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Testing the efficacy of LSN2463359, a metabotropic glutamate 5 receptor positive allosteric modulator, in animal models of schizophrenia

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Testing the Efficacy of LSN2463359, a Metabotropic Glutamate 5 Receptor Positive Allosteric Modulator, in Animal Models of Schizophrenia

By

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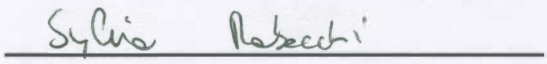

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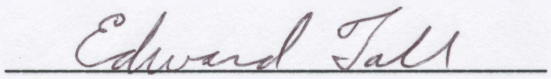

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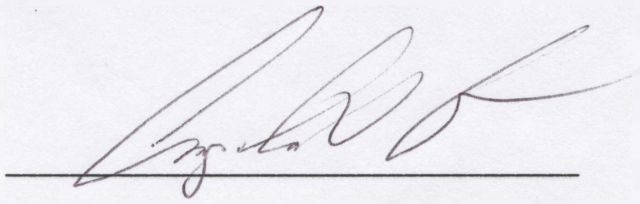

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First and foremost I would like to thank God. Without His Grace and Mercy none of my accomplishments would have been possible.

I would like to offer my sincere thanks to my mentor Dr. Rabacchi. Her thoughtful and encouraging advice was of great benefit to me throughout this process.

I would like to thank Dr. Tall, Dr. Klaus and the entire biology department for their support throughout my career here.

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ABSTRACT

For many years the dominant theory surrounding the cause of schizophrenia was focused on elevated dopamine levels found in critical areas of the brain. Recently a new theory has emerged pointing to elevated glutamate levels resulting from hypofunction of NMDA receptors and hypoactivity of GABAergic neurons which normally inhibit glutamatergic cells in a tonic manner. Therefore, while traditional antipsychotics directly block dopamine receptors, some of the newly generated compounds are designed to modulate glutamate to normal levels.

I am proposed testing the efficacy of the metabotropic glutamate 5 receptor modulator LSN2463359, previously shown to act as an indirect agonist of the NMDA receptor, in two different animal models of schizophrenia. Our study found that when LSN2463359 was administered to rats given SDZ 220-581 (an NMDA antagonist) levels of GABA, glutamate, dopamine and NAA (a marker for cell health) were modulated to normal levels. Furthermore, when the compound was administered to neuregulin-1 knockout mice, behaviors related to anxiety and social activity were also modulated to normal levels. Developing novel therapies targeting a different pathway involved with psychosis may help reduce side effects and may be beneficial in relieving symptoms not currently well treated by traditional antipsychotics.

Introduction

Schizophrenia is a debilitating, severe mental illness. It is characterized by positive and negative symptoms as well as cognitive deficits (Van Os, 2009). Positive symptoms include lack of insight, hallucinations, delusions and thought disorder. Negative symptoms include social withdrawal, self-neglect, loss of motivation, emotional blunting and paucity of speech. Cognitive deficits are found in the areas of working memory, attention, verbal learning and executive function (Van Os, 2009). There are many risk factors for acquiring schizophrenia. Risk factors include: living in an urban environment, using cannabis, immigration and genetic inheritance. Schizophrenia affects 1-2% of the population (Picchioni, 2007). Not only do individuals and families suffer from this illness, but society also suffers as the schizophrenic is unable to make a contribution and may require constant care from the mental health system.

There are two biological hallmarks of the schizophrenic brain. One hallmark is smaller brain volume and larger lateral ventricles. Another hallmark is increased dopamine synthesis, release and resting state concentrations. It has been theorized that abundance of dopamine causes an experience of salience or deep personal meaning to mundane events. The schizophrenic may create delusions and paranoid thoughts to make sense of the experience of salience (Picchioni, 2007).

Amphetamines are known to increase dopamine levels in the brain. Amphetamine misuse can lead to schizophrenic symptoms in healthy people (Bell, 1973). Antipsychotic medications which block dopamine receptors have proven very helpful to the schizophrenic community. Unfortunately dopaminergic anti-psychotics work well in patients with mostly positive symptoms rather than negative symptoms. They also have little impact on cognitive deficits. Dopaminergic antipsychotics also have harmful side effects such as increased obesity, diabetes and ataxia.

For many years the theory that increased dopamine in certain regions of the brain causes schizophrenic symptoms was the prevailing one (Howes, 2009). New theories have emerged. One theory purported that an increase in glutamate transmission in key areas of the brain, induce dopamine increases and cause over-excitation (Javitt, 2010; Moghaddam, 2012). It is well known that phencyclidine or PCP induces psychosis in humans and animals. PCP works as an N-methyl-D-aspartate (NMDA) receptor antagonist. Hypofunction of NMDA receptors leads to increased glutamate neurotransmission. It is theorized that cells with NMDA receptors excite inhibitory GABAergic neurons and hypofunction of NMDA receptors leads to decreased GABA activity and overexcitement of pyramidal cells inhibited in a tonic manner by GABAergic neurons (Homayoun, 2007; Matosin, 2013; Cohen, 2015). It is also theorized that mutations in genes related to GABA receptor activity could also play a part in excess glutamate activity in the brain (Taylor, 2015). The emerging theories

were that imbalanced glutamate neurotransmission may play a role in the development of schizophrenia. Figure 1 shows the effect hypofunction of NMDA bearing cells has on GABAergic and pyramidal cell firing. Pyramidal cells are glutamatergic.

Metabotropic glutamate 2/3 receptors (mGlu 2/3 receptors) are involved in glutamate transmission. These receptors inhibit glutamate release from the presynaptic knob. mGlu 2/3 receptors are high in density in the forebrain. Many studies have been performed which discuss the effects of mGlu 2/3 receptor agonists on schizophrenic symptoms in mice and humans. In some studies mGlu 2/3 receptor agonists have reduced psychotic symptoms in mice and humans (Patil, 2007).

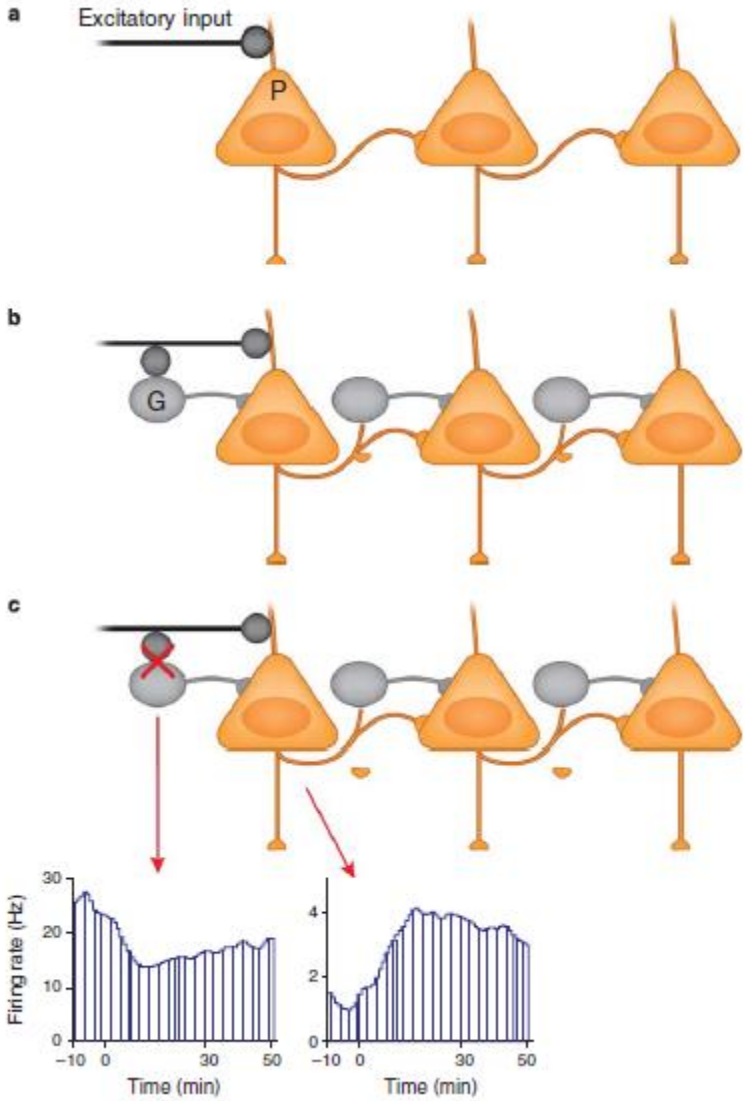


Figure 1. Schematic of the effects that hypofunction of NMDA cells exert on GABAergic (G) and Pyramidal cells (P). a. This figure shows the relationship between NMDA bearing cells and Pyramidal cells. b. This figure elucidates how GABAergic cells are also affected by NMDA bearing cells and how they exert an effect on Pyramidal cells. c. This figure shows what effect hypofunction of NMDA bearing cells have on GABAergic and Pyramidal cells. The graphs depict GABAergic cells' firing rate and Pyramidal cells' firing rate after NMDA bearing cells stop activity at time = 0. Hypofunction of NMDA bearing cells leads to hyperactivity of Pyramidal cells via disinhibition of GABAergic cells. (Moghaddam, 2012)

Metabotropic glutamate 2/3 receptor agonists in a phase II trial were found not

to be as effective as traditional antipsychotics (Kinon, 2011). A new way of altering glutamate transmission is therefore needed. Currently metabotropic glutamate 5 receptor's positive allosteric modulators (mGluR5 PAMs) are under study. Allosteric modulators are substances that indirectly influence the effects of an agonist at a target protein. Allosteric modulators bind to a site distinct from the agonist binding site and produce a conformational change in the protein. Allosteric modulators can be positive or negative. Metabotropic glutamate 5 receptors (mGluR5s) are found post-synaptically in high density in the cerebral cortex, the hippocampus, nucleus accumbens, hypothalamus and some portions of the amygdala. mGluR5s are associated with NMDA receptors in these key areas of the brain which are associated with malfunction in schizophrenia (Matosin, 2013). Not only do these receptors alter glutamate transmission, they also regulate NMDA receptors as well. mGluR5 activity leads to a cascade of intracellular signaling that potentiates or strengthens NMDA receptor activity as well as increases intracellular calcium levels leading to increased excitation from NMDA bearing cells (Matosin, 2013). mGluR5s potentiate NMDA receptors by stimulating protein kinase C which activates cell adhesion kinase β and proline rich tyrosine kinase. These phosphorylate the Src protein, which in turn directly potentiates the NMDA receptor (Matosin, 2013). Direct agonists of mGluR5s have lead to seizures and cell death, yet allosteric modulators have had a less severe effect. Research has been underway on the effect these modulators have on psychosis.

The chemical compound LSN2463359 (LSN) is an mGluR5 positive allosteric

modulator. It increases mGluR5 activity and potentiates NMDA activity indirectly (Matosin, 2013). It is well tolerated orally. We tested LSN to see its effects on a cellular and behavioral level. Finding a compound which acts differently from traditional antipsychotics is helpful to further understand and treat schizophrenic disorders.

In assessing the viability of brain cells as an indicator of a compounds efficacy there are numerous proteins/neurotransmitters one can study. For the purpose of this study, I concentrated on N-acetyl aspartate (NAA) as a marker of healthy activity in the brain. High NAA levels indicate healthy brain cells (Paslakis, 2014; Arun, 2008). Low NAA levels are linked with brain damage either by disease (such as Alzhiemer's) or by traumatic brain injury.

NAA is the second most concentrated molecule in the brain after glutamate. It is detected in the nervous system only. It has many functions, some unknown. It is believed to be involved in fluid balance as an osmolyte. It is also believed to be a source of acetate for lipid and myelin synthesis. Low levels of NAA indicate loss or damage to neuronal tissue (Tanaka, 2006; van Os, 2009; Paslakis, 2014). NAA levels in schizophrenics are markedly reduced in the frontal lobes as well as in the thalamus, basal ganglia and hippocampus (He, 2012)

One way to assess NAA levels *in vivo* or *ex vivo* is to use nuclear magnetic resonance spectroscopy (NMRS) (Jacobus, 2006). The spectroscope acts as an MRI except it records relative concentrations of brain metabolites instead of 2

dimensional images. Common biochemicals measured by the NMRS include: choline, creatine, glutamate, GABA, inositol, glucose, NAA, alanine and lactate. NAA levels can be detected by NMRS *in vivo* in humans and *ex vivo* in rats that are sacrificed and receive biopsies (Bustillo, 2012).

There are many NMDA antagonists that can be used to create a psychotic model in rats. PCP and MK-801 are most commonly used. They are non-competitive ion channel blockers. For the purposes of this study, I used the compound SDZ 220-581 (SDZ). Its chemical name is (S)-α-amino-2-chloro-5(phosphonomethyl)[1,1-biphenyl]-3-propanoic acid. It binds the NMDA receptor competitively at a site distinct from the ligand binding region. SDZ's antagonism has been shown to be more effectively altered by increased mGluR5 activity than non-competitive NMDA antagonists (Gastambide, 2013). Since SDZ has a different pharmacological profile from PCP or MK-801, it may be a better substrate to use in conjunction with LSN.

Another biological factor involved in schizophrenia is genetic inheritance. There are numerous genes linked to the development of schizophrenia. The eight most commonly linked to schizophrenia are: Neuregulin-1, Dystrobrevin-binding protein 1, D-amino acid oxidase activator, Catechol-O-methyltransferase, Disrupted in schizophrenia-1, 32 KDa dopamine and cAMP regulated phoshphoprotein, Regulator of G-protein signaling 4, and Metabotropic glutamate receptor-3. (Luo, 2014; Riley, 2009; Tan, 2014; Pelka-Wysiecka, 2013; Takahashi, 2015; Hu, 2007; Levitt, 2006 and Shibata, 2009) Depending on which gene is mutated determines how severe the

illness is and what symptomology appears. Neuregulin-1 codes for a growth factor that stimulates neuron development and differentiation. Dystrobrevin-binding protein 1 is involved in biogenesis of lysosome-related organelles. D-amino acid oxidase activator reduces NMDA receptor functioning. Catechol-O-methyltransferase degrades dopamine among other activities. Disrupted in schizophrenia 1 influences neuronal development and adult brain function. 32KDa dopamine and cAMP regulated phoshphoprotein inhibits protein phosphatase 1 and protein kinase A. Regulator of G-protein signaling 4 is involved in neuronal differentiation. Metabotropic glutamate receptor 3 modulates serotonin and dopamine transmission.

There is no one gene mutation ubiquitous to all schizophrenic patients. For the purposes of this study, I focused on neuregulin-1. Neuregulin-1 knockout mice exhibit a behavioral profile similar to that of psychotic mice (Law, 2014; Duffy, 2010; O'Tuathaigh, 2010). Neuregulins are intercellular signaling protein members of the epidermal growth factor class of neurotrophins. Neuregulin-1 plays a role in glutamatergic signaling by regulating the NMDA receptor. It also is thought to be involved in regulating synaptic plasticity (how the brain adapts to the environment and how cells adapt in strength of synaptic activity). Preventing Neuregulin-1 receptor signaling has lead to loss of NMDA activity and decreased dendritic spine numbers (Li, 2007). Since glutamatergic pathways are changed in mice with a neuregulin-1 knockout, it would be useful to see how an mGluR5 modulator would influence mice with a neuregulin-1 knockout. Mice with a neuregulin-1 knockout also exhibited a

higher sensitivity to NMDA antagonists (O'Tuathaigh, 2010). Testing the efficacy of LSN in knockout mice shows how well the mGluR5 PAM works in a system genetically predisposed to NMDA hypofunction.

Proposal

I propose to test whether LSN2463359, a metabotropic glutamate 5 receptor positive allosteric modulator, would be beneficial in increasing nerve cell viability, normalizing GABA, glutamate and dopamine levels, as well as in ameliorating psychotic behavioral readouts in schizophrenia models. Besides providing efficacy data on LSN that may help develop new treatment for schizophrenia, our study may also shed light into the validity of the new glutamatergic theory of psychosis. I propose to conduct the following studies on the SDZ-treated rat as well as on the Neuregulin 1 knockout mouse, both well-established schizophrenia models:

1. Study the effect of LSN on levels of NAA, GABA, glutamate and dopamine in the medial frontal cortex of rats after SDZ administration. This would be accomplished by nuclear magnetic resonance spectroscopy and high performance liquid chromatography. I would expect some normalization of the NAA, GABA, glutamate and dopamine levels in a dose dependent manner.
2. Study the effect of LSN on the behavior of wild type and neuregulin-1

knockout mice. Two behavioral tests commonly found altered in animal models of schizophrenia will be analyzed, including fear conditioning and social anogenital sniffing behavior. I would expect psychotic behavior to improve in a dose dependent manner.

Materials and Methods

Animals

All experiments were approved by the Institutional Animal Care and Use committee of the University Health Sciences Center and were performed according to the guidelines of the NIH.

Male Sprague-Dawley rats weighing about 300 g each were purchased from Harlan (Indianapolis, IN). They were housed in pairs and allowed to acclimatize for 2 weeks prior to the start of the study. They were kept under a 12:12 light: dark cycle and received food and water *ad libitum* (Bustillo, 2012)

Heterozygous Neuregulin 1 'knockout' mice were generated at the Victor Chang Cardiac Institute, University of New South Wales Australia. Heterozygous mutants and wildtype mice were generated from heterozygous breeding pairs and the offspring were genotyped using polymerase chain reaction. Mice were housed in groups of three to five per cage and maintained on a standard 12:12 hour light: dark cycle with *ad libitum* access to food and water (O'Tuathaigh 2010).

Drugs

SDZ was formulated in 5% (w/v) glucose solution and administered via the subcutaneous route at a dose of 1mg/kg. pH was adjusted towards neutral (Gastambide, 2013).

LSN2463359 was formulated as a suspension in a 1% (w/v)

carboxymethycellulose, 0.25% Tween 80, 0.05% antifoam vehicle and administered orally at a volume of varying doses (Gastambide, 2013).

All solutions were prepared freshly each day.

Rat brain biopsy and HR-MAS ^{1}H-MRS

The study includes the treatment of 40 rats with 1 mg/kg of SDZ 220-581 (an NMDA antagonist) suspension subcutaneously and 10 rats with vehicle for seven days. The LSN compound being tested was administered orally as a suspension once a day for two days following the SDZ treatment (1 mg/kg, 10 mg/kg and 30 mg/kg), (10 rats/group) and 10 rats will receive 5 mL of the vehicle. (Gilmour, 2012)

One day after the last injection of LSN or vehicle, rats were exposed briefly to isoflurane then sacrificed by decapitation and the medial frontal cortex was dissected for quantification of NAA, GABA and glutamate by magnetic resonance spectroscopy. Brains were rapidly removed, placed into rat brain matrix (Kent Scientific Corporation) and a 2mm coronal slices was obtained on an ice-chilled stage. One slice was selected which corresponded to the following sections of a standard rat brain atlas (+ or – refers to anterior or posterior from the Bregma): *slice 1*, +3.7 to +1.7mm. This slice contains the medial frontal cortex. Two mm circular punches were obtained from the appropriate region and immediately placed in pre-cooled plastic centrifuge tubes, frozen on solid CO_2, and then stored

at -80˚ C until HR-MAS ^{1}H-MRS analysis.

Frozen intact tissues samples were placed directly into a Bruker zirconium rotor containing 5 µL buffer. The rotor was placed into a Bruker magic angle spinning probe maintained at 4˚C in a vertical wide-bore Bruker 11.7 T magnet with an AVANCE^{TM} DRX-500 spectrometer. Rotors were spun at 4.2 $\pm$ 0.002 kHz at 54.7˚ relative to the static magnetic field B_0.

Spectra were analyzed by an operator blind to drug treatment using a custom LCModel utilizing a linear combination of 27 individual neurochemical model spectra as well as non-specific lipid signals to fit the tissue spectrum and calculate absolute concentration values for neurochemicals with signals between 1.0-4.2 ppm (Bustillo, 2012).

Microdialysis experiments

The effects of SDZ treatment on dopamine levels in the medial prefrontal cortex were measured by *in vivo* microdialysis. After 7 days of treatment with SDZ or vehicle (30 rats were given SDZ 10 were given the vehicle) one concentric dialysis probe equipped with a Cuprophan membrane (2-mm long) was implanted in the medial prefrontal cortex at coordinates (in mm): AP +2.2; L-0.2: DV-3.4 of anaesthetized mice. Microdialysis experiments were performed 20-24 hours after surgery. The aCSF (artificial cerebrospinal fluid) containing 10 µL/min (WPI model sp220i) and dialysates were collected every 20 minutes. Brain dialysates were

collected in micro vials containing 5 µL of 10 mM perchloric acid and dopamine concentration in dialyzed samples were analyzed by HPLC with amperometric detection (Hewlett Packard 1049) at +0.6 V. Baseline samples were collected before LSN treatment. LSN was administered in doses of 1 mg/kg, 10 mg/kg and 30 mg/kg to three groups of 10 rats for 2 days. After LSN treatment successive dialysate samples were collected. Baseline dopamine levels were calculated as the average of the ten predrug samples. At the end of sample collection, brains were removed and sectioned to ensure proper probe placement (Zuo, 2012).

Fear conditioning test

Varying doses of LSN (1 mg/kg, 10 mg/kg, 30 mg/kg) were administered to groups of ten neuregulin-1 knockout mice. The vehicle was administered to ten neuregulin-1 knockout mice. We also administered the vehicle and varying doses of LSN to 4 groups of 10 wild type mice. After the last administration of LSN we performed a fear conditioning test on the treated mice.

After a 2 minute habituation period a tone was presented to the mouse at a level of 80 dB for 15 seconds. A mild foot shock was administered during the last 2 seconds of the tone and co-terminates with the tone. The foot shock was 0.6 mA. After an inter-trial interval of 3 minutes a second identical trial was administered. After another interval a third trial was administered.

At the same time of day the next day of testing occurs. After a 3 minute

habituation period the same tone cue was presented for 3 minutes. A Kinder Scientific Motor Monitor was used to detect the percentage of time the mouse remains frozen (Duffy, 2010).

Anogenital sniffing test

We treated 40 neuregulin-1 knockout mice and 40 wild type mice with the same preparation protocol as that of the fear conditioning test mice and then observed anogenital sniffing in the treated mice.

Each test mouse was paired individually with an unfamiliar age-, weight- and sex-matched stranger mouse in a clear Perspex chamber. Clean bedding material was placed on the chamber floor prior to each test and the chamber floor and walls were cleaned with ethanol wipes between each test. The test mouse and the stranger were placed in the chamber simultaneously and this placement defined the start of the trial. For each animal, the trial duration was 10 minutes. Using a digital camcorder mounted above the chamber, anogenital sniffing behavior was coded using OBSERVER® video analysis software. The experimenter was blind to the genotype and treatment condition at the time of testing and coding of behavior (O'Tuathaigh, 2010).

Data analysis

Comparisons of neurochemical measures were carried out using paired *t*-

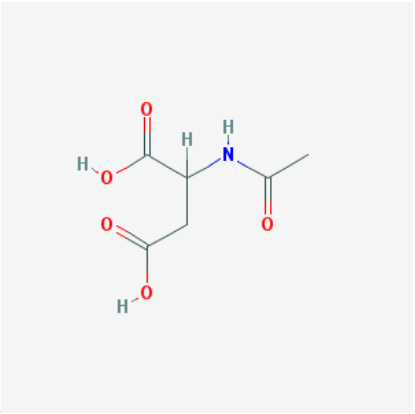
tests. LSN2463359 treated rats' neurochemical makeup was compared to untreated rats given SDZ.

Comparisons of behaviors were carried out using paired *t*-tests. Mean values were compared between LSN2463359 treated mice and vehicle treated mice.

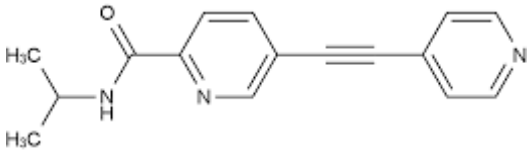
Expected Results

Section I. Determine if LSN 2463359 normalizes GABA, glutamate, dopamine and NAA levels in the medial frontal cortex of rats given SDZ.

The medial frontal cortex in rats is comparable to the prefrontal cortex in humans. This area of the brain is most heavily studied in determining the effects of psychosis or psychotic medication. Using magnetic resonance spectroscopy and high performance liquid chromatography, I determined the levels of GABA, glutamate and dopamine in the rats and assessed whether they are in accordance with the new glutamatergic theory. In addition, NAA measurements have provided useful information as to the neuroprotective actions of LSN. The chemical structure of NAA and LSN are shown below in Figure 2.



A.



B.

Figure 2. A: The chemical structure of N-acetylaspartate. B: The chemical structure of LSN2463359. *N*-(1-methylethyl)-5[2-(4-pyridinyl)ethnyl]-2-pyridinecarboximide.

The timeline for testing the neurochemical makeup via NMRS of the medial frontal cortex in rats given SDZ or the vehicle is illustrated in Figure 3.

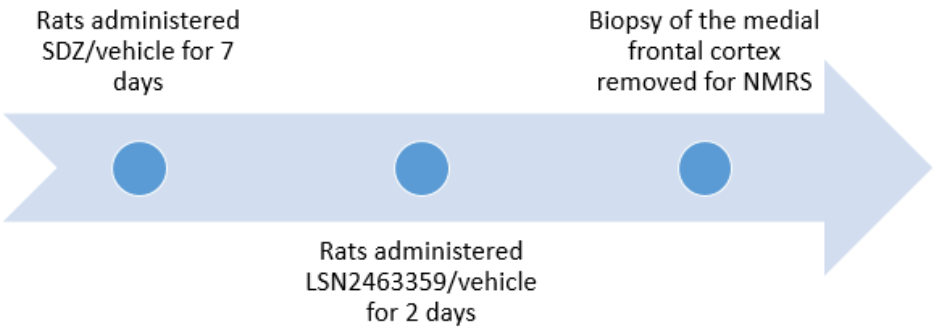


Figure 3. Experimental timeline of test for GABA, glutamate and NAA levels.

To assess LSN's effect on cell health after SDZ administration, we measured NAA levels after SDZ/vehicle and LSN/vehicle administration.

The effect of LSN on cell health is shown in Figure 4. NAA levels of rats given SDZ and LSN were compared to untreated mice and mice given SDZ alone. Here is evidence that LSN improved NAA levels (considered a sign of cell health) in rats given SDZ. The levels were not completely normalized but were near to normal. This beneficial effect was dose dependent.

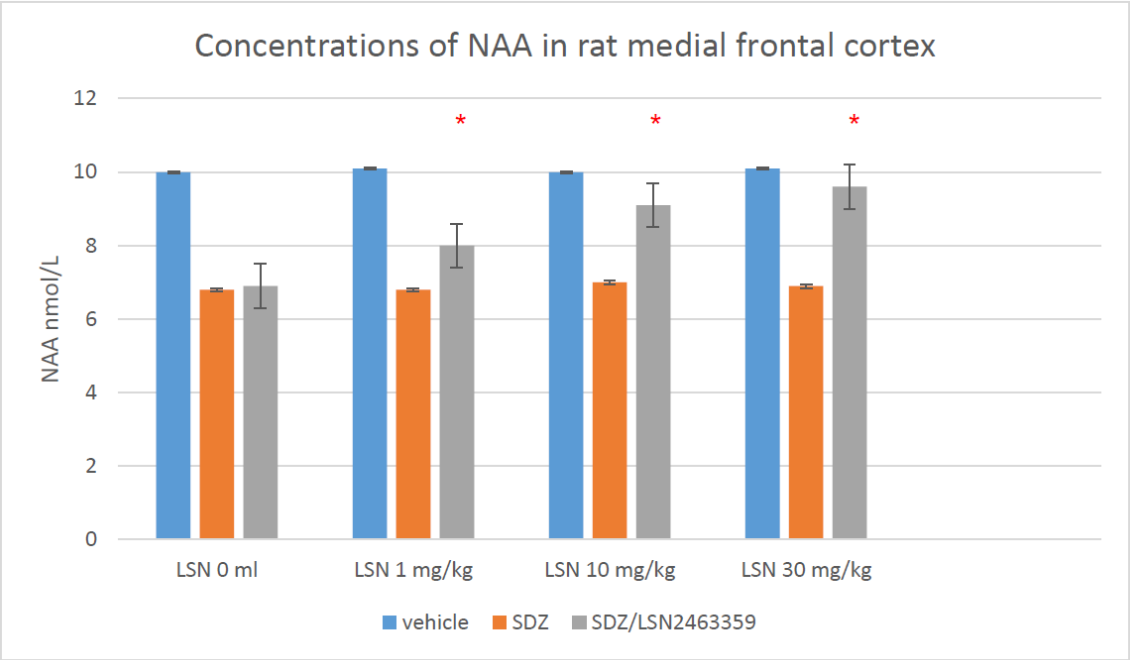


Figure 4. NAA levels in the medial frontal cortex of rats given SDZ and SDZ followed by treatment with varying doses of LSN. Statistical analysis was carried out using student's t test. Mean values of the LSN treated groups were compared to SDZ alone. (n=10) (* $p<0.05$) Error bars reflect the standard error.

GABA is an important neurotransmitter to study the effects of SDZ and LSN in the medial frontal cortex. Figure 5 shows that the NMDA antagonist SDZ had an effect on GABA levels in the medial prefrontal cortex. LSN treatment increased GABA levels after SDZ administration in a dose dependent manner. Although neurotransmitter levels were not returned to normal levels as found in the vehicle-treated rats they were raised to almost normal levels.

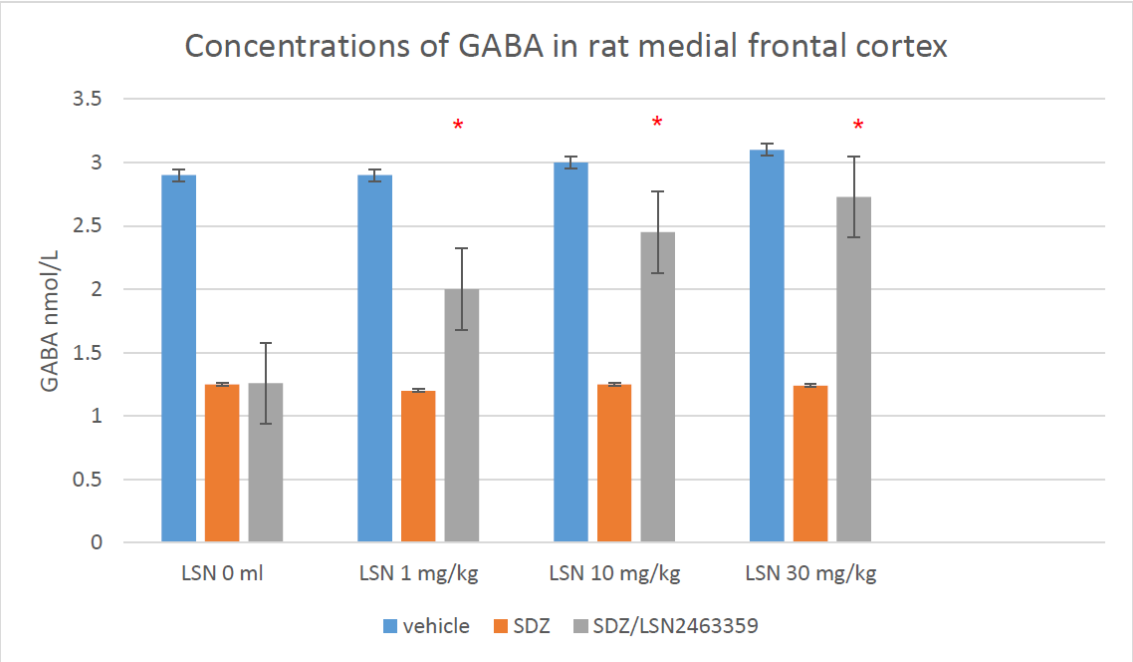


Figure 5. GABA levels in the medial frontal cortex of rats given SDZ and SDZ followed by treatment with varying doses of LSN. Statistical analysis was carried out using student's t test. Mean values of the LSN treated groups were compared to SDZ alone (n=10) (*$p<0.05$) Error bars reflect the standard error.

As glutamate levels may be changed after SDZ and LSN administration, we observed their effects in the following graph. As illustrated in Figure 6 and confirming previous data (Li, 2010) we found that SDZ administration causes marked increase in glutamate levels in the rat medial frontal cortex. This increase was dampened by LSN in a dose dependent manner. Although glutamate levels were not brought to normal levels, they were significantly reduced when compared to SDZ treatment alone.

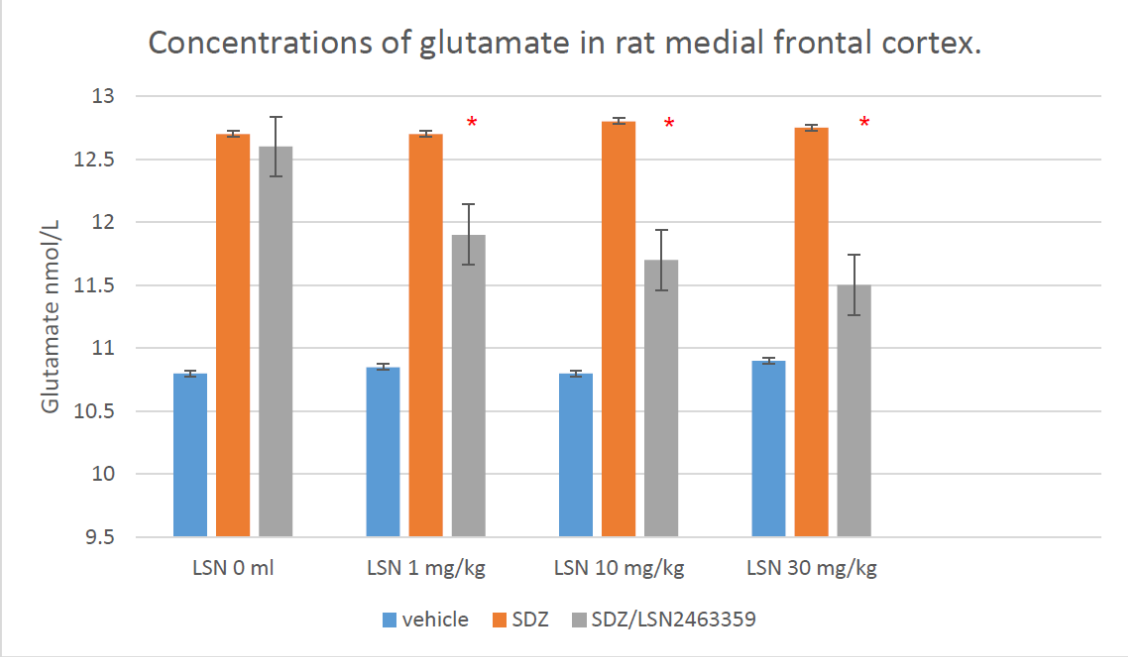


Figure 6. Glutamate levels in the medial frontal cortex of rats given SDZ and SDZ followed by treatment with varying doses of LSN. Statistical analysis was carried out using student's t test. Mean values of the LSN treated groups were compared to SDZ alone. (n=10)(*p<0.05) Error bars reflect the standard error

Using High Performance Liquid Chromatography (HPLC) we observed the change in dopamine levels in the medial prefrontal cortex of rats given SDZ and LSN or the vehicle. Due to dopamine's extremely low levels of concentration in the brain, NMRS could not be performed. Below is a timeline for methodological events.

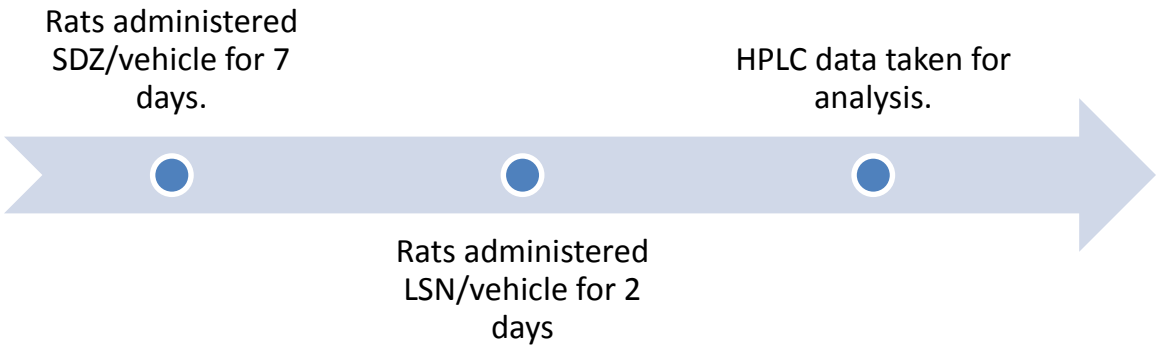


Figure 7. Timeline for testing dopaminergic activity via HPLC.

Dopamine levels in the medial frontal cortex have been shown to increase or decrease after NMDA antagonism depending on a number of variables (Zuo, 2012; Catane, 2015). We hypothesized that treatment with SDZ would cause an increase in dopamine levels.

In accordance with previous studies (Zuo, 2012), dopamine levels increased significantly upon SDZ administration. LSN decreased dopamine levels in a dose dependent manner. This is a novel finding as this experiment has not been performed previously.

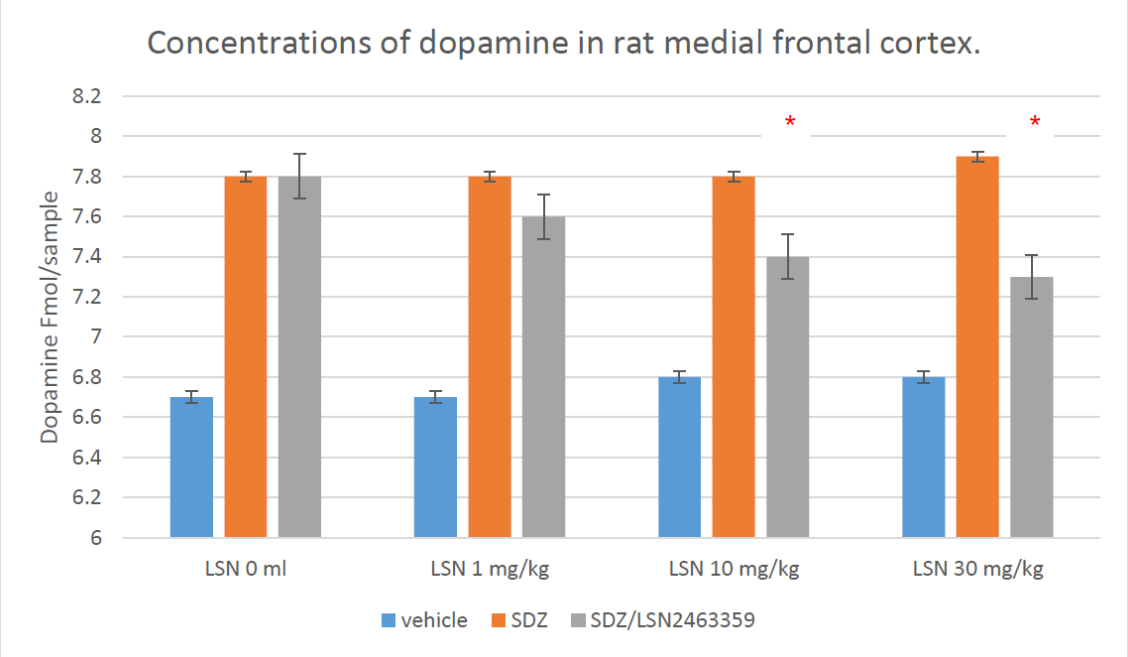


Figure 8. Dopamine levels in the medial frontal cortex of rats given SDZ and SDZ followed by treatment with varying doses of LSN. Statistical analysis was carried out using student's t test. Mean values of the LSN treated groups were compared to SDZ alone. (n=10) (*$p<0.05$) Error bars reflect the standard error.

Our findings in section 1 were consistent with previous studies in that GABA, glutamate and dopamine levels are altered by NMDA antagonism (Cohen, 2015; Bustillo, 2012) Our observations that LSN modulates to near normal levels GABA, glutamate and dopamine after SDZ administration are novel. Similarly, our finding of increased levels of NAA, indicating improved cell health, upon treatment with LSN, is novel.

Section II. Determine if LSN2463359 would diminish psychosis-like symptoms in mice that have the neuregulin-1 gene knocked out heterozygously.

Several well established murine models of schizophrenia are based on the observation that psychosis causes a decrease in social behaviors and increase in behaviors related to anxiety (O'Tuathaigh, 2010). A method commonly used to measure anxiety is to assess fear conditioning responses in mice. If a mouse is subjected to a conditioned stimulus (such as a bell) followed by a noxious stimulus (such as a foot shock) the mouse will learn to associate the conditioned response with the noxious stimulus. After the conditioning occurs, a test stimulus not followed by a noxious one is performed. A normal mouse will freeze during the test. An anxious mouse will freeze for less time.

Fear conditioning tests were carried out on 40 neuregulin-1 (NRG-1) knockout mice and 40 wild type mice. Tests were carried out with the goal to assess whether LSN would be beneficial in reducing anxiety in mice with the NRG-1 knockout. First we explored the difference in anxious behavior in wild type and NRG-1 knockout mice.

Figure 9 shows that NRG-1 knockout mice had a more anxious profile than wild type mice. The NRG-1 knockout mice spent less time frozen in the test portion of the fear conditioning test. These results were consistent with findings by Duffy et al. (2010).

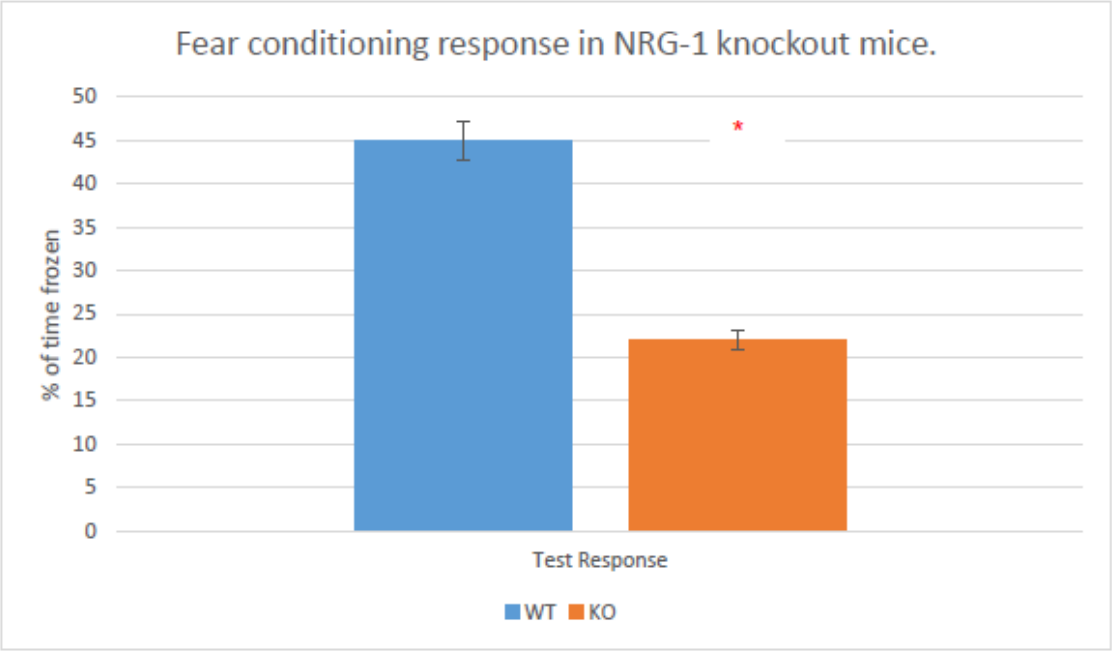


Figure 9. Fear conditioning response in wildtype and NRG-1 knockout mice. (n=10) (*p<0.05) Error bars reflect the standard error.

In order to find if LSN provides relief for anxious behavior in NRG-1 knockout mice, we performed a fear conditioning test on wild- type and NRG-1 knockout mice with varying doses of LSN (1 mg/kg, 10 mg/kg, 30 mg/kg) administered for 2 days.

As illustrated in Figure 10 our data shows that LSN modulated behaviors related to anxiety in NRG-1 knockout mice to near normal levels. LSN had little effect on the wild type mice. The psychotic mice responded to LSN in a dose dependent manner. These results are novel as fear conditioning has not been previously tested after LSN administration.

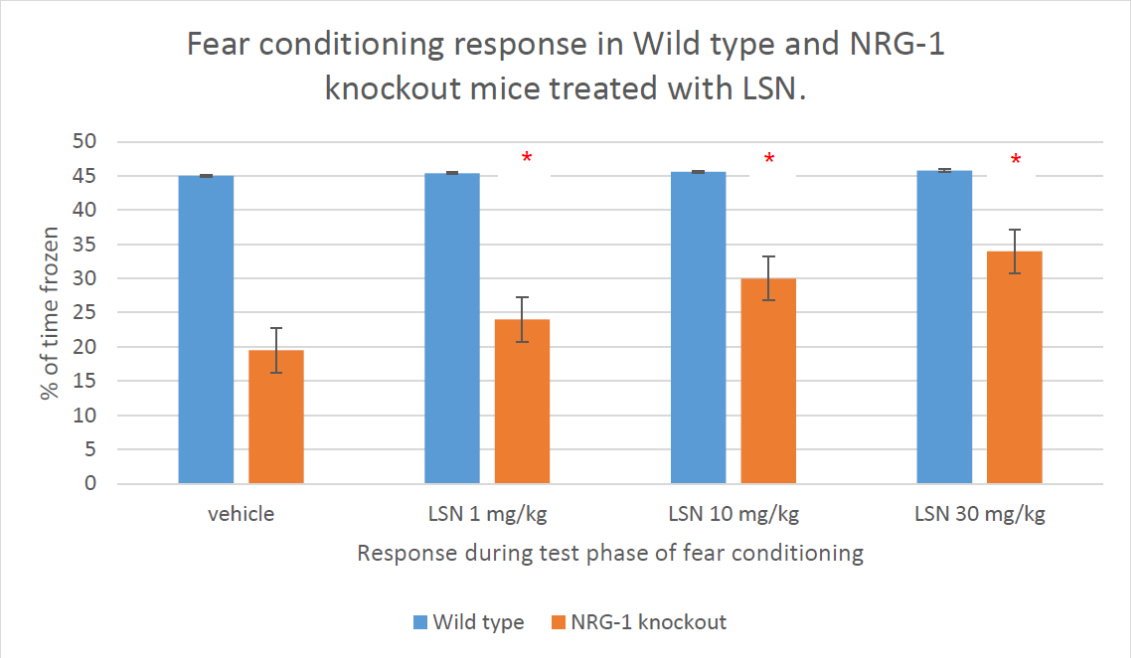


Figure 10. Fear conditioning response in wild type and neuregulin-1 knockout mice given varying doses of LSN. Statistical analysis was carried out using student's t test. Mean values of the LSN treated groups were compared to that of the vehicle treated controls. (n=10) (*p<0.05) Error bars reflect the standard error.

A method commonly used to measure social behavior is by placing a "stranger" mouse in the cage with the test mouse and measuring the time spent in anogenital sniffing. When the mouse is psychotic, usually anogenital sniffing is less intense than a nonpsychotic mouse. Our aim was to assess whether LSN would be beneficial in increasing social investigation in neuregulin-1 knockout mice. We first tested if neuregulin-1 knockout mice were more prone to social withdrawal by employing the anogenital sniffing behavioral test as previously reported by O'Tuathaigh et al. (2010).

Figure 11 confirms that NRG-1 knockout mice displayed less social investigation than wild type mice. When introduced to a stranger mouse, the NRG-1 knockout

mouse spent less time in anogenital sniffing. This finding is in accordance with previous studies (O'Tuathaigh, 2007 and 2010). We next tested whether LSN can modulate abnormal social behavior to near normal levels.

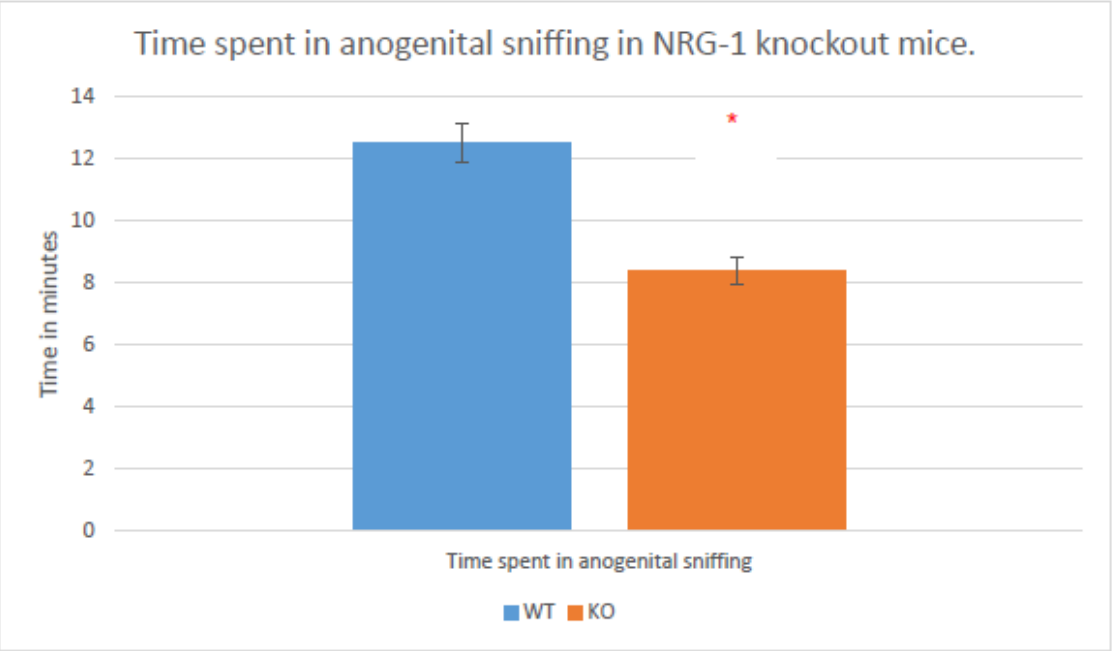


Figure 11. Anogenital sniffing test results in wild type and NRG-1 knockout mice. (n=10) (*p<0.05) Error bars reflect the standard error.

Figure 12 shows that LSN modulated social investigation in NRG-1 knockout mice in a dose dependent manner. Similar to the fear conditioning test, LSN had little effect on wild type mice. This finding is novel in that social withdrawal has yet to be tested in conjunction with LSN.

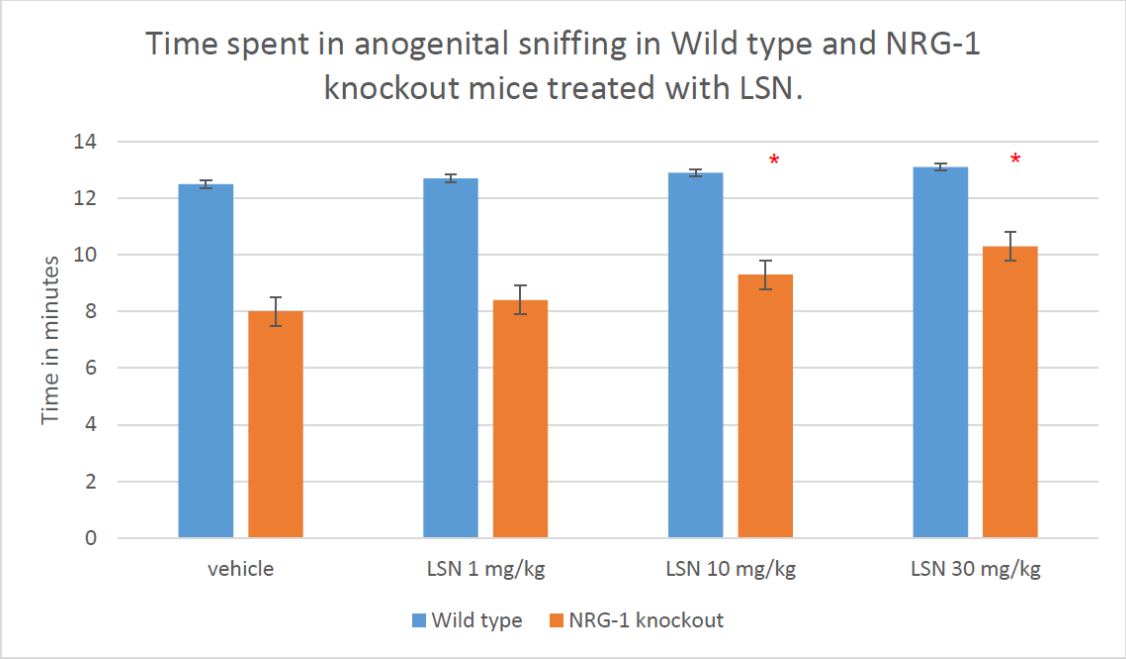


Figure 12. Time spent in anogenital sniffing by wild type and neuregulin-1 knockout mice given varying doses of LSN. Statistical analysis was carried out using student's t test. Mean values of the LSN treated groups were compared to that of the vehicle treated controls. (n=10) (*p<0.05) Error bars reflect the standard error.

Overall, our behavioral studies showed that LSN lessened anxiety and improved social activity in NRG-1 knockout mice in a dose dependent manner suggesting that LSN may improve psychosis on a behavioral level.

Discussion

We found that the chemical compound LSN2463359 exerted significant beneficial effects at the cellular and behavioral level in two different models of psychosis. LSN2463359 has shown the ability to modulate to near normal levels GABA, glutamate, dopamine and NAA in the medial frontal cortex of rats given SDZ. At a behavioral level, LSN2463359 diminished psychotic behavior in neuregulin-1 knockout mice.

Consistent with previous data (Cohen, 2015; Bustillo, 2012; Zuo, 2012) we confirmed that rats administered SDZ alone showed decreased GABA and NAA levels and increased glutamate and dopamine levels in the medial frontal cortex. This finding is in agreement with the new theory that decreased tonic GABA activity and increased glutamate excitotoxicity contribute to schizophrenia in humans (Cohen 2015). Dopamine has been shown to increase or decrease upon NMDA antagonism (Zuo, 2012; Castane, 2015). To delve further into the discrepancy, our results showed that small increases in dopamine levels were found in SDZ treated rats. LSN's ability to ameliorate dopamine levels in a dose dependent manner suggests it may be a viable tool in diminishing psychosis.

Studying LSN's effects in neuregulin-1 knockout mice helps to see their effects in a different neurological environment for NMDA receptors that may be closer to the schizophrenic model. As neuregulin-1 regulates NMDA activity it would be reasonable to suspect its mutation may affect NMDA activity and lead to

psychotic behavior. Our data showed that treatment with LSN resulted in a reduction of psychotic behavior in neuregulin-1 knockout mice as assessed by the fear conditioning and anogenital sniffing behavioral tests. The mechanisms underlying LSN's beneficial effects are possibly due to the potentiation of NMDA receptor activity elicited by LSN through the mGlu5 receptor.

Similar to our study, Gastambide et al. (2013) found that LSN brought to normal levels reversal learning deficits and deficits in instrumental responding in mice given SDZ. Both are cognitive deficits. This effect was not seen in mice given PCP or MK-801 which are non-competitive NMDA antagonists. SDZ is a competitive antagonist as it will compete with the ligand for the receptor. This led us to questions about pharmacological models and how close they are to the actual disease presentation. In contrast to our results, Bustillo et al. (2012) found that GABA **and** glutamate levels decreased after PCP treatment. In their study rats were exposed to PCP for one month unlike our choice of one week. Li et al. (2010) found that glutamate levels **increased** in the medial prefrontal cortex of rats after PCP exposure. Their study administered PCP for only 4 hours. We chose to administer SDZ for one week as too much antagonist does not serve to mimic the schizophrenic model. We chose to administer LSN for 2 days as SDZ leaves the nervous system and no longer has an effect on the medial frontal cortex. It is important to understand that excess or below par administration of antagonists or PAMs can upset the delicate balance of neural pathways. As witnessed by the

differences in LSN2463359's effects depending on the NMDA antagonist used, the length of time it was used as well as differences in genes that increase the risk of schizophrenia, it can be understood that there is no one model which would serve to mimic the complexity of schizophrenia in humans (Law, 2014).

Brody et al. (2004) discovered that mice with an mGluR5 knockout are more prone to pre-pulse inhibition deficits. Pre-pulse inhibition is the reaction to a strong noxious stimulus when preceded by a weaker stimulus. Deficits in this area show deficits in sensorimotor gating or oversensitivity to sensory stimulation. Psychotic mice are more prone to deficits in this area. This study added to our hypothesis that modulating the mGluR5 may be beneficial to alleviating psychotic symptoms.

Deng et al. (2013) reported that impaired neuregulin 1 signaling causes a decrease in GABA activity and an increase in glutamate activity in the medial prefrontal cortex of rats. This finding shows that neuregulin-1 plays an important role in the theorized pathway leading to psychosis. This would help our understanding of why neuregulin-1 knockout mice exhibit psychotic behvior.

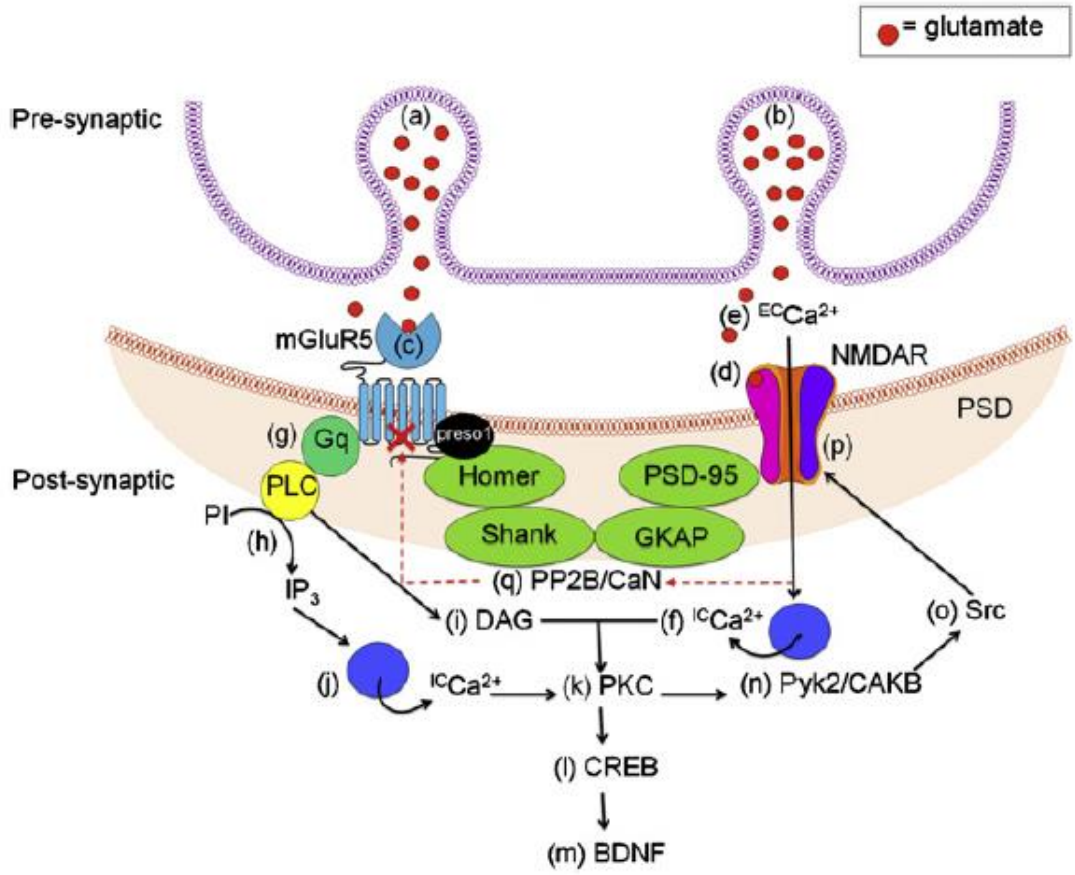


Figure 13. Schematic of the mGluR5 signaling pathway (Matosin 2013).

In Figure 13 we observe the theorized effect mGluR5 activity has on the NMDA receptor and the cell as a whole. After a signaling cascade, the NMDA receptor is potentiated by Src protein. Another effect of the cascade is the production of brain-derived neurotrophic factor or BDNF. BDNF is a neurotrophic protein that has many effects including promoting nerve health and encouraging growth and differentiation of synapses. BDNF is also a potentiator of NMDA receptors through Fyn activation. BDNF mRNA levels are decreased in cortical layers IV and V of the dorsolateral prefrontal cortex in schizophrenic patients

showing its importance in linkage to schizophrenia (Ray, 2014). Figure 13 also shows that mGluR5s are attached via scaffolding proteins to NMDA receptors which keep the receptors in close proximity to increase regulatory action. Figure 13 elucidates the close relationship between mGluR5s and NMDA receptors.

One limitation of our report is that we only administered the rats/mice LSN for two days. Many schizophrenia sufferers have chronic symptoms that need to be addressed over a long period of time. We do not know if the neuronal system would become sensitized to the compound.

Another limitation is that we only examined the neurochemical makeup of the medial frontal cortex. There are many other regions of the brain that are involved in psychosis. More detailed studies are needed to see how all the areas of the brain affected in schizophrenia are impacted by NMDA antagonism and mGluR5 PAMs.

Further studies are needed to test the validity of the new glutamatergic theory of schizophrenia pathology. I would suggest making measurements of LSN's modulating effect on neurotransmitters in other areas of the brain. I would also suggest long term studies on the effect of LSN in NRG-1 knockout mice.

Conclusions

Our findings indicate that LSN2463359 had a significant effect on the neurochemistry and behavioral characteristics of rats given SDZ and NRG-1 knockout mice. Whether the compound could be used to help humans is yet to be

determined. More studies are needed to observe its efficacy over a long period of time and in different models of psychosis. This novel treatment may be potentially used as an alternative to current antipsychotic medication. If found to have lower efficacy, it may be included in the drugs prescribed as an addition to a traditional antipsychotic.

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