsal raphe	-7.10 to –8.60
Locus coeruleus	-9.60 to –10.10
Nucleus accumbens	+2.80 to +2.15
Paraventricular nucleus	-1.53 to –2.00

The dissections were made by using a needle punch technique on frozen coronal sections. Three different needle diameters were used in accordance with the size of the area to be punched. Larger areas, such as the anterior cingulate, nucleus accumbens, paraventricular nucleus and dorsal raphe, were punched with a 16G needle (1.6 x 40mm; Sterican, B. Braun, Germany; one punch ≈ 0.23 mm^3), while the dentate gyrus, locus coeruleus (18G: 1.2 x 38mm; Sterican, B. Braun, Germany; one punch ≈ 0.19 mm^3) and the amygdala nuclei (20G: 0.9 x 40mm; Sterican, B. Braun, Germany; one punch ≈ 0.08 mm^3) were punched with smaller diameter needles. Approximately two punches were taken per side, per animal. Tissue was homogenised in 100ml (0.1 M) perchloric acid and then the suspension was centrifuged (13,500 rpm) for 10 minutes. The supernatant was stored at -80℃ until further analysis.

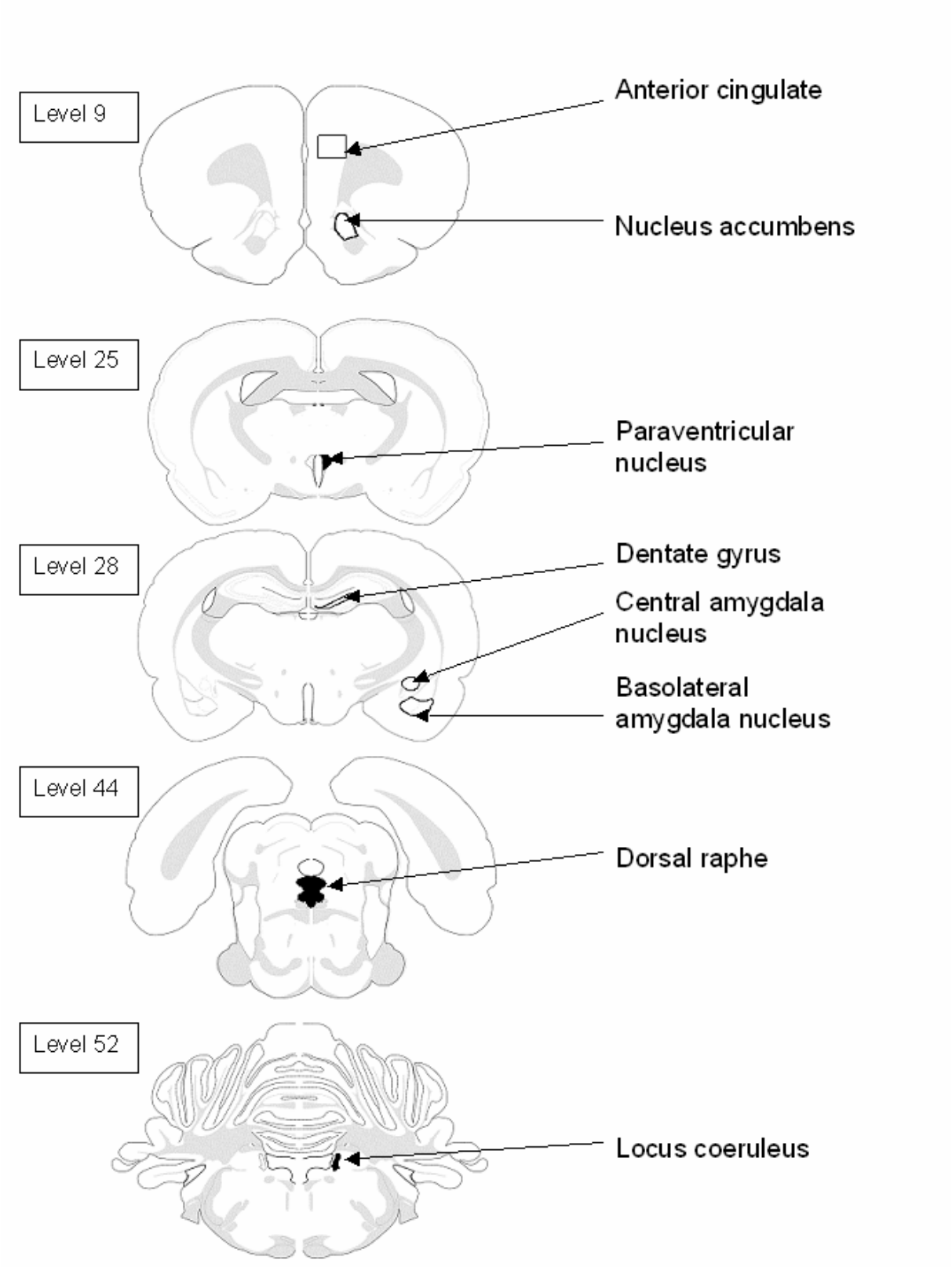

***Figure 2**: Pictorial representations of the brain areas removed for analysis of glutamate and dopamine. Delineated areas depict actual areas measured. Brain slice levels were taken from the Swanson rat brain atlas (1992), with appropriate co-ordinates listed in Table 1.*

2.6 Dopamine and glutamate analysis

Analysis of dopamine was performed by a Shimadzu LC-10 AD high performance liquid chromatograph equipped with a 15-cm reversed phase column (supelcosil 3 µm, C18, 150´4.60 mm, Bester, Amstelveen, The Netherlands) and an electrochemical detector (ESA, Chelmsford, MA,USA) at a potential setting of 300 mV. The mobile phase consisted of 10% methanol, 4.2 g sodium acetate/l, 150 mg octane sulphonic acid/l adjusted to pH 4.10. The injection volume was 20ml and the flow rate 1ml/min.

Analysis of glutamate was performed after derivatisation with ortho-phtaldehyde by a Shimadzu LC-10 AD high performance liquid chromatograph equipped with a 15-cm reversed phase column (supelcosil 3 µm, C18, 150´ 4.60 mm, Bester, Amstelveen, The Netherlands) and a fluorescence detector (Waters 470, fluorescence detection, Waters, Milford, Massachusetts, USA) with extinction and emission wavelengths set at 350 nm and 450 nm, respectively. The mobile phase consisted of 26% methanol, 10 g/l disodiumhydrogenphosphate (Na_2HPO_4), 150 mg/l EDTA and 2.19 ml/l tetrahydrofuran, and adjusted to pH 5.27. The injection volume was 20ml and the flow rate 1ml/min.

2.7 Statistics

The overall group effect was assessed via one-way analysis of variance (glutamate, dopamine) or the Kruskall-Wallis test for non-parametric data (behaviour) using SPSS (Version 12). If significant group effects were noted ($p<0.05$), pairwise parametric post-hoc comparisons (glutamate, dopamine) and Mann-Whitney U (behaviour) tests were performed, with significance determined at the $p<0.05$ level.

3 Results

3.1 Behaviour

The Kruskall-Wallis test revealed an overall group effect for the total duration of freezing behaviour ($\chi^2_{4, 24} = 23.84$; $p < 0.0001$; Fig. 3a) and frequency of freezing behaviour ($\chi^2_{4, 24} = 17.35$; $p = 0.002$; Fig. 3b). Mann-Whitney-U post hoc tests showed a significant increase in total duration spent freezing ($p = 0.004$) and an increase in frequency of freezing ($p = 0.004$) in the fear conditioned (FC) group relative to the no fear conditioned group (NFC).

Ketamine significantly blocked the effects of fear conditioning (FC + Ket versus FC) with respect to total duration (p = 0.002) and frequency of freezing behaviour (p = 0.004). Clozapine, however, was not able to restore fear conditioning, as assessed by the comparison between FC + Ket + Cloz and FC + Ket groups for either measure of freezing behaviour (duration: p = 0.240; frequency: p = 0.310). Clozapine, by itself, decreased freezing frequency (p = 0.009) but not total freezing duration (FC + Cloz versus FC).

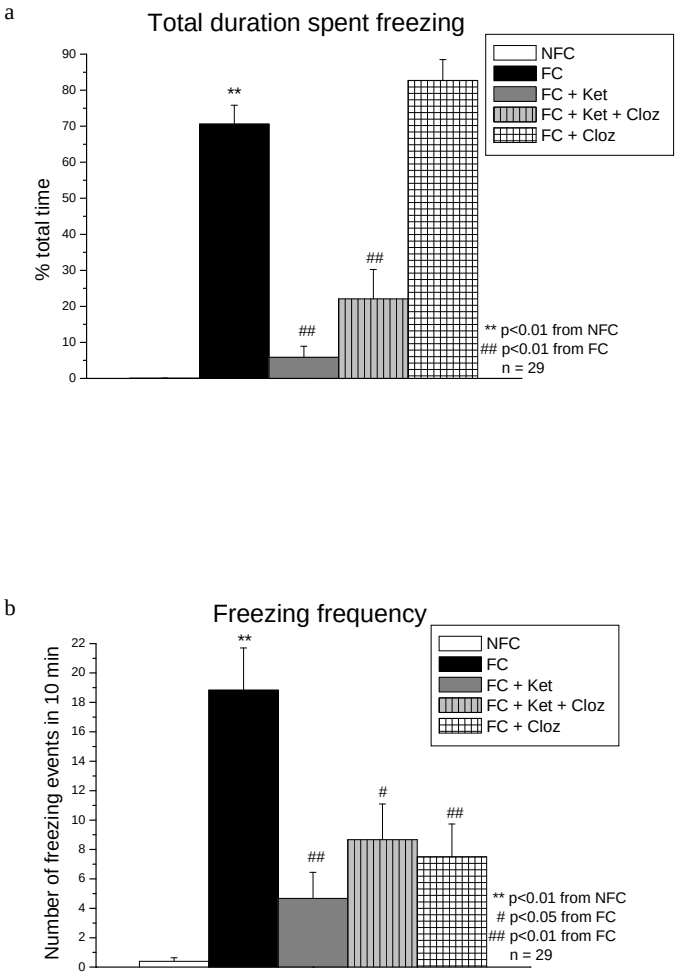

Figure 3*: Effect of ketamine and clozapine (separately and in combination) on conditioning-induced freezing behaviour. Bars represent means ± SEM. Fear conditioning increased (a) total freezing duration and (b) freezing frequency as compared to the NFC group. Ketamine blocked this effect (FC versus FC + Ket) in terms of the total duration and freezing frequency. Clozapine alone (FC versus FC + Cloz) reduced (b) freezing frequency. The FC + Ket + Cloz group were also not statistically different from the FC + Ket groups in terms of freezing behaviour. Cloz = clozapine, FC = fear conditioning; NFC = no fear conditioning, Ket = ketamine.*

3.2 Glutamate

Overall group effects were noted in all areas: anterior cingulate ($F_{4, 48} = 6.46$; $p < 0.0001$); nucleus accumbens ($F_{4, 49} = 9.29$; $p < 0.0001$), paraventricular nucleus ($F_{4, 22} = 6.29$; $p = 0.002$), central amygdala nucleus ($F_{4, 43} = 20.91$; $p < 0.0001$), basolateral amygdala nucleus ($F_{4, 48} = 8.91$; $p < 0.0001$), dentate gyrus ($F_{4, 49} = 18.20$; $p < 0.0001$), dorsal raphe ($F_{4, 23} = 94.85$; $p = 0.006$), and locus coeruleus ($F_{4, 48} = 14.45$; $p < 0.0001$).

Pairwise comparisons (least significant differences; LSD) reveal increased glutamate levels in the FC group compared to the NFC group in all areas except the anterior cingulate ($p = 0.099$; Fig. 4a), i.e. nucleus accumbens ($p < 0.0001$; Fig. 4a), paraventricular nucleus ($p = 0.012$; Fig. 4a), dentate gyrus ($p < 0.0001$; Fig. 4b), dorsal raphe ($p = 0.003$; Fig. 4b), and locus coeruleus ($p < 0.0001$; Fig. 4b). Interestingly, highly significant increases in glutamate were noted in the central amygdala nucleus ($p < 0.0001$) and basolateral nucleus ($p < 0.0001$), two areas critical to fear conditioning (Fig. 4c). Also as hypothesised, ketamine significantly reduced this effect in these two areas, the central amygdala ($p < 0.0001$) and the basolateral amygdala ($p = 0.041$), in addition to the locus coeruleus ($p = 0.008$), as revealed by the comparison between FC + Ket and FC groups.

Clozapine fully restored normal fear conditioned-induced glutamate levels in the basolateral amygdala ($p = 0.001$) and locus coeruleus ($p = 0.017$), and partially reversed the effects of ketamine in the central amygdala ($p = 0.088$), as ascertained by the comparison between FC + Ket + Cloz and FC + Ket groups. Interestingly, clozapine in combination with fear conditioning (FC + Cloz) decreased glutamate levels in the central amygdala ($p < 0.0001$) and locus coeruleus ($p = 0.007$) when compared to the FC only group, even though when ketamine was also administered (FC + Ket + Cloz) an increase in glutamate tissue content occurred in the locus coeruleus. A similar, but non-significant pattern is noted in the paraventricular nucleus, with ketamine decreasing glutamate levels induced by fear conditioning ($p = 0.478$), and clozapine restoring it significantly ($p = 0.008$). The dorsal raphe also shows a similar pattern.

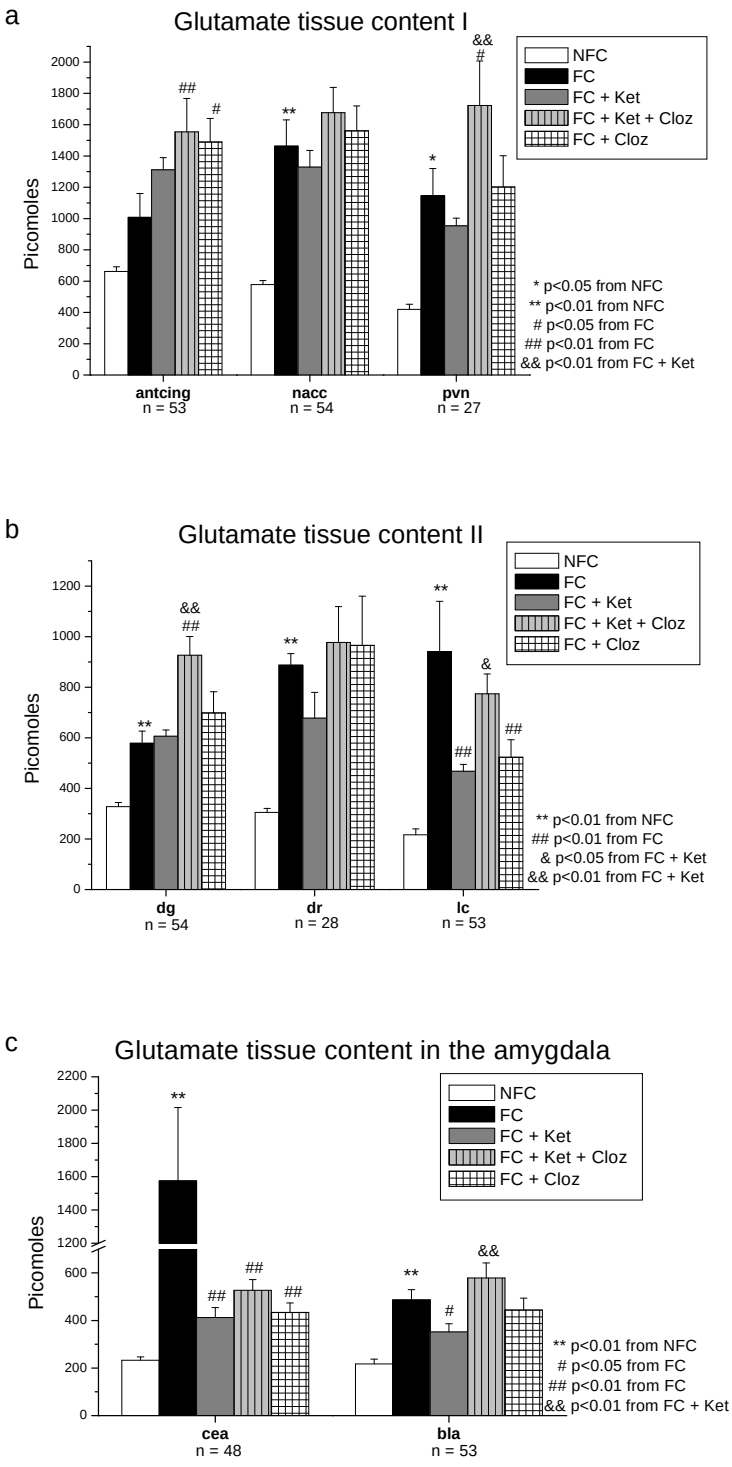

Figure 4*: Effect of drug treatments on tissue glutamate content following fear conditioning. Bars represent means ±SEM. The FC group showed increased glutamate levels in all areas except the anterior cingulate, as compared to the NFC group. Ketamine significantly reversed this effect in the central amygdala (c), basolateral amygdala (c) and the locus coeruleus (b), as revealed by the comparison between FC + Ket and FC groups. Clozapine, in turn, blocked the actions of ketamine on glutamate levels (FC + Ket versus FC + Ket + Cloz) in the central amygdala nucleus, with full restoration of normal fear conditioned-induced glutamate levels in the basolateral amygdala. Clozapine alone (FC + Cloz versus FC) decreased glutamate levels in the central amygdala, and locus coeruleus.*
antcing = anterior cingulate; bla = basolateral amygdala nucleus; cea = central amygdala nucleus; Cloz = clozapine; dg = dentate gyrus; dr = dorsal raphe; FC = fear conditioning; Ket = ketamine; lc = locus coeruleus; nacc = nucleus accumbens; NFC = no fear conditioning; pvn = paraventricular nucleus.

3.3 Dopamine

Overall group analysis shows significant changes of dopamine content in the anterior cingulate ($F_{4, 40} = 4.18$; $p = 0.006$), nucleus accumbens ($F_{4, 32} = 15.33$; $p < 0.0001$), paraventricular nucleus ($F_{4, 17} = 3.15$; $p = 0.041$), central amygdala ($F_{4, 36} = 7.09$; $p < 0.0001$), dentate gyrus ($F_{4, 17} = 5.92$; $p = 0.004$), dorsal raphe ($F_{4, 18} = 3.82$; $p = 0.02$) and locus coeruleus ($F_{4, 41} = 5.79$; $p = 0.001$). No significance differences were noted in the basolateral amygdala in terms of dopamine content ($F_{4, 40} = 7.63$; $p = 0.556$).

Comparison between FC and NFC groups revealed alterations in dopamine content due to fear conditioning. That included a decrease in dopamine content in the nucleus accumbens ($p = 0.005$; Fig. 5a) and an increase in the locus coeruleus ($p = 0.006$; Fig. 5c). A trend towards increased dopamine content in the anterior cingulate ($p = 0.056$; Fig. 5c) is also noted. Ketamine was also able to abolish this fear conditioning (FC + Ket vs. FC groups) response in the nucleus accumbens ($p < 0.0001$), while showing a trend of augmenting the response of fear conditioning in the anterior cingulate ($p = 0.053$) and locus coeruleus ($p = 0.103$). As hypothesised, clozapine counteracted the effect of ketamine in these areas (FC + Ket + Cloz vs. FC + Ket groups): anterior cingulate ($p = 0.044$), nucleus accumbens ($p = 0.011$), and locus coeruleus ($p = 0.008$). Ketamine in combination with clozapine (FC + Ket + Cloz; $p = 0.001$) or alone (FC + Ket; $p = 0.002$) increased dopamine levels in the central amygdala as compared to the FC only group (Fig. 5b). A similar pattern of results was also noted in the dorsal raphe (Fig. 5c), though not significant. Clozapine alone also showed effects (FC + Cloz), increasing dopamine content in the nucleus accumbens ($p < 0.0001$) and paraventricular nucleus ($p = 0.049$; Fig. 5c) as compared to the FC group. Not enough data was available to perform post hoc tests on the dentate gyrus.

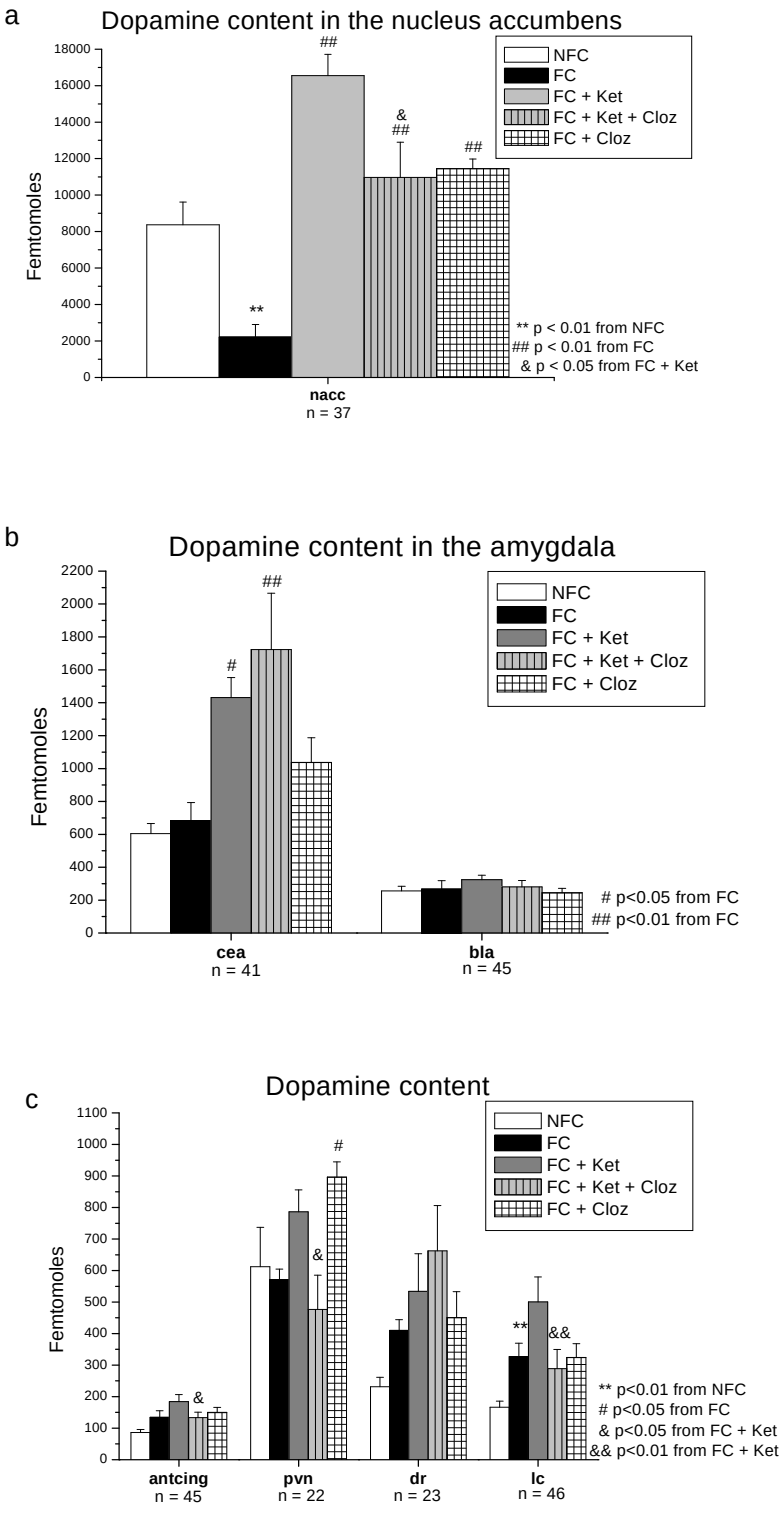

Figure 5*: Effect of drug treatments on tissue dopamine content following fear conditioning. Differences between the FC and NFC groups were manifested as a decrease in dopamine content in the nucleus accumbens (a) and an increase in the locus coeruleus (c), with a trend at increasing the dopamine content in the anterior cingulate (c). Ketamine abolished this fear conditioning response in the nucleus accumbens, while showing a trend at augmenting the response of fear conditioning in the anterior cingulate and locus coeruleus. Clozapine counteracted the effect of ketamine in the anterior cingulate, nucleus accumbens, and locus coeruleus. Clozapine alone (no ketamine) also showed effects (FC + Cloz), and increased dopamine content in the nucleus accumbens and paraventricular nucleus (c) as compared to the FC group. Ketamine in combination with clozapine (FC + Ket + Cloz; or alone (FC + Ket) increased dopamine levels in the central amygdala (b) as compared to the FC only group. Abbreviations as in previous figures.*

4 Discussion

The central aim of this study was to investigate the construct validity of a putative animal model of negative schizophrenic symptoms (Pietersen et al., 2006). The model involves compromising the functional integrity of fear conditioning circuits through ketamine administration in order to simulate impairments in aversive conditioning seen in schizophrenia (Kosmidis et al., 1999; Hofer et al., 2001). Here we investigated the more specific hypothesis that ketamine interferes with fear-conditioning-induced changes in glutamate and dopamine tissue levels in brain areas associated with fear learning. We further hypothesized that the behavioural and neurochemical effects of ketamine could be reversed by the atypical antipsychotic clozapine.

4.1 Fear conditioning affects behaviour and neurochemistry

Fear conditioning significantly increased freeze frequency and total freezing duration (FC group vs. NFC group), indicating that animals were fear conditioned (Fig. 3a, b). These behavioural changes were accompanied by increased glutamate tissue content in critical areas making up the fear circuit, especially in the central and basolateral amygdala (Fig. 4c). As these observations were made in the absence of the aversive stimulus, these data suggest that increased glutamate content is necessary for retrieval and expression of the conditioned stimulus – unconditioned stimulus (CS-US) association. Interestingly, fear conditioning did not significantly alter glutamate content in the anterior cingulate (Fig. 4a). Previous studies (Lei et al., 2004; Tang et al., 2005) have shown that glutamate receptors in the anterior cingulate are directly involved with pain stimulation, however. As no shock was administered during the test trials (on the third day) in our study, no pain would have been experienced. This may account for the lack of glutamate activation in this area.

In contrast to our results, Yokoyama et al. (2005) showed that glutamate release was enhanced in the amygdala (relative to controls) during fear learning but not during extinction trials. In our study, samples of neurotransmitter levels were obtained directly following the test session, implying that glutamate levels were elevated during extinction trials. The tissue destruction method used in our study also includes glutamate from surrounding glial cells and stored neurotransmitter, whereas the

microdialysis study of Yokoyama et al. (2005) measured only extracellular glutamate levels. As different sampling methods (tissue in our study vs. microdialysis) and shock intensities (1.5mA in our study vs. 0.8mA) were used in these studies, it makes it difficult to compare results.

The Yokoyama et al. (2005) study also shows that more dopamine is released in the amygdala during acquisition than during recall of the fearful event. We find no significant effects of fear conditioning on dopamine content in the amygdala following extinction trials (Fig. 5b). Interestingly, Suzuki et al. (2002) have shown that dopamine release in the amygdala changes significantly only 40 minutes following fear-conditioned stress, whereas we sacrificed the animals after 15 minutes. This could account for the lack of effects of fear conditioning on dopamine levels in the amygdala in our study. We did, however, find fear-conditioning effects on dopamine levels in the locus coeruleus (Fig. 5c), a main effector nucleus of the central amygdala, suggesting that the level of fear conditioning was sufficient to generate measurable effects on dopamine levels in our study.

Fear conditioning also decreased dopamine content in the nucleus accumbens in our study (Fig. 5a). It has previously been postulated that dopamine in the nucleus accumbens acts during acquisition and expression of aversively conditioned responses, by signalling the predictability of a US by a CS (Pezze and Feldon, 2004). Studies have shown that footshock (Inoue et al., 1992; Kalivas and Duffy, 1995) and anticipation of a footshock by the presentation of the CS alone (Young et al., 1993; Wilkinson et al., 1998) induces a rise of dopamine metabolites or increased dopamine release in the nucleus accumbens. Another study has shown that during the expression of conditioned fear, dopamine response to the CS was decreased in the shell, but increased in the core (Pezze et al., 2002). We suggest that the decreased nucleus accumbens dopamine content observed in our study is more indicative of the activity of the shell than the core.

4.2 Ketamine blocks indicators of fear conditioning

As indicated previously, we had expected that ketamine would block fear conditioning (FC + Ket group vs. FC group), particularly in the amygdala, through interference with NMDA receptors and dopamine function. Consistent with this hypothesis, and in

addition to the behavioural blockage of fear conditioning (Fig. 3a, b), we found that ketamine reversed fear-induced glutamate increases in the central amygdala, basolateral amygdala (Fig. 4c) and locus coeruleus (Fig. 4b), as well as reversing fear-induced glutamate decreases in the nucleus accumbens (Fig. 4a), thereby significantly interfering with fear processing. Although no effect of fear conditioning on dopamine content was found in the central amygdala (Fig. 5b), dopamine levels were increased by ketamine administration, an effect that was not reversed by clozapine (FC + Ket vs. FC + Ket + Cloz groups). This result is consistent with other studies that have indicated effects of ketamine on the dopamine system (French and Ceci, 1990; Dai et al., 1995), and may help to explain why we did not find a strong reversal effect of clozapine on behaviour even though the drug had a clear reversal effect on glutamate levels in the basolateral amygdala (Section: ‘A conceptual model to reconcile the behavioural and neurochemical data’).

4.3 Clozapine reverses ketamine’s blockade on fear processing

In agreement with our hypothesis, we found a significant reversal effect of clozapine (FC + Ket + Cloz group vs. FC + Ket group) on ketamine in tissue glutamate content in the basolateral amygdala (Fig. 4c) and locus coeruleus (Fig. 4b). A similar pattern was found in the paraventricular nucleus (Fig. 4a), although no significant effect of ketamine was noted here. Clozapine also counteracted the effect of ketamine on dopamine content in the locus coeruleus (Fig. 5c) and nucleus accumbens (Fig. 5a). Interestingly, consistent with our previous study of cFos expression (Pietersen et al., unpublished data) clozapine did not restore glutamate content in the central amygdala (Fig. 4c), the main output region of the amygdala. In terms of dopamine content, clozapine did not reverse the effect of ketamine in this area (Fig. 5b). We propose below that the lack of restoration of glutamate content in the central amygdala is responsible for clozapine’s failure to restore freezing behaviour.

4.4 The effects of clozapine in the absence of ketamine

An interesting discrepancy between behavioural and neurochemical data concerns the effects of clozapine alone (FC + Cloz group vs. FC group). On the behavioural level, clozapine administered in the absence of ketamine did not alter the affect of fear conditioning on total freezing duration, although freezing frequency was decreased (Fig. 3). The decrease in freezing frequency implies that rats froze less

often with clozapine but for longer periods at a time. We thus conclude that clozapine alone did not clearly abolish fear conditioning at the behavioural level.

On the neurotransmitter level, however, clozapine alone had an effect similar to ketamine alone. Glutamate levels induced by fear conditioning were significantly suppressed by clozapine in the central amygdala and locus coeruleus, with a trend in the basolateral amygdala (Fig. 4). These results are consistent with findings showing that clozapine directly suppresses the glutamate system—suggestive of an anxiolytic effect (Rex et al., 1998; Sharma, 2003)—and that clozapine is the most potent of the antipsychotic agents in blocking NMDA receptor antagonist-induced neurotoxicity (Farber et al., 1993; Olney and Farber, 1994).

Given the similar neurochemical effects of ketamine and clozapine, and given that ketamine alone had a powerful effect on freezing behaviour, it seems puzzling that clozapine did not also have a potent effect on behaviour. It is also puzzling to consider why the neurochemical effects of ketamine and clozapine administration do not predict their joint effects when administered together. Since ketamine and clozapine both decrease glutamate levels, we might expect that ketamine and clozapine together should produce even further decreases in glutamate levels. Yet in the basolateral amygdala and the locus coeruleus, ketamine and clozapine administered together led to relatively increased glutamate levels, comparable to the FC group. As clozapine also affects several neurotransmitter systems (Duncan et al., 1998; Johnson et al., 2005; Ma et al., 2006) including glutamate, its reversal of ketamine's effect on glutamate could also be due to its influence on other neurotransmitters in other brain regions.

4.5 A conceptual model to reconcile behavioural and neurochemical data

The model we propose is closely related to concepts introduced in Aleman and Kahn (2005) and is broadly consistent with the known anatomical and functional organization of the amygdala (Cardinal et al., 2002; Marowsky et al., 2005; Pape, 2005). We recognize that this model does not take into account the potentially important roles of regions like the nucleus accumbens in eliciting freezing behaviour.

The model may nevertheless serve as a useful basis for designing further experiments.

Aleman and Kahn (2005) speculated that positive and negative schizophrenic symptoms arise from a combination of functional lesions of the basolateral amygdala and elevated dopamine levels in the amygdala (Reynolds, 1983). Based on the results of this study and others (see references below), we adapt their conceptual model to the current context in four key ways (Fig. 6). First, we propose that glutamate-mediated fear conditioning in the basolateral amygdala drives freezing behaviour through the output nuclei of the central amygdala (Fanselow and Kim, 1994; Killcross et al., 1997; LeDoux, 1998; Koo et al., 2004). Second, we hypothesize that dopamine-modulated γ-aminobutyric acid (GABA) inhibition in the central amygdala itself modifies the outputs of the basolateral amygdala (Pare et al., 2003). Third, we interpret ketamine administration in terms of a glutamate-mediated deficit in fear conditioning in the basolateral amygdala (Monaghan and Cotman, 1985; Miserendino et al., 1990; Walker and Davis, 2002; Savonenko et al., 2003). Lastly, we propose that elevated dopamine levels in the central amygdala lead to increased GABA inhibition of the outputs of the basolateral amygdala, leading eventually to decreased freezing behaviour. It has recently been shown that ketamine also has a high affinity for dopamine D_2 receptors and may act as an agonist at this receptor (Kapur and Seeman, 2002). This scheme is also generally consistent with the finding that intercalated cells positioned between the basolateral and central amygdala (Millhouse, 1986; Cassell et al., 1999) generate feedforward GABA-mediated inhibition on central amygdala neurons (Royer et al., 1999; Pare et al., 2003). The increased dopamine in the amygdala could originate from the neurons in the ventral tegmental area, as these have been found to participate in aversive conditioning (Lamont and Kokkinidis, 1998).

The model can explain the major features of our data as follows. Fear conditioning elevates glutamate levels in the basolateral amygdala (Fig. 4c) but does not affect dopamine levels in either the basolateral or central amygdala (Fig. 5b). Output signals inducing freezing behaviour from the central amygdala are therefore strong. Ketamine decreases glutamate-related fear processing in the basolateral and central amygdala and simultaneously elevates dopamine levels in the central amygdala. The

net effect is weak output signals from the central amygdala and diminished freezing behaviour. The administration of clozapine alone (FC + Cloz group) had no statistically significant effects on glutamate levels in the basolateral amygdala or dopamine levels in the central amygdala. These data are consistent with the normal freezing behaviour (total freezing duration) following clozapine administration, since the putative circuit remained functionally intact.

The most important aspect of this conceptual model is how ketamine and clozapine act together. Our data shows that clozapine reverses the effect of ketamine on glutamate levels in the basolateral amygdala. This alone would predict normal freezing behaviour. However, we also find that clozapine does nothing to restore normal dopamine levels in the central amygdala, despite it being a dopamine D_2 antagonist. It is this elevated dopamine in the central amygdala which we propose blocks the fear-related outputs of the basolateral amygdala, by means of increased GABA inhibition, from generating high levels of freezing following combined ketamine and clozapine administration.

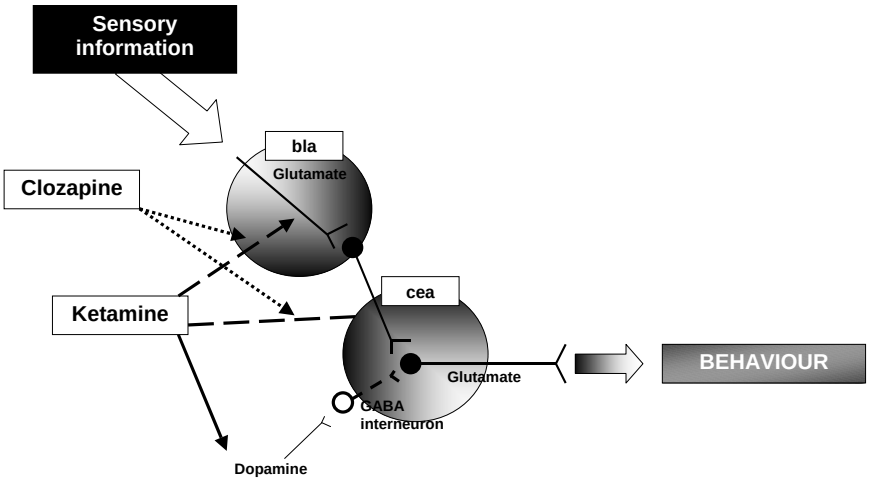

Figure 6*: Conceptual model. A schematic drawing of our conceptual model depicting interactions between dopamine and glutamate in the amygdala nuclei. Dashed lines symbolise inhibition, while solid lines represent stimulation. Sensory information (fear stimuli) is processed first by the basolateral amygdala (bla), activating the glutamate system in this area, but does not affect dopamine levels in either the basolateral or central amygdala (cea). Output signals inducing freezing behaviour from the central amygdala are therefore strong via the glutamate pathway. Ketamine decreases glutamate-related fear processing in the basolateral and central amygdala and simultaneously elevates dopamine levels in the central amygdala. The elevated dopamine in the central amygdala (together with the block of glutamate transmission from the bla to the cea) blocks the fear-related outputs by means of increased GABA inhibition. The net effect is weak output signals from the central amygdala and diminished freezing behaviour. Clozapine, while blocking the effects of ketamine on glutamate-related processing in the bla, does nothing to renormalize dopamine levels in the cea. Dopamine therefore continues to inhibit bla though-put by means of GABA, leading to normal freezing behaviour. Can chronic clozapine treatment renormalize dopamine levels and lead to long-term remediation of negative symptoms in the animal model?*

4.6 Possible modifications and extensions to the animal model

It seems plausible, in light of our conceptual model, that finding behavioural evidence for a reversal effect of clozapine on ketamine may require a chronic paradigm. Indeed, it is well known in the clinical situation that antipsychotics take a considerable period of time (up to 6 weeks) to start resolving symptoms. Enomoto et al. (2005) treated mice for 14 days with phencyclidine (PCP), an NMDA antagonist similar to ketamine, leading to impaired fear conditioning. Repeated chronic administration of olanzapine, but not haloperidol, for seven days reversed the impairment caused by PCP (Enomoto et al., 2005). A study by Sams-Dodd (1996) also showed that chronic clozapine treatment inhibited PCP-induced stereotypical behaviour and social isolation. We speculate that chronic clozapine administration may lead to normal freezing behaviour by renormalizing elevated dopamine levels in the central amygdala.

A modification of our paradigm into the chronic domain may also prove valuable in the modelling of other psychiatric disorders involving abnormal fear processing, such as anxiety disorders (Doronbekov et al., 2005; Swanson et al., 2005). For example, dysregulation of central monoamine systems and abnormal amygdala function have both been proposed to play a role in the development of post-traumatic stress disorder (Charney et al., 1993; Goldstein et al., 1994).

5 Conclusions

In summary, ketamine abolished conditioning-induced increases in glutamate levels in the amygdala, an area known to mediate fear learning. Clozapine administration reversed this effect in the basolateral amygdala, even though it did not reliably restore fearful behaviour. We present a conceptual model involving antagonistic effects of clozapine on dopamine and glutamate to reconcile this apparent discrepancy. The model suggests that chronic clozapine administration may renormalize dopamine levels in the central amygdala, thereby leading to normal expression of fearful behaviour.

6 Acknowledgements

We would like to thank Sandoz Pharma, Basel for the donation of clozapine.

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Chapter 6

Discussion

1 Summary of results

The main aim of this thesis was to investigate whether associative learning (fear conditioning) was disrupted by a general hypoglutamatergic state. We also investigated whether this paradigm could be used as an animal model of emotional blunting, as seen in schizophrenia. In the Introduction (Chapter 1), we discussed the cognitive-emotional theory according to Grossberg (2000) that describes the negative syndrome of schizophrenia. According to this theory, deficits in brain regions involved in emotion and motivational learning can lead to problems in attention. Cardinal (2002) implicated the basolateral amygdala (BLA) and anterior cingulate (ACC) as key areas involved in this process. One way of invoking these areas is through fear conditioning, which involves basic associative learning and memory processes, in addition to eliciting emotional states. We therefore investigated whether a disruption of fear conditioning in these brain areas could be indicative of emotional blunting.

Aleman and Kahn (2005) proposed that prolonged activation of the amygdala during psychotic states in the onset stages of schizophrenia could lead to glutamate excitoxicity. This would eventually result in amygdala lesions and long-term glutamate hypofunctioning (see also Heresco-Levy, 2003), thereby disrupting a primary brain area in the fear circuit. An example of simulating this glutamatergic hypofunctioning state is by administering an NMDA receptor antagonist. We therefore combined the hypotheses of Aleman-Kahn and Grossberg by inducing a hypoglutamatergic state in rats through ketamine administration and disrupting fear conditioning at a neurochemical level.

1.1 Fear conditioning

Firstly, a fear-conditioning paradigm that effectively elicited fear in rats in the absence of painful stimuli was established. This switched the focus from pain to emotion through anticipation. It was successfully achieved, as outlined in Chapter 2. Higher shock intensity gave less variable results and required fewer animals and was therefore used throughout the study. As indicated in Chapters 3-5, our fear conditioning paradigm also led to increases in 1) fear conditioned freezing behaviour, 2) cFos expression, indicative of increased neural activity and synaptic plasticity, and 3) increased glutamate tissue content in primary brain regions, including the

amygdala nuclei. Dopamine content was not affected by fear conditioning in most of the brain areas analysed, with the exception of the locus coeruleus (increased; LC) and the nucleus accumbens (decreased; Nacc). In addition, the nucleus accumbens also showed an increase in dopamine turnover (unpublished results, see Table 1).

1.2 Ketamine

The next stage was to see whether we could block these indicators of fear conditioning by inducing an overall hypoglutamatergic state through the administration of ketamine. Ketamine was chosen as it is an NMDA antagonist and it has recently been shown that fear conditioning via the amygdala acts primarily through glutamate NMDA receptors. Ketamine also exacerbates schizophrenic symptoms, including cognitive and negative symptoms, suggesting that the animal model may be suitable for studying schizophrenia.

Ketamine successfully disrupted fear conditioning, both behaviourally and in neural correlates (Chapters 3, 4). Freezing behaviour decreased almost to control levels, indicating that fear conditioning was not attained in rats receiving ketamine. Importantly, this behavioural abolishment of fear conditioning was also reflected in the BLA and ACC, through reduced cFos expression, consistent with the theory of Grossberg (2000) and Cardinal (2002). Glutamate tissue content was also attenuated down to control (no fear conditioned) levels in the amygdala nuclei, although this was not found to be the case for the ACC (Chapter 5). Dopamine content was increased by ketamine administration in the central amygdala nucleus (CEA) and the Nacc (Chapter 5).

1.3 Antipsychotics

In order to apply this model to emotional blunting as seen in those suffering with schizophrenia, we administered both an atypical and a typical antipsychotic, in addition to a metabotropic glutamate 2/3-receptor ($mGlu_{2/3}$) agonist, LY 379268 (Chapter 4). As an atypical antipsychotic, clozapine is considered to be optimal in ameliorating negative symptoms, while the typical antipsychotic, haloperidol, mostly reverses positive symptoms. Clozapine also remains the standard treatment in resistant schizophrenia today. Clinically, clozapine reduces the increases in symptoms in schizophrenics induced by ketamine (Malhotra et al., 1997a, b).

Behavioural effects in rats induced with PCP, a similar glutamate NMDA receptor antagonist, were also reversed with long-term clozapine treatment. Other animal studies have also indicated that clozapine is successful in blocking metabolic effects induced by ketamine (Duncan et al., 1998), consistent with its action on NMDA receptors. In the same experiment, haloperidol (a preferential dopamine D_2 receptor antagonist) was not able to do so. We therefore hypothesized that clozapine would be capable of reversing the effects of ketamine, while haloperidol would not. Confirmation of these hypotheses would reinforce the specificity of our model for negative symptoms, including emotional blunting.

LY 379268, an $mGlu_{2/3}$ agonist, is presently being tested for its involvement in fear learning (Walker and Davis, 2002). It is currently unclear whether LY 379268 can affect conditional fear processing in the rat. A recent paper, however, does suggest that agonists of this receptor possess anxiolytic properties (Swanson et al., 2005). The $mGlu_2$-receptor is primarily located pre-synaptically in forebrain regions, and subsequently LY 379268 has been shown to decrease glutamate release in these areas, in line with a function as an autoreceptor (Moghaddam and Adams, 1998). As the $mGlu_3$ receptor is also located post-synaptically, interaction with ketamine is more likely (Swanson et al., 2005). We therefore postulated that LY 379268 would elicit an effect on ketamine's actions in forebrain areas, possibly through interference at the post-synaptic receptor.

We hypothesized that clozapine would have the best outcome in reversing ketamine's disruption of fear conditioning. However, this was not reflected in the behavioural data (Chapter 3-5). The results of the neural correlates, however, paint a different picture. cFos expression was entirely normalized to fear conditioning levels through clozapine administration in key brain areas regulating fear processing. This included the ACC, Nacc, anterior BLA and lateral amygdala. With the exception of the ACC, no restorative effects were noted with either haloperidol or LY 379268. Glutamate tissue content levels were also restored with clozapine administration in the LC and BLA. Dopamine content was also brought back to fear conditioning levels in the LC and Nacc. Taken together, clozapine appears to reverse the disruption of fear processing by ketamine in several key brain areas. Why then, is this not

reflected in the behavioural data? In order to reconcile the neural and behavioural data, a neurochemically-based conceptual model was proposed in Chapter 5.

2 Nederlandse samenvatting

In het proefschrift staat de vraag centraal of het leren en verwerken van emoties zoals angst nog plaats kan vinden bij een (sterk) verminderde functie van het glutamaterge neurotransmitter systeem in de hersenen. Het beantwoorden van deze vraag is van belang voor de ontwikkeling van een diermodel voor de negatieve symptomen van schizofrenie, zoals bijvoorbeeld emotionele vlakheid.

Een belangrijke basis voor het onderzoek is gelegd door Grossberg, die in zijn emotionele-cognitieve theorie de negatieve symptomen van schizofrenie beschrijft (Inleiding, hoofdstuk 1). Volgens deze theorie leiden dysfuncties in hersengebieden die betrokken zijn bij het leren van emoties tot aandachtsproblemen. Er zijn aanwijzingen dat de basolaterale kern van de amygdala (BLA) en de anterior cingulate (ACC) hierbij een cruciale rol spelen. Deze hersengebieden spelen ook een belangrijke rol bij processen die essentieel zijn het conditioneren van angst (fear conditioning), zoals associatief leren, geheugen en het opwekken van emoties. Onze hypothese is dat verstoring van deze processen in de BLA en ACC een belangrijke factor is bij het ontstaan van negatieve symptomen bij schizofrenie.

Een andere pijler voor het onderzoek is de hypothese van Aleman en Kahn, die er op neer komt dat tijdens psychotische aanvallen in de beginfase van schizofrenie de amygdala voortdurend wordt geactiveerd.

Dit zou leiden tot glutamaat neurotoxiciteit, met als gevolg lesies van de amygdala en een voortdurende glutamaterge hypofunctie van dit centrale hersengebied in het angstcircuit. In ons onderzoek hebben we de hypothesen van Grossberg en Aleman&Kahn gecombineerd, en geprobeerd om via systemische toediening van een glutamaat receptor antagonist de glutamaterge hypofunctie in de amygdala na te bootsen en daardoor fear conditioning op neurochemisch nivo te verhinderen.

2.1 Fear conditioning

De eerste stap was het ontwikkelen van een diermodel waarin anticipatie-angst en psychologische stress centraal staan. Dit kan door middel van fear conditioning, waarin een geconditioneerde stimulus (CS, geluid) wordt gepaard aan een ongeconditioneerde stimulus (US, electrische schok). Na een aantal conditioneringssessies veroorzaakt het geluid, in afwezigheid van de electrische schok, een duidelijke angstreactie. Een voordeel van deze benadering is dat de perceptie van pijn geen directe rol speelt in het model. Hoofdstuk 2 laat zien dat een hogere schokintensiteit tot minder variatie leidt, waardoor de experimentele groepen kleiner kunnen zijn. In de hoofdstukken 3-5 wordt beschreven dat fear conditioning leidt tot 1) een toename van de angst (freezing behaviour), 2) een toename van cFos expressie (een maat voor neurale activiteit en synaptische plasticiteit) in een aantal relevante hersengebieden, 3) een toename van de hoeveelheid glutamaat in essentiele hersengebieden, waaronder de kernen van de amygdala. Fear conditioning blijkt echter geen effect te hebben op het dopamine gehalte van de onderzochte hersengebieden, m.u.v. de locus coeruleus (LC, toename) en de nucleus accumbens (Nacc, afname) (hoofdstuk 5). In het laatste hersengebied is er ook sprake van een duidelijke toename van de dopaminerge activiteit (turnover, hoofdstuk 5).

2.2 Ketamine

Onderzoek heeft aangetoond dat NMDA receptoren in de amygdala een belangrijke rol spelen bij fear conditioning. Daarom is vervolgens onderzocht of de aan fear conditioning gerelateerde veranderingen in gedrag en biochemie voorkomen kunnen worden door de activiteit van het centrale glutamaterge systeem te onderdrukken via systemische toediening van de NMDA receptor antagonist ketamine. Een ander argument voor het gebruik van ketamine is dat het de positieve, negatieve en cognitieve symptomen van schizofrenie verergert.

Ketamine blijkt inderdaad in staat om de aan fear conditioning gerelateerde veranderingen in gedrag en biochemie te blokkeren (hoofdstuk 3 en 4). Onze bevindingen wat betreft gedrag en cFos expressie in de BLA en ACC zijn in overeenstemming met de theorie van Grossberg. Systemische toediening van

ketamine blijkt bovendien het glutamaat gehalte in de kernen van de amygdala terug te brengen tot controle nivo's (geen fear conditioning), maar niet in de ACC (hoofdstuk 5). Het dopamine gehalte van de centrale nucleus van de amygdala en de Nacc is daarentegen verhoogd na toediening van ketamine (hoofdstuk 5).

2.3 Antipsychotica

De antipsychotica haloperidol en clozapine zijn gebruikt om de combinatie ketamine/fear conditioning te valideren als model voor negatieve symptomen van schizofrenie. Het atypische antipsychoticum clozapine wordt nog altijd beschouwd als het middel van eerste keus om de negatieve symptomen van schizofrenie te behandelen, terwijl haloperidol voornamelijk de positieve symptomen verbetert. Bovendien is clozapine nog altijd eerste keus bij therapie resistente schizofrenie. In de kliniek vermindert clozapine de negatieve effecten van ketamine bij patiënten met schizofrenie, terwijl in proefdieren de gedragseffecten van de NMDA receptor antagonist PCP duidelijk geringer zijn na chronische toediening van clozapine. Dierexperimentele studies hebben ook laten zien dat een aantal metabole effecten van ketamine geblokkeerd worden door clozapine en niet door haloperidol. Onze hypothese is dan ook dat clozapine, en niet haloperidol, in staat is om het effect van ketamine op fear conditioning te blokkeren, zowel gedragsmatig als op biochemisch niveau.

Naast de beide antipsychotica hebben we ook de metabotrobe glutamaat 2/3 receptor agonist LY 379268 gebruikt om dat deze stof het leren van emoties, zoals angst, positief zou beïnvloeden. Hoewel LY 379268 anxiolytische eigenschappen lijkt te bezitten is het niet bekend of de stof een effect heeft op fear conditioning. LY 379268 activeert zowel presynaptische als postsynaptische glutamaat receptoren, maar in neurochemisch opzicht is alleen het agonistisch effect op de postsynaptische glutamaat 2 receptoren relevant voor ons model.

We hadden verwacht dat clozapine het effect van ketamine op fear conditioning zou normaliseren, maar dit blijkt maar ten dele waar. Wat betreft het gedrag is geen van de geteste stoffen, dus ook niet clozapine, in staat om het effect van ketamine ongedaan te maken. Biochemisch gezien voldoen de stoffen min of meer aan de

verwachtingen. Clozapine normaliseert de cFos expressie in de ACC, Nacc, basolaterale en laterale amygdala, terwijl haloperidol en LY 379268 geen effect hebben met uitzondering van de ACC (hoofdstuk 4). Bovendien is clozapine in staat om de effecten van ketamine op glutamaat (BLA en LC) en dopamine (Nacc en LC) terug te brengen tot de niveaus van fear conditioning. Het is duidelijk dat gedrag en biochemie niet met elkaar in overeenstemming zijn. In hoofdstuk 5 wordt een conceptueel model voorgesteld dat deze discrepantie probeert te verklaren. Gezien de klinische en preklinische bevindingen met clozapine kan ook de acute in plaats van chronische toediening van clozapine in onze studies een rol hebben gespeeld.

3 Conceptual model

Here, we will review the model proposed in Chapter 5, incorporating the cFos data discussed in the other chapters and additional dopamine turnover data. This model focuses mainly on the amygdala nuclei and therefore forms part of the greater emotional-cognitive model proposed in the Introduction. Other brain areas will also be discussed in relation to this conceptual model. We adapt the model of Aleman and Kahn (2005) and Reynolds (1983) to the current context in four key ways. First, we propose that glutamate-mediated fear conditioning in the BLA drives freezing behaviour through the output nuclei of the CEA (Fanselow and Kim, 1994; Killcross et al., 1997; Koo et al., 2004; LeDoux, 1998). Second, we hypothesize that dopamine-modulated γ-aminobutyric acid (GABA) inhibition in the CEA modifies the outputs of the BLA (Pare et al., 2003). Third, we interpret ketamine administration in terms of a glutamate-mediated deficit in fear conditioning in the BLA (Miserendino et al., 1990; Monaghan and Cotman, 1985; Savonenko et al., 2003; Walker and Davis, 2002). Lastly, we propose that decreased dopamine turnover (see Section 2.2) due to ketamine administration in the CEA leads to increased GABA inhibition of the outputs of the BLA, leading eventually to decreased freezing behaviour.

3.1 Fear conditioning

The model can explain the major features of our data as follows. Glutamate content is increased in both amygdala nuclei as a result of fear conditioning, but dopamine content is not affected in either (Chapter 5). As a result, output signals (glutamate) inducing freezing behaviour from the BLA via the CEA remain strong, as there is no

interference of dopamine. The precise role of dopamine will be explained in the next section (see Section 2.2 Ketamine).

In terms of cFos expression, fear conditioning elevates expression in the BLA only, and not the CEA (Chapters 3, 4). Interestingly, a study by Kleim et al. (1996) shows that cFos activity is directly related to learning of a skill, and not only to the execution or maintenance of motor behaviour. This could indicate that the BLA is more concerned with processing and storage of fearful memories, while the CEA is mainly an output nucleus (Fanselow and Kim, 1994; Maren and Fanselow, 1996; Killcross et al., 1997; Shors and Matthew, 1998; Goosens and Maren, 2003; Pezze and Feldon; 2004).

3.2 Ketamine

The dopamine metabolic ratios (unpublished data, Table 1) indicate that the increase in dopamine content in the CEA after ketamine administration (FC + Ket; Chapter 5) is due to a decrease in turnover (indicative of decreased release and a subsequent increase in storage of the neurotransmitter in axon terminals). This is consistent with the results of Kapur and Seeman (2002), which indicate that ketamine acts as an agonist on the presynaptic dopamine D_2 autoreceptor, resulting in diminished dopamine release (Fig. 1). In our study, ketamine also suppresses glutamate-related fear processing in the BLA and CEA, in addition to simultaneously decreasing dopamine turnover in the CEA. We therefore propose that this decreased dopamine release liberates a tonic inhibition by GABA-ergic neurons in the intercalated cells, leading to *increased* inhibition of the glutamate signals from the BLA to the CEA.

In agreement with this, Marowsky et al. (2005) show that dopamine D_1 receptor (post-synaptic) activation disinhibits the amygdala by inhibiting GABA-ergic mechanisms within the intercalated cells. Interestingly, systemic application of dopamine D_1 agonists has been shown to retard or even reverse fear extinction (Borowski and Kokkinidis, 1998), while D_1 antagonists block either the acquisition and/or expression of fear (Greba and Kokkinidis, 2000; Inoue et al., 2000). Decreased dopamine release through ketamine's effects on the dopamine D_2 autoreceptor in our study would therefore re-activate the inhibitory control of the intercalated cells on the CEA, resulting in behavioural blockade (Fig. 1).

Contrary to these results, ketamine did not reduce cFos expression induced by fear conditioning in the BLA in Chapter 3. We therefore split the nuclei into anterior and posterior subdivisions in Chapter 4, with better results. It was discovered that the posterior nucleus did not participate in fear conditioning, while the anterior portion of the BLA did. Other studies (Sananes and Davis, 1992; Goosens and Maren, 2001; Scicli et al., 2004) have also selectively implicated the anterior portion of the basolateral nucleus in fear conditioning. Subsequently, ketamine also significantly suppressed cFos expression evoked by fear conditioning in this area, agreeing with our neurochemical conceptual model.

3.3 Antipsychotics

We hypothesised that clozapine and not haloperidol, would be able to restore normal fear-conditioned behaviour by blocking the effects of ketamine. Our data supports this as clozapine reverses the effect of ketamine more potently than haloperidol in terms of cFos expression (Chapter 4) and also restores glutamate levels (Chapter 5) in several brain areas, especially in the BLA. This alone would predict normal freezing behaviour. However, this is not the case. In terms of the conceptual model, we suggest that this is because clozapine does nothing to restore normal dopamine (and glutamate) levels in the CEA due to ketamine administration. Dopamine turnover therefore remains decreased, leaving the GABA-ergic inhibition of the CEA in tact.

Which strategies therefore could be used in order to restore normal fearful behaviour? As previously mentioned, ketamine primarily acts as an agonist at the dopamine D_2 autoreceptor, thereby inhibiting the release of dopamine. We therefore propose two methods in order to counteract ketamine's effect on fear conditioning: 1) by using a selective dopamine D_2 antagonist to block ketamine's actions on the dopamine D_2 autoreceptor or 2) by administering a selective dopamine D_1 agonist, which directly inhibits GABA-ergic function.

Initially, we chose to validate this animal model with clozapine and haloperidol as they are used in the clinical setting. As clozapine and haloperidol both act as antagonists at dopamine D_2 receptors (Farde et al., 1992), we might suppose that

they would block the effect of ketamine on dopamine transmission at this receptor, and restore normal fear-conditioned behaviour. However, both haloperidol and clozapine also have affinities for the dopamine D_1 receptor (Farde et al., 1992) and subsequently block the eventual dopamine that might be released as a result of D_2 autoreceptor blockade, leaving any behavioural blockade via the GABA-ergic cells in place. This could be one explanation why even clozapine is not particularly effective in treating negative symptoms of schizophrenia (Kane, 1989).

3.4 Possible extensions to the conceptual model

Despite this, some studies have shown that chronic administration of atypicals can block the effects of NMDA antagonists such as ketamine. For example, repeated chronic administration of olanzapine for 7 days, but not haloperidol, reversed the impairment caused by PCP, an NMDA antagonist similar to ketamine (Enomoto et al., 2005). A study by Sams-Dodd (1996) also showed that chronic clozapine treatment inhibited PCP-induced stereotypical behaviour and social isolation. This phenomenon is also seen in the clinical situation, where antipsychotics can take a considerable length of time (up to 6 weeks) to start resolving symptoms. We therefore speculate that chronic clozapine administration may also lead to normal freezing behaviour, perhaps through changes in neural plasticity, for instance through the desensitisation of D_2 autoreceptors (Giardino et al., 1991; Reuss and Unsicker, 2001). In addition, chronic treatment with typical antipsychotics such as haloperidol may not lead to desensitisation of D_2 receptors, but instead lead to (unwanted) decreased sensitivity of D_1 receptors (Imperato et al., 1994; Reuss and Unsicker, 2001).

***Table 1**: The dopac/dopamine metabolic ratios*

Brain Areas	Control		FC		FC + Cloz		FC + Ket		FC + Ket + Cloz	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
antcing	1.35	2.19	.79	49.95	.93	1.79	.64	.51	.82[&]	2.98
Nacc	.19	.16	.45*	29.08	.18[#]	.04	.20[#]	.05	.21[#]	.14
CEA	.20	.17	.19	13.28	.20	.35	.09[#]	.05	.11[$]	.10

**p < 0.01 from control #p < 0.01 from FC*
&p < 0.01 from FC + Ket $p = 0.056 from FC
antcing, anterior cingulate; CEA, central amygdala nucleus; Cloz. Clozapine; FC, Fear conditioned; Ket, ketamine; Nacc, nucleus accumbens

4 Other brain areas related to the conceptual model

Several other areas were investigated in this thesis in relation to the emotional-cognitive model of emotional blunting. Below, we try to relate some of these areas to the neurochemical conceptual model.

4.1 Anterior cingulate (ACC)

As mentioned in the Introduction, the ACC was hypothesised to play an important role in the dysregulation of attention to sensory signals in the emotional-cognitive model of Grossberg (2000) and Cardinal (2002). These sensory signals are relayed through two distinctive pathways to the amygdala: one is a faster route that arises directly from the thalamus, while the second follows a slower, but more information-rich route via the sensory cortex (Rogan et al., 1997; Tsvetkov et al., 2002) and ACC. This pathway typically imparts information regarding motivation, attention and pain stimuli to the amygdala nuclei (Bush et al., 2000; Gao et al., 2004; Sugase-Miyamoto and Richmond, 2005; Malin and McGaugh, 2006), implying a role for the ACC in fear conditioning.

Our cFos data (Chapter 3, 4) support this notion. Fear conditioning increased cFos expression in this area. This was subsequently interrupted with ketamine administration, in accordance with our hypothesis. Clozapine administration also blocked the effects of ketamine, restoring cFos expression to fear conditioning-induced levels. Unfortunately, this result was not entirely reflected in the neurochemical data. Fear conditioning only produced a slight increase in glutamate content (Chapter 5). Previous studies (Lei et al., 2004; Tang et al., 2005) have shown that glutamate receptors in the ACC are directly involved with pain stimulation. As no shock was administered during the test trials (on the third day) in our study, however, no pain would have been experienced. This may account for the lack of glutamate activation due to fear conditioning in this area. Contrary to our hypothesis, ketamine administration served only to increase both neurotransmitter levels, instead of suppressing them. This result is, however, in line with the study of Lorrain et al. (2003), which showed increased glutamate and dopamine levels in the prefrontal cortex after ketamine injections. We did not, by comparison, administer ketamine on the day of testing, ruling out the immediate effect of ketamine on glutamate and

dopamine content in our study. Another study (Lindefors et al., 1997) showed that apart from direct reactions to ketamine injections, increased basal levels of dopamine in the rat prefrontal cortex were also found after 7 days of once-daily ketamine injections. Although this study was conducted over a longer time period, it may still serve as a probable explanation for our increased neurotransmitter levels after ketamine injections, especially as these increases were minimal and did not reach significant levels.

4.2 Nucleus accumbens (Nacc)

The Nacc is intimately linked with the ACC and the amygdala, implying that it also plays a role in fear learning (Levita et al., 2002). It is speculated to be involved in adapting behavioural responses to aversive outcomes in accordance with the motivational value (McCullough et al., 1993). In particular, the Nacc dopamine levels code for the salience of the conditioned stimulus (Horvitz, 2000) in order to adjust both the strength of the association and then the behavioural response. Dopamine levels in Nacc could therefore play an important role with regards to the neurochemical infrastructure of our conceptual model.

Previously, it has been shown that the dopamine response to the conditioned stimulus leading to the expression of conditioned fear was decreased in the Nacc shell, but increased in the core (Pezze et al., 2001). The authors suggested that this decreased dopamine in the shell was due to an increase in dopamine release (Pezze et al., 2001). We also found decreased dopamine content in the Nacc, but as no microdialysis studies were done, we cannot be sure whether this is due to an increase in release or a decrease in production/storage. The dopamine metabolic ratios, however, do imply that fear conditioning increases dopamine release, as an increase in metabolism is noted. Our neural correlates are also in agreement and show increased cFos expression due to fear conditioning in the Nacc shell, with no activity noted in the core (Chapter 4). The lack of cFos expression in the core is in agreement with suggestions that the shell is involved in emotional regulation, while the core is more related to motor activity (Parkinson et al., 1999; Reynolds and Berridge, 2003, Sellings et al., 2006). In accordance with our hypothesis, ketamine abolished the effect of fear conditioning on cFos expression and dopamine content,

with clozapine restoring it, thereby implicating a role for the Nacc shell in our emotional-cognitive model.

4.3 Paraventricular nucleus (PVN)

The PVN forms a critical part of the stress pathway (hypothalamus-pituitary-adrenal: HPA axis) that typically leads to the secretion of certain hormones mediating the physiological responses underlying fear. More specifically, studies have illustrated that HPA axis activation leads to the release of CRF, ACTH and cortisol (Nash and Maickel, 1988; Udelsman and Chrousos, 1988). Neuronal projections connect the amygdala to the PVN. The PVN is therefore an effector nucleus of the amygdala via glutamate/GABA-ergic pathways (Herman et al., 2002; Herman et al., 2005), and events affecting the one should project to the other.

Some studies suggest that the main output nucleus of the amygdala, the CEA, primarily projects to the PVN (Gray et al., 1989). Our data (both cFos and glutamate) do not agree with this notion, as the effects of ketamine and clozapine in the PVN reflect more the activity of the BLA than the CEA (Chapters 3, 4, 5). Other studies also indicate that BLA projections influencing affective behaviour pass through non-CEA (medial) regions to the hypothalamus and brainstem areas (Luiten et al., 1983; van der Kooy et al., 1984). We therefore suggest that the main amygdala nucleus projecting to the PVN is in fact the BLA.

As the BLA is critically involved in fear memory storage, while the CEA modulates mainly behavioural outputs, we may put forward another speculation. Perhaps the stress signals and hormonal release via the PVN prepare the animal in anticipation of the stressful event and aid in fear memory storage, and do not actually contribute to the execution of the fearful behaviour. The observation that the outputs of the PVN in this study do not correlate with the behaviour measured supports this notion. As the PVN is not the only nucleus conveying the output of the amygdala, the lack of fearful behavioural restoration with clozapine could therefore have resulted from the inhibitory signals from the CEA (see Section 2) to other regulatory effector nuclei more intimately involved in the execution of behaviour, such as the PAG.

4.4 Locus coeruleus (LC)

The LC is the primary brain stem nucleus producing noradrenalin. It is also linked to the amygdala, implying a role in fear learning. For example, within the BLA itself, noradrenalin enhances glutamatergic synaptic plasticity (Ferry et al., 1997), which is thought to underlie learning and memory functions. In fact, noradrenalin infused directly into the amygdala attenuates memory impairment (Liang et al., 1995).

It is therefore not surprising that the LC is implicated in our emotional-cognitive model of emotional blunting. Our cFos results indicate that fear conditioning increases and ketamine suppresses expression in this area (Chapter 3). Glutamate tissue content is also increased and then attenuated by ketamine, with clozapine fully restoring the fear conditioning effect on glutamate in the locus coeruleus. These patterns are also reflected in the BLA, consistent with the connection between the two areas. From these results, we speculate that noradrenalin projecting from the LC is necessary for enhancing fearful memory storage in the BLA, and that disruption thereof could contribute to emotional blunting.

5 Main conclusions of original hypotheses

In the Introduction, several main hypotheses were constructed with regards to our emotional-cognitive model of emotional blunting in schizophrenia. These were as follows:

1) Fear conditioning will lead to:

a. Increases in behaviour associated with fear (e.g. freezing).

b. Increases in cFos expression in those brain areas involved in fear conditioning, including the ACC and BLA.

c. Increased glutamate content and dopamine modulation in those same brain areas.

2) Ketamine administration will abolish all the above-mentioned effects of fear conditioning to baseline levels of behaviour, cFos expression and neurotransmitter levels.

3) Administration of clozapine, but not haloperidol or LY 379268 (an anxiolytic), will block the effects of ketamine in all measured output parameters, either fully or partially.

As outlined in the summary above, all requirements were fulfilled, except with respect to the behavioural data mentioned under point 3 above. We then constructed a conceptual model in order to explain why clozapine did not fully restore behaviour, while neural correlates indicated a positive outcome. We also describe improvements that may extend the model and allow for full behavioural restoration. Taken together, the current thesis supports the notion that glutamatergic hypofunctioning in amygdala and related brain areas underlie deficits in the processing of emotions as seen with negative schizophrenic symptoms, especially emotional blunting. The present thesis therefore might pave the way for future studies to explore novel drug treatments of these notoriously drug-resistant symptoms, such as selective dopamine D_2 antagonists or selective D_1 agonists.

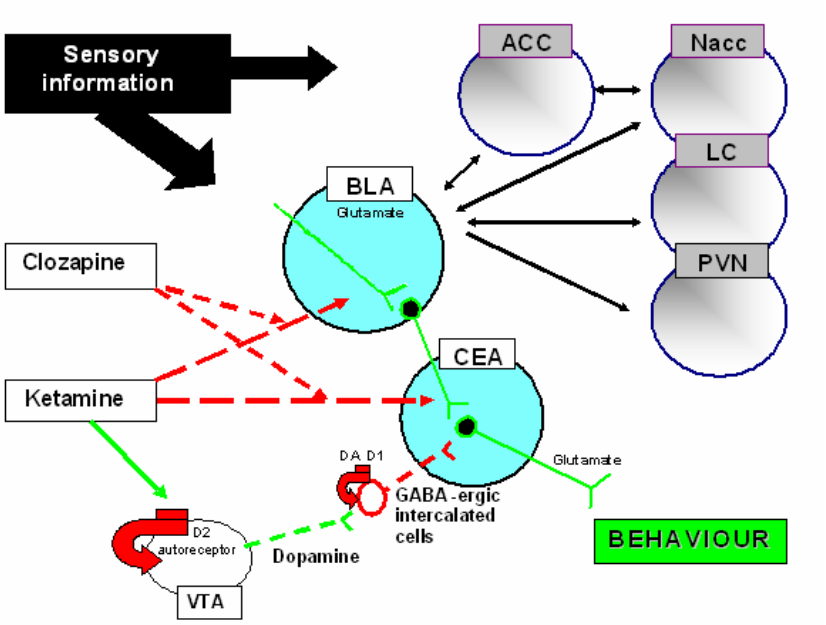

***Figure 1**: Conceptual model. A schematic drawing of our conceptual model depicting interactions between dopamine and glutamate in the amygdala nuclei. The interactions between other brain areas studied and the amygdala conceptual model are also indicated. Dashed lines symbolise inhibition, while solid lines represent stimulation. Lines between brain areas represent functional connectivity between the regions. Sensory information (fear stimuli) is processed first by the basolateral amygdala (BLA), activating the glutamate system in this area, but does not affect dopamine levels in either the BLA or central amygdala (CEA). Output signals inducing freezing behaviour from the CEA are therefore strong via the glutamate pathway. Ketamine decreases glutamate-related fear processing in the BLA and CEA and simultaneously elevates dopamine content (storage) in the CEA, by blocking dopamine release via the dopamine D_2 autoreceptor located on the cell body (possibly the VTA). The decreased dopamine release (together with the block of glutamate transmission from the BLA to the CEA) blocks the fear-related outputs by means of increased GABA inhibition via intercalated cells projecting onto the CEA. The net effect is weak output signals from the CEA and diminished freezing behaviour. Clozapine, while blocking the effects of ketamine on glutamate-related processing in the BLA, does nothing to renormalize dopamine levels. GABA-ergic intercalated cells therefore continue to inhibit CEA/and or BLA and cannot restore behaviour. Can chronic clozapine treatment renormalize dopamine levels and lead to long-term remediation of negative symptoms in the animal model? ACC, anterior cingulate; DA, dopamine; LC, locus coeruleus; Nacc, nucleus accumbens; PVN, paraventricular nucleus; VTA, ventral tegmental area*

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I began my PhD journey in 2001, when I arrived in Groningen for my interview. It was also my first time in The Netherlands, and Europe for that matter, and I was very nervous. I remember bugging this old lady on the train from schiphol, asking her several times if this was the train to Groningen, and not the one going to Leeuwaarden. She must have wondered who this strange person was speaking in a very heavily accented Dutch dialect that sounds very melodic and childlike – a language us South-Africans know as Afrikaans.

Rob Visser was the one who picked me up from the train station in Groningen. In fact, he immediately picked me out from the crowd. I wonder why? It was then I also had my first back-of-the-bike-seat bicycle ride and I screamed the entire journey. Despite this, Rob Visser has helped me out tremendously, from tax returns, loaning money to rent an apartment, to finding a last minute flight to South Africa when I heard my dad was passing away. He also delivered me to the train station to catch that flight the next day at 6am. For this and all your help, Rob, I am truly grateful. You are the backbone of many a PhD here in Groningen.

My first day in Groningen (somewhere in January 2002), was marked by the disappearance of my luggage; inevitably lost by British Airways. Thankfully, there were a lot of wonderful students at the International Student house, Kraneweg 4, who helped me out with extra clothes and clean linen. I would like to thank Melanie Ruger, in particular, who picked me up from the airport with hot tea waiting in the car, and who lent me clean clothes and took me in for that night to comfort me with chocolate for my loss.

To my main girls at the international student house, Stephanie and Lisa, thank you for all our wonderful talks and goings out to e.g. Sally's, on International Mondays! I had such a blast with you guys! We will always have Ireland. I especially want to thank my best friend for the first 4 months in Groningen. Zuzka (or Zabka/Froggy), without you, my first months in Groningen would not be as colourful as they were with you around. I wish you happiness and fulfilment in your marriage and career.

Unfortunately, everyone has to grow up sometime, so I had to leave the student house and seek other accommodation. I stayed in a variety of places, and must have

moved 6 times during my stay here. Lets just say, Direct Wonen made a killing. Finally, I found a suitable dwelling located on the Schuitendiep. Living with 8 other people, Dutchies, was quite an eye-opener, and I was quickly initiated into the ways of Dutch student living. Despite this, we still all had a pretty good time. Job and Tim, thank you for those rainy-day games of Kolonisten. Job, I really enjoyed our heart-felt conversations about relationships and life. Thank you also for escorting me to the doctor when I really needed you. Wasn't our trip to South Africa fabulous! No wonder you want to return. Sorry about the robbery.

At this juncture, I would like to thank Cedric. Thank you for your love and support, especially when my dad was suffering from cancer. Those were tough times and I am glad you stuck by me. I wish you well in all your endeavours.

After a lot of sad good byes to international students who only stayed a short while in Groningen, I decided to start making more permanent friends, by hanging out with fellow PhD students. We had a lot of parties, especially at Sonja's original apartment. I had great fun with you guys, and I am glad we had this time together. Here I would like to thank Sonja, Joyce, Branislava, Graeme, Eleanora, Stasinos, Francisco, Paola, Gilles, Alarm, Monica, Andrea, Lenny, Lena, Mylene, Laurent, Beatriz, and Teresa. At least this is in writing, so you can finally understand what I am saying!

Some friends have been with me since the beginning; since the first day we took Dutch level II together. Even when classes ended, we still remained friends and even started a dinner club, where we would cook each other meals from our respective countries. Lavinia, Esther, you have been wonderful friends and wonderful chefs. I miss our dinner parties very much. Good luck, Lavinia with your job and best wishes, Esther, with your marriage and children ;)

Of course, I have also had some Dutch friends. Irma, Gonnie, Marjolein, Martijn, Minke, Marieke, Simone, Evelien, Janine, Lieke, Walther, Bernard, and Marit: thank you for the times we had together. Irma, my paranimph, you are my rock; where would I be without you? Lost somewhere I am sure. Thank you for your love, friendship, and organisational skills. Martijn, we had a lot of unforgettable adventures in South Africa! Marjolein, Lisbon would not have been the same without you. Minke,

due to similar circumstances, we bonded in sorrow. I would never wish it upon anyone, but I am glad I had to chance to get to know you better. Marieke, USA will be remembered because of your excellent driving skills (jokes)! I also want to thank my students who helped “Super Lab Girl” (my alter-ego) with her experiments. I definitely could not have done it without you. Well, at least not with as much enjoyment.

Somewhere in the middle of my time here, this blonde-haired South African girl appeared in my life. Miranda at first tried to speak Afrikaans to me. When she realised that all that came out of my mouth was Dutchified Afrikaans, we swapped to English. We have been friends ever since. Miranda, you are my link to South Africa and one of my best friends (even though you break a lot of my things). I can’t summarise here everything that we have been through, but just know that when I am down, you are always able to cheer me up. So I don’t mind feeding you every now and then ;)

Speaking of South Africans, I would also like to thank my former supervisor, now professor and head of department (in other words, a VIP), Willie Daniels. You always supported me in the endeavours to go overseas and always have my interests at heart. It is through your guidance that I am where I am today. I would also like to thank Ingrid Webster, for her constant friendship, even though we live so far apart.

I would also like to thank my current supervisor and promoter. Thank you Fokko, for your constant support, enthusiasm for my ideas and for believing in me. Thank you also for your education on good food and good wines. Thank you, Hans, for giving me this opportunity. I would also like to mention some other psychiatry staff members that have been a constant help during my time here: Margo, Jacqueline, Folkert, Tineke, Kor, Jan Bakker, and Eite Oosterveld. Also thanks to Joseph Haas from Eli Lilly, for the statistical guidance.

Back to my apartment hopping, one of my apartments I shared with a Hungarian vegetarian, Gabor. In fact, he promoted 1 month ahead of me this year. I really enjoyed living with you, Gabor, even when you were hopping around like a Duracell bunny and got annoyed at me with my butt glued to the couch. You are a great friend, and I miss you a lot. Tell Anca I say HI! Our bbq’s will always be in my

memory: 1) because they were infamous, and 2) because you provided me with many photos documenting them. South Africa and USA will never be the same again. Respect and love yo-yo!

Another completely different aspect of my time here, involved my religion, Catholiscm. I joined the Bonifatius group, where I first met Irma, Francesco, Mathijs, Bélen, and Sylvain amongst others. I like to think that my faith has grown from our discussions and meetings. The meetings were good forums to express myself and have some interesting theological debates. It was also the springboard to going to the World Youth Days in Cologne and the Philippines, which really strengthened my faith and enhanced my life experiences. Lord, thank you for the strength and peace when I needed it most.

I also met Livvy in Cologne, who at first failed to recognise that I was a native English speaker. Don't worry girl, I forgive you. "Wees stil aan de overkant". Thank you for your friendship and for being my running partner. Thank you also very much for proof reading this thesis. It was a huge task. Good luck with your future endeavours, and keep dreaming☺. To all the Philippines participants, our time together was unforgettable. I want to send a special shout-out to Bram, Edu, Jolein, Jocine (aka Christine), Imelda, Rick, Jos, Tony, Anneke, Vincent, Madel and Hao de Carabou. Op de Achterbank!

Now we get to the really mushy part. I met Tony a year or so before we got together. We were friends first before we got together after a particularly good roast chicken and a lot of laughs with flirtatious undertones. Tony, you are more than just a boyfriend to me. You are also my paranimph, thesis editor, psychologist, supervisor, employer, physiotherapist, comedian, dishwasher, housemate, best friend and life partner. You mean more to me than I can express, and you have made my life so far, very special. I could not have done this without you. I have seen my life without you, and I hope I will never have to live it that way. Our family (you, me, tutti and patchy) are everything to me and always will be.

I also want to thank the ones I left behind, my family. Mom, you are still the voice inside my head that reasons with me and tells me when I am being silly. Thank you

for your constant calls, love and support. Thank you also, for being here today. Janine, my sister and clothing advisor, your love and support are also deeply appreciated. You are hilarious girl! And I miss you so much. Things are so *random* without you. I just know that things will work out for you. Just don't move too far away, ok? Or should I tell that to myself? Thanks also for the support from my extended family, especially Uncle Gerald, Lionel and Keith, and Aunty Mary. You are the link that binds me to my father.

Dad, wherever you are out there, I know you are supporting me and guiding me still. I also know that you are very proud of me. I love and miss you so much! You were always my biggest supporter. I will never forget you. I therefore dedicate this thesis in your memory.