Development of a Double-hit model of schizophrenia in male Sprague Dawley rats.

By

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Preface

Positive symptoms of schizophrenia can be eradicated using available typical and atypical antipsychotic drugs, while negative and cognitive symptoms are resistant to the current available therapeutics. Animal models of psychiatric disorders are clinical tools used to investigate neurobiological changes of the disorder. Animal models of schizophrenia allow researchers to better monitor the progression of the disorder, they also provide an opportunity to conduct invasive techniques to study the molecular and structural changes involved in the disorder. Developing an animal model of schizophrenia capable of inducing all symptoms of schizophrenia will assist researcher to better understand mechanisms involved in the pathophysiology of schizophrenia.

DECLARATION

I, Khanyiso Bright Shangase hereby declare that the dissertation entitled:

"Development of a Double-hit model of schizophrenia in male Sprague Dawley rats" is the result of my own investigation and research and that it has not been submitted in part or in full for any other degree or to any other university. Where the work of others was used, it is duly acknowledged in the text.

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PLAGIARISM DECLARATION

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List of abbreviations

α	**Alpha**
ANOVA	**Analysis of Variance**
AREC	**Animal Research Ethics Committee**
β	**Beta**
BDNF	**brain derived neurotrophic factor**
BRU	**Biomedical Research Unit**
cDNA	**complementary deoxyribonucleic acid**
D2	**Dopamine receptor 2**
DNA	**deoxyribonucleic acid**
ELISA	**enzyme-linked immunosorbent assay**
GABA	**gamma-amino-butyric-acid**
GAD1	**Glutamate decarboxylate 1**
GAPDH	**Glyceraldehyde 3-phosphate dehydrogenase**
GH	**Group housed**
GHK	**Group housed + ketamine**
GPx1	**Glutathione peroxidase**
Hipp	**Hippocampus**
IL-6	**interleukin 6**
K	**Ketamine**
KG	**kilogram**
MDA	**malondialdehyde**

µ	**micro**
µg	**microgram**
µl	**microlitre**
mg	**milligram**
ml	**millilitre**
mRNA	**messenger ribonucleic acid**
NMDAR	**N-methyl-D-aspartate receptor**
NORT	**novel object recognition test**
OD	**Optical density**
PCR	**polymerase chain reaction**
PFC	**Prefrontal cortex**
PND	**Postnatal day**
PVALB	**Parvalbumin**
r	**Peason correlation**
RNA	**ribonucleic acid**
ROS	**reactive oxygen species**
RT PCR	**Real-time polymerase chain reaction**
S	**Saline**
S.C.	**Subcutaneous injection**
SI	**Social isolated**
SIK	**Social isolated + ketamine**
SOD	**superoxide dismutase**
TBARs	**thiobarbituric acid reactive substances**

TNF-α	**tumour necrosis factor α**
UKZN	**University of KwaZulu-Nata**

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Study outline

The current thesis is presented in manuscript format, consisting of 7 sections viz. Abstract, Chapter 1: Introduction/Literature Review, Chapter 2: Systematic Review, Chapter 3: Manuscript 1, Chapter 4: Manuscript 2, Chapter 5: Manuscript 3, Chapter 6: Synopsis and Appendices, Chapter 7: References. The abstract summarizes the purpose and findings of the present study. Chapter 1 is a brief background and features the relevant literature review in problem solving and current gaps, and how the aims of the current research fill the gaps in the literature. Chapter 2 comprises a systematic review study titled "***Investigating the robustness of a rodent "double hit" (post-weaning social isolation and NMDA receptor antagonist) model as an animal model for schizophrenia: A Systematic Review*****".** This systematic review is published in the journal "**Brian sciences**" and is available online (**Appendix 1**). Chapter 3 comprises the first research study in manuscript format that seeks to investigate the "***Effects of combined postweaning social isolation and ketamine administration on schizophrenia-like behaviour in male Sprague Dawley rats*****".** This manuscript has been published in the journal "**Behavioural Brain Research**" and available online, the manuscript has been formatted according to the journal's guidelines for authors **(Appendix 2).** Chapter 4 comprises the second research study in manuscript formal that seeks to investigate the **"*Evaluating neurochemical changes and associated locomotor activity in a double hit schizophrenia model following exposure to ketamine and social isolation*".** Chapter 5 comprises the third research study in manuscript formal that seeks to investigate the **"*The role of GABAergic dysfunction and oxidative stress in schizophrenia*".** Chapter 6 is the discussion that links the findings of the four studies with the aims of the project. Appendices contain the ethical clearance letter, symposium presentation certificates and publications.

Abstract

Schizophrenia is a debilitating neuropsychiatric disorder that affects approximately 1% of the world's population. Schizophrenia symptoms are classified as positive, negative, and cognitive. Patients with schizophrenia may present with a combination of symptoms, which causes complications in the diagnosis and treatment. Available drugs used to treat schizophrenia have shown high efficacy against the positive symptoms, while the negative and cognitive symptoms proved to be more resistant against the available treatment. There is an urgent need to develop an animal model of schizophrenia that will assist researchers in investigating the pathophysiology of schizophrenia. In this study we aim to develop a double hit animal model of schizophrenia that will be able to mimic behavioural and molecular changes similar to those observed from positive, negative, and cognitive symptoms of schizophrenic patients. The study objectives are divided into three manuscripts, manuscript 1 will characterise behavioural and molecular changes associated with the negative and cognitive symptoms of schizophrenia using our double hit model. Furthermore, manuscript 2 will evaluate neurotransmitter changes and receptor changes associated with the positive symptoms of schizophrenia using our double hit model. Lastly, manuscript 3 will examine or explore mechanisms or pathway involved the pathophysiology of schizophrenia.

Animal models of schizophrenia provide a platform to screen the progression of the disease. They further offer researchers the opportunity to conduct invasive monitoring of molecular and structural changes involved in the disease. These models are divided into four categories viz: neurodevelopmental, pharmaceutical, lesion, and genetical. In this study we will focus on the neurodevelopmental (post-weaning social isolation) and pharmacological (ketamine) animal models of schizophrenia. Social isolation is not always a reliable model of schizophrenia as the behavioural impairments induced can be easily reversed when the animals are handled frequently during experimentation. Researchers have shown that ketamine causes psychotic behaviour in normal human volunteers and exacerbates symptoms in individuals with schizophrenia. Even though ketamine model is considered reliable, there are weaknesses associated with the model. Researchers used different dosages, and this may result in divergent or contradictory results. Due to weaknesses associated with the social isolation and ketamine models, researchers have tried combining the two models to produce what is known as the double hit model. We conducted a systematic review to evaluate the effectiveness of the rodent "double hit" (post-weaning social isolation and N-methyl-D-aspartate (NMDA) receptor antagonist, ketamine) model in producing symptoms of schizophrenia. This Review aimed to

evaluate the effectiveness of the rodent “double hit” (post-weaning social isolation and N-methyl-D-aspartate (NMDA) receptor antagonist) model to produce symptoms of schizophrenia. The MEDLINE (PubMed) and Ebscohost databases were used to search for studies. The systematic review is based on quantitative animal studies. Studies in languages other than English that can be translated sufficiently using Google translate were also included. Data extraction was performed individually by two independent reviewers and discrepancies between them were resolved by a third reviewer. SYRCLE’s risk of bias tool was used to test the quality and biases of included studies. Our primary search yielded a total of 47 articles. Seventeen articles met the inclusion criteria for this systematic review. Ten of the seventeen studies found that the “double hit” model was more effective in developing various symptoms of schizophrenia. Five studies showed that the “double hit” model was robust and capable of inducing cognitive impairments and positive symptoms of schizophrenia than either treatment alone. In our lab we designed a double hit model of schizophrenia combining post-weaning social isolation and ketamine (SIK) in order to induce negative and cognitive symptoms of schizophrenia.

A total of 32 male Sprague Dawley rats (21 days old) were acquired from the breeding unit of the University of KwaZulu-Natal. The animals were collected on PND21 because at that age they are independent not depending on dams for survival. On postnatal day (PND) 23 the animals were randomly separated to 4 different groups as follows: 8 group housed male rats + saline (0.9% NaCl) injected subcutaneously (GH), 8 grouped housed male rats + ketamine (16 mg/kg) injected subcutaneously (GHK), 8 socially isolated male rats + saline (0.9% NaCl) injected subcutaneously (SI), and 8 socially isolated male rats + ketamine (16 mg/kg) injected subcutaneously (SIK). The Shapiro-Wilk test was used to test for normality. Two-way ANOVA, followed by Bonferroni’s multiple comparisons test was used to analyse the data. Multiple comparisons will be conducted even in the absent of significant interaction effects because we want to compare the effect of the double hit model when compared to other groups. Our SIK animals group showed high anxiety like behaviour, with increased adrenocorticotropic hormone (ACTH) concentration (GH vs. SIK, $p<0.0001$; GHK vs. SIK, $p<0.0001$; SI vs. SIK, $p<0.0001$), corticosterone concentration (GH vs. SIK, $p<0.0001$; GHK vs. SIK, $p<0.0001$; SI vs. SIK, $p<0.0001$) and norepinephrine concentration (GH vs. SIK, $p<0.0001$; GHK vs. SIK, $p<0.0001$; SI vs. SIK, $p=0.0021$) when compared to the other groups. SIK animals showed reduced social interaction (GH vs. SIK, $p<0.0001$; GHK vs. SIK, $p=0.0022$; SI vs. SIK, $p<0.0001$) and decreased oxytocin concentration (GH vs. SIK, $p<0.0001$; GHK vs. SIK,

p<0.0001; SI vs. SIK, p<0.0001) in the amygdala. The SIK group of animals were more aggressive toward a juvenile intruder (GH vs. SIK, p<0.0001; GHK vs. SIK, p<0.0001; SI vs. SIK, p=0.0401) but had less testosterone concentration (GH vs. SIK, p<0.0001; GHK vs. SIK, p=0.0004; SI vs. SIK, p=0.0009). The SIK group showed impaired visual learning and memory and increased expression of proinflammatory cytokines IL 6 (GH vs. SIK, p<0.0001; GHK vs. SIK, p<0.0001; SI vs. SIK; p<0.0001), TNFα (GH vs. SIK, p<0.0001; GHK vs. SIK, p<0.0001; SI vs SIK, p<0.0001). We suggest that our double hit model, SIK, is more robust in inducing negative and cognitive symptoms of schizophrenia than each treatment alone.

We further investigated the behavioural and neurochemical changes associated with locomotor activity in schizophrenia using the double-hit model. The SIK group showed high locomotor hyperactivity (GH vs. SIK, p<0.0001; GHK vs. SIK, p<0.0001; SI vs. SIK, p=0.0003), which was accompanied by high dopamine D2 mRNA expression (GH vs. SIK, p<0.0001; GHK vs. SIK, p<0.0001; SI vs. SIK, p=0.028), high acetylcholine concentration (GH vs. SIK, p<0.0001; GHK vs. SIK, p=0.0004; SI vs. SIK, p<0.0001), lower glutamate concentration (GH vs. SIK, p<0.0001; GHK vs. SIK, p=0.0001; SI vs. SIK, p<0.0001), and GABA concentration (GH vs. SIK, p<0.0001; GHK vs. SIK, p=0.0006; SI vs. SIK, p=0.0001). We proposed that our double hit model is more robust in inducing behavioural and molecular changes associated with positive symptoms of schizophrenia. To improve our knowledge on the mechanisms involved in the pathophysiology of schizophrenia, we investigated the effect of the model on GABAergic function and oxidative stress. Group housed animal injected with ketamine (GHK) and social isolated animals injected with saline (SI) showed reduced mRNA expression of genes such as glutamate decarboxylate 1 (GAD1) (GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001), brain derived neurotrophic factor (BDNF) (GH vs. GHK, p<0.0001; GH vs. SI, p=0.0002), and parvalbumin (PVALB) (GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001), which are involved in GABAergic neurotransmission. The expression of these genes was reduced even further in the SIK group (GH vs. SIK, p<0.0001) in both the prefrontal cortex (PFC) and the hippocampus. The concentration of the antioxidants superoxide dismutase (SOD) (GH vs. SIK, p<0.0001; GHK vs. SIK, p=0.0005; SI vs. SIK, p<0.0001) and glutathione peroxidase (GPx1) (GH vs. SIK, p<0.0001; GHK vs. SIK, p=0.0003; SI vs. SIK, p<0.0001) was reduced in the SIK group while malondialdehyde (MDA) concentration (GH vs. SIK, p<0.0001; GHK vs. SIK, p<0.0001; SI vs. SIK, p<0.0001) was increased. In conclusion SIK showed that oxidative stress and dysfunction in the GABAergic pathway are involved in the pathophysiology of schizophrenia. Our double hit model was able to successfully induce behavioural and molecular

changes similar to those observed on positive, negative, and cognitive symptoms of schizophrenia. This double hit model will assist researchers to better understand the pathophysiology of schizophrenia, and this will assist researchers to develop better therapeutic drugs to treat schizophrenia.

Chapter 1: Literature review

1.1 Schizophrenia

Schizophrenia is a neuropsychiatric disorder that affects brain function, specifically individual thoughts and feelings (Millier et al., 2014). Schizophrenia symptoms are divided into three categories viz: positive symptoms, such as conceptual disorganisation, hallucinations, delusions, thought disorder; negative symptoms, such as avolition, social withdrawal, poverty of thought, anhedonia, emotional blunting, and cognitive symptoms, such as impaired executive function, impaired working memory (Andreasen, 1995, Canetta and Kellendonk, 2018). Globally, the prevalence of schizophrenia is approximately 1%, however this figure can potentially vary in different regions and social groups (van Os and Kapur, 2009, Keshavan et al., 2020). In the Sub-Saharan African region, in total the incidence of cases is roughly 1.3 million (Charlson et al., 2018). Schizophrenia has a severe impact on patients and their caretakers; it also causes a huge financial burden on the health care system (Winship et al., 2019). Symptoms progress either gradually or abruptly, and differ from one patient to another (Jones et al., 2011). Schizophrenia inception is usually post-adolescent (16-25 years); males present with a greater incidence of psychotic symptoms while females show a later onset of these symptoms (Jones et al., 2011). Schizophrenic patients show different symptom combination, which makes diagnosis and treatment very difficult (Jones et al., 2011).

Even though the aetiology of schizophrenia is debatable, it is a multivariable neurodevelopmental disorder affected by both environmental and genetic factors (Lewis and Lieberman, 2000, van Os et al., 2010). The first-generation antipsychotic drugs (haloperidol and chlorpromazine) are used to treat the psychotic symptoms (Remington, 2003, Jones et al., 2011). The atypical antipsychotic or second generation (clozapine, olanzepine, risperidone, and aripiprazole) have a reduced tendency to produce unwanted side effects (Remington, 2003). Available typical and atypical antipsychotic drugs have shown high effectiveness against positive symptoms of schizophrenia, while negative and cognitive symptoms showed resistance against available treatment (Hunter and Barry, 2012, Leucht and Davis, 2017, Saleh et al., 2023). Lack of understanding of mechanisms and pathways underlying negative and cognitive symptoms contribute to the reduced effects of typical and atypical antipsychotic drugs (Saleh et al., 2023). Due to this problem, there is an urgent need to develop an animal model of schizophrenia that will allow researchers to investigate pathways involved in the pathophysiology of schizophrenia. Understanding the pathophysiology of schizophrenia will

allow researchers to develop more effective drugs that can be used to treat all symptoms of schizophrenia. Animal models of schizophrenia are important preclinical tools that are used to investigate the neurobiological and molecular basis of schizophrenia (Powell and Miyakawa, 2006).

1.2 Animal models of schizophrenia

Different animal models of complex psychiatric disorders play a huge role in studying the neurobiological basis of the disorder (Winship et al., 2019). Animal models can assist in the identification of new drugs of interest that can be further investigated for more effective treatment in the future (Jones et al., 2011). The animal models offer a speedier podium to screen disease progression and the chance to conduct invasive monitoring of molecular and structural alterations that trigger the cause of the disease (Jones et al., 2011). All animal models of schizophrenia should have reliable symptom homology, must be able to imitate the theoretical neurobiological validation and pathology, and finally must be able to produce the anticipated pharmacological response, or lack of it to known treatment (Jones et al., 2011). Animal models of schizophrenia are divided into four categories: developmental, pharmacological, lesion and genetic manipulation (Carpenter and Koenig, 2008, Jones et al., 2011). Even though the lesion models of schizophrenia are reliable and able to induce most symptoms, there are shortfalls associated with these models (Chambers and Self, 2002, Richtand et al., 2006). Lesioning of animals is time-consuming and laborious because it requires surgery. Furthermore, this model is usually performed on 7-day's old pups and this requires the use of anaesthesia and operation, which increases the level of mortality (Richtand et al., 2006, Białoń and Wąsik, 2022). Genetic models of schizophrenia can induce brain morphological and physiological impairments similar to those observed in schizophrenia patients. Even though genetic models of schizophrenia are reliable, the production of the model is very expensive, time consuming, and complicated (Białoń and Wąsik, 2022). Both neurodevelopmental and pharmacological models of schizophrenia are easy to execute in the lab, they are inexpensive, and have a low mortality rate (Białoń and Wąsik, 2022). In this study, we will focus on the neurodevelopmental and pharmacological models. The neurodevelopmental models are used by researchers to explain the fact that exposing the neonate whether during gestation or the perinatal period or harsh environmental conditions increases susceptibility of an individual to schizophrenia.

1.3 Neurodevelopmental models of schizophrenia

Harsh environmental conditions such as maternal stress, infections, and malnutrition during the early life stages are some of the unfortunate conditions that increase an individual's susceptibility to schizophrenia. This is explained as a neurodevelopmental origin of the disorder (Lewis and Levitt, 2002). While the exact type of early life stress event may not be important, the time at which the event occurs is of importance (Jones et al., 2011). The neurodevelopmental models of schizophrenia are based on the idea that changes in the development and maturation of the hippocampus and the prefrontal cortex during prenatal and perinatal life cause long term impairment in the brain (Białoń and Wąsik, 2022). This animal model of schizophrenia utilizes the changes in the environment during the very sensitive perinatal period to induce permanent disruptions in the development of the central nervous system (Jones et al., 2011). The few neurodevelopmental models of schizophrenia available include post-weaning social isolation, maternal immune activation (MIA) and methylazoxymethanol acetate (MAM). There are disadvantages associated with MIA and MAM such as the fact that the day of gestation should be accurately identified and following intraperitoneal injection, the possibility of miscarriage increases (Białoń and Wąsik, 2022). Stressful conditions such as post-weaning social isolation during a critical growing period causes permanent disruptions in neurogenesis, and have also been proven to replicate core symptoms of schizophrenia (Jones et al., 2011).

1.3.1 Social isolation

1.3.1.1 Post-weaning social isolation

Post-weaning social isolation refers to the single housing of rat or mouse pups in individual cages from their age of weaning (between postnatal days 21 to 26) (Fone and Porkess, 2008). Post-weaning social isolation of rodents induces a variety of behavioural abnormalities such as locomotor hyper-activity, deficits in sensorimotor gating, increased fear to novelty (neophobia), and increased anxiety-like behaviour and aggression (Valzelli, 1973, Einon and Morgan, 1977, Heidbreder et al., 2000, Weiss et al., 2004, Marsden et al., 2011). Together these behavioural changes are called the isolation syndrome; furthermore, some of these features resemble key symptoms of schizophrenia (Fone and Porkess, 2008, Jones et al., 2011). Socially isolated rats are consistently more active than group housed littermates when placed in a novel environment (Fone et al., 1996, Silva-Gómez et al., 2003, Del Arco et al., 2004). This locomotor hyper-activity is observed as an increase in horizontal activity which can happen minutes after placement in the open field test apparatus and shows an inability to habituate in

an environment (Jones et al., 2011). Locomotor hyperactivity is believed to be caused by mesolimbic dopamine hyperactivity and this may be used as an index for the positive symptoms in schizophrenia (Jones et al., 2011).

Socially isolated animals can also produce behavioural impairments similar to those observed in cognitive symptoms of schizophrenia. Researchers have shown that socially isolated rodents were unable to discriminate between familiar and novel objects (King et al., 2009, McLean et al., 2010, Marsden et al., 2011). Failure to recognise the novel object is associated with the impaired visual learning and memory observed in schizophrenia patients (McClure et al., 2007). Both rodents and humans have a similar innate curiosity to explore novel objects over familiar ones and this is thought to investigate the visual episodic memory (Winters et al., 2008). In humans, this is translated to the visual learning and memory which is greatly affected in schizophrenia patients (Young et al., 2009). Social isolation is inexpensive, easy to execute and greatly mimic what exactly happened to human (Jones et al., 2011). The major shortfall of social isolation is fragility of behaviour produced that can be easily reversed by repeated handling or exposure to a number of tests during the development period (Jones et al., 2011). Furthermore, social isolation has proven to be weak in modelling negative symptoms of schizophrenia. Another model of schizophrenia that has showed to be more effective and reliable is the pharmacological model.

1.4 Pharmacological model of schizophrenia

Pharmacological models of schizophrenia produce outcomes that have promising translational significance across rodents, non-human primates, and humans (Jones et al., 2011). Treating rodent with psychotomimetic drugs induces hallucinations and psychotic episodes. These drugs have also been found to exacerbate schizophrenia symptoms in patients (Tsai and Coyle, 2002, Coyle et al., 2003). They include amphetamine (dopaminergic activator) and PCP or ketamine (glutamatergic antagonist) and they are widely used in rodent models of schizophrenia (Jones et al., 2011). Dopamine enhancers such as amphetamine, provide limited understanding to schizophrenia pathophysiology since the drug can successfully induce impairments related to positive symptoms of the disorder (Białoń and Wąsik, 2022). PCP is a schedule ll drug under the controlled substances act. Therefore the drug is not easily accessible to researchers (Shields et al., 2019). The glutamate idea in schizophrenia is derived from the already available information suggesting that acute administration of NMDAR inhibitor such as ketamine induces schizophrenia-like symptoms in humans (Coyle et al., 2003). Ketamine has been

shown to induce psychotic behaviour in normal human volunteers, and also exacerbates symptoms in individuals with schizophrenia (Coyle et al., 2012).

1.4.1 Ketamine

Ketamine (2-chlorphenyl-2-methylamino-cyclohexanone) is a PCP derivative. It was initially produced by Calvin Stevens of the Parke-Davis pharmaceutical firm in 1962 and officially described in 1965 (Domino et al., 1965, Rowland, 2005). Due to PCP's neurotoxicity and emergence symptoms, such as confusion, hallucinations, and delirium, researchers proposed ketamine as a safer substitute to PCP (Rowland, 2005). Currently, ketamine is internationally used as a general dissociative anaesthetic in both human and veterinary medicine, but it is not considered as a primary anaesthetic (Rowland, 2005). Schizophrenic patients injected with a subanaesthetic dose of ketamine showed degeneration of mental status (Malhotra et al., 1997, Lahti et al., 2001). Healthy individuals treated with ketamine developed signs of both positive and negative symptoms of schizophrenia (Beck et al., 2020). Studies on humans, monkeys, and rodents reported that subchronic and acute ketamine injection induce memory impairments (Ranganathan et al., 2017, Białoń et al., 2020, Kozela et al., 2020, Cao et al., 2021, Roussy et al., 2021). The ketamine model of schizophrenia is found to be more reliable for studying neurotransmitter disturbance and other features related to the disorder (Frohlich and Van Horn, 2014).

Ketamine can induce hyperlocomotion, impair sensorimotor gating, anxiety-like behaviour, and impair social behaviour (Damazio Pacheco et al., 2019, Wąsik et al., 2019, Xu et al., 2019, Zoupa et al., 2019, Fujikawa et al., 2021, Sedky and Magdy, 2021, Azimi Sanavi et al., 2022). Ketamine has been reported to induce changes in the molecular structures, such as changes in the brain derived neurotrophic factor (BDNF) which sustains cell viability and physiological neuronal function (Becker et al., 2008). The expression of BDNF positive cells was reported to be reduced in the frontal cortex, striatum, hippocampus and posterior cingulate cortex of animals treated with subchronic levels of ketamine (Ben-Azu et al., 2018, Xu et al., 2019). Furthermore, expression of BDNF protein was decreased in the hippocampus, striatum, frontal cortex, and amygdala of animals treated with subchronic levels of ketamine (Canever et al., 2018, de Araújo et al., 2021). Similar changes in the BDNF have been observed in schizophrenia patients (Carlino et al., 2011).

The ketamine model is inexpensive, very simple, and it is able to produce negative and cognitive symptoms of schizophrenia (Białoń and Wąsik, 2022). Despite all these benefits, the

ketamine model has some shortfalls. Researchers have used different doses and regiments which produce varying results. Ketamine is usually given to adult rats, which does not reflect the construct validity of the proposed neurodevelopmental genesis of schizophrenia (Jones et al., 2011). The glutamate hypothesis provides an explanation for the onset of schizophrenia, which is early adulthood, and this is the reason why researchers found it interesting (Coyle et al., 2012).

1.5 Neurotransmitter dysfunction in schizophrenia

1.5.1 The role of glutamate in schizophrenia

The amino acid glutamate is the primary excitatory neurotransmitter through the glutamatergic mechanism in the central nervous system (Meldrum, 2000). Glutamatergic neurons approximately use between 60 and 80 percent of total brain metabolic activities (Rothman et al., 2003). The glutamate hypothesis of schizophrenia developed two decades ago, is based on the idea that NMDA receptor antagonist such as ketamine will induce symptoms similar to those observed in schizophrenic patients (Frohlich and Van Horn, 2014). Studies conducted on patients with chronic symptoms of schizophrenia showed reduced glutamate concentration (Ongür et al., 2009, Tayoshi et al., 2009, Natsubori et al., 2014). However, results on glutamate concentrations in schizophrenia patients have not been consistent because other studies reported increased glutamate concentration in the brain (de la Fuente-Sandoval et al., 2011, Tandon et al., 2013). Glutamatergic neurotransmission is carried out through ionotropic and metabotropic glutamate receptors. NMDAR is an ionotropic glutamate receptor defined after N-methyl-D-aspartate, which is the selective activator of the glutamate receptor (Frohlich and Van Horn, 2014). Besides NMDAR, there are other two groups of ligand-gated ionotropic glutamate receptors such as, α-animo-3-hydroxy-5-methyl-4-isoxazoleproprionic acid receptors (AMPAR) and kainite receptors (Frohlich and Van Horn, 2014). Even though other glutamate receptors have been implicated in schizophrenia, the dominant hypothesis is for the dysfunction observed in the NMDA receptor (Stone et al., 2007).

The NMDAR dysfunction hypothesis in schizophrenia comes about following the observation that non-competitive NMDAR antagonists such as phencyclidine (PCP), ketamine, and MK801 produce immediate psychological effects, which resemble symptoms that are observed in schizophrenia (Jones et al., 2011). A study performed by Olney and Farber shows that animals treated with NMDAR antagonist produce neurotoxicity changes in cortical brain regions which mimic negative and cognitive symptoms, which is proposed to be similar to what is observed

in schizophrenia patients (Olney and Farber, 1995). One study proposed that NMDAR antagonist might have a preferential effect on NMDAR expressed on GABA neurons, but another study challenged that proposal (Homayoun and Moghaddam, 2007, Rotaru et al., 2012). NMDAR antagonists have been proven to reduce GABAergic interneuron function, and this is believed to cause a rise in pyramidal cell firing due to disinhibition (Behrens et al., 2007, Korotkova et al., 2010).

1.5.2 The role of GABA in schizophrenia

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system (Benes, 2015). Impaired GABAergic signalling can induce imbalances between the excitatory and inhibitory neurotransmission which is believed to be the major contributor to the pathophysiology of schizophrenia (Benes and Berretta, 2001, Guidotti et al., 2005, Tso et al., 2015). Dysfunction in GABA may induce all symptoms of schizophrenia (Tso et al., 2015). Dysfunction in GABA biomarkers such as Ca^{2+} binding protein parvalbumin, the enzyme glutamic acid decarboxylase of 67 kDa (GAD1/GAD67), and brain derived neurotrophic factor (BDNF) have been implicated in the pathophysiology of schizophrenia (Curley et al., 2011, Chung et al., 2016, Porcher et al., 2018).

Glutamic acid decarboxylase 67 (GAD67) is the synthesizing enzyme that controls the secretion of GABA by converting glutamic acid to GABA (Asada et al., 1997). Reduced expression of GAD67 mRNA in grey matter has been reported on schizophrenic patients when compared to normal controls (Lewis et al., 2012). Furthermore, decreased GAD67 protein expression measured by western blot and immunofluorescence was reported in schizophrenia patients (Curley et al., 2011). Binding protein parvalbumin is responsible for calcium transport, calcium buffering, and protection of neurons against calcium overload. This enzyme is mainly housed in the GABA neurons (Permyakov and Uversky, 2022). Deficits in the mRNA expression of the calcium binding protein parvalbumin have been reported in schizophrenia patients (Chung et al., 2016, Volk et al., 2016). Brain derived neurotrophic factor (BDNF) is responsible for the development and maturity of the neuronal circuit, as well as the manufacturing of inhibitory neurons throughout the growth period (Gubellini et al., 2005, Gottmann et al., 2009). A dysfunction in BDNF processing is associated with psychiatric diseases such as schizophrenia (Carlino et al., 2011, Garcia et al., 2012). $GABA_A$ receptor agonist (muscimol) reverses increased extracellular dopamine in the prefrontal cortex induced by NMDA receptor antagonists (Yonezawa et al., 1998). Furthermore, another $GABA_A$

receptor agonist (TPA023) reverses working memory deficits induced by ketamine in rhesus monkeys (Castner et al., 2010). Researchers have proposed that GABA can be utilized as treatment for schizophrenia because of its ability to inhibit dopamine signalling (Garbutt and van Kammen, 1983).

1.5.3 The role of dopamine in schizophrenia

Dopamine belongs to the catecholamine family of neurotransmitters located in the central nervous system (Hornykiewicz, 1966). The dopamine effect in the pathophysiology of schizophrenia was observed when researchers found that treating animals with amphetamine and other compounds that increase extracellular concentration of dopamine can induce psychotic symptoms identical to those seen in schizophrenia patients (Lieberman et al., 1987). Researchers have shown that patients with prodromal symptoms of schizophrenia (early signs of schizophrenia that precedes the manifestation of full symptoms) have elevated dopamine synthesis capacity (Howes et al., 2009, Egerton et al., 2013). This increased capacity was observed on individuals with psychotic symptoms of schizophrenia (Howes et al., 2011a, Howes et al., 2011b). Other studies showed that injecting animals with drugs that inhibit dopamine such as reserpine can reduce psychotic symptoms (Carlsson et al., 1957). The major impairments and dysfunction observed in schizophrenic disorder are caused by negative and cognitive symptoms of the disorder (Howes et al., 2015). Research conducted on individuals with prodromal indicators of schizophrenia showed increased striatal dopamine production which was directly proportional to the weakened performance on cognitive tasks (Howes et al., 2009). Furthermore, this increase in dopamine production was positively correlated with the impaired cortical function during the onset of schizophrenia (Fusar-Poli et al., 2010, Allen et al., 2012).

The support for dopamine involvement in schizophrenia was strengthened by the fact that the effectiveness of antipsychotic drugs directly depends to their affinity for dopamine receptors (Creese et al., 1976). Postmortem studies showed that neurobiological alterations in schizophrenia involved the increase in striatal dopamine concentration and an increase in D2 receptors (Owen et al., 1978, Mackay et al., 1982). An increase in tyrosine hydroxylase, an enzyme involved in the production of dopamine has been reported in the substantial nigra of patients with schizophrenia compared to their counterparts (patients with depression and healthy controls) (Howes et al., 2013). Furthermore, this means that there is an increased production of dopamine in the midbrain section of dopamine neurons including their striatal

terminals (Howes et al., 2015). All current available antipsychotic treatments inhibit D2 dopamine receptors. They also target other dopamine receptors and receptors for histamine, norepinephrine, serotonin, and acetylcholine in the brain (Howes et al., 2015). One third of patients with schizophrenia is resistant to non-clozapine antipsychotic drugs. They also do not respond to drugs that inhibit presynaptic dopamine receptors (Mortimer et al., 2010, Remington et al., 2012). Even though there is enough evidence about dopamine and glutamate on schizophrenia, other researchers are investigating the effect of acetylcholine neurotransmitter or cholinergic neurotransmission on schizophrenia pathophysiology (Raedler et al., 2007).

1.5.4 The role of acetylcholine in schizophrenia

Acetylcholine controls a number of physiological and behavioural processes in the peripheral and central nervous system (Jones et al., 2012). In the central nervous system, acetylcholine controls sensory perception, sleep/wake cycle, cognitive processing, and arousal (Abrams et al., 2006). The central cholinergic neurotransmission controls brain function in a number of regions thought to be affected by schizophrenia (Jones et al., 2012). Acetylcholine accomplishes its neurotransmission by the activation of its cell surface muscarinic and nicotinic receptors (Jones et al., 2012). Changes in the muscarinic cholinergic neurotransmission is implicated in the pathophysiology of schizophrenia (Jones et al., 2012). Muscarinic acetylcholine receptor antagonists in health induce cognitive impairments and psychotic like behaviour similar to those observed in schizophrenic patients (Hamborg-Petersen et al., 1984, Tandon et al., 1991). Muscarinic acetylcholine receptor agonists improve cognitive function and reverse cognitive impairment induced by a cholinergic lesion (Decker and Majchrzak, 1992, Jones et al., 2012). Dysfunction in the nicotinic acetylcholine receptor may be involved in the pathophysiology of schizophrenia (Jones et al., 2012). Individuals with schizophrenia have high incidence of smoking cigarette when compared to normal individuals or people with other psychiatric diseases (Campo-Arias et al., 2006). Acute treatment with a nicotinic agonist, has been shown to improve cognitive function specifically in behaviours related to attention and vigilance (AhnAllen et al., 2008, Jubelt et al., 2008). Even though the effect of dysfunction of different neurotransmitters in schizophrenia has been highly documented, other researchers are investigating the role of different hormones in the pathophysiology of schizophrenia.

1.5.5 The role of oxytocin in schizophrenia

Oxytocin is a hydrophilic cyclic neuropeptide that is produced in the hypothalamus and released through the posterior pituitary (Goh et al., 2021). Responses to emotional processing

such as fast cue detection and emotional recognition are referred to as lower order social cognition processes (Guastella et al., 2015). Oxytocin plays an important role in social cognition, by working with the dopaminergic neural pathway to facilitate the reward system and the inhibition of defensive behaviour (Ellenbogen, 2018). Abnormal changes in the signalling of oxytocinergic and dopaminergic reward systems in the amygdala can induce dysfunction in emotional processing and lead to impaired social responses (Rosenfeld et al., 2011). Meta-analysis study reported that an intranasal oxytocin treatment improved the emotional recognition of both happy and fearful faces, this supports the idea that oxytocin affects social interaction (Van and Bakermans-Kranenburg, 2012, Shahrestani et al., 2013).

1.6 Neuropathology of schizophrenia

1.6.1 Proinflammatory cytokines and schizophrenia

Neuroinflammation is induced by the activation of microglia cells located in the brain. They secrete proinflammatory cytokines (Doorduin et al., 2009, Monji et al., 2009). Furthermore, few studies have been conducted in trying to investigate the relationship between immune markers and symptoms of schizophrenia (Khandaker et al., 2015). Cytokines are soluble polypeptides that activate proteins which are responsible for initiating and sustaining the immune response (Potvin et al., 2008). Pathogens stimulate proinflammatory cytokines and other important immune factors, such as: interleukin 1β, interleukin 6, and tumour necrosis factor α (TNFα) which play a vital function in the early defence mechanism against infections (Ashdown et al., 2006, Meyer et al., 2009).

In the adult brain, proinflammatory cytokines have been found to be involved in neuronal death after injury or neurodegenerative injury (Allan and Rothwell, 2003). Previous studies that have investigated the cytokines in the cerebrospinal fluid have reported an increased concentration of interleukin 6 in schizophrenia patients (Khandaker et al., 2014). Increased interleukin 6 concentration in the brain was reported in schizophrenic patients, which suggested the involvement of inflammation in the pathophysiology of schizophrenia (Potvin et al., 2008). Furthermore, increased interleukin 6 was reported in the cerebrospinal fluid of schizophrenic patients (Garver et al., 2003, Hayes et al., 2014). Another cytokine of interest is TNFα, which has been found to be increased in the plasma schizophrenia and remains high even after treatment (Goldsmith et al., 2016). In animal models, ketamine injections caused an imbalance between pro and anti-inflammatory cytokines (Białoń and Wąsik, 2022). A single ketamine injection in mice induced increased concentration of the proinflammatory cytokine interleukin

6 (IL-6) in the entire brain, specifically increased mRNA and protein expression of IL-6, and TNF-α was recorded in the hippocampus (da Silva Araújo et al., 2017, de Araújo et al., 2021). Interestingly, another study reported no significant changes in the concentration of IL-6 and TNF-α in different brain regions of rats that were subchronically treated with ketamine (Réus et al., 2017). We would like to investigate if the double hit model of schizophrenia will be able to induce changes in cytokine concentration and expression like those observed in schizophrenic patients. Cytokines can potentially increase oxidative stress by inducing the secretion of toxic nitric oxide which stimulate the hypothalamic pituitary adrenal axis to secrete cortisol in humans and corticosterone in animals (Dantzer et al., 2008, Miller et al., 2009).

1.6.2 Oxidative stress and schizophrenia

The brain is the most actively metabolic organ of the body, and it also produces high levels of reactive oxygen particles (Valko et al., 2007). This burden is made worse by several factors, such as: oxidative potential of monoamines like dopamine and excitatory neurotransmitters (glutamate), and the susceptibility of brain lipid elements to oxidation (Valko et al., 2007). Even though free radicals are needed for several physiological functions, if their metabolism is being dysregulated, they can cause damage to a number of cell components, such as lipids via peroxidation, protein via carboxylation and nucleic acids via oxidative damage (Valko et al., 2007). The body can easily control its oxidative status through homeostasis, oxidative stress can be caused by increased production of reactive oxygen species (hydrogen peroxide or superoxide) or reactive nitrogen species (nitric oxide) (Valko et al., 2007). Antioxidant such as glutathione (GSH) scavenges on reactive oxygen species and leads to the protection of neuronal cells and inhibits the effect of oxidative stress (Upthegrove and Khandaker, 2020). Reduced concentrations of GSH have been reported in individuals suffering from acute psychosis when compared to their normal health counterparts (Wood et al., 2009, Raffa et al., 2011).

Dysfunction in the synthesis of GSH causes oxidative stress and reduced parvalbumin immunoreactivity in the hippocampus (Steullet et al., 2010). One study reported a reduction in GSH in relapse schizophrenic patients, furthermore reduction in other antioxidants such as superoxide dismutase (SOD) and catalase (CAT) was also reported (Flatow et al., 2013). Studies on schizophrenic patients and postmortem brain tissue showed decreased GSH concentration in the brain and the cerebrospinal fluid (Do et al., 2000, Yao et al., 2006). The functional outcomes caused by increased oxidative stress or impaired defence mechanisms against oxidative stress in the brain still need to be further investigated (Upthegrove and

Khandaker, 2020). According to available information on neurochemical alteration in schizophrenia, it has been suggested that oxidative stress causes over stimulation of dopamine receptor function (Upthegrove and Khandaker, 2020). Negative symptoms of schizophrenia have been linked to low concentration of GSH (Upthegrove and Khandaker, 2020).

Under stressful events, the body activates the HPA axis which is the primary response to a stressful stimulus (Stephens and Wand, 2012). The activity of the HPA axis begins in the hypothalamus where the paraventricular nucleus secretes corticotrophin-releasing hormone (CRH) (Jedema and Grace, 2004). The CRH stimulates the secretion of adrenocorticotrophic hormone (ACTH) in the pituitary gland (Jedema and Grace, 2004). ACTH induces the secretion of glucocorticoids (cortisol or corticosterone) from the adrenal gland (Stephens and Wand, 2012). Hormones of the HPA axis act in a negative feedback loop, as produced glucocorticoid inhibits its own production by signalling a negative feedback to the hypothalamus and the pituitary gland to reduce the secretion of CRH and ACTH (Stephens and Wand, 2012). Both clinical and biological data showed an impaired biological response to stress in individuals with schizophrenia (Walker and Diforio, 1997), this is linked to the dysfunction of the HPA axis (Sinclair et al., 2011, Ciufolini et al., 2014). Studies investigating the relationship between cortisol and schizophrenia showed that patients suffering from schizophrenia have a tendency of having high basal cortisol concentration when compared to normal controls (Garner et al., 2011, Corcoran et al., 2012). Furthermore, available antipsychotic drugs reduce ACTH and cortisol in schizophrenic patients (Cesková et al., 2006). A negative correlation exists between cognition and glucocorticoids in animal models and patients suffering from schizophrenia (Walder et al., 2000, Wolf, 2003, Lee et al., 2007a). Increased corticosterone concentration is associated with decreased structural volume of the hippocampus and the prefrontal cortex (Sapolsky et al., 1990, Carrion et al., 2010). This volume loss in the hippocampus and the prefrontal cortex is highly observed in schizophrenic patients (Webster et al., 2002, Sinclair et al., 2011).

Conclusion

Available typical and atypical antipsychotic drugs can reverse positive symptoms of schizophrenia, while negative and cognitive symptoms remain resistant to the available treatment (Nuechterlein et al., 2004, Keefe et al., 2007). Lack of understanding of mechanisms underlying negative and cognitive symptoms contribute to the resistant of these symptoms on the available drugs used to treat schizophrenia. Developing an animal model that will be able

to induce negative and cognitive symptoms of schizophrenia will assist researchers to better understand the pathophysiology of schizophrenia. Using an animal model of schizophrenia to further investigate or study the pathophysiology of schizophrenia will assist in the development of new drugs that can be used in the treatment of schizophrenia. Social isolation as an animal model of schizophrenia has weaknesses such as reversal of behavioural impairments by repeated handling of the animals (Fone and Porkess, 2008). The ketamine model is usually used in older animals, and this does not properly represent what is observed in schizophrenia as it is for a neurodevelopmental psychiatric disorder. Ketamine is an NMDA receptor antagonist (Krystal et al., 1994, Morgan and Curran, 2006), but its effects on glutamate dysfunction is not fully explained. There is plausible evidence of increased dopamine concentration and dopamine D2 receptor density in the pathophysiology of schizophrenia (Howes et al., 2015), but their role on negative and cognitive symptoms of schizophrenia is still not clear. Developing a double hit animal model of schizophrenia that will be able to induce most the symptoms of schizophrenia will assist researchers in further investigating behavioural and molecular changes involved in the disorder.

Aim of the study

In this study we aim to develop a double hit animal model of schizophrenia that will be able to mimic behavioural and molecular changes similar to those observed from positive, negative, and cognitive symptoms of schizophrenic patients.

Objectives of the study

Systematic review will be conducted to investigate the face, construct, and predictive validity of the double hit model of schizophrenia. Manuscript 1 will characterise behavioural and molecular changes associated with the negative and cognitive symptoms of schizophrenia using our double hit model. Furthermore, manuscript 2 will evaluate neurotransmitter changes and receptor changes associated with the positive symptoms of schizophrenia using our double hit model. Lastly, manuscript 3 will use the double hit model to explore mechanisms and pathways involved the pathophysiology of schizophrenia.

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PROLOGUE

Chapter 2 of this thesis is prepared in manuscript format. It is a systematic review focusing on the different double hit models used in research. There are several animal models of schizophrenia that are available which can be used to study behavioural and molecular changes involved in the disease. Most models of schizophrenia do not cover all symptoms of schizophrenia. Hence, researchers have tended to use the double hit models, where two models of the disease are sequentially in order to enhance the effect. In this study we conducted a systematic review in order to investigate the effectiveness of the double hit models when compared to single hit models. The manuscript is titled "***Investigating the robustness of a rodent "double hit" (post-weaning social isolation and NMDA receptor antagonist) model as an animal model for schizophrenia: A Systematic Review*".** This systematic review was published in the journal "**Brain Sciences**" and is available online **(Appendix 1),** the chapter has been formatted according to the journal's guidelines for authors.

Chapter 2

Investigating the robustness of a rodent "double hit" (post-weaning social isolation and NMDA receptor antagonist) model as an animal model for schizophrenia: A Systematic Review

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Abstract: Schizophrenia is a debilitating psychiatric disorder comprising positive, negative, and cognitive impairments. Most of the animal models developed to understand the neurobiology and mechanism involved in schizophrenia do not produce all the symptoms of the disease. Therefore, researchers need to develop new animal models with greater translational reliability, and the ability to produce most if not all symptoms of schizophrenia. This Review aimed to evaluate the effectiveness of the rodent "double hit" (post-weaning social isolation and N-methyl-D-aspartate (NMDA) receptor antagonist) model to produce symptoms of schizophrenia. This systematic review was developed according to the PRISMA guidelines and checklist of 2020. The MEDLINE (PubMed) and Ebscohost databases were used to search for studies. The systematic review is based on quantitative animal studies. Studies in languages other than English that can be translated sufficiently using Google translate were also included. Data extraction was performed individually by two independent reviewers and discrepancies between them were resolved by a third reviewer. SYRCLE's risk of bias tool was used to test the quality and biases of included studies. Our primary search yielded a total of 47 articles, through different study selection processes. Seventeen articles met the inclusion criteria for this systematic review. Ten of the seventeen studies found that the "double hit" model was more effective in developing various symptoms of schizophrenia. Most studies showed that the "double hit" model is robust and capable of inducing cognitive impairments and positive symptoms of schizophrenia.

Keywords: Post-weaning social isolation (SI); NMDA receptor antagonist; Ketamine; Phencyclidine (PCP); Dizocilpine (MK801); animal models; basic research, rodents; schizophrenia

1. Introduction

Schizophrenia is a severe psychiatric disorder that is generally characterized by profound impairment in thinking which affects language, sense of self, and perception (McGrath et al., 2008). Schizophrenia affects more than 21 million individuals globally (McGrath et al., 2008). The symptoms of the disease are divided into three categories viz. positive, negative, and cognitive (Crow, 1980, Andreasen, 1995). Positive symptoms include hallucinations, and delusions) and negative symptoms include avolition, deficits in social functioning, anhedonia, and blunted effects, while cognitive symptoms are deficits in attention and perception, in executive control, working and long-term memory (Bowie and Harvey, 2005, Pratt et al., 2012). Schizophrenia onset usually manifests during post adolescence (16-25 years) (Jones et al., 2011). Males usually show significantly higher incidences of schizophrenia than females (McGrath, 2006). Schizophrenia treatment focuses on the stage when the affected patients show clear clinical symptoms such as psychosis (Kahn and Sommer, 2015). The well documented mechanisms of action for the antipsychotic treatment are to correct the high levels of dopamine turnover in the striatum (Lin et al., 2011). Schizophrenic patients with first episode of the disorder respond reasonably well to the treatment, the challenge is to keep them in a good state (Kahn et al., 2008, Robinson, 2010). Previous studies have proposed that D-serine and glycine can improve negative and positive symptoms, but that has not been observed on cognitive impairments (Singh and Singh, 2011, Heresco-Levy and Javitt, 2004). α 2/3-selective agonist and α 5-selective inverse agonist are selective GABAergic drugs that have been proposed to improve cognition in schizophrenia patients (Kahn and Sommer, 2015). Animal models of schizophrenia are used to study multifactorial psychiatric disorders including the neurobiological basis of different disorders (Jones et al., 2011).

When compared to humans, animal models produce a speedy platform examine structural and molecular alterations that trigger the pathology, and to test innovative therapeutics that are not possible to investigate in humans (Jones et al., 2011). There are more than 20 different animal models of schizophrenia, and they are divided into four categories: developmental, lesion, genetic manipulation, and drug induced (Jones et al., 2011). Rodents are social mammals and housing in a group or in isolation has been shown to affect behaviour (Brown and Grunberg, 1995, Arakawa, 2005). Post-weaning social isolation of rats by placing them individually, results in social deprivation that causes permanent changes in brain development which may lead to behavioural deficits in adulthood (Lapiz et al., 2001, Fone and Porkess, 2008). Post-weaning social isolation in rodents, produces spontaneous hyper-locomotor activity,

sensorimotor gating deficits, enhanced response to novelty, heightened anxiety states, aggression, and cognitive impairment (Fone and Porkess, 2008, Valzelli, 1973, Einon and Morgan, 1977, Heidbreder et al., 2000, Weiss et al., 2004, Marsden et al., 2011). All these changes are collectively called isolation syndrome, and some resemble the positive symptoms of schizophrenia. When placed in an averse novel arena, socially isolated rats are more active than non-isolated counter parts (Silva-Gómez et al., 2003, Del Arco et al., 2004). The robustness of social isolation can be reduced by type of rat strain, gender, caging condition, and routine handling (Weiss et al., 1999). Positive symptoms can be treated with antipsychotics drugs such as haloperidol and olanzapine, while negative and cognitive symptoms remain resistant to the available antipsychotic treatment (Jones et al., 2011).

Studies have shown that glutamatergic pathway dysfunction is a fundamental pathological shift seen in schizophrenia (Olney and Farber, 1995, Tsai and Coyle, 2002, Coyle et al., 2003). The N-methyl-D-aspartate receptor (NMDAR) has been shown to play a role in the pathophysiology of schizophrenia (Coyle et al., 2003). Treating rats with NMDA glutamate receptor antagonists (PCP or MK801 or ketamine) induces several behavioural abnormalities such as impairments in reversal learning, social interaction, prepulse inhibition (PPI), and working memory (Martinez et al., 1999, Rung et al., 2005, Abdul-Monim et al., 2007, Beninger et al., 2009). These are similar to those observed in schizophrenia. Most rodent models of schizophrenia tend to replicate aspects of the positive symptoms. Less than 20 percent of animal models of schizophrenia can produce all symptoms. Therefore, there is an urgent need for the development of a schizophrenia animal model that will adequately replicate positive, negative, and cognitive symptoms of the disorder. Researchers have started designing the "double hit" model by combining post-weaning social isolation and NMDA receptor antagonist to develop a strong animal model of schizophrenia that has the potential to replicate positive, negative, and cognitive symptoms (Castillo-Gómez et al., 2017, Liu et al., 2017, Garcia-Mompo et al., 2020).

1.2 Study aim.

1.2.1 The present review aimed to evaluate work published on the effectiveness of the "double hit" model in producing all the symptoms of schizophrenia.

1.3 Study objectives

1.3.1 To determine the effectiveness of "double hit" model of schizophrenia in producing positive, negative, and cognitive symptoms of schizophrenia.

1.3.2 To assess the strength of the “double hit” model of schizophrenia as a reliable developmental model of schizophrenia.

2. Methods

This systematic review is registered at the International Prospective Register of Systematic reviews (PROSPERO) (CRD42021247585). This systematic review was developed according to the preferred reporting items for systematic review and meta-analysis (PRISMA) guidelines and checklist of 2020 (Page et al., 2021).

2.1. Search Strategy

Medical search headings (MeSH) were used in the formulation of a systematic search strategy. PubMed, and Ebscohost, databases were utilized to search for published studies. Literature search focus only to the English language and other languages easily translated by Google Translate, and animals (rodent) subjects, all databases were searched until 30 May 2023. All included studies reference lists were scanned to confirm the literature saturation. Lastly, the bibliography of all the included studies was circulated to the systematic review team, and other schizophrenia expects chosen by the team. The literature search was done by two independent authors (KBS and ML) and (MM) was approached for arbitration. The literature search was based on these keywords and rodent subject headings: “schizophrenia”, AND “social isolation”, AND “NMDA receptor antagonists”, AND “basic research”, AND “animal models”.

2.2. Selection Process

The systematic review is based on quantitative animal studies. Studies in other languages that were successfully and sufficiently translated to English by Google Translate were also included. The process of screening studies was conducted by two authors independently (KBS and ML) to eliminate any inconsistencies in terms of the fitness of studies. The screening of studies was done in the following order: tittle, abstract, keywords and synonyms followed by full-text screening. All studies that induce schizophrenic-like symptoms using a “double hit” rodent model (post-weaning social isolation and NMDA receptor antagonist) were included. Any disagreements between two authors (KBS and ML) were resolved by allowing the third author (MM) to screen those studies and then after discussions the agreement was reached. The Prisma flow diagram was utilized to document the final selection process. All included studies were subjected to data collection, and quality assessment.

2.3. Data extraction

Data extraction was executed by two authors independently (KBS and ML), and the third author (MM) was approached in case of any disagreements between two authors. All important information such as: the authors, country, year of publication, study design, sample size, rodent characteristics (strain, gender, and age), NMDA receptor antagonists used and dosage, period of isolation, symptoms severity, types of control used, and control of symptoms if included were recorded in a Microsoft excel table created by two authors (KBS and ML). The third author (MM) read and approved the content of Microsoft Excel table. Included studies consisted of different social isolation periods, different NMDA receptor antagonists and different NMDA receptor antagonist dosages. Groups from various arms of the study were merged into a single group to avoid the possibility of introducing the bias caused by multiple statistical comparisons with one control group.

2.4. Risk of bias and quality assessment

SYRCLE's risk of bias tool was utilized to test the quality and possibility of bias in all included studies (Hooijmans et al., 2014). SYRCLE risk of bias tool is a derived version of the Cochrane risk of bias tool. Evidence from included studies was critically appraised by SYRCLE risk of bias tool. SYRCLE risk of bias is made up of ten entries, and these entries answers questions that are related to the following topics: selection bias, detection bias, performance bias, reporting bias, attrition bias, and other biases (Hooijmans et al., 2014). To assist in reaching the strong judgment additional signaling questions are included. "Yes" means low risk of bias; "no" means high risk of bias; and "unclear" means an unclear risk of bias (Hooijmans et al., 2014). If one of the questions is answered with "no," this means high risk of bias for that entry (Hooijmans et al., 2014). Two independent authors (KBS and ML) evaluated the quality of each included studies. In case of disagreements between two authors the third author (MM) was asked to adjudicate.

3. Results

3.1 Study selection

Our primary search yielded a total of 47 articles (Figure 1). Through different study selection processes, 17 articles met the inclusion criteria for this systematic review (Castillo-Gómez et al., 2017, Liu et al., 2017, Garcia-Mompo et al., 2020, Tuboly et al., 2009, Ashby et al., 2010, Simpson et al., 2010, Hickey et al., 2012, Simpson et al., 2012, Hawken et al., 2013, Inta et al.,

2013, Petrovszki et al., 2013, Gaskin et al., 2014, Gaskin et al., 2016, Wu et al., 2016, Shortall et al., 2020, Hamieh et al., 2021, Klimczak et al., 2021). Eight studies used Sprague Dawley rats (Liu et al., 2017, Ashby et al., 2010, Simpson et al., 2010, Hickey et al., 2012, Simpson et al., 2012, Hawken et al., 2013, Inta et al., 2013, Wu et al., 2016), three studies used Lister-hooded rats (Gaskin et al., 2014, Gaskin et al., 2016, Shortall et al., 2020), three studies used Wistar rats (Tuboly et al., 2009, Petrovszki et al., 2013, Hamieh et al., 2021), and another three studies used mice (Castillo-Gómez et al., 2017, Garcia-Mompo et al., 2020, Klimczak et al., 2021). All studies used male animals except one that used both male and female animals (Petrovszki et al., 2013). Most studies originated from Europe with the United Kingdom (Gaskin et al., 2014, Gaskin et al., 2016, Shortall et al., 2020), and Spain (Castillo-Gómez et al., 2017, Garcia-Mompo et al., 2020, Klimczak et al., 2021) each had three studies, Hungary (Tuboly et al., 2009, Petrovszki et al., 2013) had two, while France (Hamieh et al., 2021) and Germany (Inta et al., 2013) had one each. North America (Canada) produced five papers (Ashby et al., 2010, Simpson et al., 2010, Hickey et al., 2012, Simpson et al., 2012, Hawken et al., 2013), and Asia (China) two papers (Liu et al., 2017, Wu et al., 2016).

3.2 Risk of bias and quality assessment.

The quality scores of each study assessing the risk of bias are displayed in table 1. All studies showed high level of quality. There is one study with "no" response to the question, this response negatively affects the quality of the study (Wu et al., 2016). All studies included the control group or animals without treatment (group housed + saline/vehicle), and all studies used the control group as the normal group.

3.3 Effectiveness and robustness of the "double hit" (post-weaning social isolation and NMDA receptor antagonist) model of schizophrenia.

Ten studies found that the "double hit" model is more effective when compared to the intervention using social isolation on its own or an NMDA receptor antagonist (Castillo-Gómez et al., 2017, Garcia-Mompo et al., 2020, Simpson et al., 2010, Inta et al., 2013, Petrovszki et al., 2013, Gaskin et al., 2014, Gaskin et al., 2016, Shortall et al., 2020, Hamieh et al., 2021, Klimczak et al., 2021). Three of these studies were able to produce positive symptoms of schizophrenia, which is the impairment on the locomotor activity and molecular changes associated with this symptom (Simpson et al., 2010, Gaskin et al., 2016, Hamieh et al., 2021). Other studies showed that the "double hit" model produced impairments on the novel object discrimination test which is related to cognitive symptom (Gaskin et al., 2014, Gaskin et al.,

2016), furthermore another study showed impairment on the social recognition test which is related to negative symptom (Hamieh et al., 2021). Another study reported present of heat shock protein 70 in brain regions of animals treated with "double hit" model of schizophrenia (Inta et al., 2013). "Double hit" model induce reduction in prepulse inhibition and reduced cingulate 1 cortex volume (Garcia-Mompo et al., 2020). Seven studies did not find the "double hit" model to be more effective when compared to each treatment alone (Liu et al., 2017, Tuboly et al., 2009, Ashby et al., 2010, Hickey et al., 2012, Simpson et al., 2012, Hawken et al., 2013, Wu et al., 2016).

3.4 The effect of "double hit" (post-weaning social isolation and NMDA receptor antagonist) model of schizophrenia on neurotransmitters.

One study reported disturbance in excitatory/inhibitory neurotransmitter balance in the key brain region (Castillo-Gómez et al., 2017). The study of Shortall et al. (Shortall et al., 2020) reported reduced glutamate release. Two studies reported decreased hippocampus and prefrontal cortex GABA release (Simpson et al., 2010), and reduced Gad67 expression (Garcia-Mompo et al., 2020). Another study reported a significant decrease in the number of PV+ interneurons, perineuronal nets when compared to normal control (Klimczak et al., 2021).

3.5 Used of "double hit" (post-weaning social isolation and NMDA receptor antagonist) model as a developmental model of schizophrenia.

Ten studies found that the "double hit" model is more effective when compared to the intervention using social isolation on its own or an NMDA receptor antagonist (Castillo-Gómez et al., 2017, Garcia-Mompo et al., 2020, Simpson et al., 2010, Inta et al., 2013, Petrovszki et al., 2013, Gaskin et al., 2014, Gaskin et al., 2016, Shortall et al., 2020, Hamieh et al., 2021, Klimczak et al., 2021). Four of these studies injected phencyclidine (PCP) on PND7, PND9, and PND11 (Gaskin et al., 2014, Gaskin et al., 2016, Shortall et al., 2020, Hamieh et al., 2021), three studies injected MK801 on PND7 (Castillo-Gómez et al., 2017, Garcia-Mompo et al., 2020, Klimczak et al., 2021). Post-weaning social isolation period varied per study, but seven studies started social isolation on PND21 (Castillo-Gómez et al., 2017, Garcia-Mompo et al., 2020, Simpson et al., 2010, Inta et al., 2013, Shortall et al., 2020, Hamieh et al., 2021, Klimczak et al., 2021), while other three studies started the social isolation on PND23 (Petrovszki et al., 2013, Gaskin et al., 2014, Gaskin et al., 2016).

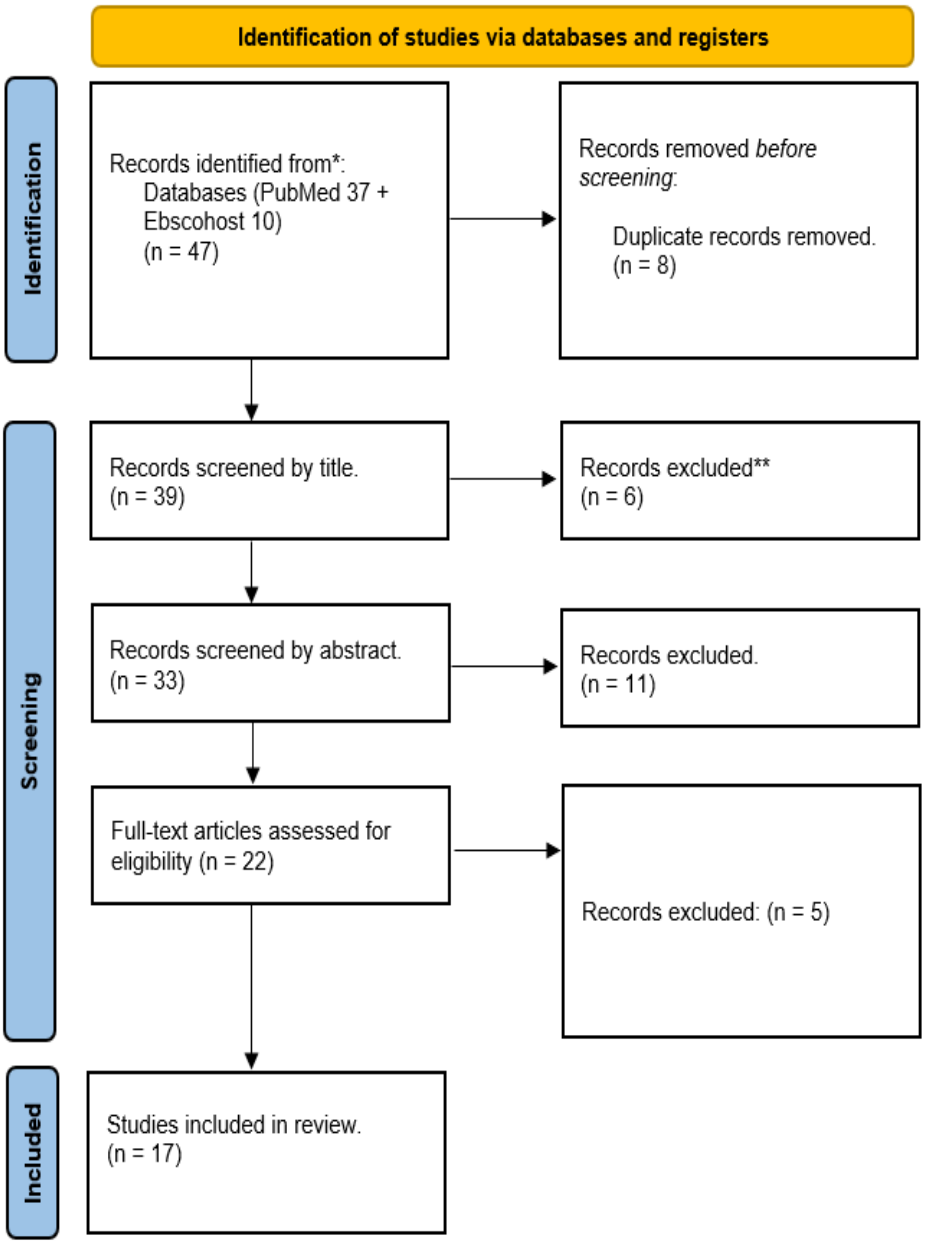

Figure 1. Flowchart of the study selection process and the characteristics of the included studies are summarized in Table 2.

Table 1: shows the risk of bias for all selected articles.

Studies	Selection bias			Performance bias		Detection bias		Attrition bias	Reporting bias	Other	Judgement/ Risk of bias
	Was the allocation sequence adequately generated and applied?	**Were the groups similar at baseline or were they adjusted for confounders in the analysis?**	**Was the allocation adequately concealed?**	**Were the animals randomly housed during the experiment?**	**Were the caregivers and/or investigators blinded from knowledge which intervention each animal received during the experiment?**	**Were animals selected at random for outcome assessment?**	**Was the outcome assessor blinded?**	**Were incomplete outcome data adequately addressed?**	**Are reports of the study free of selective outcome reporting?**	**Was the study apparently free of other problems that could result in high risk of bias?**	
(Tuboly et al., 2009)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
(Ashby et al., 2010)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
(Simpson et al., 2010)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
(Hickey et al., 2012)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low

(Simpson et al., 2012)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
(Hawken et al., 2013)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
(Inta et al., 2013)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
(Petrovszki et al., 2013)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
(Gaskin et al., 2014)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
(Gaskin et al., 2016)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
(Wu et al., 2016)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Low
(Castillo-Gómez et al., 2017)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
(Liu et al., 2017)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low

(Garcia-Mompo et al., 2020)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
(Shortall et al., 2020)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
(Hamieh et al., 2021)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
(Klimczak et al., 2021)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
(Shangase et al., 2024)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low

“Yes” means low risk of bias, “No” means high risk of bias, “Low” it’s a judgement meaning low risk of bias for the paper.

Table 2. Characteristics for all included studies.

Study	Location of the study	Year of publication	Species	Sex	NMDA receptor antagonist, Dosage, period of injection, type of injection	Commencement of social isolation, period of social isolation	Research groups per study	Outcome of the "double hit" model
(Hickey et al., 2012)	Canada	2012	Sprague Dawley rat	Male	MK801, 0.5 mg/kg, (PND 56-62) 2 times daily for 7 days, i.p. injection	From PND21-PND78	GH + Sal, GH + MK, SI + Sal, and SI + MK.	Combined post-weaning social isolation and subchronic MK801 treatment did not produce additive or synergistic effects on locomotor behaviour or Gaba signalling, but rather induce differential effects on GABAa receptor binding.

(Inta et al., 2013)	Germany	2013	Sprague Dawley rat	Male	MK801, (2 mg/kg) one injection, on PND64, i.p. injection	On PD21 (weaning age corresponding to pre-adolescence), rats were housed either individually or in groups of three in cages for a 6-week period from PND21 to PND63	GH + Sal, GH + MK, SI + Sal, and SI + MK	Juvenile rats exposed to chronic isolation had increased MK801-triggered expression of heat shock protein 70, a marker of neuronal injury, in the retrosplenial cortex. This suggests an additive effect of juvenile stress and NMDA receptor blockade, with possible relevance for schizophrenia.
(Simpson et al., 2012)	Canada	2012	Sprague Dawley rat	Male	MK801, 0.5 mg/kg, (PND56-PND62) was injected twice daily, i.p. injection.	On PND21-PND73 animals were socially isolated and rats remained in their assigned housing for the duration of the experiment.	GH + Sal + Sal, GH + MK + Sal, SI + Sal + Sal, SI + MK + Sal, GH + Sal + PLZ, GH + MK + PLZ, SI + Sal + PLZ, and SI + MK + PLZ	The combination of social isolation and subchronic MK801 did not produce greater behavioural changes than either treatment alone.
(Gaskin et al., 2016)	United Kingdom	2016	Lister-hooded rat	Male	PCP, 10mg/kg on post-natal days (PND7, PND9, and	Started on PND 23 till the end of the study	V + GH + V, V + GH + L15, PCP + SI + V, PCP + SI + L10, and PCP + SI + L15	Acute lamotrigine (10–15mg/kg i.p.) reversed the hyperactivity and novel object recognition impairment induced by “double

					PND11), s.c. injection			hit" model (PCP-SI) but had no effect on the prepulse inhibition deficit.
(Garcia-Mompo et al., 2020)	Spain	2020	GIN mice	Male	MK801, 1 mg/kg on PND 7 pups received one injection, i.p. injection	PND21 till the end of the study-PND133	CTRL + V, CTRL + THC, (SI + MK) + V, and (SI + MK) + THC	We found that "double hit" had reductions in prepulse inhibition of the startle reflex (PPI), GAD67 expression and cingulate 1 cortex volume.
(Petrovszki et al., 2013)	Hungary	2013	Wister rat	Both male and female	Ketamine, 30 mg/kg 5 times/week, 15 injections in total) from PND35-PND56 of age, i.p. injection	After weaning at 3 weeks of age (PND21–23 days), Rats were housed individually for 28 days (between 4 and 7 weeks of age)	NaNo, NaTr, SelNo, and SelTr	Selective breeding after juvenile isolation and ketamine treatment produces several signs which resemble those found in schizophrenia.
(Ashby et al., 2010)	Canada		Sprague Dawley rat	Male	MK801, 0.5 mg/kg, (PND56-	Social isolation started at postnatal	GH + Sal, GH + MK, SI + Sal, and SI and MK	The lack of additive or synergistic effects in the

		2010			PND62) 2 × day for seven days, i.p. injection.	day PND21 until PND56		"double hit model" suggests that combining isolation and subchronic MK801 treatment does not necessarily produce greater behavioural or physiological dysfunction than that seen with either treatment alone.
(Hamieh et al., 2021)	France	2020	Wister rat	Male	PCP (10 mg/kg) on (PND7, PND9, and PND11), s.c. injection	At the weaning day (PND 21), male rat pups were housed individually until the end of the study	V + GH + V, PCP + SI + V, and PCP + SI + Clo	The PCP-SI model presents with enduring and robust deficits (hyperactivity and social recognition impairment) associated with positive symptoms and cognitive/social deficits of schizophrenia, respectively. These deficits are normalized by chronic treatment with clozapine, thereby confirming the predictive validity of this animal model.

(Tuboly et al., 2009)	Hungary	2009	Wister rat	Male	Ketamine, 30 mg/kg, (PND28-PND42) one injection per day, i.p. injection	Wistar rats after weaning (PND21–PND23 days old) were either housed individually or grouped for 21 days.	GH + Sal, GH + Ket, SI + Sal, and SI + Ket	Since both social isolation and NMDA treatment are well-known animal models of schizophrenia, our results showed that juvenile isolation but not ketamine administration can stimulate hypoalgesia associated with this disease.
(Liu et al., 2017)	China	2017	Sprague Dawley rat	Male	MK801, 0.1, 0.3, and 0.5 mg/kg in PND7-PND21, s.c. injection	At PND21, rats were social isolated for four weeks (on PND49)	GH + Sal, SI + Sal, GH + MK0.1, SI + MK0.1, GH + MK0.3, SI + MK0.3, GH + MK0.5, and SI + MK0.5	Administration of MK801 and social isolation are two independent factors on the neurodevelopmental defects. Combining social isolation and subchronic MK801 treatment does not necessarily produce greater behavioural or physiological dysfunction than that seen with either treatment alone.
(Simpson et al., 2010)	Canada	2010	Sprague Dawley rat	Male	MK801, 0.5 mg/kg, injected twice	Rats were obtained at weaning (PND21); they were	GH + Sal, GH + MK, SI + Sal, and SI + MK	Locomotor activity was increased in social isolated rats. This activity was exacerbated in

					per day for 7 days from PND56-PND62, i.p. injection.	socially isolated, or group housed according to their randomly assigned housing groups and remained in their assigned groups for the duration of the experiment.		MK801-SI rats suggesting a possible decrease in hippocampal and/or prefrontal cortex GABA function.
(Gaskin et al., 2014)	United Kingdom	2014	Lister-hooded rat	Male	PCP, 10 mg/kg, on post-natal day (PND7, PND9, and PND11), s.c. injection	Rats were socially isolated on PND23, and animals remain isolated for 6 weeks.	GH + CTRL, GH + PCP, SI + CTRL, and SI + PCP	Neonatal PCP and social isolation both produced behavioural deficits in adult rats resulting in severe cognitive impairment (visual recognition memory impairment). This provided a comprehensive preclinical model that can be used to determine the neurobiological aetiology of schizophrenia than either treatment alone.

(Hawken et al., 2013)	Canada	2013	Sprague Dawley rat	Male	MK801 0.5 mg/kg, (PND62-PND68) twice daily for 7 days, i.p. injection	Rats reared in groups or in isolation beginning at PND21.	GH + Sal, GH + MK, SI + Sal, and SI + MK	Results showed that polydipsia is a schizophrenia-like behavioural effect caused by social isolation. The "double hit" model did not yield a more pronounced polydipsia effect than each treatment alone.
(Shortall et al., 2020)	United Kingdom	2020	Lister-hooded rat	Male	PCP-HCL, 10 mg/kg on (PND 7, PND9, and PND11), s.c. injection	Animals were socially isolated on PND21-PND63.	GH + V, GH + PCP, SI + V, and SI + PCP	Glutamate release was reduced in a "double hit" model, this reduced interneuron firing and caused impairment in the novel object discrimination task.
(Wu et al., 2016)	China	2016	Sprague Dawley rat	Male	MK801, 0.2mg/kg, (PND7-PND10), i.p. injected	Animals were socially isolated on PND21 and remained isolated for 8 weeks.	GH + Sal, GH + MK, and SI + Sal	Both socially reared rats with neonatal exposure to the NMDA receptor antagonist MK-801 and isolation-reared rats exhibited augmented startle responses.

(Castillo-Gómez et al., 2017)	Spain	2017	Transgenic strain mice	Male	MK801, 1mg/kg on PND7, once off/one injection, i.p. injection	Rats were socially isolated on PND21 and remained isolated for 10 weeks.	GH + Sal, GH + MK, SI + Sal, and SI + MK	The "double hit" model showed that the change in E/I balance in the key brain regions as one of the underlying causes of schizophrenia.
(Klimczak et al., 2021)	Spain	2021	FVB mice	Male	MK801, 1mg/kg on PND7, once off/one injection, i.p. injection	Rats were socially isolated on PND21 and remained isolated for 10 weeks.	GH + Sal, GH + MK, SI + Sal, and SI + MK	The "double hit" model showed a significant decrease in the number of PV+ interneurons, perineuronal nets (PNNs), and PNNs+PV+ cells when compared to control grouped mice.
(Shangase et al., 2024)	South Africa	2024	Sprague Dawley rats	Male	Ketamine, 16mg/kg, 52 days, subcutaneous injection	Rats were socially isolated on PND23 and remained isolated for 9 weeks	GH + Sal, GH + Ket, SI + Sal, and SI + Ket	The double hit model was able to induce aspect of positive, negative, and cognitive symptoms of schizophrenia than either treatment alone.

Abbreviations: SI (social isolation), GH (group housed), MK (MK801), Sal (saline), Plz (phenelzine), L (lamotrigine), PCP (phencyclidine), CTRL (control), V (vehicle), THC (9-tetrahydrocannabinol), NaNo (naïve rats without treatment), NaTr (naïve rats with post-weaning social isolation and ketamine treatment), SelNo (selectively bred animals without any treatment), SelTr (selectively bred rats with both post-weaning social isolation and ketamine treatment), Clo (clozapine), Ket (ketamine), i.p. (intraperitoneal), s.c. (subcutaneous), PND (postnatal day).

4. Discussion

Studies have shown that schizophrenia incidence in human is significantly higher in male than in female [52, 53]. Furthermore, these studies proposed that the overall male:female risk ratio is 1.4:1. This difference could not be explained by methodological factors connected to age or diagnostic criteria [7, 52, 53]. In this review, we showed that most pre-clinical studies used male rodents. We found that from the studies that used rats, 47.2% of them used Sprague Dawley rats, while 17.6% of them used lister-hooded rats, another 17.6% used Wistar rats, and another 17.6% used mice. Rats are commonly used as animal models of schizophrenia, and their behaviour better mimic the behaviour seen in humans, because their physiological function is similar to those observed in humans [54]. The main reason why fewer studies used mice as animal models of schizophrenia could be that, sometimes it is difficult to work with mice as they are more aggressive compared to rats [54]. Different studies used different NMDA receptor antagonists, ten studies used MK801 while four used phencyclidine and two used ketamine. Scientists prefer ketamine over phencyclidine because phencyclidine produces high levels of neurotoxicity and severe hallucination [55]. Neuroimaging and neuropsychological studies concluded that based on ethical reasons MK801 and ketamine are preferred over phencyclidine [56]. MK801 produces much longer lasting symptoms than ketamine and phencyclidine, and the fact that it is safer than phencyclidine is the reason most studies ended up using it.

4.1. "Double hit" (post-weaning social isolation and NMDA receptor antagonist) model on neurotransmitters.

A study found an imbalance on excitatory and inhibitory neurotransmission in the prefrontal cortex and amygdala of "double hit" (Mk801-SI) mice [33]. Furthermore, imbalances in excitatory and inhibitory neurotransmission on vital brain regions has been found to be an underlying factor in psychiatric disorders such as schizophrenia [33]. The "double hit" model has also been reported to have an influence in the release of different neurotransmitters. One study on "double hit" model found reduction in the number of PV+ interneurons, fast-spiking GABAergic neurons that modulated inhibitory control in both cortical and subcortical circuits [51]. The authors also reported reduced perineuronal nets, which provide a protective layer, maintain optimum local ionic homeostasis, and provide neuronal protection against oxidative stress [51]. A study on the (PCP-SI) model reported downregulation of several GABA-associated genes such as PVALB gene encoding parvalbumin and three GABA receptor subunit

genes (GABBR1, GABRA4, GABRB2) [47]. One study proposed that the increase in locomotor activity may be caused by decreased on the inhibitory neurotransmitter (GABA) function in the mesolimbic region and prefrontal cortex [40]. Six genes encoding enzymes that control glutamate metabolism were significantly down-regulated in PCP-SI rats [47]. It was not immediately evident if the downregulation of these genes is a compensatory outcome of lower basal glutamate in (PCP-SI) rats or a direct cause of the developmental manipulation [47].

4.2. "Double hit" (post-weaning social isolation and NMDA receptor antagonist) model on positive symptoms of schizophrenia

Ten studies showed that the "double hit" model produced enhanced behavioural and physiological deficits compared to either treatment alone [33, 35, 40, 44-50]. Three studies found that the "double hit" model caused locomotor hyperactivity which is a positive symptom of schizophrenia [40, 47, 50]. Locomotor hyperactivity is expressed as increased horizontal activity and is easily noticeably after the first 15 minutes in the open field test. This is caused by an increase in mesolimbic dopamine and serves as a baseline for the positive symptoms of schizophrenia [24]. Two studies found that the "double hit" model caused impairment in the PPI, linked to sensorimotor gating deficits [35, 48]. It has been shown that this PPI impairment is linked to the mesolimbic dopamine system because injection of 6-hydroxydopamine to deplete dopamine in the nucleus accumbens reversed PPI impairment [57].

4.3. "Double hit" (post-weaning social isolation and NMDA receptor antagonist) model on cognitive symptoms of schizophrenia

Three studies showed that the "double hit" model (PCP-SI) causes cognitive impairments similar to those observed in patients with schizophrenia [46, 49, 50]. Two studies found that animals exposed to the "double hit" model failed to identify the difference between a familiar and a novel object in a novel discrimination paradigm [46, 49]. Novel object recognition test measures visual recognition memory performance. This has translational relevance to one of the cognitive domains present in schizophrenia [58]. A study reported that the "double hit" model (PCP-SI) rats were unable to recognize a juvenile rat already contacted 30 minutes previously, possibly suggesting a lack of motivation to interact socially [50]. It has been reported that in the MK801-SI model, rats showed an increased expression of heat shock protein 70, a marker for neuronal damage in the neocortical regions [44]. The NMDA receptor antagonist, which is used to induce schizophrenia has been also proven to induce cortical injury

(cell necrosis) in the neocortical regions [59]. Other studies have gone further to investigate if available drugs used to treat schizophrenia would be able to reverse different symptoms of schizophrenia induced by a "double hit" model.

4.4. Response of "double hit" (post-weaning social isolation and NMDA receptor antagonist) model on drugs used to reverse symptoms of schizophrenia

A study showed that a drug (lamotrigine) was able to reverse hyperactivity caused by PCP-SI on rats placed in a novel arena [47]. Lamotrigine achieves this by reducing excitability in the striatal neurons which cause inhibition of pre-synaptic voltage-gated sodium channels which reduce glutamate release [60, 61]. Lamotrigine at a dose of (10 mg/kg i.p) was able to reverse the PCP-SI induced impairments in a novel object recognition test [47]. Furthermore, another study showed that clozapine was able to reverse locomotor hyperactivity and social recognition impairment caused by PCP-SI [50].

5. Conclusions, limitations, and future directions

In conclusion most studies showed that "double hit" (post-weaning social isolation and NMDA receptor antagonist) model is robust and capable of inducing wider spectrum of more severe cognitive impairments and positive symptoms of schizophrenia when compared to either model alone. "Double hit" model has also proven to be a robust and reliable developmental model of schizophrenia, since it was able to successfully induce all symptoms of schizophrenia. Different drugs (lamotrigine and clozapine) were able to reverse different impairments that were caused by the "double hit" model, this showed the predictive validity of this model and its potential as a translational model. More research is still needed to investigate the effect of the "double hit" model on negative and cognitive symptoms of schizophrenia. According to our knowledge this is the first study to review the "double hit" models of schizophrenia, hence will contribute positively to the development of effective animal models of schizophrenia that produce all symptoms of schizophrenia. Nevertheless, there are limitations to our study. We only considered studies on studies in rodents' models of schizophrenia. We excluded other animal subjects and human. Furthermore, there is a possibility that we might have missed some papers because of our search strategy design. The study only focused on post-weaning social isolation and NMDA receptor antagonist "double hit" model of schizophrenia, while there are many possible "double hit" models of schizophrenia that have valuable data. Choosing NMDA receptor antagonists in our "double hit" model make our paper more focused on glutamate

neurotransmitter, whereas there are other neurotransmitters that are involved in schizophrenia. Another study is needed that will cover all “double hit” models not only postweaning social isolation and NMDA receptor antagonist.

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Network, IB is adjunct director of the French EQUATOR Centre and TCH is co-director of the Australasian EQUATOR Centre, which advocates for the use of reporting guidelines to improve the quality of reporting in research articles. JMT received salary from Evidence Partners, creator of DistillerSR software for systematic reviews; Evidence Partners was not involved in the design or outcomes of the statement, and the views expressed solely represent those of the author.

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PROLOGUE

Chapter 3 examines a number of parameters that result in the development of the robust double hit model we developed. Negative and cognitive symptoms of schizophrenia are resistant to available antipsychotic treatment. The previous systematic review showed that ten studies out of seventeen studies reported that the double hit model of schizophrenia is more effective and robust in producing all symptoms of schizophrenia than single hit models. In this study we combined postweaning social isolation and NMDA receptor antagonist (ketamine) to establish a double hit model of schizophrenia. We used our double hit model of schizophrenia to investigate behavioural and molecular changes involved in negative and cognitive symptoms of schizophrenia. This chapter is written in manuscript format and is titled "***Effects of combined postweaning social isolation and ketamine administration on schizophrenia-like behaviour in male Sprague Dawley rats*".** This manuscript was published in the journal "**Behavioural Brain Research**" and is available online **(Appendix 2),** the chapter has been formatted according to the journal's guidelines for authors.

Chapter 3

Effects of combined postweaning social isolation and ketamine administration on schizophrenia-like behaviour in male Sprague Dawley rats.

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The pathophysiology behind negative and cognitive symptoms of schizophrenia is not well understood, thus limiting the effectiveness of treatment on these symptoms. Developing reliable animal model of schizophrenia is vital to advance our understanding on the neurobiological basis of the disorder. Double hit is used to refer to the use of two schizophrenia inducing interventions viz ketamine exposure and social isolation. In this study we aim to investigate the robustness of double hit model of schizophrenia in inducing negative and cognitive symptoms of schizophrenia. On postnatal day (PND) 23, thirty-two male Sprague Dawley rats were randomly grouped into four equal groups as follows: group housed + saline (GH), group housed + ketamine (GHK), isolated + saline (SI), and isolated + ketamine (SIK). A single ketamine dose (16 mg/kg) was administered 3 times a week for four weeks. Isolated animals were housed singly throughout the study. The following behavioural tests were carried out: elevated plus maze, three chamber social interaction, resident intruder tests, and novel object recognition (NOR). The SIK group exhibited high anxiety levels, with increased ACTH, corticosterone and norepinephrine concentration when compared to the other groups. The SIK animals also presented with reduced social interaction and decreased oxytocin concentration. SIK rats were more aggressive towards a juvenile intruder but had low testosterone concentration. The SIK group or double hit model showed impaired visual learning and memory and increased expression of proinflammatory cytokines. This suggest that the double hit model is more robust in inducing negative and cognitive symptoms of schizophrenia than each treatment alone.

Keywords: Schizophrenia, group housed, isolation, ketamine, double hit, and aggressive behaviour.

Introduction

Schizophrenia is a heterogenous neuropsychiatric disorder that affects approximately 1% of the population worldwide (Goldner et al., 2002). Schizophrenia is characterized by positive symptoms (hallucinations and delusions), negative symptoms (impaired social interaction, deficits in emotional expression, and lack of motivation), and cognitive dysfunction (impaired problem solving, impaired visual learning and memory, and impaired processing speed) (Gomes et al., 2016). Antipsychotic treatment has been shown to be effective in attenuating positive symptoms but has limited effect on negative and cognitive symptoms (Harvey and McClure, 2006, Keefe, 2007). Animal models of schizophrenia provide valuable insight in investigating features of the disease that may be difficult to do in human studies (Jones et al., 2011). A developmental model of schizophrenia is achieved by social isolation of rat weanlings (housing rodents individually in single cages) (Lapiz et al., 2003, Fone and Porkess, 2008). Social isolation induces alterations in brain development and causes behavioural impairments similar to those observed in schizophrenia (Fone and Porkess, 2008). Even though social isolation is considered a reliable model of schizophrenia, the shortfall of the model is that all behavioural impairments and molecular dysfunction induced can be easily reversed by repeated handling of animals by the researcher (Jones et al., 2011).

Ketamine is a pharmacological model that has been proven to induce psychotic behaviour in normal human volunteers and animals (Coyle et al., 2012, Ben-Azu et al., 2022). Furthermore ketamine has been found to exacerbate symptoms in individuals with schizophrenia (Coyle et al., 2012). The ketamine model of schizophrenia is found to be more reliable in the investigation of all symptoms of schizophrenia. More interestingly the model is also reliable in studying neurotransmitter disturbance and any other features related to the disorder (Frohlich and Van Horn, 2014, Ben-Azu et al., 2019, Ben-Azu et al., 2023, Ben-Azu et al., 2024). Researchers used different doses and regimes of ketamine, and this causes the researchers to report contradicting results, and the injection of ketamine in adult animals has been shown not to mimic the onset of the schizophrenia disorder. Negative (social withdrawal) and cognitive (memory impairment) symptoms of schizophrenia are associated with neuroprogressive hypothesis (progression of cognitive decline and increased severity of symptoms) (Ribeiro et al., 2013, da Silva Araújo et al., 2017, Ben-Azu et al., 2019). Furthermore, the neuroprogressive hypothesis indicates that schizophrenia is likely more associated with neuroinflammation as also proven by other studies which have reported elevated brain concentrations of inflammatory cytokines (Monji et al., 2009, Monji et al., 2013).

Increased oxytocin binding in the amygdala can reverse social withdrawal. Oxytocin receptor binding is more localised in the amygdala, and amygdala dysfunction have been implicated in exacerbating negative symptoms of schizophrenia (Lee et al., 2007b). It is proposed that oxytocin achieved this by working together with the dopaminergic neural pathway to facilitate the reward system and inhibition of defensive behaviour (Ellenbogen, 2018). Impaired social interaction and defensive behaviour observed in rats may be linked to the agitation and aggressive behaviour observed in schizophrenic patients (Volavka and Citrome, 2011). It has been proposed that agitation in individuals with schizophrenia is associated with exacerbated psychotic symptoms and poor impulse control, and if left untreated this might escalate to aggressive behaviour (Volavka and Citrome, 2008, Volavka and Citrome, 2011). Schizophrenic patients have showed impaired ability to adapt under stressful conditions, demonstrated by reduced norepinephrine response to stress (Kudoh et al., 1999). Animals and humans are susceptible to developing aggressive behaviour under stressful conditions (Volavka and Citrome, 2011, Takahashi, 2022). Increase in testosterone concentration has been shown to potentiate aggressive behaviour in males that possess dominant and impulsive personality behaviour (Carré et al., 2017). Stress has been suggested to be behind the development of aggressive behaviour in schizophrenic patients, although the mechanism of action has not been fully studied (Volavka and Citrome, 2011).

Both social isolation and NMDA receptor antagonists induce behavioural, structural, and functional changes in animals similar to those found in schizophrenic patients (Beninger et al., 2002, Day-Wilson et al., 2006, Fone and Porkess, 2008, Hickey et al., 2012). Combining social isolation and NMDA receptor antagonist models into a double hit model results in a robust model of schizophrenia (Shangase et al., 2023). Other studies that investigated double hit model did not find it to be robust when compared to either treatment alone (Ashby et al., 2010, Wu et al., 2016, Liu et al., 2017). In this study we used the double hit (post-weaning social isolation and NMDA receptor antagonist (ketamine)) model to investigate behavioural and molecular changes associated with negative and cognitive symptoms of schizophrenia. We aim to investigate the combined effects of postweaning social isolation and ketamine on modelling social withdrawal and cognitive impairment-like symptoms of schizophrenia in male Sprague Dawley rats. In this study we will investigate the effect of the double hit model on stress related hormones (corticosterone, ACTH, and norepinephrine), oxytocin, and inflammatory cytokines (IL6 and TNFα)

Methods and materials

A total of 32 male Sprague Dawley rats (21 days old) were acquired from the breeding unit of the University of KwaZulu-Natal. On postnatal day (PND) 23 the animals were randomly separated to 4 different groups as follows: 8 group housed male rats + saline (0.9% NaCl) injected (GH), 8 grouped housed male rats + ketamine (16 mg/kg) injected (GHK), 8 socially isolated male rats + saline (0.9% NaCl) injected (SI), and 8 socially isolated male rats + ketamine (16 mg/kg) injected (SIK). The rats were housed under standard laboratory conditions of 21-24 °C room temperature, 55% humidity and maintained on a 12:12 hour light-dark cycle (lights on at 06h00 and off at 18h00). No environmental enrichment was added to the cages with the socially isolated groups so as to prevent attenuating the social isolation effect (Brenes et al., 2020). All handling and experimental manipulation were carried out in the light phase (Richetto et al., 2019). Food and water were available *ad libitum*. Experiments were conducted in full compliance with the guidelines, rules and regulations specified by the Public Health Service policy on humane care and use of laboratory animals. The University of KwaZulu-Natal Animal Research Ethics Committee (AREC) approved all procedures (AREC/00002812/2021).

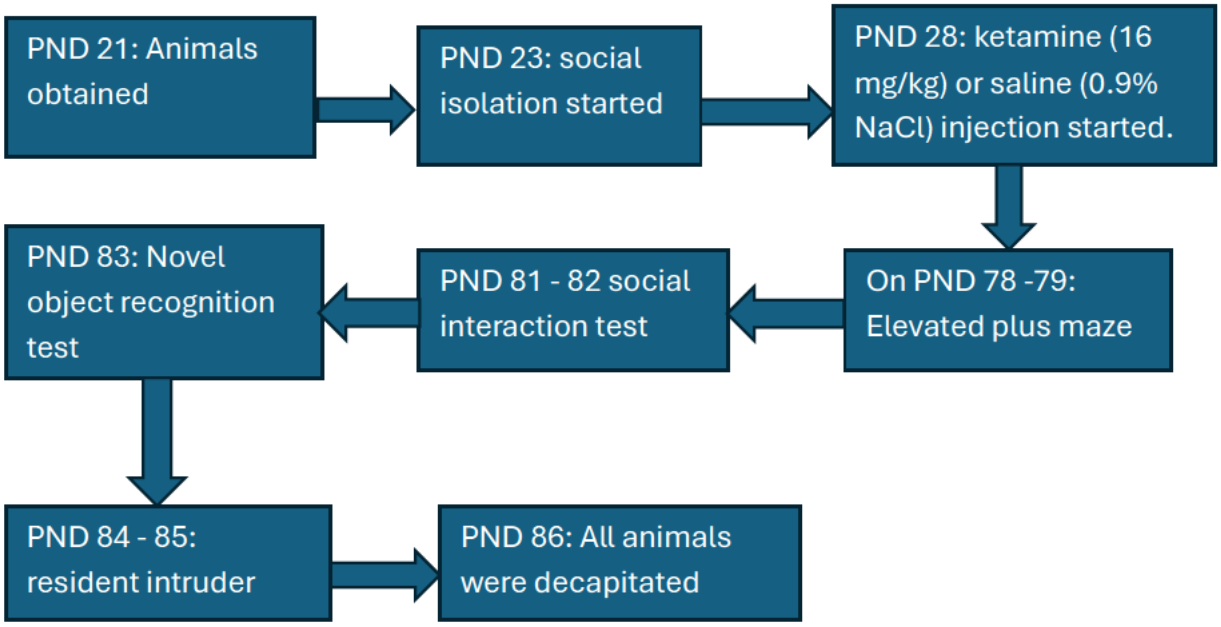

Figure 1: Flow diagram showing the summary of the experimental design.

Ketamine exposure and social isolation

We used the same ketamine dose as (Koh et al., 2016). Ketamine (100 mg/ml concentration) was diluted to 1.6 mg/ml using saline (0.9% NaCl) as a solvent and injected subcutaneously at a volume of 10 ml/kg of body weight equivalent to a dosage of 16 mg/kg [Dosage = Concentration x Volume] (Schobel et al., 2013). Due to ethical considerations the lowest published dosage of ketamine was used which is 16 mg/kg. A repeated single dose of ketamine (16 mg/kg, subcutaneously) treatment regimen began on PND 28 to PND 85 and was done as follows: Animals that received ketamine were injected subcutaneously 3 times a week (Monday, Wednesday, and Friday) at 09:00 h for 4 weeks (Koh et al., 2016). Thereafter on PND 57, the animals were injected 2 times a week (Monday and Friday) at 09:00 until the end of the experimental period (PND) 85. Animals that were not injected with ketamine were injected with the same volume of saline (0.9% NaCl). On PND 23 animals that were isolated in the socially isolated groups were house singly in transparent cages with dimensions (40 cm long x 25 cm wide x 35 cm high) for 62 days (animals were able to hear, smell and see other animals but physical contact with them was not allowed), Group housed animals were housed 4 per group in standard group cages with dimensions 55 cm long x 35 cm wide x 20 cm high.

Elevated Plus Maze (EPM)

The EPM test is used to assess anxiety-like behaviour. We followed the protocol of (Qi et al., 2016, Bruijnzeel et al., 2019). Briefly, the test apparatus consisted of four black polypropylene arms. The EPM was divided into five zones (two open arms, two closed arms, and a centre zone). The EPM consisted of a plus-shaped platform, the open arms were placed opposite of each other. The arms were 10 cm wide, 50 cm long, and were on 55 cm tall acrylic legs. The two “open” arms had 0.5 cm ledges, and the two “closed” arms had 30 cm walls. On PND 78 and PND 79 the animals were tested (animals were tested for two days in order to record the average score). After the ketamine injection at 09:00 in the morning animals were allowed to rest and approximately at 13:00 animals were transferred to the EPM room to habituate for an hour to the new environment and at 14:00H the test started. Rats were placed in the centre square facing an open arm. Between each trial the apparatus was wiped with 70% ethanol to

remove any residual odours. The following parameters were recorded during the 5-min test using a video camera: time spent in the open and closed arms, time spent in the centre space, number of entries into the open and closed arms, latency to enter an open arm or the closed arm. The videos were used to score behaviour by the researcher and an observer who was blind to the experimental groups. Anxiety-like behaviour was measured as the percentage of time spent on the closed arms and calculated as follows: [time on close arms] / [time on open + closed arms] x100%.

Social preference test

The three-chamber social preference test was used to assess social impairment in rodent animal models (Rein et al., 2020). On PND 81 and PND 82 the animals were tested (animals were tested for two days in order to record the average score). After the ketamine injection at 09:00 in the morning animals were allowed to rest and approximately at 13:00H the animals were transferred to the behavioural room to habituate to the novel environment, and at 14:00H the test started. Two clean two empty small-wired cages were placed into the three-chamber apparatus, each was centred approximately halfway between the midline and the far wall. One empty cage was then exchanged by an identical small-wired cage containing an unknown same-sex conspecific rat to serve as the social stimulus. The social stimulus rat was unfamiliar to the test rat, the social stimulus rat was interchanged regularly to avoid exhaustion or social fatigue of the social stimulus rat. Another wire small cage was left empty and serves as the non-social stimulus. The location of the social or non-social stimulus in either side chamber was counterbalanced between tests. The experimental animal was placed into the apparatus containing the social stimulus rat and non-social stimuli and allowed to explore for 10 minutes. The amount of time spent interacting with the social stimulus rat and the non-social stimulus was recorded. After 10 minutes the test rat and social stimulus rat were returned to their respective home cages. Between each trial the apparatus was wiped with 70% ethanol to remove any residual odours that may affect subsequent tests. All phases were videotaped, and videos were scored afterwards by an observer who was blind to the experimental groups. The results were expressed using the Social Preference index: [time investigating the social stimulus] / [total time investigating both stimuli] x100%.

Novel object recognition (NOR) test

The NOR test was conducted using the open field chamber to investigate the authenticity of recognition memory (Bevins and Besheer, 2006, Watson et al., 2012a) with some minor

changes. After the ketamine injection at 09:00 in the morning animals were allowed to rest and approximately at 13:00H animals were moved to the behavioural room for acclimatization, and at 14:00H the behavioural test started. On PND 83, rats were placed in a chamber for a 5-minute acclimatization period, after that they were returned to their home cage for 1 minute, during that time two identical objects (plastic bottles, 8 cm high and 5 cm in diameter, coated in white masking tape) were introduced into the chamber. Rats were returned to the chamber for their first familiarisation trial and exploration of each object was recorded for 3 min (trial 1). For 2h rats were returned to their home cage, and one object selected in a pseudo-random manner replaced with a visually distinct novel object (identical size bottle covered in three rings of 2cm black tape). A second, 3-min choice trial (trial 2) was performed, and exploration of each object (defined as directed attention to the object with the nose ≤1 cm away and active vibrissae) recorded using Canon PowerShot SX700 HS camera (South Africa) by an individual unaware of the treatment sitting 1 m from the chamber. Climbing on or chewing the object was not recorded, as these behaviours do not represent directed attention to the object (Gaskin et al., 2014).

Resident intruder test

The resident-intruder test is a standardised method used to measure offensive aggressive behaviour and defensive behaviour in male rats (Koolhaas et al., 2013). The test was conducted On PND 84 and PND 85 by introducing an intruder to the resident home cage, (the test was conducted for two days in order to record the average score). For 5 days before the resident-intruder test, the sawdust in the home cage of the socially isolated and group-housed male rat (resident) was not changed to allow the animals to establish and maintain territoriality within their home cage (Trainor et al., 2008, Koolhaas et al., 2013). Intruders were younger (juvenile), well-socialized, never have been isolated and never have been in contact with the resident (Koolhaas et al., 2013). After the ketamine injection at 09:00 in the morning animals were allowed to rest and approximately at 12:00 in the afternoon, the resident males were moved to a clear cage with their old bedding, and they were allowed to habituate for 1 hour in their new cage in their home room. At 13:00 both resident males and intruders were moved to the experimental room where they were allowed to habituate for another hour. Before the resident intruder test was run, an extra 15 minutes was allowed for the resident male and the intruder to calm down and make sure that the experimenter and the camera did not stress the animals. An intruder was then introduced into the resident male cage. Observation of dominant and subordinate status, latency to the first aggressive attack and the number of attacks of the

resident towards the intruder were recorded for 10 minutes. For the resident male, the duration and frequency of the following behavioural parameters were determined - attack latency: the time between the introduction of the intruder and the first clinch attack; lateral threat, upright posture, clinch, attack, keeping down, chase, non-social exploration, rest or inactivation (Trainor et al., 2008, Koolhaas et al., 2013). All residents that attacked the intruder were considered aggressive.

Decapitation

On PND 86 all animals were decapitated, 24 hours after the last behavioural test (resident intruder test). A sharp guillotine was used to humanely guillotine the head of the rat, sharp stainless steel surgical scissors were used to remove the tissue and pelage covering the top part of the rat skull. Once the skull was clearly exposed, the sharp small surgical scissors were used to cut the skull in the middle from back to the front part of the skull. Debakey Tweezer-X1 was used to peel the skull from the point of the incision until the brain was fully exposed. The brain was scooped with Kelly forceps CVD-X2 and placed in a cold sludge of 0.9% saline for a few minutes. After 5 minutes the brain was moved to the cutting board and the scalpel was used to harvest the amygdala, prefrontal cortex, and hippocampus. The amygdala has an almond shape and is located deep within the temporal lobes, medial to the hypothalamus and adjacent to the hippocampus. The brain tissue was snap-frozen in liquid nitrogen and stored at -80 °C in a Biofreezer for few days and the benchwork analysis started and the tissues were used. Trunk blood was collected to vacuette heparin tubes and spun in a centrifuge (BioRad, USA) at 10 000 g. Plasma was collected in 2.0 ml microcentrifuge tubes and stored at -80 °C in a Biofreezer.

Plasma adrenocorticotropic hormone (ACTH) concentration

Plasma ACTH concentration was measured with an ELISA kit (Elabscience Biotech (E-EL-R0048, Texas, USA). A 50µl aliquot of standard or sample was added to a 96 well ELISA plate, followed immediately by the addition of 50µl of Biotinylated Detection AB, followed by incubation for 45 minutes at 37°C HettCube 200 incubator. After the incubation period, the plate was aspirated and washed 3 times with the wash buffer provided. Then 100µl of HRP conjugate was added followed by 30 minute's incubation at 37 ºC in the HettCube 200 incubator. The plate was then aspirated and washed 5 times. 90µl of substrate was added followed by a 15 minute incubation period at 37°C in the HettCube 200 incubator. After the 15 minute incubation, a stop solution (50µl), was added to terminate the reaction. The optical

density (OD) was measured using a micro-plate reader set at 450 nm wavelength. All the duplicated OD reading for each standard and samples were averaged. All averaged OD values were blank corrected (OD of standard or sample – OD of the blank). The XY table was created on GraphPad Prism 8 with the standard concentration on the X-axis and OD on the Y-axis. The standard curve was created, and the actual concentration was extrapolated from the standard curve. The sensitivity levels of the ELISA are 10-1000 pg/ml. The samples were measured in duplicates; outliers were replaced through imputation with values identical to the mean. A best-fit curve was constructed using GraphPad Prism version 8, and its equation was used to calculate the sample concentration. The samples were randomised in the ELISA plate to eliminate bias. We chose the ELISA because we wanted to measure the concentration, in our laboratory, we mostly use the ELISA to measure the concentration.

Plasma corticosterone concentration

Plasma corticosterone concentration was measured with an ELISA kit (Elabscience Biotech (E-E-OSEL-R0002, Texas, USA). A 50µl aliquot of standard or sample was added to a 96 well ELISA plate, followed immediately by the addition of 50µl HRP linked to Biotinylated Detection AB, followed by incubation for 60 minutes at 37°C in the HettCube 200 incubator. The plate was then aspirated and washed 5 times. A 90µl of substrate was added followed by a 15 minute incubation period at 37°C HettCube 200 incubator. After the 15 minute in the incubation, a stop solution (50µl), was added to terminate the reaction. The optical density (OD) was measured using a micro-plate reader set at 450 nm wavelength. All the duplicated OD reading for each standard and samples were averaged. All averaged OD values were blank corrected (OD of standard or sample – OD of the blank). The XY table was created on GraphPad Prism 8 with the standard concentration on the X-axis and OD on the Y-axis. The standard curve was created, and the actual concentration was extrapolated from the standard curve. The sensitivity levels of the ELISA are 12.50-800 ng/ml. The samples were measured in duplicates; outliers were replaced through imputation with values identical to the mean. A best-fit curve was constructed using GraphPad Prism version 8, and its equation was used to calculate the sample concentration. The samples were randomised in the ELISA plate to eliminate bias. We chose the ELISA because we wanted to measure the concentration, in our laboratory, we mostly use the ELISA to measure the concentration.

4.8 Plasma testosterone concentration

Plasma testosterone concentration was measured with an ELISA kit (Elabscience Biotech (E-EL-0155, Texas, USA). A 50µl aliquot of standard or sample was added to a 96 well ELISA plate, followed immediately by the addition of 50µl of Biotinylated Detection AB, followed by incubation for 45 minutes at 37°C in the HettCube 200 incubator. After the incubation period, the plate was aspirated and washed 3 times with the wash buffer provided. Then 100µl of HRP conjugate was added followed by 30 minutes incubation at 37 ºC HettCube 200 incubator. The plate was then aspirated and washed 5 times. A 90µl of substrate was added followed by a 15 minute incubation period at 37°C HettCube 200 incubator. After the 15 minute in the incubation, a stop solution (50µl), was added to terminate the reaction. The optical density (OD) was measured using a micro-plate reader set at 450 nm wavelength. All the duplicated OD reading for each standard and samples were averaged. All averaged OD values were blank corrected (OD of standard or sample – OD of the blank). The XY table was created on GraphPad Prism 8 with the standard concentration on the X-axis and OD on the Y-axis. The standard curve was created, and the actual concentration was extrapolated from the standard curve. The sensitivity levels of the ELISA are 0.1-20 ng/ml. The samples were measured in duplicates; outliers were replaced through imputation with values identical to the mean. A best-fit curve was constructed using GraphPad Prism version 8, and its equation was used to calculate the sample concentration. The samples were randomised in the ELISA plate to eliminate bias. We chose the ELISA because we wanted to measure the concentration, in our laboratory, we mostly use the ELISA to measure the concentration.

Amygdala oxytocin concentration

Amygdala oxytocin concentration was measured with an ELISA kit (Elabscience Biotech (E-EL-0029, Texas, USA). A 50µl aliquot of standard or sample was added to a 96 well ELISA plate, followed immediately by the addition of 50µl of Biotinylated Detection AB, followed by incubation for 45 minutes at 37°C in the HettCube 200 incubator. After the incubation period, the plate was aspirated and washed 3 times with the wash buffer provided. Then 100µl of HRP conjugate was added followed by 30 minutes incubation at 37 ºC HettCube 200 incubator. The plate was then aspirated and washed 5 times. A 90µl of substrate was added followed by a 15 minute incubation period at 37°C HettCube 200 incubator. After the 15 minute in the incubation, a stop solution (50µl), was added to terminate the reaction. The optical density (OD) was measured using a micro-plate reader set at 450 nm wavelength. All the duplicated

OD reading for each standard and samples were averaged. All averaged OD values were blank corrected (OD of standard or sample – OD of the blank). The XY table was created on GraphPad Prism 8 with the standard concentration on the X-axis and OD on the Y-axis. The standard curve was created, and the actual concentration was extrapolated from the standard curve. The sensitivity levels of the ELISA are 10-1000 pg/ml. The samples were measured in duplicates; outliers were replaced through imputation with values identical to the mean. A best-fit curve was constructed using GraphPad Prism version 8, and its equation was used to calculate the sample concentration. The samples were randomised in the ELISA plate to eliminate bias. We chose the ELISA because we wanted to measure the concentration, in our laboratory, we mostly use the ELISA to measure the concentration.

Plasma norepinephrine concentration

Plasma norepinephrine concentration was measured with an ELISA kit (Elabscience Biotech (E-EL-0047, Texas, USA). A 50µl aliquot of standard or sample was added to a 96 well ELISA plate, followed immediately by the addition of 50µl of Biotinylated Detection AB, followed by incubation for 45 minutes at 37°C in the HettCube 200 incubator. After the incubation period, the plate was aspirated and washed 3 times with the wash buffer provided. Then 100µl of HRP conjugate was added followed by 30 minutes incubation at 37 ºC HettCube 200 incubator. The plate was then aspirated and washed 5 times. A 90µl of substrate was added followed by a 15 minute incubation period at 37°C HettCube 200 incubator. After the 15 minute in the incubation, a stop solution (50µl), was added to terminate the reaction. The optical density (OD) was measured using a micro-plate reader set at 450 nm wavelength. All the duplicated OD reading for each standard and samples were averaged. All averaged OD values were blank corrected (OD of standard or sample – OD of the blank). The XY table was created on GraphPad Prism 8 with the standard concentration on the X-axis and OD on the Y-axis. The standard curve was created, and the actual concentration was extrapolated from the standard curve. The sensitivity levels of the ELISA are 0.1-100 ng/ml. The samples were measured in duplicates; outliers were replaced through imputation with values identical to the mean. A best-fit curve was constructed using GraphPad Prism version 8, and its equation was used to calculate the sample concentration. The samples were randomised in the ELISA plate to eliminate bias. We chose the ELISA because we wanted to measure the concentration, in our laboratory, we mostly use the ELISA to measure the concentration.

RNA isolation for real-time PCR (RT PCR)

Real-time PCR was used to quantify the expression levels of different genes (GAD1, PVALB, BDNF, TNFα, and IL 6), RNA was isolated from the dissected PFC and hippocampus tissue. The homogenization buffer (Maxwell simpleRNA tissue kit, Promega, USA) was used to homogenize the samples and RNA was extracted according to the manufacturer's instructions. The integrity of the sample was determined using an Agilent 2100 Bioanalyzer. The purity of isolated RNA was evaluated using a NanoDrop Spectrophotometer (thermoFisher SCIENTIFIC, USA), and the samples were considered pure if RNA absorbance values were between 1.7 and 2.0. All isolated RNA with a concentration of 100 ng/µl and above were converted to cDNA using the iScripTM cDNA Synthesis kit (BioRad, USA), the negative control (RNA free water was used instead of RNA sample) was applied during the cDNA synthesis, and 2 steps SYBR green master mix was used for all reactions (FastStart, USA). The cDNA was stored at -20 °C; the primers used in this study are shown in table 1. GAPDH was used as our reference gene. These primers were supplied by Inqaba Biotechnical Industries (South Africa), IL-6, TNFα, and GAPDH were amplified and analysed using the Roche Light Cycler® 480 real-time PCR system (Roche Diagnostics, USA). The following PCR conditions were used: 30 cycles; with preincubation of 95 °C for 30 seconds; 3 step amplification of 95 °C for 15 seconds, 60 ºC for 60 seconds, 72 ºC for 5 seconds; melting of 95 ºC for 10 seconds, 97 ºC for 1 seconds; cooling of 37 ºC for 30 seconds. GAPDH primer annealing 65 °C for 1 minute; IL-6 primer annealing 65 ºC for 1 minutes; TNFα primer annealing 62 ºC for 1 minutes. The samples were run in duplicates.

Table 1: Primer sequences for RT-PCR analysis.

Gene Name	Forward Primer	Reverse Primer	Product size (bp)
GAPDH	ggcaagttcaatggcacagt	tggtgaagacgccagtagactc	183
TNFα	ttctgtctactgaacttcggggt	gtatgagatagcaaatcggctgacggt	310
IL 6	aacgatgatgcacttgcaga	gagcattggaaattggggta	298

Statistical analysis

Data was analysed using the software, GraphPad Prism version 8 (San Diego, California, USA). All results are presented as the mean ± SEM. The Shapiro-Wilk test was used to test for normality. Two-way ANOVA, followed by Bonferroni's multiple comparisons test was used to analyse the data. Multiple comparisons will be conducted even in the absent of significant interaction effects because we want to compare the effect of the double hit model when compared to other groups.

Results

Elevated Plus Maze (EPM)

There was an overall housing effect in the percentage time spent in the closed arms of the EPM in the group housed saline (GH), group housed ketamine (GHK), isolated saline (SI), and isolated ketamine (SIK), $F_{(1,28)} = 70.55$, $p<0.0001$. There was an overall ketamine effect in the percentage time spent in the closed arms of the EPM in the GH, GHK, SI, and SIK, $F_{(1,28)} = 31.10$, $p<0.0001$. There was a statistically significant interaction between social isolation and ketamine in percentage time spent in the close arms $F_{(1,28)}= 62.70$, $p=0.0080$. Multiple comparisons showed that there was a ketamine and isolation effect on percentage time spent in the closed arms of the EPM in the GHK and SI when compared to GH group *(GH vs. GHK, P=0.0047; GH vs. SI, $p<0.0001$, Bonferroni's multiple comparisons test, Figure 2). Multiple comparisons showed that isolation enhanced the ketamine effect on percentage time spent in the closed arms of the EPM α(GHK vs SIK, $p<0.0001$, Figure 2). Multiple comparisons showed that there was a ketamine effect in social isolated animals α(SI vs. SIK, $p=0.0018$, Figure 2).

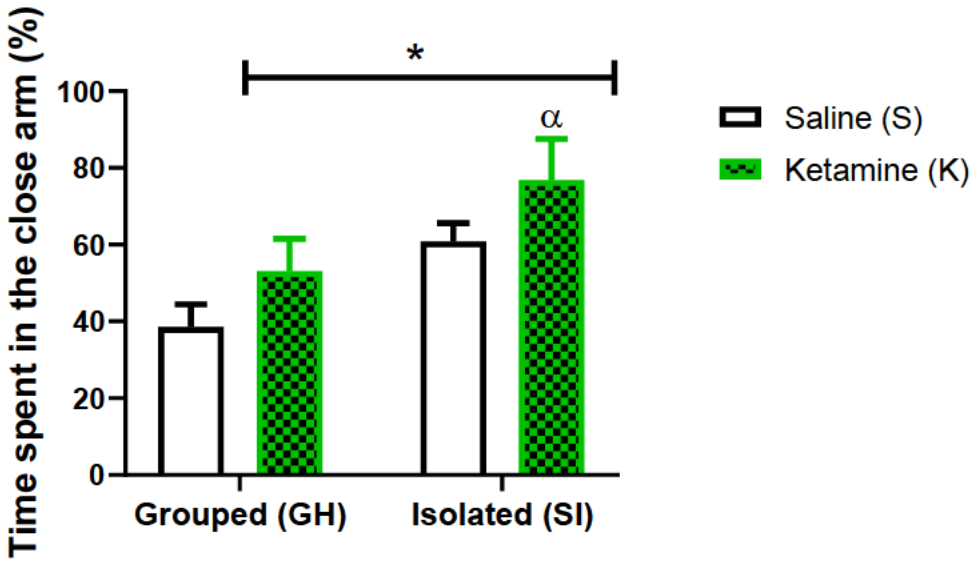

Figure 2. percentage time spent in the closed arms of the Elevated Plus Maze in the GH, GHK, SI, SIK. *(GH vs. GHK, p=0.0047; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001); α(GHK vs. SIK, p<0.0001; SI vs. SIK, p=0.0018). Data presented as mean ± SEM, n = 8 per group.

Social interaction

There was an overall housing effect in the percentage time spent socializing in the social interaction test in the GH, GHK, SI, and SIK, $F(1,28) = 53.07$, $p<0.0001$. There was an overall ketamine effect in the percentage time spent socializing in the social interaction test in the GH, GHK, SI, and SIK, $F(1,28) = 94.54$, $p<0.0001$. There was a statistically significant interaction between social isolation and ketamine in the percentage time spent socializing in the social interaction test $F(1,28)= 241$, $p=0.0013$. Multiple comparisons showed that there was a ketamine effect and isolation effect on percentage time spent socializing in the social interaction test in the GHK and SI compared to the GH group *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001, Figure 3). Multiple comparisons showed that isolation enhanced the ketamine effect on percentage time spent socializing in the social interaction test α(GHK vs SIK, p=0.0022, Figure 3). There was a ketamine effect in the social isolated animals α(SI vs. SIK, p<0.0001, Figure 3).

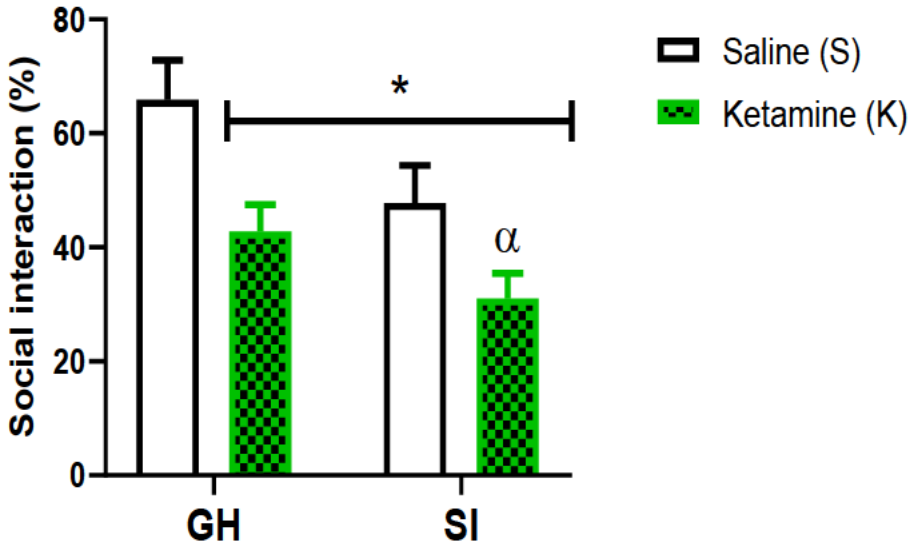

Figure 3. Graph showing percentage time spent socializing for GH, GHK, SI, and SIK. *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001); α(GHK vs. SIK, p=0.0022); SI vs. SIK, p<0.0001). Data presented as mean ± SEM, n = 8 per group.

Aggressive behaviour

There was an overall housing effect in the percentage time spent showing an aggressive behaviour towards a juvenile in the GH, GHK, SI, and SIK, $F(1,28) = 292.1$, $p<0.0001$. There was an overall ketamine effect in the percentage time spent showing an aggressive behaviour towards a juvenile intruder in the GH, GHK, SI, and SIK, $F(1,28) = 5.86$, $p=0.0222$. There was a statistically significant interaction between social isolation and ketamine in the percentage time spent showing an aggressive behaviour, $F(1,28) = 24.99$, $p=0.0013$. Multiple comparisons showed that there was an isolation effect on percentage time spent showing an aggressive behaviour towards a juvenile intruder in the SI and SIK compared to the GH group *(GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001, Figure 4). However, social isolation showed more effect on aggressive behaviour than ketamine #(GHK vs. SI, p<0.0001, Figure 4). Multiple comparisons showed that there was a ketamine effect in social isolated animals α(SI vs. SIK, p=0.0401, Figure 4). Multiple comparisons showed that social isolation enhanced the ketamine effect α(GHK vs. SIK, p<0.0001, Figure 4).

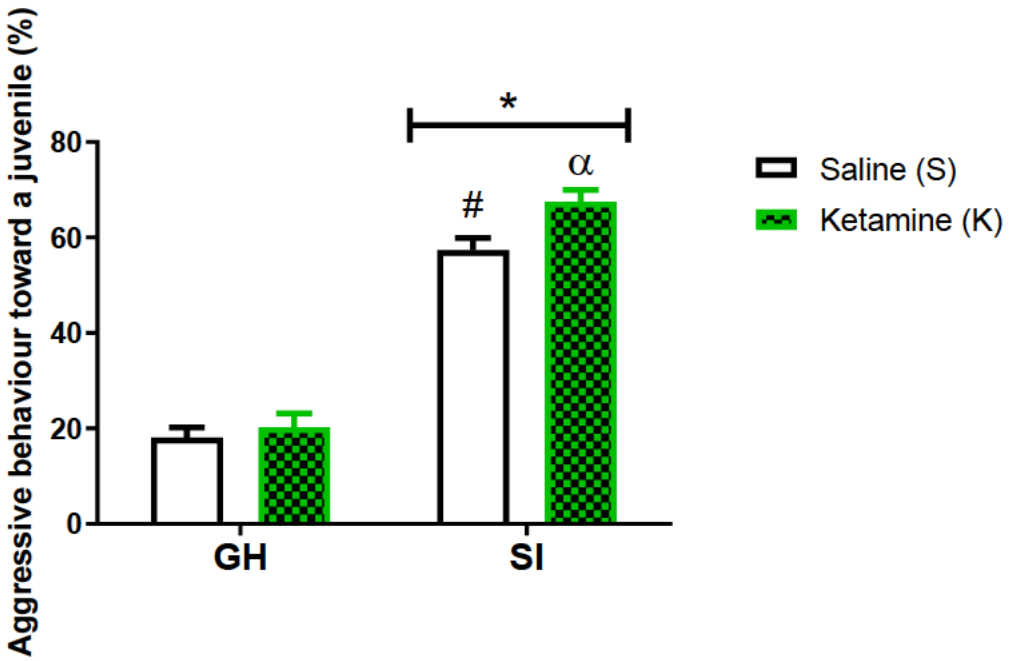

Figure 4. Graph showing percentage time spent showing an aggressive behaviour for GH, GHK, SI, and SIK. *(GH vs. SI, $p<0.0001$; GH vs. SIK, $p<0.0001$); #(GHK vs. SI, $p<0.0001$); α(GHK vs. SIK, $p<0.0001$; SI vs. SIK, $p=0.0401$). Data presented as mean ± SEM, n = 8 per group.

Novel object recognition test

Impairment in the novel object recognition test (NORT) was measured in the following groups: GH, GHK, SI, and SIK. There was an overall housing effect in time spent exploring the familiar and the novel object in the GH, GHK, SI, and SIK, $F(3,48) = 22.48$, $p<0.0001$. There was an overall ketamine effect in time spent exploring the familiar and the novel object in the GH, GHK, SI, and SIK, $F(3,48) = 13.04$, $p=0.0007$. Multiple comparisons showed that the animals in the GH group were able to differentiate between the familiar and the novel object *(GH novel vs. GH familiar, $p=0.0004$, Figure 5).

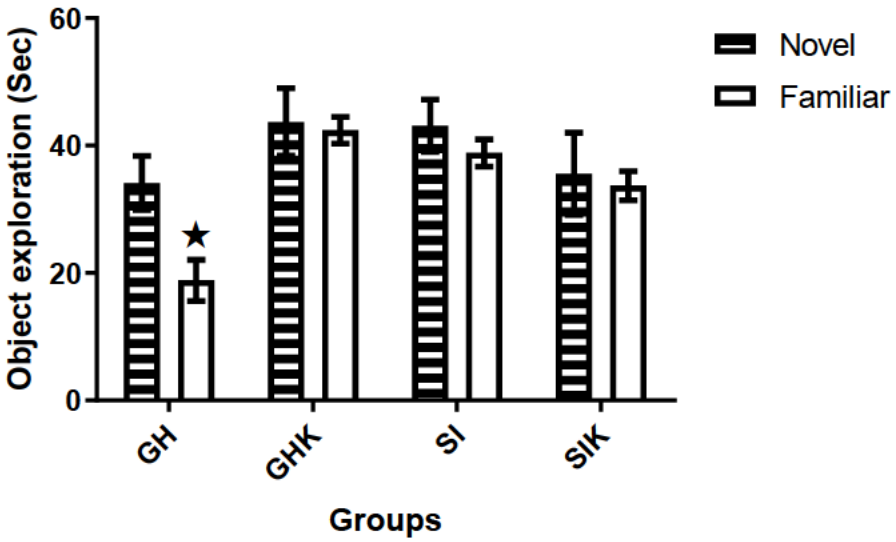

Figure 5: Graph showing impairment in the novel object recognition test (NORT) in the following groups: GH, GHK, SI, and SIK. *(GH novel vs. GH familiar, P=0.0004). Data presented as mean ± SEM, n = 8 per group.

Adrenocorticotropic hormone (ACTH) concentration

There was an overall housing effect in plasma ACTH concentration in the GH, GHK, SI, and SIK, $F(1,28) = 164.5$, $p<0.0001$. There was an overall ketamine effect in plasma ACTH concentration in the GH, GHK, SI, and SIK, $F(1,28) = 34.84$, $p=0.0027$. There was a statistically significant interaction between social isolation and ketamine in plasma ACTH concentration $F(1,28)= 20.26$, $p=0.0001$. Multiple comparisons showed that there was an isolation effect on plasma ACTH concentration in the SI and SIK compared to the GH group *(GH vs. SI, $p<0.0001$; GH vs. SIK, $p<0.0001$, Figure 6). However, social isolation showed more effect on plasma ACTH concentration than ketamine #(GHK vs. SI, $p=0.0002$, Figure 6). Multiple comparisons showed that there was a ketamine effect in social isolated animals α(SI vs. SIK, $p<0.0001$, Figure 6). Multiple comparisons showed that social isolation enhanced the ketamine effect α(GHK vs. SIK, $p<0.0001$, Figure 6).

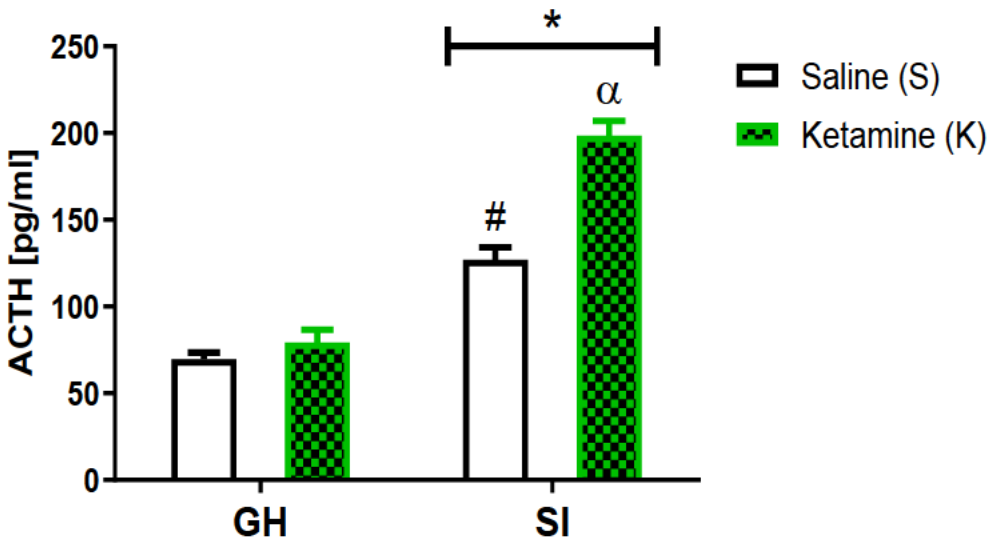

Figure 6. Graph showing plasma ACTH concentration for GH, GHK, SI, and SIK. *(GH vs. SI, $p<0.0001$; GH vs. SIK, $p<0.0001$); #(GHK vs. SI, $p=0.0002$); α(GHK vs. SIK, $p<0.0001$; SI vs. SIK, $p<0.0001$). Data presented as mean ± SEM, n = 8 per group.

Plasma corticosterone concentration

There was an overall housing effect in plasma corticosterone concentration in the GH, GHK, SI, and SIK, $F(1,28) = 216.8$, $p<0.0001$. There was an overall ketamine effect in plasma corticosterone concentration in the GH, GHK, SI, and SIK, $F(1,28) = 23.29$, $p=0.0001$. There was a statistically significant interaction between social isolation and ketamine in plasma corticosterone concentration, $F(1,28)=12.10$, $p=0.0017$. Multiple comparisons showed that there was an isolation effect on plasma corticosterone concentration in the SI and SIK compared to the GH group *(GH vs. SI, $p<0.0001$; GH vs. SIK, $p<0.0001$, Figure 7). However, social isolation showed more effect in plasma corticosterone concentration than ketamine #(GHK vs. SI, $p<0.0001$, Figure 7). Multiple comparisons showed that there was a ketamine effect in social isolated animals α(SI vs. SIK, $p<0.0001$, Figure 7). Multiple comparisons showed that social isolation enhanced the ketamine effect in plasma corticosterone concentration α(GHK vs. SIK, $p<0.0001$, Figure 7).

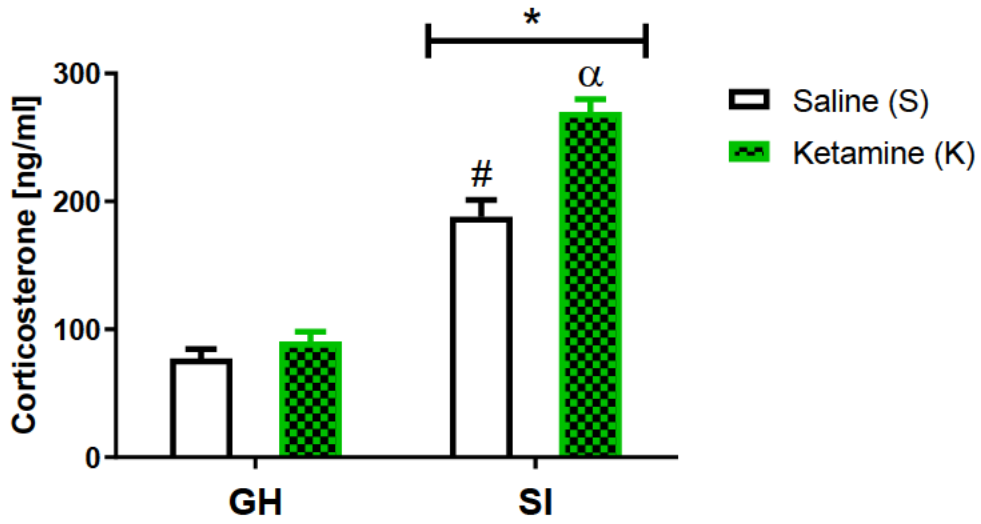

Figure 7. Graph showing plasma corticosterone concentration for GH, GHK, SI, and SIK. *(GH vs. SI, $p<0.0001$; GH vs. SIK, $p<0.0001$); #(GHK vs. SI, $p<0.0001$); α(GHK vs. SIK, $p<0.0001$; SI vs. SIK, $p<0.0001$). Data presented as mean ± SEM, n = 8 per group.

Plasma testosterone concentration

There was an overall housing effect in plasma testosterone concentration in the GH, GHK, SI, and SIK, $F(1,28) = 27.75$, $p<0.0001$. There was an overall ketamine effect in plasma testosterone concentration in the GH, GHK, SI, and SIK, $F(1,28) = 23.84$, $p=0.0001$. Multiple comparisons showed that there was a ketamine effect in social isolated animals α(SI vs. SIK, $p=0.0009$, Figure 8). Multiple comparisons showed that social isolation enhanced the ketamine effect in plasma testosterone concentration α(GHK vs. SIK, $p=0.0004$, Figure 8).

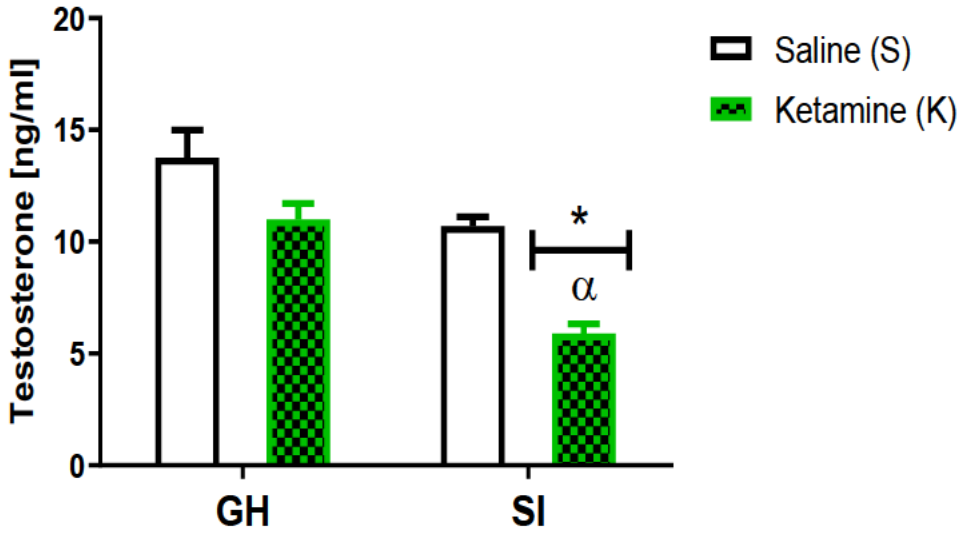

Figure 8. Graph showing plasma testosterone concentration for GH, GHK, SI, and SIK. *(GH vs. SIK, p<0.0001); α(GHK vs. SIK, p=0.0004; SI vs. SIK, p=0.0009). Data presented as mean ± SEM, n = 8 per group.

Oxytocin concentration in the Amygdala

There was an overall housing effect in amygdala oxytocin concentration in the GH, GHK, SI, and SIK, $F(1,28) = 494.6$, $p<0.0001$. There was an overall ketamine effect in amygdala oxytocin concentration in the GH, GHK, SI, and SIK, $F(1,28) = 110.9$, $p<0.0001$. There was a statistically significant interaction between social isolation and ketamine in oxytocin concentration $F(1,28) = 4.489$, $p=0.0431$. Multiple comparisons showed that there was a ketamine and isolation effect on amygdala oxytocin concentration in the GHK and SI compared to the GH group *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001, Figure 9). However, social isolation showed more effect in amygdala oxytocin concentration than ketamine #(GHK vs. SI, p<0.0001, Figure 9). Multiple comparisons showed that there was a ketamine effect in social isolated animals in amygdala oxytocin concentration α(SI vs. SIK, p<0.0001, Figure 9). Multiple comparisons showed that social isolation enhanced the ketamine effect in amygdala oxytocin concentration α(GHK vs. SIK, p<0.0001, Figure 9).

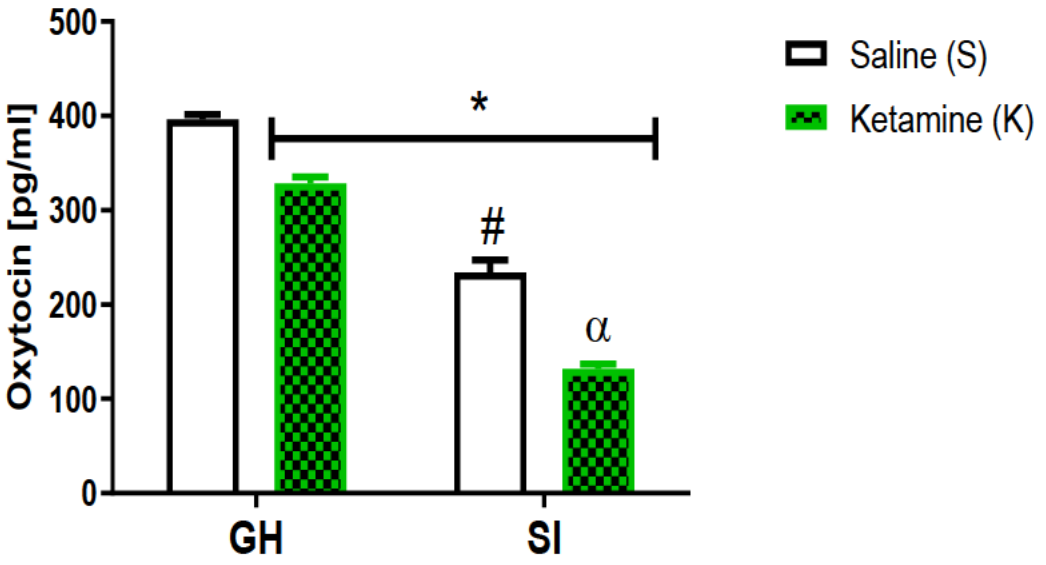

Figure 9. Graph showing amygdala oxytocin concentration for GH, GHK, SI, and SIK. *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001); #(GHK vs. SI, p<0.0001); α(GHK vs. SIK, P<0.0001; SI vs. SIK, P<0.0001). Data presented as mean ± SEM, n = 8 per group.

Plasma norepinephrine concentration

There was an overall housing effect in plasma norepinephrine concentration in the GH, GHK, SI, and SIK, $F(1,28) = 180.5$, $p<0.0001$. There was an overall ketamine effect in plasma norepinephrine concentration in the GH, GHK, SI, and SIK, $F(1,28) = 20.45$, $p=0.0001$. Multiple comparisons showed that there was an isolation effect on plasma norepinephrine concentration in the SI and SIK compared to the GH group *(GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001, Figure 10). However, social isolation showed more effect in plasma norepinephrine concentration than ketamine #(GHK vs. SI, p<0.0001, Figure 10). Multiple comparisons showed that there was a ketamine effect in social isolated animals in plasma norepinephrine concentration α(SI vs. SIK, p=0.0021, Figure 10). Multiple comparisons showed that social isolation enhanced the ketamine effect in plasma norepinephrine concentration α(GHK vs. SIK, p<0.0001, Figure 10).

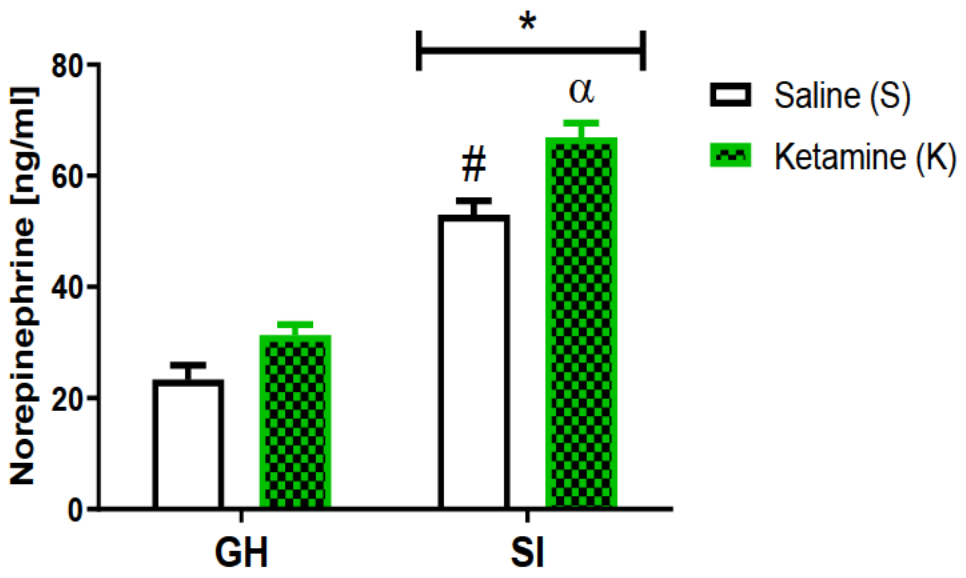

Figure 10. Graph showing plasma norepinephrine concentration for GH, GHK, SI, and SIK. *(GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001); #(GHK vs. SI, P<0.0001); α(GHK vs. SIK, p<0.0001; SI vs. SIK, p=0.0021). Data presented as mean ± SEM, n = 8 per group.

TNFα mRNA expression in the PFC

There was an overall housing effect in TNFα mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 117.0$, $p<0.0001$. There was an overall ketamine effect in TNFα mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 127.8$, $p<0.0001$. There was a statistically significant interaction between social isolation and ketamine in TNFα mRNA expression $F(1,28) = 92.08$, $p<0.0001$. Multiple comparisons showed that there was a ketamine effect and isolation effect on TNFα mRNA expression in the SIK compared to the GH group *(GH vs. SIK, p<0.0001, Bonferroni's multiple comparison test, Figure 11). Multiple comparisons showed that isolation enhanced the ketamine effect on TNFα mRNA expression α(GHK vs SIK, p<0.0001, Figure 11). Multiple comparisons showed that there was a ketamine effect in the social isolated animals on TNFα mRNA expression α(SI vs. SIK, p<0.0001, Figure 11).

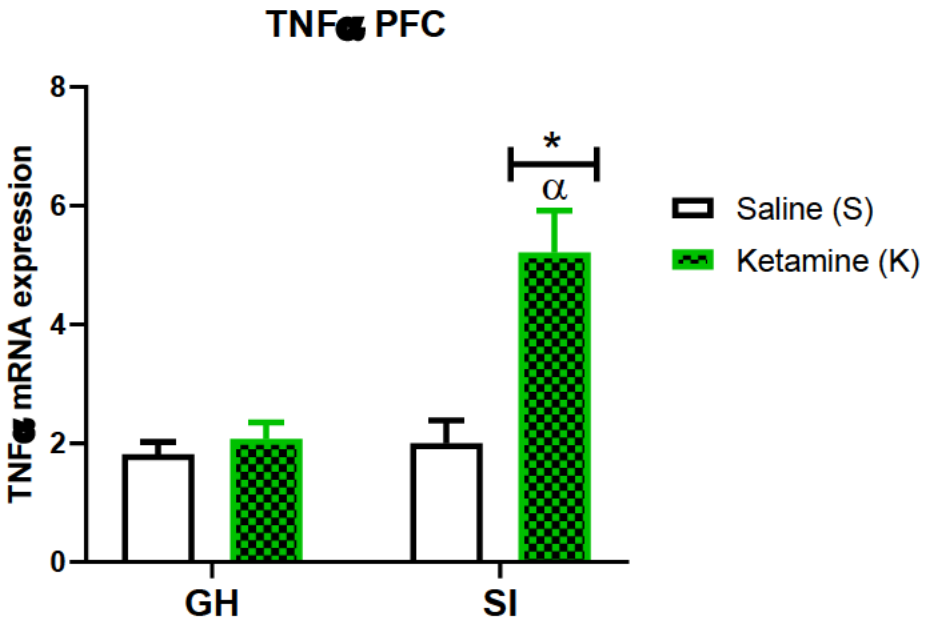

Figure 11. Graph showing TNFα mRNA expression in the GH, GHK, SI, SIK. *(GH vs. SIK, P<0.0001); α(GHK vs. SIK, P<0.0001; SI vs. SIK, P<0.0001). Data presented as mean ± SEM, n = 8 per group.

TNFα mRNA expression in the hippocampus

There was an overall housing effect in TNFα mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 171.4$, $p<0.0001$. There was an overall ketamine effect in TNFα mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 220.9$, $p<0.0001$. There was a statistically significant interaction between social isolation and ketamine in TNFα mRNA expression $F(1,28) = 19.25$, $p=0.0001$. Multiple comparisons showed that there was a ketamine effect and isolation effect on TNFα mRNA expression in the GHK, SI, and SIK compared to the GH group *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001, Bonferroni's multiple comparison test, Figure 11). Multiple comparisons showed that isolation enhanced the ketamine effect on TNFα mRNA expression α(GHK vs SIK, p<0.0001, Figure 12). Multiple comparisons showed that there was a ketamine effect in the social isolated animals in the TNFα mRNA expression α(SI vs. SIK, p<0.0001, Figure 12).

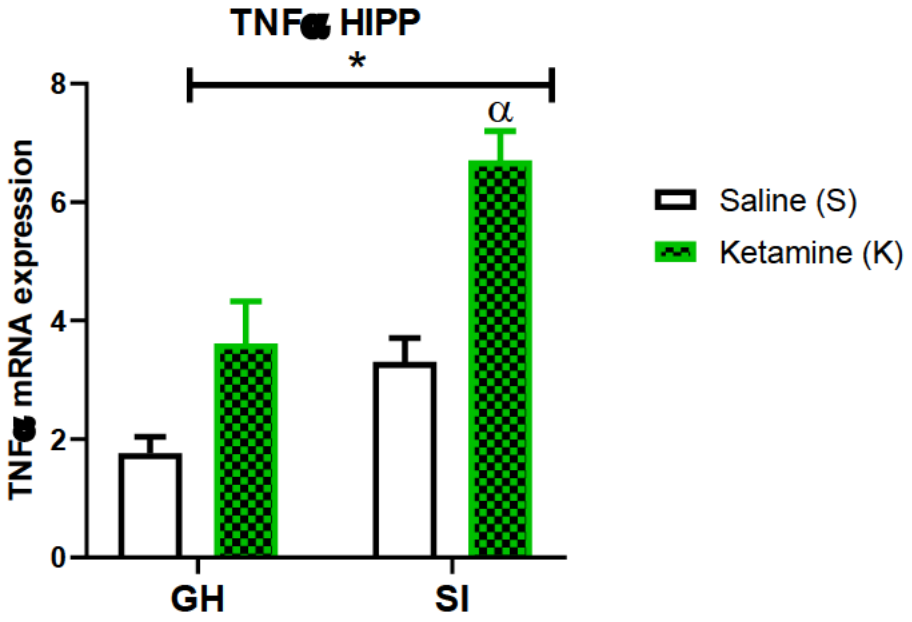

Figure 12. Graph showing TNFα mRNA expression in the GH, GHK, SI, SIK. *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001); α(GHK vs. SIK, p<0.0001; SI vs. SIK, p<0.0001). Data presented as mean ± SEM, n = 8 per group.

IL 6 mRNA expression in the PFC

There was an overall housing effect in IL6 mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 64.67$, $p<0.0001$. There was an overall ketamine effect in IL6 mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 106.6$, $p<0.0001$. There was a statistically significant interaction between social isolation and ketamine in IL6 mRNA expression $F(1,28) = 13.19$, $p=0.0011$. Multiple comparisons showed that there was a ketamine effect and isolation effect on IL6 mRNA expression in the GHK, SI, and SIK compared to the GH group *(GH vs. GHK, p=0.0003; GH vs. SI, p=0.0251; GH vs. SIK, p<0.0001, Bonferroni's multiple comparison test, Figure 13). Multiple comparisons showed that isolation enhanced the ketamine effect on IL6 mRNA expression α(GHK vs SIK, p<0.0001, Figure 13). Multiple comparisons showed that there was a ketamine effect in the social isolated animals in IL6 mRNA expression α(SI vs. SIK, p<0.0001, Figure 13).

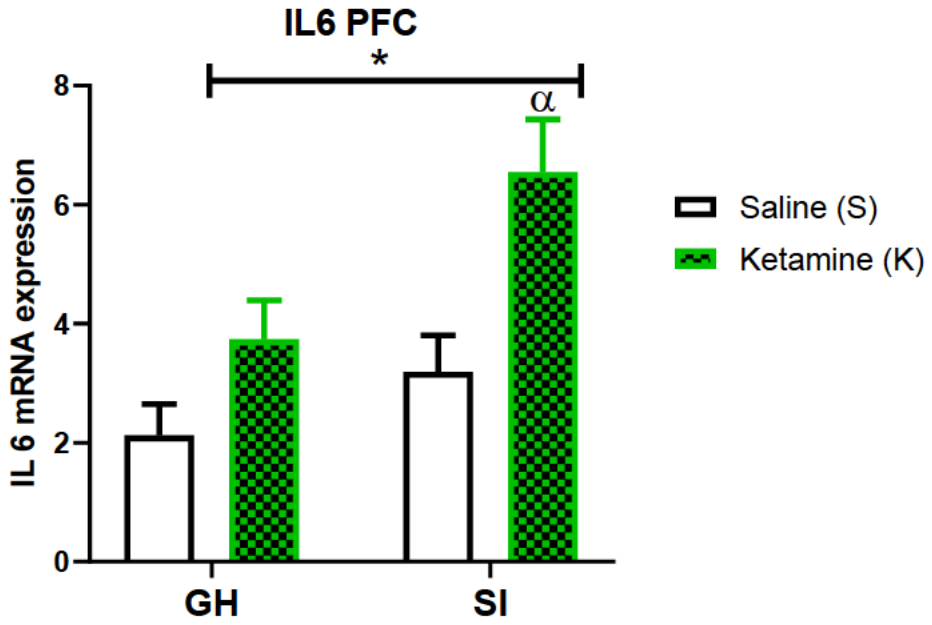

Figure 13. Graph showing IL 6 mRNA expression in the GH, GHK, SI, SIK. *(GH vs. GHK, p=0.0003; GH vs. SI, p=0.0251; GH vs. SIK, p<0.0001); α(GHK vs. SIK, p<0.0001; SI vs. SIK, p<0.0001). Data presented as mean ± SEM, n = 8 per group.

IL 6 mRNA expression in the hippocampus

There was an overall housing effect in IL6 mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 145.0$, $p<0.0001$. There was an overall ketamine effect in IL6 mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 225.9$, $p<0.0001$. There was a statistically significant interaction between social isolation and ketamine in IL6 mRNA expression $F(1,28) = 6.576$, $p=0.0160$. Multiple comparisons showed that there was a ketamine effect and isolation effect on IL6 mRNA expression in the GHK, SI, and SIK compared to the GH group *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001, Bonferroni's multiple comparison test, Figure 14). Multiple comparisons showed that isolation enhanced the ketamine effect on IL6 mRNA expression α(GHK vs SIK, p<0.0001, Figure 14). Multiple comparisons showed that there was a ketamine effect in the social isolated animals on IL6 mRNA expression α(SI vs. SIK, p<0.0001, Figure 14).

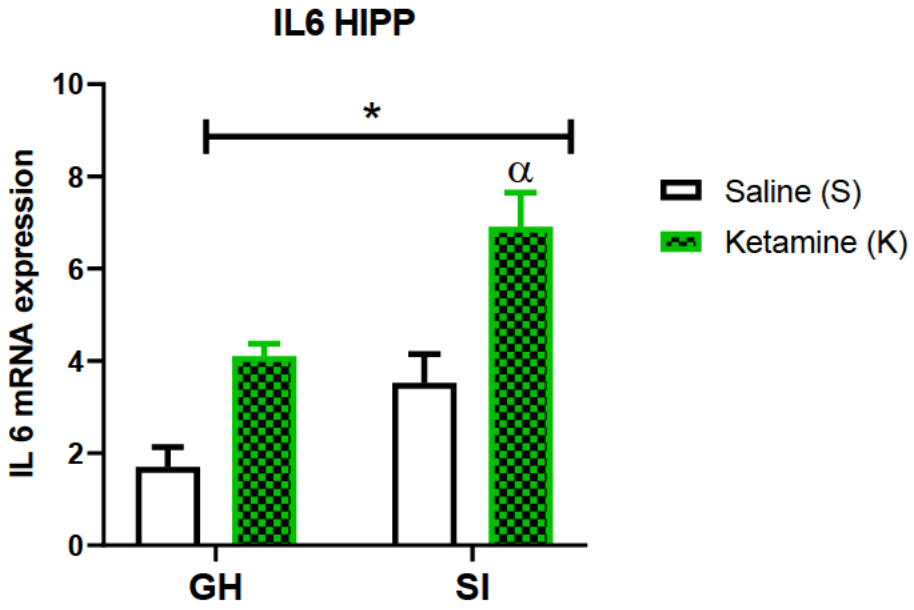

Figure 14. Graph showing IL 6 mRNA expression in the GH, GHK, SI, SIK. *(GH vs. GHK, $p<0.0001$; GH vs. SI, $p<0.0001$; GH vs. SIK, $p<0.0001$); α(GHK vs. SIK, $p<0.0001$; SI vs. SIK, $p<0.0001$). Data presented as mean ± SEM, n = 8 per group.

Discussion

In this study, we investigated the effect of the double hit (post-weaning social isolation and NMDA receptor antagonist (ketamine)) model in inducing negative and cognitive symptoms of schizophrenia in male Sprague Dawley rats. The present study showed that the double hit model of schizophrenia was able to induce negative (impaired social interaction) and cognitive (impaired visual learning and memory) symptoms of schizophrenia than either treatment alone. Our findings reveal that GHK, SI, and SIK rats showed reduced levels of social interaction when compared to the GH. This is similar to the social withdrawal which is a negative symptom of schizophrenia (Wilson and Koenig, 2014). Studies have reported that rats that were treated with chronic NMDA receptor antagonist (phencyclidine) showed reduced social interaction (Sams-Dodd, 1996, Lee et al., 2005). In the current study SIK (double hit) rats showed less social interest when compared to other groups. A double hit (isolation-phencyclidine) model showed deficits in a social interaction test (Hamieh et al., 2021). These studies agree with our findings that rats with schizophrenia-like symptoms show impaired social interaction.

The role of oxytocin in negative symptoms of schizophrenia mainly focuses on social interaction. Our study showed that the schizophrenia model groups (GHK, SI and SIK rats) had reduced oxytocin concentration in the amygdala. We showed reduced oxytocin concentration in isolated ketamine rats compared to other groups. A previous study showed that an infusion of oxytocin in the central nucleus of the amygdala successfully reversed the social interaction deficits caused by NMDA receptor antagonist (phencyclidine) in rats (Lee et al., 2005). Furthermore, it has been reported that reduced plasma oxytocin concentration is positively correlated with greater severity of antisocial behaviour in schizophrenic patients (Strauss et al., 2015). These studies agree with our findings that reduced oxytocin concentration is associated with reduced social interaction.

Our study revealed that both SI and SIK rats showed high levels of aggressive behaviour toward a juvenile intruder. The SIK group showed highest levels of aggressive behaviour when compared to other groups. Studies have reported that schizophrenia is associated with increased risk for aggressive behaviour (Swanson et al., 1990, Fazel et al., 2009). Patients with schizophrenia experience stressful events such as economic deprivation, childhood conduct problems, and violent victimization are more susceptible to develop aggressive behaviour (Swanson et al., 2008). Stress is one of the pathophysiological factors in aggressive behaviour in individuals with schizophrenia, but its mode of action is not fully understood (Volavka and Citrome, 2011). Post-weaning social isolation has proven to be a reliable and robust animal model of stress which can lead to exacerbated aggressive behaviour in adulthood (Toth et al., 2011). Our study showed that GHK, SI and SIK rats spent more time in the closed arms of the plus maze. SIK rats spent more time in the closed arms of the EPM than all other groups. Increased anxiety-like behaviour in the EPM has been reported in socially isolated rats, specifically spending more time on the closed arms of the maze (Bickerdike et al., 1993, Hellemans et al., 2004). A study on a double hit model of schizophrenia reported that NMDA receptor antagonist and social isolation are independent models, but that social isolation is mostly associated with anxiety-like behaviour (Liu et al., 2017). The literature agrees with our findings in which we find that socially isolated animals show high anxiety-like behaviour which was reflected by more time spent in the closed arms of the plus maze.

This anxiety-like behaviour was further supported by high levels of stress hormones such as corticosterone and adrenocorticotropic hormone (ACTH). Our study reveals that high concentration of ACTH was observed on SI and SIK rats when compared to their group housed counterparts. Furthermore, our study showed high concentration of corticosterone in SI and

SIK rats when compared to group housed rats. SIK showed high concentrations of ACTH and corticosterone when compared to other groups. During a stressful event, the hypothalamic pituitary-adrenal (HPA) axis is activated; this further stimulates the body and brain-wide physiological changes through the production of glucocorticoids (Herman et al., 2016). This leads to the activation of CRH which stimulates the production of ACTH in the anterior pituitary, in turn promoting glucocorticoid production and release from the adrenal cortex (Ulrich-Lai and Herman, 2009, Herman et al., 2016). Glucocorticoids, such as corticosterone stimulate the brain through rapid action of cellular signalling mechanisms as well as through slower-acting effects on cellular adaptation by control of gene expression (Evanson et al., 2010, Joëls et al., 2012). Corticosterone can have rapid effects on neural circuit during the time of stress exposure, this can manifest in the long-term effect on neural circuit in the form of functional or structural plasticity (Kinlein et al., 2019).

These changes in the neural function and structural plasticity can significantly influence how the organism responds to stress (McEwen et al., 2015, McEwen, 2017). Under stressful conditions both humans and animals are more likely to develop aggressive behaviour (Volavka, 2008). Schizophrenia patients lack the ability to cope with stress in a non-aggressive way, this can be attributed to the fact that schizophrenia patients have reduced ability to control their impulses and may misunderstand social situations (Morris et al., 2009, Volavka and Citrome, 2011). Our results showed high concentration of norepinephrine in SI and SIK rats. SIK rats showed higher concentration of norepinephrine than other groups. This observation supports the hypothesis that norepinephrine plays a physiological role in response to a stressful event. Exposure to stress has been shown to result in increased catecholamine (norepinephrine) biosynthesis and storage capacity in the peripheral tissues (Kvetňanský, 1973, Sanchez and Pereira, 2002). The literature agrees with our findings because social isolated (IS and IK) rats showed high concentrations of stress hormones ACTH, corticosterone, and norepinephrine.

Our results showed that group housed animals (GH and GHK) showed less aggressive behaviour towards the juvenile male rats. Even though GHK animals spent more time in a closed arm of plus maze, the same animals showed low levels of the stress hormones such as ACTH, corticosterone, and norepinephrine. Removal of stress and paying active attention on patients' needs has potential to reduce overt physical aggression (Volavka and Citrome, 2011). Furthermore, patient environment should be enriched such as increasing social interaction may assist in reversing persistent aggression in schizophrenia patients (Volavka and Citrome, 2011). GH and GHK showed high concentration of testosterone when compared to isolated male rats

(SI and SIK) as shown in results. High testosterone concentration can lead to aggressive behaviour and animals with high testosterone levels are likely to win mating competitions and become dominant in a colony (Muller, 2017). The aggressive behaviour in this study was not caused by testosterone, as isolated rats had lower testosterone concentration, but they were aggressive. Activation of the HPA axis inhibits the hypothalamic-pituitary-gonadal (HPG) activation; the HPG axis is responsible for reproductive physiology and associated behaviours (Lumley et al., 1999, Sapolsky et al., 2000, Retana-Márquez et al., 2003). Our findings agree with previous studies because isolated rats showed lower concentration of testosterone and higher concentration of stress hormones.

GHK animals which were group housed and treated with NMDA receptor antagonist (ketamine) showed impaired visual learning and memory in the NOR test when compared to GH group. Other studies have reported impaired visual learning and memory on animals that were treated with NMDA receptor antagonist (McKibben et al., 2010, Grayson et al., 2016). SI animals showed impaired visual learning and memory when compared to GH animals, this outcome is supported by other studies that reported the same cognitive impairment in isolated animals (Watson et al., 2012b, McIntosh et al., 2013). SIK animals showed impaired visual learning and memory when compared to GH animals. Our double hit model of schizophrenia did not exacerbate the impaired visual learning and memory since GHK and SI showed the same effect as SIK. Previous studies on double hit model of schizophrenia reported impaired visual learning and memory on animals (Gaskin et al., 2014, Gaskin et al., 2016, Shortall et al., 2020). Lui and colleagues conducted a study on double hit model, they concluded that NMDA receptor antagonist and social isolation are two independent models and further show that NMDA receptor antagonist is more associated with impaired visual learning and memory (Liu et al., 2017). The NOR test for visual learning and memory performed in this study has a translational relevance to visual recognition memory impairment, which is observed in schizophrenia patients (Rajagopal et al., 2014). In the current study SIK showed increased TNFα mRNA expression when compared to all other groups in the PFC.

Animals that were treated with ketamine (GHK and SIK) showed increased TNFα mRNA expression in the hippocampus. Animals that were socially isolated (SI and SIK) showed increased TNFα mRNA expression in the hippocampus. SIK showed increase in TNFα mRNA expression when compared to all other groups in the hippocampus and the PFC. Animals that were treated with ketamine (GHK and SIK) showed increased IL-6 mRNA expression in the PFC and in the hippocampus. Ketamine has been found to exacerbate schizophrenia symptoms

through microglia activation (Koh et al., 2016). The activation of microglia cells induces the secretion of proinflammatory cytokines such as TNFα and IL-6 which regulate neuroinflammation (Jung et al., 2017). Animals that were socially isolated (SI and SIK) showed increased IL 6 mRNA expression in the PFC and the hippocampus. SIK showed increased IL-6 mRNA expression when compared to all other groups in the PFC and the hippocampus. Increased concentration of IL-6 in the brain has been reported in schizophrenia (Lin et al., 1998, Monji et al., 2013). Previous studies have shown that neuroinflammation further exacerbates schizophrenia-like symptoms such as impaired visual learning and memory (Monji et al., 2009, Kirkpatrick and Miller, 2013). Increased IL-6 and TNFα mRNA expression were reported in postmortem PFC tissues of schizophrenic patients when compared to normal control (Volk et al., 2015, Pandey et al., 2018). Levels of IL-6 and TNFα have been significantly higher in chronic schizophrenia patients compared to the normal controls (Goldsmith et al., 2016). In adult brains, proinflammatory cytokines have been found to be involved in neuronal death after injury or neurodegenerative injury (Allan and Rothwell, 2003). A previous study found that juvenile social isolation exacerbate NMDA receptor antagonist-triggered expression of heat shock protein 70, which is the marker for neuronal injury (Inta et al., 2013). Even though GHK and SI showed increase TNFα and IL-6 mRNA expression, SIK showed more increased mRNA expression of TNFα and IL-6 when compared to all other groups.

Conclusion

The SIK group exhibited higher anxiety-like behaviour, reduced social interaction and more aggressive-like behaviour when compared to other groups. Furthermore, the SIK displayed impaired visual learning and memory accompanied by higher TNFα and IL 6 gene expression in the PFC and the hippocampus when compared to other groups. This suggest that post-weaning social isolation and ketamine when combined was more robust in modelling the selected behavioural impairments observed in schizophrenia patients than each treatment alone. Our double hit model failed to exacerbate the impaired visual learning and memory since GHK and SI showed the same effect as SIK.

Author contribution

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Conceptualization (KBS, ML, and MM); Data curation (KBS); Formal analysis (KBS); Funding acquisition (ML and MM); Investigation (KBS, ML, and MM); Methodology (KBS, ML, and MM); Project administration (KBS); Resources (ML and MM); Software (KBS); Supervision (ML and MM); Validation (ML and MM); Visualization (KBS, ML, and MM); Roles/Writing - original draft (KBS); and Writing – review (ML and MM) & editing (KBS, ML, and MM).

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Data availability statement

Data will be provided to the Journal if requested.

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Conflict of Interest

The authors declare no conflicts of interest.

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PROLOGUE

In the previous study we were able to show that the double hit model of schizophrenia is able to induce negative and cognitive symptoms of schizophrenia. Further, we were able to show behavioural and molecular changes involved in negative and cognitive symptoms of the disease. Understanding behavioural and molecular changes involved in the positive symptoms of schizophrenia is important and it will assist researchers in improving the treatment of schizophrenia. We used the double hit model of schizophrenia to investigate behavioural and molecular changes underlying positive symptoms of schizophrenia. This manuscript is titled "***Evaluating neurochemical changes and associated locomotor activity in a double hit schizophrenia model***". This manuscript is submitted in the journal "**Brain Research**" to be considered for publication**,** the chapter has been formatted according to the journal's guidelines for authors.

Chapter 4

Evaluating neurochemical changes and associated locomotor activity in a double hit schizophrenia model

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Abstract

Our double hit model has been shown to be more robust in inducing behavioural and molecular changes involved in the negative and cognitive symptoms of schizophrenia when compared to each single hit model. Disrupted dopamine, glutamate, GABA, and cholinergic functions are implicated in the pathophysiology of positive symptoms of schizophrenia. In this study, we investigated the behavioural and neurochemical changes associated with locomotor activity in schizophrenia using the double-hit model of schizophrenia. On postnatal day (PND) 23, rats were grouped (n=8) as follows: group-housed + saline (GH), group-housed + ketamine (GHK), socially isolated + saline (SI), and socially isolated + ketamine (SIK). On PND 28, a single ketamine dose (16 mg/kg) was administered three times a week for four weeks. Thereafter, the animals were injected twice a week for the duration of the study. Isolated animals were housed singly throughout the study. On PND 86 the open field test was conducted to assess locomotor activity. On PND 88 the study was terminated, and the striatum was harvested for the measurement of the concentration of dopamine, glutamate, GABA, and acetylcholine and dopamine D2 mRNA expression. The SIK group showed more hyperactivity than the other groups (GH vs SIK, $p<0.0001$; GHK vs SIK, $p<0.0001$; SI vs SIK, $p=0.0003$). This was accompanied by the overexpression of dopamine D2 mRNA (GH vs SIK, $p<0.0001$; GHK vs SIK, $p<0.0001$; SI vs SIK, $p=0.0281$), with increased acetylcholine concentration (GH vs SIK, $p<0.0001$; GHK vs SIK, $p=0.0004$; SI vs SIK, $p<0.0001$), and a decreased glutamate concentration (GH vs SIK, $p<0.0001$; GHK vs SIK, $p=0.0001$; SI vs SIK, $p<0.0001$) and GABA concentration (GH vs SIK, $p<0.0001$; GHK vs SIK, $p=0.0006$; SI vs SIK, $p=0.0001$). Taken together, these changes suggest that the SIK group highly represent the positive symptoms observed in schizophrenia patients. Our double hit model was able to induce neurotransmitter changes similar to those observed from positive symptoms of schizophrenia patients.

Keywords: Schizophrenia, social isolation, ketamine, locomotor activity, dopamine, GABA, and acetylcholine.

Introduction

Schizophrenia is a neuropsychiatric disorder characterised by positive, negative, and cognitive symptoms (Andreasen, 1995, Gomes et al., 2016). In animals, positive symptoms of schizophrenia include delusions, auditory and visual hallucinations, and are characterised by hyper-locomotor activity (Andreasen, 1995, Pratt et al., 2012). Researchers have used hyper-locomotor activity as an indication of positive symptoms when investigating different parameters of schizophrenia (Gaskin et al., 2016, Hamieh et al., 2021). Researchers used these animal models to reproduce aetiological factors related to schizophrenia such as neurochemical and physiological changes (Jones et al., 2011). Post-weaning social isolation is a neurodevelopmental animal model of schizophrenic-like symptoms. The social isolation of rat pups immediately after weaning on postnatal day (PND)21, is achieved by housing rats singly in clear plastic cages that enable them to see, smell and hear conspecifics but do not have physical contact (Simpson et al., 2010).

Post-weaning social isolation of rodents induces locomotor hyperactivity and this behavioural impairment resembles the positive symptoms of schizophrenia (Fone and Porkess, 2008). Studies have shown that socially isolated rats are more active (increased ambulation) in a novel arena compared to their group housed counterparts (Hickey et al., 2012, Gaskin et al., 2014). A shortcoming of social isolation studies is that behaviours produced by isolation can be easily reversed by frequent handling of the animals or when exposing them to a variety of behavioural tests (Jones et al., 2011). This locomotor hyperactivity is increased horizontal movement activity which is explained as an inability to habituate, which hypothetically reflects mesolimbic dopamine hyperactivity (Fabricius et al., 2011). Social isolation in rats increases both dopamine release and dopamine receptor sensitivity in the nucleus accumbens and in the striatum (Owen et al., 1978, Jones et al., 1992). Post-mortem studies showed that a neuropathological pathway involved in schizophrenia includes increases in both striatal dopamine D2 receptor and dopamine concentration (Owen et al., 1978, Mackay et al., 1982). It has also been shown that dysfunction in the glutamatergic pathway is involved in the pathophysiology of schizophrenia (Tsai and Coyle, 2002, Coyle et al., 2003, Stone, 2011).

The pharmacological model of schizophrenia has shown that blockade of N-methyl-D-aspartate (NMDA) receptor by non-competitive antagonists such as ketamine can induce an increase in locomotor activity (Kalinichev et al., 2008, Jones et al., 2011). Glutamate concentration has been reported to be increased in different brain regions in individuals at high

risk of schizophrenia and untreated individuals with schizophrenia (de la Fuente-Sandoval et al., 2011, Tandon et al., 2013). NMDA receptor hypofunction has also been shown to be involved in the pathophysiology of schizophrenia (Egerton et al., 2020, McCutcheon et al., 2020). Data from post-mortem studies of people with schizophrenia showed changes in the binding and transcription of the glutamate NMDA receptor (Lewis and Moghaddam, 2006). Non-competitive NMDA receptor antagonist such as dizocilpine (MK801), phencyclidine (PCP), and ketamine induce psychological effects that are identical to the core symptoms of schizophrenia (Morgan and Curran, 2006, Javitt, 2007). NMDA receptor antagonists are usually given to adult animals, hence this schizophrenia model cannot be used as a neurodevelopmental model as it lacks construct validity (Jones et al., 2011). The pathophysiology of schizophrenia not only depends on the excitatory neurotransmitter glutamate, but also the inhibitory neurotransmitter GABA, which has also been proven to play a part in the pathophysiology of schizophrenia (Guidotti et al., 2005). Any disturbance in GABA or glutamate signalling can cause an imbalance between the inhibitory and excitatory networks (Benes and Berretta, 2001, Guidotti et al., 2005). NMDA receptor antagonists have been shown to decrease GABAergic function and this is likely to cause an increase in pyramidal cell firing due to disinhibition (Olney and Farber, 1995, Homayoun and Moghaddam, 2007). Studies have also proposed that acetylcholine is associated with the aetiology of schizophrenia (Lacey et al., 1990, Higley and Picciotto, 2014). Acetylcholine is a vital regulator of neuronal activity in the central nervous system and the periphery (Changeux, 2010, Picciotto et al., 2012). There is increasing evidence from preclinical and clinical studies pointing to the dysfunction of acetylcholine signalling in playing a role in the pathophysiology of schizophrenia (Higley and Picciotto, 2014).

More studies are still needed to investigate the changes in various neurotransmitters involved in motor control on locomotor activity in a rat model for schizophrenia. There is a paucity of studies investigating changes in the concentration of different neurotransmitters (dopamine, glutamate, GABA, and acetylcholine) and their effect on locomotor activity in a schizophrenia model. Post-weaning social isolation and NMDA receptor antagonist have their shortcomings but combining both models into one can improve robustness. This study aims to use the double hit (postweaning social isolation and NMDA receptor antagonist (ketamine)) model of schizophrenia to investigate changes in neurotransmitters (dopamine, glutamate, acetylcholine, and GABA) concentration that may lead to increased locomotor activity, a feature of positive symptoms of schizophrenia in animals.

Methods and materials

Animals

A total of 32 male Sprague Dawley rats (21 days old (92 g)) were acquired from the breeding unit of the University of KwaZulu-Natal. On PND23, animals were randomly separated to 4 different groups (n=8/group) as follows: group housed rats + vehicle (0.9% NaCl) injected, grouped housed rats + ketamine (16 mg/kg) injected, socially isolated rats + vehicle (0.9% NaCl) injected, and socially isolated rats + ketamine (16 mg/kg) injected. The rats were housed under standard laboratory conditions of 21-24 °C room temperature, 55% humidity and maintained on a 12:12 hour light-dark cycle (lights on at 06h00 and off at 18h00). No environmental enrichment was added to the cages with the socially isolated animals so as to prevent attenuating the social isolation effect (Brenes et al., 2020). All handling and experimental manipulation was carried out in the light phase (Richetto et al., 2019). Food and water were available ad libitum. Experiments were conducted in full compliance with the guidelines, rules and regulations specified by the Public Health Service policy on humane care and use of laboratory animals. The University of KwaZulu-Natal Animal Research Ethics Committee (AREC) approved all procedures (AREC/00002812/2021).

Experimental design

On PND21-PND22, animals were acclimatized (housed in groups of 8 animals per cage). On PND23, animals were assigned to 4 different groups (group housed + saline (GH), group housed + ketamine (GHK), socially isolated + saline (SI), and socially isolated + ketamine (SIK)). Thereafter, on PND23 social isolation commenced in appropriate groups (SI and SIK) and lasted throughout the study. On PND28, ketamine (16 mg/kg) treatment commenced in the appropriate groups (GHK and SIK) and lasted throughout the study. On PND86 the open field test was performed for each animal in a behavioural test room for 10 minutes after an hour of habituation. On PND88 all animals were decapitated to collect the striatum. The striatum was processed and stored at –80 ºC in a biofreezer until ready for the biochemical analyses of dopamine, GABA, glutamate, and acetylcholine concentration, as well as dopamineD2 mRNA receptor expression.

Ketamine treatment and social isolation

We used the same ketamine dose as (Koh et al., 2016). Ketamine (100 mg/ml concentration) was diluted to 1.6 mg/ml using saline (0.9% NaCl) as a solvent and injected subcutaneously at

a volume of 10 ml/kg of body weight equivalent to a dosage of 16 mg/kg [Dosage = Concentration x Volume] (Schobel et al., 2013). Due to ethical considerations the lowest published dosage of ketamine was used, which is 16 mg/kg. Animals that received ketamine were injected subcutaneously 3 times a week (Monday, Wednesday, and Friday) at 09:00 h for 4 weeks. After 4 weeks, and at PND 57, the number of injections were reduced due to ethical considerations, the animals were injected twice a week (Monday and Friday) at 09:00 until the end of the experimental period PND 88. Animals that were not injected with ketamine were injected with the same volume of 0.9% NaCl as ketamine every time ketamine was administered. On PND 23 animals that were isolated in the socially isolated groups were housed singly throughout the study in transparent cages with dimensions (40 cm long x 25 cm wide x 35 cm high). Animals were able to hear, smell and see other animals but physical contact with them was not allowed, Group housed animals were housed 4 per group in standard home cages with dimensions 55 cm long x 35 cm wide x 20 cm high.

Open field test (locomotor activity)

The open field test was performed on PND 86 to measure locomotor activity by observing the number of crossings (calculating times the line of a square is crossed with all 4 legs). The open field apparatus was a clear plexiglass (72 cm x 72 cm x 36), with 16 squares at the bottom of the apparatus (18 cm x 18 cm). On the test day, at 09:00, animals were moved to the experimental room and allowed to habituate for an hour. After the habituation period, each experimental rat was placed in the open field apparatus (the animal was placed in the corner square of the apparatus). and the number of horizontal crossings performed by each animal was recorded for 10 minutes. Between each trial, the apparatus was wiped clean with 70% ethanol to remove any residual odours that may affect subsequent tests. All tests were recorded using a camera (Canon PowerShot SX70 HS), and the videos were scored afterwards by an observer who was blind to the experimental groups.

Decapitation

The day after the last behavioural test on PND 88, all animals were decapitated using a sharp guillotine. Striatum was harvested into 2ml centrifuge tubes and snap-frozen in liquid nitrogen and stored at -80 °C in a biofreezer until ready for benchwork analysis.

Neurotransmitter analysis

Striatal acetylcholine (E-EL-R0081), and dopamine (E-EL-R0343) concentrations were measured with an ELISA kit strictly adhering to the manufacturer's instructions (Elabscience Biotech, Texas, USA). The samples were measured in duplicates; outliers were replaced through imputation with values identical to the mean. A best-fit curve was constructed using GraphPad Prism version 8, and its equation was used to calculate the sample concentration. The samples were randomised in the ELISA plate to eliminate bias. We chose the ELISA because we wanted to measure the concentration, in our laboratory, we mostly use the ELISA to measure the concentration.

Striatal GABA (E-BC-K852-M) and glutamate (MET-5080) concentrations were assessed using a colorimetric assay kit strictly adhering to manufacturer's instructions (Elabcience Biotech, Texas, USA). The samples were measured in duplicates; outliers were replaced through imputation with values identical to the mean. A best-fit curve was constructed using GraphPad Prism version 8, and it equation was used to calculate the sample concentration. The samples were randomised in the ELISA plate to eliminate bias. We chose the ELISA because we wanted to measure the concentration, in our laboratory, we mostly use the ELISA to measure the concentration.

RNA isolation for real-time PCR (RT PCR)

Real-time PCR was used to quantify the expression levels of dopamine receptor D2, and total RNA isolated from the dissected striatum tissue. The homogenization solution (Maxwell RSC simplyRNA Tissue Kit, Promega, USA) was used to homogenize the samples and RNA was extracted according to the manufacturer's instructions. The purity of isolated RNA was evaluated using a NanoDrop Spectrophotometer (thermoFisher SCIENTIFIC, USA), and the samples were considered pure if RNA absorbance values is ideally 2.0 but can vary from between 1.8 and 2.0. All isolated RNA with a concentration of 100 ng/µl and above were converted to cDNA using the iScripTM cDNA Synthesis kit (Bio-Rad, USA), and 2 steps SYBR green master mix was used for all qPCR reactions (Bio-Rad, USA). The cDNA was stored at -20 °C; the primers used in this study are shown in table 1. These primers were supplied by Inqaba Biotechnical Industries (South Africa), the primer sequence were obtained from the previous published study, and the standard was run to optimise the primers. dopamine receptor D2 and GAPDH were amplified and analysed using the Roche Light Cycler® 480 real-time PCR system (Roche Diagnostics, USA). In our lab we used GAPDH as a reference

gene and its optimal conditions are well established in our lab. In our lab we have published studies where we used GAPDH as a reference gene. The following PCR conditions were used: 30 cycles at 95 °C for 15 seconds gene denaturation, 60 °C for 60 seconds GAPDH primer annealing, 61 °C for 1 minute dopamine receptor D2 primer annealing, and 72 °C for 8 minutes final extension. The reactions were run in triplicate, fortunately we did not have outliers in our data. In our reaction we did have negative and positive control. The positive control was used to prove that the RT qPCR conditions were optimal. The negative control known as no template control was also included in our reaction, the negative control contains all the RT qPCR reaction reagents and nuclease-free water or PCR graded water instead of a cDNA template. The expression was calculated by measuring the actual concentration. Actual concentration=concentration of the sample/concentration of the reference gene.

Table 1: Sequences of primers used in the determination of Dopamine receptor D_2 mRNA expression using RT PCR. The primer sequence was obtained from the previous published study.

Primer	**Gene Sequence**
GAPDH forward	5′-GGCATTGCTCTCAATGACAA-3′
GAPDH reverse	5′-ATGTAGGCCATGAGGTCCAC-3′
Dopamine receptor D_2 forward	5′-AGACACCACTCAAGGGCAAC-3′
Dopamine receptor D_2 reverse	5′-CGCCTGTTCACTGGGAAACT-3′

Statistical analysis

Data was analysed using the software, GraphPad Prism version 8 (San Diego, California, USA). All results are presented as the mean ± SEM. The Shapiro-Wilk test was used to test for normality. Our data was normally distributed. Two-way ANOVA, followed by the Bonferroni multiple comparisons test was used to analyse the data. The $p< 0.05$ was considered significant. Multiple comparisons will be conducted even in the absent of significant interaction effects because we want to compare the effect of the double hit model when compared to other groups. The correlation coefficient was tested using the parametric Pearson correlation.

Results

Locomotor activity

Locomotor activity was measured in the group housed saline (GH), group housed ketamine (GHK), isolated saline (SI), and isolated ketamine (SIK) groups following placement in the open field. There was an overall housing effect in locomotor activity in the GH, GHK, SI, and SIK groups, $F(1,28) = 162.7$, $p<0.0001$. There was an overall ketamine effect in locomotor activity in the GH, GHK, SI, and SIK, $F(1,28) = 32.31$, $p=0.0027$. Multiple comparisons showed that there was a ketamine effect and isolation effect on locomotor activity in the GHK, SI, and SIK compared to the GH group *(GH vs. GHK, $p=0.0159$; GH vs. SI, $p<0.0001$; GH vs. SIK, $p<0.0001$, Figure 1). However, social isolation showed a greater effect on locomotor activity than ketamine #(GHK vs. SI, $p=0.0002$, Figure 1). Social isolation enhanced the ketamine effect on locomotor activity α(GHK vs SIK, $p<0.0001$; SI vs. SIK, $p=0.0003$, Figure 1).

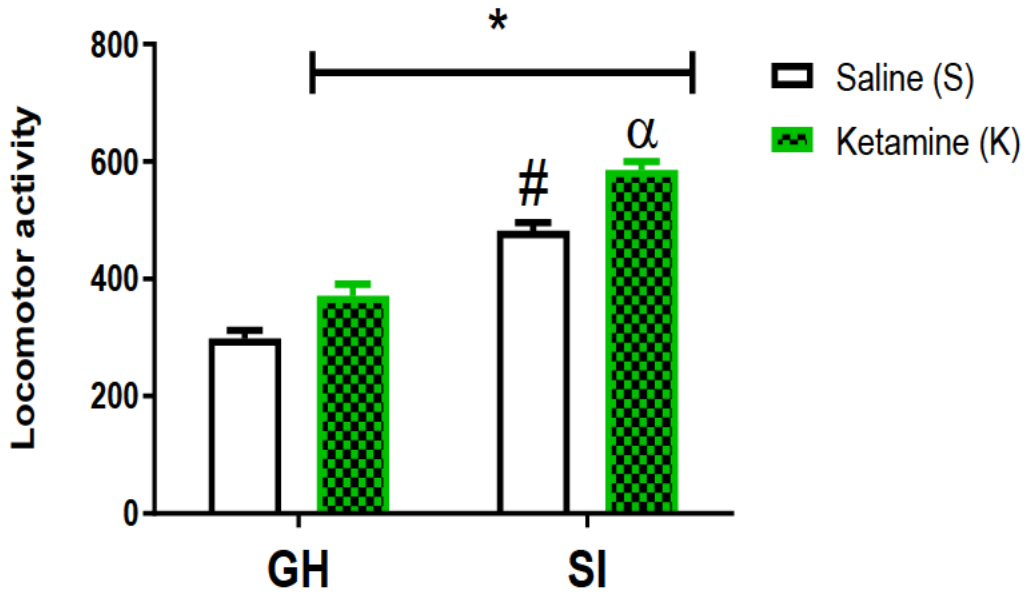

Figure 1. Locomotor activity in the GH, GHK, SI, SIK groups (n = 8 per group) as observed during the open field test. Data presented as mean ± SEM. *(GH vs. GHK, $p=0.0159$; GH vs. IS, $p<0.0001$; GH vs. SIK, $p<0.0001$); #(GHK vs. SI, $p=0.0002$; GHK vs. SIK, $p<0.0001$); α (IS vs. SIK, $p=0.0003$).

Dopamine D2 mRNA receptor expression

There was an overall housing effect on dopamine D2 mRNA receptor expression in the GH, GHK, SI, and SIK, $F(1,28) = 55.93$, $p<0.0001$. There was an overall ketamine effect on dopamine D2 mRNA receptor expression in the GH, GHK, SI, and SIK, $F(1,28) = 8.71$, $p=0.0027$. There was an isolation effect on dopamine (D2) mRNA receptor expression #(GHK vs. SI, $p=0.0204$, Figure 2). There was a ketamine effect on D2 mRNA receptor expression in the isolation animals α(SI vs. SIK, $p=0.0281$, Figure 2).

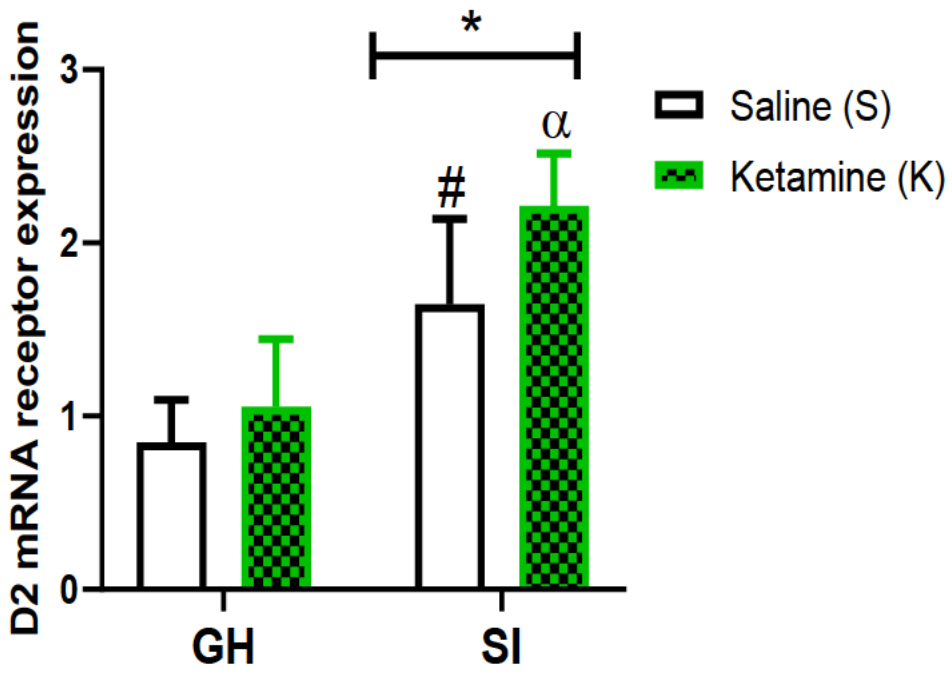

Figure 2. Dopamine D2 mRNA receptor expression in the GH, GHK, SI, SIK groups (n = 8 per group). Data presented as mean ± SEM. *(GH vs. SI, $p=0.0011$; GH vs. SIK, $p<0.0001$); #(GHK vs. SI, $p=0.0204$; GHK vs. SIK, $p<0.0001$) α(IS vs. SIK, $p=0.0281$).

Dopamine concentration

There was an overall housing effect on dopamine concentration in the GH, GHK, SI, and SIK, $F(1,28) = 441.9$, $p<0.0001$. There was an overall ketamine effect on dopamine concentration in the GH, GHK, SI, and SIK, $F(1,28) = 10.83$, $p=0.0027$. There was a ketamine effect and isolation effect on dopamine concentration in the GHK, SI, and SIK groups compared to the

GH group *(GH vs. GHK, p=0.0079; GH vs. SI, p<0.0001, GH vs. SIK, p<0.0001, Figure 3). There was an isolation effect on dopamine concentration #(GHK vs. SI, p<0.0001; GHK vs. SIK, p<0.0001, Figure 3).

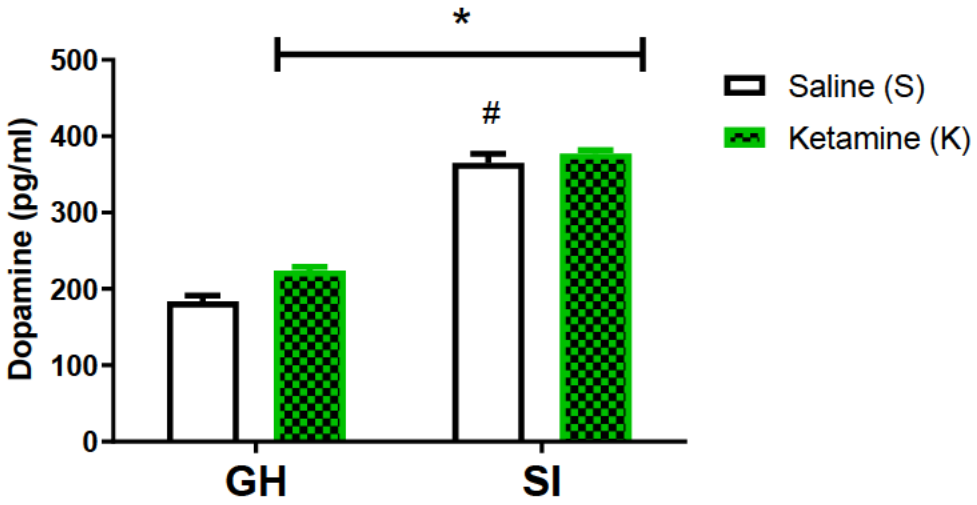

Figure 3. Dopamine concentration in the GH, GHK, SI, SIK groups (n = 8 per groups). Data presented as mean ± SEM. *(GH vs. GHK, p=0.0079; GH vs. SI, p<0.0001; GH vs. IK, p<0.0001); #(GHK vs. SI, p<0.0001; GHK vs. SIK; p<0.0001).

Acetylcholine concentration

There was an overall housing effect in acetylcholine concentration in the GH, GHK, SI, and SIK, $F(1,28) = 115.0$, $p<0.0001$. There was an overall ketamine effect in acetylcholine concentration in the GH, GHK, SI, and SIK, $F(1,28) = 185.2$, $p=0.0027$. There was a significant interaction between social isolation and ketamine in acetylcholine concentration, $F(1,28) = 16.73$, $p=0.0003$. There was a ketamine effect and isolation effect on acetylcholine concentration in the GHK and IS groups compared to the GH group *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001, Figure 4). Social isolation enhanced the ketamine effect α(GHK vs. SIK, p=0.0004; SI vs. SIK, p<0.0001, Figure 4).

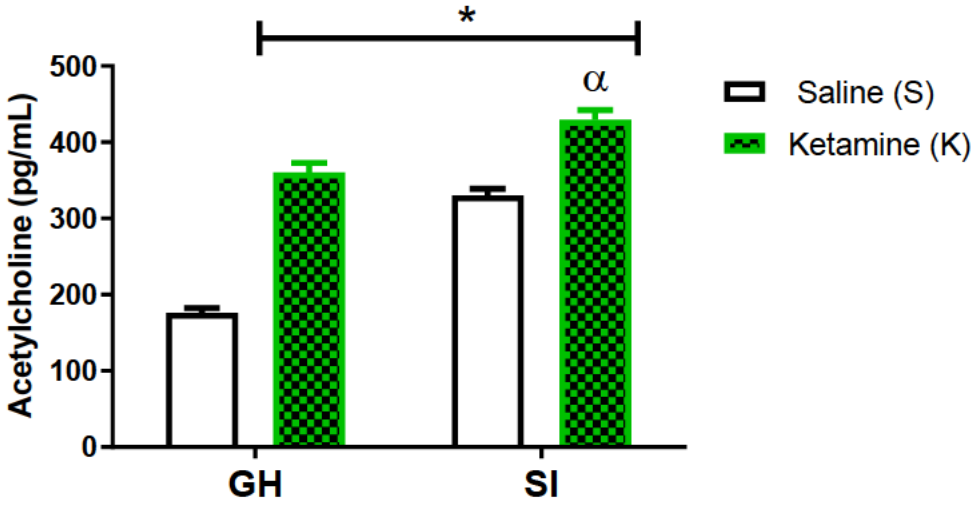

Figure 4. Acetylcholine concentration in the GH, GHK, SI, SIK groups (n = 8 per group). Data presented as mean ± SEM. *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001); α(GHK vs. SIK, p=0.0004; SI vs. SIK, p<0.0001).

Glutamate concentration

There was an overall housing effect in glutamate concentration in the GH, GHK, SI, and SIK, $F(1,28) = 71.44$, $p<0.0001$. There was an overall ketamine effect in glutamate concentration in the GH, GHK, SI, and SIK, $F(1,28) = 34.65$, $p=0.0027$. There was a ketamine effect and isolation effect on glutamate concentration in the GHK and SI groups compared to the GH group *(GH vs. GHK, p=0.0001; GH vs. SI, p=0.0167, Figure 5). Social isolation enhanced the ketamine effect α(GHK vs. SIK, p=0.0001; SI vs. SIK, p<0.0001, Figure 5).

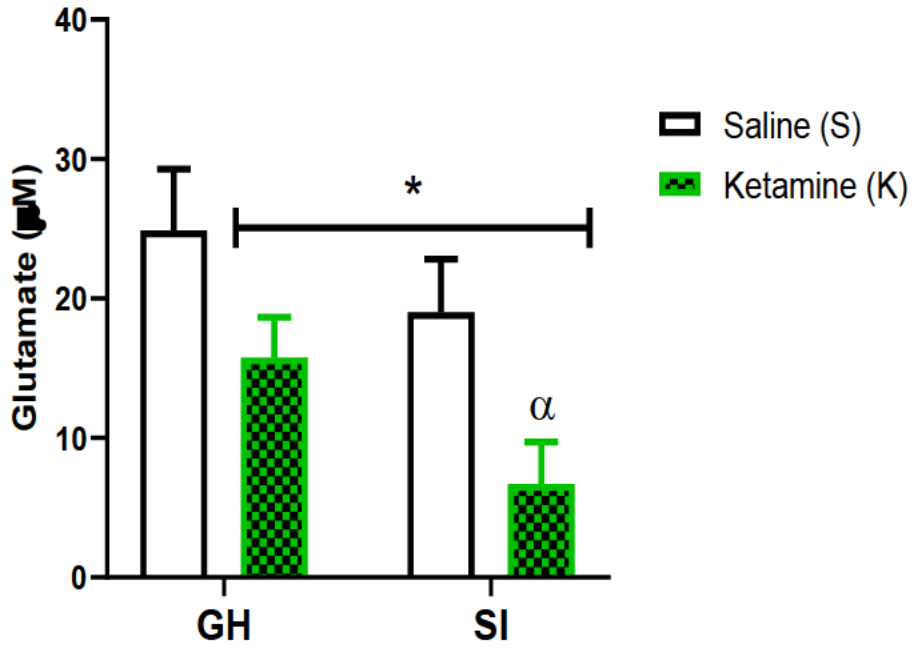

Figure 5. Glutamate concentration in the GH, GHK, SI, SIK groups (n = 8 per group). Data presented as mean ± SEM. *(GH vs. GHK, p=0.0001; GH vs. SI, p=0.0139; GH vs. SIK, p<0.0001); #(GHK vs. SIK', p=0.0001); α(SI vs. SIK, p<0.0001).

γ-Aminobutyric Acid (GABA) concentration

There was an overall housing effect in GABA concentration in the GH, GHK, SI, and SIK, $F(1,28) = 60.56$, $p<0.0001$. There was an overall ketamine effect in GABA concentration in the GH, GHK, SI, and SIK, $F(1,28) = 73.42$, $p=0.0027$. There was a ketamine effect and isolation effect on GABA concentration in the GHK and SI groups compared to the GH group *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001, Figure 6). Social isolation enhanced the ketamine effect α(GHK vs. SIK, p=0.0006; SI vs. SIK, p=0.0001, Figure 6).

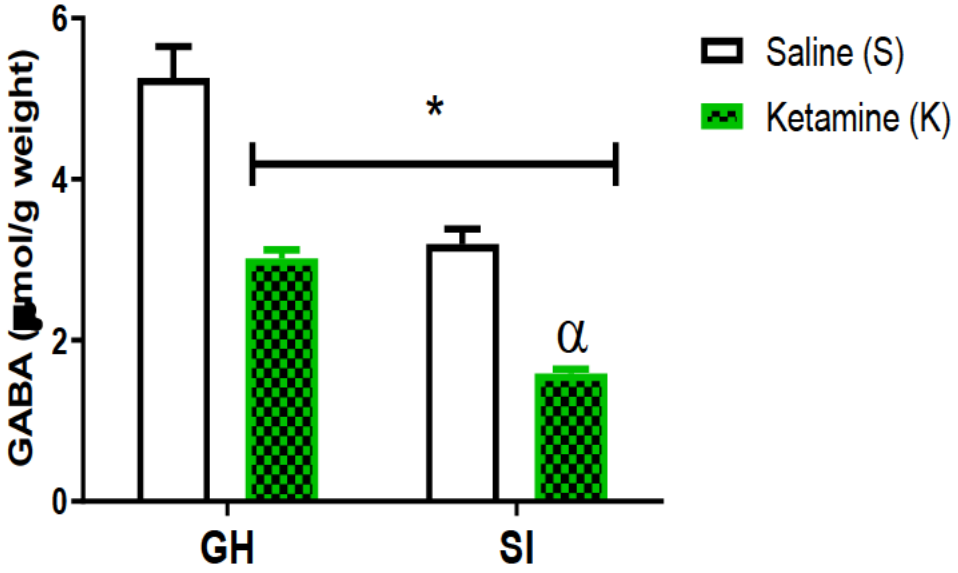

Figure 6. GABA concentration in the GH, GHK, SI, SIK groups (n = 8 per groups). Data presented as mean ± SEM. *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001); # (GHK vs. SIK, p=0.0006); α (SI vs. SIK, p=0.0001).

Correlation

Pearson correlation between locomotor activity and dopamine (refers to dopamine striatal levels) was found to be positive and statistically significant (r = 0.8438, p<0.0001), this shows that an increase in dopamine concentration will lead to an increase in locomotor activity. Pearson correlation between locomotor activity and mRNA expression of dopamine D2 receptor was found to be positive and statistically significant (r = 0.8244, p<0.0001), this shows that an increase in dopamine D2 mRNA receptor expression will lead to an increase in locomotor activity. Pearson correlation between locomotor activity and glutamate was found to be negative and statistically significant (r = -0.7220, p<0.0001), this shows that a decrease in glutamate concentration will lead to an increase in locomotor activity. Pearson correlation between locomotor activity and GABA was found to be moderately negative and statistically significant (r = -0.6665, p<0.0001), this shows that a decrease in GABA concentration will lead to an increase in locomotor activity. Pearson correlation between locomotor activity and acetylcholine concentration was found to be highly positive and statistically significant (r = 0.7031, p<0.0001), this shows that an increase in acetylcholine concentration will lead to an increase in locomotor activity.

Table 2: Pearson correlation analysis between locomotor activity and (dopamine concentration, glutamate concentration, GABA concentration, acetylcholine concentration, and dopamine D2 mRNA expression).

	Locomotor vs. Dopamine	**Locomotor vs. Dopamine D2**	**Locomotor vs. Glutamate**	**Locomotor vs. GABA**	**Locomotor vs. Acetylcholine**
Peason correlation (r)	0.8438	0.8244	-0.7220	-0.6665	0.7031
P (two-tailed)	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001
Significant (alpha <0.05)	Yes	Yes	Yes	Yes	Yes
N-value	32	32	32	32	32

Discussion

In this study we used the double hit model of schizophrenia to investigate its effects on neurotransmitter concentration and dopamine D2 receptor mRNA expression whether these led to changes associated with positive symptoms in schizophrenia. Increased locomotor activity is associated with the animal's inability to habituate; they continuously and actively explore the testing apparatus. This has been described as an index for the positive symptoms of schizophrenia (Fone and Porkess, 2008, Jones et al., 2011). In this instance, we used locomotor activity as a measure of schizophrenia-like behaviour in animals. We showed that animals treated with both ketamine and social isolation (SIK) had increased locomotor activity when compared to other groups. The double hit model was able to induce one of the positive symptoms of schizophrenia. This was accompanied by changes in dopamine, acetylcholine, glutamate, and striatal GABA concentration as well as striatal dopamine D2 mRNA receptor

expression. The outcomes of the study will assist researchers to better understand the molecular underpinnings of positive symptoms of schizophrenia. Our findings are supported by other studies that reported increased locomotor activity in animals that were treated with the double hit model of schizophrenia (Simpson et al., 2010, Gaskin et al., 2016, Hamieh et al., 2021). The GHK animals were treated with an NMDA receptor antagonist (ketamine). This group was also highly mobile when compared to the control group (GH). This is a novel finding for group housed rats as other studies did not find differences in locomotor activity (Sams-Dodd, 2004, Beninger et al., 2009, Simpson et al., 2010). This may be explained by the fact that these studies used the NMDA receptor antagonist MK-801 while we used ketamine. The dosage and the duration of treatment could have been a factor as well. Our ketamine injections were maintained throughout the study duration, while these studies did not therefore that can possible have caused this effect.

In the current study rats that were socially isolated and treated with saline (SI), showed increased locomotor activity when compared to the GH group and GHK group. Watson et al, reported increased locomotor activity in socially isolated animals that were treated with saline, the authors conducted a single hit model of social isolation and comparing it with group housed animals (Watson et al., 2012b). This shows that social isolation is more of a factor in locomotor activity when compared to ketamine. Dopamine D2 receptor mRNA expression in the SIK animals was higher than in the other groups. The idea that dopamine D2 receptor is altered in schizophrenia patients is supported by genetic studies that showed positive correlation between schizophrenia and the dopamine D2 receptor gene (2014). We also found a positive correlation between locomotor activity and dopamine D2 mRNA expression. This suggests that an increase in dopamine D2 mRNA receptor expression may lead to an increase in locomotor activity. Positive symptoms of schizophrenia such as locomotor hyperactivity are associated with striatal dopamine D2 receptor overexpression which is caused by hyperactive mesolimbic dopamine projections (Davis et al., 1991, Lau et al., 2013). Antipsychotic drugs used to treat schizophrenia such as lamotrigine act as dopamine D2 receptor antagonists and have proven to be effective against the positive symptoms of schizophrenia (Gaskin et al., 2016).

We also showed that socially isolated animals (SIK and SI) had increased striatal dopamine concentration when compared to group-housed animals (GH and GHK). This has also been shown in psychotic patients and those exhibiting positive symptoms of schizophrenia (Meyer-Lindenberg et al., 2002, McGowan et al., 2004, Howes et al., 2009, Howes et al., 2013). We found a positive correlation between locomotor activity and striatal dopamine concentrations.

We also showed a ketamine effect in the grouped housed animals with the GHK having higher dopamine concentration. NMDA receptor antagonists such as ketamine or phencyclidine can evoke the production of dopamine (Ary and Komiskey, 1982, Seeman and Lasaga, 2005). This increase in dopamine concentration is thought to induce certain core symptoms of schizophrenia such as psychosis and locomotor hyperactivity which are part of the positive symptoms of schizophrenia (Deutch et al., 1987, Jentsch et al., 1998, Kalinichev et al., 2008).

Even though evidence points towards dopamine dysfunction as the pathogenesis of schizophrenia, dysfunction of glutamatergic system is also thought to be involved in the pathogenesis of schizophrenia (Goff and Coyle, 2001). In our study both social isolation and NMDA receptor antagonist decreased glutamate concentration. Social isolation has been reported to result in a decrease in glutamate concentration in the brain (Shao et al., 2015). Social isolation is a neurodevelopmental model of schizophrenia (Jones et al., 2011). The SIK group showed reduced glutamate concentration when compared to the other groups. Furthermore, we found that the NMDA receptor antagonist (ketamine) reduces glutamate concentration. These findings are supported by other studies that have reported a decrease in glutamate concentration in different brain regions of individuals with chronic schizophrenia (Ongür et al., 2009, Rowland et al., 2009, Tayoshi et al., 2009, Lutkenhoff et al., 2010, Natsubori et al., 2014). Animals treated with an NMDA receptor antagonist or are socially isolated showed decreased expression of glutamate decarboxylase (GAD67), which converts glutamate to GABA (Hashimoto et al., 2003, Benes et al., 2007, Gilabert-Juan et al., 2012). The effect of ketamine on glutamate has the potential to disturb the GABAergic system, GABA levels are altered between the groups because of the effect of ketamine on glutamate.

Evidently, GHK and SI showed reduced GABA concentration when compared to GH group, SIK showed reduced GABA concentration when compared to other groups. GABA is the main inhibitory neurotransmitter in the CNS, it stimulates fast neuronal inhibition through GABA receptors (Whiting, 2003). NMDA receptor antagonist such as ketamine have been reported to decrease both GABAergic interneuron function and striatal GABA concentration (Olney and Farber, 1995, Homayoun and Moghaddam, 2007). The SIK group had high acetylcholine concentration, furthermore, both GHK and SI had high acetylcholine concentration when compared to GH. Acetylcholine controls the dopamine neuron burst firing, rewarding processes, and locomotor activity in the dopamine mesolimbic region (Chapman et al., 1997, Lodge and Grace, 2006). Methamphetamine induced an increase in acetylcholine production, which caused dopaminergic firing, which may contribute to an increase in locomotor activity

(Mereu et al., 1987, Lacey et al., 1990). Increased acetylcholine concentration in the striatum and prefrontal cortex is associated with adaptive copying behaviours, and stressors such as social isolation can lead to adaptive behavioural response mediated through acetylcholine release (Higley and Picciotto, 2014). This response to stress mediated by acetylcholine release in the striatum and PFC induces the release of dopamine (Rougé-Pont et al., 1998, Marinelli and Piazza, 2002), and this dopamine can contribute to the increase in locomotor activity. Our results showed a positive correlation between locomotor activity and acetylcholine concentration, suggesting that an increase in acetylcholine concentration may lead to increased locomotor activity. This study only focused on one positive symptom of schizophrenia (locomotor activity) and molecular changes associated with it; another study is needed which will investigate the role of SIK on cognitive impairment and molecular changes associated with it.

Conclusion

Both models (social isolation and ketamine) were individual successful in inducing both behaviour and molecular changes similar to those observed in positive symptoms of schizophrenia. The double hit model (SIK) was able to induce robust neurochemical and behavioural changes that were more aligned to the positive symptoms of schizophrenia than either treatment alone. This was evidenced by higher locomotor activity, striatal dopamine D2 mRNA expression, striatal dopamine concentration, and striatal acetylcholine concentration as well as lower striatal GABA concentration and striatal glutamate concentration when compared to other groups. The outcomes of the study will add insight in the behavioural and molecular changes involved in the positive symptoms of schizophrenia. The current study only investigated dopamine receptor; future studies should focus on other receptors related to other neurotransmitters.

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PROLOGUE

In the previous chapters we were able to show that the double hit model of schizophrenia is more robust and is able to induce behavioural and molecular changes observed in schizophrenia patients when compared to each single hit model (social isolation or Ketamine). Understanding the mechanisms involved in the pathophysiology of schizophrenia will assist researchers in designing drugs that will be more successful in the treatment of the disease. There is a paucity of studies investigating mechanisms involved in the pathophysiology of schizophrenia. Therefore, in this chapter we investigated **"The role of GABAergic dysfunction and oxidative stress in schizophrenia".** This manuscript is submitted in the journal **"Neuroscience"** to be considered for publication, the chapter has been formatted according to the journal's guidelines for authors.

Chapter 5

GABAergic dysfunction and oxidative stress in schizophrenia

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Abstract

Few studies have investigated the mechanisms involved in the pathophysiology of schizophrenia. Changes in genes involved in GABAergic neurotransmission and oxidative stress can induce or exacerbate schizophrenia symptoms. This study aims to investigate the effect of a double hit model of schizophrenia on GABAergic function and oxidative stress in the prefrontal cortex (PFC) and hippocampus. To achieve our aims, rats were grouped as follows: 8 group-housed + saline (GH), 8 group-housed + ketamine (GHK), 8 isolated + saline (SI), and 8 isolated + ketamine (SIK). On post-natal day (PND) 28, a single ketamine dose (16 mg/kg) was administered three times a week for four weeks on GHK and SIK groups. The isolated animals were housed singly throughout the study. On PND 86 all animals were decapitated, and the prefrontal cortex (PFC) and hippocampus tissue collected. In both the PFC and hippocampus there was a reduction on glutamate decarboxylate 1 (GAD1), brain derived neurotrophic factor (BDNF), parvalbumin (PVALB), and mRNA gene expression in the GHK and SI groups. The expression of these genes was further reduced in the SIK group in both the PFC and the hippocampus. We also found a decrease in superoxide dismutase (SOD) and glutathione peroxidase (GPx1) concentration and an increase in the concentration of malondialdehyde (MDA) in the SIK group. These results suggest that oxidative stress and dysfunction in the GABAergic pathway are involved in the pathophysiology of schizophrenia.

Keywords: Schizophrenia, ketamine, double hit model, cognition, GABA biomarkers, and oxidative stress

Introduction

Schizophrenia is a debilitating psychiatric disorder that affects approximately 1% of the global population, with onset around the period of brain development that comes after puberty (Kirkbride et al., 2012). Schizophrenia is characterized by positive (hallucinations and delusions), negative (social withdrawal and apathy) and cognitive (poor memory and executive function) symptoms (Pratt et al., 2012). Current antipsychotic drugs have limited effect on negative and cognitive symptoms (Keefe et al., 2007, Mintz and Kopelowicz, 2007). A better understanding of neurophysiological and molecular alterations involved in schizophrenia will assist in the development of new effective drugs that can be used to treat schizophrenia. The double hit model of schizophrenia (postweaning social isolation and NMDA receptor antagonist) has been shown to be more robust in modelling the disorder than either treatment alone (Shangase et al., 2023, Shangase et al., 2024). The hippocampus and the prefrontal cortex (PFC) play a critical role in cognition, specifically memory integration and relational processing (Schlichting et al., 2015, Mack et al., 2018). Findings on postmortem studies from patients suffering from schizophrenia have reported a decline in gamma-amino-butyric-acid (GABA) releasing interneurons in the prefrontal cortex (PFC) and the hippocampus (Benes et al., 1991, Benes et al., 1998).

GABA is the major inhibitory neurotransmitter in the CNS and is vital for CNS suppression (Benes, 2015, Tso et al., 2015). Other studies have reported a reduction in the expression of genes involved in GABA neurotransmission, such as the gene encoding the enzyme glutamate decarboxylase 1 (GAD 1), the PVALB gene which encodes the calcium-binding protein, and brain derived neurotrophic factor (BDNF) gene in neuropsychiatric disorders (Hashimoto et al., 2003, Benes et al., 2007, Lisman et al., 2008, Heckers and Konradi, 2010, Gilabert-Juan et al., 2012). NMDA receptor antagonists cause a reduction in parvalbumin-containing GABAergic neurons (Radonjić et al., 2013, Jevtić et al., 2016). Reduction in BDNF concentration has been observed in psychiatric disorders such as schizophrenia (Carlino et al., 2011).

The pathophysiology of schizophrenia is caused by NMDA receptor dysfunction in GABAergic interneurons which leads to the disinhibition of pyramidal cells and excitotoxin damage in the hippocampus (Schobel et al., 2013). Furthermore, NMDA receptor antagonists induce necrosis and neuronal vocalization in certain neocortical brain regions in rats (Olney et

al., 1989, Gass and Herdegen, 1995). Molecular and genetic studies have shown that impaired redox reactions response to oxidative stress does play a role in the pathophysiology of schizophrenia (Prabakaran et al., 2004, Li et al., 2006). The imbalance between reactive oxygen species (ROS) and antioxidant production is referred to oxidative stress (Flatow et al., 2013). Increased production of ROS and reduced antioxidant protection have been reported in schizophrenic patients, suggesting the role of oxidative stress in the pathophysiology of schizophrenia (Salim, 2014). The failure of antioxidants such as superoxide dismutase (SOD) and glutathione peroxidase (GPx1)) to provide defence against ROS production induces cell membrane damage, DNA damage, protein carbonylation, lipid peroxidation and promotes apoptosis (Yao and Keshavan, 2011). Malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS) are byproducts of lipid peroxidation (Flatow et al., 2013). There is a paucity of studies investigating the mechanisms involved in the pathophysiology of schizophrenia. In this study, we aim to investigate the role of oxidative stress and the dysfunction in GABAergic biomarkers in the pathophysiology of schizophrenia.

Methods and Materials

Animals

A total of 32 male Sprague Dawley rats were acquired from the breeding unit of the University of KwaZulu-Natal. On PND 23, the animals were randomly separated into 4 different groups (n=8/GROUP) as follows: group housed rats + vehicle (0.9% NaCl) injected subcutaneously, grouped housed rats + ketamine (16 mg/kg) injected subcutaneously, socially isolated rats + vehicle (0.9% NaCl) injected, and socially isolated rats + ketamine (16 mg/kg) injected. The rats were housed under standard laboratory conditions of 21-24 °C room temperature, 55% humidity and maintained on a 12:12 hour light-dark cycle (lights on at 06h00 and off at 18h00). No environmental enrichment was added to the cages with the socially isolated animals so as to prevent attenuating the social isolation effect (Brenes et al., 2020). All handling and experimental manipulation were carried out in the light phase (Richetto et al., 2019). Food and water were available ad libitum. Experiments were conducted in full compliance with the guidelines, rules and regulations specified by the Public Health Service policy on humane care and use of laboratory animals. The University of KwaZulu-Natal Animal Research Ethics Committee (AREC) approved all procedures (AREC/00002812/2021).

Experimental design

On PND21-PND22, animals were acclimatized (housed in groups of 8 animals per cage). On PND23, the animals were assigned to their groups viz: group housed + saline (GH), group-housed + ketamine (GHK), socially isolated + saline (SI), and socially isolated + ketamine (SIK). Thereafter, social isolation commenced in the SI and SIK groups and lasted throughout the study. On PND28, ketamine (16 mg/kg) treatment commenced in the GHK and SIK groups and lasted throughout the study. On PND 86, all animals were decapitated, and the prefrontal cortex (PFC) and hippocampal tissue was collected. The tissue was processed and stored at –80 ºC in a biofreezer until ready for biochemical measurement of GAD1, BDNF, and PVALB mRNA expression.

Ketamine treatment and social isolation

We used the same ketamine dose as (Koh et al., 2016). Ketamine (100 mg/ml concentration) was diluted to 1.6 mg/ml using saline (0.9% NaCl) as a solvent and injected subcutaneously at a volume of 10 ml/kg of body weight equivalent to a dosage of 16 mg/Kg [Dosage = Concentration x Volume] (Schobel et al., 2013). Due to ethical considerations the lowest published dosage of ketamine was used which is 16 mg/kg. A repeated single dose of ketamine (16 mg/kg,S.C.) treatment regimen began on PND 28 to PND 85 and was done as follows: Animals that received ketamine were injected subcutaneously 3 times a week (Monday, Wednesday, and Friday) at 09:00 h for 4 weeks (Koh et al., 2016). Thereafter, on PND 57, the animals were injected 2 times a week (Monday and Friday) at 09:00 until the end of the experimental period (PND) 85. Animals that were not injected with ketamine were injected with the same volume of saline (0.9% NaCl). On PND 23 animals that were isolated in the socially isolated groups were house singly in transparent cages with dimensions (40 cm long x 25 cm wide x 35 cm high) for 62 days (animals were able to hear, smell and see other animals but physical contact with them was not allowed), Group housed animals were 4 per group in standard group cages with dimensions 55 cm long x 35 cm wide x 20 cm high.

Decapitation

On PND 86 all animals were decapitated. A sharp guillotine was used to humanely behead the rat. Sharp stainless steel surgical scissors were used to remove the tissue and pelage covering the top part of the rat skull. Once the skull was clearly exposed, the sharp small surgical scissors were used to cut the skull in the middle from back to the front part. Debakey Tweezer-X1 was used to peel the skull from the point of the incision until the brain was fully exposed. The brain was scooped with Kelly forceps CVD-X2 and placed in a cold sludge of 0.9% saline for a few

minutes. After 5 minutes, the brain was moved to the cutting board and the scalpel was used to cut the prefrontal cortex and hippocampus. Brain tissue was snap-frozen in liquid nitrogen and stored at -80 °C in a Biofreezer for a few days before the benchwork analysis was undertaken.

RNA isolation for real-time PCR (RT PCR)

Real-time PCR was used to quantify the expression levels of different genes (GAD1, PVALB, and BDNF), RNA was isolated from the dissected PFC and hippocampus tissue. The homogenization buffer (Maxwell simpleRNA tissue kit, Promega, USA) was used to homogenize the samples and RNA was extracted according to the manufacturer's instructions. The purity of isolated RNA was evaluated using a NanoDrop Spectrophotometer (thermoFisher SCIENTIFIC, USA), and the samples were considered pure if RNA absorbance values is ideally 2.0 but can vary from between 1.8 and 2.0. All isolated RNA with a concentration of 100 ng/µl and above were converted to cDNA using the iScripTM cDNA Synthesis kit (BioRad, USA), and 2 steps SYBR green master mix was used for all reactions (FastStart, USA). The cDNA was stored at -20 °C; the primers used in this study are shown in table 1. These primers were supplied by Inqaba Biotechnical Industries (South Africa). GAD1, PVALB, BDNF, and GAPDH were amplified and analysed using the Roche Light Cycler® 480 real-time PCR system (Roche Diagnostics, USA). the primer sequences were obtained from the previous published study, and the standard was run to optimise the primers. In our lab we used GAPDH as a reference gene and its optimal conditions are well established in our lab. In our lab we have published studies where we used GAPDH as a reference gene. The efficiency of the primer sets was 100%. The following PCR conditions were used: 30 cycles; with preincubation of 95 °C for 30 seconds; 3 step amplification of 95 °C for 15 seconds, 60 ºC for 60 seconds, 72 ºC for 5 seconds; melting of 95 ºC for 10 seconds, 97 ºC for 1 seconds; cooling of 37 ºC for 30 seconds. GAPDH primer annealing 65 °C for 1 minute; GAD1 primer annealing 60 ºC for 1 minute; PVALB primer annealing 58 ºC for 1 minute; BDNF primer annealing 63 ºC for 1 minute. The reactions were run in triplicate, fortunately we did not have outliers in our data. In our reaction we did have negative and positive control. The positive control was used to prove that the RT qPCR conditions were optimal. The negative control known as no template control was also included in our reaction, the negative control contains all the RT qPCR reaction reagents and nuclease-free water or PCR graded water instead of a cDNA template. The expression was calculated by measuring the actual concentration. Actual concentration=concentration of the sample/concentration of the reference gene.

Table 1: Primer sequences for RT-PCR analysis.

Gene Name	Forward Primer	Reverse Primer	Product size (bp)
GAPDH	ggcaagttcaatggcacagt	tggtgaagacgccagtagactc	183
GAD 1	cacaaactcagcggcataga	gccttgtcccctgtatcgta	194
PVALB	gagtgcggatgatgtgaaga	gtcagcgccacttagctttc	228
BDNF	ggttcgagaggtctgacgac	caaaggcacttgactgctga	159

PFC and hippocampus MDA concentration

PFC and hippocampus MDA concentration was measured with an ELISA kit (Elabscience Biotech (E-EL-0060, Texas, USA). A 50µl aliquot of standard or sample was added to a 96 well ELISA plate, followed immediately by the addition of 50µl of Biotinylated Detection AB. The plate was incubated for 45 minutes at 37°C in the HettCube 200 incubator. After the incubation period, the plate was aspirated and washed 3 times with the wash buffer provided. Then 100µl of HRP conjugate was added followed by 30 minutes incubation at 37 ºC in the HettCube 200 incubator. The plate was then aspirated and washed 5 times. A 90µl of substrate was added followed by a 15 minute incubation period at 37°C in the incubator. After the 15 minute incubation period, a stop solution (50µl), was added to terminate the reaction. The optical density (OD) was measured using a micro-plate reader set at 450 nm wavelength. All the duplicated OD readings for each standard and samples were averaged. All averaged OD values were blank corrected (OD of standard or sample – OD of the blank). The XY table was created on GraphPad Prism 8 with the standard concentration on the X-axis and OD on the Y-axis. The standard curve was created, and the actual concentration was extrapolated from the standard curve. The sensitivity levels of the ELISA are 10-1000 ng/ml. The samples were measured in duplicates; outliers were replaced through imputation with values identical to the mean. A best-fit curve was constructed using GraphPad Prism version 8, and it equation was used to calculate the sample concentration. The samples were randomised in the ELISA plate to eliminate bias. We chose the ELISA because we wanted to measure the concentration, in our laboratory, we mostly use the ELISA to measure the concentration.

PFC and hippocampus GPx1 concentration

PFC and hippocampus GPx1 concentration was measured with an ELISA kit (Elabscience Biotech (E-EL-M0950, Texas, USA). A 50µl aliquot of standard or sample was added to a 96 well ELISA plate, followed immediately by the addition of 50µl HRP linked to Biotinylated Detection AB, followed by incubation for 60 minutes at 37°C in a HettCube 200 incubator. The plate was then aspirated and washed 5 times. 90µl of substrate was added followed by a 15 minute incubation period at 37°C. After the 15 minute incubation, a stop solution (50µl), was added to terminate the reaction. The optical density (OD) was measured using a micro-plate reader set at 450 nm wavelength. All the duplicated OD reading for each standard and samples were averaged. All averaged OD values were blank corrected (OD of standard or sample – OD of the blank). The XY table was created on GraphPad Prism 8 with the standard concentration on the X-axis and OD on the Y-axis. The standard curve was created, and the actual concentration was extrapolated from the standard curve. The sensitivity levels of the ELISA are 10-1000 pg/ml. The samples were measured in duplicates; outliers were replaced through imputation with values identical to the mean. A best-fit curve was constructed using GraphPad Prism version 8, and it equation was used to calculate the sample concentration. The samples were randomised in the ELISA plate to eliminate bias. We chose the ELISA because we wanted to measure the concentration, in our laboratory, we mostly use the ELISA to measure the concentration.

PFC and hippocampus SOD1 concentration

PFC and hippocampus SOD1 concentration was measured with an ELISA kit (Elabscience Biotech (E-EL-R1424, Texas, USA). A 50µl aliquot of standard or sample was added to a 96 well ELISA plate, followed immediately by the addition of 50µl HRP linked to Biotinylated Detection AB, followed by incubation for 60 minutes at 37°C HettCube 200 incubator. The plate was then aspirated and washed 5 times. 90µl of substrate was added followed by a 15 minute incubation period at 37°C HettCube 200 incubator. After the 15 minute incubation, a stop solution (50µl), was added to terminate the reaction. The optical density (OD) was measured using a micro-plate reader set at 450 nm wavelength. All the duplicated OD reading for each standard and samples were averaged. All averaged OD values were blank corrected (OD of standard or sample – OD of the blank). The XY table was created on GraphPad Prism 8 with the standard concentration on the X-axis and OD on the Y-axis. The standard curve was created, and the actual concentration was extrapolated from the standard curve. The sensitivity levels of the ELISA are 0.1-10 ng/ml. The samples were measured in duplicates; outliers were replaced through imputation with values identical to the mean. A best-fit curve was constructed

using GraphPad Prism version 8, and it equation was used to calculate the sample concentration. The samples were randomised in the ELISA plate to eliminate bias. We chose the ELISA because we wanted to measure the concentration, in our laboratory, we mostly use the ELISA to measure the concentration.

Statistical analysis

Data was analysed using the software, GraphPad Prism version 8 (San Diego, California, USA). All results are presented as the mean ± SEM. The Shapiro-Wilk test was used to test for normality. All our data was normally distributed. Two-way ANOVA, followed by the Bonferroni multiple comparisons tests was used. $p < 0.05$ was considered significant. Multiple comparisons will be conducted even in the absent of significant interaction effects because we want to compare the effect of the double hit model when compared to other groups.

Results

GAD 1 mRNA expression in the PFC

There was an overall housing effect in GAD1 mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 176.1$, $p<0.0001$. There was an overall ketamine effect in GAD1 mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 312.6$, $p<0.0001$. There was a significant interaction between social isolation and ketamine in GAD1 mRNA expression, $F(1,28)= 5.685$, $p=0.0241$. There was a ketamine effect and isolation effect on GAD1 mRNA expression in the GHK, SI, and SIK compared to the GH group *(GH vs. GHK, $p<0.0001$; GH vs. SI, $p<0.0001$; GH vs. SIK, $p<0.0001$, Figure 1). However, social isolation showed more effect on GAD1 mRNA expression than ketamine #(GHK vs. SI, $p=0.0252$, Figure 1). Social isolation enhanced the ketamine effect on GAD1 mRNA expression α(GHK vs SIK, $p<0.0001$, Figure 1). There was a ketamine effect in the social isolated animals α(SI vs. SIK, $p<0.0001$, Figure 1).

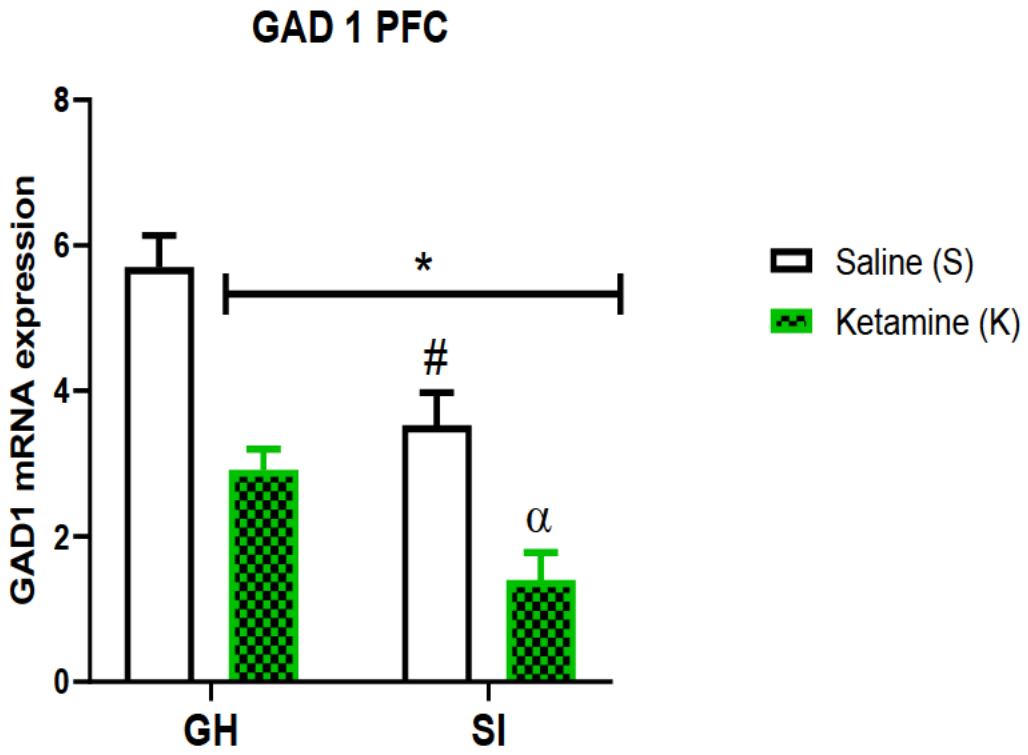

Figure 1. Graph showing PFC GAD 1 mRNA expression in the GH, GHK, SI, SIK. *(GH vs. GHK, $p<0.0001$; GH vs. SI, $p<0.0001$; GH vs. SIK, $p<0.0001$); #(GHK vs. SI, $p=0.0252$); α(GHK vs. SIK, $p<0.0001$; SI vs. SIK, $p<0.0001$). Data presented as mean ± SEM, n = 8 per group.

GAD 1 mRNA expression in the hippocampus

There was an overall housing effect in GAD1 mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 87.89$, $p<0.0001$. There was an overall ketamine effect in GAD1 mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 279.5$, $p<0.0001$. There was a ketamine effect and isolation effect on GAD1 mRNA expression in the GHK, SI, and SIK compared to the GH group *(GH vs. GHK, $p<0.0001$; GH vs. SI, $p<0.0001$; GH vs. SIK, $p<0.0001$, Figure 2). However, social isolation showed more effect on GAD1 mRNA expression than ketamine #(GHK vs. SI, $p<0.0001$, Figure 2). Social isolation enhanced the ketamine effect on GAD1 mRNA expression α(GHK vs SIK, $p<0.0001$, Figure 2). There was a ketamine effect in the socially isolated animals α(SI vs. SIK, $p<0.0001$, Figure 2).

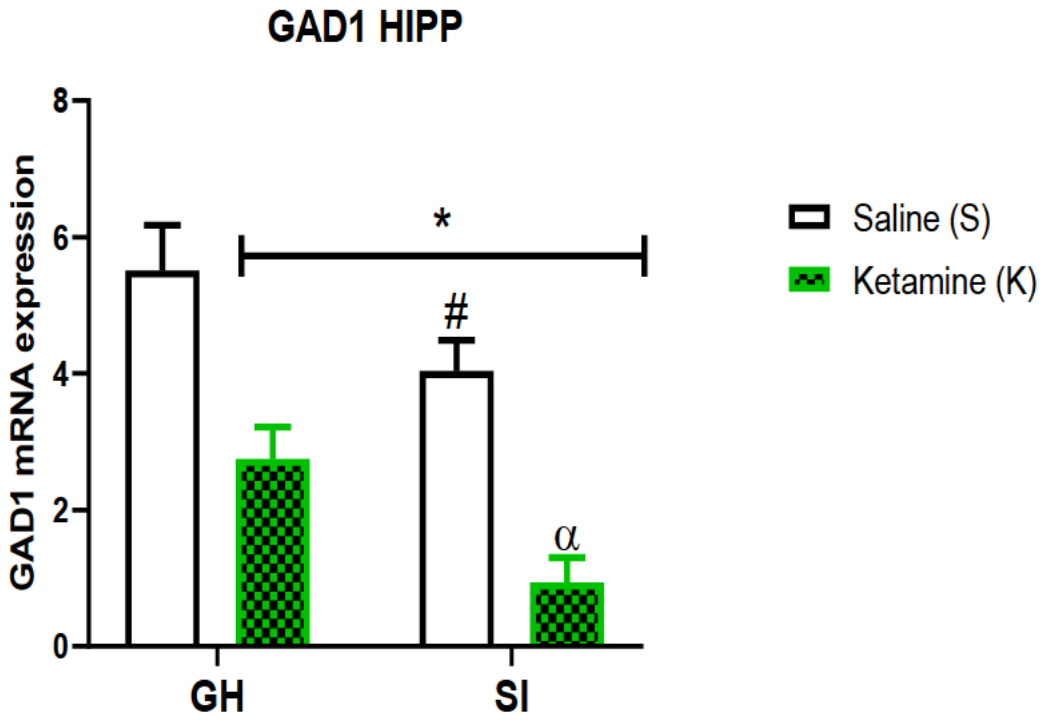

Figure 2. Graph showing GAD 1 mRNA expression in the GH, GHK, SI, IK. *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001); #(GHK vs. SI, p<0.0001; GHK vs. SIK, p<0.0001); α (SI vs. SIK, p<0.0001). Data presented as mean ± SEM, n = 8 per group.

PVALB mRNA expression in the PFC

There was an overall housing effect in PVALB mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 40.68$, $p<0.0001$. There was an overall ketamine effect in PVALB mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 38.14$, $p<0.0001$. There was a ketamine effect and isolation effect on PVALB mRNA expression in the GHK, SI, and SIK compared to the GH group *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001, Bonferroni's multiple comparison test, Figure 3). Social isolation enhanced the ketamine effect on PVALB mRNA expression α(GHK vs SIK, p=0.0232, Figure 3). There was a ketamine effect in the social isolated animals α(SI vs. SIK, p=0.0332, Figure 3).

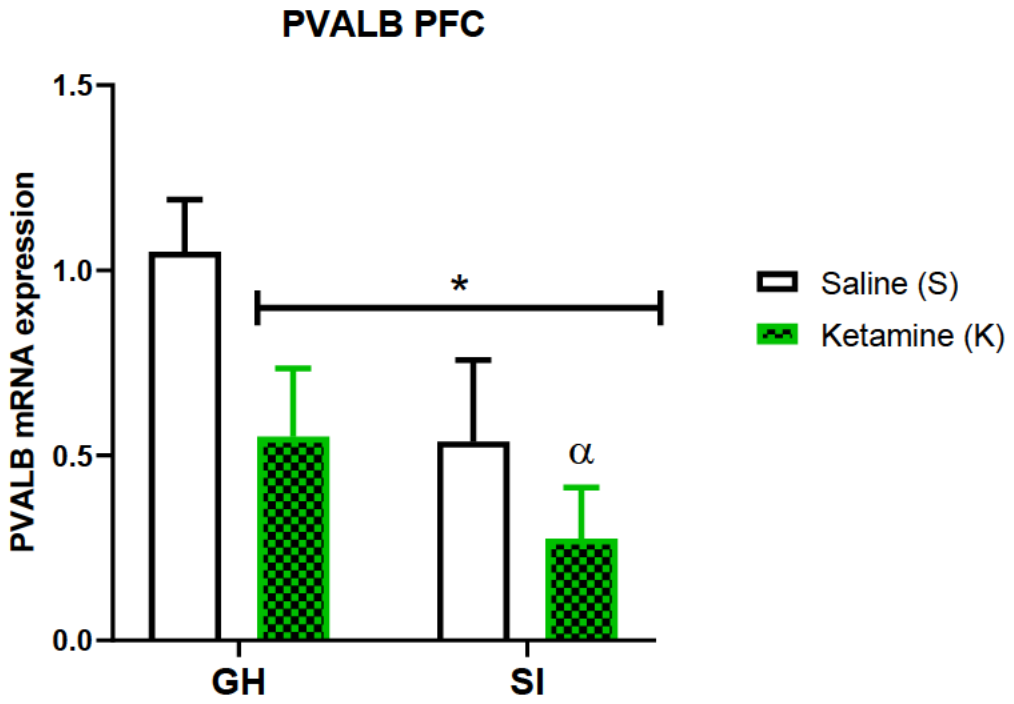

Figure 3. Graph showing PVALB mRNA expression in the GH, GHK, SI, SIK. *(GH VS. GHK, p<0.0001; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001); α(GHK vs. SIK, p=0.0232); SI vs. SIK, p=0.0332). Data presented as mean ± SEM, n = 8 per group.

PVALB mRNA expression in the Hippocampus

There was an overall housing effect in PVALB mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 58.91$, $p<0.0001$. There was an overall ketamine effect in PVALB mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 24.00$, $p<0.0001$. There was a ketamine effect and isolation effect on PVALB mRNA expression in the GHK, SI, and SIK compared to the GH group *(GH vs. GHK, p=0.0140; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001, Bonferroni's multiple comparison tests, Figure 4). Social isolation enhanced the ketamine effect on PVALB mRNA expression α(GHK vs. SIK, p<0.0001, Figure 4). There was a ketamine effect in the social isolated animals α(SI vs. SIK, p=0.0077, Figure 4).

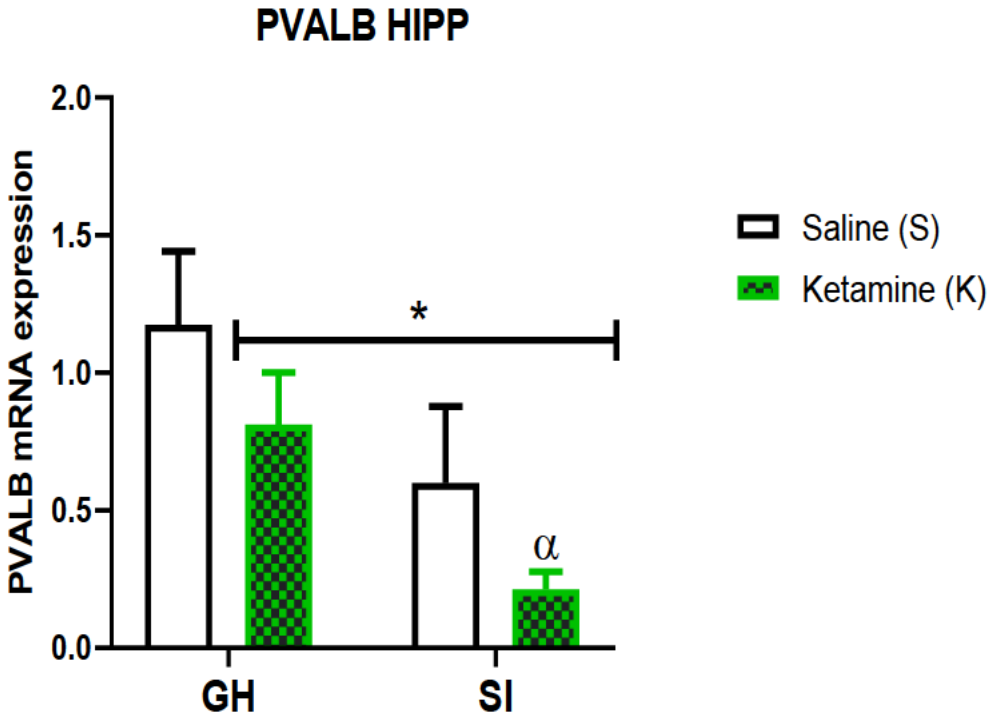

Figure 4. Graph showing PVALB mRNA expression in the GH, GHK, SI, SIK. *(GH vs. GHK, p=0.0140; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001); α(GHK vs. SIK, P<0.0001; SI vs. SIK, p=0.0077). Data presented as mean ± SEM, n = 8 per group.

BDNF mRNA expression in the PFC

There was an overall housing effect in BDNF mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 34.97$, $p<0.0001$. There was an overall ketamine effect in BDNF mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 69.11$, $p<0.0001$. There was a ketamine effect and isolation effect on BDNF mRNA expression in the GHK, SI, and SIK compared to the GH group *(GH vs. GHK, p<0.0001; GH vs. SI, p=0.0002; GH vs. SIK, p<0.0001, Figure 5). Social isolation enhanced the ketamine effect on BDNF mRNA expression α(GHK vs SIK, p=0.0125, Figure 5). There was a ketamine effect in the social isolated animals α(SI vs. SIK, p=0.0001, Figure 5).

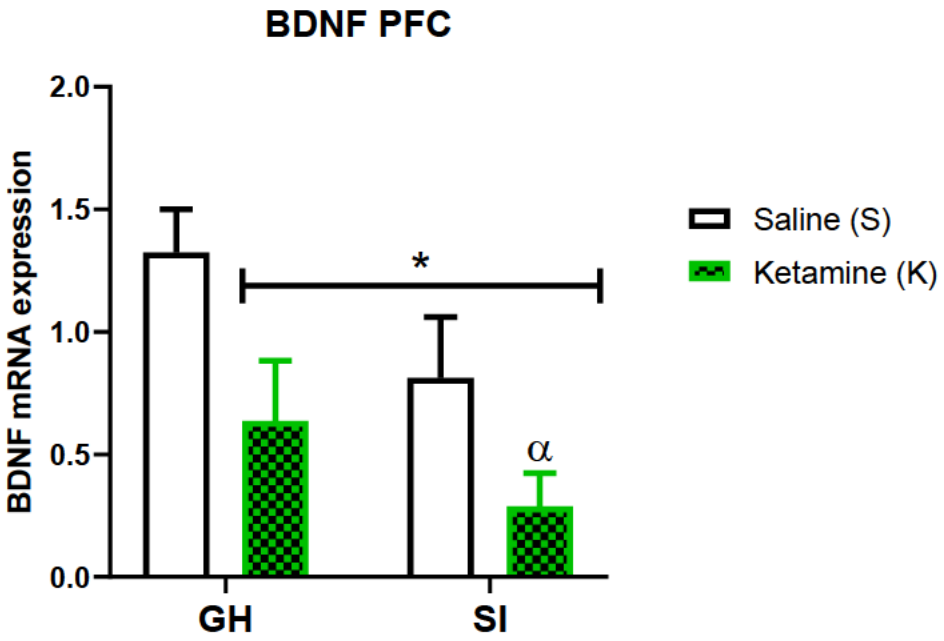

Figure 5. Graph showing BDNF mRNA expression in the GH, GHK, SI, SIK. *(GH vs. GHK, p<0.0001; GH vs. SI, p=0.0002; GH vs. SIK, p<0.0001); α(GHK vs. SIK, p=0.0125; SI vs. SIK, p=0.0001). Data presented as mean ± SEM, n = 8 per group.

BDNF mRNA expression in the Hippocampus

There was an overall housing effect in BDNF mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 119.0$, $p<0.0001$. There was an overall ketamine effect in BDNF mRNA expression in the GH, GHK, SI, and SIK, $F(1,28) = 66.93$, $p<0.0001$. There was a ketamine effect and isolation effect on BDNF mRNA expression in the GHK, SI, and SIK compared to the GH group *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001, Figure 6). Social isolation enhanced the ketamine effect on BDNF mRNA expression α(GHK vs SIK, p<0.0001, Figure 6). There was a ketamine effect in the social isolated animals α(SI vs. SIK, p<0.0001, Figure 6).

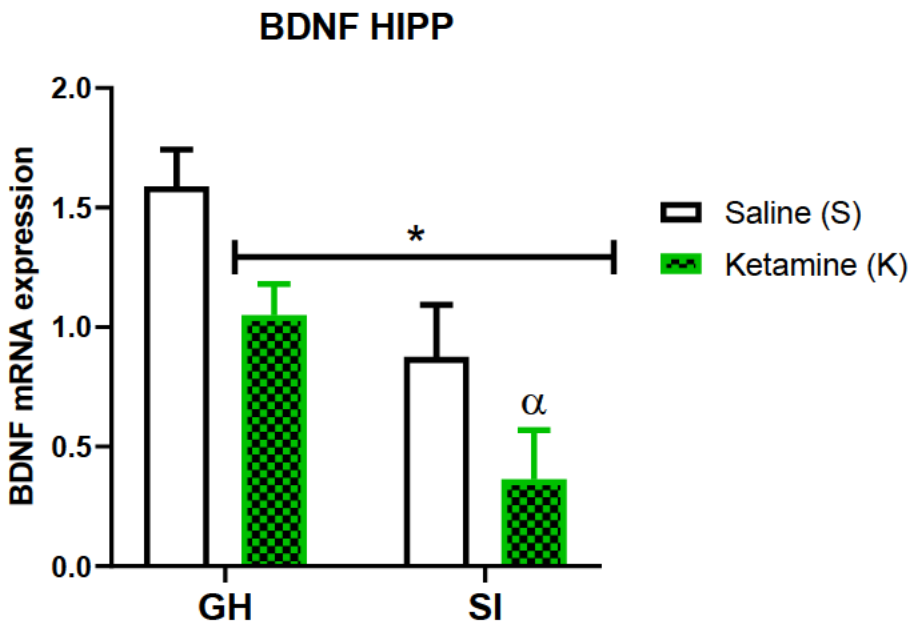

Figure 6. Graph showing BDNF mRNA expression in the GH, GHK, SI, SIK. *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001); α(GHK vs. SIK, p<0.0001; SI vs. SIK, p<0.0001). Data presented as mean ± SEM, n = 8 per group.

MDA concentration in the PFC

There was an overall housing effect in MDA concentration in the GH, GHK, SI, and SIK, $F(1,28) = 452.6$, $p<0.0001$. There was an overall ketamine effect in MDA concentration in the GH, GHK, SI, and SIK, $F(1,28) = 1671$, $p<0.0001$. There was a statistically significant interaction between social isolation and ketamine in MDA concentration, $F(1,28) = 30.13$, $p<0.0001$. There was a ketamine and isolation effect on MDA concentration in the GHK, SI and SIK compared to the GH group *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001, Figure 7). However, ketamine showed more effect on MDA concentration than isolation #(GHK vs. SI, p<0.0001, Figure 7). There was a ketamine effect in social isolated animals on MDA concentration α(SI vs. SIK, p<0.0001, Figure 7). Social isolation enhanced the ketamine effect on MDA concentration α(GHK vs. SIK, p<0.0001, Figure 7).

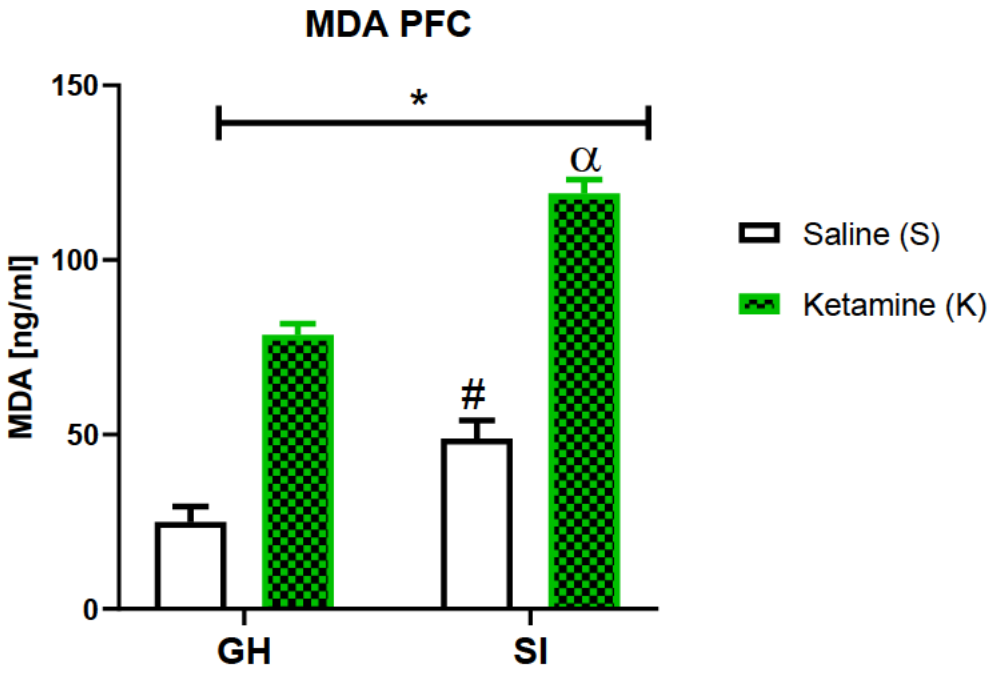

Figure 7. Graph showing MDA concentration for GH, GHK, SI, and SIK. *(GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001); #(GHK vs. SI, p<0.0001); α(GHK vs. SIK, p<0.0001; SI vs. SIK, p<0.0001). Data presented as mean ± SEM, n = 8 per group.

MDA concentration in the hippocampus

There was an overall housing effect in MDA concentration in the GH, GHK, SI, and SIK, $F(1,28) = 396.9$, $p<0.0001$. There was an overall ketamine effect in MDA concentration in the GH, GHK, SI, and SIK, $F(1,28) = 1538$, $p<0.0001$. There was a statistically significant interaction between social isolation and ketamine in MDA concentration, $F(1,28) = 67.52$, $p<0.0001$. There was a ketamine and isolation effect on MDA concentration in the GHK, SI and SIK compared to the GH group *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001, Figure 8). However, ketamine showed more effect on MDA concentration than isolation #(GHK vs. SI, p<0.0001, Figure 8). There was a ketamine effect in social isolated animals on MDA concentration α(SI vs. SIK, p<0.0001, Figure 8). Social isolation enhanced the ketamine effect on MDA concentration α(GHK vs. SIK, p<0.0001, Figure 8).

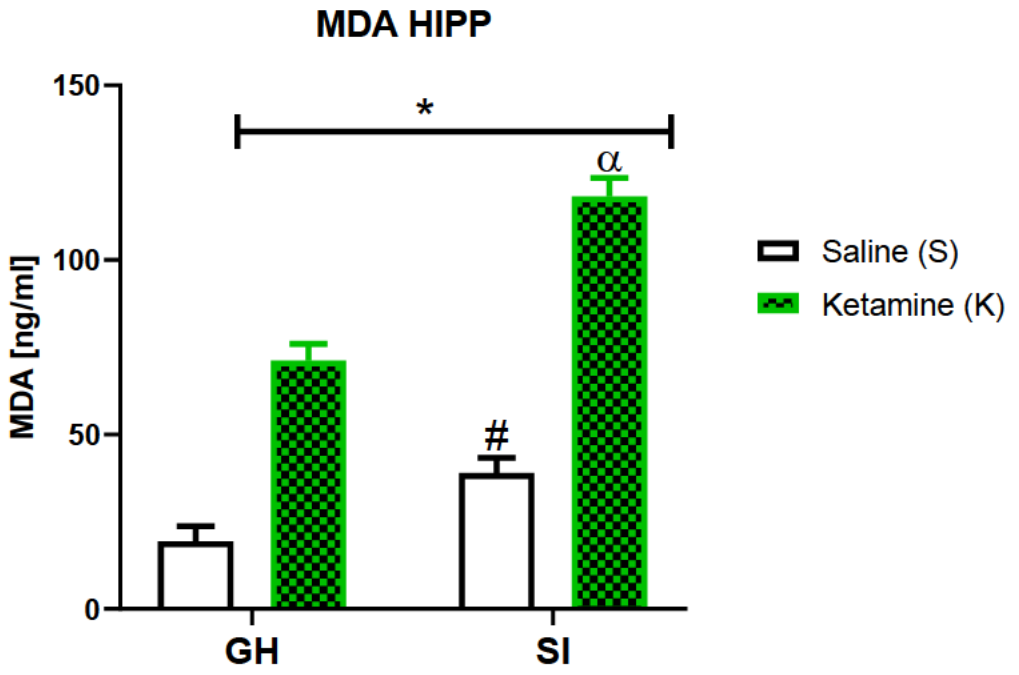

Figure 8. Graph showing MDA concentration for GH, GHK, SI, and SIK. *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001); #(GHK vs. SI, p<0.0001); α(GHK vs. SIK, p<0.0001; SI vs. SIK, p<0.0001). Data presented as mean ± SEM, n = 8 per group.

GPx1 concentration in the PFC

There was an overall housing effect in GPx1 concentration in the GH, GHK, SI, and SIK, $F(1,28) = 118.1$, $p<0.0001$. There was an overall ketamine effect in GPx1 concentration in the GH, GHK, SI, and SIK, $F(1,28) = 287.7$, $p<0.0001$. There was a statistically significant interaction between social isolation and ketamine in GPx1 concentration, $F(1,28) = 17.01$, $p=0.0003$. There was a ketamine and isolation effect on GPx1 concentration in the GHK, SI and SIK compared to the GH group *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001, Figure 9). However, ketamine showed more effect on GPx1 concentration than isolation #(GHK vs. SI, p=0.0011, Figure 9). There was a ketamine effect in social isolated animals on GPx1 concentration α(SI vs. SIK, p<0.0001, Figure 9). Social isolation enhanced the ketamine effect on GPx1 concentration α(GHK vs. SIK, p=0.0003, Figure 9).

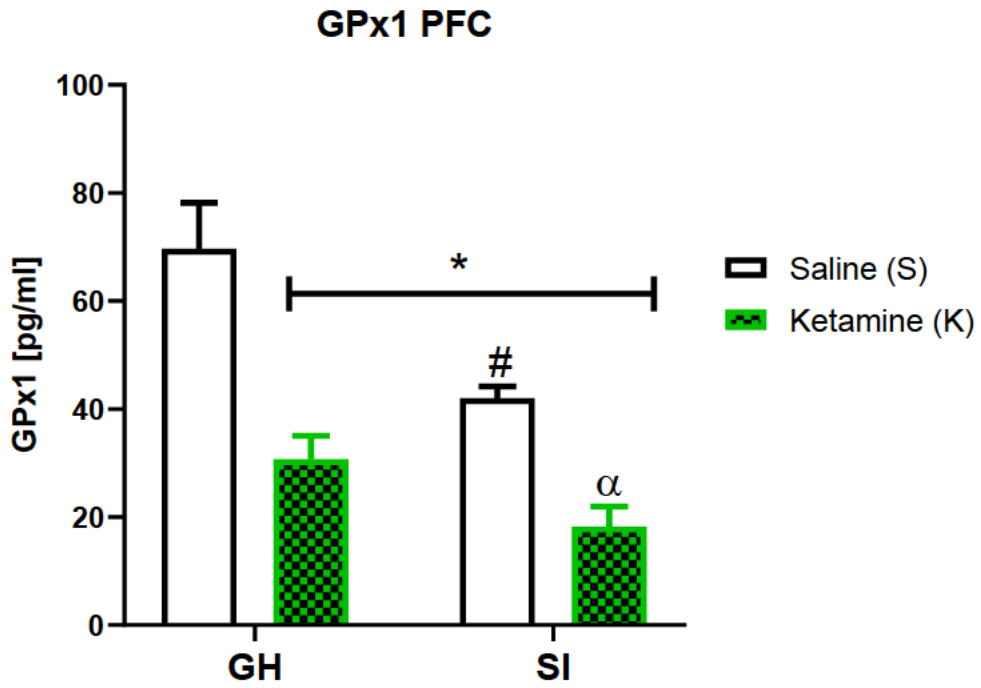

Figure 9. Graph showing GPx1 concentration for GH, GHK, SI, and SIK. *(GH vs. SI, $p<0.0001$; GH vs. SIK, $p<0.0001$); #(GHK vs. SI, $p=0.0011$); α(GHK vs. SIK, $p=0.0003$; SI vs. SIK, $p<0.0001$). Data presented as mean ± SEM, n = 8 per group.

GPx1 concentration in the Hippocampus

There was an overall housing effect in GPx1 concentration in the GH, GHK, SI, and SIK, $F(1,28) = 147.6$, $p<0.0001$. There was an overall ketamine effect in GPx1 concentration in the GH, GHK, SI, and SIK, $F(1,28) = 626.7$, $p<0.0001$. There was a statistically significant interaction between social isolation and ketamine in GPx1 concentration, $F(1,28) = 29.02$, $p<0.0001$. There was a ketamine and isolation effect on GPx1 concentration in the GHK, SI and SIK compared to the GH group *(GH vs. GHK, $p<0.0001$; GH vs. SI, $p<0.0001$; GH vs. SIK, $p<0.0001$, Figure 10). However, ketamine showed more effect on GPx1 concentration than isolation #(GHK vs. SI, $p<0.0001$, Figure 10). There was a ketamine effect in social isolated animals on GPx1 concentration α(SI vs. SIK, $p<0.0001$, Figure 10). Social isolation enhanced the ketamine effect on GPx1 concentration α(GHK vs. SIK, $p=0.0003$, Figure 10).

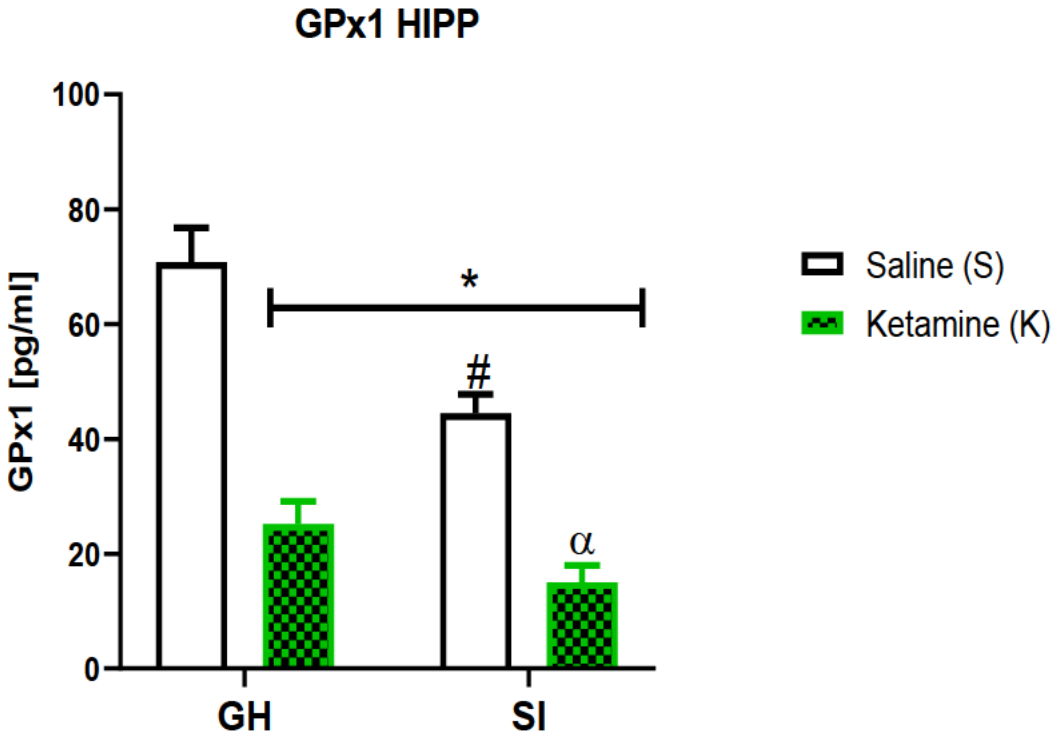

Figure 10. Graph showing GPx1 concentration for GH, GHK, SI, and SIK. *(GH vs. GHK, $p<0.0001$; GH vs. SI, $p<0.0001$; GH vs. SIK, $p<0.0001$); #(GHK vs. SI, $p<0.0001$); α(GHK vs. SIK, $p=0.0003$; SI vs. SIK, $p<0.0001$). Data presented as mean ± SEM, n = 8 per group.

SOD1 concentration in the PFC

There was an overall housing effect in SOD1 concentration in the GH, GHK, SI, and SIK, $F(1,28) = 57.40$, $p<0.0001$. There was an overall ketamine effect in SOD1 concentration in the GH, GHK, SI, and SIK, $F(1,28) = 182.1$, $p<0.0001$. There was a ketamine and isolation effect on SOD1 concentration in the GHK, SI and SIK compared to the GH group *(GH vs. GHK, $p<0.0001$; GH vs. SI, $p<0.0001$; GH vs. SIK, $p<0.0001$, Figure 11). However, ketamine showed more effect on SOD1 concentration than isolation #(GHK vs. SI, $p=0.0015$, Figure 11). There was a ketamine effect in social isolated animals on SOD concentration α(SI vs. SIK, $p<0.0001$, Figure 11). Social isolation enhanced the ketamine effect on SOD concentration α(GHK vs. SIK, $p=0.0005$, Figure 11).

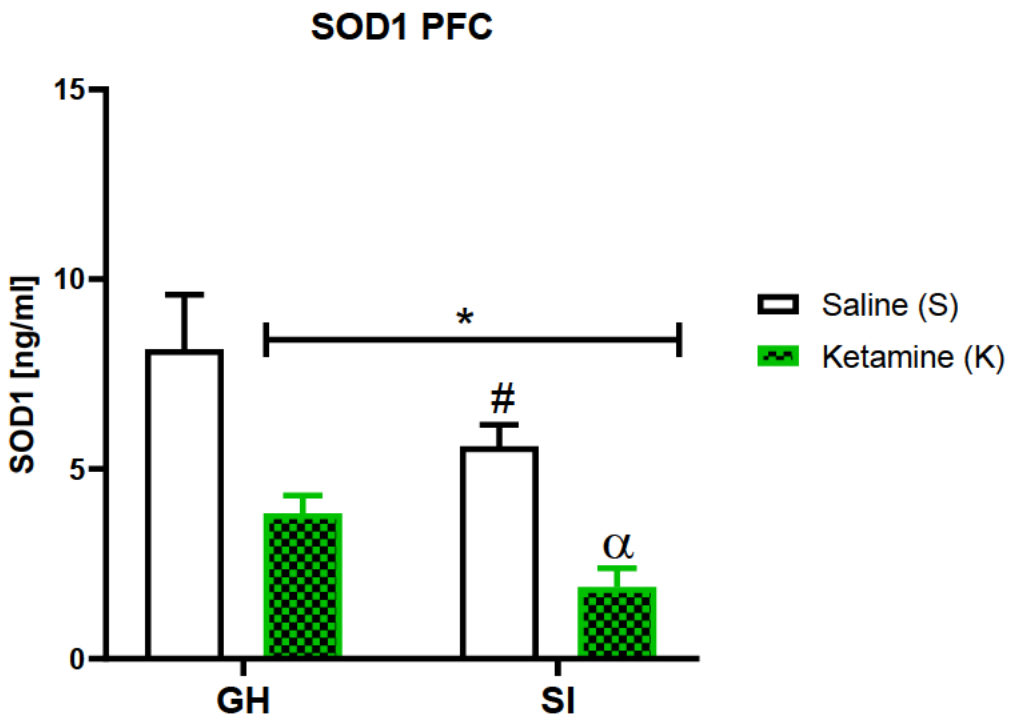

Figure 11. Graph showing SOD1 concentration for GH, GHK, SI, and SIK. *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001); #(GHK vs. SI, p=0.0015); α(GHK vs. SIK, p=0.0005; SI vs. SIK, p<0.0001). Data presented as mean ± SEM, n = 8 per group.

SOD1 concentration in the hippocampus

There was an overall housing effect in SOD1 concentration in the GH, GHK, SI, and SIK, $F(1,28) = 120.40$, $p<0.0001$. There was an overall ketamine effect in SOD1 concentration in the GH, GHK, SI, and SIK, $F(1,28) = 499.5$, $p<0.0001$. There was a statistically significant interaction between social isolation and ketamine in SOD1 concentration, $F(1,28) = 26.60$, $p<0.0001$. There was a ketamine and isolation effect on SOD1 concentration in the GHK, SI and SIK compared to the GH group *(GH vs. GHK, p<0.0001; GH vs. SI, p<0.0001; GH vs. SIK, p<0.0001, Figure 12). However, ketamine showed more effect on SOD1 concentration than isolation #(GHK vs. SI, p<0.0001, Figure 12). There was a ketamine effect in social isolated animals on SOD concentration α(SI vs. SIK, p<0.0001, Figure 12). Social isolation enhanced the ketamine effect on SOD concentration α(GHK vs. SIK, p=0.0019, Figure 12).

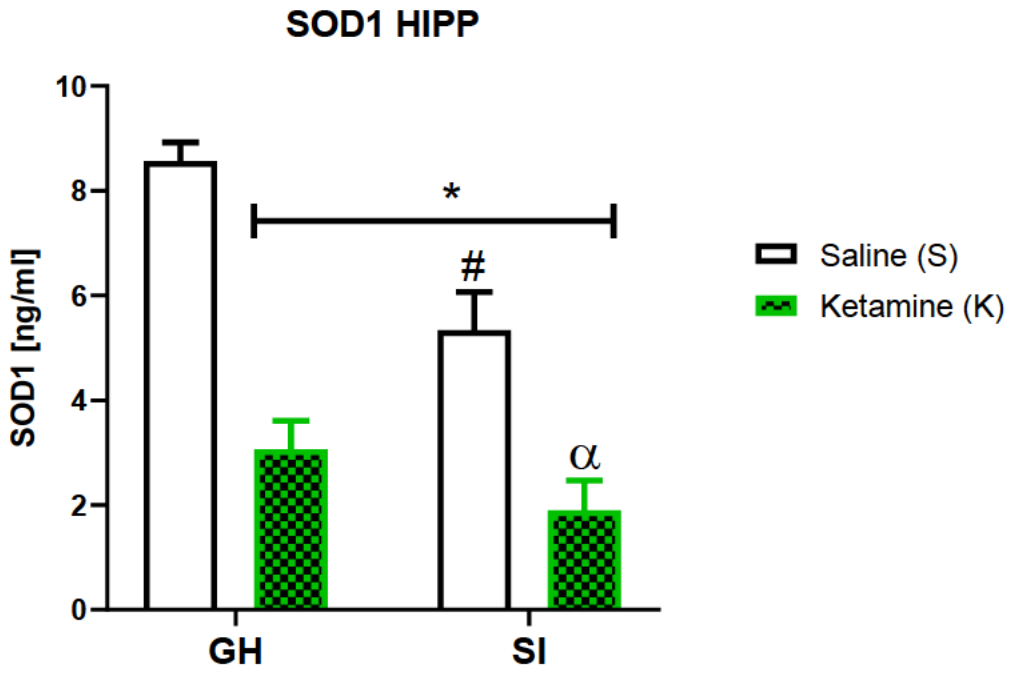

Figure 12. Graph showing SOD1 concentration for GH, GHK, SI, and SIK. *(GH vs. GHK, $p<0.0001$; GH vs. SI, $p<0.0001$; GH vs. SIK, $p<0.0001$); #(GHK vs. SI, $p<0.0001$); α(GHK vs. SIK, $p=0.0019$; SI vs. SIK, $p<0.0001$). Data presented as mean ± SEM, n = 8 per group.

Discussion

This study explores changes in molecular levels of a schizophrenia animal model by investigating the association between gene expression and protein concentration of components within the oxidative stress pathways and GABAergic neurotransmission on schizophrenia. SIK showed reduced GAD1 mRNA, PVALB mRNA, and BDNF mRNA gene expression when compared to other groups in the PFC and the hippocampus. SIK showed increased MDA concentration and reduced SOD and GPx1 concentration when compared to other groups in the PFC and the hippocampus. Optimal balance between excitatory and inhibitory neurotransmission plays a crucial role in plasticity and maturation of the inhibitory neurotransmitter network (Castillo-Gómez et al., 2017). The balance between excitatory and inhibitory neurotransmission can be affected by the alterations in the expression of genes involved in GABAergic neurotransmission such as glutamate decarboxylase (GAD1), brain

derived neurotrophic factor (BDNF), and parvalbumin (PVALB) that influence the development and physiology of the inhibitory circuit (Tao et al., 2014, Mitchell et al., 2015).

Our results showed that animals treated with ketamine (GHK and SIK) had reduced GAD1 gene expression while the socially isolated animals (SI and SIK) showed reduced GAD1 gene expression. SIK showed decreased GAD1 gene expression when compared to all the other groups. The GAD1 gene encodes for the enzyme that converts glutamate to GABA (Benes et al., 2007). GAD1 has been reported to be downregulated in the hippocampus and cortices of schizophrenia patients (Hashimoto et al., 2003, Benes et al., 2007). Decreased GAD1 mRNA expression was present in the PFC on postmortem studies of schizophrenic patients (Volk et al., 2000, Knable et al., 2002). Bullock and colleagues reported a reduction in the mRNA expression of GABA synthesizing enzyme in animals after chronic injection of an NMDA receptor antagonist (Bullock et al., 2009). Reduced expression of GAD 1 was observed in cultured hippocampal neurons treated with ketamine (Kinney et al., 2006). Reduced GAD 1 expression may lead to reduced production of GABA from glutamate (Jones et al., 2011).

Our results showed that animals treated with ketamine (GHK and SIK) had reduced BDNF mRNA expression. Socially isolated animals (SI and SIK) showed reduced BDNF mRNA expression. Furthermore, SIK the lowest BDNF mRNA expression when compared to the other groups. It has been shown that BDNF mRNA expression is reduced in brain regions of rats treated with an NMDA receptor antagonist (Liu et al., 2011). BDNF expression has been shown to be reduced in the hippocampus of social isolated male rats (Scaccianoce et al., 2006). Amongst other functions, BDNF controls the development, differentiation, and maturation of inhibitory neurons (Carmona et al., 2006, Gottmann et al., 2009). Furthermore, BDNF is responsible for the control of GABAergic synapse formation and maintenance (Hong et al., 2008, Sakata et al., 2009). Reduced BDNF may lead to impaired brain development, synaptic dysconnectivity, and reduced neuroplasticity, which is similar to the neurochemical and morphological changes observed in schizophrenia patients (Shoval and Weizman, 2005, Buckley et al., 2007).

In the PFC, animals treated with ketamine (GHK and SIK) showed reduced PVALB mRNA expression. Animals acutely treated with an NMDA receptor antagonist showed decreased PVALB expression (Olney and Farber, 1995, Grunze et al., 1996, Braun et al., 2007, Morrow et al., 2007). As a result, we propose that NMDA receptor antagonists act on the NMDA receptors expressed by GABAergic interneurons. Socially isolated animals (SI and SIK)

showed reduced PVALB mRNA expression in the PFC. SIK showed further reduction of PVALB mRNA expression in the PFC when compared to the other groups. Social isolation in rats has been found to reduce expression of PVALB cells in the hippocampus (Harte et al., 2007). There was a reduction in hippocampal PVALB mRNA expression in the SIK. GHK and SI also showed reduced PVALB mRNA expression in the hippocampus. PVALB plays a role in gamma oscillation, which is housed in different regions of the brain including the hippocampus where they play a major role in attentional selection and memory regulation, PVALB was reduced in schizophrenic patients (Colgin and Moser, 2010, Dienel and Lewis, 2019).

Postmortem studies on schizophrenic brain tissues showed reduced expression of GABAergic biomarkers such as GAD1, BDNF, and PVALB (Hashimoto et al., 2003). These changes are associated with impaired cognition, and this is hypothesized to be the main pathophysiology of schizophrenia (Rao et al., 2000, Constantinidis et al., 2002). Disruptions in GABA signalling induces an imbalance between excitation and inhibition in the cerebral cortex which is a major factor in the pathophysiology of schizophrenia (Benes and Berretta, 2001, Guidotti et al., 2005). Schobel and colleagues suggested that schizophrenia pathophysiology is initiated by the dysfunction of NMDA receptors localised on GABAergic interneurons which causes disinhibition of pyramidal cells and excitotoxic injury (Schobel et al., 2013). Oxidative stress has been found to induce deficits in the parvalbumin inhibitory interneurons, which is highly associated with schizophrenia pathophysiology (Steullet et al., 2017). Our results showed high concentration of MDA in the GHK and SI when compared to the GH group in both the PFC and the hippocampus. The double hit model (SIK) showed high MDA concentrations when compared to all other groups in both the PFC and the hippocampus. Studies on schizophrenia patients and animals with schizophrenia-like symptoms showed increased MDA concentrations (Padurariu et al., 2010, Estaphan et al., 2021). Estaphan et al. suggested that there is an increase in oxidative stress in the double hit model of schizophrenia as shown by the presence of high MDA concentration (Estaphan et al., 2021).

MDA is an important end product of lipid peroxidation and a marker of oxidative stress, oxidative have been implicated in the pathophysiology of schizophrenia (Flatow et al., 2013). Schizophrenia is linked to impaired antioxidant defence against reactive oxygen species (Estaphan et al., 2021). Our results found reduced GPx1 concentration in the GHK and SI. The SIK group showed reduced GPx1 concentration in both the PFC and the hippocampus. The double hit model has been shown to have reduced GPx1 concentration in the cortex and the

hippocampus (Estaphan et al., 2021). A significant reduction in GPx1 activity was reported in schizophrenia patients (Padurariu et al., 2010). GPx1 is an intracellular antioxidant enzyme that oxidises hydrogen peroxide to water to hinder its harmful effect, in schizophrenic patients GPx1 activity is reduced (Huang et al., 2018). In the current study, we observed reduced SOD1 concentration in the GHK and SI groups when compared to the GH group. The SIK group showed greater reduction in the SOD concentration. Studies on schizophrenia patients reported reduced SOD activity (Sinet et al., 1983, Mukherjee et al., 1996). The decrease in antioxidants indicate the presence of oxidative stress.

Conclusion

SIK showed reduced mRNA expression of genes involved in the GABAergic signalling (GAD1, BDNF, and PVALB). This downregulation of these genes might be the cause for induce dysfunction in the GABAergic neurotransmission, which can potentially induce an imbalance between the excitatory and inhibitory network. SIK showed high MDA concentration in the PFC and the hippocampus when compared to other groups, the same group of animals showed reduced antioxidants SOD and GPx1 concentration. The current study showed that the double hit model (SIK) induce dysfunction in the GABAergic neurotransmitter and oxidative stress does play a role in the pathophysiology of schizophrenia.

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Chapter 6

Synthesis

The aim of this project was to develop an animal model of schizophrenia that would be able to induce all symptoms of schizophrenia. Animal models of schizophrenia are divided into four classes: developmental, genetic, drugs, and lesion. Genetic animal models of schizophrenia are very expensive, while lesion models have a high mortality rate when compared to other models. Post-weaning social isolation is a neurodevelopmental animal model of schizophrenia which is achieved by housing rats individually. Social isolation of rodents induces sensorimotor gating deficits, spontaneous hyper-locomotor activity, heightened anxiety states, and cognitive impairment. The weakness of the social isolation model is that behavioural impairments induced by the model can be easily reversed by repeated handling of the animals during the experimental period. The NMDA receptor antagonist ketamine is used to induce a pharmacological model of schizophrenia. Injecting rodents with ketamine induces behavioural deficits similar to those observed in schizophrenic patients such as impaired social interaction and learning and memory.

The ketamine model has weaknesses. When different doses of ketamine treatment are used, the results vary and are contradictory. Due to the shortcomings of the social isolation and ketamine models, researchers proposed combining these models to form what is known as the double hit model of schizophrenia. In our laboratory we focused on the social isolation and the NMDA receptor antagonist models. We conducted a systematic review study to assess the effectiveness or robustness of the double hit model of schizophrenia. We concluded that the double hit model of schizophrenia is effective and robust.

It has been shown that positive symptoms are easily treated with the available typical and atypical antipsychotic drugs, while negative and cognitive symptoms remain resistant. We investigated behavioural and molecular changes involved in negative and cognitive symptoms of schizophrenia. Reduced social interaction is related to the social withdrawal which is the negative symptom observed in schizophrenia patients. Reduced plasma oxytocin concentration is positively correlated with antisocial behaviour in schizophrenia patients. We showed that our double hit model of schizophrenic had reduced levels of social interaction during the social preference test and a lower oxytocin concentration in the amygdala. Our double hit model was able to induce behavioural and molecular changes associated with negative symptoms of schizophrenia.

Socially isolated animals showed anxiety-like behaviour during the elevated plus maze test which was further supported by a high concentration of the stress hormone. Under stressful conditions, both humans and animals are more likely to develop aggressive behaviour. Grouped housed animals did not show aggressive behaviour even when exposed to ketamine. We propose that after giving treatment to schizophrenia patients, we must also provide a strong support structure for these patients. Our double hit model showed high anxiety-like behaviour, reduced social interaction and was more aggressive when compared to other groups. The double hit model also exhibited impaired visual learning and memory during the novel object recognition test, which was further supported by high expression levels of proinflammatory cytokines (IL-6 and TNFα) genes in the PFC and the hippocampus. Furthermore, this shows that our double hit model was able to induce inflammation in the PFC and the hippocampus. This suggests that our double hit model was more robust in modelling the negative and cognitive symptoms observed in schizophrenia patients than either treatment alone.

We further investigated the effects of the double hit model on behavioural and neurochemical changes involved in positive symptoms of schizophrenia. The double hit model did present with increased striatal dopamine. An increase in striatal dopamine production has been reported in schizophrenic patients. Furthermore, dopamine D2 receptor mRNA expression was upregulated. NMDA receptor antagonists such as ketamine have shown reduced GABAergic interneuron function and GABA concentration. The GABA concentration was greatly reduced in our double-hit model. This model was able to induce behavioural and molecular changes associated with positive symptoms of schizophrenia.

We further investigated mechanisms involved in the pathophysiology of schizophrenia. GHK, SI, and SIK showed reduced expression of genes (GAD1, BDNF, and PVALB) involved in the GABAergic neurotransmission. Reduction in gene expression involved in GABAergic function is associated with impaired cognitive function and this is believed to play a role in the pathophysiology of schizophrenia. A disruption in GABA neurotransmission led to an imbalance between excitation and inhibition in the cerebral cortex (Guidotti et al., 2005). This imbalance is a major contributor in the pathophysiology of schizophrenia (Benes and Berretta, 2001). Dysfunction of NMDA receptors localised on GABAergic interneurons result in the disinhibition of pyramidal cells and excitotoxic injury (Schobel et al., 2013). Oxidative stress has been found to induce deficits in the parvalbumin inhibitory interneurons, which is highly associated with schizophrenia pathophysiology (Steullet et al., 2017). The double hit model showed a high MDA concentration in the PFC and the hippocampus. The double hit model also

showed reduced antioxidants SOD and GPx1 concentration. We suggest that the dysfunction in GABAergic neurotransmission and oxidative stress plays a role in the pathophysiology of schizophrenia. This information may assist researchers in designing effective therapeutic drugs that can be used in the treatment of schizophrenia, but more studies need to be conducted, and results must be compared with the results of the current study. In the current study we have 8 animals per group, this sample size provided the study with reliable and effective statistical power and effect sizes. Behavioural impairments caused by social isolation and ketamine can be reversed. In the current study we maintained social isolation and ketamine injections throughout the study to prevent the reversal of behavioural impairments caused by both models. The double hit group (SIK) were not responding well to ketamine injections some animals have scratches and sores from injections. The veterinary practitioner based on our breeding centre was asked to come and observed the animals, and the vet observed the animals and prescribed medications which was used to treat the sores. In trying to refine the model, next time we will use 20mg/kg instead of 16mg/kg, furthermore in the current study we used ELISA and RT-PCR as our analysis techniques, next time we intend to use western blot and histochemistry analysis.

CONCLUSION

This study showed that the double hit model of schizophrenia was robust and effective in inducing some aspects of positive, negative, and cognitive symptoms of schizophrenia. Our double hit model of schizophrenia showed reduced social interaction, anxiety-like behaviour, aggressive behaviour, high locomotor activity and impaired visual learning and memory. This was accompanied by increased concentration of ACTH, corticosterone, norepinephrine, MDA, dopamine, and acetylcholine, and enhanced mRNA expression of proinflammatory cytokines (IL6 and TNFα). Furthermore, decreased concentration of oxytocin, testosterone, GABA, glutamate, SOD, and GPx1 and reduced mRNA expression of GAD1, PVALB, BDNF, SOD, and GPX1. The use of this double hit animal model of schizophrenia can assist researchers in designing more effective therapeutic drugs that can be used to better treat schizophrenia, but more work needs to be done.

Appendix 1

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Khanyiso Bright Shangase; Mluleki Luvuno; Musa V. Mabandla

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Appendix 2

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Research article

Effects of combined postweaning social isolation and ketamine administration on schizophrenia-like behaviour in male Sprague Dawley rats

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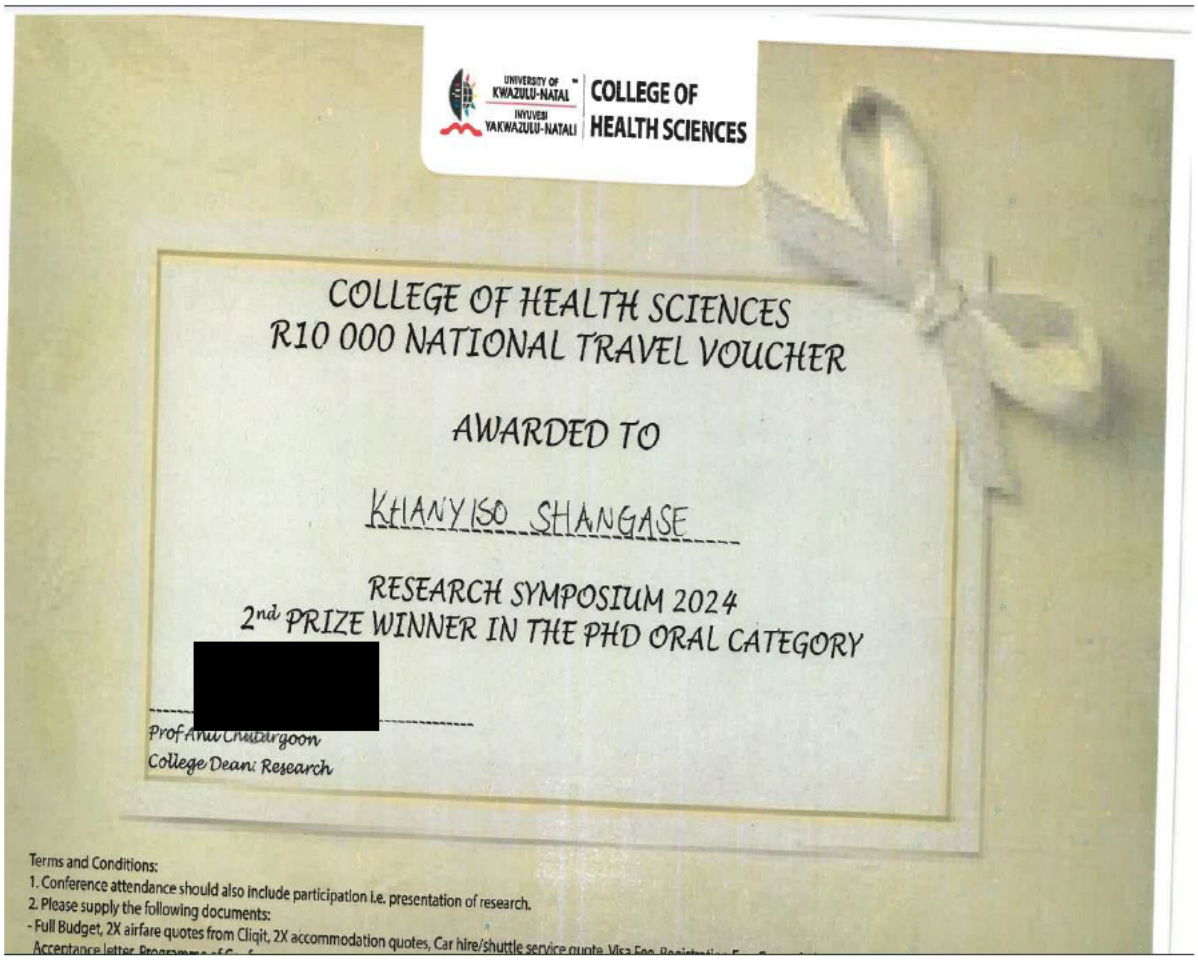
ABSTRACT

The pathophysiology behind negative and cognitive symptoms of schizophrenia is not well understood, thus limiting the effectiveness of treatment on these symptoms. Developing reliable animal model of schizophrenia is vital to advance our understanding on the neurobiological basis of the disorder. Double hit is used to refer to the use of two schizophrenia inducing interventions viz ketamine exposure and social isolation. In this study we aim to investigate the robustness of double hit model of schizophrenia in inducing negative and cognitive symptoms of schizophrenia. On postnatal day (PND) 23, thirty-two male Sprague Dawley rats were randomly grouped into four equal groups as follows: group housed + saline (GH), group housed + ketamine (GHK), isolated + saline (SI), and isolated + ketamine (SIK). A single ketamine dose (16 mg/kg) was administered 3 times a week for four weeks. Isolated animals were housed singly throughout the study. The following behavioural tests were carried out: elevated plus maze, three chamber social interaction, resident intruder tests, and novel object recognition (NOR). The SIK group exhibited high anxiety levels, with increased ACTH, corticosterone and norepinephrine concentration when compared to the other groups. The SIK animals also presented with reduced social interaction and decreased oxytocin concentration. SIK rats were more aggressive towards a juvenile intruder but had low testosterone concentration. The SIK group or double hit model showed impaired visual learning and memory and increased expression of proinflammatory cytokines. This suggest that the double hit model is more robust in inducing negative and cognitive symptoms of schizophrenia than each treatment alone.

Appendix 3



Chapter 7

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