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2020

Signaling Mechanism Responsible for 5-HT2A Receptor Tolerance to Psychedelic Induced Head-Twitch Behavior in Mice

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Thesis Defense Date: May 18, 2020

Acknowledgments:

I would like to express my sincere gratitude to my family and friends who have supported me throughout this year and remained a source of guidance at every turn. I'd like to thank Dr. Mario de la Fuente Revenga for his patience and assistance in teaching me and also for setting a model example for what it means to be dedicated to the pursuit of scientific inquiry. To all of my other lab members, I am honored to have spent this time seeing the amazing work that you contribute to this important field. I'd like to thank my committee members, Dr. Vijay Lyall and Dr. Jennifer Wolstenholme, for their support and willingness to help me with this process. Thank you also to the Wolstenholme lab for allowing me to share space and utilize their lab's equipment to run my experiments! Finally, I'd like to thank Dr. Javier González-Maeso for his kindness and intellectual guidance during this process. Your mentorship has helped me to grow as a student and as an individual and challenged me to always think more critically about the world around me. Thank you all for the support, I could not have done this without you!

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A. List of Abbreviations:

1,2 DAG	1,2 Diacylglycerol
5-HT	5-Hydroxytryptamine or Serotonin
5-HTP	5-Hydroxytryptophan
5-HT$_{2A}$R	Serotonin 2A Receptor
5-HT$_{2C}$R	Serotonin 2C Receptor

Bisindolylmaleimide- II:
- 3-(1*H*-Indol-3-yl)-4-[1-[2-(1-methyl-2-pyrrolidinyl)ethyl]-1*H*-indol-3-yl]-1*H*-pyrrole-2,5-dione

DMSO	Dimethyl sulfoxide
DOI	2,5-dimethoxy-4-iodoamphetamine
ERK	Mitogen Activated Protein Kinase
GPCR	G Protein Coupled Receptor
HTR	Head Twitch Response
IP$_3$	Inositol 1,4,5-trisphosphate
LSD	Lysergic acid diethylamide
NMDA	*N*-methyl-D-aspartate

M100,907:
- (*R*)-(2,3-dimethoxyphenyl)-[1-[2-(4-fluorophenyl)ethyl]piperidin-4-yl]methanol, Volinanserin

MEF	Mouse Embryonic Fibroblasts
MEK1/2	Mitogen Activated Protein Kinase Kinase

SL327
- (Z)-3-Amino-3-(4-aminophenylthio)-2-(2-(trifluoromethyl)phenyl)acrylonitrile

U73122
- 1-(6-((3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione

QQ Plot Quantile Quantile Plot

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Chapter 4: Discussion

C. Abstract

In recent years, there has been a growing interest in psychedelic compounds stemming from their promising use as therapeutic agents and research tools that can be used to treat and study several neuropsychiatric disorders. Psychedelics have proven useful in a broad range of these diseases - they serve as models for psychosis in schizophrenia but have also had promising results in treating major depressive disorder, anxiety, and other common disorders. Decreased stigmatization surrounding psychedelics has further increased their use in research and clinical settings. In light of these trends and the promising nature of their use, a thorough understanding of the mechanisms underlying their action is necessary. The Serotonin 2A Receptor, or 5-HT$_{2A}$R, has been shown to play a major role in neuropsychiatric disorders and also serves as the primary receptor mediating psychedelic action. In rodent models, administration of psychedelics such as the phenethylamine-derived compound 2,5-dimethoxy-4-iodoamphetamine (DOI) produces a behavior known as the head twitch response. This characteristic head movement occurs as the specific result of 5-HT$_{2A}$R activation by psychedelic substances and in tandem with characteristic psychedelic genotypic expression pathways, and can therefore be used to quantify 5-HT$_{2A}$R activation.

Repeated administration of psychedelics has also been shown to lead to tolerance, producing diminished therapeutic effects for otherwise promising treatments. In animal models, tolerance can be demonstrated using the HTR model, quantified through progressive decreases in the number of head twitch behaviors elicited upon psychedelic administration. While several theories have attempted to explain the mechanisms behind tolerance, they remain unknown and are worthy of further study. My project aimed to examine signaling pathways underlying psychedelic action, the head twitch response, and tolerance in order to more fully understand the

mechanisms behind these processes. My results indicate that the Mitogen Activated Protein

Kinase, ERK, plays a role in mediating the head twitch response acutely, highlighting this

signaling pathway as a mediator of this behavioral response. However, signaling blockade at the

level of ERK failed to prevent tolerance development. Interestingly, signaling blockade at the

level of Protein Kinase C (PKC) failed to diminish head twitch acutely and also failed to prevent

tolerance. Finally, pre-treatment with the 5-HT$_{2A}$R specific antagonist M100,907 attenuated head

twitch acutely but similarly failed to prevent tolerance. Collectively, these results point towards

the MAP Kinase pathway as important in mediating psychedelic induced head twitch behavior,

but further study is needed to investigate the source of tolerance.

Signaling Mechanism Responsible for 5-HT2A Receptor Tolerance to Psychedelic Induced Head-Twitch Behavior in Mice

By Audrey Rebecca McMurtrie

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology and Biophysics at Virginia Commonwealth University

Virginia Commonwealth University, 2020

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D. Chapter 1: Introduction

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I. Introduction to Hallucinogens

Hallucinogens are chemical substances that affect complex integrated neurological processes such as cognition, perception, and mood (Charney & Nestler, 2011, pg. 38). Though hallucinogens have recently become more commonly used in scientific research, their use in human society is far from novel. Historical and anthropological records show that human civilizations have incorporated naturally occurring hallucinogens into religious rituals, healing methods, and other cultural practices for thousands of years (Nichols, 2016). Hallucinogens served as tools that enabled humans to expand consciousness, connect with spiritual traditions, and alter their perception of reality (Nichols, 2004). Jaffe's modern definition of hallucinogens aligns with their historical use, characterizing hallucinogens as any substance that can "reliably… induce states of altered perception, thought, and feeling that are not experienced otherwise except in dreams or at times of religious exaltation" (Nichols, 2016). Though hallucinogens share an ability to affect complex neurological processes, the mechanisms through which they mediate effects are distinct and allow for further classification. For example, psychedelics such as psilocybin (found in "magic mushrooms") and the synthesized compound lysergic acid diethylamide (LSD) all serve as serotonin 2A receptor (5-HT$_{2A}$R) agonists or partial agonists (Smith et al, 2014). Dissociative hallucinogens such as ecstasy act as non-competitive N-methyl-D-aspartate (NMDA) receptor antagonists, while stimulant hallucinogens act as dopamine reuptake inhibitors (Mori & Suzuki, 2016). Deliriant hallucinogens such as scopolamine and atropine have been shown to induce dream-like hallucinations and hyperactivity through antagonism of muscarinic receptors (Volgin et al., 2018).

In recent years, interest in studying psychedelic hallucinogens, in particular, has grown dramatically as the result of a few factors. Firstly, these compounds have proven useful in the

study and treatment of a broad array of neuropsychiatric disorders such as schizophrenia, anxiety, major depressive disorder, Parkinson's, alcoholism, addiction, and borderline personality disorder (Charney & Nestler, 2011, pg. 38). These disorders place a tremendous burden on individuals afflicted with them and on healthcare systems, creating incentive for the discovery of more effective treatments (Nichols, 2016). Previously, concerns over psychedelics' ability to modify cognition and thought created safety concerns and stigmas that manifested as legal barriers preventing their use in research and treatment (Nichols, 2016). In the 1960's, for example, fears over psychedelic use in the United States grew due to their association with political protest, counterculture beliefs, and other deviations from convention. Psychedelics were subsequently classified as Schedule I drugs in the Controlled Substances Act of 1970, placing them under the strictest legal regulation possible (Nichols, 2016). However, psychedelics are considered relatively safe in part because they have little to no affinity for targets that mediate critical cardiovascular, renal, or hepatic functions (Nichols, 2004). Further, psychedelics prove to be non-reinforcing substances that do not induce physiologic dependence (O'Brien, *Ch. 24,* 2011). When administered in proper doses and in controlled settings, psychedelics have proven to be safe and non-addictive. Together, these promising findings have aided in shifting attitudes and increasing demand for psychedelics' use in research and therapy (Charney & Nestler, 2011, pg. 39). As such, a thorough understanding of the mechanisms underlying their action is necessary.

II. The 5-HT$_{2A}$R is the Primary Target of Psychedelic Action

As researchers explored the underlying mechanisms of psychedelic action, the serotonin hypothesis emerged as a leading contender. This theory argues that the 5-HT$_{2A}$R plays a critical role in mediating psychedelic action (Charney & Nestler, 2011, pg. 38). This theory originated

after structural similarities were noted between the 5-HT2AR's endogenous agonist, serotonin or

5-HT, and LSD, a synthesized psychedelic with 5-HT2AR agonist activity (Nichols, 2016). Both

structures share a common indole ring, which early researchers theorized allowed them to have

comparable receptor affinity and action (Nichols, 2016).
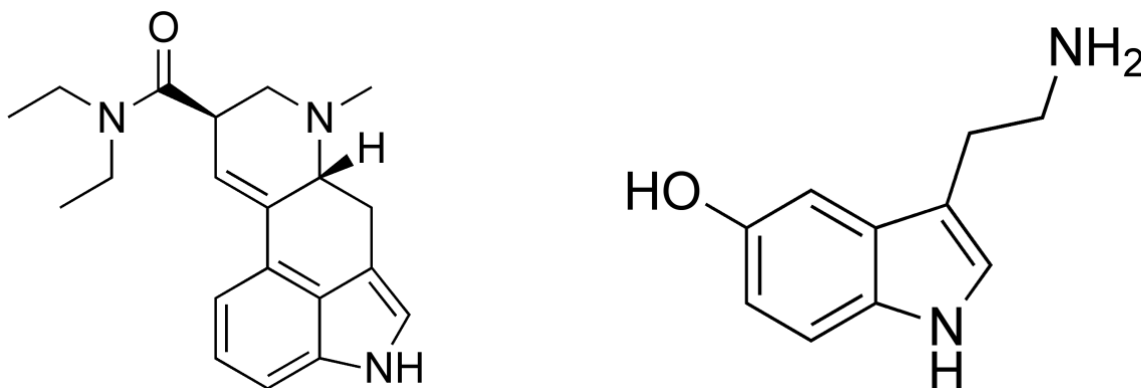
Figure 1: Structural similarities between lysergic acid diethylamide (LSD) and Serotonin (5-HT) provided early evidence for serotonin hypothesis of psychedelic action. Chemical structures of LSD, (left) and 5-HT (right). Early researchers first hypothesized that LSD mediated effects through a serotonin receptor due to structural similarities between the two molecules.

Within this theory, the sensory, cognitive, and behavioral changes that occur after taking

psychedelics result from selective stimulation of the 5-HT2AR, particularly in apical dendrites in

neocortical pyramidal cells of cortex layer V (Nichols, 2016). This theory is supported by studies

such those that used receptor-specific antagonists to study the 5-HT2AR's role in psychedelic's

effects. In 1998, Vollenweider pre-treated human subjects with the 5-HT2AR specific antagonist,

ketanserin, prior to administering psilocybin (Nichols, 2004). Those subjects who received

ketanserin reported a significant or complete reduction in metrics that quantified altered states of

consciousness (Nichols, 2004). In a more recent study, human subjects that were treated with

LSD or psilocybin described symptoms that included altered consciousness, "cognitive

bizarreness", and visual alterations (Kraehenmann et al., 2017). However, subjects that were pre-treated with ketanserin failed to report the same LSD and psilocybin-induced effects (Kraehenmann et al., 2017). Similarly, a 1968 study by Anden noted that rats who were pre-treated with ketanserin failed to exhibit psychedelic discriminative cues in a drug discrimination model (Nichols, 2016, pg. 280). Although the complete mechanisms of psychedelic action remain unknown, these studies highlight the necessary role that the 5-HT$_{2A}$R plays in mediating psychedelic action in humans and rodents.

It should also be noted that while the 5-HT$_{2A}$R has been highlighted as playing a necessary role in mediating psychedelic action, it is not the only receptor that is acted upon by these drugs. In fact, it is thought that these unique affinities and binding identities are at least partially responsible for the array of intracellular, cognitive, and behavioral effects induced by various psychedelics (Ray, T., 2010). In 2010, Ray published a study testing the affinity, selectivity, and binding breadth of 35 different psychedelic compounds at 51 different sites that include receptors, membrane receptors, and ion channels (Ray, T., 2010). Ray found that tested psychedelics had interactions with a large number of receptors, with varying affinities, such that forty-two tested sites showed psychedelic action (Ray, T., 2010). For example, LSD was also found to have strong affinity with the Serotonin 7 Receptor (5-HT$_{7}$R), DOI for the alpha 2A (α_{2A}), and psilocin for the Dopamine 1 receptor (D1). Additionally, Ray assessed the proportional breadth (B$_{p}$) of these psychedelics for serotonergic systems, which compares a compound's interaction with one receptor or one receptor class in proportion to its interaction with all other receptors (Ray, T., 2010). This assessment was performed on serotonergic systems to determine what proportion of interactions occur at some sort of 5-HT receptor. Results for various psychedelic compounds varied widely, with LSD possessing a B$_{p}$ of 0.719 whereas the

tryptamine derived psychedelic dipropyltryptamine (DPT) had a B_P score of 0.426 (Ray, T. 2010). These scores indicate that LSD and DPT's interactions with serotonergic receptors accounts for only 71.9% and 42.6% of all interactions, respectively. These varied results point towards the complexity of psychedelic action in a physiologic system and could account for the spectrum of effects observed upon different psychedelic administration.

III. The 5-HT2AR: Clinical Correlations

The 5-HT2AR is implicated in a wide array of conditions that collectively place an immense burden on those afflicted with them and on healthcare systems. These disorders include schizophrenia, psychosis, depression, Parkinson's disease, anxiety, substance use disorder, bipolar disorder, and more (Smith, et al., 2014). Here I will cover a few of these disorders and discuss the 5-HT2AR's applicability in studying and treating them.

Schizophrenia is a psychiatric disorder that is characterized by three classes of symptoms: positive, negative, and cognitive (Fatemi & Clayton, 2016). Positive symptoms include those which are typically associated with schizophrenia such as visual, auditory, and tactile hallucinations as well as delusions (Fatemi et al., 2016). Negative symptoms involve decreased psychiatric function such as difficulty planning, loss of emotional expression, and difficulty finding pleasure (Fatemi et al., 2016). Cognitive symptoms are varied but can include things such as memory dysfunction, decreased attention span, disordered or confused speech, and memory issues. Globally, 1% of the population is afflicted with schizophrenia and the World Health Organization ranks schizophrenia as one of the top ten illnesses contributing to the global burden of disease (Fatemi & Clayton, 2016).

The 5-HT2AR is thought to play a significant role in schizophrenia for several reasons. A 2014 study by the Schizophrenia Working Group of the Psychiatric Genomics Consortium

utilized genome wide association study arrays to assess over 1,000 genes for their association with schizophrenia. Out of those tested, the gene encoding the 5-HT$_{2A}$R, *HT2RA,* ranked in the top 12 schizophrenia associated genes (Ripke et al., 2014). Additionally, post-mortem studies in human subjects have provided insight into the neurostructural and physiologic changes that the 5-HT$_{2A}$R plays in disorders such as schizophrenia. For example, [$_3$H] ketanserin binding studies with schizophrenic subject's post-mortem brain tissue found significantly altered expression of the 5-HT$_{2A}$R as compared to healthy individuals (García-Bea et al., 2019). It is also thought that the 5-HT$_{2A}$R plays a role in mediating hallucinations- benchmark clinical symptoms of schizophrenia. Hallucinations, which are mediated through the 5-HT$_{2A}$R, can be induced by psychedelics in healthy individuals and exacerbated by psychedelics in schizophrenic subjects (Charney et al., 2011, pg. 39). This overlapping site of action also points towards the 5-HT$_{2A}$R as important in mediating characteristic positive symptoms of schizophrenia.

Schizophrenia is commonly treated through prescribed antipsychotic medications, which fall into two categories: typical (or first generation) and atypical (or second generation) (Charney & Nestler, 2011, pg. 40). Many typical and nearly all atypical antipsychotics have been shown to act at the 5HT$_{2A}$R (Panicker, Raote, & Bhattacharya, 2007). Clozapine, an atypical antipsychotic, is considered among the most effective antipsychotic medications available and has also been shown to have an increased affinity for the 5-HT$_{2A}$R compared to other antipsychotics (Browning et al., 2005).

In addition to schizophrenia, the 5-HT$_{2A}$R has been implicated in other disorders, both in their origin and in how they are treated. For example, post-mortem studies examining brain tissue of human subjects diagnosed with major depressive disorder have shown decreased levels of hippocampal 5-HT$_{2A}$Rs compared to healthy individuals (Raote et al, 2007). Further, tri-cyclic

antidepressants, which exert effects at the 5-HT$_{2A}$R, are commonly prescribed as a treatment for major depressive disorder (Panicker et al., 2007). Chronically, antidepressants have also been shown to lead to decreased cortical density of the 5-HT$_{2A}$R, indicating that tolerance develops in response to sustained exposure to the receptor's endogenous agonist.

Visual hallucinations are also sometimes seen in Parkinson's disease and are thought to result from dysregulation of the 5HT$_{2A}$R. A 2010 post-mortem study compared *in vivo* binding [$_{18}$F]setoperone PET scans in Parkinson's patients who did and did not experience hallucinations. In these binding assays, it was found that those subjects with a history of visual hallucinations had significantly increased 5-HT$_{2A}$R expression and binding in several regions of the cortex, including the ventral visual pathway (Nichols, 2016, pg. 280). 5-HT$_{2A}$R inverse agonists, such as pimavanserin, were subsequently tested in a clinical trial with Parkinson's patients, with promising clinical efficacy (Cummings et al., 2014). These studies further demonstrate the role that the 5-HT$_{2A}$R plays in mediating hallucinations, even outside the scope of schizophrenia and psychosis. Additionally, it highlights the 5HT$_{2A}$R as a therapeutic target that is worthy of investigation and thorough understanding.

Although post-mortem studies have allowed researchers to study the relationship between the 5-HT$_{2A}$R, psychedelics, diseases, and therapies in human tissue, there are limitations to these studies. Typically, these result from variability between the pre-mortem and peri-mortem conditions of these human subjects. For example, it is estimated that the lifetime rate of substance abuse among schizophrenics is close to 50% and that substance abuse is typically common among those who suffer from chronic mental illnesses (Dixon et al., 1991). These substances include alcohol and tobacco, both of which have been found to alter dendritic length and complexity as well as gene expression (Charney & Nestler, 2011, pg. 205). Furthermore,

post-mortem study results can be confounded by the patients' age, presence of other comorbid physical or psychiatric diseases, and history of psychiatric treatment such as what treatment they were on (if any) at the time of death (Charney & Nestler, 2011, pg. 205).

IV. Psychedelics Serve as Models of Disease and as Therapeutic Agents

Psychedelics induce cognitive and behavioral effects that mirror those in schizophrenic subjects, including hallucinations and disrupted thought. In schizophrenic human subjects, psychedelic administration has been shown to worsen trademark symptoms of schizophrenia such as visual and auditory hallucinations and paranoia (Paparelli et al., 2011). Because of their ability to induce symptoms that resemble an acute psychotic state in healthy individuals and to exacerbate acute psychosis in schizophrenic subjects, psychedelics can be used to model psychosis and schizophrenia (Halberstadt & Geyer, 2013). As such, they have become increasingly valuable tools for modeling disease in neuropsychiatric research.

In addition to their ability to induce hallucinations and thereby model psychosis, psychedelics such as LSD and psilocybin have displayed promising antidepressant and anxiolytic effects (Muttoni et al., 2019). Psychedelics are now being studied as therapeutic agents for individuals who have failed traditional treatment methods (Muttoni et al., 2019). In a recent clinical trial studying the effects of psychedelics on depression, subjects were administered a controlled, one-time dose of a psychedelic compound and then subsequently assessed for effects on mood, outlook, and other factors (Muttoni et al., 2019). 87% of subjects experienced lasting increased life satisfaction or wellbeing following a one time dose (Muttoni et al., 2019). These changes were attributed to psychedelics' ability to induce mental flexibility and enduring positive changes in attitudes, moods, perspective, values and behaviour (Muttoni et al., 2019).

Psychedelics' use as therapeutic agents are highly promising, yet an incomplete understanding of the precise mechanisms through which these effects are mediated demands further investigation.

Several recent studies have also examined the effect of psychedelics of human subjects suffering from anxiety, depression, and dysthymic disorder secondary to terminal cancer or terminal illness (Griffiths et al., 2015; Ross et al., 2016). These studies each found that psychedelic treated subjects experienced a significant reduction in anxiety and depression compared to placebo groups (Nichols, 2016). These effects occurred acutely but, more importantly, were found to be sustained without additional treatment even six months following treatment (Nichols, 2016). These subjects, most of whom were in late stages of terminal illness, reported improved overall quality of life, better spiritual well being, and improved attitudes towards their disease progression and death, topics with which they had previously struggled greatly (Nichols, 2016).

Animal studies have also supported findings from clinical trials in humans. A 2003 study by Dhonnchadha found that DOI administration in mice had a significant anxiolytic effect on mice behavior (Nichols, 2016). Animal studies remain a highly valuable tool to elucidate the mechanisms underlying these promising clinical findings; several of these will be discussed next.

V. Behavioral Models of Psychedelic Action

Animal models provide a valuable research tool to better understand the mechanisms behind physiologic and behavioral phenomena *in vivo*. As such, animal behavior can also be used to study psychedelic action and to model psychosis (Charney & Nestler, 2011, pg. 39). Although there are certain challenges in using animal models (lacking face validity and varied doses/ regimens between animals and humans), they remain important tools that enable study of the mechanisms underlying the aforementioned neuropsychiatric disorders and the mechanisms

that can be used to treat them (Charney & Nestler, 2011, pg. 39). Additionally, animal models can be used to study the mechanisms of psychedelic-associated phenomena such as tolerance, which develops in both humans and animals after repeat administration of a constant dose (Smith et al., 2014). The mechanisms behind this well-observed phenomena are not fully understood, and demand further study. Several models are currently employed to study the mechanisms of psychedelic action. For my project, I primarily used the head twitch response behavioral assay and locomotion studies in mouse models.

I first utilized locomotion studies to investigate the behavioral and motor effects of various compounds on mice. In 2013, Dr. Mark Geyer studied the effects of LSD and DOI on mice and determined that these psychedelics induced several characteristic and 5-HT$_{2A}$ R dependent behaviors at all doses other than very low doses (Hanks & González-Maeso, 2012). These changes included a decrease in the overall amount of activity (measured as horizontal locomotion), decrease in exploratory behavior (measured through number of rears and nose pokes in a hole board), and path stereotypy (measured through observing animals' tendency to follow similar paths) (Hanks & González-Maeso, 2012). However, the non-psychedelic 5-HT$_{2A}$ agonist lisuride induced a biphasic dose response curve such that low doses suppressed locomotor function and high doses enhanced locomotor function (Hanks & González-Maeso, 2012). In an earlier, 2009 study, Halberstaft noted that the $_{2A}$ R agonist, DOI, induced a U shaped dose-response curve on locomotor activity (Halberstadt et al., 2009). While they noted that low doses of DOI increased locomotion in mice, 5-HT$_{2A}$ R KO mice did not display this change (Halberstadt et al., 2009). For my particular project, locomotion studies provided a tool that I used in concert with the head twitch response assays. By examining mice's overal motor function in drug-treated mice compared to vehicle-treated mice, I could test for significant

differences in horizontal activity. Any evidence that a drug significantly altered overall locomotion may indicate that their overall motor function is suppressed. In such a case, diminished head movement occurring during the head twitch may be due to suppressed motor function rather than a head twitch response-specific effect.

The Head Twitch Response (HTR) is a rodent behavior that was observed in mice as early as 1963, after mice were injected with 5-hydroxytryptophan or serotonin (Corne et al., 1963). Physically, the HTR is characterized by a rapid, paroxysmal side to side head rotation that occurs in mice and in rats (Halberstadt & Geyer, 2013). On average, each HTR consists of 7.2 head rotations that change directions with a characteristic frequency of 90.3 Hz (Halberstadt & Geyer, 2013). A HTR typically initiates while mice are balanced on hind paws or on all fours, with their body hunched backward and head extended forward (Halberstadt & Geyer, 2013). Throughout the course of the HTR, the mouse's head extends away from the body, whereas the torso is not typically involved (Halberstadt & Geyer, 2013). However, head twitches that occur in synchrony with torso movement typically do so during a full body shake (Halberstadt & Geyer, 2013). For many years, these characteristic physical movements were recorded using video devices and then painstakingly processed and scored by trained individuals. This required both extensive training and time, and was at risk for bias or inaccurate recordings depending on the viewer. However, recent improvements to the HTR assay system, detailed in Revenga's 2019 article, utilize characteristic head movement frequencies and magnetometer-detected voltage changes (Revenga et al., 2019; Revenga et al., 2020). These changes allow for fully automated detection of the head twitch response and a high throughput behavioral assay to study psychedelic action at the $5\text{-HT}_{2A}\text{R}$ level.

Since the 1960s, understanding of the mechanisms underlying the HTR has increased and, with this, so has its utility as a behavioral model. It is thought that the HTR is mediated by neurons located in the prefrontal cortex (Willins & Meltzer, 1997). Although psychedelics may exert effects on multiple receptors, it is well established that interaction with the 5-HT$_{2A}$R is necessary in order for this response to occur. In 2007, Dr. González-Maeso demonstrated that psychedelic action at the 5-HT$_{2A}$R is necessary to induce HTR by showing that 5-HT$_{2A}$R KO mice failed to exhibit HTR upon psychedelic stimulation (González-Maeso et al., 2007). Conversely, WT mice displayed a robust response when administered psychedelics (González-Maeso et al., 2007). Additionally, it was found that non-psychedelic 5-HT$_{2A}$R agonists such as lisuride or ergotamine did not induce head twitches in mice, but psychedelic compounds such as LSD, DOI, psilocybin, and DMT did (González-Maeso et al., 2007). This study further highlighted the key role that the 5-HT$_{2A}$R plays in mediating psychedelic action. As such, the HTR assay has become a reliable model on which research can better understand the mechanisms of psychedelic action at the 5-HT$_{2A}$R. The HTR also serves as a useful model for psychosis in rodents that can be used to study schizophrenia and other psychosis-associated psychiatric disorders. In this study, HTR has also been used to examine psychedelic-induced tolerance. Although lacking face validity poses a downside to utilization of the HTR, this assay serves as a valuable rodent model of human psychedelic action (Hanks & González-Maeso, 2012). As such, the HTR is a behavioral assay that can measure activation of the 5-HT$_{2A}$R and its downstream signaling pathways, serve as a behavioral proxy of psychedelic effects in humans, and be used to differentiate whether compounds are likely to exert psychedelic effects or not (Revenga et al., 2019). In this study, it has particularly been used to examine the signaling pathway's role in mediating had twitch response behavior and tolerance.

VI. The 5-HT$_{2A}$ R: Structure, Function, and Signaling

The 5-HT$_{2A}$R is one of fourteen types of serotonin (5-HT) receptors found throughout the body (Mccorvy & Roth, 2015). In addition to their varied roles in neurologic function, 5-HT receptors are also highly concentrated in the gastrointestinal system, where they assist with peristalsis and gut motility, and in the circulatory system, where they aid with coagulation and blood vessel vasoconstriction (Mccorvy & Roth, 2015). Thirteen of these (excluding the serotonin 3 (5-HT$_3$) receptor) are classified as G Protein Coupled Receptors (GPCRs) (Mccorvy & Roth, 2015). GPCRs such as the 5-HT$_{2A}$R are a class of membrane receptors that transduce extracellular stimuli into intracellular signals (Wettschureck & Offermanns, 2005). In order to mediate these effects, GPCR receptors utilize different signaling pathways- Gα_i, G$\alpha_{q/11}$, and Gα_s, each associated with distinct intracellular signaling cascades (Mccorvy & Roth, 2015). The 5-HT$_2$ family of receptors includes the 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors and uses G$\alpha_{q/11}$ pathways (Mccorvy & Roth, 2015). These receptors can all be activated through their endogenous agonist, serotonin. Additionally, as discussed earlier, the 5HT$_{2A}$R also serves as a site for psychedelic binding (González-Maeso et al., 2007).

GPCRs possess characteristic structural and functional motifs, including seven transmembrane domains that are flanked by an extracellular N-terminus and intracellular C-terminus (Mccorvy & Roth, 2015). This receptor forms a complex with three G proteins- an α subunit and a β/ γ subunit (Wettschureck & Offermanns, 2005). In an inactive state, the α subunit is bound to guanosine diphosphate (GDP) and the β/ γ subunit, forming a heterotrimeric protein complex that is coupled to the receptor (Wettschureck & Offermanns, 2005). Ligand binding induces promotes the exchange of GDP for guanosine triphosphate (GTP) on the α subunit,

leading to dissociation of the α subunit from the β/ γ subunit (Wettschureck & Offermanns, 2005).

Upon psychedelic binding, the $G_{q/11}$ coupled 5-HT_{2A} R initiates a series of intracellular signaling cascades that begins with GDP/GTP exchange and dissociation of the α and β/ γ protein subunits (Raote et al., 2007). Phospholipase C (PLC) is activated by G_q and leads to subsequent increases in intracellular Inositol 1,4,5-trisphosphate (IP_3) and 1,2 diacylglycerol (DAG), in addition to activation of Protein Kinase C (PKC) (Raote et al., 2007). IP_3 increases induces $Ca2+$ release from endoplasmic reticulum stores, a well characterized signal transduction pathway associated with G_q coupled receptors (Raote et al., 2007). In addition, PLC activation leads to phosphorylation and activation of ERK, a protein in the Mitogen Activated Protein Kinase (MAPK) pathway (Raote et al., 2007). In order to activate ERK, PLC must first activate Ras, which activates Raf, which activates Mitogen Activated Protein Kinase Kinase (MEK) (Raote et al., 2007). MEK sits just upstream of ERK and functions as a kinase to phosphorylate and activate ERK. From there, ERK goes on to mediate additional effects through continued signaling and genetic expression patterns (Raote et al., 2007).
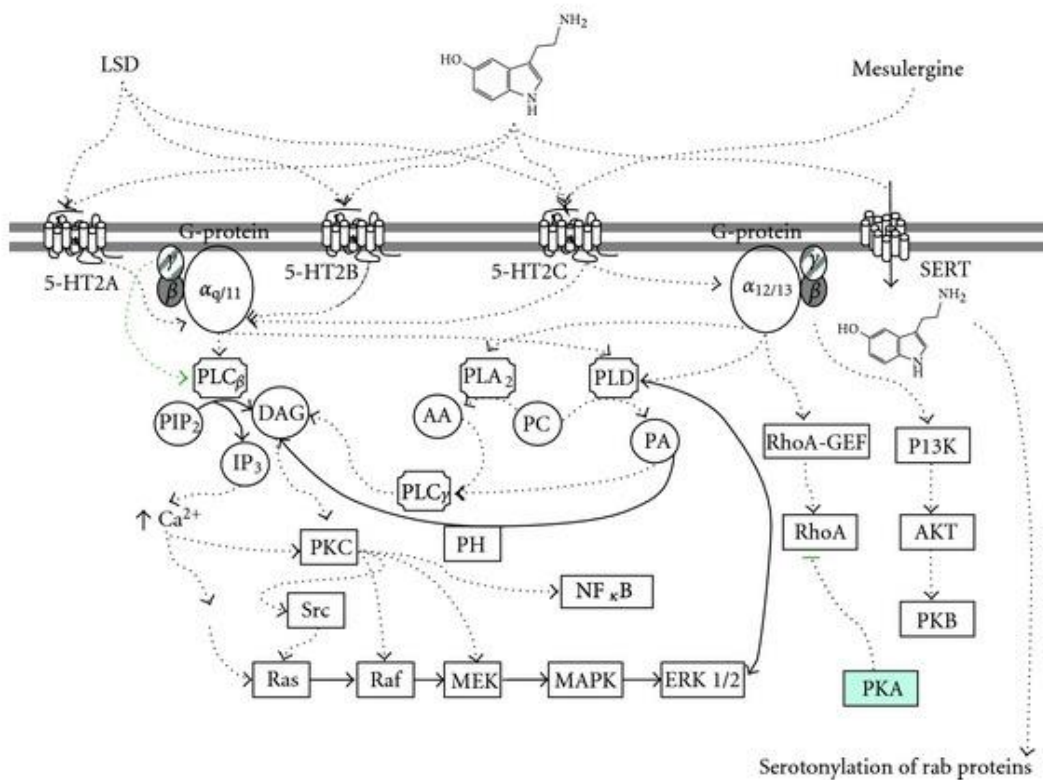
Schematic diagram demonstrating 5-HT$_{2A}$R signaling pathways. The 5-HT$_{2A}$R functions according to G$_{q/11,}$ signaling, which classically activates PLC and leads to increases in DAG, intracellular calcium release, and PKC activation. The MAPK pathway can also be activated through G$_q$ signaling, as is shown here. Signaling pathways of the Serotonin 2B (5-HT$_{2B}$) and Serotonin 2C (5-HT$_{2C}$) receptors are also shown.

Although understanding the complex interplay between signaling pathways proves challenging, there is evidence that it is intrinsic signaling rather than neuronal circuitry regulation that is responsible for psychedelic's characteristic genotypic and behavioral responses (González-Maeso et al., 2007). In 2007, González-Maeso et al demonstrated that selective 2A R expression in rodent cortex was sufficient to mediate signaling and induce characteristic psychedelic behavioral responses such as head twitch (González-Maeso et al., 2007). However, treatment with tetrodotoxin, which prevents neuron and neuronal circuitry activation, had no

effect on transcription patterns that are associated with psychedelics (González-Maeso et al., 2007). Thus, exploring intrinsic 5-HT$_{2A}$R signaling may provide a means of mechanisms of understanding psychedelic action.

VII. Tolerance Results from Repeat Action at the 5-HT$_{2A}$R

Tolerance is defined as a progressive decrease in effects- behavioral or physiologic- with repeated administration of a constant dose (Buchborn et al., 2018). This phenomenon, sometimes referred to as tachyphylaxis, has been observed in both animals and in humans who have been treated with psychedelic (Nichols, 2016, pg. 281). It is thought the tolerance broadly results from a decreased availability of the 5HT$_{2A}$R and decreased sensitization to agonist binding, evidenced by studies that showed selectively diminished 5-HT$_{2A}$R density in rat brains after animals were repeatedly treated with LSD (Buckholtz et al., 1988). Similar studies testing repeated doses of the phenethylamine-derived psychedelic, DOI, have also shown downregulation of 5-HT$_{2A}$R in rats (McKenna et al., 1989). Interestingly, downregulation and tolerance has been observed in response to agonists such as DOI or LSD, 5-HT$_{2a}$R antagonists such as the antipsychotic clozapine, and antidepressants (García-Bea et al., 2019). Because tolerance can result from both antagonism and agonism of the 5-HT$_{2A}$R by therapeutic agents and psychedelics, understanding the mechanism behind this phenomena is highly important.

Repeat administration of DOI led to a 24% and 30% decrease in phospholipase C (PLC) activity following 4 and 7 days' administration, respectively (Damjanoska et al., 2004). Seven repeated days of DOI treatment decreases G$_{\alpha/q}$ by 47% but does not change G$_{\alpha/11}$ (Psychedelics, 282). In 2008, Shi et. al further showed that chronic treatment with DOI leads to tolerance and functional uncoupling of the 5-HT$_{2A}$R. Shi's group tested 5-HT$_{2A}$R binding in rat hypothalamic paraventricular nucleus (PVN) and found that repeat DOI treatment over 4 or 7 days led to a

50% decrease in 5-HT$_{2A}$R specific binding, and that DOI induced oxytocin and acetylcholine release also decreased with repeat administration (Nichols, 2016, pg. 282). In animal models, tolerance has been observed through progressive diminishments in psychedelic-induced head twitch responses in both ergoline and phenethylamine derived psychedelics (Smith et al., 2014).

Although well established in literature, the precise mechanisms underlying tolerance are not understood (Smith et al., 2014). Many GPCRs are thought to be downregulated through the internalization of agonist bound receptors into intracellular vesicles through scaffolding proteins such as β-arrestin (Bohn & Schmid, 2010). Several studies have examined the role of β-arrestins in downregulating and internalizing 5-HT$_{2A}$, with interesting results. Stimulation with the endogenous agonist, 5-HT, leads to internalization of the receptor only in WT mouse embryonic fibroblast (MEF) cells but not in β-arrestin1/2 KO MEFs (Schmid, Raehal, & Bohn, 2008). However, there were no differences in receptor internalization noted between WT and β-arrestin1/2 KO MEFs upon stimulation by the psychedelic 5-HT$_{2A}$R agonist DOI (Schmid et al., 2008). Additionally, *in vivo* studies in WT mice found that both DOI and 5-HTP administration led to increased phosphorylation of ERK1/2 in the prefrontal cortex. However, in β-arrestin2 KO mice, DOI alone led to increased ERK1/2 phosphorylation (Schmid et al., 2008). Finally, WT and β-arrestin2 KO mice displayed differential behavioral cues when treated with the psychedelic DOI versus the endogenous agonist, 5-HT. Namely, pre-treatment with DOI produces head twitch responses in both WT and KO mice, whereas head twitches were only observed with 5-HTP administration to WT mice (Schmid et al., 2008). These findings indicate that receptor signaling and trafficking is dependent on the type of ligand binding, a concept known as ligand directed signaling (Bohn & Schmid, 2010). Further, it argues that β-arrestins may not be accountable for psychedelic induced tolerance at the 5-HT$_{2A}$R.

Therefore, my study aimed to utilize a 5-HT$_{2A}$ R specific behavioral assay in order to assess behavior that sheds light on receptor activity. Within the head twitch response assay, tolerance can be demonstrated through sequential administration of a psychedelic compound, over the course of several days, during which time animals are monitored for head twitch response behavior. Should the animals exhibit decreasing head twitch responses over the course of these repeat administrations, desensitisation and or tolerance has developed. The use of various kinase and receptor inhibitors and antagonists in combination with psychedelic compounds allowed me to examine the pathway in a somewhat stepwise manner. In doing so, I aimed to identify key regulatory points that might play a role in mediating tolerance.

While there may be limitations to the use of chronic psychedelic administration due to their significant cognitive and neurologic effects, the proper administration and use of these compounds requires a more thorough and well investigated understanding of the mechanisms that underlie the function.

VIII. The Mitogen Activated Protein Kinase, ERK, is a Possible Target for Preventing Tolerance Development

The signaling pathways that mediate the varied genotypic, physiologic, and behavioral changes from psychedelic action are complex and not yet fully understood. However, several studies have identified the Mitogen Activated Protein Kinase (MAPK) pathway as playing a potentially critical role in mediating psychedelic-specific effects. In particular, the MAPK, ERK, has been identified as a potentially significant player in psychedelic action.

In 2005, Dr. Jeff Browning utilized both behavioral and molecular studies to examine the MAP kinase pathway's role in clozapine's antipsychotic function. The MAP Kinase signaling cascade is known to impact several pathways, including MEK/ERK, JNK, and p38 pathways

(Browning et al., 2005). MAPK activation is known to mediate acute, intermediate, and chronic effects on physiologic function. Acutely, MAPK pathways can modulate K+ channels, alter slow Na+ channel inactivation, and impact glutamate receptor functioning (Browning et al., 2005). Intermediately, MAPK affects phosphorylation of the synaptic structural proteins MAP-2 and synapsin I (Browning et al., 2005). In the long term, this pathway also has implications on cell growth, transcription factor activation, cell differentiation, long term potentiation, and learning and memory (Browning et al., 2005). Additionally, it has been shown that serotonergic agents, including the atypical antipsychotic clozapine and the 5-HT$_{2A}$R agonist DOI both influence MAPK, which in turn alters cell function and behavior (Browning et al., 2005). For example, pre-treatment with the MEK1/2 inhibitor SL327 reversed the atypical antipsychotic clozapine's effects in Conditioned Avoidance Response (CAR) studies in mice (Browning et al., 2005). Browning argued that ERK signal transduction also plays an important role in mediating antipsychotic function. Collectively, the varied roles that MAPK plays in regulating physiologic functions and its specific response to 5-HT$_{2A}$R modifications point towards this pathway as a one that warrants further study. While the mechanisms behind psychosis, antipsychotic medications, and tolerance are not fully understood, various studies have pointed towards the MAP Kinase pathway as playing a key regulatory role. Because of this, I hypothesized that ERK may also play a role in mediating the downregulation and/ or desensitization of the 5-HT$_{2A}$R upon psychedelic activation. This hypothesis was the jumping off point for a series of studies examining the 5-HT$_{2A}$R signaling pathway's role in tolerance development.

IX. Rationale for this Study:

Psychedelic have become more frequently studied as both therapeutic targets and research tools to treat and study neuropsychiatric disorders. Despite promising findings, the

mechanisms behind certain psychedelic-mediated effects and phenomena such as tolerance are not fully understood. This project aims to utilize the robust, 5-HT$_{2A}$R specific Head Twitch Response behavioral assay to study tolerance *in vivo* in order to shed light on these mechanisms, and to hopefully contribute towards the scientific community's overall understanding of psychedelic action. Interestingly, tolerance at the 5-HT$_{2A}$R has been shown to occur as the result of both agonist and antagonist action at the receptor in human and in animal models. Thus, better understanding of the cellular mechanisms that contribute to tolerance would increase the overall understanding of the 5-HT$_{2A}$R's function. Additionally, psychedelic-induced tolerance poses obstacles towards the use of chronic therapeutic psychedelic use. Thus, tolerance at the level of the 5-HT$_{2A}$R must be better understood.

Several promising studies have pointed towards ERK as an important signaling checkpoint in mediating short, intermediate, and long term effects related to 5-HT$_{2A}$R activation. Therefore, my study started by hypothesizing that this intracellular signaling point may play a role in mediating or preventing tolerance. Through follow-up studies, I then examined various points along the 5-HT$_{2A}$R's signaling pathway to assess the role they played in mediating behavioral effects and psychedelic-induced tolerance. While the promising nature of psychedelic compounds' use as research tools and therapeutic agents, it is necessary to fully understand the complex signaling mechanisms that mediate the physiologic responses.

E. Chapter 2: Materials and Methods

Animals:

Adult (10-20 weeks old) male WT c57bl/6 mice were used for all locomotion and head

twitch response studies. Mice were ordered from Charles River. All mice were housed in a

vivarium in cages with up to five other mice on a twelve-hour light/dark cycle at 23 °C,

excluding experimental days. Mice were provided with food and water *ad libitum* at all times

other than during experimental testing. Routine monitoring and veterinary care were provided

with the assistance of Virginia Commonwealth University's Division of Animal Research staff.

All experiments and housing guidelines followed NIH guidelines and were approved by Virginia

Commonwealth University's Institutional Animal Care and Use Committee. All efforts were

made to minimize animal suffering and the number of animals used in these experiments.

Drugs:

The IUPAC names and sources of drugs used for these procedures and experiments are as
follows:
1. DOI:
    a. IUPAC Name: 2,5-Dimethoxy-4-iodoamphetamine
    b. Manufacturer: Sigma Aldrich
2. SL327
    a. IUPAC Name: (Z)-3-amino-3-(4-aminophenyl)sulfanyl-2-[2-
       (trifluoromethyl)phenyl]prop-2-enenitrile
    b. Manufacturer: Tocris
3. Clozapine -
    a. IUPAC Name: 8-chloro-11-(4-methylpiperazin-1-yl)-5H-
       dibenzo[b,e][1,4]diazepine
    b. Manufacturer: Tocris
4. U73122 -
    a. IUPAC Name: 1-(6-((3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-
       pyrrole-2,5-dione
    b. Manufacturer: Apex Bio
5. Bisindolylmaleimide II -
    a. IUPAC Name: 3-(1*H*-Indol-3-yl)-4-[1-[2-(1-methyl-2-pyrrolidinyl)ethyl]-1*H*-
       indol-3-yl]-1*H*-pyrrole-2,5-dione

        b.   Manufacturer: Tocris
6. M100,907
        a.   IUPAC Name: (R)-(2,3-Dimethoxyphenyl)(1-(4-fluorophenethyl)piperidin-4-yl)methanol, Volinanserin
        b.   Manufacturer: Tocris
7. Ketamine:
        a.   IUPAC Name: 2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one
8. Xylazine:
        a.   IUPAC Name: N-(2,6-dimethylphenyl)-5,6-dihydro-4H-1,3-thiazin-2-amine

Drugs were dissolved in either 0.9% NaCl or DMSO based on solubility guidelines. Injection volumes and concentrations were determined by the mice's body weight (0.001-0.05 mL/gram). All drugs were administered i.p., based on previous literature demonstrating each drug's successful i.p. administration. Any reference to vehicle refers to the solvent into which drugs were dissolved - either 0.9% NaCl or DMSO. Each vehicle dose was administered at an equivalent volume during experimental testing.

Locomotion Studies:

All newly delivered mice were allowed at least three days acclimation time before undergoing any locomotion studies. This acclimation period was done to ensure that stress from the transport process was minimized and did not impact locomotor function or other behaviors. Locomotion chambers were composed of 16 x 16 inch open-field plastic cages with an automated detection system composed of 16 infrared light beam arrays in the X and Y axes ("Open Field- Locomotor Activity", 2016). These chambers were connected to measurement software, Fusion, which measures motion via disruptions to these light beams as the mouse moves throughout the chamber. These interrupts are detected and can be quantified into metrics such as horizontal/ vertical activity, rearing, turning, and total distance ("Open Field- Locomotor Activity", 2016).

Animals were placed into the open-field cages and monitored for 5 minutes to allow them to acclimate to the environment. Mice were then injected with either vehicle or SL327 50 mg/kg i.p. and then monitored for 90 minutes to examine the effect of this compound on overall motor activity. Cages were thoroughly cleaned with a diluted Roccal-D solution and water between trials to eliminate odor cues from other animals. On Day 2, mice were again allowed 5 minutes to acclimate and were then monitored for 90 minutes to assess whether SL327 treatment on Day 1 had an impact on locomotion on Day 2. Data from these locomotion studies was collected on Fusion Software.

Ear Tag Production and Placement:

In order to run HTR assays, mice require placement of magnetic devices that allow for voltage change detection. Recent refinements to the HTR behavioral assay were made by Dr. Mario de la Fuente Revenga, which further improved upon modifications made by Dr. Halberstadt a few years prior (de la Fuente Revenga, 2019; Halberstadt, 2013). These modifications both decreased the invasiveness required to collect HTR data and improved the rate at which data can be processed (de la Fuente Revenga, 2019). Previously, HTR detection systems required the surgical implantation of a magnetic device onto the top of mice's skulls, an invasive procedure that requires longer recovery time (de la Fuente Revenga, 2019). However, recently validated modifications to the measurement system allowed for equivalent accuracy by placing magnetic devices onto colored animal identification ear tags (de la Fuente Revenga, 2019).

Magnetic ear tag devices were created by attaching small, circular neodymium magnets to the end of standard colored ear tags with superglue. These were affixed to the ear tags with north polarity facing upwards on the tag. In an attempt to mitigate inflammatory processes

around the ear from implantation of the magnetic devices, each tag was coated in a clear coating of Seche Vite brand fingernail polish. This additional step was added due to concerns over inflammatory processes around the ears impacting experimental results. We believed this additional layer may slow the onset of these changes and allow for longer animal testing.

Once the tags were created, mice were weighed and placed under anesthesia using ketamine 100mg/kg i.p. as anesthetic and xylazine 10mg/kg i.p. as an analgesic. Ophthalmic ointment was applied to the mice's eyes to prevent ocular dryness or injury during the procedure and recovery stage. After anesthesia was confirmed using foot pinch, the magnetic ear-tag devices were attached bilaterally to the animals' ears. Mice were monitored and provided with warming during the anesthesia recovery process. Mice were then allowed one week to recover from the procedure before experimental testing began.

Head Twitch Response:

HTR experiments previously required trained coders to view and manually record each head twitch incidence during an experimental trial. This method, while effective, was time-intensive and prone to error due to bias or false positive/ negative HTR identification. A newly updated system provides a fully-automated and high throughput measurement system that takes advantage of characteristic frequency and voltage changes that occur during a head twitch. These patterns have been demonstrated to differ from other activities such as grooming, jumping, and rearing (de la Fuente Revenga, 2019).

The HTR chambers are composed of 11 cm diameter x 14 cm tall plastic chambers that have been wrapped in non-overlapping ~500-turn 30 AWG enameled wire. The terminals of each coil are connected to a phono preamplifier (Pyle PP444). At this time, a total of 6 mice can

be run during one trial, one mouse per chamber. Mice are placed into these chambers and recorded for 30 minutes to measure basal activity and allow for acclimation to the chamber. Mice were then administered drug combinations and monitored for 90 minutes. During their time in the chambers, head and body movements are amplified and recorded as changes in voltage occurring with varying frequencies. Dr. de la Fuente Revenga utilized spectrography and identified two characteristic frequency ranges corresponding with HTR, at 40-50 Hz and 80-100 Hz (de la Fuente Revenga, 2019). The higher frequency (80-100 Hz) detected during each HTR event had no overlapping behaviors such as jumping, grooming, rearing, or ambulation. However, the 40-60 Hz range does overlap with these behaviors (de la Fuente Revenga, 2019). Thus, the amplified signal filters and searches for HTR using a higher, 70-110 Hz, frequency range. The amplified signal is recorded through MATLAB software. These wavelet signals are transformed into unipolar peaks that are then identified as true HTR events based on three criteria: 1. The peak voltage prominence is greater than 0.075 V, 2. The event is separated from any other event by at least 200ms, 3. The width of the signal is less than 90 ms at half the maximum value of prominence (de la Fuente Revenga, 2019).

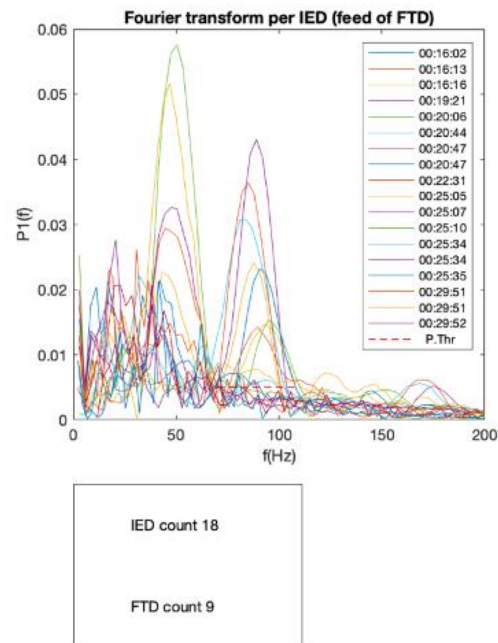**Detection of raw head twitch response versus filtered signal**

Figure 3: Characteristic frequency ranges that lead to automated detection of head twitch response in mice Image taken from a processed signal on MATLAB, demonstrating the frequency range and time markers for HTR events during a fifteen-minute fraction. Frequency ranges (X axis) for each line indicate head rotations occurring due to a given head movement. The HTR characteristically occurs within the 40-60 and 80-100 Hz range, as is demonstrated here. However, the automated HTR system searches for signals that fall within a 70-110 Hz frequency range. These raw signals are denoted as IED. IEDs are then filtered through three additional criteria to ensure accurate signals, denoted as FTDs. These nine signals correspond to nine validated head twitches that occurred during this fifteen minute time span.

Mice were tested at least one week apart to ensure that undesired tolerance did not develop. Previous studies by Smith in 2014 showed that tolerance to DOI induced HTR did not develop when DOI 1mg/kg was administered every seven days (Smith et al., 2014).

Statistical Analysis:

Data from the HTR system was first processed using Matlab software. Data from Locomotion studies were analyzed by Fusion software. Data from Fusion was compiled into a

large data set that measured activity based on several different metrics, including horizontal activity count. This metric was selected as a measure of locomotor function. Results from both HTR and locomotion studies were input into Prism Graphpad version 8 for image processing and statistical analysis.

In order to assess the effect of time and treatment type (my independent variables) on HTR (my dependent variable), 2-Way ANOVA was used. When appropriate, Bonferroni multiple comparisons post hoc test was used for additional statistical analysis to strengthen the statistical power and to examine several points of comparison. When comparing locomotor activity by treatment type, t-tests were used to compare total locomotor activity in drug versus vehicle treated animals. Significance level set at $p < 0.05$. All data, except where otherwise indicated, will be presented as mean $\pm$ S.E.M.

F. Chapter 3: Results

Figure 4: SL327's Impact on Locomotive Function in Male c57bl/6 WT Mice

Prior to initiating HTR experiments, I tested a 50mg/kg dose of SL327 on male c57bl/6 WT mice in a locomotion study. This test was performed in order to assess whether SL327 alone impacted locomotor activity on the same day of treatment and 24 hours following treatment when compared to vehicle treated animals. Because the HTR assay measures head movement in an automated fashion, locomotion studies aided in evaluating whether changes to the HTR could be due to generally diminished or enhanced motor activity versus a behavior-specific effect. Mice (n=4) were placed in a 16x16-inch open field locomotion chamber and monitored for 5 minutes while acclimating to cages. Mice were then injected with either 50mg/kg SL327 i.p. or vehicle (DMSO) and monitored for 90 minutes. Mice were then returned to their cages until re-testing 24 hours later.

In order to assess whether SL327 treatment exerted residual locomotive effects, mice (n=4) were placed into 16x16 inch locomotor chambers 24 hours after treatment with either SL327 or vehicle and allowed to acclimate for 5 minutes. Mice were not given any treatment on Day 2, and were again monitored for 90 minutes to test for changes in total horizontal activity between treatment groups on Day 1. Of note, outlier tests identified one outlier in the vehicle treated group.
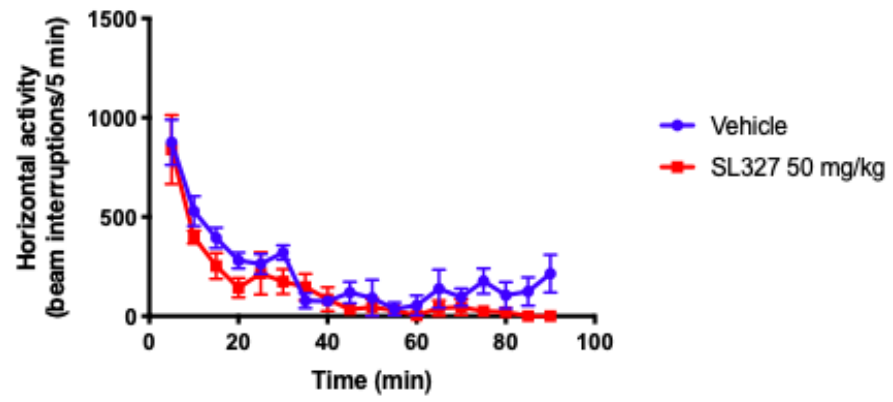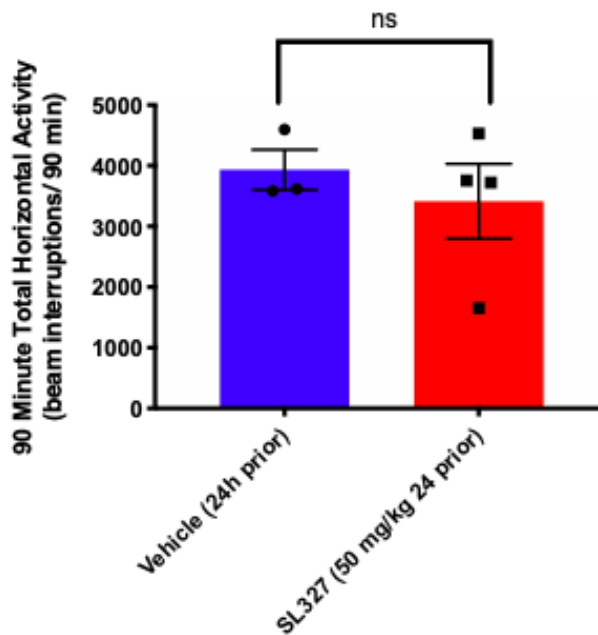
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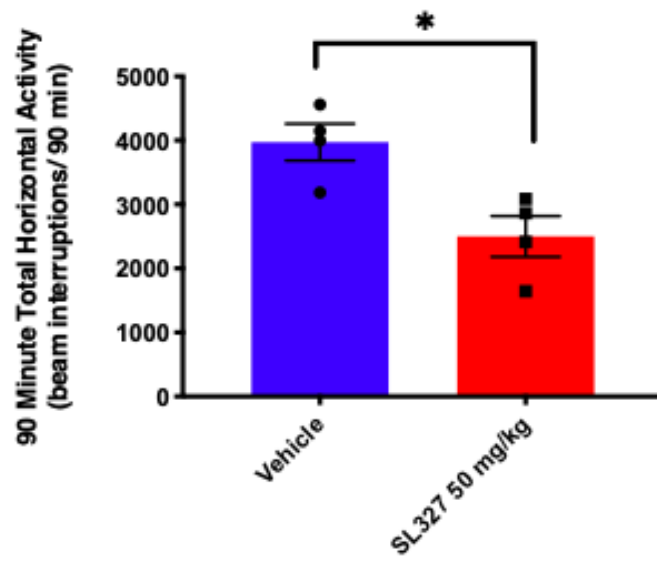
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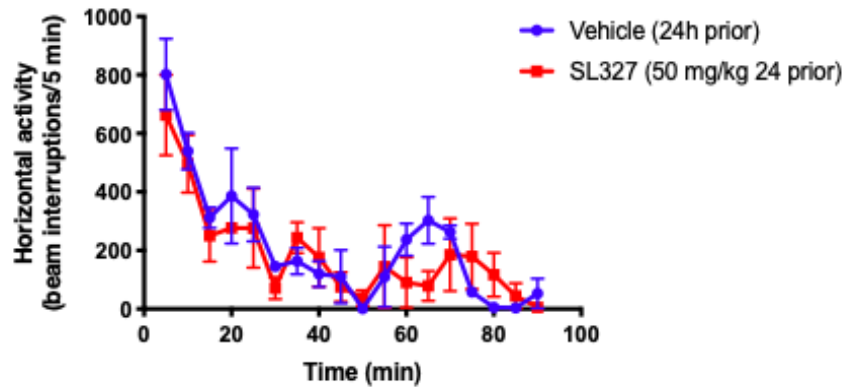
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Figure 4: Male c57bl/6 WT mice were monitored in locomotion chambers for 90 minutes immediately following treatment with either SL327 50mg/kg i.p. or vehicle. **(A)** Horizontal activity over a 90-minute time course, measured in five minute fractions**. (B)** 90-minute total horizontal locomotor activity. Locomotor activity was measured using horizontal activity on Fusion software by Omnitech Electronics. Unpaired t-test analysis comparing vehicle versus SL327 treated animals on Day 1 reveals a statistically significant difference (p= 0.0141). **(C)** 90-minute time course was measured and plotted in 5-minute fractions, showing total horizontal activity. **(D)** 90 minute total horizontal activity 24 hours following treatment with either SL327 or vehicle revealed similar locomotor activity between groups, with no statistically significant difference between groups (p=0.5361) by unpaired t-test. Data are presented as mean ±S.E.M.

Despite the small sample size (n=4) in this trial, my results indicate that SL327 treatment did have a significant impact on overall total locomotion on Day 1. Mice treated with SL327 50mg/kg had an overall diminished amount of horizontal activity over 90 minutes when compared to mice treated with vehicle, DMSO. This was demonstrated through an unpaired t test comparing treatment type's effect on locomotor activity, which revealed a significant p value of 0.0141 However, 24 hours following treatment with SL327 or vehicle, there was no statistically significant difference between treatment groups' locomotor activity by unpaired t test, with a p value of 0.5361.

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Figure 5: Residual Effect of SL327 treatment on DOI induced HTR in male c57bl/6 WT mice

I wanted to test if treatment with SL327 alone resulted in any modulation of the HTR in mice. Additionally, I hoped to assess whether treatment with SL327 would have any residual effects when both treatment groups were administered DOI 24 hours later. Male c57bl/6 mice (n=3) were placed into HTR coils and monitored for 30 minutes to assess basal activity. On Day 1, mice were treated with either SL327 50 mg/kg i.p. or an equivalent volume of vehicle i.p., then monitored for 90 minutes in HTR chambers to assess function. On Day 2, mice were again monitored for 30 minutes to assess basal function. All mice were then treated with DOI 2mg/kg and monitored for 90 minutes to assess HTR over this time course. I hypothesized that on Day 1, in the absence of any psychedelic to induce HTR, both treatment groups would exhibit minimal head twitch response behavior. However, I hypothesized that SL327 treatment may lead to
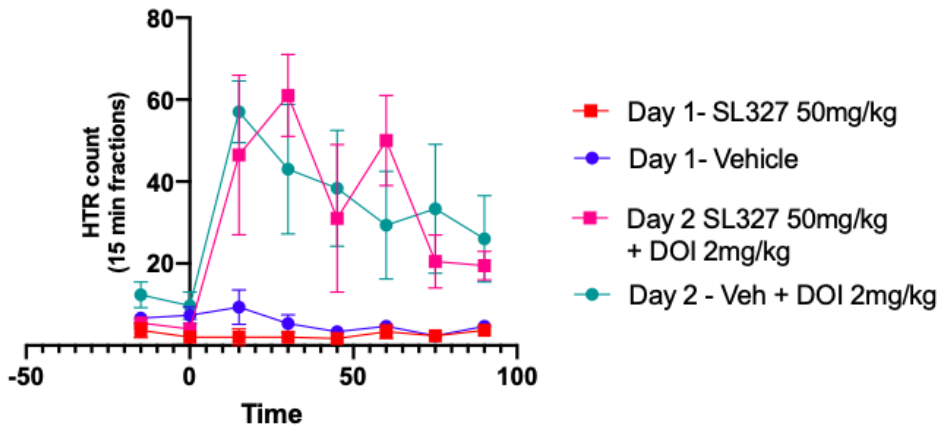
residual signaling effects such that 24 hours later, DOI administration would have a diminished

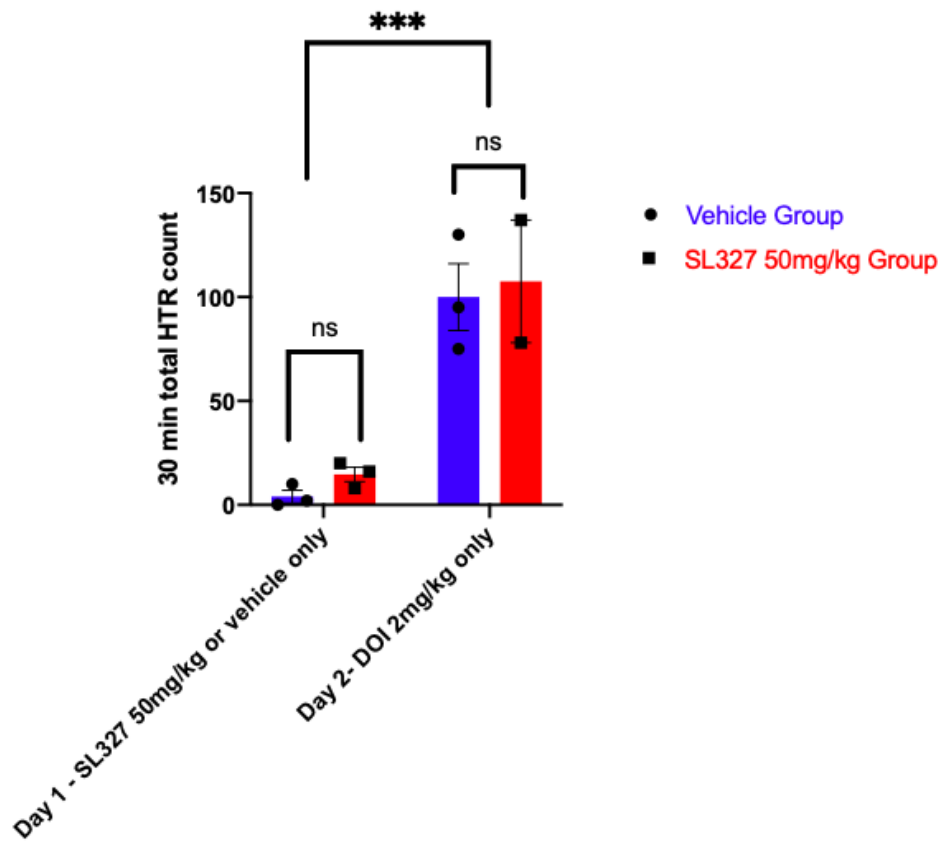effect compared to vehicle treated mice.
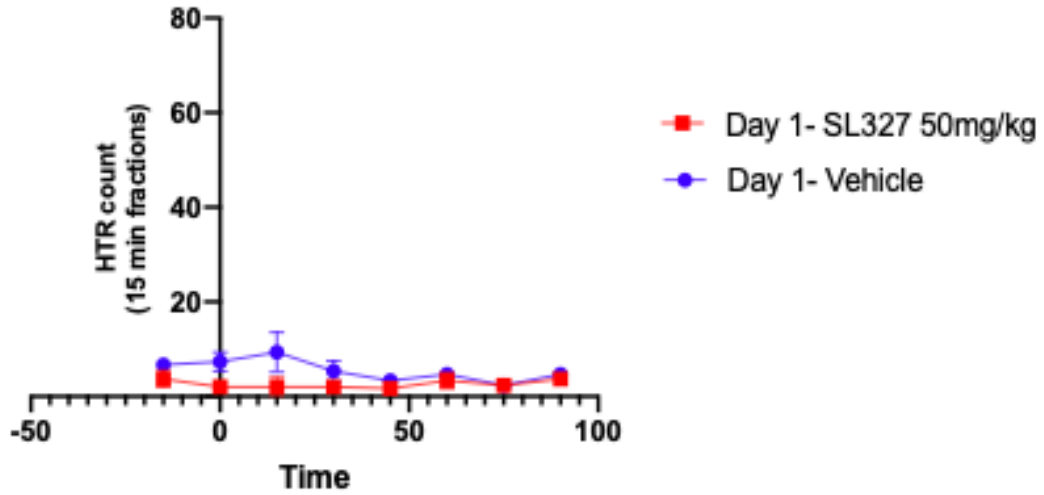
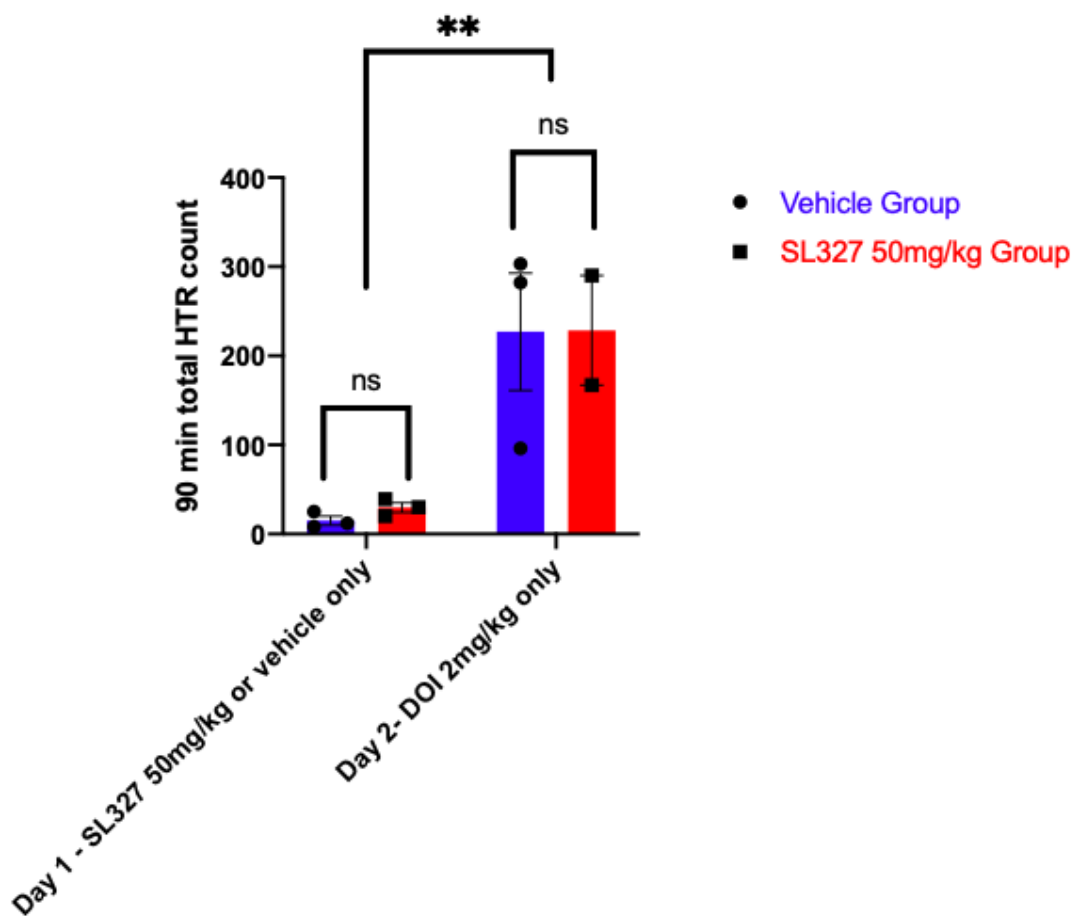

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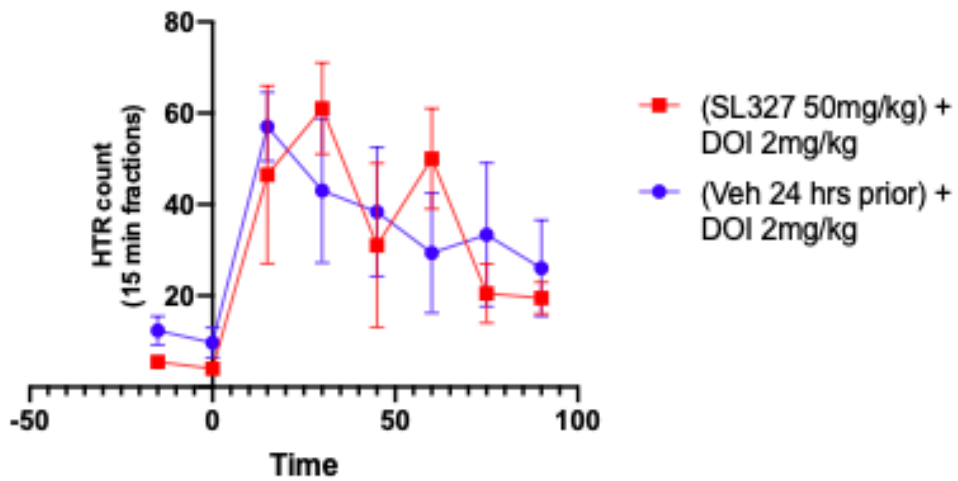
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Figure 5: Control experiment testing the effect of SL327 vs vehicle treatment alone on WT male c57bl/6 mice (Day 1) and post DOI 2mg/kg treatment 24 hours later (Day 2) **(A):** Time course displaying pre-treatment HTR activity and post-treatment HTR activity, in 15 minute fractions; **(B)** Day 2 90 minute time course, displayed in 15 minute fractions; **[C]** Combined 90 minute time course, Day 1 and Day 2, in 15 minute fractions; **(D)** 30 minute total HTR on Day 1 versus Day 2. 2 Way ANOVA revealed a significant difference by treatment Day [$F_{(1,7)}$ 48.6, p = 0.0002], but insignificant changes between treatment groups on each day [$F_{(1,7)} = 0.45$, p=0.5238]; **(E):** 90 minute total HTR on Day 1 vs Day 2. 2 Way ANOVA revealed an overall statistically significant difference with respect to treatment Day [$F_{(1,7)}= 23.26$, p = 0.0019]. However, treatment type had a statistically insignificant effect [$F_{(1,7)}=0.03602$, p = 0.8549] by 2 Way ANOVA. Data are presented as group mean ±S.E.M.

Statistical analysis of the 30 and 90 minute total HTR via 2 Way ANOVA showed that

treatment day had a statistically significant effect on HTR over 30 minutes (p=0.0002) and 90

minutes (p=0.0019). Treatment day correlated with whether or not animals received DOI, and as expected mice only displayed large amounts of HTR behavior when administered a psychedelic 5-HT$_{2A}$R agonist. Pre-treatment type (vehicle versus SL327) had statistically insignificant effect on HTR by 2 Way ANOVA over 30 (p= 0.5238) and 90 minutes (p= 0.8549). Bonferroni post-hoc analysis tested for statistical significance between various treatment groups and days, and found that although there was an insignificant difference between treatment groups on Day 1 and on Day 2, the differences between Day 1 and Day 2 were statistically significant. These findings indicate that SL327 treatment alone does not induce HTR behavior when compared to vehicle treated animals. Additionally, because animals had similar responses when DOI was given on Day 2, my results point towards SL327 as not exerting residual effects that lasted 24 hours and altered DOI-induced behavior. However, it should be noted that this study similarly had a small n (n=3), and thus further testing and validation is required.

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Figure 6: Effect of SL327 pre-treatment on DOI-induced HTR and DOI induced tolerance in male c57bl/6 Mice

Wild Type male c57bl/6 mice (n=12) were placed into HTR chambers and monitored for 30 minutes to assess basal HTR activity and allow for acclimation to the chambers. Mice were then pre-treated with either vehicle (DMSO) or SL327 50mg/kg i.p.. 5 minutes later, DOI 2mg/kg i.p. was administered to all animals. Mice were monitored in the HTR chambers for 90 minutes. 24 hours following their initial treatment with either SL327 50mg/kg + DOI 2mg/kg or vehicle + DOI 2mg/kg, mice were again tested to assess if SL327 impacted the development of tolerance that is known to occur with repeat DOI treatment. To test this, mice were placed into HTR chambers and monitored for 30 minutes. Mice were then administered 2mg/kg of DOI i.p. And monitored for 90 minutes. Taken together, my results from Day 1 indicate that SL327 does

significantly attenuate the HTR in mice who are treated with DOI. However, this MEK inhibitor fails to significantly impact the tolerance induced after repeat administration of DOI on Day 2. This finding led me to explore other compounds that impacted signaling along the 5-HT$_{2A}$ pathway in order to determine whether or not one of these steps serves as a potential regulatory point.
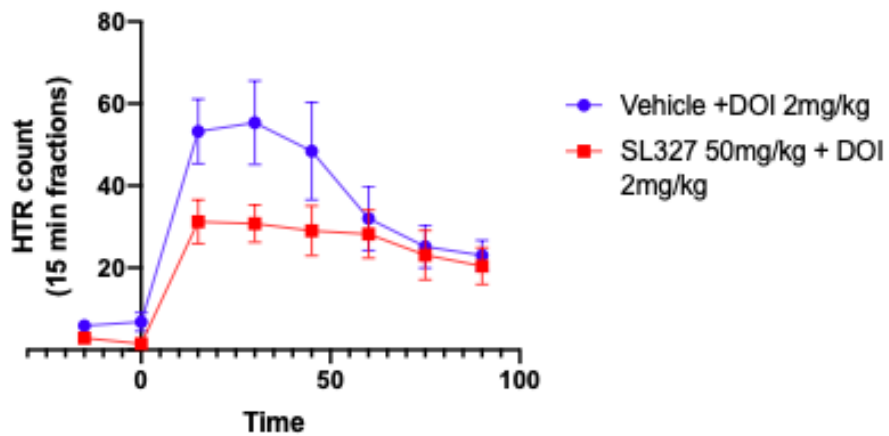
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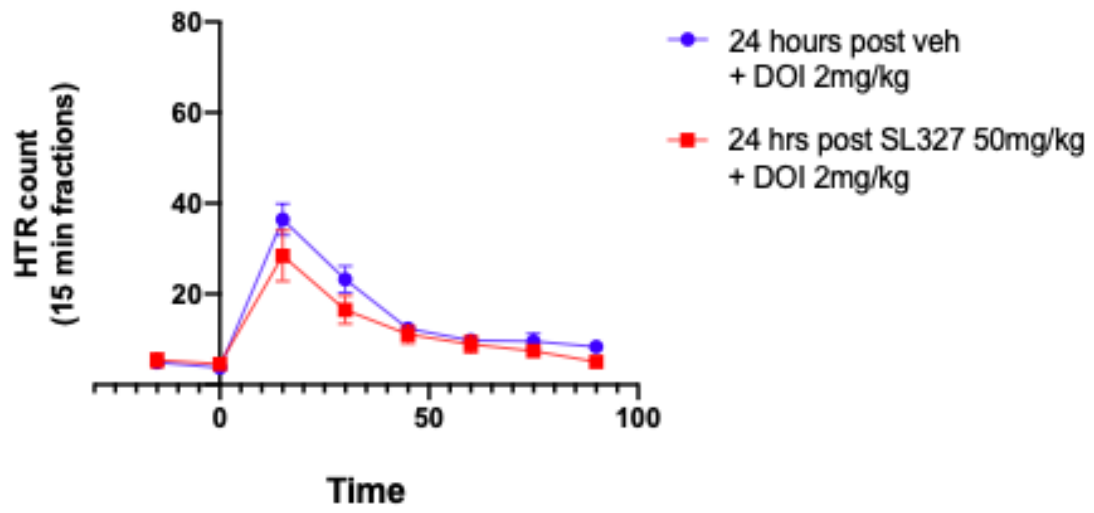
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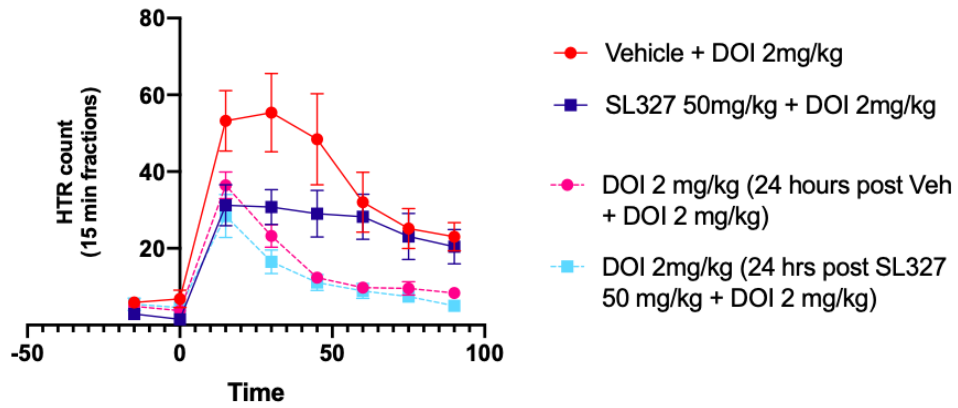
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Figure 6: Effect of SL327 50mg/kg pre-treatment on DOI-induced HTR in male WT c57bl/6 mice. Male c57bl/6 WT mice were administered SL327 50mg/kg or vehicle and monitored for 30 minutes. All mice were then injected with DOI 2mg/kg i.p. And monitored for 90 minutes. On

Day 2, mice received no pre-treatment but were again monitored for 30 minutes, after which they were again treated with DOI. **(A)** Time course displaying DOI induced HTR over 90 minutes on Day 1, displayed in 15 minute fractions; **(B)** Time course di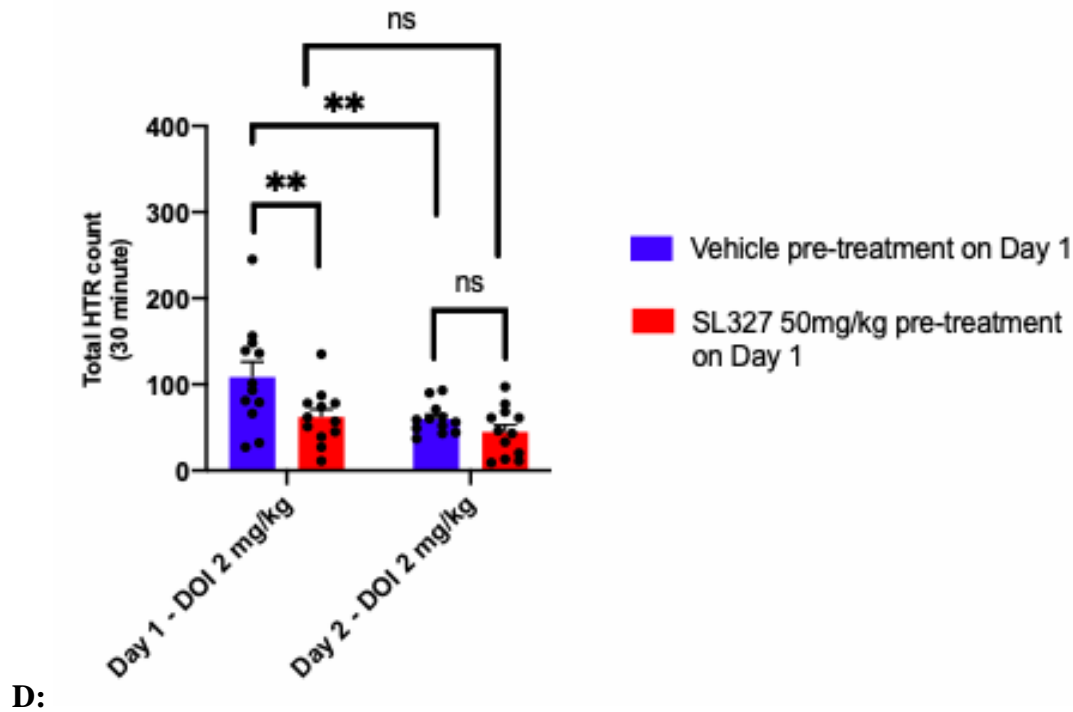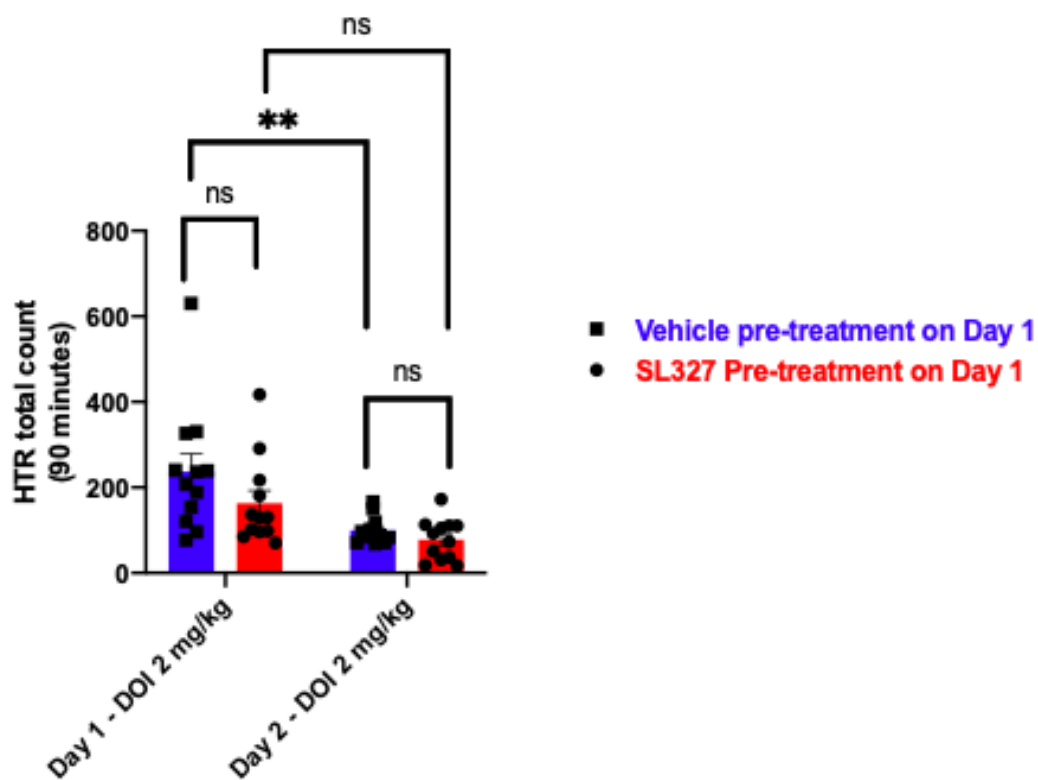splaying DOI induced HTR over 90 minutes on Day 2, displayed in 15 minute fractions; **[C]** Combined Day 1 and Day 2 90 minute time course; **(D)** 30 minute total HTR over 30 minutes following DOI administration on Day 1 and Day 2. 2 Way ANOVA reveals that pre-treatment with SL327 led to a statistically significant attenuation of HTR [$F_{(1,44)} = 7.739$, p= 0.0079] and a significant difference between HTR elicited on Day 1 versus Day 2 [$F_{(1,44)}$ 8.961, p= 0.0045]. Post hoc analysis confirms a significant difference between treatment groups on Day 1 (p= 0.0092) but no significant difference between treatment groups on Day 2 (p=0.6985); **(E)** Total HTR over 90 minutes, Day 1 and Day 2. On Day 1, SL327 pre-treated animals had a diminished but not statistically significant difference over 90 minutes [$F_{(1,44)}$= 3.169, p= 0.0820]; post-hoc analysis similarly revealed no statistically difference between treatment groups on Day 1 (p= 0.1185) or Day 2 (p= > 0.9999). Data are presented as mean +/- S.E.M.

Statistical analysis assessing for Time (treatment day) and Pre-treatment type (SL327 or vehicle) effects on HTR were tested for using 2 Way ANOVA. This revealed a statistically significant effect with regard to treatment type over 30 minutes (p=0.0079). Over 90 minutes, pre-treatment type had a noticeable but not statistically significant difference (p=0.0820). Time, however, had a significant effect over both 30 (p=0.0045) and 90 (p=0.0002) minutes. Bonferroni post-hoc analysis was run to allow further comparisons between treatment groups, indicated in Figures 5D and 5E. The significant difference between vehicle and SL327 pre-treated groups over 30 minutes on Day 1 (p=0.0275) aligns with ANOVA analysis and indicates that MEK1/2 inhibition did translate to a diminished behavioral effect. Additionally, the significant difference between the vehicle group on Day 1 and Day 2 (30 minute total p=0.0182) indicate that tolerance did develop with repeat DOI administration. Overall, my results indicate that MEK1/2 inhibition successfully attenuates the acute HTR behavior elicited by DOI. This points towards the MAP Kinase pathway as potentially important in mediating HTR behavior, and possibly psychedelic action. However, we were unable to successfully prevent tolerance

development, and thus aimed to examine other signaling points to see whether or not they could

prevent tolerance.

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Figure 7: Effect of Bisindolylmaleimide-II pre-treatment on DOI-induced HTR and DOI induced tolerance in male c57bl/6 mice

I first examined the role that Protein Kinase C (PKC) plays in mediating HTR behavior

acutely and in mediating tolerance. This particular checkpoint was selected due to earlier studies

that pointed towards its role in mediating 5-HT$_{2A}$R internalization. Firstly, studies have shown

that PKC activation is sufficient to produce 5-HT$_{2A}$R internalization (Nichols, 2016).

Additionally, other studies have shown that DOI requires PKC activation in order to cause

receptor internalization (Nichols, 2016). Because 5-HT$_{2A}$R internalization is commonly seen

with tolerance, thereby leading to a diminished number of available receptors, we hypothesized

that PKC inhibition would both acutely diminish HTR behavior and would prevent tolerance
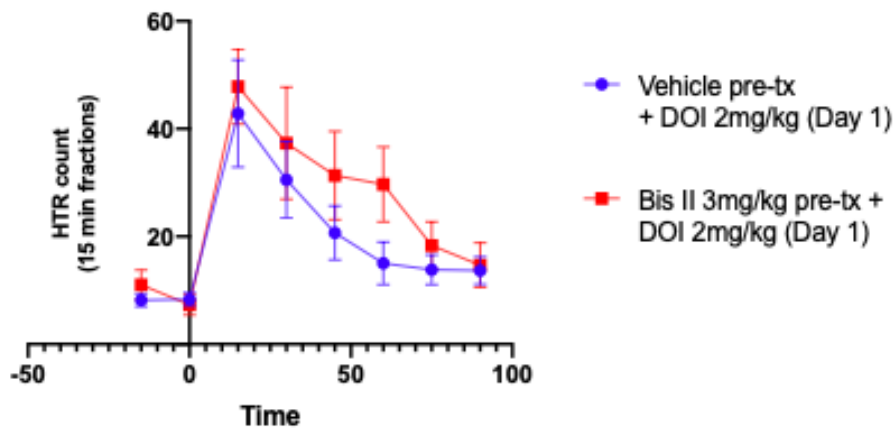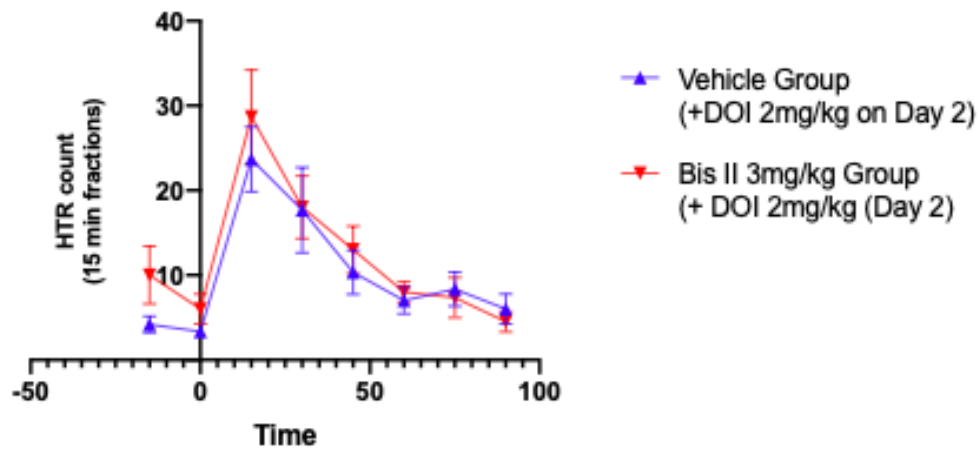
development.

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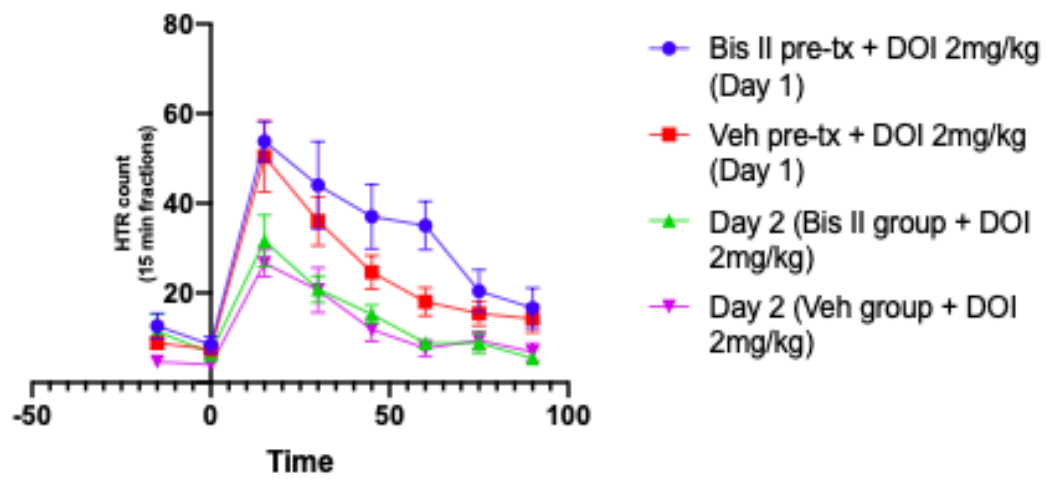
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Figure 7: Effect of Bisindolylmaleimide-II pretreatment on DOI-induced HTR in male c57bl/6 WT mice. HTR results are shown for mice pre-treated with vehicle (n=12) or Bisindolylmaleimide-II (5mg/kg) i.p., on Day 1 followed by DOI (2mg/kg) i.p. 5 minutes later. 24 hours later, mice were again monitored to assess basal activity for 30 minutes, and then again
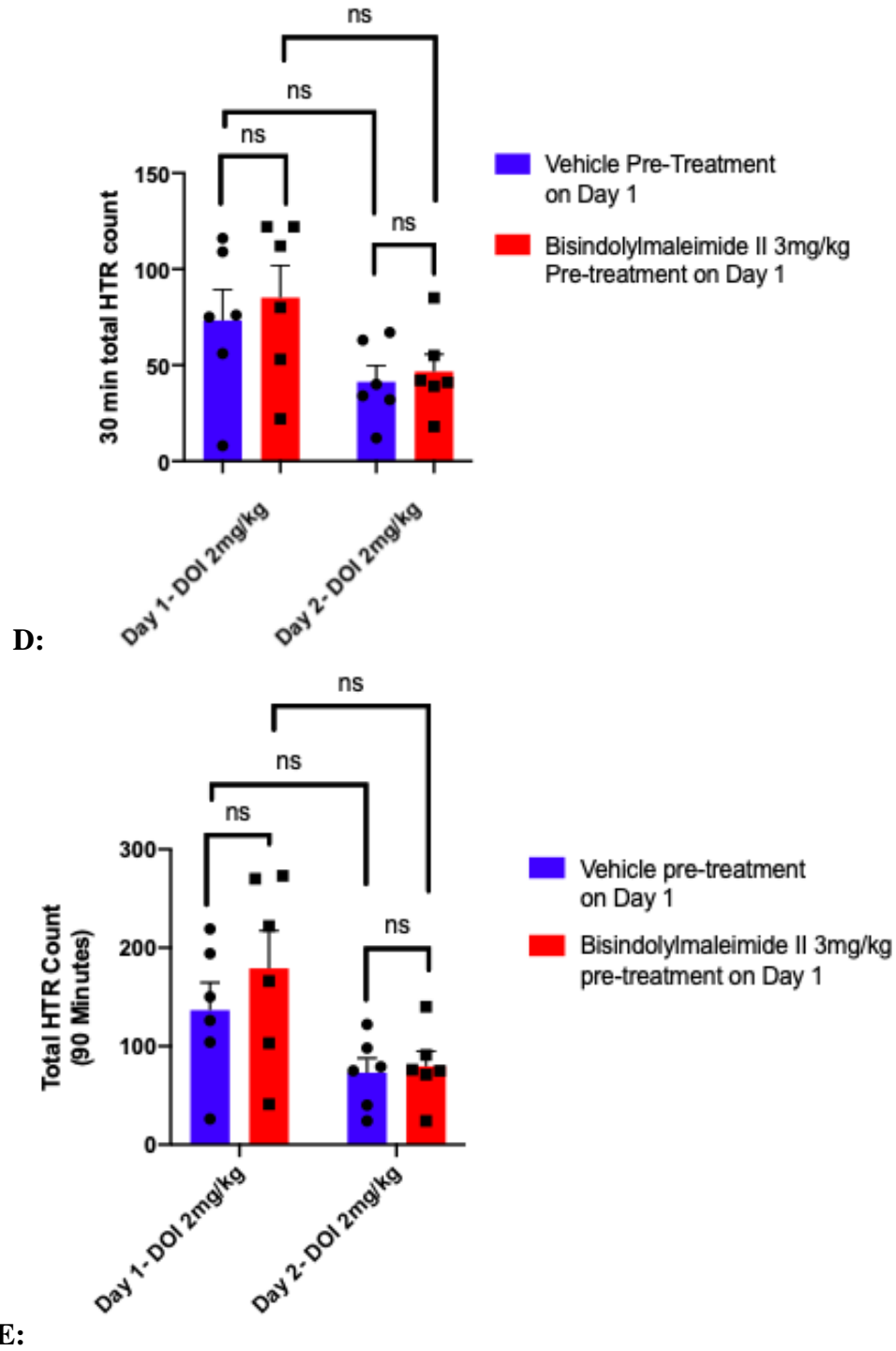
administered DOI 2mg/kg i.p. Mice were then immediately monitored for 90 minutes. **(A)** 90-minute time course with tracings from Day 1; **(B)** 90 minute time course, in fifteen minute fractions, for HTR elicited o**n** Day 2; **[C]** Combined 90 minute time course for HTR elicited on Day 1 and Day 2; **(D)** Total HTR over the first 30 minutes following drug administration, Day 1 vs. Day 2. 2 Way ANOVA reveals no significant difference due to pre-treatment type [$F_{(1,20)}$ = 0.4258, p=0.5215] However, there was a significant difference due to time (treatment day), indicating that tolerance had developed [$F_{(1,20}$ = 7.182, p=0.0144]; **(E)** Total HTR count over 90 minutes following drug administration, Day 1 vs Day 2**.** Two-way ANOVA revealed no significant difference due to pre-treatment type [$F_{(1,20)}$ = 0.8936, p=0.3558]. However, a significant difference was noted due to time [$F_{(1,20)}$=9.841, 0.0052]; Data are presented as group mean ± S.E.M.

Over both 30 and 90 minutes, time (treatment day) did have a statistically significant effect on HTR by 2 Way ANOVA (30 minute p=0.0144, 90 minute p= 0.0052). Both treatment groups showed an overall reduction in HTR behavior between Day 1 and Day 2, indicating that tolerance developed. However, pre-treatment type was found to be insignificant over both 30 and 90 minutes by 2 Way ANOVA (30 minute p= 0.5215; 90 minute p=0.5909). Bonferroni post-hoc analysis allowed for further analysis between treatment groups and days. Interestingly, this analysis revealed no statistically significant relationships across all tested relationships.

Effect of U73122 pre-treatment on DOI-induced HTR:

I originally planned to test the impact that a further upstream blockade had the acute HTR on Day 1 and on tolerance on Day 2. I planned to use U73122, a PLC inhibitor, to investigate PLC's role in mediating HTR behavior and tolerance. Previous studies by Schmid (2008) tested U73122 in MEFs and found that PLC inhibition with U73122 completely prevented PLC signaling activated by DOI administration (Schmid et al., 2008). Additionally, they found that DOI stimulates ERK phosphorylation in a PLC dependent manner (Schmid et al, 2008). Given

promising findings regarding ERK's role in acutely mediating HTR and these earlier studies, we hypothesized that PLC may play a regulatory role in mediating tolerance.

Unfortunately, the results from this study do not shed much light on tolerance but do hint that either the dose of U73122 or the combination of this dose and large DMSO volume required to administer U73122 i.p. is likely toxic in mice. Mice displayed little to no HTR activity based on the automated detector and, based on my visual observation, very little overall motor activity during the 90 minutes they were tested. I continued to monitor mice after testing and noted them to be awake and responsive but slow to move. The day after testing, 3 out of 5 mice that were treated with U73122 died and one of the vehicle-treated mice died. It should be noted that the results of these trials were likely impacted by the solvent that had to be used for this highly hydrophobic drug, U73122. To both administer the proper dose and ensure proper solubility in DMSO, mice received anywhere from 250-300 µL of U73122 in DMSO or vehicle (DMSO). This produced visibly significant impacts on behavior and overall mobility within minutes after mice were treated on day 1. It was decided to not proceed with further testing on Day 2.

Given the solubility issues I faced with this compound, I would consider using lower doses of the U73122, alternate administration methods, or a different type of PLC inhibitor with better solubility.

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Figure 8: Effect of M100,907 effect on DOI induced HTR and DOI induced tolerance in male c57bl/6 WT mice

M100,907 is a highly selective 5-HT$_{2A}$R antagonist that was used to study the effects of blockade at the receptor level. It has been shown to block HTR acutely, and I hypothesized that pre-treatment with this 5-HT$_{2A}$R antagonist would acutely decrease HTR due to decreased

efficacy of DOI, our receptor agonist. Additionally, I hypothesized that tolerance would fail to

develop on Day 2 due to M100,907's blockade 24 hours prior. This study was performed as a

sort of control, based on the theory that preventing DOI binding at the 5-HT$_{2A}$R acutely on Day 1

would translate to a stronger response on Day 2, when compared to vehicle treated groups.
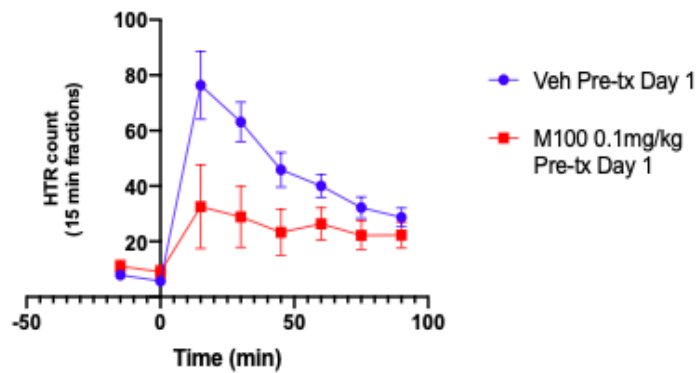
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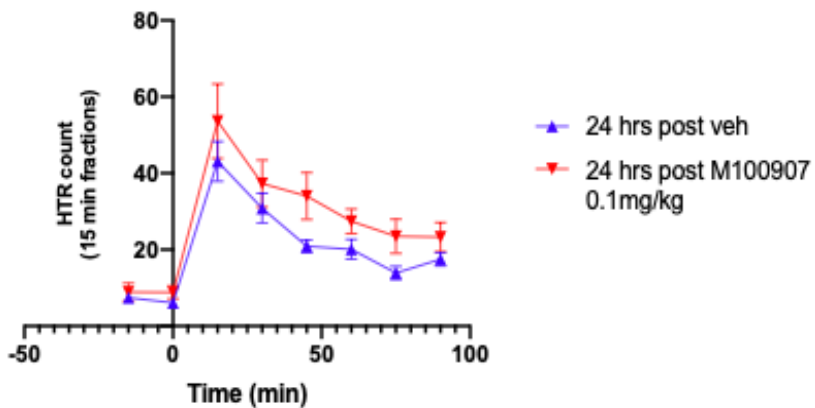
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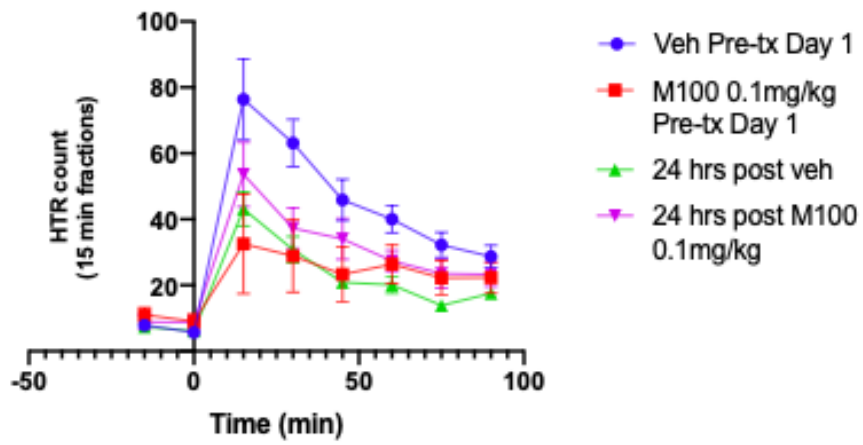
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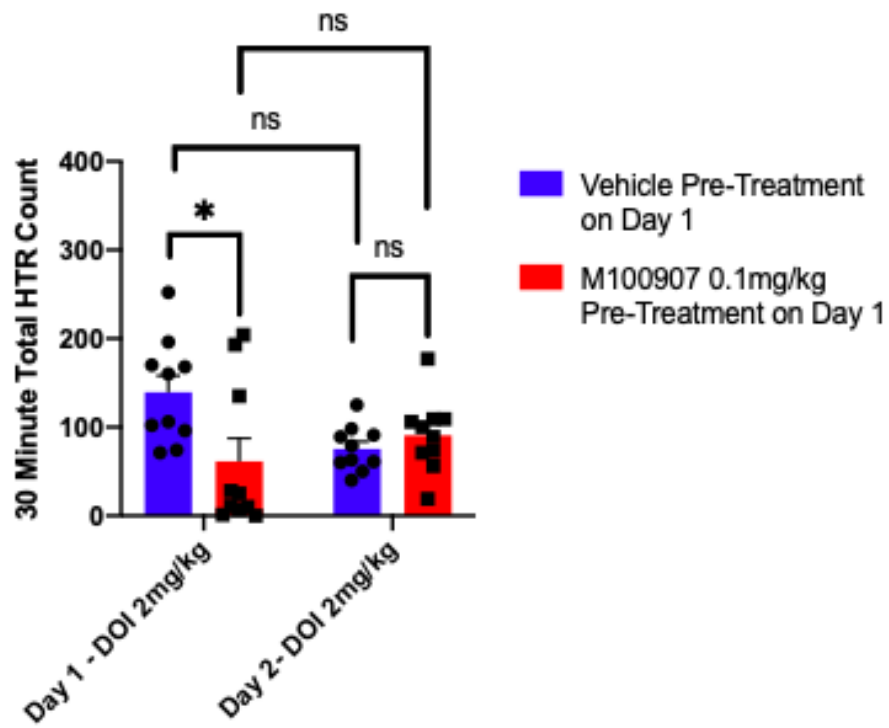
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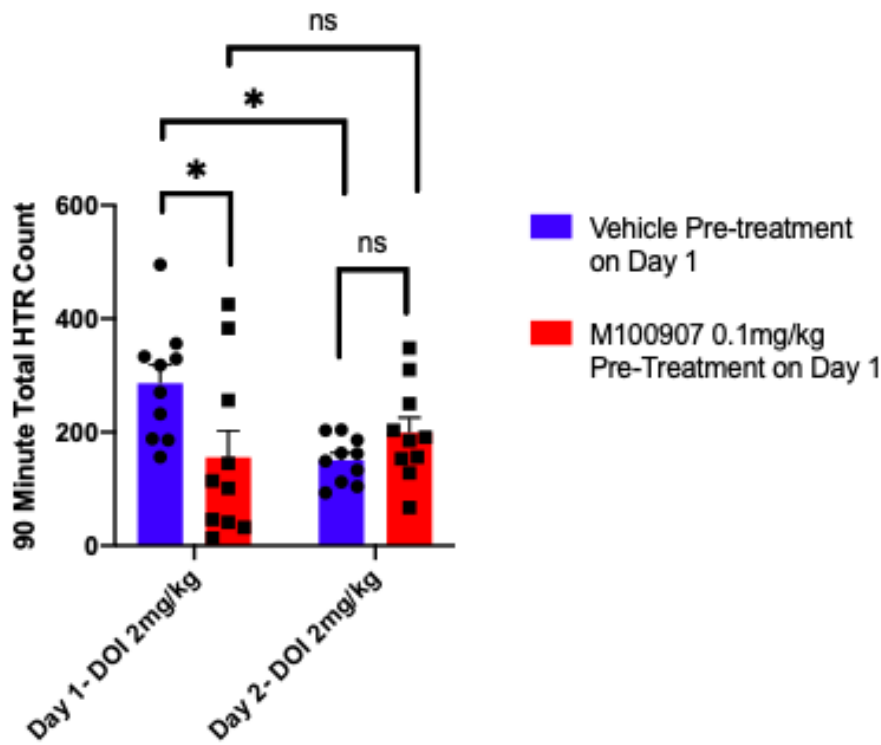
E.

Figure 8: Male c57bl/6 WT mice (n= 10) were treated on Day 1 with either vehicle or 0.1mg/kg M100907, followed by 2mg/kg DOI 10 minutes later. Mice were recorded for 90 minutes following treatment. 24 hours later, mice were again administered 2mg/kg DOI. **(A)** 90 minute time course for M100,907 or vehicle pre-treated mice on Day 1, measured in 15 minute fractions; (B) 90 minute time course for mice measured in 15 minute fractions **[C]** Combined Day 1 and Day 2 90 minute time courses measured in 15 minute fractions; **(D)** 30 minute HTR totals from Day 1 and Day 2. Although 2 Way ANOVA revealed no significant difference between treatment groups [f(1,36)=3.117, p=0.0860], post hoc analysis revealed a statistically significant difference between vehicle and M100,907 pre-treated animals on Day 1 only (p= 0.0222); **(E)** 90 Minute total HTR from Day 1 and Day 2. On Day 1, pr-treatment type had a statistically significant effect on HTR by 2 Way ANOVA [F (1,36)=1.649, p=0.2073]. Time (treatment day) also failed to exhibit a statistically significant effect by 2 Way ANOVA [F (1,36)=2.046, p=0.1612]. Bonferroni post-hoc analyses tested for additional statistical relationships, which are marked on figures. Data are presented as mean ±S.E.M.

Our results indicate that over 30 minutes, pre-treatment type had a noticeable but not statistically significant effect on HTR on Day 1 by 2 Way ANOVA (p=0.0860). However, Bonferroni post-hoc analysis revealed statistically significant differences between vehicle and M100,907 pre-treated groups over both 30 minutes (p=0.0222) and 90 minutes (p=0.0403).

Time (treatment day) similarly failed to exert a statistically significant difference by 2 Way ANOVA for both 30 Minute (p=0.3400) and 90 Minute Totals (p=0.1612). These results indicate that while M100,907 successfully attenuated the response, it was to a lesser degree than expected based on literature with similar experiments. Additionally, our current trial shows that 5-HT$_{2A}$R antagonism via M100,907 failed to prevent tolerance.

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Figure 9: Effect of pre-treatment with the atypical antipsychotic, clozapine, on DOI induced HTR in male c57bl/6 WT mice

This study was originally performed as a part of another project, but was included to serve as a contrast to my current M100,907 findings. Clozapine is a commonly prescribed atypical antipsychotic that has antagonist activity at the 5-HT$_{2A}$R and the Dopamine 2 (D2) receptor. Its effects have been shown to attenuate HTR behavior, and my findings confirm this. Although this was not tested for in this current study, clozapine itself has also been shown to lead to tolerance at the 5-HT$_{2A}$R, decreasing its long-term clinical efficacy.

Mice were monitored for 30 minutes in HTR chambers to assess basal activity. Mice (n=4) were then injected with either vehicle or clozapine 5mg/kg. Ten minutes later, mice were administered DOI 2mg/kg and immediately placed into HTR chambers for a 90 minute monitoring phase. Results from this experiment are displayed below.
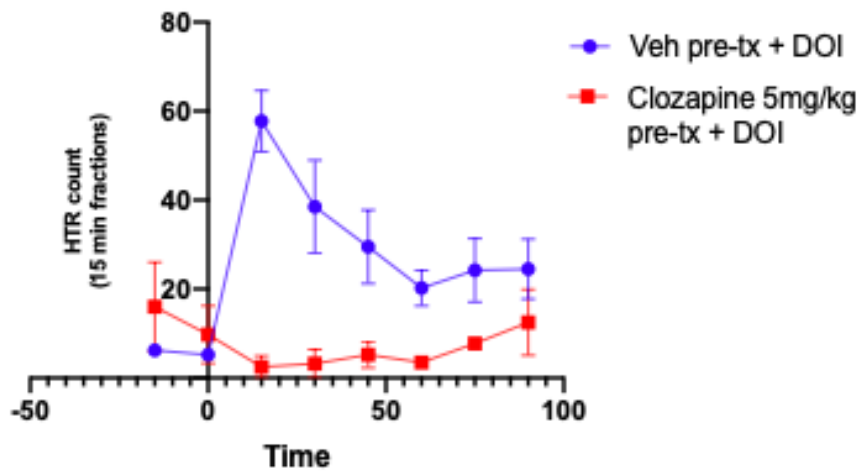
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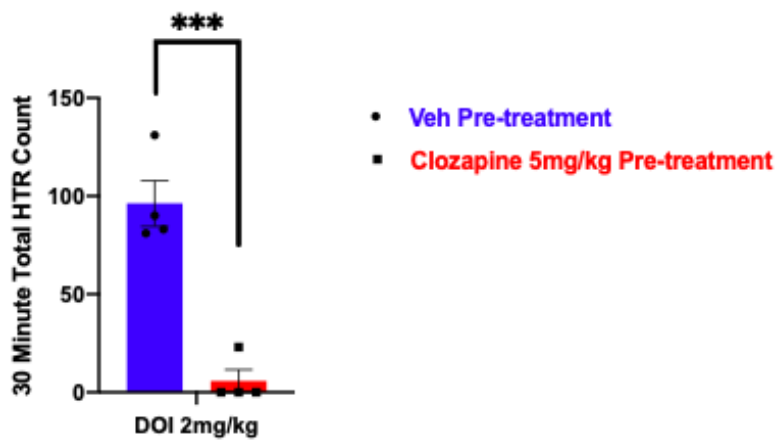
B.
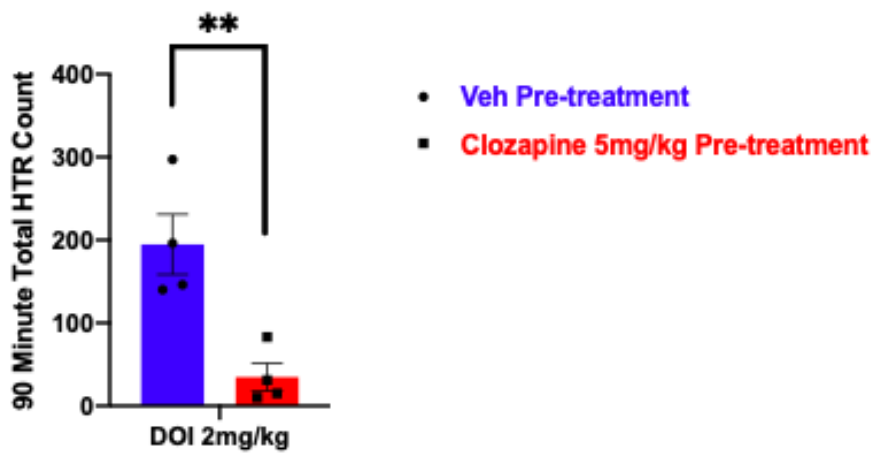
C. .

Figure 9: Male c57bl/6 WT mice were placed into monitoring chambers and monitored for 30 minutes to assess basal HTR activity and allow time to acclimate. Mice were then administered either clozapine 5mg/kg i.p. or vehicle. 10 minutes later, all mice were administered DOI 2mg/kg to assess clozapine's ability to acutely prevent HTR. **(A)** Time course for treatment groups over 90 minutes, displayed in 15 minute fractions; **(B)** 30 minute total HTR count for clozapine and vehicle pre-treated mice. Unpaired t test reveals a statistically significant difference (p=0.0005) between groups over 30 minutes; **[C]** 90 minute total HTR count for clozapine and vehicle pre-treated mice. Unpaired t test reveals a statistically significant difference (p=0.0071) between groups over 90 minutes. Data are presented as mean ± S.E.M.

My findings confirmed clozapine's ability to acutely block HTR behavior in a significant manner. However, it should also be noted that these results are preliminary and require further validation, since groups only included 4 animals each. Total HTR was measured over 30 and 90 minutes and analyzed via unpaired t test. Statistical analysis indicated that clozapine pre-treatment resulted in a statistically significant reduction of HTR behavior over 30 (p= 0.0005) and 90 (p=0.007) minutes.

G. Chapter 4: Discussion

Collectively, my results indicate that signaling blockade downstream of the 5-HT$_{2A}$ R may have the ability to acutely attenuate DOI induced HTR, but were insufficient to prevent the development of tolerance. My studies with SL327 demonstrated that inhibiting ERK phosphorylation, which thereby inhibits activation of this kinase and its downstream targets, will acutely attenuate the DOI induced head twitch. This highlights the MAP Kinase pathway as important in mediating the head twitch behavior. Interestingly, however, similar effects were not seen using the PKC inhibitor, Bisindolylmaleimide-II. Furthermore, M100,907 pre-treatment failed to prevent tolerance and had a smaller than expected effect on HTR, contrasted by the significant effect that clozapine had in acutely blocking HTR. Here I will start by discussing each experiment's results in more depth. I will then discuss limitations or room for improvement to these studies.

In considering the locomotion studies with SL327, our results indicate that SL327 has a significant effect when compared to control groups on Day 1. Despite the small sample size (n=4), there was also a statistically significant difference in total horizontal activity between SL327 and vehicle treated mice, with the SL327 treated group displaying less horizontal activity. Because our HTR measurement assay does not typically involve visual recordings to validate results, it is possible that the lower HTR response on Day 1 could be secondary to an overall loss of motor function rather than a HTR specific effect. However, it is also possible that decreased horizontal activity may occur without reductions to reflexive head movements. Further testing on this topic is warranted to evaluate the origin of this statistically significant difference. However, when mice were tested 24 hours later without repeat dosing, there was no statistically significant

difference between groups. Because of this, comparisons regarding SL327's effect on tolerance could be more easily discerned from any motor suppressive or enhancing effects.

SL327's residual effect on DOI induced HTR:

I hypothesized that both treatment groups would exhibit minimal head twitch response behavior on Day 1 because no psychedelics were administered to induce HTR. On Day 1, treatment with either SL327 or vehicle yielded supports this hypothesis, evidenced by comparable, minimal HTR shown by both treatment groups. It is worth noting that although there was not significant activity, both treatment groups still displayed some HTR activity. However, this is in line with other studies that show mice to exhibit small amounts of naturally occurring HTR activity.

On the second day of testing, I hypothesized that SL327 administration the day prior would have disrupted signaling pathways such that DOI induced agonism of the $5HT_{2a}R$ would elicit diminished HTR when compared to vehicle treated mice. However, both treatment groups had comparable responses to treatment with DOI 2mg/kg, with no statistically significant difference between their total responses over both 30 and 90 minutes. This study indicates that treatment with SL327 alone does not cause long lasting changes that, in turn, affect DOI's ability to induce HTR. However, it must be noted that one outlier was identified in the Day 2 SL327 treated group, which had a HTR count far higher than the other mice tested. This diminished my sample size and the power of the statistical analysis, and so additional trials to validate these results are necessary. Unfortunately, time and animal restrictions prevented this from occurring.

SL327: MEK1/2 Inhibition's Effect on HTR Behavior and Tolerance

Several studies have pointed towards the important role that ERK plays in mediating psychedelic-specific behavioral and transcriptional effects in rodent models. Therefore, we hypothesized that treatment with this MEK1/2 inhibitor would prevent phosphorylation (and activation) of ERK and therefore lead to a diminished HTR in mice. Additionally, we hypothesized that preventing ERK phosphorylation would prevent the development of tolerance with repeat DOI administration. While this was not tested for these studies, this diminished behavioral response is thought to correspond to a diminished psychedelic-specific gene expression pattern, *egr-1* and *egr-2*. If true, the SL327 pre-treated group would be expected to have a comparable number of head twitches between Day 2 and Day 1.

Collectively, my results show that preventing ERK phosphorylation via inhibition of the upstream kinase, MEK, will lead to a statistically significant attenuation of psychedelic induced HTR behavior. This effect was most noticeable over the first 30-45 minutes following DOI treatment. Time courses displayed in Figures 5A, 5B, and 5C display a rise, then fall, of HTR during the 90 minutes animals were monitored. While DOI has been shown to have a 16-30 hour duration of action in the body (Smith et al, 2014), previous HTR studies employing DOI as the psychedelic of choice show similar time courses. A 2007 study hypothesized that ERK signaling may play a critical role in opioid induced tolerance and dependence by using SL327 to inhibit ERK phosphorylation and activation in mice. However, this study found that treatment with SL327 does significantly attenuate the levels of phosphorylated ERK on Western Blot analysis, tolerance and dependence were both unaffected in tested mice. Opioids and psychedelics act through different receptors, but my results similarly indicate that ERK signaling does not mediate tolerance.

Bisindolylmaleimide-II: PKC Inhibition's Effect on HTR Behavior and Tolerance

Because signaling blockade at the level of ERK failed to exhibit the phenotypic response I hypothesized it would, I looked further upstream in the signaling pathway to assess whether blockade at these levels would impact HTR regulation and tolerance. For one trial, we chose the Protein Kinase C (PKC) inhibitor, Bisindolylmaleimide-II, and similarly hypothesized that PKC inhibition would lead to an acutely diminished HTR on Day 1 and disrupt the development of tolerance seen on Day 2. Interestingly, acute treatment with Bisindolylmaleimide-II did not attenuate the DOI-induced HTR on Day 1, actually increasing and slightly sustaining the DOI-induced HTR throughout the 90 minute time course (Fig 6A). It is possible that the slightly increased response on Day 1 results from a sort of positive feedback that reinforced DOI's behavioral effects. However, I find this less likely because signaling blockade both upstream (at the level of the $5HT_{2A}R$ via M100,907) and downstream (at the level of ERK via SL327) did acutely attenuate HTR in a statistically significant manner. Therefore, I suspect that Bisindolylmaleimide-II may have limited blood brain barrier permeability, and therefore may not have reached cortical $5\text{-}HT_{2A}R$ at all. In this case, both Bisindolylmaleimide-II and vehicle pre-treated groups would be expected to have similar effects, as is seen in my results. With more time, I would perhaps have attempted ICV administration of the compound to ensure it reached its desired targets.

On Day 2, both the vehicle and Bisindolylmaleimide-II treated groups were noted to have similar head twitch counts over the 90 minute time course following repeat DOI treatment. These behavioral responses were significantly diminished from day 1, indicating that tolerance was not prevented.

U73122: PLC Inhibition' Effect on HTR Behavior and Tolerance

This drug proved to be highly insoluble and difficult to work with. After reviewing solubility guidelines, it was decided to proceed with a 5mg/kg dose dissolved in DMSO. Unfortunately, 60% (3 out of 5) of the mice dosed with the 5mg/kg dose of U73122 died overnight following acute treatment, rendering a second day's trial unreliable with only two remaining mice. HTR results from the first day's trial are of questionable utility and are not displayed. I believe it would be worthwhile to re-try this experiment with a smaller dose and with different solvent and injection methods. I suspect that the DMSO primarily resulted in the inactivity during the monitoring period, but that the dose of U73122 was too high and resulted in a toxic effect.

Additionally, it is worth noting that data from this trial may have been otherwise unreliable based on the basal activity that was recorded for 30 minutes before any treatment. Mice are testable for only about 4 weeks following magnetic ear tag placement, after which they begin to develop scabbing and inflammatory processes around the ears. Mice have been shown to exhibit a small amount of basal HTR activity, even without psychedelic treatment (Halberstadt et al, 2013). Because the magnetometer monitoring system is located on the animals ears, it is possible that irritation surrounding the magnetic devices may lead to increased ear and head movement, including increased head twitch. This particular group of animals had a higher than normal level of basal activity and had been ear tagged just over 4 weeks prior. Repeating this trial with improved administration methods on more recently tagged mice could lead to more reliable results.

M100,907: Selective 5HT$_{2A}$R Antagonism's Effect on HTR Behavior and Tolerance

This drug is a selective 5-HT$_{2A}$R antagonist and has been used in studies to help elucidate distinctions between the 5-HT$_{2A}$R and 5-HT$_{2C}$R. DOI has been shown to induce HTR in a dose-dependent, inverted U shaped curve, with maximum HTR around the 1-2 mg/kg dose (Halberstadt et al., 2013). With this experiment, I hypothesized that M100,907 pre-treatment would have a significant effect on DOI induced head twitch such that the HTR would be nearly entirely diminished. Further, I hypothesized that M100,907 would prevent tolerance when mice were treated again with DOI. I decided to run this trial given that we did not have clear success with the other drugs that were used. While the dose that was used (0.1mg/kg) was not the maximum dose that has been used in *in vivo* studies, we settled on this dose due to its common use in experiments, and felt this dose would produce successful but also relevant results (i.e. using a higher, 1mg/kg dose might be more effective but does not tell us much or help much moving forward with other studies).

A total of ten animals were tested per treatment group. Results from Day 2 were consistent within treatment groups, showing that DOI-induced tolerance was not blocked with specific inhibition of the 5-HT2AR. Statistical analysis revealed no significant difference between treatment groups on Day 2. However, Day 1's measured HTR varied within the M100,907 treated group, and I believe this warrants further investigation. Seven out of the 10 animals pre-treated with M100,907 displayed a drastic decrease in the number of head twitches measured. However, the remaining three displayed head twitch numbers that were comparable to mice that were pre-treated with vehicle. To assess whether M100,907 pre-treatment leads to a bimodal rather than normally distributed behavioral response, I ran a Quantile Quantile (QQ) analysis of the ten animals tested (Fig 7).
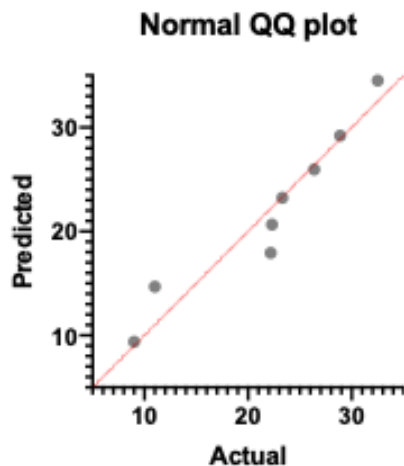
Figure 10: Normal QQ Plot displaying actual quantile versus predicted quantiles. Despite some variance, close apposition over the red line indicates that these values were normally distributed rather than bimodally or otherwise distributed.

QQ analysis showed 74.16% probability that my results had a normal, Guassian distribution. Nonetheless, I believe this study would benefit from a larger n to increase statistical power. Additionally, repeating the study with either a larger, 1mg/kg dose of M100,907 or smaller dose of DOI may allow for the effect of M100,907 to overcome DOI. Additionally, it is possible that the three animals who did not have a noticeable difference between vehicle treated animals did not receive the drug properly, either through erroneous subcutaneous administration or another incorrect administration technique. To further ensure these results were not the result of improper software analysis, I performed a review of the raw matlab signal files and found no evidence that low voltage waveforms were missed by the detector.

M100,907's ability to acutely attenuate DOI induced HTR was statistically significant but to a lesser degree than expected. This is particularly evident when contrasted with the effect of clozapine pre-treatment on HTR (Fig. 6). Clozapine acts at various receptors, including as a $5HT_{2A}$ R antagonist, and had a dramatic effect on DOI's ability to induce head twitch acutely. I

hypothesized that pre-treatment with M100,907 would have a similar impact. This was not the case, yet it is worth using a larger dose of M100,907 to compare for a larger effect.

Collectively, my results shed light on the mechanisms underlying both the HTR in mice but failed to find a significant target for preventing tolerance. Although blockade with SL327 pre-treatment did attenuate the HTR occurring on Day 1, upstream blockade of PKC with Bis-II pre-treatment did not lower the HTR in our group, and in fact had a slightly increased HTR in mice on Day 1. This may indicate that regulation of this phenotypic expression occurs either at the level of ERK or at some point between PKC and ERK. However, it is probably that this particular inhibitor was unable to cross the blood brain barrier, which provides a reasonable explanation for the comparable effect between inhibitor treated and vehicle treated animals. This is an area for future study that could further explore and validate these results, either through more direct intracerebroventricular (ICV) administration or the use of other drug compounds.

If these results are consistent in repeat trials, it is possible that blockade of the 5-HT2A signaling pathway on Day 1 led to a compensatory mechanism from the 2A's pathway such that stimulation with a psychedelic 5-HT2A agonist led to a more robust response. However, due to SL327's potency as a MEK1/2 inhibitor, concerns about repeat administration effects on systems throughout the body, continued repeat administration poses risks that could impact more widespread behaviors and the health of the animals. Interrupting 5-HT2A signaling at the level of the receptor and downstream of receptor activation both failed to impact tolerance, indicating that tolerance is likely mediated by more than 5-HT2A signaling alone. Given findings regarding the 5-HT2AR's heterodimerization of the metabotropic glutamate receptor 2 (mGluR2), it is possible that cross- talk between these two GPCRs plays a role in mediating tolerance. This relationship, and the interplay between other receptors and the 5HT2AR, are all areas worthy of

further investigation. In the following section, I will highlight some potential areas that I believe could improve upon my current studies and serve as future directions for research into this topic.

Clozapine: **Effect on HTR Behavior and Tolerance**

In contrast to my M100,907 studies, pre-treatment with clozapine resulted in a drastic attenuation of HTR behavior on Day 1. The contrast between these two compounds, which share 5-HT$_{2A}$R antagonist activity, could point towards a few other considerations for further studies. Firstly, because clozapine also has antagonist activity at the Dopamine 2 receptor and had a much more effective ability to decrease HTR behavior, it is possible that other receptors may also play roles in upregulating and downregulating the complex signaling pathways that underlie behavior. Alternatively, it is possible that this difference resulted from dosing differences, and that a larger dose of M100,907 may have been able to have the same effectiveness as clozapine. Finally, this study could also point towards distinctions between various antagonists and their intrinsic efficacy. These considerations are all worthwhile, and could be better investigated through follow up studies.

H. Chapter 5: Future Directions/ Follow Up Studies

In this section I will discuss a few possible future directions that I believe would help to substantiate my current findings and further the investigation into tolerance. Additionally, I will highlight some limitations to these current studies, which were particularly affected by lab closures due to the COVID-19 outbreak.

With more time available, I would first perform additional trials with M100,907 in order to gather a more statistically powerful set of data with a larger n. My current results align with other studies in which M100,907 had an attenuating effect on behavioral cues such as head twitch, although this effect was less robust than I hypothesized. In addition to running additional trials with the current doses, I would also like to repeat these trials with a larger dose of M100,907 to determine if a more robust blockade could be achieved with higher doses. Additionally, it is worth considering whether or not M100,907 itself may induce tolerance, as tolerance has been observed in response to both repeat agonist and antagonist administration. Further, because DOI induces HTR in accordance with an inverted U shaped dose curve (Halberstadt et al., 2009), it would be interesting to run these experiments using varying doses of DOI.

Testing with Multiple Inhibitors to confirm ERK's role in mediating HTR:

Although my experiments failed to identify a reliable signaling candidate that may play a role in the development of tolerance, my results point towards the MAP Kinase pathway as important in mediating HTR. As a translational behavioral model, this points towards ERK as also playing a role in mediating hallucinations and psychosis. To confirm this, I believe it would be worthwhile to compare different MEK1/2 inhibitors' effects on HTR. Head twitch and tolerance development could be impacted by factors including the half life of a given drug,

pharmacokinetics and binding, interaction with other receptors, and other factors. Utilizing several types of inhibitors could allow more confident identification of signaling pathways such as ERK's role in mediating head twitch behavior or tolerance. Similar findings in multiple HTR trials would substantiate these findings and enhance their validity and replicability.

Functional Selectivity and Cross Tolerance:

The 5-HT$_{2A}$R has been shown to exhibit functional selectivity depending on the nature of ligand binding. Functional selectivity complicates our understanding of 5-HT$_{2A}$R function as it can lead to differential levels of signaling (such as Ca$_{2+}$ release), distinct behavioral cues (such as HTR), and even varying patterns of genetic expression (*egr-1, egr-2, c-fos)*. Therefore, testing a variety of psychedelic compounds in tandem with inhibitors such as SL327 would allow further identification of how agonist directed signaling functions between classes of psychedelics. I would therefore aim to recreate my study using at least one tryptamine derived psychedelic such as N,N-dipropyltryptamine and one ergoline derived psychedelic such as LSD. Smith (2014) demonstrated that tryptamine derived psychedelics such as N,N-dipropyltryptamine (DPT) have lower potential for inducing tolerance compared to phenethylamine derived psychedelics such as DOI. This study also found that cross tolerance exists between phenethylamine and tryptamine derived hallucinogens such that substitution with DPT sustained tolerance that had been induced through repeat DOI administration. Perhaps it is also worthwhile to reproduce some of these studies to determine how signaling blockade affects cross tolerance development.

Functional selectivity is also evident in studies highlighting differences between β-arrestin's role in receptor internalization. While it appears that hallucinogens induce HTR in a β-arrestin independent manner, other proteins that traditionally mediate receptor internalization may play roles in this process. For example, one study utilized green fluorescent protein (GFP)

to tag 5-HT2AR and to study the role that dynamin plays in internalizing the 5-HT2AR upon

agonist and antagonist stimulation (Bhatnagar et al., 2000). Dynamin is traditionally involved in

the formation of endosomes that contain internalized receptors (Bhatnagar et al., 2000). This

study found that agonist induced internalization of GFP-tagged 5HT2ARs in WT versus dynamin

dominant negative mutants was dynamin dependent. Therefore, further exploration into the

signaling responsible for recruiting and activating dynamin may be another promising lead

towards preventing tolerance via receptor internalization.

In addition to studying the 5-HT2AR specifically, I believe there are a few other

membrane receptors that are worthy of consideration and investigation. The 5-HT2AR has

recently been shown to form a heterodimer with the metabotropic glutamate receptor 2

(mGluR2), another class of GPCR (González-Maeso et al., 2008). It is thought that interactions

between these two receptors lead to cross talk and signal modification. Therefore, it is highly

possible that this cross-talk is also responsible for differences in agonist directed signaling and

may also play a role in mediating tolerance. Buchborn additionally argued that glutamate

receptors may play a role in inducing tolerance to prevent overstimulation of the cortex (Nichols,

2016 pg. 283). To test for this, I could use a selective mGluR2/3 antagonist such as LY379268 or

LY354740 as pre-treatment prior to administering DOI. Both of these compounds have been

shown to attenuate DOI induced HTR, but their effect on DOI induced tolerance is less

investigated (Nichols, 2016).

Additionally, some studies have similarly pointed towards cross-talk between the 5-HT2A

and 5-HT2C receptor such that the 5-HT2CR can modulate signaling (Fiorella et al, 1995b;

Fantegrossi et al., 2010). Fiorella utilized drug discrimination studies and found that the distinct

effects of phenethylamine and indolalkyamine derived psychedelics are indeed mediated through

the 5-HT$_{2A}$R (Fiorella et al, 1995). However, they also found that the 5-HT$_{2C}$R was responsible for modulating this signal (Fiorella et al, 1995). Fantegrossi utilized HTR and similarly found that 5-HT$_{2C}$R agonist activity appears to drive inhibition of HTR behavior. Therefore, it is possible that this inhibitory effect may provide insights into other mechanisms behind tolerance.

Alternate Signaling Points:

In addition to the signaling mediators investigated in this study, there are several other signaling candidates and pathways that I believe are worthy of further study. Firstly, I would aim to investigate the specific role that G$_q$ signaling plays in mediating HTR acutely and in mediating tolerance. Using a similar experimental design, I would use the G$_q$ inhibitor YM-254890. Utilizing this inhibitor would particularly supplement my studies with M100,907 and could provide further insights into the nuanced signaling that occurs between receptor activation and G protein signaling initiation.

Additionally, several pieces of literature pointed towards another member of the MAP Kinase/ERK pathway as a potentially promising target for mediating tolerance, specifically. This protein is known as RSK2, or p90 ribosomal S6 kinase (Nichols, 2016). Given my study's findings that support the MAP Kinase pathway's role in mediating HTR behavior acutely, I feel this is a pathway that should be further investigated. Additionally, other studies have found that RSK2 plays important roles in 5-HT$_{2A}$R signaling. In 2006, Sheffler found that RSK2 possesses a tonic "braking" function on 5-HT$_{2A}$R signaling (Nichols, 2016). Additionally, Strachan found that RSK2 regulates both acute and extended signaling from the 5-HT$_{2A}$R in a negative fashion (Nichols, 2016). Tolerance has been shown to occur in tandem with downregulated receptor signaling. Thus, it is possible that RSK2's "braking" role may function to mediate tolerance. Finally, a 2010 study from Strachan also showed that RSK2 deletion allows for increased DOI

efficacy, demonstrated through increased ERK phosphorylation, increased intracellular Ca_{2+} release, and increased IP accumulation (Nichols, 2016). RSK2's role in mediating tolerance has not yet been fully explores, but I believe these studies and my own findings with the MAP Kinase pathway all point towards it as a worthy study target.

Overall, this project has demonstrated that the signaling mechanisms driving psychedelic action are inherently complex and are further complicated by cross signaling and crosstalk. Nonetheless, valuable research tools such as the HTR assay enable high throughput testing that can study these pathways and serve as a supplement to other behavioral, *in vitro*, and molecular studies. With continued effort, I believe we can translate better understanding of these signaling pathways into more thorough understanding of disease pathophysiology and treatment options.

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