EFFECTS OF THE NEGATIVE ALLOSTERIC MODULATION OF $GABA_A$ RECEPTORS ON THE MONOAMINE AND GLUTAMATE SYSTEMS IN THE RAT: RELEVANCE TO THE ANTIDEPRESSANT RESPONSE

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ABSTRACT

Background: Negative allosteric modulators (NAMs) of gamma-aminobutyric acid A ($GABA_A$ receptors bind with high affinity to the α5 subunit located primarily on glutamate pyramidal neurons, thereby directly increasing AMPA throughput. Their specificity limits off-target effects, reducing the possibility of adverse side effects. The α5-preferring $GABA_A$-NAM L-655,708 has been shown to exert rapid and sustained antidepressant-like action in rats.

Aim: This study aims to examine the effects of L-655,708 on the activity of monoamine and glutamate systems in rats in relation to the antidepressant response.

Methods: Experiments were performed in male Sprague-Dawley rats anesthetized using chloral hydrate. In vivo electrophysiological recordings of neuronal activity were carried out, in the dorsal raphe nucleus (DRN), locus coeruleus (LC) and ventral tegmental area (VTA) and prefrontal cortex (PFC) respectively, within two hours, one day, one week and two weeks after a single administration of vehicle solution or L-655,708 (3 mg/kg; i.p.). The responsiveness of AMPA and NMDA receptors in pyramidal neurons in the CA1 region of the hippocampus was also assessed using extracellular recording techniques with microiontophoresis.

Results: One day post injection, L655,708 elicited a three fold enhancement in the population activity of DA neurons in the VTA compared to controls. This increase was attenuated after administration of flumazenil, a competitive $GABA_A$ receptor antagonist, and NBQX, an AMPA receptor antagonist. The effect was sustained for up to one week following injection of L-655,708, but was back at baseline after two weeks. Additionally, NMDA receptor responsiveness in hippocampal CA1 neurons was significantly reduced, while AMPA responsiveness remained unchanged.

Conclusion: L-655,708 caused a robust increase in the population activity of DA neurons in the VTA one day and one week after injection, surpassing the duration of the effects of ketamine. These findings support the potential of α5 GABA-NAMs as fast-acting, targeted antidepressant agent.

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5-HT	5-hydroxytryptamine, serotonin
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
BDNF	Brain-derived neurotropic factor
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
DA	Dopamine
DH	Dorsal hippocampus
DRN	Dorsal raphae nucleus
FDA	Food and Drug Administration
FST	Forced swim test
GABA	Gamma-aminobutyric acid
GPCR	G protein-coupled receptor
HPA-axis	Hypothalamic pituitary adrenal
i.p.	Intraperitenal
i.v.	Intravenous
LC	Locus coeruleus
LTP	Long-term potentiation
MAO	Monoamine oxidase inhibitor
MDD	Major depressive disorder
MRS	Magnetic Resonance Spectroscopy
mTOR	Mammalian target of rapamycin
NAc	Nucleus accumbens
NAM	Negative allosteric modulator
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione

NE	Norepinepherine
NET	Norepinephrine transporter
NMDA	N-methyl-D-aspartate
PAM	Positive allosteric modulator
PFC	Prefrontal cortex
PPD	Postpartum depression
RCT	Randomized clinical trial
SERT	Serotonin transporter
SNpc	Substantial nigra pars compacta
SNRI	Serotonin norepinephrine reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
TCAs	Tricyclic Agents
TRD	Treatment resistant depression
TrkB	Tropomyosin receptor kinase B
VH	Ventral hippocampus
VP	Ventral palladium
VTA	Ventral tegmental area
WHO	World Health Organization

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PREFACE

Areebah Ahmed (author) contributed to all aspects of the study, including data collection, analysis, and manuscript writing.

Dr. Pierre Blier (supervisor) provided mentorship on all aspects of the study. He provided guidance through the research process, clinical and scientific oversight, and expert advice on literature and writing.

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Chapter 1: Introduction

1. Major Depressive Disorder

1.1. Background

Major depressive disorder (MDD), also referred to as depression, is one of the most common psychiatric disorders with a high economic burden and a lifetime prevalence of about 8.3% in Canada as of 2021 (Li et al., 2021). Depression has been determined to be the third largest cause of disease burden by the World Health Organization (WHO) since 2008 and is projected to reach the unassailable first rank by 2030 (Li et al., 2021). The disorder is known to disrupt biopsychosocial relationships and quality of life for individuals, further perpetuating symptoms of the illness. In accordance to the *Diagnostic and Statistical Manual of Mental Disorders,* a diagnosis of MDD is made based on at least two weeks of depressed mood and/or anhedonia combined with five other symptoms including; increases or deacreases in weight, appetite, and psychomotor activity, feelings of guilt, low energy, difficulties in concentration and in severe cases, suicidal ideation (DSM-5-TR; APA, 2022). In addition, the absence of a past history of manic or hypomanic episodes is required to confirm the diagnosis of MDD.

1.2. Etiology of MDD

As depicted by the diathesis-stress model, there is no one primary cause of MDD as its onset is multifactorial, involving genetic, neurobiological, psychological and environmental factors. Studies indicate that genes may influence the heritability of MDD. In particular, twin studies show that genes may be responsible for 40-50% of the risk for developing depression in the general population (Pasquini et al., 2014). Additionally, first-degree relatives of individuals

with MDD are about three times more likely to develop the disorder, indicating the involvement of genetic factors (Pasquini et al., 2014). Environmental factors such as adverse childhood experiences, low socioeconomic circumstances and life stressors can also contribute to the development of MDD. These stress-inducing experiences can cause epigenetic modifications which may lead to increases in the risk of developing MDD (Li et al., 2021). In particular, stress-induced dysfunction of the hypothalamic pituitary adrenal (HPA) axis is present in 40-60% of patients with MDD, highlighting the role of stress in the progression of the disease (Li et al., 2021).

2. Neurochemical Systems in Depression

MDD is a highly complex disease that has very heterogeneous presentations from one patient to another, and its exact mechanisms remain elusive. For instance, two patients can both meet the criteria for depression and not have a single symptom in common. Nonetheless, over the past decades, research has shed light on a few hypotheses and brain systems that may explain the manifestation of depression.

2.1. The Monoamine Systems

The monoamine system is comprised of dopamine (DA), serotonin (5-HT) and norepinephrine (NE) neurons with cell bodies located primarily in the ventral tegmental area (VTA), dorsal raphe nucleus (DRN) and locus coeruleus (LC), respectively, and projections throughout the brain.

2.1.1. The Dopamine System

Dopamine neurons, located in the ventral midbrain, play a central role in motivation, cognition and movement. Dysfunction of the dopaminergic pathways has been linked to numerous psychiatric and neurodegenerative disorders (Van den Heuvel & Pasterkamp, 2008). The neurotransmitter DA is synthesized from L-tyrosine by tyrosine hydroxylase to L-DOPA, and then decarboxylated to dopamine (Van den Heuvel & Pasterkamp, 2008).

Dopamine receptors are G protein-coupled receptors (GPCRs) which bind DA to trigger downstream cellular signalling. There are 5 types of DA receptors characterized into two classes; D_1-like receptors (D_1 and D_5) that couple to stimulatory G protein Gs/olf, which activates adenylyl cyclase to increase downstream cAMP production, and D_2-like receptors (D_2, D_3 and D_4) that generally couple to inhibitory G protein Gi/o to inhibit adenylyl cyclase and downregulate cAMP production (Beaulieu et al., 2015). These receptors are involved in motivation, movement, hormone regulation and cognition and their dysfunction is linked to various psychiatric disorders (Beaulieu et al., 2015). These receptors are also often the target of many medications used for the treatment of MDD.

There are four major dopamine pathways, all possessing distinct functions. The first is the **mesocortical pathway** in which dopaminergic neurons originate in the VTA and project to the prefrontal cortex (PFC) (Klein et al., 2019). It is involved in cognition and emotional regulation (Klein et al., 2019). The second pathway is known as the **mesolimbic pathway** which projects from the VTA to the nucleus accumbens, amygdala and hippocampus and plays a central role in motivation, reward prediction and pleasure (Dunlop & Nemeroff, 2007). This pathway is heavily involved in cognition and decision-making and its overactivity has been linked to the positive symptoms of schizophrenia (Klein et al., 2019). In the **third pathway,** dopamine

originates in the substantia nigra pars compacta (SNpc) and projects to the dorsal striatum, playing a central role in movement and motor coordination (Klein et al., 2019). A loss of 80% of dopamine neurons in the nigrostriatal pathway gives rise to the characteristic tremors of Parkinson's disease (Van den Heuvel & Pasterkamp, 2008). In fact, the potential involvement of DA in depression has been postulated on a basis of patients with Parkinson's disease, a significant number of whom experience depressive symptoms due also to the decrease in catecholamines and 5-HT availability associated with the disease, sometimes before the appearance of tremors (Lemke et al., 2004; Cummings, 1992; Santamaria et al., 1986). When given medications such as L-dopa to enhance DA levels, a significant decrease in depressive symptoms may be observed (Lemke et al., 2004). Lastly, the **tuberoinfundibular dopaminergic** pathway innervates the hypothalamus to the pituitary gland and regulates lactation (Klein et al., 2019). While these pathways are distinct, they are heavily involved in crosstalk which modulates movement, cognition and motivation behaviours.

Dopamine neurons exhibit two primary patterns of firing: tonic firing, which maintains dopamine tone and phasic firing, which is associated with reward-predicting transient stimuli (Grace et al., 2007). DA population activity is a key player in motivation, as a strong phasic response to a motivationally relevant cue is contingent on the number of spontaneously active DA neurons (Grace, 2016; Floresco et al., 2001). Hence, a decrease in DA population activity leads to a blunted dopamine response to reward stimuli, contributing to anhedonia and motivational deficits observed in depression.

2.1.2. The Serotonin System

Serotonin is a central nervous system (CNS) monoamine neurotransmitter primarily located in the DRN. It is synthesized from its precursor tryptophan by the enzyme tryptophan

hydroxylase to 5-HTP and then decarboxylated into 5-HT (Shu et al., 2025). The 5-HT network plays a crucial role in mood regulation, cognition and behaviour and its dysregulation is a central mechanism in the pathology of depression. More recently, the direct involvement of 5-HT in patients with depression was shown by measuring the release capacity of 5-HT in response to d-amphetamine administration, where depressed patients were found to have diminished release capacity of 5-HT as compared to healthy controls (Erritzoe et al., 2022). The serotonin transporter (SERT) is responsible for 90% of the clearance of 5-HT in the neuronal synaptic cleft (Shu et al., 2025). Studies indicate dysfunctions SERT expression in individuals susceptible to depression which can also be negatively impacted by stress (Shu et al., 2025). There are approximately 17 known 5-HT receptor subtypes that are classified into seven subfamilies; 5-HT_1 through to 5-HT_7, all of which have varying roles and distributions throughout the brain (Nautiyal & Hen, 2017). All 5-HT receptors are GPCRs with the exception of the 5-HT_3 receptor which is a ligand gated ion channel (Nautiyal & Hen., 2017). In particular, 5-HT_1 receptors couple to the inhibitory Gi/o proteins to inhibit adenylyl cyclase and decrease cAMP production. In contrast, the 5-HT_2 receptors are excitatry in nature and couple to the Gq protein to activate phospholipase C, thereby increasing downstream inositol triphosphate and calcium levels (Nautiyal & Hen., 2017).

Of particular importance in the pathology of MDD are the 5-HT_{1A} and 5-HT_{1B} receptors. 5-HT_{1A} act as autoreceptors in the DRN by inhibiting 5-HT release, and are also heteroreceptors in the PFC and limbic areas of the brain (Nautiyal & Hen., 2017). Increased 5-HT_{1A} autoreceptor density has been linked to depression and the targeting of these receptors is central to many conventional antidepressant therapies (Nautiyal & Hen., 2017). 5-HT_{1B} receptors are located on axon terminals in the brain and act as autoreceptors, but also postsynaptically and denoted as

heteroreceptors (Nautiyal & Hen, 2017). Decreased 5-HT_{1B} binding and expression have been linked to depression and suicide, and assessed with this methodology mainly represent the heteroreceptor population because attenuated 5-HT_{1B} autoreceptors appears crucial to the therapeutic benefits of selective serotonin reuptake inhibitors (SSRIs) (Hamon & Blier, 2013). Indeed, their knockout in rodent models has been shown to enhance SSRI-induced 5-HT increases because of their absence from 5-HT terminals (Nautiyal & Hen, 2017).

5-HT_{2A} and 5-HT_{2B} receptors are also highly involved in mood regulation through differing mechanisms. 5-HT_{2A} receptors are highly enriched in PFC pyramidal neurons as well as gamma-aminobutyric acid (GABA) interneurons in this region (Celada et al., 2004). Pharmacological activation of the 5-HT_{2A} receptor leads to an increase in glutamate transmission and neuroplasticity, however, excessive activation of this receptor has been linked to depressive-like symptoms (Zhang & Stackman, 2015; Celada et al., 2004). Antagonism of the 5-HT_{2A} receptor may play a role in the antidepressant response (Zhang & Stackman, 2015; Celada et al., 2004). In contrast, 5-HT_{2B} receptors are primarily enriched on astrocytes and some 5-HT neurons (Hertz et al., 2015; Belmer et al., 2018). These receptors may play a central role in the antidepressant response to SSRIs by driving astrocytic metabolic and gene-expression changes and enhancing DRN firing activity (Hertz et al., 2015; Belmer et al., 2018).

2.1.3. The Norepinephrine System

Norepinephrine neurons are primarily located in the LC and project to key areas in the limbic system of the brain that are responsible for regulating emotion (Stahl, 2021). NE plays an important role in modulating attention, cognition, energy, sleep and arousal and dysfunction of the NE system has been linked to anhedonic symptoms commonly seen in MDD. Norepinephrine synthesis begins with the conversion of tyrosine to L-DOPA via the rate-limiting enzyme

tyrosine hydroxylase. L-DOPA is converted to dopamine and then to NE. Norepinephrine receptors are GPCRs and are classified into two main subfamilies; α- and β-adrenergic receptors. In particular, α- receptors are further classified into α_1 receptors which couple to the excitatory Gq protein to increase intracellular calcium levels, and α_2 receptors which couple to inhibitory Gi proteins, inhibiting adenylyl cyclase and reducing cAMP activity (Maletic et al., 2017). Further, β-adrenergic receptors are also subcategorized into β_1, β_2 and β_3 receptors which all couple to the excitatory Gs protein, thereby stimulating adenylyl cyclase and increasing cAMP production (Maletic et al., 2017). Both α- and β-adrenergic receptors are primarily found on nerve fibers originating from the LC and projecting to various areas of the forebrain (Maletic et al., 2017). These receptors are involved in the antidepressant response by regulating NE signaling and synaptic plasticity. In particular, α_2-adrenergic receptors suppress cyclic adenosine monophosphate (cAMP) and have a dampening effect on cell signalling while α_1- and β-adrenergic receptors have stimulatory effects on cell signalling as they increase cAMP levels (Maletic et al., 2017). Studies have shown that genetic variations in the NE transporter (NET), such as the NET-T182C polymorphism, have been linked to and increased risk for depression (Moret & Briley, 2011). Depletion studies have shown that when NE levels are experimentally decreased in patients in remission treated with NE medications, depressive-like symptoms begin to arise, highlighting the importance of NE as a therapeutic target for the treatment of MDD (Moret & Briley, 2011).

2.1.4. The Monoamine Hypothesis

Traditional medications used to treat MDD lead to the formation of the classical monoamine hypothesis. This hypothesis implies that the underlying mechanism for depression is a lowering of one or more of the monoamine neurotransmitters. The relationship between

monoamine depletion and the antidepressant response has been well-established by numerous studies (Delgado, 2000). While the traditional monoamine hypothesis provided significant insight into the mechanism of action of medications used to treat depression, it remaines incomplete and does not account for the dpathophysiology of depression. For instance, the mere depletion of 5-HT and/or NE/DA does not reproduce depression in healthy individuals (Leyton et al., 2000; Ruhe et al., 2007). However, it is important to note that while 5-HT depletion does not cause depression, it can induce replase in patients that are in remission (Ruhe et al., 2007). As such, a more refined hypothesis known as the modern monoamine receptor hypothesis was developed. This hypothesis posits that antidepressant efficacy is linked to the adaptive regulation of presynaptic autoreceptors on monoamine neurons, thereby enhancing transmission possibly even in the absence of a prior decrease (Hamon & Blier, 2013). Despite this rebranding, the monoamine hypothesis alone remains insufficient to explain the etiology of a disease as complex as MDD and suggests that other neurological systems may also be involved in the pathology of depression.

2.2. Glutamate and GABA Systems

In recent years, there has been a shift toward exploring the roles of GABA and glutamate in depression. Glutamate is the predominant excitatory neurotransmitter of the brain, synthesized via the enzyme glutaminase from its precursor glutamine (Mathews et al., 2012). It is the facilitator of over 90% of synaptic transmission and is used by over 80% of neurons (Mathews et al., 2012). Glutamate acts primarily on ionotropic receptors, which include amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA) and kainic acid, as well as metabotropic receptors to regulate synaptic plasticity, long-term potentiation (LTP) and neurodevelopment (Sanacora et al., 2012). The glutamate hypothesis

states that depression is the result of the disturbance of the excitatory-inhibitory imbalance in the brain caused by the dysregulation of the glutamate neurotransmitter (Sanacora et al., 2012).

In contrast, GABA is the primary inhibitory neurotransmitter of the brain and also plays a crucial role in balancing neurotransmission in various brain systems including the monoamine systems. The effects of GABA are exerted through the activation of its two receptors; ionotropic $GABA_A$ receptors ($GABA_A$Rs) and the metabotropic $GABA_B$Rs (Luscher & Sahir, 2011). In particular, ionotropic $GABA_A$ Rs conduct chloride ions, which typically cause hyperpolarization of the cell membrane, leading to a decrease in neuronal firing. Alterations in $GABA_A$R functioning and GABAergic dysfunction have been linked to the pathology of depression. MRS studies indicate reduced levels of GABA in the PFC and occipital cortex in depressed patients (Sanacora et al., 1999; Hasler et al., 2007). Additionally, lowered levels of GABA have been found in the plasma, cerebrospinal fluid and cortical tissue of depressed patients, further solidifying its role in MDD (Luscher & Sahir, 2011). Genetic studies have shown decreased expression of $GABA_A$R α1, α3, α4 and δ subunit mRNA in the frontal cortex of suicidal patients with concurrent depressive disorders (Luscher & Sahir, 2011).

The hippocampus and PFC are two central structures linked to mood regulation, reward processing and motivation. They contain glutamatergic and GABAergic neurons which distally mediate the activity of the monoamine system in two distinct ways. The first is through the presence of bidirectional projections to the VTA, DRN and LC, which allow for the downstream distal mediation of DA, 5-HT and NE levels (Jones & Moore, 1977; Vasquez-Borsetti et al., 2008; Santana et al., 2004; Celada et al., 2001; Perez et al., 2022). The second way in which distal control is exerted is through the activation and inhibition of monoamine receptors. The majority of glutamate pyramidal neurons in the PFC express 5-HT_{1A} and 5-HT_{2A} receptors,

dopamine D_1 and D_2 receptors and α_1- and α_2-adrenoceptors (Santana & Artigas, 2017; Santana et al., 2004). It has been established that 5-HT_{2A} and D_1 pyramidal receptors can exert excitatory activity on monoamine systems,whereas 5-HT_{1A} and D_2 receptors on pyramidal neurons suppress glutamate activity, thereby decreasing monoamine excitation (Figure 1; Vazquez-Borsetti et al., 2008; Santana & Artigas, 2017; Santana et al., 2004; Celada et al., 2001).

Ultimately, disruptions in the GABA-glutamate balance may play a significant role in the pathology of MDD and therapeutic strategies that work to restore this imbalance can potentially work to reduce depressive symptoms.

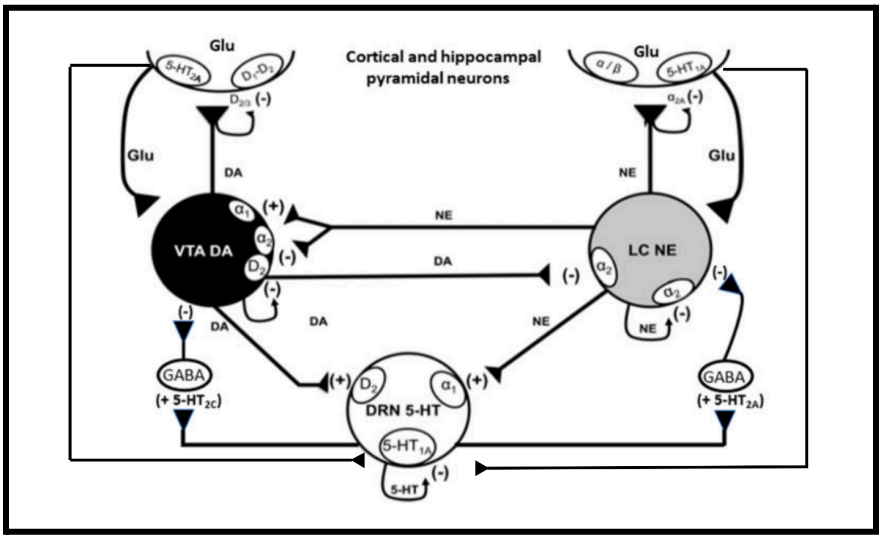

Figure 1: Representation of the reciprocal interactions between glutamate pyramidal neurons and 5-HT, NE and DA neurons as well as interplay within the monoamine system. Modulation of different neurotransmitters on auto- and heteroreceptors is shown with (+) signs indicating a stimulatory effect and (−) signs indicating an inhibitory one. Adapted from Daniels et al., 2023.

2.2.1. AMPA and NMDA Involvement in the Antidepressant Response

AMPA and NMDA receptors are two subtypes of glutamate receptors centrally involved in eliciting antidepressant-like effects in response to numerous treatment interventions for depression. The delicate balance of AMPA and NMDA receptors would be disturbed in MDD,

specifically caused by an overactivation of NMDA receptors and underactivation of AMPA receptors, which leads to the expression of depressive-like behaviours (Zarate et al., 2006). Some rapid-acting antidepressants exert their therapeutic effects by restoring the AMPA-NMDA receptor ratio towards excitatory AMPA-mediated glutamate neurotransmission. AMPA to NMDA receptor activity, a well-established correlate of synaptic strength, glutamate transmission and is responsible for promoting brain-derived neurotrophic factor (BDNF) release and mediating neuronal plasticity (Fischelle et al., 2015; Tizabi et al., 2012; Andreasen et al., 2013; Li et al., 2004). For instance, at subanesthetic doses, ketamine exerts its effects by temporarily blocking NMDA receptors on GABAergic neurons in the PFC and hippocampus. This disinhibits the glutamate neurons in these regions, leading to a surge in glutamate release that is AMPA-receptor mediated (Duman et al., 2019; Li et al., 2010). The direct involvement of AMPA receptor activation in the antidepressant response elicited by traditional and fast acting antidepressant treatments is well documented by studies in which the administration of the AMPA antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX) abolishes the antidepressant-like response, indicating its reliance on AMPA receptor activity (Fischelle et al., 2015; Tizabi et al., 2012; Andreasen et al., 2013).

3. Current Treatment Landscape for Depression

While there has been a considerable effort over the past few decades to develop various pharmacological therapies for depression, the heterogeneous nature of the disease and the shortfalls in translational research remain obstacles in the progression of the field. The two main challenges with medications to treat MDD on the market today are their delayed onset of action and the lack of response in a significant proportion of patients. Between 2009 and 2019, there were 828 compounds under development; however, only a fraction of them were potentially

efficacious, while the rest had been discontinued or failed (Li et al., 2021). Overall, less than 50% of patients enter complete remission with personalized treatment therapies and numerous antidepressant trials (Li et al., 2021). This highlights the imminent need for different approaches in our search for new and effective therapies for depression.

3.1. Monoamine Modulators

Prior to the 1950s, the main treatments for depression were opioids and amphetamines; however, due to their risk for addiction and numerous adverse effects, those approaches were abandoned. The observation that tricyclic agents (TCAs) and monoamine oxidase inhibitors (MAOIs) have antidepressant properties was later attributed to their capacity to increase the availability of 5-HT, NE and/or DA (Alexander & Young, 2023). More specifically, TCAs prevent monoamine reuptake while MAOIs prevent monoamine catabolism (Alexander & Young, 2023). Despite their efficacy, these medications have a slow onset of action, are not well tolerated, and possess a potentially lethal risk of overdose. As such, the pursuit for more selective and targeted antidepressant strategies began.

The late 1980s marked the emergence of SSRIs, such as fluoxetine, which have since remained the first line of treatment for patients with MDD (Hamon & Blier, 2013). SSRIs exert their therapeutic effects by more selectively targeting SERT, thereby increasing the level of 5HT in the synaptic cleft, leading to cell body and terminal autoreceptor desensitization, resulting in a subsequent sustained release of 5-HT levels and consequently, their antidepressant effect (Blier & De Montigny, 1983). Despite their promising outlook, these medications have a slow rate of onset, and a third of patients still do not respond to initial treatment (Hamon & Blier, 2013). In addition, SSRI use is often accompanied by side effects such as emotional blunting and a

decreased sex drive, which frequently leads to medication noncompliance (Alexander & Young, 2023).

The 1990s marked the approval of serotonin norepinephrine reuptake inhibitors (SNRIs) for the treatment of MDD. Venlafaxine was one of the first non-TCA SNRIs to be marketed and was found to be more effective than conventional SSRIs due to its dual action (Poirier and Boyer 1999; Hillhouse & Porter, 2015). Other SNRIs such as milnacipran, duloxetine, desvenlafaxine and levomilnacipran have also entered the market (Hillhouse & Porter, 2015; Blier, 2013). SNRIs exhibit their antidepressant-like effects by inhibiting 5-HT and NE reuptake at the respective transporters, allowing for an elevation of these two neurotransmitters (Hillhouse & Porter, 2015; Blier, 2013). Despite their promising effects, this class of medication still leaves some patients with inadequate responses.

Ultimately, the incomplete effectiveness of monoamine medications and their delayed onset still highlight the need for more effective and fast-acting therapeutic strategies for the treatment of depression.

3.2. Treatment-Resistant Depression

While adjunctive therapies have been proven to be successful in patients who do not respond to SSRIs, they still remain ineffective for a subset of the depressed patient population that is characterized as treatment resistant. While a unanimous consensus has not been reached regarding its definition, treatment-resistant depression (TRD) is generally defined as having MDD that has failed 2 or more trials of medications from different classes at an appropriate dose and sufficient duration (Gaynes et al., 2020; McIntyre et al., 2023). Approximately 30% of patients with MDD are characterized as treatment-resistant (McIntyre et al., 2023). These patients experience greater impairments in psychosocial functioning, increased risk for suicide

and comorbidities such as diabetes and cardiovascular diseases (McIntyre et al., 2023). Therapeutic intervention and efficacy can be complicated for patients with TRD; however, research has recently pointed towards a few promising treatments.

3.3. Adjunctive Strategies

Adjunctive strategies are commonly used for the treatment of MDD for patients who do not respond to a single conventional treatment, as they target additional brain systems. Often, previously called "second-generation antipsychotics" intended for the treatment of schizophrenia and mania can be used as adjunctive antidepressant treatments at low doses due to their high affinity for various monoamine targets without blocking DA D_2 receptors (Blier & Blondeau, 2011). In particular, D_2 partial agonists such as aripiprazole, brexpiprizole and cariprazine are known to stabalize dopamine levels as they have the ability to function as both agonists and antagonists depending on DA levels (Lieberman, 2004). Additionally, they also act as partial agonists at 5-HT_{1A} receptors and as antagonists at 5-HT_{2A} receptors, both actions which are thought to contribute to their antidepressant-like effects (Pae et al., 2008).

In parallel, adjunctive risperidone treatment with conventional SSRIs was shown to improve response rates and increase remission in randomized controlled trials (RCTs) (Ostroff & Nelson, 1999; Mahmoud et al., 2007; Keitner et al., 2009). Early studies tested olanzapine with fluoxetine and showed promising results in patients who did not respond to an SSRI treatment (Shelton et al., 2001). In addition, aripiprazole, brexpiprazole and cariprazine are also effective, evidence-based augmentation strategies that have been approved for use in MDD, supported by multiple RCTs demonstrating improved response remission versus monotherapies (Luan et al., 2018; Thase et al., 2019; Durgam et al., 2016). Ultimately their success in RCTs allowed for adjunctive strategies to become widely accepted approaches for TRD.

Interestingly, pramipexole, a full DA receptor agonist initially intended for the treatment of Parkinson's disease has shown effectiveness in combating depression in RCTs and is now in treatment guidelines for MDD (Browning et al., 2025; Tundo et al., 2019; Lam et al., 2024). Unlike partial agonists of DA receptors which also target other monoamine receptors, pramipexole has a high affinity for DA receptors, in particular the D_3 receptor which has been implicated in depression, thereby exerting its effects primarily through DA modulation, but also enhances 5-HT transmission through reciprocal connections (Tundo et al., 2019; Chernoloz et al., 2009).

While these adjunctive medications are efficacious and generally well-tolerated, a subset of patients still do not respond to this therapeutic approach.

3.4. Ketamine

In recent years, there has been a push towards the development of novel fast-acting medications for depression, predominantly based on the antidepressant-like action of the NMDA antagonist, ketamine, an anesthetic agent re-tooled for MDD (Li, 2020). Recently, a fast-acting treatment has been approved for patient use by the FDA and nearly world-wide: the s-enantiomer of ketamine, esketamine, for treatment-resistant depression (Li et al., 2021).

While racemic ketamine and esketamine have revealed immense potential as a fast-acting strategy, it is accompanied by aversive side effects due to its unlocalized targeting of NMDA receptors throughout the brain (Tropolli et al., 2022). At low doses, esketamine has been hypothesized to exert its antidepressant effects through the blockade of NMDA receptors on inhibitory GABA interneurons, thus inducing a surge of glutamate, which leads to the activation of AMPA receptors on post-synaptic glutamate neurons (Figure 3; Sanacora & Schatzberg, 2015; Moghaddam et al., 1997). This, in turn, leads to increases in BDNF levels, which stimulate

downstream tropomyosin receptor kinase B (TrkB) receptors and activate the mammalian target of rapamycin (mTOR) pathways, thus leading to synaptogenesis and the reversal of anhedonic symptoms (Sanacora & Schatzberg, 2015).

While the latter two steps in the hypothesized mechanism of action for ketamine are supported in animal models, human studies have produced contradictory findings. To test the role of the mTOR pathway in the ketamine-induced antidepressant response, a study used rapamycin, an mTOR inhibitor, that was administered to depressed patients prior to treatment with ketamine (Abdallah et al., 2020). The rapamycin pretreatment actually did not inhibit the antidepressant response and even prolonged the antidepressant effect of ketamine, thereby indicating that responsiveness to ketamine is perhaps not mediated through the mTOR pathway (Abdallah et al., 2020). Animal studies show that BDNF Val66Met knock-in mice prevent the antidepressant-like effects of ketamine in the forced swim test (FST), however, such a polymorphism in humans does not prevent the therapeutic antidepressant response (Liu et al., 2012). More specifically, the effects of ketamine treatment were examined in depressed Taiwanese patients, with a subset of the population known to have less functional Met alleles of the Val^{66}Met polymorphism of the BDNF gene (Su et al., 2017). It was found that these patients responded adequately to ketamine treatment, thus pointing to the fact that BDNF may not be essential in the antidepressant efficacy of ketamine in humans (Su et al., 2017). To provide evidence for the essential role of AMPA in the antidepressant effects of ketamine, a study used the AMPA antagonist NBQX in rats and subjected them to the FST (Zhou et al., 2014). The results indicated that the inhibition of AMPA receptor activation blocked the antidepressant-like effects of ketamine observed through an increase in immobility, thus indicating the central role of AMPA in the anhedonic effects of ketamine (Zhou et al., 2014). Ultimately, it may be concluded

that the rapid antidepressant-like effects of ketamine are induced by an AMPA-evoked surge in glutamate activity. While ketamine and its more potent enantiomer esketamine, have established the possibility of fast-acting strategies, their lack of NMDA receptor specificity and widespread effects result in marked side effects, such as dissociation and the potential risk of abuse, thus indicating the need for more targeted therapeutic agents.

3.5. Brexanolone and Zuranalone

Brexanolone and zuranolone are fast-acting $GABA_A$ receptor positive allosteric modulators (PAMs), recently approved by the FDA for the treatment of post-partum depression (PPD). Allopreganolone is a derivative of progesterone that rises during pregnancy and sharply declines thereafter, leading to the hypothesis that it may be the underlying cause of PPD (Cornette et al., 2021; Dacarett-Galeano & Diao., 2019). Brexanolone and zuranolone mimic the actions of allopreganolone by directly targeting $GABA_A$ receptors, which mediate tonic inhibition and phasic inhibition to produce a net inhibitory effect on neurotransmission, thus resulting in its therapeutic effects (Cornette et al., 2021; Dacarett-Galeano & Diao., 2019; Rubin, 2023). While brexanolone and zuranolone have been shown to be successful in the treatment of PPD and has indicated effective clinical application as a fast-acting GABA therapeutic strategy, the need for a similar drug based on this GABA mechanism of action for the treatment of MDD still remains. Notably, however, zuranolone has recently shown promising results in clinical trials for the treatment of MDD, with remission rates ranging from 40–64 % after the completion of a 14-day treatment period (Meshkat et al., 2023; Rubin, 2023).

4. α5 $GABA_A$ Negative Allosteric Modulators (NAMs)

There is heightened interest in NAMs of $GABA_A$ receptors as a potential fast-acting alternative to the ketamines. $GABA_A$Rs are classified as pentameric ligand-gated ion channels

composed of two α (1-6), two β (1-3) and one γ (1-3) subunits, which play a central role in synaptic plasticity and cognition (Jacob, 2019). α5 $GABA_A$R-NAMs are a class of drugs that specifically target $GABA_A$Rs, which are highly concentrated on the glutamate neurons in the hippocampus and the PFC, two significant brain structures involved in reward processing, motivation, and mood regulation (Serwanski et al., 2006; Jacob, 2019). In fact, this class of drugs binds selectively to the benzodiazepine site located at the α/γ subunit interface of $GABA_A$ receptors, with 100 times higher affinity for the α5 subunit as opposed to the α1, α2 and α3 subunits (Quirk et al., 1996). The specificity of this drug class theoretically limits the occurrence of off-target effects, thereby allowing the possibility of replicating the therapeutic effects of ketamine without exerting undesired side effects, such as dissociation. L-655,708 is a selective α5 $GABA_A$R-NAM that is currently being tested preclinically as a potential novel medication for the treatment of depression and is the treatment used in this study.

4.1. Allosteric Modulation of $GABA_A$ Receptors

The positive or negative modulation of α5 $GABA_A$ receptor NAMs can elicit distinct effects on neuronal excitability, its downstream effects, as well as the antidepressant response. Under normal conditions, GABA binds to the α5 $GABA_A$, increasing the frequency of opening and allowing for an greater influx of Cl- ions, which hyperpolarize the membrane potential and make it less likely to fire action potentials (Soh & Lynch, 2015). In the case of NAMs, such as L-655,708, these agents bind to a site separate from the GABA binding site called the allosteric or benzodiazepine site. This causes the ion channel to decrease in its frequency of opening, limiting the influx of Cl- ions. This leads to depolarization of the membrane, causing an increase in action potentials and a release of glutamate (Soh & Lynch, 2015). The opposite is true in the case of PAMs, where the PAM binds to the allosteric site, causing the ion channel to open at a

faster rate, increasing the influx of Cl- ions and leading to neuronal hyperpolarization, preventing an action potential and eliciting a net inhibitory effect on glutamate transmission (Figure 2; Soh & Lynch, 2015). Essentially, L-655,708 functions as an 'inverse agonist' at the α5 site on the $GABA_A$ receptor, thereby decreasing its baseline activity. This is unlike an antagonist such as flumazenil, which binds to the same site but has no intrinsic effect on receptor activity, rather it blocks the binding of both agonists and inverse agonists, preventing them from modulating receptor activity.

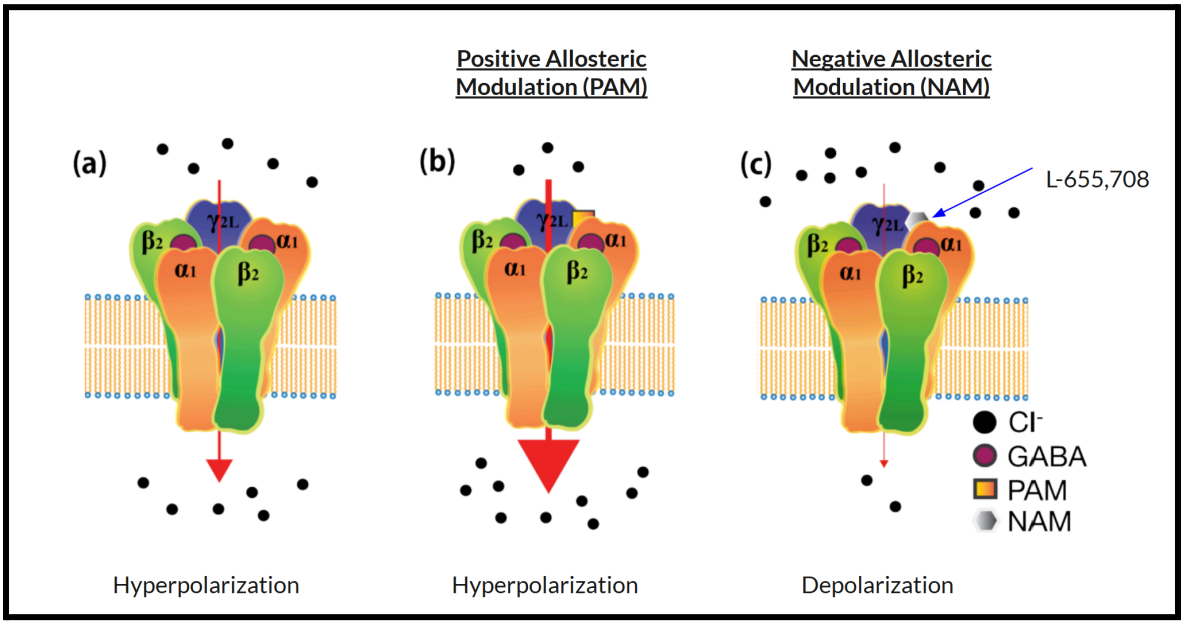

Figure 2: Depiction of the actions of GABA, PAMs and NAMs on $GABA_A$ receptors. Allosteric modulators bind at secondary binding sites on $GABA_A$ receptors, causing a change in the frequency of channel opening such that chloride ion current is increased or decreased through the channel, leading to hyperpolarization or depolarization. Adapted from Soh & Lynch, 2015.

4.2. Behavioural Studies

Preclinical behavioural studies have indicated promising antidepressant-like effects promoted by α5 $GABA_A$R-NAMs, particularly in the FST. Rodents subjected to the FST exhibited a decrease in immobility one day after the administration of L-655,708 that persisted

for up to one week with a single injection (Carreno et al., 2017; Bugay et al., 2020). As well, a marked increase in swimming behaviour, an indicator of increased serotonin levels was observed one week after administration of L-655,708 (Carreno et al., 2017; Bugay et al., 2020). These results were rapidly reversed following the administration of NBQX, a potent AMPA receptor antagonist, suggesting that the mechanism of action of L-655,708 may be AMPA dependent (Bugay et al., 2020). NE-dependent climbing in FST was unchanged 30 minutes after administration of L-655,708, although an increase in climbing behaviour was found 24 hours following the direct administration of L-655,708 into the ventral hippocampus (VH) (Bugay et al., 2020). No locomotive or psychomimetic side effects were observed in response to L-655,708 (Carreno et al., 2017; Bugay et al., 2020). Ketamine has also been reported to decrease immobility and increase swimming and climbing behaviour acutely for up to 24 hours after administration; however, its effects are short-lived and dissipate beyond the first day (Gigliucci et al., 2013; López-Gil et al., 2019).

Furthermore, behavioural studies have demonstrated that L-655,708 reverses the DA-dependent anhedonic deficits in the sucrose preference test (SPT) one day, but not immediately after its injection (Fischell et al., 2015; Bailey et al., 2023; Bugay et al., 2020). These results are similar to the antidepressant-like effects of ketamine observed in the SPT (Jiang et al., 2017).

In addition, EEG studies indicate analogous increases in gamma power in both human and animal models in response to ketamine and α5 $GABA_AR$-NAM drug treatment, thus suggesting a similar mechanism of antidepressant effects (Tropolli et al., 2022; de la Salle et al., 2022; Vlisides et al., 2018).

4.3. Basmisanil

Basmisanil is a highly selective α5 $GABA_AR$ NAM with a mechanism of action that is similar to that of L-655,708 (Figure 3). This drug has been tested in humans as a cognitive enhancer for Down's syndrome, as it reduces tonic inhibition in the hippocampus and PFC, thereby increasing neurotransmission and cognitive abilities (Hipp et al., 2021). While Basmisanil has not yet been tested in depression, studies are indicative of its good tolerability, lack of any major aversive side effects, and selective target engagement in the human brain (Hipp et al., 2021). These findings point to the potential efficacy of other α5 $GABA_AR$ NAMs in MDD, such as L-655,708, which show promising antidepressant-like effects in preclinical studies and may be translated to humans.

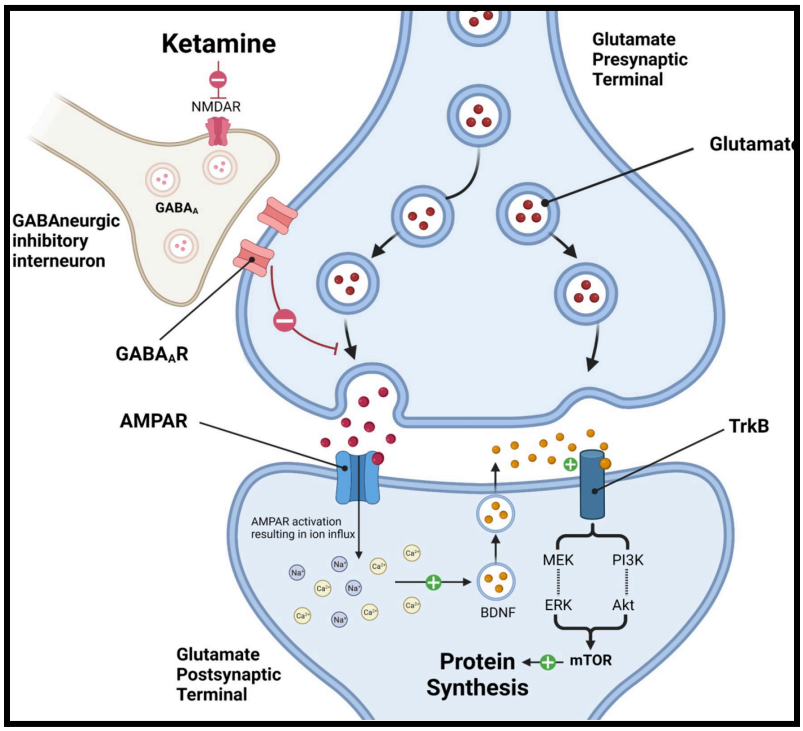

Figure 3: Hypothesized targets and mechanisms of α5 $GABA_AR$-NAMs and ketamine. Adapted from Dao & Aggarwal, 2023.

5. Objectives

Recent studies have investigated the effects of α5 $GABA_A$R-NAMs on mice and rats using the FST and the SPT to determine its antidepressant-like effects on behaviour (Tropolli et al., 2022; Zanos et al., 2017). However, limited work has been done to investigate the electrophysiological impact of α5 $GABA_A$R-NAM activation. To bridge this gap in the literature, the present study aimed:

1) To determine whether acute administration of L-655,708 has any effect on the firing and burst activity of 5-HT, NE and DA neurons;

2) To determine whether potential L-655,708-induced changes in monoamines neurons activity are mediated through:

a) the benzodiazepine site and,

b) by AMPA receptors, as ketamine does;

3) To explore the duration of any changes induced by L-655,708 on the firing activity of monoamine neurons;

4) To study the AMPA and NMDA-induced responses on pyramidal neurons in the hippocampus;

5) To examine the effects of L-655,708 on the spontaneous firing activity of pyramidal neurons in the PFC.

Chapter 2: Article- AMPA receptors modulate increased dopamine neuronal activity up to one week after a single injection of a negative allosteric modulator of α5 $GABA_A$ receptors

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Abstract

Background: Negative allosteric modulators (NAMs) of $GABA_A$ receptors targeting the α_5-subunit, primarily expressed on glutamate pyramidal neurons in hippocampus and cortex, reduce inhibitory tone, and enhance AMPA receptor throughput. The α_5-$GABA_A$-NAM, L-655,708, has shown rapid and sustained antidepressant-like effects in rodents.

Aim: This study aimed to investigate the effects of L-655,708 on monoamine and glutamate systems in rats in relation to the antidepressant-like response.

Methods: Male Sprague-Dawley rats received a single dose of L-655,708 (3 mg/kg, i.p.) or vehicle. In vivo extracellular recordings were conducted in the dorsal raphe nucleus (DRN), locus coeruleus (LC), ventral tegmental area (VTA), and medial prefrontal cortex (mPFC) acutely, one day, one week, and two weeks post-injection. AMPA and NMDA responsiveness in the hippocampal CA1 region was assessed using microiontophoresis.

Results: The NMDA-induced response in the CA1 hippocampus was significantly reduced one day post-injection of L655,708, while the AMPA response remained unchanged. Although mPFC pyramidal neurons firing was not changed, there was a two-fold increase in population activity of VTA dopamine (DA) neurons one day post-injection, lasting up to one week. Flumazenil, the benzodiazepine site antagonist, and NBQX, an AMPA receptor antagonist, blocked this effect. L-655,708 had no acute effect on firing activity of serotonin or norepinephrine neurons.

Conclusion: L-655,708 enhanced VTA DA neuron population activity for up to one week after a single injection. This effect was dependent on AMPA and took place through action on the benzodiazepine site.

Key Words

Antidepressant; L-655,708; dopamine; norepinephrine; serotonin

Introduction

Selective serotonin (5-HT) reuptake inhibitors (SSRIs) have traditionally been the first line of treatment for patients with major depressive disorder (MDD). They have, however, a slow rate of onset of action, numerous side effects and a third of patients fail to respond to an initial trial (Hamon & Blier, 2013). While SSRIs increase 5-HT transmission, they concurrently dampen norepinephrine (NE) and dopamine (DA) neuronal activity (Dremencov et al., 2009; Ghanbari et al., 2010).

Although there has been a shift toward treatments primarily targeting the glutamate system, monoamines remain implicated in the antidepressant response due to the well-established reciprocal interaction between the two systems (Millan, 2006; Pralong et al., 2002). For instance, the effects of ketamine, which acts by blocking N-methyl-D-aspartate (NMDA) receptors on gamma-aminobutyric acid (GABA) neurons and increasing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor throughput (Li et al., 2011; Miller et al., 2016; Widman and McMahon, 2018; Gerhard et al., 2020), do affect the monoamine systems. Administration of ketamine increases DA neuron population activity and NE neuron firing activity without any action on 5-HT neuronal activity (El Iskandrani et al., 2015; Witkin et al., 2016; Daniels et al., 2023). Nevertheless, microdialysis studies show elevated extracellular concentrations of 5-HT, DA and NE in the medial prefrontal cortex (mPFC) and ventral hippocampus, following a single injection of ketamine (Moghaddam et al., 1997; Lopez-Gil et al., 2019; Lorrain et al., 2003).

While ketamine indirectly enhances pyramidal neuron activity through GABAergic inhibition, similar increases in excitatory glutamate transmission can be directly induced by targeting α_5-subunits on pyramidal neurons (Brunig et al., 2002) using negative allosteric

modulators (NAMs) of $GABA_A$ receptors (α_5-$GABA_A$R-NAMs; Glykys and Mody, 2006; Prenosil et al., 2006; Zorrilla de San Martin et al., 2020).

$GABA_A$Rs are pentameric ligand-gated ion channels located on glutamate neurons, highly concentrated in hippocampal CA1 and CA3 regions and, to a lesser extent, in the PFC (Sur et al., 1999; Serwanski et al., 2006; Jacob, 2019). α_5-$GABA_A$R-NAMs are a class of drugs that specifically target the benzodiazepine site of α_5-$GABA_A$Rs. Their binding causes a decrease in the frequency of ion channel opening, limiting the influx of Cl- ions and thereby leading to depolarization and a subsequent release of glutamate that allows for an increase in AMPA receptor throughput (Sigel & Steinmann, 2012; Jacob, 2019). L-655,708 was reported to be a selective α_5 $GABA_A$R-NAM exhibiting 50- to 100-fold selectivity for the α_5-subunit (Quirk et al.,1996). It was also been shown to exert antidepressant-like effects in the forced swim test (FST) and sucrose preference test (SPT) through the blockade of the α_5-subunit on $GABA_A$ Rs (Troppoli et al., 2022, Bugay et al., 2020; Carreno et al., 2017; Zanos et al., 2017; Xiong et al., 2018).

Given the predominant expression of α_5 $GABA_A$ receptors in the hippocampus, the present study aimed at determining whether their blockade by L-655,708 alters AMPA- and NMDA responses in this brain region. Since α_5-$GABA_A$ receptors are also present, albeit in moderate density in the mPFC, it was deemed important to determine the effect of L-655,708 on the firing activity of pyramidal neurons in this structure. Given the results in behavioural studies, and taking into account the fact that FST (swimming and climbing) and SPT, respectively inform on 5-HT, NE and DA systems, the present study aimed at determining whether: 1) acute administration of L-655,708 has any effect on the firing and burst activity of 5-HT, NE and DA neurons; 2) potential L-655,708-induced changes in monoamines neurons activity are mediated

through a) the benzodiazepine site and b) by AMPA receptors, as ketamine does; 3) determine whether AMPA is involved in sustaining any changes in neuronal activity induced by a single injection of L-655,708.

Methods

Animals

In vivo electrophysiological recordings were conducted during the light phase in male Sprague-Dawley rats obtained from Charles River (St. Constant, Quebec, Canada) weighing 250-350 grams at the time of the experiment. Upon arrival, rats were housed in pairs under standard laboratory conditions (12 hour dark/light cycle) with access to food and water. Rats were acclimated to the novel environment for 7 days prior to the experiments. All animals were handled in accordance with the Canadian Council on Animal Care (CCAC) guidelines, and study protocols were approved by the local Animal Care Committee (University of Ottawa Animal Care Committee, Ottawa, Canada).

Drug administration

L-655,708 was purchased from (Cayman Chemical, MI, USA) and dissolved in 75% DMSO and 25% water. A single 3 mg/kg dose of L-655,708 was administered intraperitoneally (i.p.) 30 minutes, one day, one week and two weeks prior to electrophysiological recordings, which lasted for up to two hours. Control rats were injected with a vehicle solution. The dose of L655,708 used in the present study is based on previous work by Attack et al. (2006) showing that a dose of 3 mg/kg results in approximately 75% occupancy at α_5-$GABA_A$Rs and 33% at α_1, α_2, and α_3 receptor subtypes; this indicates an optimal balance for achieving maximal α_5-selective engagement while minimizing off-target effects.

NBQX (10 mg/kg) was purchased from Cayman Chemical (MI, USA) and dissolved in 0.9% aqueous saline solution. Flumazenil (3 mg/kg) was purchased from (Cayman Chemical, MI, USA) and dissolved in 80% DMSO and 20% water. Both NBQX and flumazenil were administered i.p. 30 - 60 minutes prior to recording in treated and control rats. Rats were not randomized, and experimenter was not blinded.

in vivo electrophysiological recordings

In vivo extracellular recordings were conducted in rats anesthetized using chloral hydrate (400 mg/kg, i.p) and mounted on a stereotaxic apparatus (David Kopf, CA, USA). Periodic administration of supplemental chloral hydrate (100 mg/kg, i.p) was carried out during the experiment to maintain anesthesia and body temperature of 37°C was maintained using a heating pad. For each brain structure of interest, a burr hole was drilled in the skull according to standard rat brain stereotaxic coordinates, using the atlas by Paxinos and Watson, 1987. Neuronal activity was captured in real time using the Spike2 program (Cambridge Electronic Design, Cambridge, UK).

Extracellular recordings and microiontophoresis in hippocampus CA1 pyramidal neurons

Microiontophoresis and extracellular recordings of pyramidal neurons in the dorsal CA1 region of the hippocampus were conducted using five-barreled glass micropipettes and positioned in accordance to the following coordinates: 4.0 to 4.2 mm anterior to lambda and 4.0 to 4.2 mm lateral to the midline. Pyramidal neurons were typically encountered at a depth of 2.5 to 3.0 mm below the surface of the brain. The central barrel used for electrophysiological recordings contained 2 M NaCl solution, while the peripheral barrels contained AMPA (5 mM in 200 mM NaCl, pH 8), NMDA (10 mM in 200 mM NaCl, pH 8) and 2 M NaCl for current balancing. Neurons were identified based on the following properties: 1) a large amplitude (0.5 -

1.2 mV), 2), long-duration (0.8 - 1.2 ms) action potentials alternating with complex spike discharges (Kandel and Spencer, 1961). Ejection currents of AMPA (-1 and -2 nA) and NMDA (-3, -5 and -8 nA) were used to activate pyramidal neurons, which are not firing spontaneously in the hippocampal CA1 region of rats under chloral hydrate anesthesia. The currents of AMPA and NMDA for microiontophoretic ejections of 40 seconds in duration were kept constant for both vehicle and rats that received L-655,708. A retention current of +15 nA was used in between ejections that lasted 40s. AMPA and NMDA responses were assessed in an average of six neurons per rat.

Electrophysiological recordings of mPFC pyramidal neurons

Neurons in the mPFC were recorded by positioning a single-barrel glass micropipette at 3.2 - 3.4 mm anterior to Bregma and 0.6 - 0.8 mm lateral to the midline suture at a depth of 2.5 to 5.0 mm from the surface of the brain. mPFC pyramidal neurons were identified in accordance with the following electrophysiological criteria: 1) irregular firing with rates ranging between 0.01 and 3 Hz, 2) long duration (0.8 - 1.2 ms) and 3) biphasic or triphasic waveforms (Riga et al., 2017).

Electrophysiological recordings of ventral tegmental area (VTA) DA neurons

For DA neuron recordings, a single-barrel glass micropipette was positioned at 3.0 - 3.6 mm anterior to lambda and 0.6 - 1.0 mm lateral to the midline suture. DA neurons were typically encountered at a depth of 7.5 to 8.5 mm from the surface of the brain. DA neurons were classified based on well-established characteristic features including: 1) regular and irregular, slow firing rates (2 - 10 Hz), 2) biphasic or triphasic waveforms, 3) the distinctive presence of a notch on the depolarization phase of the spike, 4) the distance from the spike initiation to the trough is equal or greater than 1.1 ms and 5) a characteristic low-pitch sound on the audio

monitor (Ungless and Grace, 2012). The number of spontaneously active DA neurons found per track, referred to as population activity, was determined by recording multiple trajectories in a grid of 6-9 tracks per rat.

Electrophysiological recordings of dorsal raphe nucleus (DRN) 5-HT neurons

Serotonin neurons were recorded using single-barrel glass micropipettes positioned on the midline at 0.9–1.2 mm anterior to lambda and +/- 0.2 mm lateral to the midline suture. Neurons were typically encountered at a depth of 4.5 - 5.5 mm from the surface of the brain. Serotonin neurons were identified based on the following electrophysiological criteria: 1) regular and slow firing rates (0.5 - 2.5 Hz), 2) long duration (2 - 5 ms), and 3) biphasic or triphasic waveforms (Vandermaelen & Aghajanian, 1983).

Electrophysiological recordings of locus coeruleus (LC) NE neurons

Norepinephrine neurons were recorded by positioning a single barrel glass micropipette 0.8 -1.1 mm posterior to lambda and 0.9 – 1.3 mm lateral from the midline suture at a depth of 4.5 – 6.0 mm from the surface of the brain. Norepinephrine neurons were identified by the presence of: 1) regular firing rates (0.5 - 5 Hz), 2) long-duration (>2 ms) and 3) a volley of discharge of action potentials in response to pinching of the contralateral paw, followed by a transient silent period (Marwaha & Aghajanian, 1982).

Burst Analysis

The beginning of a burst was determined by the occurrence of two spikes with interspike intervals (ISI) < 0.08 s for NE and DA neurons and <0.01 s for 5-HT neurons. Burst termination was defined as an ISI >0.16 s for NE and DA (Grace and Bunney, 1983) and ISI >0.01 s for 5-HT neurons (Hajós and Sharp, 1996). For mPFC pyramidal neurons, burst identification was

based on the following criteria: a series of two or more spikes, with ISI <0.045 s from the initiation and >0.045 s for termination of burst (Laviolette et al., 2005).

Statistical Analyses

Data are presented as mean values ± SEM. Statistical comparisons between control and L-655,708-treated groups were carried out using the two-tailed t test when normality passed with the Shapiro-Wilk test. When normality failed, the nonparametric Mann-Whitney test was utilized, and is based by comparing medians of the groups. One-way and two-way analysis of variance (ANOVA) with a post-hoc analysis were also used when appropriate. Statistical comparisons were conducted using the software SigmaPlot 12.5 (Systat Software Inc, CA, USA).

Results

Effects of a single administration of L-655,708 on AMPA- and NMDA-induced responses in CA1 region of the hippocampus

The AMPA- and NMDA-evoked response of CA1 pyramidal neurons was assessed by determining the number of spikes generated when ejecting AMPA at currents of -1 and -2 nA, and NMDA at currents -3, -5, and -8 nA, in controls and rats that received L-655,708. There was no significant effect of treatment (two-way repeated measures ANOVA $F[1,10] = 0.2$; $p = 0.6$), current ($F[1,10] = 4$; $p = 0.1$) and interaction ($F[1,10] = 1.4$; $p = 0.3$) on AMPA-evoked responses (Figure 1A and 1C). In contrast, a two-way repeated measures ANOVA on NMDA-induced response revealed a robust decrease in responsiveness of these neurons ($F[1,20] = 17$; $p = 0.002$) and current ($F[2,20] = 8$; $p = 0.003$). There was, however, no significant interaction between treatment and current intensity ($F[2, 20] = 1.4$; $p = 0.3$). Holm-Sidak post hoc analysis showed that in the L-655,708-administered group, there was a statistically

significant decrease in the NMDA-induced response that was independent of intensity of current ($p = 0.002$; figure 1D).

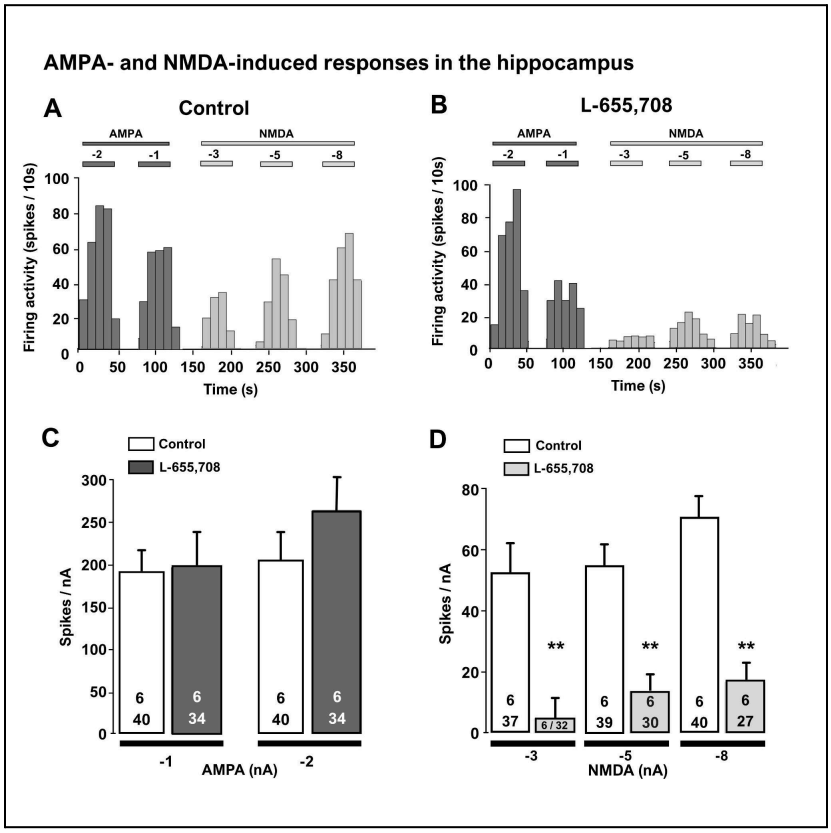

Figure 1: Representative integrated firing rate histograms illustrating the effects of L-655,708 (3 mg/kg, i.p) on AMPA- and NMDA-induced response in CA1 region of the hippocampus, one day after administration **(A, B)**. Overall change in the number of spikes generated by nA by AMPA and NMDA in hippocampal CA1 pyramidal neurons following injection of L-655,708 compared to control **(C, D)**. Horizontal bars indicate the duration of iontophoretic applications of AMPA and NMDA. Number of rats and neurons are indicated at the bottom of the histogram. Statistical significance is indicated where it applies. * $p < .05$ and ***$p < 0.001$ when compared to control.

Effects of a single administration of L-655,708 on the activity of mPFC pyramidal neurons

The mean firing rate and the percentage of spikes in bursts of a population of mPFC pyramidal neurons was determined before and at least 30 minutes after L-655,708 injection. One

day after a single administration of L-655,708, there was no significant alteration of pyramidal neuronal firing (two-tailed t-test, t[8] = -0.5; p = 0.7; Figure 2A). There was also no change in the percentage of spikes occurring in bursts (two-tailed t-test, t[8] = 0.8; p = 0.4; Figure 2B).

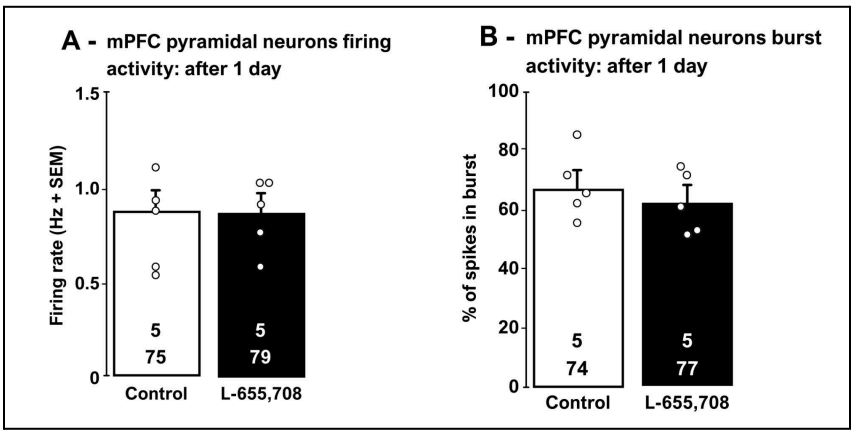

Figure 2: The effects of L-655,708 (3 mg/kg, i.p) administration on mPFC pyramidal neurons assessed one day after administration. Histograms show data as mean values ± S.E.M. Each dot represents the mean of firing **(A)** and % of spikes in burst mode **(B)** of mPFC pyramidal neurons, assessed one day after administration of vehicle and L-655,708. Number of rats and neurons are indicated at the bottom of the histogram.

Effects of a single administration of L-655,708 on the activity of VTA DA neurons

When injected acutely, L-655,708 had no effect on the firing rate of DA neurons (two-tailed t-test, t[8] = -0.6; p = 0.6; supplementary table 1), the percentage of spikes in burst (control: 18 ± 3.7%, L-655,708: 21 ± 6.1%; two-tailed t-test, t[8] = -0.4; p = 0.7; supplementary table 1), and population activity of DA neurons (control: 1.4 ± 0.2 neurons/tract, L-655,708: 1.9 ± 0.2 neurons/tract; two-tailed t-test, t[8] = -1.8; p = 0.1; figure 3A). One day following the administration of L-655,708 injection, however, there was a robust two-fold increase in the number of spontaneously active DA neurons compared to rats administered with the vehicle (control: 1.3 ± 0.1 neurons/tract, L-655,708: 3.3 ± 0.1 neurons/tract, one-way ANOVA F[4,24] =

37; p = 0.001; supplementary table 1, figure 3A). However, following Kruskal-Wallis test on ranks the firing activity (H[4] = 8; p = 0.1), the percentage of spikes occurring in bursts was not significantly altered by L655,708 (one way ANOVA F[4,24] = 0.4; p = 0.8; supplementary table 1).

The Holm-Sidak post-hoc test revealed that the increase in DA neuron population activity was abolished when the AMPA receptor antagonist NBQX (10 mg/kg) was administered 30 minutes before L-655,708 (p = 0.7; Figure 3B). It is important to note that, at this dose, NBQX on its own was previously shown to be devoid of action on this parameter (El Iskandrani et al., 2015).

To ascertain that L-655,708 was acting through α_5-$GABA_A$Rs to increase DA neuron population activity, the competitive benzodiazepine site antagonist flumazenil (3 kg/mg) was injected 30 minutes prior to recordings. Indeed, administration of the latter drug, which has no effect on its own, abolished the increase in DA neuron population activity induced by injection of L-655,708 (Holm-Sidak post-hoc, p = 0.9; figure 3B).

To evaluate whether the effects of a single L655,708 administration were prolonged beyond the drug's elimination, its effects were examined one week after injection. Indeed, a significant increase in the number of spontaneously active DA neurons in the VTA was still present one-week post-injection, when compared to control (one way ANOVA F[2,14] = 44; p = 0.001; supplementary table 1, figure 3A). In order to assess if AMPA receptors are involved in maintaining this effect, NBQX was administered 30 minutes before the recordings. In fact, the increase of DA neuron population activity induced by L-655,708 was abolished by NBQX (Holm-Sidak method, p = 0.5; figure 3C). No changes from baseline were observed in DA neurons firing activity (one way ANOVA F[2,15] = 2; p = 0.2; supplementary table 1) and the

percentage of spikes occurring in burst one week after administration of L-655,708 (Kruskal-Wallis test H[2] = 0.8; p = 0.6; supplementary table 1).

To determine for how long the effects of L-655,708 on DA neurons were sustained, VTA DA neurons population activity was assessed two weeks after a single injection. The enhancement in the number of spontaneously active DA neurons produced by L-655,708 was no longer present (control: 0.9 ± 0.1 neurons/tract; L-655,708: 1.1 ± 0.2 neurons/tract; two-tailed t-test, t[10] = 1; p = 0.3; supplementary table 1; figure 3A). Firing and burst activity were unchanged 2 weeks following a single injection of L655,708.

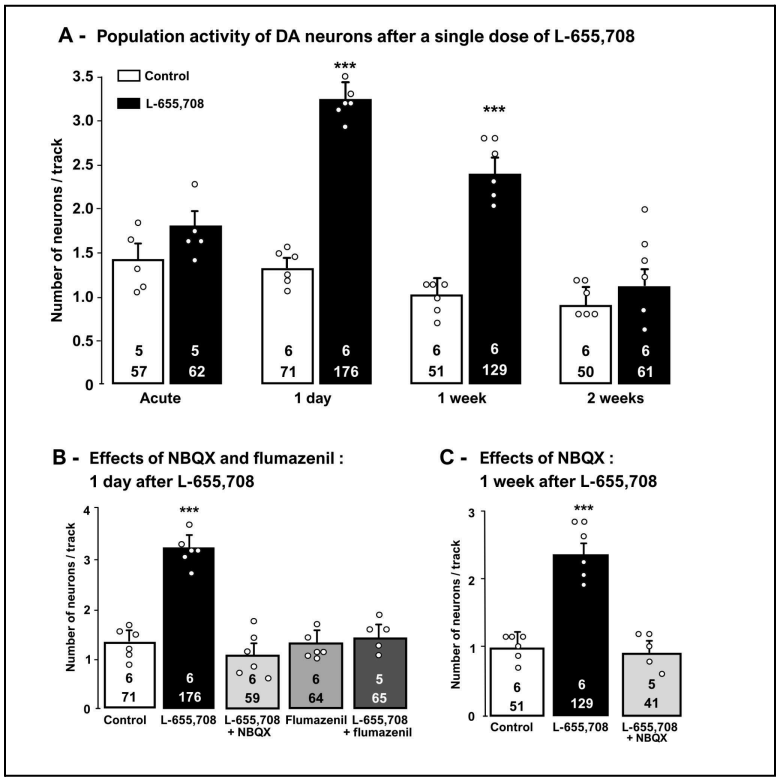

Figure 3: Effects of a single dose of L-655,708 (3 mg/kg, i.p.) on VTA DA neurons, 30 minutes, one day, one week and two weeks following its administration **(A)**. Histograms show data as mean values ± S.E.M. Each dot is from one rat and represents the average of number of DA neurons found per track. **(B)** effects of flumazenil (3 mg/kg, i.p) and NBQX (10 mg/kg, i.p) on increase in the number of DA neurons found per track, that was induced one-day following

injection of L-655,708. **(C)** Reversal by a pre-treatment with NBQX of the effects of L-655,708 on population activity assessed one week following its administration. Histograms show data as mean values ± S.E.M. Statistical significance is indicated where it applies. ***$p < 0.001$ when compared to control.

Effects of a single administration of L-655,708 on the activity of DRN 5-HT neurons

When injected 30 minutes prior to electrophysiological recordings, a single dose of L-655,708 did not significantly change the firing activity of 5-HT neurons during the 2 hour recording session (Mann Whitney test $U = 7$; $p = 0.2$; Figure 4A). Additionally, there was no alteration in the number of neurons firing in bursts (two-tailed t-test, $t[10] = 1$; $p = 0.3$; supplementary table 1). Similarly, one day after its administration, L-655,708 did not induce any significant changes in the firing rate (two-tailed t-test, $t[11] = 1.4$; $p = 0.2$; Figure 4B) and burst activity (two-tailed t-test, $t[11] = 0.4$; $p = 0.7$; supplementary table 1).

Effects of a single administration of L-655,708 on the activity of LC NE neurons

As depicted in figure 4C, there were no significant changes in NE neurons firing activity (two-tailed t-test, $t[12] = 0.2$; $p = 0.8$; figure 4C) and the number of NE neurons with burst activity (two-tailed t-test, $t[12] = -0.4$; $p = 0.7$; supplementary table 1), 30 minutes after the administration of L-655,708, for up to 2 hours. Additionally, no alterations in neuronal firing activity were observed one day post administration (two-tailed t-test, $t[10] = 0.07$; $p = 0.9$; figure 4D), or in the bursting activity of neurons (two-tailed t-test, $t[10] = 0.2$; $p = 0.9$; supplementary table 1).

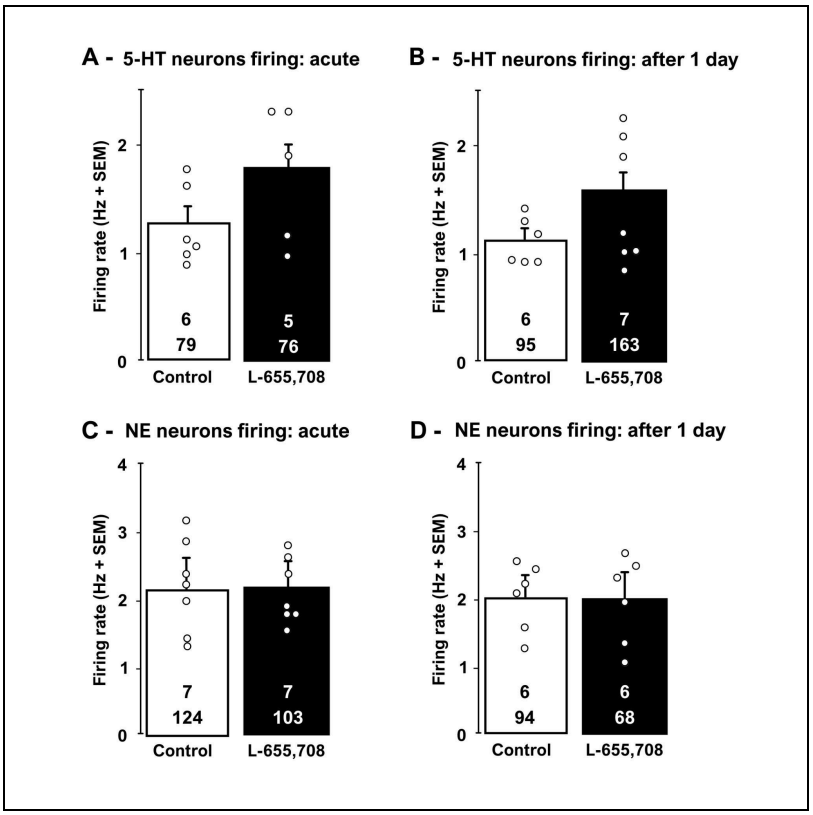

Figure 4: Effects of a single dose of L-655,708 (3 mg/kg, i.p) on firing activity of DRN 5-HT **(A, B)** and LC NE neurons **(C, D)**, 30 minutes and one day following its injection. Histograms show data as mean values ± S.E.M. Each dot is from one rat and represents the mean of the firing rate of 5-HT and NE neurons. Number of rats and neurons are indicated at the bottom of the histogram.

Discussion

The present study documented that L-655,708 significantly reduced NMDA but not AMPA receptor responsiveness in the hippocampus. Furthermore, a striking two-fold enhancement in VTA DA neuron population activity was observed one day following a single injection, which persisted for up to one week before returning to baseline by week two. The reversal of these effects by flumazenil and NBQX confirmed the involvement of the benzodiazepine site of α_5-$GABA_A$Rs and an AMPA receptor-dependent mechanism, respectively.

Notably, either acutely or one day post administration, L-655,708 had no effect on the firing activity of 5-HT and NE neurons, nor glutamate neurons in the mPFC.

α_5-subunit-containing $GABA_A$ receptors, the primary target of L-655,708, are highly expressed in the hippocampus and moderately in the frontal cortex (Fritschy and Mohler, 1995; Sur et al., 1999; Sotiriou et al., 2005). Indeed, local application of L-655,708 in the ventral hippocampus decreased immobility and increased climbing in the FST (Bugay et al., 2020), while these effects were blocked when lidocaine was directly injected into the hippocampus (Carreno et al., 2017). Together, this suggests that the antidepressant-like effects of L-655,708 originate in the hippocampus. While NMDA receptor responsiveness was reduced, AMPA activity remained unchanged in the present study, previous work in animal models of depression reported that L-655,708 significantly increased the hippocampal AMPA/NMDA ratio—a key measure of synaptic strength (Fischell et al., 2023). In the same model, L-655,708 rapidly restored weakened AMPA (GluA1)-mediated excitatory transmission, an effect associated with the reversal of depressive-like behavioural phenotypes (Fischell et al., 2015). Despite these synaptic changes in the hippocampal-mPFC pathway, L-655,708 did not alter the firing activity of pyramidal neurons in the mPFC one day post-administration (Bugay et al., 2020). Given that modulation of α_5-$GABA_A$Rs is known to relieve inhibitory control over mPFC pyramidal neurons (Sur et al., 1999), an increase in firing activity might have been expected. Contrarily, the direct disinhibition of GABAergic interneurons by the enantiomer S-ketamine has been shown to enhance pyramidal firing, an effect not observed with either ketamine or L-655,708 (Blier and El Mansari, 2025). The mechanisms underlying these differential effects on mPFC pyramidal neuron activity remain to be elucidated. Nonetheless, L-655,708 elevated levels of the AMPA receptor GluA1 subunit in mPFC synaptosomal fractions and altered the AMPA-to-NMDA ratio

at temporo-ammonic–CA1 synapses, suggesting the induction of glutamatergic plasticity within the mPFC, possibly originating from hippocampal inputs (Bugay et al., 2020; Troppoli et al., 2022). The critical role of mPFC AMPA receptor signalling to the antidepressant-like effects of L-655,708 is further supported by findings that local infusion of the AMPA receptor antagonist NBQX blocked both AMPA-mediated excitatory transmission and behavioural antidepressant-like effects in the FST (Bugay et al., 2020). Similarly, inactivation of the infralimbic (IL) region of the PFC completely abolished the antidepressant and anxiolytic effects of systemic ketamine in rodents, whereas direct microinfusion of ketamine into the IL-PFC reproduced these effects and enhanced the number and function of layer V pyramidal neuron spines (Li et al., 2011; Fuchikami et al., 2015; Moda-Sava et al., 2019). Collectively, these findings highlight the central role of mPFC circuitry—particularly AMPA receptor–mediated plasticity—in both initiating and sustaining the antidepressant-like responses induced by L-655,708 and related compounds such as ketamine.

In addition to regulating mPFC activity, the ventral subregions of the hippocampus also modulate DA neurotransmission through polysynaptic circuitry (Floresco et al., 2001; Grace et al., 2007). In the VTA, it is assumed that half DA neurons are tonically inhibited through ventral hippocampus-accumbens (NAc)-ventral pallidal (VP)-VTA feedback pathway (Grace et al., 2007). Since intra-ventral hippocampus injection of L655,708 increases DA neurons population activity (Perez et al., 2023), it is reasonable to assume that the ability of L-655,708 to increase DA neuron population activity in the present study, is mediated via its induced-increase in neuron excitability in the ventral hippocampus (Bugay et al., 2020). This in turn enhanced neuronal firing in the NAc, which yielded a GABAergic inhibition of firing of the VP, relieving DA neurons from inhibition and increasing DA neuron population activity herein (Floresco and

Grace, 2003; Floresco et al., 2001). This constitutes a stable baseline level of extrasynaptic DA in projection areas, hence allowing the hippocampus to modulate novelty-gated information storage (Lisman and Grace, 2005). Remarkably, the increase in the population activity of DA neurons persisted for up to one week after a single administration of L-655,708 but returned to baseline levels two weeks after a single injection. This enhancement both after one day and one week took place through AMPA activation because their antagonism with NBQX blocked this effect. In light of these results, and although the mechanisms involved in maintaining this response remain to be elucidated, two explanations can be proposed based on the hippocampus-VTA loop (Lisman & Grace, 2005). First, an increase in the AMPA/NMDA ratio and AMPA-dependent excitatory synaptic strength in the hippocampus (Fischell et al., 2015) may underlie the sustained augmentation observed in the VTA, as this effect was abolished by local hippocampal lidocaine injection (Carreno et al., 2017). Second, the sustained response may reflect the involvement of long-term potentiation (LTP) within the VTA , as a similar and long-lasting form of synaptic plasticity induced for instance by a single cocaine injection has previously been reported in the VTA, lasting for up to five days (Ungeless et al., 2001; see also Argilli et al., 2008). The increase in DA neuron population activity, along with behavioural data showing the reversal of DA-related hedonic deficits in the SPT by L-655,708 (Fischell et al., 2015), emphasize the important role of DA modulation though AMPARs in triggering and maintaining the antidepressant response of this drug. Despite a DA transmission enhancement, L655,708 does not induce any addiction in rats, in paradigms of self-administration, which are highly predictive of drug potential for abuse (Carreno et al., 2017). Interestingly in the clinic, the enantiomer of ketamine, S-ketamine, did not yield any addictive tendencies for addiction in patients with major depressive disorder over a five-year period (Zaki et al., 2025).

Flumazenil abolished the L-655,708-induced enhancement in DA neuron population activity herein, which is in line with its blunting of improvement seen in preference for sucrose in the SPT and the female urine sniff test following injection of the NAM MRK-016 (Troppoli et al., 2022). Altogether, these data show that, in addition to allosteric modulation of the benzodiazepine site on the effect on anhedonia, it also mediates the L655,708-induced action on DA neurons population activity.

The present study showed that L-655,708 had no impact on 5-HT neuronal firing after 30 minutes and 24 hours post-administration. The FST results support these findings as L-655,708 showed no effect on 5-HT-dependent swimming at those time points. A significant increase in swimming was, however, observed one week after the direct injection of L-655,708 into the ventral hippocampus (Carreno et al., 2017; Bugay et al., 2020). Since α_5-$GABA_A$ receptors are not expressed in the DRN and LC, direct effects of L-655,708 on the firing activity of 5-HT and LC neurons can be excluded (Sur et al., 1999; Fritschy and Mohler, 1995; Corteen et al., 2011). As the hippocampus has no known projections to the DRN and LC, indirect modulation of the mPFC by L-655,708 may still influence 5-HT and NE neuronal firing through blockade of α_5-$GABA_A$Rs, although these represent only 13% of receptor in this region (Sur et al., 1999; Lee et al., 2003; Peyron et al., 1997). While stimulation of the mPFC inhibits most 5-HT neurons, up to 17% can be activated (Hajos et al., 1998; Celada et al., 2001). Although the overall mean firing activity of 5-HT neurons recorded following L-655,708 was unchanged, it is possible that its administration induced an increase in a subpopulation of neurons that can be activated by mPFC stimulation. Nevertheless, whether mPFC neurons containing α_5-$GABA_A$Rs innervate 5-HT neurons that are inhibited or activated by stimulation of PFC remains to be elucidated. Similarly, while it is unknown whether mPFC neurons containing α_5-$GABA_A$Rs innervate LC

NE neurons, the present findings show no effect of L-655,708 on the average firing rate of NE neurons. This is in line with results showing that NE-dependent climbing in FST was also unchanged 30 minutes after administration of L-655,708, although an increase in climbing behaviour was found 24 hours following the direct administration of L-655,708 into the ventral hippocampus (Bugay et al., 2020). Nonetheless, while the current study showed no change in mean firing activity of 5-HT and NE neurons, alterations in 5-HT and NE receptor functions in postsynaptic areas may contribute to the antidepressant-like effects and warrant further investigation.

While both L-655,708 and ketamine can increase DA population activity, ketamine does so acutely and its action dissipates after 24 hours, whereas the effects of L-655,708 become apparent after 24 hours. While the enhancing effect of L-655,708 is sustained for up to one week with a single injection, that of ketamine requires two-week repeated injections to produce a sustained increase, and it lasts only three days (El Iskanderani et al., 2015; Iro et al., 2021). The delay in onset of L-655,708 compared to ketamine remain to be determined. However, although rapid synthesis of hippocampal BDNF / TrkB is suggested to be involved in the rapid antidepressant response to ketamine (Autry et al., 2011; Adashi et al., 2008), these proteins did not increase in the hippocampus 30 minutes and one day after L655,708 administration (Bugay et al., 2020). It is important to note also that in the clinic, some patients respond to ketamine only after hours or days, and this is considered as rapid therapeutic response.

In summary, the present study demonstrated that a single injection of L-655,708 produced a sustained increase in the population activity of VTA DA neurons that lasted for up to one week. These results indicate that modulation of α_5-$GABA_A$-NAM could exert a rapid and sustained

antidepressant action, in part, by prolonging enhanced DA transmission through AMPA activation.

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Authorship contribution statement

MEM. and PB designed the study and wrote the protocol. AA carried out the experiments and undertook the statistical analysis. AA, MEM, and PB wrote the manuscript.

Declaration of conflicting interests

AA and MEM declare no conflict of interest. P.B. received grant funding and/or honoraria for lectures, expert testimony, and/or participation in advisory boards for Allergan, Eisai, Idorsia, Janssen, Lundbeck, Otsuka, Pfizer, Valeant, and Takeda.

	Firing Activity (Hz)		Bursting Activity (% neurons firing in burst)		Population Activity (neurons/track)		Rats (N)	Neurons (n)
	Mean	Median	Mean	Median	Mean	Median		
A. DRN								
Control (acute)	1.2 ± 0.2	1.1	24 ± 3.7	_	_	_	6	79
L-655,708 (acute)	1.8 ± 0.3	1.9	19 ± 3.3	_	_	_	5	76
Control (1 day)	1.1 ± 0.1	_	20 ± 5.7	_	_	_	6	95
L-655,708 (1 day)	1.6 ± 0.3	_	18 ± 3.8	_	_	_	7	163
B. LC								
Control (acute)	2.2 ± 0.2	_	16 ± 4.6	_	_	_	8	124
L-655,708 (acute)	2.1 ± 0.2	_	19 ± 5.8	_	_	_	7	103
Control (1 day)	2.0 ± 0.2	_	19 ± 4.4	_	_	_	6	94
L-655,708 (1 day)	2.0 ± 0.2	_	18 ± 6.5	_	_	_	6	68
C. VTA								
Control (acute)	3.7 ± 0.1	_	18 ± 3.7	_	1.4 ± 0.2	_	5	57
L-655,708 (acute)	4.1 ± 0.7	_	21 ± 6.1	_	1.9 ± 0.2	_	5	62
Control (1 day)	3.6 ± 0.1	4.5	24 ± 2.8	_	1.3 ± 0.1	_	6	71
L-655,708 (1 day)	4.5 ± 0.1	3.6	31 ± 2.6	_	3.3 ± 0.1***	_	6	176
Control (1 week)	3.7 ± 0.2	_	22 ± 7.1	_	1.0 ± 1.0	_	6	51
L-655,708 (1 week)	5.4 ± 0.6	_	28 ± 3.2	_	2.4 ± 0.2***	_	6	129
Control (2 weeks)	3.8 ± 0.5	_	33 ± 4.1	_	0.9 ± 0.1	_	6	50
L-655,708 (2 weeks)	4.7 ± 0.4	_	43 ± 6.0	_	1.1 ± 0.2	_	6	61
D. PFC								
Control (1 day)	0.8 ± 0.1	_	67 ± 4.8	_	_	_	5	75
L-655,708 (1 day)	0.9 ± 0.1	_	62 ± 4.1	_	_	_	5	79

Supplementary Table 1. Summary table of the effects of a single administration of L-655,708 (3 mg/kg, i.p.) treatment on the firing rate and bursting activity of **(A)** DRN 5-HT, **(B)** LC NE, **(C)** VTA DA and **(D)** PFC pyramidal neurons as well as the population activity of **(C)** VTA DA neurons. Data are expressed as means ± S.E.M. Median values are indicated in instances where the Mann-Whitney test was used. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

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Chapter 3: Discussion

The two primary challenges with the treatment of depression remain to be a slow rate of therapeutic onset and low response rates. While initially the discovery of SSRIs was a breakthrough in treatment for MDD for improved tolerability and safety, it did not meet criteria for both treatment challenges. As such, the advent of fast acting strategies with the introduction of ketamine changed the landscape for research in the treatment context. While ketamine proved to be successful, it is not without drawbacks and it was apparent that continued efforts for more targeted therapies is necessary. The common underlying factor for the discovery and implementation of these antidepressant therapies throughout the past few decades was the translation from preclinical discoveries to the clinical setting. Indeed, basic research is the foundation of shaping and directing clinical care for patients with MDD and the present study was conducted with the goal of broadening the understanding of α5 $GABA_A$ receptor targeting strategies so that it may lead to therapeutic application in the patient population.

The primary aim of this electrophysiological study was to examine the effects of L-655,708 on the firing activity of monoamine and glutamate neurons which are centrally involved in the antidepressant response.

In the present study, L-655,708 caused a sustained increase in the population activity of DA neurons in the VTA for up to one week after a single injection, but not acutely, and its effect disappeared after two weeks. These results align with previous findings indicating that this drug produces antidepressant-like effects in the FST at 24 hours and one week post-treatment, but not acutely (Carreno et al., 2017; Bugay et al., 2020). The reversal of these effects by the administration of flumazenil, a benzodiazepine site antagonist, and NBQX, an AMPA receptor agonist, confirms that L-655-708 acts on the benzodiazepine site on $GABA_A$Rs and that its

sustained antidepressant-like response is AMPA receptor dependant. Notably, L-655,708 had no effect on the activity of 5-HT and NE neurons either acutely or one day post administration. In addition, the population activity for these neurons was also examined, however, no changes were observed. This study also looked at the effects of L-655,708 on glutamate system modulation. Although PFC pyramidal neuronal activity remained unchanged, a significant decrease in NMDA receptor responsiveness was observed in hippocampal CA1 pyramidal neurons 24 hours post treatment, while AMPA responsiveness remained unchanged.

1. Role of Dopamine in the Antidepressant Response

Dopamine population activity is an electrophysiological measure of the number of spontaneously active DA neurons per track within the VTA (Ungless & Grace, 2012). Under basal conditions, about 50% of DA neurons in the rodent brain are held in a hyperpolarized, non-firing state due to inhibitory GABAergic inputs from the ventral pallidum (VP) to the VTA (Grace et al., 2007). Population activity is a key player in motivation, as a strong phasic response to a motivationally relevant cue is contingent on the number of spontaneously active DA neurons (Grace, 2016; Floresco et al., 2001). Hence, a decrease in DA population activity leads to a blunted dopamine response to reward stimuli, which may contribute to anhedonia and motivational deficits observed in depression.

Indeed, ketamine and α5 $GABA_AR$-NAMs have been shown to enhance dopamine transmission, though the manner in which they do so differs mechanistically. For instance, ketamine has been shown to elicit a robust increase in DA transmission in the VTA as well as in the PFC, nucleus accumbens (NAc) and striatum, likely through NMDA-blockade induced glutamate transmission (Iro et al., 2021; Cola et al., 2021). Likewise, the present study also

documented that the antidepressant-like response seen after L-655,708 administration is likely attributed, in part, to the significant increase in DA neuron population activity. This phenomenon has been shown to occur via the disinhibition of the VH and subsequent NAc-VP pathway (Bugay et al., 2020; Perez et al., 2022).

Mechanistically, L-655,708 acts selectively on α5-containing $GABA_A$ receptors heavily enriched in the CA1 and CA3 regions of the hippocampus and, to a lesser extent, in the PFC, making them an ideal target for hippocampus-specific modulation (Sur et al., 1999; Serwanski et al., 2006; Sotiriou et al., 2005). More specifically, the concentration of α5 $GABA_A$ receptors is only slightly higher in the CA1 region of the VH as compared to the dorsal hippocampus (DH) (Sotiriou et al., 2005). Nevertheless, recordings in this study were obtained from the DH as it is not possible to lower large five-barrelled micropipettes dead into the hippocampus to reach the VH. There is evidence suggesting that the activation of the CA1 region of the VH can directly increase VTA DA population activity. By inhibiting α5-$GABA_A$ receptors on CA1 neurons in the hippocampus, L-655,708 would effectively reduce inhibitory tone. Patch-clamp studies confirmed that one day after administration, CA1 pyramidal neurons in the VH have higher input resistance and fire more readily, indicating sustained disinhibition and elevated excitability (Bugay et al., 2020; Perez et al., 2020). This disinhibition triggers a cascade through hippocampus neuronal circuits. Increased hippocampus activity stimulates glutamatergic afferents to the NAc, which in turn activate inhibitory GABAergic projections to the VP (Bugay et al., 2020; Perez et al., 2022). This reduces VP-mediated inhibition of VTA DA neurons, effectively releasing them from their hyperpolarized state and enabling spontaneous firing, leading to an increase in population activity (Perez et al., 2022; Ungless & Grace, 2012). Inactivation of the VH with lidocaine effectively blocks this circuit and the behavioural

antidepressant-like effects of L-655,708 (Carreno et al., 2017), while an injection of L-655,708 directly into the VH has been shown to produce a subsequent antidepressant-like effect (Bugay et al., 2020; Perez et al., 2022), further confirming that the actions of L-655,708 are dependent on hippocampal excitation.

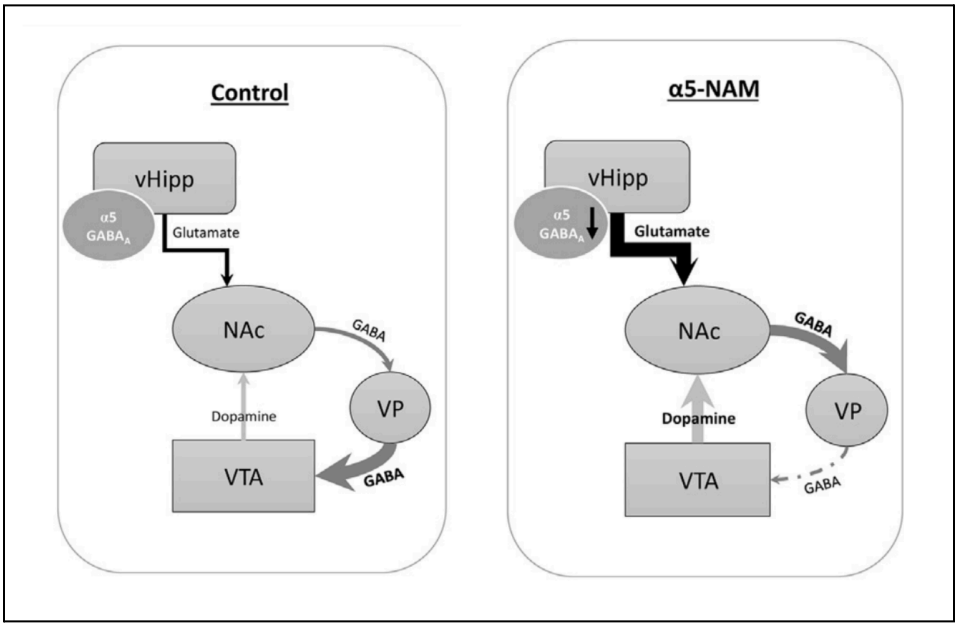

Figure 4: Depiction of the hippocampal NAc-VP pathway under normal conditions and in the case of NAMs of α5 $GABA_A$ receptors. Adapted from Perez et al. 2022.

2. AMPA-NMDA Ratio

In contrast to α5 $GABA_A$ receptor concentrations, the density of AMPA and NMDA receptors is higher in the CA1 region of the DH as compared to the VH (Pandis et al., 2006). As such, we aimed to examine the effects of L-655,708 on receptor responsiveness in the DH. Importantly, hippocampal disinhibition and elevated neuronal excitability in this region are associated with a shift in AMPA to NMDA receptor activity (Li et al., 2004; Andreasen et al., 2013).

AMPA receptor activity is at the heart of the antidepressant response evoked by therapeutic interventions. AMPA is a necessary component required to induce the glutamate surge associated with antidepressant efficacy (Moghaddam et al., 1997). Importantly, hippocampal disinhibition and elevated neuronal excitability are associated with a shift in AMPA to NMDA receptor activity, a well-established correlate of synaptic strength, plasticity and glutamate transmission (Li et al., 2004; Andreasen et al., 2013; Du et al., 2006). NMDA receptors have been shown to increase excitatory postsynaptic potentials in GABA interneurons throughout the brain, however, NMDA receptor antagonists, such as ketamine, increase the excitation of pyramidal neurons at a delayed rate by decreasing the activity of GABA interneurons (Homayoun & Moghaddam, 2007).

In preclinical models of chronic stress, the AMPA-NMDA ratio is reduced, reflecting weakened excitatory transmission. Both L-655,708 and a related α5 $GABA_AR$-NAM, MRK-016, have been shown to restore the AMPA-NMDA balance at hippocampal CA1 synapses (Fischell et al., 2015; Troppoli et al., 2022). The present study supports these findings by showing a decrease in NMDA receptor responsiveness in CA1 hippocampal neurons in the presence of unchanged AMPA receptor activity following L-655,708 administration. This results in a net shift towards an overall increase in AMPA receptor activity, indicating improved synaptic strength and enhanced glutamate transmission, both outcomes consistent with an antidepressant-like response (Fischell et al., 2015). These findings have been confirmed by a previous study which demonstrated a decrease in NMDA receptor subunit expression in the hippocampus after L-655,708 administration, without altering AMPA subunit levels (Nuwer et al., 2021).

Interestingly, this mechanism is not unique to L-655,708. Ketamine and SSRIs have also been shown to elevate the AMPA-NMDA ratio, though through distinct pathways. Ketamine shifts the balance towards enhanced AMPA-mediated transmission by directly blocking NMDA receptors, while chronic SSRI treatment promotes AMPA receptor upregulation and NMDA downregulation over time (Tizabi et al., 2012; Andreasen et al., 2013). These converging effects on glutamate transmission and plasticity highlight a shared downstream mechanism of action among diverse classes of medications used to treat depression.

3. $GABA_A$ Receptors as a Common Target for Antidepressant Efficacy

Preclinical testing has supported the notion of $GABA_A$ receptors as a successful target for therapeutic strategies in the treatment of depression. While the present study and other previous work shows that α5 $GABA_A$ receptors can directly modulate $GABA_A$ receptor activity, ketamine and SSRIs have also been shown to affect GABA transmission through this receptor. SSRIs primarily exert their effects by inhibiting the SERT and increasing extracellular 5-HT, however, they may also have an effect on GABA neurotransmission. In particular, the SSRI fluoxetine has been shown to increase $GABA_A$ receptor activity by acting as a positive allosteric modulator through a novel modulatory site located on the α subunit of the GABA receptor complex (Robinson et al., 2003). The SSRI fluoxetine also increases sensitivity of $GABA_A$ receptors to low concentrations of GABA neurotransmitters, which are seen in patients with MDD (Robinson et al., 2003).

While ketamine primarily antagonizes NMDA receptors on GABA interneurons in the brain, it has also been shown to have peripheral impacts on $GABA_A$ receptors. For instance, chronic ketamine administration upregulates α5 $GABA_A$ receptor subunit expression in the PFC (Tan et al., 2011). As well, ketamine has been shown to increase tonic inhibitory currents that are

mediated by $GABA_A$ receptors in hippocampal neurons (Wang et al., 2017). This signifies that ketamine can potentially regulate excitatory neuronal activity through the modulation of $GABA_A$ receptors. Both the upregulation of $GABA_A$ receptor activity as seen with NAMs, such as L-655,708, and the downregulation of these receptors as seen with fluoxetine and ketamine is essential in maintaining the delicate excitatory-inhibitory (E:I) balance in the brain that is contributing to the antidepressant-like effects of these varying therapeutic strategies.

4. Limitations

The present study had a few limitations. Firstly, extracellular recordings were conducted under 400 mg/kg of chloral hydrate anesthesia in both control and vehicle rats to ensure similar baseline conditions in both groups. As such, it can be assumed that any difference between these two groups would be attributable to effects induced by L-655,708. Chloral hydrate, through its metabolite trichloroethanol, enhances $GABA_A$-mediated neurotransmission, which is responsible for its anesthetic effects (Lovinger et al., 1993). In addition, its potency to enhance this GABA effect appears to be greater than to depress excitatory transmission (Lovinger et al., 1993). Moreover, chloral hydrate inhibits the AMPA response by 50% (Fischer et al., 2000). On the other hand, L-655,708 reduces the activity of the $GABA_A$ receptor through negative allosteric modulation, which results in an increase in excitatory transmission and the AMPA-NMDA ratio. Therefore, it is possible that effects obtained after L-655,708 administration herein are even more likely to be under-estimated because of the opposing action of chloral hydrate on the impact of L-655,708.

In addition, this study does not use an animal model of depression and experiments were conducted in healthy rats. This is primarily because this study set out to examine the impact of L-655,708 on monoamine and glutamate circuitry independent of circuitry downregulation often

observed in animal models of depression. Further, previous behavioural studies have confirmed the antidepressant-like effects of L-655,708 in chronic stress rodent model (Carreno et al., 2017; Troppoli et al., 2022).

5. Concluding Remarks

MDD is expected to be the leading cause of disease burden by 2030 (Li et al., 2021). This high prevalence and profound impacts on both health and social systems highlight the need for more selective and fast-acting drugs for the treatment of depression. The experience of adverse side effects and lack of perceived efficacy surrounding therapeutics to treat depression is one of the leading causes of medication noncompliance and relapse in mental health disorders, including MDD which ultimately worsens clinical and economic outcomes for patients (Ho et al., 2016).

Traditional medications, such as SSRIs, are limited due to their delayed onset, modest efficacy, and undesirable side effects. Even rapid-acting treatments such as ketamine, though promising, possess a broad side effect profile related to dissociation, abuse potential, and vast receptor targets. Against this backdrop, the search for more targeted and selective interventions has become imperative. The shift away from a monoamine-centric framework of depression toward a broader view that includes GABA-glutamate dynamics and DA circuitry reflects the nature of MDD as a multisystemic disorder. The ability of the NAM L-655,708 to selectively target α5 $GABA_A$ receptors—highly localized in hippocampal and prefrontal circuits involved in depression—highlights the therapeutic value of precise neural targeting to minimize off-target effects and maximize efficacy.

By advancing the understanding of the mechanisms behind the antidepressant-like efficacy of α5 $GABA_A$R NAMs, such as L-655,708, on the monoamine and glutamate systems

of the brain, the present study offers a promising preclinical framework that can be used to inform the application of these selective, fast-acting strategies in the clinical setting. This will fundamentally work to improve outcomes for patients with MDD.

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