A frightening view on schizophrenia. Combining fear conditioning and ketamine administration to investigate emotional blunting in an animal model of schizophrenia
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Chapter 4

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

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\textit{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 hypoglutationergetic 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

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 excitotoxicity, 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
Effects of antipsychotics on a putative animal model

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°C) 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.

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). CLOZ, clozapine; FC, Fear conditioning; HALO, haloperidol; KET, ketamine; LY, LY379268; NFC, no fear conditioning; SAL, saline.

**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.
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).
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 °C for two 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. In preparation for cFos staining, whole brains were dehydrated in a 30% sucrose solution overnight and subsequently frozen with gaseous CO₂ at –80 °C.
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 3×5 min. After pre-incubation with 0.3 % H₂O₂ (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, 1:10000 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% H₂O₂ (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 mm²). 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

<table>
<thead>
<tr>
<th>Area</th>
<th>Mm from Bregma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior cingulate</td>
<td>+2.80 to +2.15</td>
</tr>
<tr>
<td>Nucleus accumbens: core and shell</td>
<td>+2.80 to +0.45</td>
</tr>
<tr>
<td>Paraventricular nucleus</td>
<td>-1.53 to -2.00</td>
</tr>
<tr>
<td>Central nucleus of amygdala</td>
<td>-2.45 to -2.85</td>
</tr>
<tr>
<td>Anterior basolateral nucleus amygdala</td>
<td>-2.45 to -2.85</td>
</tr>
<tr>
<td>Posterior basolateral nucleus amygdala</td>
<td>-2.45 to -2.85</td>
</tr>
<tr>
<td>Lateral nucleus amygdala</td>
<td>-2.45 to -2.85</td>
</tr>
</tbody>
</table>

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
Chapter 4

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
Effects of antipsychotics on a putative animal model

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), 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; pvm, 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 LY379268 (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 D₂ 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 D₂ 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-amygda 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 4


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Chapter 4


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Effects of antipsychotics on a putative animal model


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