ABSTRACT

POTENTIAL ANTIDEPRESSANT EFFICACY OF PSILOCYBIN AND RELATED TRYPTAMINES

by Oscar Sandoval

Clinical trials on the use of psilocybin to treat depression are promising but are confounded by the presence of hallucinations which may not be necessary for effectiveness. Preliminary data suggests that when administered to rats, related tryptamines baeocystin, norbaeocystin, and aeruginascin don't cause hallucination-like acute behavioral responses (i.e., head twitch response) at any dose. This thesis explores whether related tryptamines have similar or better antidepressant efficacy than psilocybin and selective serotonin reuptake inhibitor fluoxetine, through both behavioral and biological markers of depression-associated processes in rats. Therapeutic potential of these tryptamines was assessed by administering baeocystin, norbaeocystin, aeruginascin, psilocybin, vehicle, or fluoxetine via gavage to male Long Evans rats (n=10) and assessed immobility in the forced swim test (FST) to measure antidepressant efficacy. Additionally, Brain Derived Neurotrophic Factor expression was assessed using ELISA and Western Blot. Like fluoxetine, rats given psilocybin and norbaeocystin had significantly decreased immobility time compared to vehicle ($p < 0.05$). ELISAs showed increased Brain Derived Neurotrophic Factor expression in rat hippocampus after psilocybin treatment, which was not replicated by Western Blot. These results suggest norbaeocystin may have similar antidepressant effects to psilocybin, alternative to traditional antidepressants, warranting further investigation by preclinical and clinical scientists.

POTENTIAL ANTIDEPRESSANT EFFICACY OF PSILOCYBIN AND RELATED TRYPTAMINES

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Table of Contents

Page
Abstract……………………………………………………………………………….i
List of Figures…………………………………………………………………….....iv
Dedication……………………………………………………………………………v
Acknowledgements………………………………………………………………...vi

Chapters
1. Chapter 1: Introduction………………………………………………………..1
2. Chapter 2: Methods……………………………………………………………4
3. Chapter 3: Results……………………………………………………………..7
4. Chapter 4: Discussion/Conclusions…………………………………………...8
5. Chapter 5: Supplemental Data………………………………………………...18
6. Chapter 6: Figures and Tables………………………………………………...…21

List of Figures

Figure Page

Figure 1: Head Twitch Response…………………………………………………………..22

Figure 2: Experimental Timeline/Drug Regime………………………………………23

Figure 3: Forced Swim Test Results………………………………………………..24

Figure 4: ELISA Results ………………………..………………………………..……25

Figure 5: Western Blot Results/Images………………...………………………..……26

Dedication

I dedicate this project and all countless hours of literature review and project management to my father Armando Sandoval for being an unwavering rock of support and showing me what it means to work hard. I also dedicate this to my mother Monica Sandoval for being the most caring mother and constantly being my emotional support throughout my life. To my brother Armando Sandoval Jr. for being my inspiration on continuing my higher education and setting the standard of greatness in academia for me outside of being my best friend.

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Chapter 1: Introduction

Depression is the leading cause of disability with an increased risk of all-cause mortality. In 2018 approximately 10% of the adult population in the United States was diagnosed with major depressive disorder (MDD) (Gumnick & Nemeroff, 2000). According to the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5), symptoms of depression are highly variable and can include but are not limited to depressed moods, loss of interest/pleasure, and suicidal ideation (American Psychiatric Association, 2013). Despite an increasing number of treatments for depression becoming more accessible, they have limited efficacy.

There are currently multiple forms of medication used to treat depression, most of which target the serotonin system. It is thought that increasing the level of neurotransmitters like serotonin and dopamine could be what drives their therapeutic efficacy; any way that these neurotransmitters could be upregulated could potentially be therapeutic. Selective serotonin reuptake inhibitors (SSRIs) such as Prozac® are thought to elicit their therapeutic effects by blocking reuptake of serotonin, resulting in increased serotonin levels in the synapse, leading to circuit-level adaptations. However, about 50% of people do not respond to SSRIs, and as many as 10% to 30% would be considered treatment resistant (Arroll et al., 2005). Other forms of medications used are tricyclic antidepressants (TCAs) such as Anafranil®/Clomipramine®, which similarly block the reuptake of serotonin and norepinephrine, and monoamine oxidase inhibitors (MAOIs) like Isocarboxazid®/Marplan® that increase monoamines like dopamine and serotonin by inhibiting the enzyme that breaks down these neurotransmitters. On average, TCAs and MAOIs also have low (50% to 60%) response rates with active treatment. Treatment plans vary among people and often rely on trial and error before deciding the best one (Amsterdam & Shults, 2005). On average there is a 1 in 4 chance that changing from one antidepressant type to another will have improved therapeutic effects (Sinyor et al., 2010). Additionally, most antidepressants have delayed onset of action, often causing patients to prematurely stop treatment (Tylee & Walters, 2007). Guidelines from the National Institute for Health and Clinical Excellence (NICE, 2022) suggest SSRIs as the first line of pharmacological treatment for patients, while being warned about the delay onset of effect over a 6-week period. A combination of lack of efficacy, delayed onset of therapeutic effects, and severe side effects (e.g., suicidal ideation, weight gain, sexual dysfunction) makes current treatments for depression controversial. Thus, there is increasing social pressure for the development of alternatives to these medications.

In addition to the effects of traditional antidepressants on neurotransmitter levels, an increasing amount of research suggests that many adaptive neurobiological changes also occur with treatment (Taylor et al., 2006). Examples of these changes are the potential desensitization and internalization of serotonin receptors and increased expression of proteins such as Brain Derived Neurotrophic Factor (BDNF). These changes can occur as early as the first week of treatment with serotonin reuptake inhibitors (Blier et al., 1987; Duman et al., 1997). Increased BDNF expression modifies gene expression and can cause signaling cascades related to reduced expression of depressive-like behavior. Likewise, decreased expression of BDNF is related to an increase in depressive like behaviors (Yang et al., 2020). Brain regions such as the hippocampus and medial prefrontal cortex experience alterations in BDNF level, which could be the primary sites of action given their role in behavioral flexibility, emotional regulation, and motivation

(Bittar & Labonté, 2021; Rosas-Vidal et al., 2018; Sheline, 2011). The delayed onset effects of SSRIs, TCAs, and MAOIs can be attributed to changes to biological markers like BDNF, which may take time to occur.

Recent studies on novel antidepressants have highlighted the therapeutic potential of psychedelic drugs, especially psilocybin. Psilocybin, which is broadly a serotonin receptor agonist, is a naturally occurring compound found in *psilocybe* mushrooms. One distinct property of psilocybin is its hallucinogenic activity, thought to be mediated by its action on the serotonin 2A receptor (5HT2A)(Madsen et al., 2019). However, psilocybin is rapidly metabolized via alkaline phosphatase into psilocin, which has much higher affinities for a variety of receptor targets (Dinis-Oliveira, 2017), including 5HT2A, 5HT2C, 5HT1A, 5HT1B, and 5HT5, and others aside from serotonin (Halberstadt & Geyer, 2011). Thus, most of the effects of psilocybin are likely mediated by psilocin. Preclinical studies suggest that psilocybin could have therapeutic antidepressant efficacy (Carhart-Harris et al., 2017; Davis et al., 2021). However, understanding psychedelics such as psilocybin is complicated by the hallucinogenic activity they elicit, making it difficult to consider the use of psychedelics as potential antidepressants. There are two opposing opinions regarding the value of psilocybin-induced hallucinations. One opinion holds that hallucinations may be necessary for psychedelics to have their effects; the idea being that psilocybin may moderate the clinical efficacy of other interventions (Cavarra et al., 2022). For example, hallucinations may allow patients to build stronger bonds with their therapists during treatment, improving therapy efficacy, perhaps by helping patients rethink habits after those hallucinations. The other perspective is that hallucinations have no role in the therapeutic efficacy of these drugs since there are hallucinogens with no therapeutic value. Most psychedelics are DEA Schedule 1 substances, a classification reserved for compounds with high abuse potential and no medicinal value, which has been questioned as accurate (Johnson et al., 2018). This barrier would suggest that researchers must find alternatives that are non-hallucinogenic and further expand the field to provide evidence of psychedelic therapeutic efficacy (Belouin & Henningfield, 2018) without the confound of hallucinations. Therefore, there is a need to research these psychedelics in detail with both preclinical trials and their effects in animal models. The use of animal models in psychedelic research is limited, but behavioral assays such as the Head Twitch Response (HTR) in rodents can act as a proxy for hallucinations (Corne et al., 1963). HTR is the most commonly accepted behavioral method of showing activation of the 5HT2A receptor. However, this activation is only a proxy for psychedelic activity in rodents and therefore should not be considered as a marker for hallucinations (Glatfelter et al., 2021). The debate about the use of psychedelics in a potential therapeutic manner is ongoing and only a select number of researchers with the proper schedule 1 licensing are allowed to work with these compounds due to their complex US regulations. However, there are other compounds found in these mushrooms that may be alternatives to psilocybin.

Related tryptamines norbaeocystin, baeocystin, and aeruginascin are all compounds found in magic mushrooms that could have similar therapeutic effects to psilocybin. These related tryptamines have similar chemical structures to psilocybin (variations in methylation), but of those studied, they show different affinities to serotonin receptors, including weaker affinity for 5HT2A (Glatfelter et al., 2022). These tryptamines could potentially elicit therapeutic effects based on these different receptors they bind to, which would potentially eliminate the hallucinogenic confounds of psilocybin. Along with Glatfelter et al, 2022, preliminary studies

from our lab (not a part of this Thesis) suggest there are fewer head twitch responses in rodents after administration of these tryptamines, compared to psilocybin (**Figure 1**). This proxy for hallucinations could suggest that these related tryptamines might not be hallucinogenic. No prior research has evaluated the potential antidepressant efficacy of these related tryptamines in animal models. Therefore, the purpose of this Thesis is to determine the potential antidepressant efficacy of these related tryptamines.

There are many animal behavioral assays used to characterize the therapeutic efficacy of potential antidepressants (e.g. sucrose preference, tail suspension test). The Forced Swim Test (FST) is perhaps the most common, used to assess despair in animal models by measuring immobility time (i.e., floating with the absence of movement outside of keeping their nose above the water) along with other behaviors such as swimming and climbing. Previous studies using the FST looked at the antidepressant efficacy of chronic administration of SSRIs (e.g. fluoxetine), and showed a decrease in immobility, which suggested potential antidepressant efficacy (Dulawa et al., 2004). This allows fluoxetine to serve as a positive control for comparison with other potential therapeutics (i.e. psilocybin). The use of hallucinogenic compounds like psilocybin in the forced swim test have shown positive results suggesting psilocybin decreases immobility time in the FST similar to fluoxetine (de Gregorio et al., 2021). Furthermore, it is known that the FST is a stressful assay to rodents and can have a variety of side effects from increased stress levels to changes in biological markers (Gourley et al., 2008; Mezadri et al., 2011). Up regulation of BDNF in the medial prefrontal cortex and the hippocampus promotes synaptic plasticity and reduces immobility in the forced swim test, which suggests that BDNF could be a biological marker of antidepressant activity (Rosas-Vidal et al., 2018). By assaying behavior in the FST and BDNF express, we can examine therapeutic potential in the related tryptamines by comparing them to a psilocybin control and a common SSRI (fluoxetine).

There is an urgent need to better understand how psilocybin-related tryptamines, which show potential non-hallucinogenic activity, might elicit similar therapeutic effects in comparison to psilocybin. The present study looked for potential antidepressant efficacy of related tryptamines norbaeocystin, baeocystin, and aeruginascin using the FST, with analysis of BDNF expression in the medial prefrontal cortex and hippocampus via western blot and enzyme-linked immunosorbent assay (ELISA). Psilocybin and related tryptamines were compared to an established pharmacological treatment of depression, fluoxetine, through a modified version of the FST (Slattery & Cryan, 2012). I hypothesized that rats given psilocybin, norbaeocystin, and baeocystin would exhibit decreased immobility time in the FST and increased BDNF expression in the medial prefrontal cortex and hippocampus based on previous literature revolving the potential use of these tryptamine's (Glatfelter et al., 2022). Such results would be indicative of antidepressant potential, justifying further studies of these compounds

Chapter 2 Methods:

Subjects
A total of 58 male adult (PND 90-150) Long Evans rats were purchased from Charles River Laboratories (Raleigh, NC). All rats were pair-housed in standard rat cages on a 12 hr light/dark cycle (on at 0700 hr) with free access to food and water. All experiments were conducted during the light cycle between 0900 and 1700 hr. Rats habituated for five days upon arrival to the animal facility. No experimental interventions occurred during this habituation period.

All procedures were conducted in accordance with our protocols and the National Institute of Health's guidelines for the use of animals in research (National Research Council, 2011) and were approved by Miami University's Institutional Animal Care and Use Committee.

Drug Regimen
All tryptamines used in this study were provided by Dr. J. Andrew Jones (Miami University Department of Chemical, Paper, and Biomedical Engineering) and were synthesized via bioengineered *E. coli*. Purification of these tryptamines was done through semi-preparative High-Performance Liquid Chromatography (HPLC) followed by concentration under reduced pressure and precipitation in a super saturated aqueous solution. Purity was determined by ^{1}H NMR. Rats were given one of 6 conditions: vehicle (distilled water), fluoxetine (20 mg/kg), psilocybin (1 mg/kg), norbaeocystin (1 mg/kg), baeocystin (1 mg/kg), or aeruginascin (1 mg/kg). Dosages were determined based on previous HTR data suggesting psilocybin's effects are strongest at 1 mg/kg. Subjects were administered drug via intragastric infusion (gavage) of 1 mL/kg. All drugs were administered 3 times during a 24-hour period; 23.5, 5, and 1 hour before testing, matching existing dosing paradigms for this test (Cryan et al., 2005) (**Figure 2**).

Forced Swim Test (FST) Habituation
Methods for the forced swim procedure were adapted from those previously published (Slattery & Cryan, 2012). Rats were brought into the procedure room 30 minutes prior to the FST habituation stage. After the 30 minutes elapsed, subjects were placed into a plastic tube (22 x 8 in) filled two-thirds of the way (~16in) with room temperature (23 - 26°C) water. The temperature of the water was measured between every forced swim test session to ensure consistency across subjects. The water was exchanged every third session, and any animal waste was removed between each session. During the habituation phase, rats were placed inside the plastic tube to swim for 10 minutes. This habituation phase is thought to induce a learned helplessness state that is then modulated by antidepressant compounds (Cryan et al., 2005). Once animals have completed the 10-minute swim, rats were dried in a fresh, clean towel and placed into a dry cage set on top of a heating pad until internal body temperature was regulated. No data was recorded on the habituation day of the forced swim task.

Forced Swim Test (FST) Test Day
After treatment was administered over the span of 24 hours, as described above, rats completed another forced swim test in similar conditions to the habituation task. Subjects were brought into the procedure room for 30 minutes to habituate to the room. After the room habituation period, rats were placed in the forced swim tubes, as described above, but for 5 minutes. The test was recorded using an iPhone 12 Pro, held 30 cm directly above the tubes so that all edges were

observable on the recording for future analysis. Rats were pulled out, dried, warmed, and sacrificed for tissue collection 30 minutes after the test. Video recordings were then coded by 2 trained observers, blind to animal conditions, for the following behaviors: swimming, climbing, and immobility. Swimming is described as a rat swimming in horizontal movements or from one quadrant to the other. Climbing is an upward directed movement of the forepaws against the cylinder. Immobility consists of the rat floating in the water without struggling, only making necessary movements to stay above the water (Slattery & Cryan, 2012). Observer scores were required to match within 90%, and were averaged to obtain a final value for that subject.

Tissue Collection

Following completion of FST, rats were sacrificed and tissue was collected to assess levels of Brain Derived Neurotrophic Factor protein (BDNF) in the dorsal and ventral hippocampus (CA1, CA2, CA3) and medial prefrontal cortex (mPFC, cingulate gyrus). All subjects were euthanized with sodium pentobarbital; brains were extracted and drop frozen in 2-methylbutane, then stored in -80C prior to slicing via cryostat. Coronal slices of 80µm from hippocampus (Hipp: CA1, 2, 3 and DG) and medial prefrontal cortex (PrL, CG, and IL) were collected (Paxinos & Watson, 1986) and brain punches (2.0 mm) taken from each slice, resulting in a total of ~50 µg of tissue per region. Supernatant of tissue samples from the same vial were used for both ELISA and western blot procedures.

Western Blot

Hippocampal and prefrontal cortical protein samples were denatured by heating to 100°C for 5 minutes; samples were prepared by homogenizing the tissue at 4 degrees Celsius in 75 µL phosphate buffered saline (PBS), 150 µL of 2X Laemmli sample buffer, 50µL 2-mercaptoethanol, and 50 µL protease inhibitor cocktail (Bio-Rad, CA, USA). Samples were vortexed for 30 seconds and centrifuged at 3,000 x g for 10 minutes. Total protein concentration per well was measured utilizing Bradford Assay. Prior to electrophoresis, total protein per lane was determined and normalized based on Bradford Assay result (e.g., 5 µg per lane). I then separated the proteins in the samples using electrophoresis at 200V for ~25 minutes. The proteins traveled through a Tris-Glycine eXtended Stain-Free Protein Gel, which was imaged and electro-transferred to a polyvinylidene fluoride membrane (PVDF) (Bio-Rad, CA, USA). The PVDF membrane was also imaged and quantified for later analysis. Membranes were incubated in EveryBlot Blocking Buffer for 60 min at room temperature to block non-specific binding. Primary antibody Rabbit anti-BDNF was incubated (1: 2000; Bio-Rad, CA, USA) for an hour. I washed the membrane post primary antibody 5 times for 5 minutes each time in 0.05% Tween Tris-Buffered Saline solution. Secondary antibody Goat anti-Rabbit StrB700 (1:5000; Bio-Rad, CA, USA) was used to detect the primary antibody via fluorescence and incubated for another hour. Membranes were washed 6 times for 5 minutes with 0.05% Tween-TBS solution. Immunopositive bands were visualized utilizing the Chemi-Doc Imaging System (Bio-Rad; CA, USA). Results are expressed as fold change from Vehicle expression of BDNF.

Enzyme-linked Immunosorbent Assay

Supernatant rat BDNF levels were determined by commercially available assay kits. The detection limit of each kit is 12 pg/mL for BDNF (Invitrogen, Carlsbad, CA, USA). The wells were loaded with 50 µL of standards and sample (~5 µg total protein) and incubated at room temperature for 2 hours with gentle shaking. The provided diluent acted as a zero standard to

accommodate for background for our standard curve. I added 100 µL of BDNF biotinylated antibody and incubated them for 1 hour at room temperature with gentle shaking. Wells were washed with reverse osmosis water, followed then by adding 100 µL SAV-HRP (Streptavidin-Horseradish Peroxidase) to each well for 1 hour. Next, 100 µL of Chromogen was added in each well for 30 minutes before adding the stop solution. Wells were protected from light post substrate addition. Absorbance of each well was measured using an automated microplate reader (BioTek Instruments, Winooski, VT, USA) set to 450 nm and we subtracted it from 550 nm to account for any noise the machine may have emitted or any light-scattering particulates that could have offset our absorbance, within 30 minutes post stopping solution. Standard curve was made through mean absorbance of standard against the concentration and multiplied by the dilution factor. All samples were recorded in duplicate.

Statistical analysis

Data were analyzed in GraphPad Prism (v.8.4.3). Forced swim data were analyzed using one-way ANOVA. *Post-hoc* two-stage linear set-up method of Benjamini, Krieger, and Yekuteili (Benjamini et al, 2006) was conducted where appropriate to compare vehicle and treatment groups. Western blot data were analyzed using an unpaired one sample t-test to compare treatment from vehicle after normalizing protein concentrations by lane and using fold-change from vehicle as the main measure. ELISAs were also analyzed as fold change from vehicle using one sample t test. A significant result was defined as $p < 0.05$. All analyses were expressed as mean ± SEM.

Chapter 3 Results

Forced Swim Test

To assess potential antidepressant efficacy of our tryptamines, rats underwent the FST, which included 3 measurable behaviors: immobility, swimming, and climbing. Immobility was used to assess helplessness states and was characterized as floating with the absence of movement, aside from what was necessary to keep their nose above the water. As shown in **Figure 3A**, a one-way ANOVA showed there was a statistically significant effect of group on immobility [$F(5,52) = 0.475$, $p = 0.005$], with post hoc tests finding significant differences between vehicle and psilocybin ($p = 0.040$), norbaeocystin ($p = 0.05$), and fluoxetine ($p = 0.004$). Additionally, swimming was characterized as consistent movement of the forepaws or circular movement in the tube, which assessed active struggling and engagement in the task. A one-way ANOVA revealed (**Figure 3B**) another significant effect of group [$F(5,52) = 0.5413$, $p = 0.006$], with post hoc tests showing that psilocybin ($p = 0.044$) and fluoxetine ($p = 0.004$) differed from vehicle. The final behavior assessed was climbing, which was characterized by the forepaws breaking the surface water level and actively trying to move upwards in the tube. One-way ANOVA revealed no significant effect of group [$F(5,52) = 0.1791$, $p = 0.969$] (**Figure 3C**).

BDNF Western Blot

Western blot images and protein quantification of BDNF were recorded post FST and drug administration as density/intensity via fold-change from vehicle. BDNF level was normalized by lane to total protein to account for inconsistent sampling or sample preparation. One-sample t tests revealed only a significant decrease in mature-BDNF (12-14Kd) expression in the medial prefrontal cortex of rats that were administered norbaeocystin [$t(6) = 3.736$, $p = 0.0135$] (**Figure 5A**). There were no significant effects found in the hippocampus (**Figure 5B**) for both mature and pro-BDNF (32-37Kd) **(Figure 5C)** as well as between the ratio of both isoforms of BDNF **(Figure 5D)**.

BDNF ELISA

Protein quantification was recorded post FST and drug administration and transformed as a fold-change from the vehicle treatment condition. One-sample t tests for each group (comparing group means to a fold change of 1) revealed that rats in the psilocybin condition had significantly higher expression of BDNF in the hippocampus compared to vehicle [$t(5) = 2.891$, $p = 0.034$] (**Figure 4B**). There were no significant differences in BDNF levels in the remaining treatment conditions in the hippocampus. One sample t tests also found no significant effects on BDNF expression in the medial prefrontal cortex (**Figure 4A**).

Chapter 4: Discussion/Conclusion

This study determined the potential antidepressant efficacy of psilocybin and related tryptamines in comparison to a positive control of fluoxetine, using the forced swim test. These related tryptamines showed decreased HTR in comparison to psilocybin, suggesting potential non-hallucinatory states in rats. We hypothesized that related tryptamines would elicit similar antidepressant effects as psilocybin and SSRI fluoxetine in the FST, along with up-regulation of BDNF, a biomarker of antidepressant action. I found that psilocybin and norbaeocystin showed similar behavioral effects as the common SSRI fluoxetine, in that they all decreased immobility time (**Figure 3A**)., while baeocystin and aeruginascin caused non-significant decreases (**Figure 3A**). Other measurable behaviors (swimming and climbing) showed only a significant increase in swimming behavior for the fluoxetine and psilocybin groups and no effects on climbing (**Figures 3B and 3C**). However, similar non-significant trends can be seen in the other tryptamine conditions, suggesting that they might elicit their mechanisms differently from psilocybin, despite sharing chemically similar structures. Overall, these results support the claim that at least one of these compounds may have antidepressant potential (norbaeocystin), based on the similar trend of decreasing immobility time.

The FST is commonly used to assess behavioral despair in rodent models and is widely accepted to be the standard when assessing antidepressant efficacy of pharmacological agents (Yankelevitch-Yahav et al., 2015). However, there are multiple factors such as rat strain, environment, and gender that could influence results in the modified FST (Bogdanova et al., 2013). This study utilized male Long Evans rats, a popular strain for assessing a plethora of behaviors. Other strains have been shown to exhibit more or stronger depressive-like behaviors (e.g., Sprague Dawley) (Bielajew et al., 2003). Therefore, my results might only be revealing strong drug effects, with smaller behavioral differences obscured. By using more resilient animals, I might not be showing the full potential of these compounds. Biological sex of rodents has also shown to play an influential role in the FST, as well as in baseline levels of expression of BDNF in the hippocampus (Chan & Ye, 2017; Kokras et al., 2015). My use of male rats allowed me to measure all observable behaviors in the FST without having to account for estrous stages, which are known to induce higher immobility times in diestrus and proestrus in female rats (Consoli et al., 2005). However, future studies must assess the antidepressant potential of these compounds in female rats, especially considering the higher prevalence of depression in women (Albert, 2015). Rats can easily change observable behaviors if they are under stress prior to the FST (Commons et al., 2017), and variance in animal handling could alter anxiety/depressive like behaviors (Song et al., 2021). Throughout my study, rats had to be transported and handled daily, so we must consider the possibility of enhanced stress levels from necessary movement and handling (including the multiple gavages). The mechanisms of these related tryptamines have not been fully studied in the literature and could suggest alternative routes when interpreting our results together with psilocybin.

As a potential biomarker of antidepressant efficacy, I assessed BDNF expression level in the hippocampus and medial prefrontal cortex after the forced swim test, as well as different isoforms of it present in my western blots (Mature vs Pro-BDNF) (Supplementary Data 2A/2B). The ELISAs showed that there was an increase in BDNF expression in the hippocampus in the psilocybin condition compared to vehicle, but not in the medial prefrontal cortex (**Figures 4A**

and 4B). In contrast, data from our western blots found a significant decrease in mature-BDNF levels in rat medial prefrontal cortex that were given norbaeocystin (**Figure 5A)**, and no significant differences in BDNF expression outside of this (**Figure 5B**). I hypothesized that there would be an increase in BDNF expression in both the rat hippocampus and medial prefrontal cortex in both the ELISAs and Western Blots. I also hypothesized that we would see similarities in BDNF levels between both methodologies. However, some differences were observed, which could have arisen from combination of issues when completing these assays. The use of western blots also requires more processing steps compared to ELISAs, which could lead to an aggregation of user errors that individually may not be consequential. Both assays also utilize antibodies in order to detect the specific target protein, in this case BDNF. However, there is a higher risk antibody specificity being varied by each individual band (Buchwalow et al., 2011). The western blot protocol used here was developed recently in our lab, which also raises questions about its reliability. However, instructions based on previous established protocols (Bio-Rad Laboratories, 2020) were used to reduce possible errors, and the equipment and antibody manufacturer were consulted throughout the protocol development process. Both methodologies were novel in practice for this Thesis, but the lack of similarities/correlation between both quantification methods leaves many questions and could lead to a misinterpretation of my results.

The results from my ELISAs suggest psilocybin up regulates expression of BDNF in the hippocampus. I believe the results of the ELISA are more reliable in this situation due to my ability to test each sample in duplicate (N=10-12), which reduces variance in our results. Only enough protein remained for one replicate per sample to be run in the western blots (N=5-6). Additionally, my ELISA kits had a stronger citation base, suggesting greater reliability. Lastly, a combination of user error and possible antibody specificity leads me to believe that the group differences found by the ELISA could be more reliable if in future research we account for both isoforms of BDNF in the ELISAs by using more specific antibodies for both isoforms. However, despite differences in effect directions and size, both methods provided confirmation that BDNF expression occurred in both frontal cortex and hippocampus, aligning with previous work. However, beyond their disagreeing effects, there are other considerations regarding both methods. The FST is often considered a proxy for antidepressant efficacy and BDNF promotes neuroplasticity in the brain regions associated with depression through increased neurogenesis (Davoudian et al., 2022; Gourley et al., 2008; Leal et al., 2017; Yang et al., 2020). However, based on my power analysis in order to obtain 80% power a total of 10 samples per group is needed for both western blots and ELISAs. In this current study we have an achieved power of 0.23 with a sample size of 6. Therefore, there is a need to collect data from 4-6 additional animals in future studies to ensure we get a power of 80%. There is a lack of statistical power; my power analysis suggests that I need a total of 10 samples for each condition in both western blots and ELISA in order to obtain $\beta = .8$. Additionally, we see that different isoforms of BDNF existed in our protein samples, in line with other literature (**Figure 5A and 5C**)(Je et al., 2012.).

The mature form of BDNF was the primary focus of this thesis, as it is thought to be the isoform that promotes antidepressant efficacy. It is unclear how these different isoforms of BDNF are accounted for in each of my methodologies but is likely related to the specific antibodies used. With a single antibody, ELISAs may account for these different isoforms as a sum or ratio, but this is unclear and would depend on the relative specificity of the antibody for each target. In

future studies, specific antibodies for each isoform of BDNF could be used, to determine their ratio and total amount more accurately. (**Figure 5D;** dependent on antibody specificity). In the western blot analysis, bands for both pro- and mature forms can be easily visualized, but again, the specificity of the single antibody used may differ for each isoform. Bands for both isoforms of BDNF were found in western blots from hippocampus tissue, while only the mature band was visible in frontal cortex samples. More research on our antibody specificity and total BDNF vs isoform specific expression in our western blots is needed to confirm our findings in this assay. Additionally, re-evaluation of our methodologies, based on lack of similarities between my ELISAs and western blots, is needed. Psychedelics, like psilocybin, increase expression of BDNF and promote synaptogenesis and plasticity (de Vos et al., 2021). Therefore, I hypothesize that this increase in BDNF found by the ELISA could underlie decreased immobility time in rats after psilocybin or related tryptamine treatment. The reason being is because there is an increase in BDNF levels, which could promote synaptogenesis in brain regions that are thought to mediate antidepressant effects. My results suggest there could be increases in BDNF expression, but a lack of reliability of my quantification methods (no similarities between ELISA and WB) means we cannot confirm my hypothesis at this time.

Psilocybin and related tryptamines are thought to elicit their effects through their activation of serotonin receptors. Psilocybin's active metabolite psilocin has a high affinity to 5HT2A, while the active metabolites of baeocystin and aeruginascin have a lower affinity to the 5HT2A receptor in comparison to psilocin (Sherwood et al., 2020, Glatfelter et al., 2022). Norbaeocystin's metabolite has not yet been studied. If the proxy of HTR being a marker for hallucinations is true, this means related tryptamines like norbaeocystin, which significantly decreased immobility time in the FST, could elicit antidepressant effects without the need for 5HT2A activation. Recent literature has explored whether 5HT2A receptor activation and hallucinations are needed to elicit the antidepressant effects in psilocybin (Hesselgrave, 2021). Non-hallucinogenic drugs such as lisuride (a 5HT2A and 1A agonist) elicit antidepressant effects similar to ketamine, suggesting that hallucinations might not be necessary for the tryptamines explored here to elicit their therapeutic effects (Qu et al., 2023). Additionally, psilocin binds directly to TrkB receptors unlike fluoxetine. This suggests that SSRIs might up regulate BDNF expression and thus elicit their antidepressant effects while these tryptamines directly bind to TrkB which by itself might be leading the antidepressant effects (Moliner et al., 2023). This could explain why we see an increase in BDNF with fluoxetine, but not as much in our tryptamines. Furthermore, rats given norbaeocystin show few HTRs (**Figure 1**), suggesting it could be non-hallucinogenic, but still elicit similar antidepressant effects as psilocybin and fluoxetine. Preliminary results from our lab has also looked at blood brain barrier penetration of the active forms of these tryptamines, and found that 4-hydroxy-*N,N,N*-trimethyltryptamine (aeruginascin) was the only one that did not cross the blood brain barrier (data not shown). These results may suggest that psilocybin, norbaeocystin, and baeocystin could act more directly in the central nervous system, while aeruginascin might elicit its effects more peripherally. This could also explain why I see increased immobility time in aeruginascin compared to the other tryptamines. Additionally, I saw a trend in increased expression of BDNF after administration of psilocybin, which is also known to regulate neural circuits involved in mood disorders like depression (Martinowich & Lu, 2007). ELISAs and western blots revealed no real difference from vehicle in aeruginascin, meaning that modulation of BDNF could be more peripherally mediated unlike the rest of the tryptamines

Recent literature has speculated the downstream effects of psilocybin though other neurotransmitters like dopamine (Lowe et al., 2021). If dopamine were to be up regulated due to the downstream effects of these tryptamines, I could consider taking a different approach from the perspective of mobility stimulation through dopaminergic pathways (Adams et al., 2022). The 5HT1A receptor, which these related tryptamines are known to have a higher affinity to, is also known to increase release of dopamine in the medial prefrontal cortex and hippocampus in rats (Marona-Lewicka & Nichols, 2011). Ultimately, activation of other serotonin receptors may have increased dopamine release, which could explain why these related tryptamines are performing better in the FST, instead of purely relying on the effects on the 5HT2A receptor. All of these results suggests that norbaeocystin and maybe some of these other related tryptamines could be an alternative non-hallucinogenic therapeutic similar to psilocybin based on their lack of activation of the 5HT2A receptor and its effects on the FST. Our behavioral data suggest the use of related tryptamines as a potential antidepressant is supported by our results in the FST and the upregulation of BDNF in specific brain regions associated with depressive like activity.

The oral route of administration used here could have a different mechanism of action than other studies that have relied on injection, including differing CNS, PNS, and even in the gut microbiome effects. A large concentration of serotonin and dopamine receptors lie in the gut microbiome with a majority of peripheral serotonin coming from the gut microbiome, and their activation could cause direct activation of the gut brain axis (Gershon & Tack, 2007) or have effects on the vagus nerve. If a large concentration of receptors are found peripherally, it begs the question on how things such as permeability outside of the blood brain barrier may affect the mechanisms and onset effects of our tryptamines. Oral administration of psilocybin has been shown to alter hallucinogenic activity and have delayed onset effects that could contribute to understanding treatment options for mental health disorders (Kelly et al., 2023). I also must consider how peripheral mechanisms can alter results in the FST and expression of biological markers in the brain (Luo et al., 2018). Effects revolving around receptor modulation both peripherally and centrally suggest effects of drugs might elicit different effects on our methodologies (Strandwitz, 2018).

The use of the FST to evaluate potential antidepressant efficacy, along with protein quantification, has its advantages (high throughput antidepressant screening methodology) and its disadvantages (very specific in many materials/subjects). These methodologies have been used to previously assess other antidepressants and biological markers (Martinowich & Lu, 2007; Yankelevitch-Yahav et al., 2015). Determining if other factors such as gut microbiome bacterial levels change with administration of these tryptamines and if, in turn, they alter the behavioral paradigms presented here, could be beneficial for future research. Future directions should also look at how gut permeability can affect the behavioral response of psilocybin and novel tryptamines in these behavioral assays, and how it could regulate biological markers in specific brain regions. The use of alternative pharmacological treatments such as ketamine could be beneficial to understand how these tryptamines may be similar when eliciting antidepressant efficacy. Despite these tryptamines being considered non-hallucinogenic, I might consider evaluating the potential hallucinogenic effects of these novel tryptamines through a more biphasic model of behaviors, like the drug discrimination paradigm to further screen these related tryptamines more reliably.

My thesis showed that psilocybin and norbaeocystin have potential therapeutic effects using both behavioral paradigms and biological markers in brain tissue, similar to the common SSRI fluoxetine. These results may suggest I need other physiological markers to fully understand if baeocystin and aeruginascin truly had no effects or if other factors concealed their efficacy (i.e. route of administration). Understanding how these related tryptamines may share some but not all mechanisms with psilocybin is key in broadening the field's view. The field requires that we understand the mechanisms of these tryptamines outside of just behavior, and for that we require more in-depth understanding of how these tryptamines elicit their effects through different mechanisms both behaviorally and biologically.

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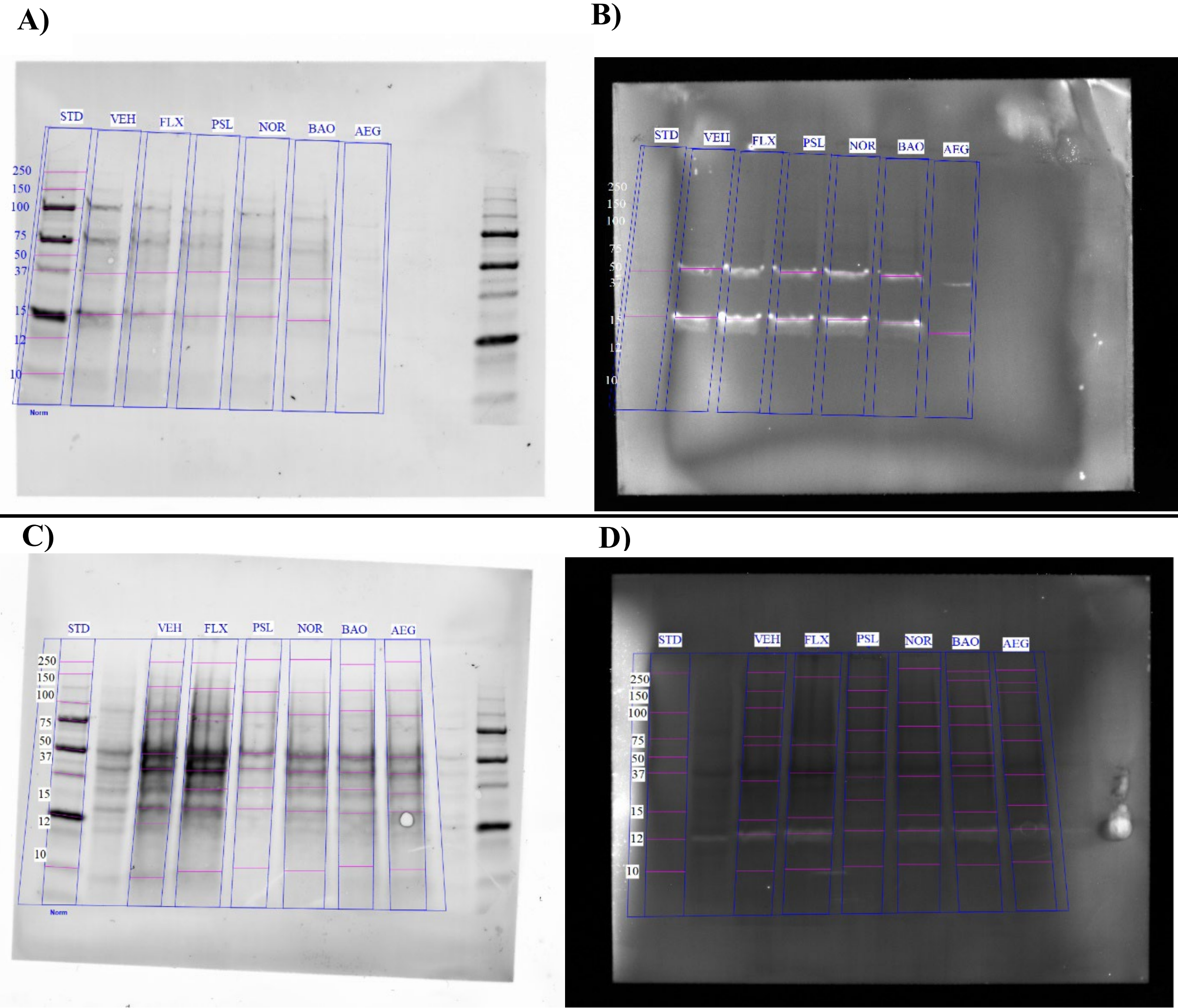
Chapter 5: Supplemental Data

	Ki (nM)					
Receptors	Psilocybin	Psilocin	Baeocystin	Norpsilocin	Aeruginascin	4-HO-TMT
5-HT1A	5284	164	-	86	-	-
5-HT1B	4983	580	370	99	-	-
5-HT1D	186	130	394	194	-	-
5-HT1E	601	155	1352	161	-	-
5-HT2A	-	180	-	391	-	-
5-HT2B	259	8	134	57	-	720
5-HT2C	808	175	-	243	-	-
5-HT5A	-	116	-	365	-	-
5-HT6	89	38	-	54	-	2267
5-HT7A	138	75	117	68	-	-

SD1: Receptor affinities for most tryptamines and their dephosphorylated forms that were used in this study. (Glatefelter et al., 2022)

Tryptamine	Dephosphorylated Form
Psilocybin	Psilocin
Norbaeocystin	4-HT
Baeocystin	Norpsilocin
Aeruginascin	4-HO-TMT

SD2: Chemical structure and metabolites for all the tryptamines that were used in this study.



SD 2A/2B: Images of SDS-Page and blot of rat hippocampus total protein expression and BDNF expression. A) SDS-Page showing total protein expression in all lanes for hippocampus. B) Blot showing BDNF expression in both the pro (top lane) and mature (bottom lane) isoforms in hippocampus only. C) SDS-Page showing total protein expression in all lanes for medial prefrontal cortex. D) Blot showing BDNF expression in all lanes for medial prefrontal cortex.

Chapter 6: Figures and Tables

Figure Legends

Figure 1: Total amount of Head Twitch Response elicited by psilocybin and related tryptamines (n = 5-10 per group). Psilocybin produces a dose-response curve, but related tryptamines do not. One-way ANOVA found significant effect of HTR in rats given 1mg/kg of psilocybin when compared to 0mg/kg. $^{**}p<.01$.

Figure 2: Overall timeline of dosing paradigm and modified FST.

Figure 3: Forced Swim Test revealed differences in immobility, swimming and climbing times in rats treated with psilocybin, and related tryptamines that were similar to rats treated with fluoxetine. A) One-way ANOVA revealed psilocybin and norbaeocystin reduced immobility time similar to rats that were given fluoxetine. B) One-way ANOVA showed that fluoxetine and psilocybin increased swimming time when compared to vehicle treatment rats. C) Climbing behavior was recorded but showed no significant differences between treatment conditions. $^{*}p<0.05$, $^{**}p<0.01$

Figure 4: Quantification of BDNF expression in rat medial prefrontal cortex and hippocampus utilizing ELISAs. A) Unpaired t test showed there was no significant differences in ELISA quantification of BDNF in rat frontal cortex. B) Unpaired t test revealed that ELISA showed increased BDNF expression in rat hippocampus when administered psilocybin. $^{*}p < 0.05$

Figure 5: A) Unpaired t test showed in our Western Blots decreasing amounts of BDNF in rat frontal cortex when norbaeocystin is administered. B) There were no significant differences in my Western Blot imaging and quantification of BDNF expression in rat hippocampus. C) No significant differences in Western Blot imaging and quantification of Pro-BDNF levels found in n=3 of our hippocampus blots. D) Ratio for amount of Mature BDNF over Pro-BDNF in 3 samples. $^{*}p < 0.05$

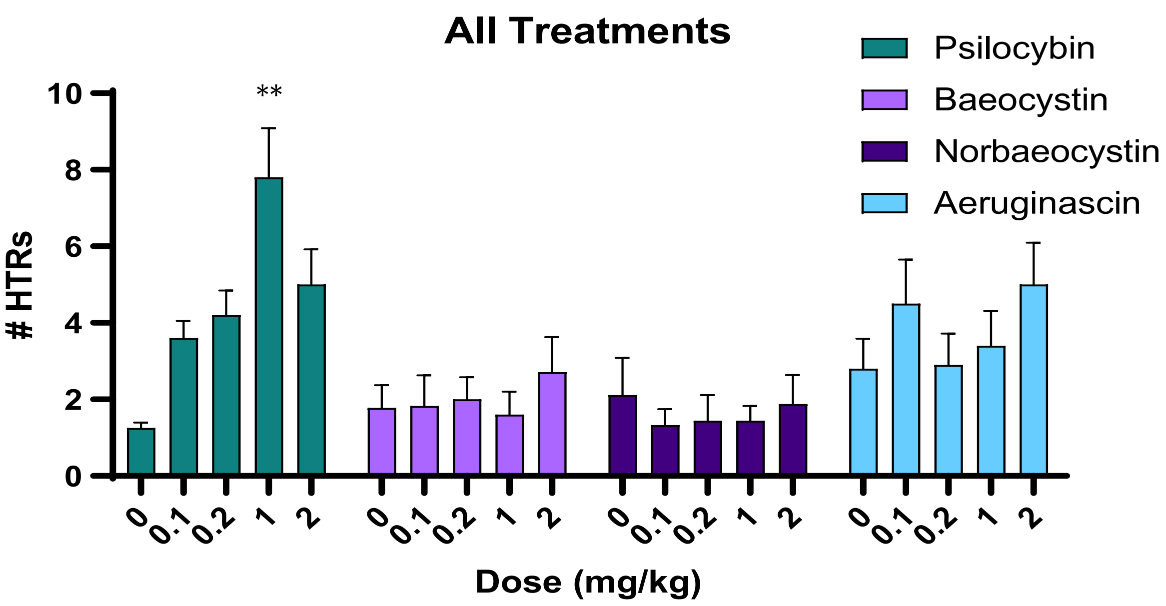
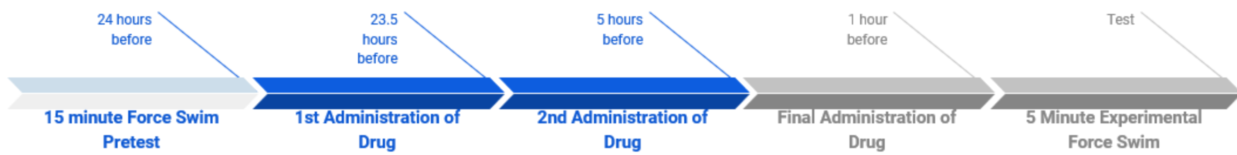


Figure 1: Total amount of Head Twitch Response elicited by psilocybin and related tryptamines (n = 5-10 per group). Psilocybin produces a dose-response curve, but related tryptamines do not. One-way ANOVA found significant effect of HTR in rats given 1mg/kg of psilocybin when compared to 0mg/kg. **$p < .01$.



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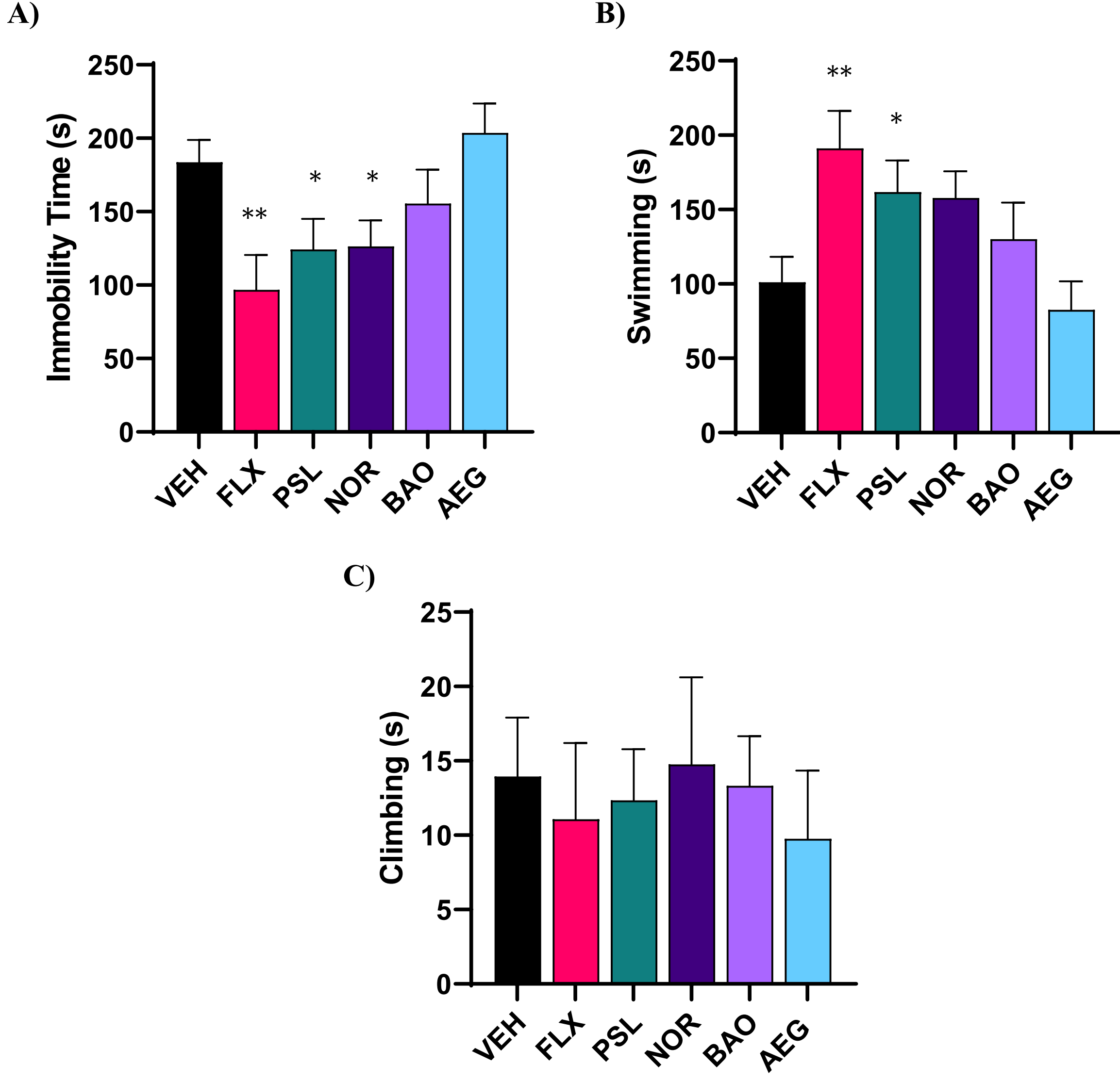

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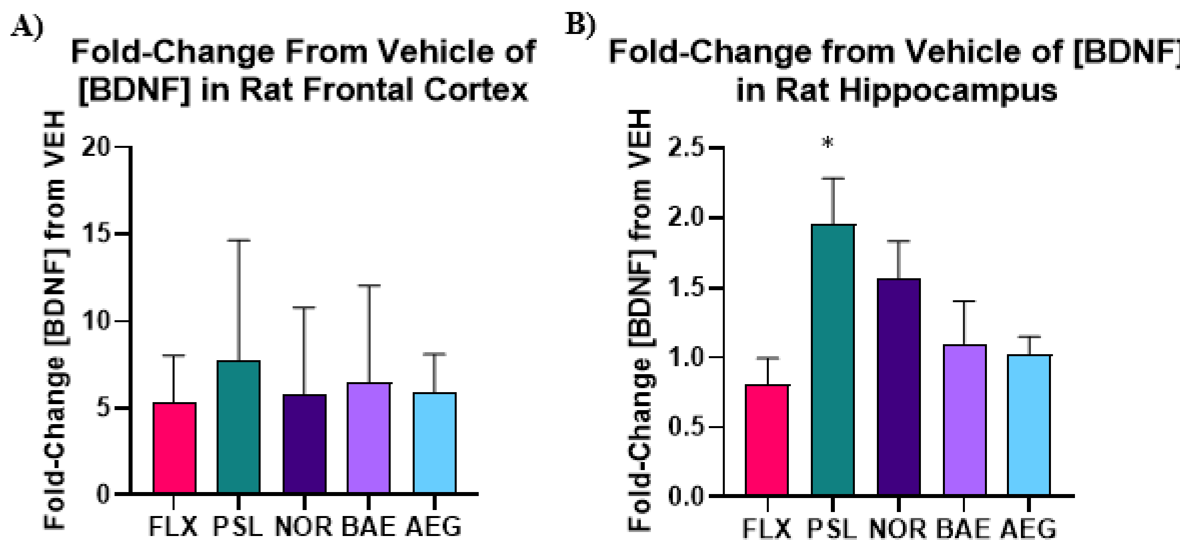

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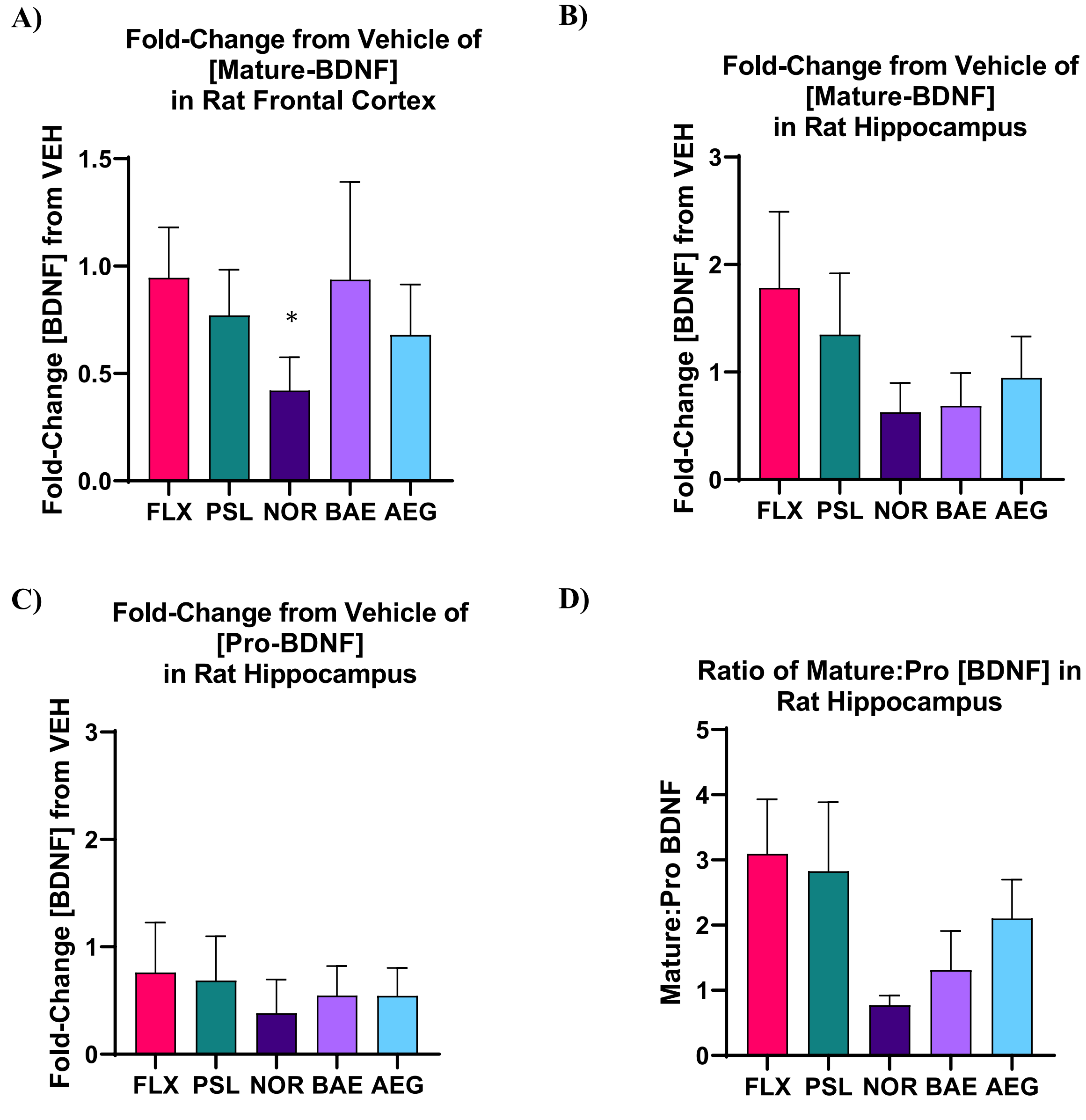

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