UNIVERSITY OF CALIFORNIA SAN DIEGO

Mechanisms of Ketamine's Sustained Antidepressant Effect

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Shae Beverly Galli

Committee in charge:

Professor Yimin Zou, Chair
Professor Jill Leutgeb, Co-Chair
Professor Stacey Glasgow

2022

The thesis of Shae Beverly Galli is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2022

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PREFACE

The research described in this thesis was performed under the supervision of Professor Dr. Yimin Zou and Assistant Project Scientist Dr. Andiara E. Freitas. The project was carried out as a part of Dr. Andiara E. Freitas' primary research project. She performed the stereotactic surgeries and injections, as well as designed the experimental paradigms and carried out the behavioral tests. She taught me how to setup and perform the behavioral tests, and how to maintain the mouse colony including genotyping and post-operative follow-up. With her help and guidance, I was able to perform transcardial perfusions and brain tissue collection + sectioning, carry out excitatory synapse analysis and behavioral video analysis, and help with raw data collection for analysis.

ACKNOWLEDGEMENTS

I would like to first thank Dr. Yimin Zou for his ongoing mentorship, encouragement, and inspiration throughout my time as a student. From the first office hours I attended as an undergraduate to my last defense practice as a master's student, his discussion, passion, and deep knowledge within the field of neuroscience has sparked my own curiosity and my passion for scientific discovery. I am most sincerely thankful for his invitation to me to volunteer in the lab as an undergraduate and for his guidance and advice that has made it possible for me to progress throughout the master's program!

I would also like to sincerely thank Dr. Andiara E. Freitas for her wonderful mentorship, training, and wisdom that has guided me throughout my time as a master's student. Her incredible knowledge and long term dedication to the field of depression research has shown me the magnitude to which scientific discovery and passion can reach. I am deeply grateful for her time and commitment to my training in the lab and for her leadership in experimental design, her invitation to collaborate in conducting experiments and discussing results, and for graciously answering my countless questions about research methods, literature, and the neurobiology of depression. This program would not have been possible without her and I am most sincerely thankful!

I would like to thank lab technician Clayton Baker for patiently teaching me a myriad of lab techniques, for offering wonderful graduate school advice, and for bringing happiness and positivity into each day. I would also like to thank my committee members Dr. Jill Leutgeb and Dr. Stacey Glasgow for their inspiration as professors in my undergraduate classes and for their feedback and support as members of my master's thesis committee.

I would like to sincerely thank my parents, Mark and Krissy, for their unconditional love, their persistent encouragement of my lifelong passion for biology, and their wholehearted support. They have built the foundation of who I am today and have enthusiastically lifted me up with every step of my journey as a student and a young adult. I have an immense amount of love and gratitude for them, and my process would not have been possible without them! I would like to thank the Lord for His great love and for my salvation and life in Jesus Christ. I would like to thank my brother, Roen, for his optimistic love and encouragement, and I would like to thank my boyfriend, Jack, for his love and support, his belief in me, and for the joy and light he's brought into every day.

Figures 1.5, 1.6, 1.7, 2.3, 2.4, 3.2, 3.3, 3.4, and 4.2 are currently being prepared for submission for publication of the material. Freitas, A. E., Feng, B., Baker, C., Galli, S., Ban, Y., Zou, Y. (2022). *Ketamine reverses stress-induced behavior by restoring excitatory synapses in the basolateral amygdala-projecting infralimbic prefrontal cortex neurons.* (In preparation). The thesis author was a co-author of this material.

ABSTRACT OF THE THESIS

Mechanisms of Ketamine's Sustained Antidepressant Effect

by

Shae Beverly Galli

Master of Science in Biology

University of California San Diego, 2022

Professor Yimin Zou, Chair
Professor Jill Leutgeb, Co-Chair

Depression is characterized by a loss of synaptic connections in regions of the brain involved in emotional regulation and cognitive function, and antidepressant treatments generally result in a compensatory increase in the number of these connections. Previous studies have

shown that a single sub-anesthetic dose of ketamine, a rapid-acting antidepressant, increases synaptic connectivity in excitatory neurons projecting from the prefrontal cortex to other brain regions involved in cognitive regulation and function, countering the destabilization and loss of synapses observed in depression. However, there is little understanding of the molecular pathways and mechanisms involved in synapse restoration at the synapse structure level. Due to the role of proteins involved in synapse formation and maintenance, we sought to determine whether ketamine's antidepressant mechanism is dependent on these proteins in projecting neurons of excitatory circuits that contribute to emotional regulation. Mice were divided into groups with or without chronic stress and with conditional knockouts for the proteins of interest. They were then subjected to a series of behavioral tests and their brain tissue was analyzed for changes in excitatory synapse number. We found that ketamine's promotion of synapse formation and sustained antidepressant-like effect on behavior was hindered in knockout mice. These findings revealed that ketamine is dependent on proteins involved in synapse formation and maintenance for its rescue of depressive-like behavior and restoration of excitatory synaptic connectivity.

Introduction/Background

Depression is one of the most prominent diseases worldwide and is characterized among other symptoms by depressed mood, impaired concentration, feelings of worthlessness, appetite changes, fatigue, and loss of interest or pleasure in daily activities (World Health Organization, 2021, Bains & Abdijadid, 2022). When these symptoms persist consistently beyond two weeks and significantly interfere with a person's ability to function in day-to-day life, this is diagnosed as a major depressive disorder (MDD). Postmortem studies of brain tissue from patients diagnosed with depression have revealed that depression is characterized by reduced volume in the hippocampus and prefrontal cortex, brain regions involved in cognition and emotional regulation. Further investigation of these tissues and of animal models has shown minimal changes in cell number, leading researchers to hypothesize that the reduction in volume is due to reduction in synapse number and an overall decrease in connectivity in these brain regions (Duman, 2014). Thus, current antidepressants produce a compensatory increase in excitatory neurotransmission by generating an overall increase in the concentration of neurotransmitters in excitatory synapses. Normally, after excitatory neurotransmission takes place, monoamine reuptake transporters in presynaptic neurons act to clear the synapse of neurotransmitter, restoring the concentration to baseline and preventing excitotoxicity. Antidepressants such as selective serotonin reuptake inhibitors (SSRIs) therefore work by blocking this reuptake, or reabsorption, of monoamines such as serotonin by the presynaptic neuron. This method ultimately leads to an increase in monoamine concentration in the synapse, increasing the level of excitatory neurotransmission taking place (Hasselman, 2014). Due to the pharmacological mechanism of these drugs, hypotheses for depression have historically been based on the monoamine hypothesis, which describes depression as a deficiency in neurotransmitters involved

in excitatory neurotransmission, including serotonin and norepinephrine (Sanacora et al., 2012). This mechanism seems feasible, as antidepressants such as SSRIs have been relatively effective in patients since their introduction to psychiatry's treatment arsenal (Hasselman, 2014). However, antidepressants typically take four to six weeks to exert their effect, an observation inconsistent with the proposed antidepressant mechanism being an acute increase in excitatory neurotransmission. Additionally, remission of depressive symptoms begins long after patients start experiencing negative side effects such as increased anxiety and even increased suicidality in younger patients (Bratsos & Saleh, 2019). This often leaves patients under the impression that antidepressants don't work for them because they stop taking the drugs due to these adverse side effects without giving the medication the necessary amount of time for it to work. Furthermore, only one third of patients respond to the first prescribed antidepressant, followed by another third responding to additional trials of different antidepressants. This unfortunately leaves one third of patients diagnosed with treatment-resistant depression, as their symptoms don't improve after various trials of current medication options (Duman, 2014, Hasselman, 2014). Thus, there is a major need for deeper understanding of the mechanisms of currently prescribed antidepressants and for further development of potential therapeutics targeting such molecular mechanisms as they are further elucidated.

As commonly prescribed antidepressants, such as SSRIs, exert changes in neurotransmitter levels and alter synaptic connectivity, they provide insight into the mechanisms underlying depression (Duman, 2014, Harmer et al., 2017). For example, treatment resistant patients are sometimes referred to alternative treatments including rapid-acting antidepressants, such as ketamine, which induce synaptic changes by creating an increase in glutamatergic neurotransmission (Bratsos & Saleh, 2019). Unlike typical antidepressants which take four to six

weeks to exert their effect, a single sub-anesthetic dose of ketamine produces an antidepressant effect within hours that lasts for about a week (Duman & Aghajanian, 2012). Synchronous with findings that patients with depression have reduced hippocampal and prefrontal volume due to decreased synaptic connectivity, ketamine has been shown to increase synaptic connectivity in the prefrontal cortex by promoting the formation and stabilization of excitatory synapses (Duman, 2014).

Depression is commonly modeled in mice exposed to chronic stress, as depression is a form of chronic stress. The depressive-like behavior and physiological changes in these mouse models has provided insight into the neurobiological mechanisms of antidepressants. In such mouse models, ketamine has been shown to increase the length and number of dendritic spines, neuronal processes that form connections between excitatory neurons. The excitatory synaptic connections that were lost in these models of depression, specifically in the medial prefrontal cortex, were restored by ketamine administration (Moda-Sava et al., 2019). This evidence demonstrates why ketamine is useful as a rapid-acting antidepressant in humans with treatment resistant depression. However, to optimize drug development and create safer, more effective therapeutics, a deeper understanding of ketamine's mechanism of action is needed. Specifically, since ketamine's effect is associated with an increase in synapse number and connectivity, investigation of its molecular mechanisms at a circuit-specific level is needed to understand this synapse formation and maintenance.

Proteins involved in mechanisms of synapse formation and stabilization provide insight into ketamine's ability to increase the number of dendritic spines. Excitatory synapses are formed during development and are strengthened and maintained throughout adulthood, and a multitude of proteins and mechanisms are involved in these processes. One of these mechanisms

involves Planar Cell Polarity (PCP) proteins, which function in the adult brain to maintain structure and stability of excitatory synapses. Planar cell polarity proteins have long been known to mediate apical basal polarity in epithelial tissue, such as the orientation of stereocilia of the inner ear. They play a highly critical role in axonal growth cone guidance in central nervous system development of vertebrates (Zou, 2020). Research from the lab of Dr. Yimin Zou in the UCSD Department of Neurobiology has uncovered the prominent role of planar cell polarity proteins in the developing and adult brain, as they were found to be critical in the formation and maintenance of excitatory synapses. Primary investigation revealed that glutamatergic synapses, the main excitatory synapses in the adult brain, are dependent on PCPs Celsr3, Prickle1, and Prickle2 for their formation and maintenance in the hippocampus and prefrontal cortex. In 2017 in collaboration with Dr. Yimin Zou, Dr. Thakar et al. demonstrated that during development, Celsr3 is critical in the formation of excitatory synapses in the mouse hippocampus while Vangl2 interferes with synapse formation. (Thakar et al., 2017). Later, in 2021, two papers were published from Dr. Yimin Zou's lab, bringing to light the critical role of PCP proteins in excitatory synapse maintenance in the adult brain. Dr. Bo Feng, Dr. Andiara E. Freitas, and their colleagues in the Zou lab found that in the adult mouse CA1 hippocampus, Celsr2 and Celsr3 promote synapse stability while Vangl2 destabilizes excitatory synapses. These proteins thus play an important and opposing role in synapse regulation in the adult brain in addition to synapse formation during development (Feng et al., 2021). Additionally, Dr. Yue Ban and their colleagues in the Zou lab demonstrated that knockout of Prickle1 and Prickle2 in the adult mouse medial prefrontal cortex significantly reduced and nearly eliminated glutamatergic synapse number (70-80% reduction). Their results also showed that PK2 promotes synapse formation by stabilizing transsynaptic Celsr3 complexes, specifically by antagonizing Vangl2 and thus

preventing synapse destabilization. Ultimately, these various studies from Dr. Zou's lab demonstrate that PCP proteins are critical in the formation and maintenance of excitatory synapses in adulthood (Ban et al., 2021).

As ketamine increases excitatory synapse number, it is dependent on molecules involved in synapse formation and stabilization, including the well known and prominent brain derived neurotrophic factor BDNF (Duman, 2014). Ketamine is an NMDAR antagonist, known to increase glutamate levels in the prefrontal cortex. One possible mechanism is that through the blockade of NMDA receptors on GABAergic interneurons, ketamine creates an overall disinhibition of excitatory synapses in the medial prefrontal cortex, thus enabling their activity and ultimately promoting and strengthening synaptic connections (Gerhard et al., 2020). This mechanism would thus underlie the overall increase in excitatory neurotransmission seen in ketamine treated models (Duman, 2014). As ketamine functions by restoring synaptic connectivity and dendritic spines lost in depression, we wanted to investigate whether ketamine's mechanism is dependent on the PCP proteins involved in the formation and maintenance of excitatory glutamatergic synapses, specifically in neurons projecting from the infralimbic prefrontal cortex (IL-PFC) to the basolateral amygdala (BLA), a circuit that mediates emotional regulation.

As depression is a form of chronic stress, mice that are chronically exposed to stressful conditions can be used to model depressive-like physiology and behavior (Dieterich et al., 2019). In our research, mice are chronically exposed to corticosterone (CORT), a compound similar to the biological stress hormone cortisol (Dieterich et al., 2019). Motivational behavior is evaluated using the tail suspension test, in which mice are suspended by the tail and immobility time is used to identify lack of motivation to escape the uncomfortable suspended position (Steru et al.,

1985). The open field test, consisting of a square frame in which mice can freely move, serves to control for changes in locomotion that could be attributed to chronic stress or ketamine treatment (Seibenhener & Wooten, 2015). Anhedonia, the lack of pleasure, is measured using the food preference test in which we measure the amount of food consumed by mice following a short period of food deprivation (Freitas et al., 2022). We looked specifically at neurons projecting from the infralimbic prefrontal cortex to the basolateral amygdala to study ketamine's sustained effect on behavior and excitatory synapses in the circuit mediating emotional regulation. The prefrontal cortex is one of the main brain regions affected in patients suffering with depression, and the amygdala serves as a primary center for emotional processing (Duman, 2014). We evaluated the role of these circuits in depressive-like behavior via iDREADD (designer receptors exclusively activated by designer drugs) silencing of their pathways and subsequent behavioral tests. Then, through use of the CRISPR/Cas9 system, we knocked out Celsr2, Celsr3, and Prickle2 in IL PFC > BLA projecting neurons to investigate whether they're required in ketamine's ability to restore lost synapses and rescue behavior in chronic stress models of depression. By determining the role of planar cell polarity proteins in synapse formation and maintenance as well as behavior following rapid acting antidepressant treatment, major insight is provided into potential targets for future therapeutics and will thus move treatments towards a more circuit-specific and direct mechanistic approach.

Chapter 1 - The IL PFC > BLA circuit regulates motivational behavior and pleasure

Experimental Design

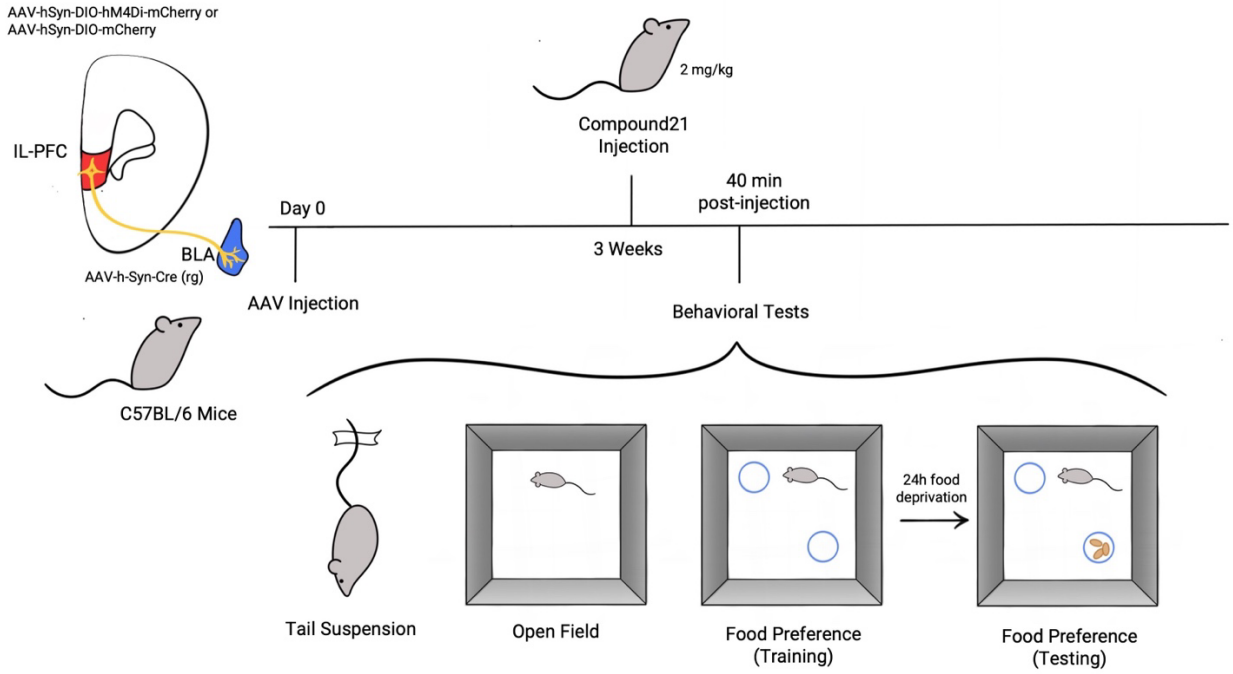

Figure 1.1. A schematic illustration of the surgical and behavioral paradigm for mCherry control and iDREADD experimental mice.

Methods

Andiara E. Freitas carried out the surgeries in control and experimental groups consisting of stereotactic injections. She also performed the Compound 21 injections prior to behavioral testing and performed and recorded the behavioral tests. Shae Galli helped carry out the behavioral tests and performed the video analysis using SMART Video Tracking System software. Shae collected the raw data from analysis, and Andiara performed statistical analysis and graph generation using GraphPad Prism.

In order to establish the behavioral role of excitatory neurons projecting from the IL PFC to the BLA, neurons in this circuit were silenced. C57BL/6 mice were stereotactically injected into the infralimbic prefrontal cortex with the inhibitory DREADD AAV-hSyn-DIO-hM4Di-mCherry and AAV-h-Syn-Cre was injected retrogradely into the basolateral amygdala. hSyn means these viruses are synapsin-driven; as synapsin is a neuron-specific protein, the hSyn promoter ensures the viruses are expressed specifically in neurons (Jackson et al., 2016). DIO means double-floxed inverse open reading frame, meaning the iDREADD and mCherry are flanked with lox sites and will only be expressed when interacting with Cre, which reorients the gene of interest (iDREADD and mCherry), allowing circuit-specific transcription. The control group consisted of C57BL/6 mice injected with the viral vector AAV-hSyn-DIO-mCherry into the infralimbic prefrontal cortex and AAV-h-Syn-Cre injected into the basolateral amygdala. Three weeks later, Compound 21 was injected intraperitoneally (2mg/kg) to activate the iDREADDs and silence the IL PFC > BLA circuit. Since findings show that the commonly used iDREADD agonist CNO has off-target behavioral effects, Compound 21 was chosen so that behavioral effects would not be affected by the compound itself and could be attributed to pathway silencing alone (Roy et al., 2021, MacLaren et al., 2016). Forty minutes post-injection, mice were subjected to a series of behavioral tests to determine the role of IL PFC > BLA pathway activity in motivational and pleasure-oriented behavior.

First, the tail suspension test (TST) was used to evaluate motivational behavior. In conditions with visual and acoustic isolation, adhesive tape was attached 1cm from the tail tip of mice who were then suspended 50cm above the floor (PanLab Harvard Apparatus, MA, USA; Steru et al., 1985). Activity was recorded for a duration of 6 minutes by an experienced researcher in double blinded conditions, and videos were analyzed double blinded using SMART

Video Tracking System to determine immobility time (PanLab Harvard Apparatus, MA, USA; Juszczak et al., 2006).

Figure 1.2. A representative image of the use of SMART Video Tracking System software for analysis of immobility time in the TST. Data outlined in blue indicates the immobility time readout given by the software.

The video software distinguishes between periods of immobility and low/high activity, resulting in a readout from the total 6 minute experiment separated into time periods of immobility, low activity, and high activity. The software also allows arenas to be placed over the video, allowing the researcher to specify the parameter in which the software should detect activity (Figure 1.2). Within the arena, throughout the 6 minute experiment duration, the software generated a heatmap of each mouse's activity, with dark blue representing lowest activity and dark red representing highest activity. The immobility time raw data was collected from each mouse and then averaged and statistically analyzed in GraphPad Prism using an unpaired Student's t-test for comparison (Figure 1.5).

This was followed by the open field test (OFT) to determine the effect of the experimental conditions on mice's locomotor activity (Seibenhener & Wooten, 2015). Mice were placed in an open-field arena (45x45cm apparatus, cleaned with 70% ethanol between each test; PanLab Harvard Apparatus, MA, USA) for a duration of 6 minutes and recorded double blinded by an experienced researcher. Videos were analyzed double blinded using SMART Video Tracking System to determine total distance traveled.

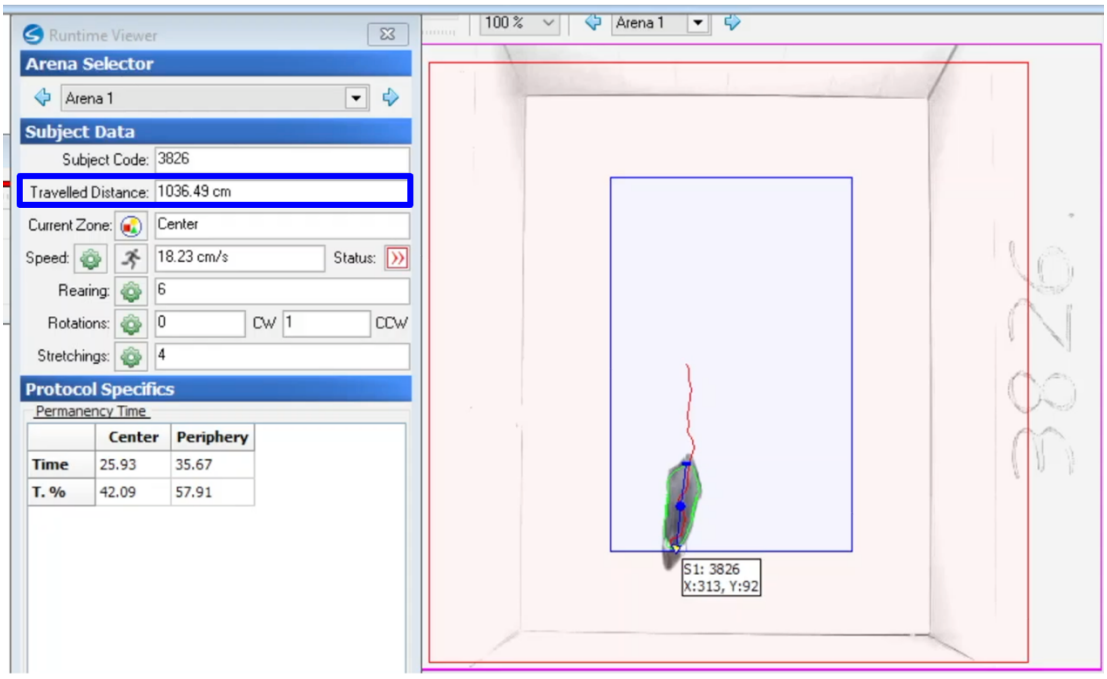

Figure 1.3. A representative image of the use of SMART Video Tracking System software for analysis of total locomotion, or travelled distance, in the OFT. Data outlined in blue indicates the travelled distance readout given by the software, detected based on the position of the mouse's head, body center, and tail.

Based on the detected position of the mouse's head, body center, and tail, the software records the total distance traveled and also generates a heatmap representing the mouse's trajectory throughout the 6 minute experiment. The blue arena in the software image above indicates the center of the maze, and the region outside of this arena indicated in red is considered the periphery. These parameters are useful for analyzing anxiety behavior, but in this

experiment, we were only interested in locomotion and thus only collected raw data of total distance travelled for each mouse (Figure 1.3). The data set was averaged and statistically analyzed in GraphPad Prism using an unpaired Student's t-test (Figure 1.6).

Finally, mice were subjected to the food preference test (FPT) in order to evaluate their response to pleasure from normal daily activities. This test was developed by Andiara to identify anhedonic-like behavior in mice, in which they lack the ability to feel pleasure from common enjoyable activities, such as consumption of their regular everyday food. The sucrose preference test is commonly used to study pleasure-related behavior in mice, but since sucrose is not a common everyday food for mice, Andiara developed the FPT to use mice's regular food pellets, since depression is characterized by a loss of pleasure in normal daily activities (Bains & Abdijadid, 2022, Freitas et al., 2022).

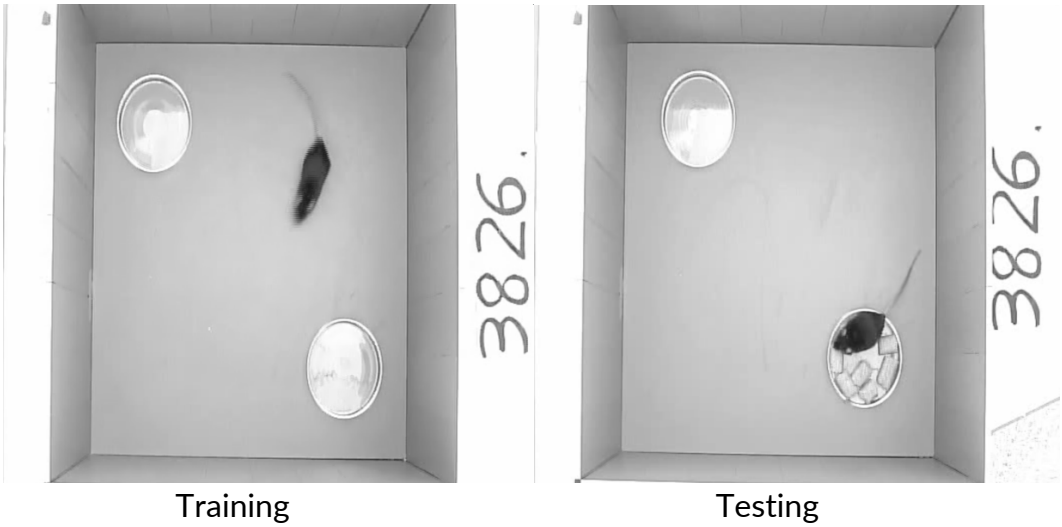

Figure 1.4. A representative image of the experimental setup for analysis of total food consumed in the FPT. The mass of the food dish was collected for each sample before and after the testing period to determine total amount of food consumed.

Prior to testing day, mice are habituated for a 5 minute duration to the testing apparatus, an open field arena in which two empty petri dishes are placed (PanLab Harvard Apparatus, MA,

USA). This habituation period allows mice to become accustomed to the testing environment and serves to eliminate any anxiety related to a new environment, which may interfere with and affect their eating behavior. Mice are then deprived of food for 24 hours and again subjected to the apparatus, this time for a total of 15 minutes and with regular food pellets added to one of the petri dishes (Figure 1.4). The food dish was weighed before and after the testing period, and the difference in mass was used to determine total amount of food consumed. The data set was then averaged and statistically analyzed in GraphPad Prism using an unpaired Student's t-test (Figure 1.7).

The IL PFC > BLA circuit regulates motivational behavior and pleasure

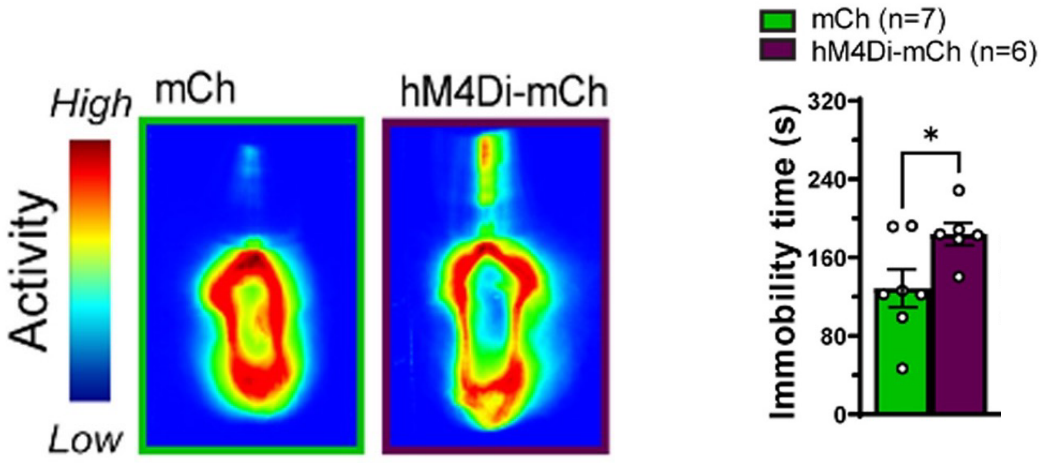

Figure 1.5. Immobility time is significantly increased in iDREADD silencing of the IL PFC > BLA circuit. Shown are representative heatmaps from the tail suspension test, used to visually represent the difference in immobility between the two groups. The immobility time mean for each group is represented in the graph in terms of seconds. Each bar represents the mean +/- S.E.M. of 6-7 mice. An unpaired Student's t-test was performed for statistical analysis (*$p<0.05$ compared with the control group).

Starting with the TST, the graph in Figure 1.5 shows that mice whose IL PFC > BLA pathways were silenced exhibited a statistically significant increase in immobility time as compared to control mice, indicated by a p-value < 0.05.

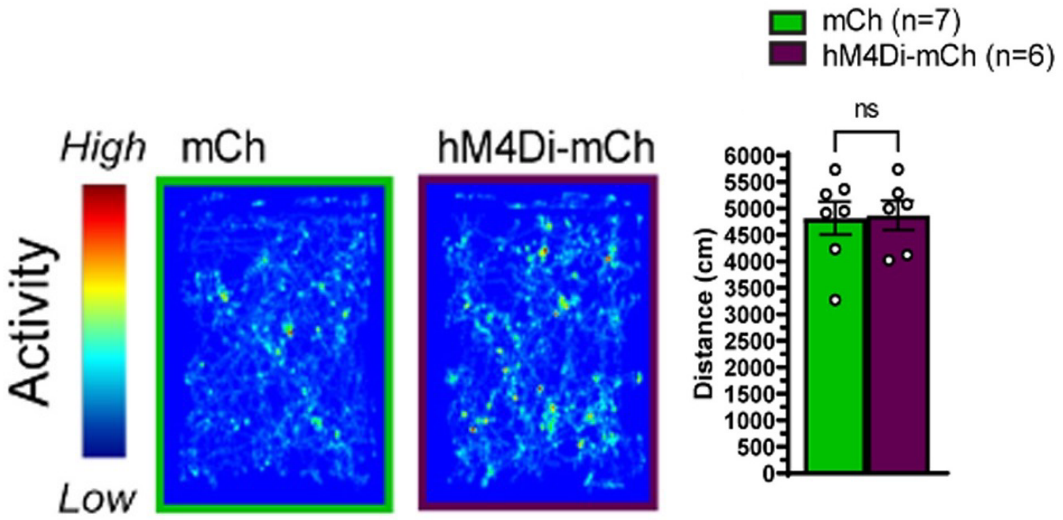

Figure 1.6. Locomotion is not significantly affected by iDREADD silencing of the IL PFC > BLA circuit. Shown are the results of the open field test. Representative heatmaps illustrate locomotion in each of the two groups, and the graph shows the data from total distance traveled in centimeters. Each bar represents the mean +/- SEM of 6-7 mice. An unpaired Student's t-test was performed for statistical analysis (ns = no significance compared with the control group).

The graph in Figure 1.6 of the OFT results demonstrates that there was no significant difference in the total distance traveled between the control and experimental groups, as the Student's p-value was > 0.05.

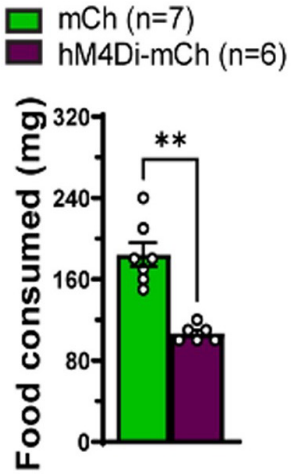

Figure 1.7. Food consumption is reduced during iDREADD silencing of the IL PFC > BLA circuit. The graph represents the amount of food consumed in milligrams in each of the groups. Each bar represents the mean +/- SEM of 6-7 mice. An unpaired Student's t-test was performed for statistical analysis (**p<0.01 compared with the control group).

Lastly, Figure 1.7 of the FPT data illustrates that mice whose IL PFC > BLA pathways were silenced consumed significantly less food than mice in the control group, as indicated by a p-value < 0.01.

Discussion

From the behavioral experiments performed in mice whose IL PFC > BLA circuits were silenced via Compound 21 activation of iDREADDs, it was demonstrated that silencing neuronal activity in this pathway reduced motivational behavior and induced anhedonia. First, the tail suspension test results showed an increase in immobility time in the experimental group. In this test, the mouse is highly active when attempting to escape the uncomfortable tail-suspended position, so immobility time indicates periods in which the mouse loses motivation to escape. These results therefore demonstrate that IL PFC > BLA pathway activity mediates motivational behavior and silencing this pathway contributes to loss of motivation, one of the hallmarks of depression. Next, the open field test was used to investigate whether circuit-specific silencing of

the IL PFC > BLA had any locomotor effects. Results of the OFT showed no significant difference in locomotion between the two groups, indicating that silencing did not cause any changes in locomotor activity. This confirms that the difference in immobility time seen in the TST is indeed due to a change in motivational behavior and cannot be attributed to locomotor impairment from iDREADD silencing or from Compound 21 itself. Lastly, the food preference test demonstrated the emergence of anhedonia, a lack of pleasure in normal daily activities and another hallmark of depression. As mice consumed significantly less food when the IL PFC > BLA pathway was silenced, the FPT results indicated that mice were experiencing less pleasure in consuming their everyday food pellets. This further illuminated the role that these projecting neurons have in depressive-like behavior. Overall, the behavioral results of silencing neurons projecting from the IL PFC to the BLA demonstrates that activity in this pathway facilitates motivation and pleasure, and disruption of this circuitry does indeed underlie the behavioral changes observed in chronic stress models of depression.

Figures 1.5, 1.6, and 1.7 are currently being prepared for submission for publication of the material. Freitas, A. E., Feng, B., Baker, C., Galli, S., Ban, Y., Zou, Y. (2022). *Ketamine reverses stress-induced behavior by restoring excitatory synapses in the basolateral amygdala-projecting infralimbic prefrontal cortex neurons.* (In preparation). The thesis author was a co-author of this material.

Chapter 2 – Ketamine's restoration of synaptic connectivity in IL PFC > BLA projecting neurons is prevented by knockout of Celsr2/3 or PK2

Experimental Design

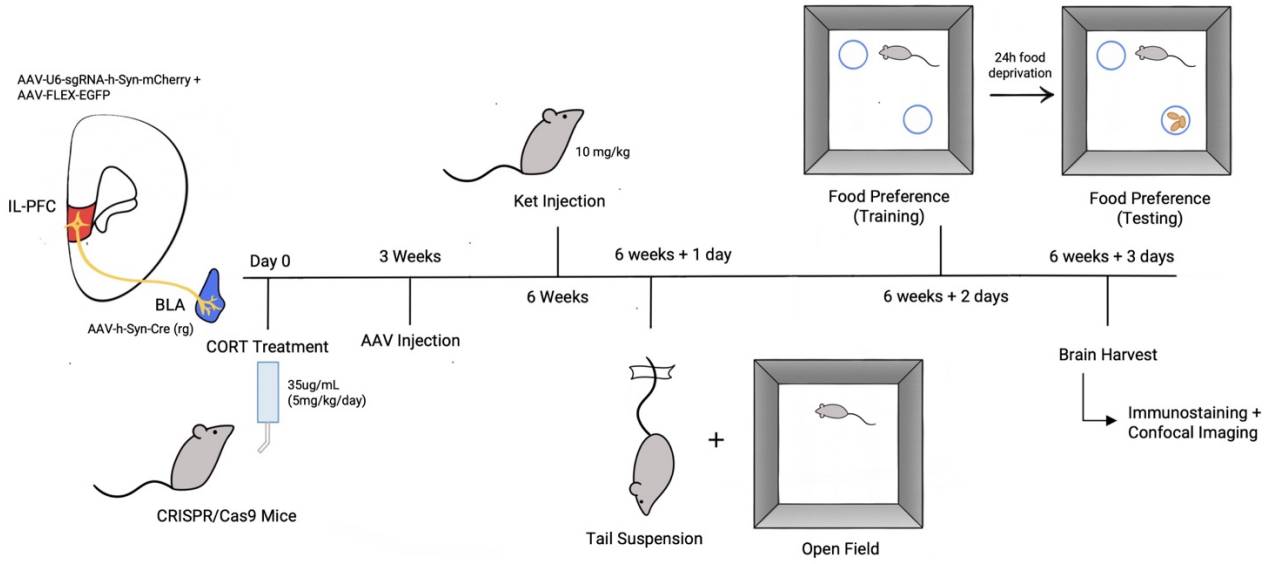

Figure 2.1. A schematic illustration of the surgical and behavioral paradigm for control sgRNA and Celsr2/3 or PK2 sgRNA mice.

Methods

Shae Galli and Andiara E. Freitas prepared and administered CORT in the drinking water of mice throughout the experimental paradigm. Andiara performed the surgeries in control and experimental groups consisting of stereotactic injections. She also performed the Ketamine and Saline injections prior to behavioral testing. Shae performed transcardial perfusions followed by brain harvest, and carried out some of the brain sectioning and mounting. Andiara also carried out perfusions and brain harvest and sectioning, and she performed the immunostaining protocol as well as section mounting and imaging using Zeiss 880 confocal microscope. Both Shae and

Andiara performed image analysis and quantification using Fiji ImageJ and collected the raw data, and Andiara carried out statistical analysis and graph generation using GraphPad Prism.

Next, to investigate the significance of PCP proteins in ketamine's mechanism of synapse restoration, mice were subjected to a chronic stress model of depression and underwent circuit-specific knockouts of different PCP proteins to determine whether ketamine's effect was perturbed. To model chronic stress, CRISPR/Cas9 mice were chronically exposed to corticosterone (CORT), which is similar to the biological stress hormone cortisol (Dieterich et al., 2019). CORT was administered at a concentration of 35ug/mL (equivalent to 5mg/kg/day) dissolved in 0.45% β-Cyclodextrin in the drinking water (mice in the control, unstressed group were exposed to β-Cyclodextrin only). After three weeks, mice were stereotactically injected with AAV-U6-sgRNA-h-Syn-mCherry and AAV-FLEX-EGFP into the IL PFC and AAV-h-Syn-Cre retrogradely into the BLA (viruses obtained from Feng et al. 2021). Mice in the β-Cyclodextrin control groups received control sgRNAs only. Mice in CORT control groups received control sgRNAs, while mice in CORT experimental groups received either sgRNAs simultaneously knocking out both Celsr2 and Celsr3 PCP proteins, or sgRNAs knocking out Prickle2 PCP proteins. Since the mice are genetically bred with the Cre-dependent CRISPR/Cas9 system, and because sgRNAs and FLEX-EGFP were injected into the IL PFC, only neurons projecting to the BLA interacted with the retrograde Cre, resulting in a circuit-specific knockout of each PCP protein and circuit-specific expression of GFP. Three weeks following surgery, mice were injected intraperitoneally with either saline or ketamine (at a concentration of 10mg/kg). Organization of control and experimental groups are show in the schematic below (Table 1.1).

Table 1.1. A representation of the treatment of various control and experimental groups. β-CD treatment is a non-stressed condition as control, while CORT treatment is a chronic stress condition. Control sgRNA injections serve as a control for Celsr2/3 and PK2 sgRNAs, which knock out Celsr2 + Celsr3 or Prickle2, respectively, in a circuit-specific manner. Lastly, each group was subdivided and treated with either saline as a control or ketamine.

β-CD	Control sgRNA	Saline
	Control sgRNA	Ketamine
CORT	Control sgRNA	Saline
	Control sgRNA	Ketamine
	Celsr23 sgRNA	Saline
	Celsr23 sgRNA	Ketamine
	PK2 sgRNA	Saline
	PK2 sgRNA	Ketamine
n = 13-16 for each group		

One day after saline/ketamine injection, mice were subjected to the series of behavioral tests established in the iDREADD experiments: TST, OFT, and FPT to evaluate the various treatment effects. Behavioral tests were carried out and recorded in double blinded conditions. After behavioral tests, mice were transcardially perfused with 1x PBS followed by 4% PFA, and their brains were harvested. Brains were immersed in 4% PFA for 24h following perfusion, and then transferred to 30% sucrose in which they were immersed for one week for dehydration. Brains were then embedded in OCT medium and sectioned in a cryostat at 30um thickness.

In order to quantify the number of excitatory synapses in each group, brain sections were stained with primary antibodies against pre-synaptic bassoon and post-synaptic PSD95. Secondary antibodies were then used to label bassoon with AlexaFluor 405nm blue and PSD95 with AlexaFluor 647nm far red. Since the sgRNA viruses AAV-U6-sgRNA-h-Syn-mCherry

contained an mCherry reporter, far red was chosen to distinguish postsynaptic markers in a different channel. IL PFC > BLA circuit specific dendrites are visible as the green fluorescence expressed by Cre-dependent FLEX-EGFP. Following staining, slices were mounted and imaged in layer 2/3 pyramidal neurons of the IL PFC using Zeiss 880 confocal microscope at 63x magnification, and their images were used to quantify protein expression and colocalization in double blinded conditions using Fiji ImageJ software with synapse counter plugin (Figure 2.2).

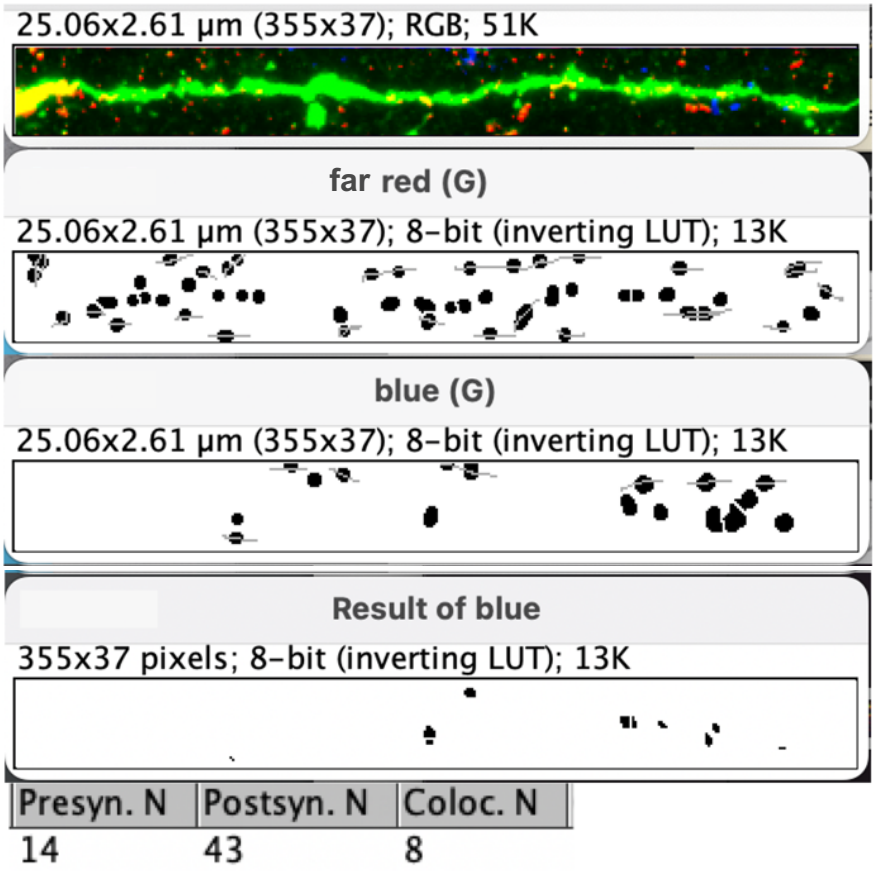

Figure 2.2. A representation of outputs from synapse counter plugin Fiji ImageJ, used to identify and quantify excitatory synapses. Blue indicates the blue channel in which presynaptic bassoon puncta are visible, while far red represents the far red channel in which postsynaptic PSD95 puncta are visible. Result of blue is the channel in which blue and far red puncta were colocalized, indicating excitatory synapses.

Synapse counter plugin in Fiji ImageJ software gives an output of each fluorescent channel, allowing specific puncta to be counted or eliminated based on their anatomical location.

Puncta were eliminated if they were not located on the main dendritic shaft or dendritic spines; those overlapping or adjacently touching a shaft or spine were included. Proximity to the dendrite was the primary consideration for including or eliminating puncta; any puncta located farther from the dendrite were quantified only if connected by the visible neck of a dendritic spine. Following identification, bassoon and PSD95 puncta and their colocalization were quantified. Fiji ImageJ software generates a "Result of blue" channel, resulting from the colocalization of the blue and far red channels and thus indicating points where bassoon and PSD95 puncta overlap. If these puncta were determined to be located on the dendrite shaft or spines, they were considered. These colocalization data were then normalized to 100% of the synapse number in control samples and statistically analyzed via a two-way ANOVA followed by Tukey's test in GraphPad Prism. Their results were graphed in Figure 2.4 as means of each condition +/- S.E.M., and statistical significance is indicated by the symbols above each bar.

Ketamine's restoration of synaptic connectivity in IL PFC > BLA projecting neurons is prevented by knockout of Celsr2/3 or PK2

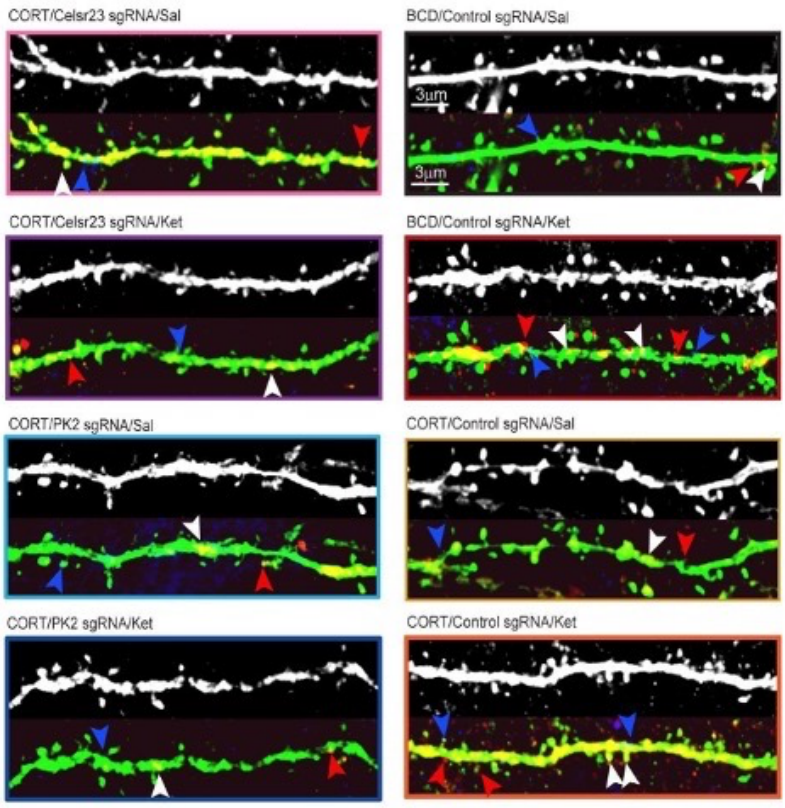

Figure 2.3. Ketamine is dependent on Celsr2/3 and PK2 for its restoration of excitatory synapse number following chronic stress. Shown are representative images of the dendrites of IL PFC > BLA projecting neurons in each experimental group, with blue arrows indicating bassoon puncta, red arrows indicating PSD95 puncta, and white arrows indicating colocalized bassoon/PSD95 puncta. Dendrites are 30um in length (scale bar indicates a length of 3um) and images are taken at 63x magnification of 30um brain slices.

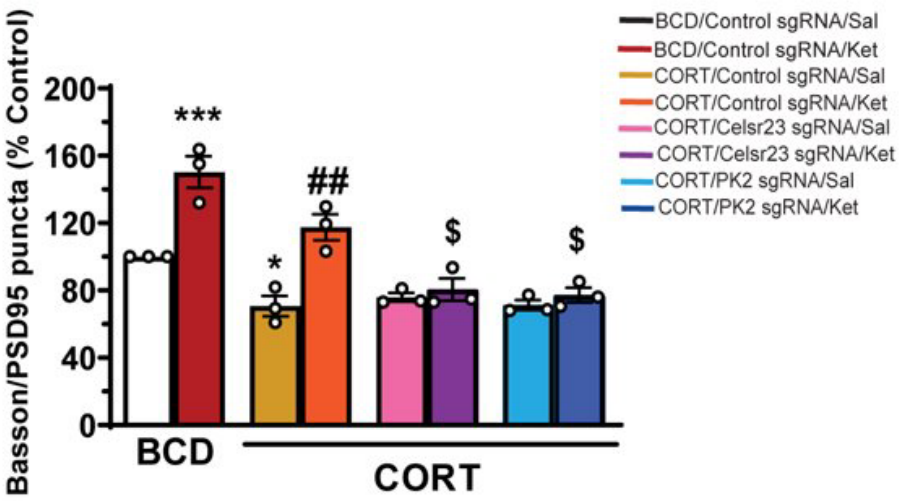

Figure 2.4. Ketamine is dependent on Celsr2/3 and PK2 for its restoration of excitatory synapse number following chronic stress. The graph shows the quantification of colocalized puncta, giving synapse number, normalized to 100% of synapse number in control samples (β-Cyclodextrin treated, control sgRNA, saline-injected). Each bar represents the mean +/- S.E.M. of 3 mice with 8-10 dendrites each, and symbols represent the p-values resulting from a two-way ANOVA followed by Tukey's test (* = $p<0.05$, *** = $p<0.001$ compared with β-CD/control sgRNA/Sal, ## = $p<0.01$ compared with CORT/control sgRNA/Sal, $ = $p<0.05$ compared with CORT/control sgRNA/Ket).

As shown in Figure 2.4, ketamine treated mice in non-stressed conditions demonstrate a significant increase in the number of excitatory synapses in IL PFC > BLA projecting neurons compared to saline-treated non-stressed mice. Alternatively, mice in chronically stressed conditions show a significant reduction in the number of these synapses when compared with unstressed mice. This synapse reduction is restored by subsequent ketamine treatment, seen in ketamine-treated chronically stressed mice. Interestingly, in chronically stressed mice with PCP proteins Celsr2/3 or PK2 knocked out in a circuit-specific manner, ketamine treatment did not restore the number of excitatory synapses lost during chronic stress.

Discussion

Synapse quantification of IL PFC > BLA projecting neurons revealed that ketamine treatment induces synaptogenesis in these neurons, consistent with existing literature (Duman & Aghajanian, 2012, Moda-Sava et al., 2019). Alternately, chronic stress causes a reduction in excitatory synapse number, which is reversed by the synaptogenesis induced by subsequent ketamine treatment. However, knocking out Celsr2/3 or PK2 prevents this synaptogenesis-inducing effect, indicating that when these proteins were knocked out, ketamine's ability to restore synapses lost under chronic stress was prevented. This shows that ketamine is dependent on these proteins for its sustained restoration of circuit-specific excitatory synapses lost due to chronic stress, indicating that planar cell polarity proteins play a critical role in facilitating excitatory synaptogenesis in ketamine's antidepressant mechanism.

Figures 2.3 and 2.4 are currently being prepared for submission for publication of the material. Freitas, A. E., Feng, B., Baker, C., Galli, S., Ban, Y., Zou, Y. (2022). *Ketamine reverses stress-induced behavior by restoring excitatory synapses in the basolateral amygdala-projecting infralimbic prefrontal cortex neurons.* (In preparation). The thesis author was a co-author of this material.

Chapter 3 – Ketamine's antidepressant-like behavioral effect is prevented by circuit-specific knockout of Celsr2/3 and PK2

Experimental Design

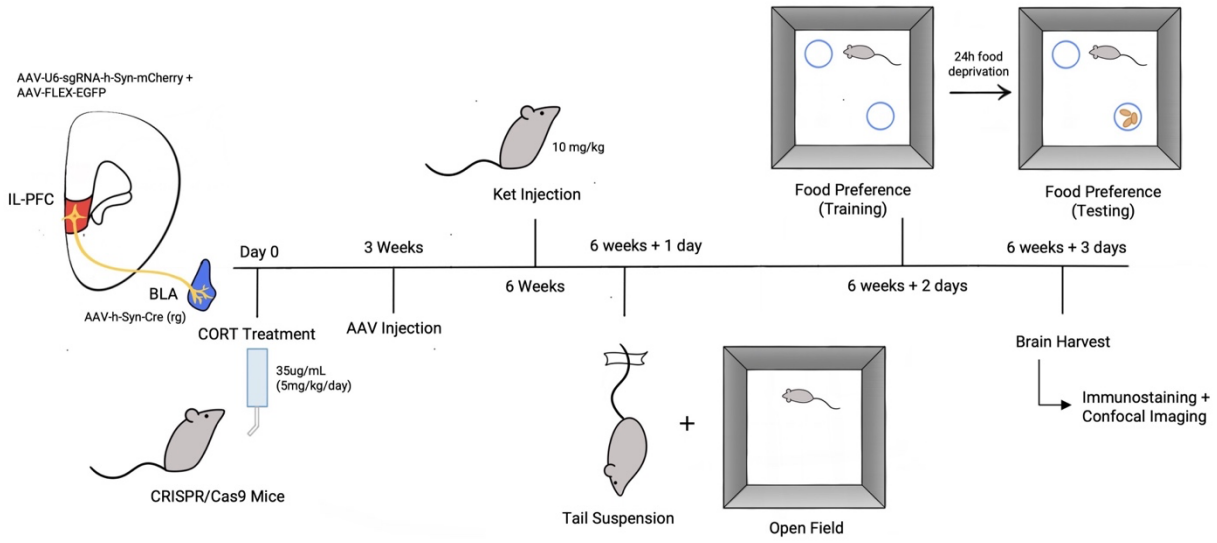

Figure 3.1. A schematic illustration of the surgical and behavioral paradigm for control sgRNA and Celsr2/3 or PK2 sgRNA mice.

Methods

Shae Galli and Andiara E. Freitas prepared and administered CORT in the drinking water of mice throughout the experimental paradigm. Andiara performed the surgeries in control and experimental groups consisting of stereotactic injections. She also performed the Ketamine and Saline injections prior to behavioral testing, and performed and recorded the behavioral tests. Shae helped carry out the behavioral tests and performed the video analysis using SMART Video Tracking System software. Shae collected the raw data from analysis, and Andiara performed statistical analysis and graph generation using GraphPad Prism.

Then, to investigate the significance of PCP proteins in ketamine's antidepressant-like behavioral effect, the behavioral tests were analyzed from the mice in the previous section that

underwent unstressed and stressed conditions with various sgRNA injections and Sal/Ket treatments. Following exposure to chronic stress, circuit-specific knockouts of different PCP proteins, and intraperitoneal injections of either Saline or Ketamine, mice were subjected to the series of behavioral tests established in the iDREADD experiments: the TST, OF, and FPT. These experiments were carried out and analyzed to determine whether ketamine's effect on behavior was perturbed in the circuit-specific absence of PCP proteins Celsr2/3 or PK2. The TST and OFT behavioral results were performed and recorded in double blinded conditions by an experienced researcher. Video recordings from behavioral tests were then analyzed double blinded with SMART video software to determine changes in immobility time and distance traveled. The FPT results were determined by weighing the food dish before and after the testing paradigm on FPT testing day. Finally, the results of these three behavioral experiments were statistically analyzed in GraphPad Prism using a two-way ANOVA followed by Tukey's test to determine significance. Results are graphed in Figure 3.2-3.4 as means of each experimental condition +/- S.EM., and statistical significance is indicated by the asterisks above each bar-to-bar bracket.

Ketamine's antidepressant-like behavioral effect is prevented by circuit-specific knockout of Celsr2/3 and PK2

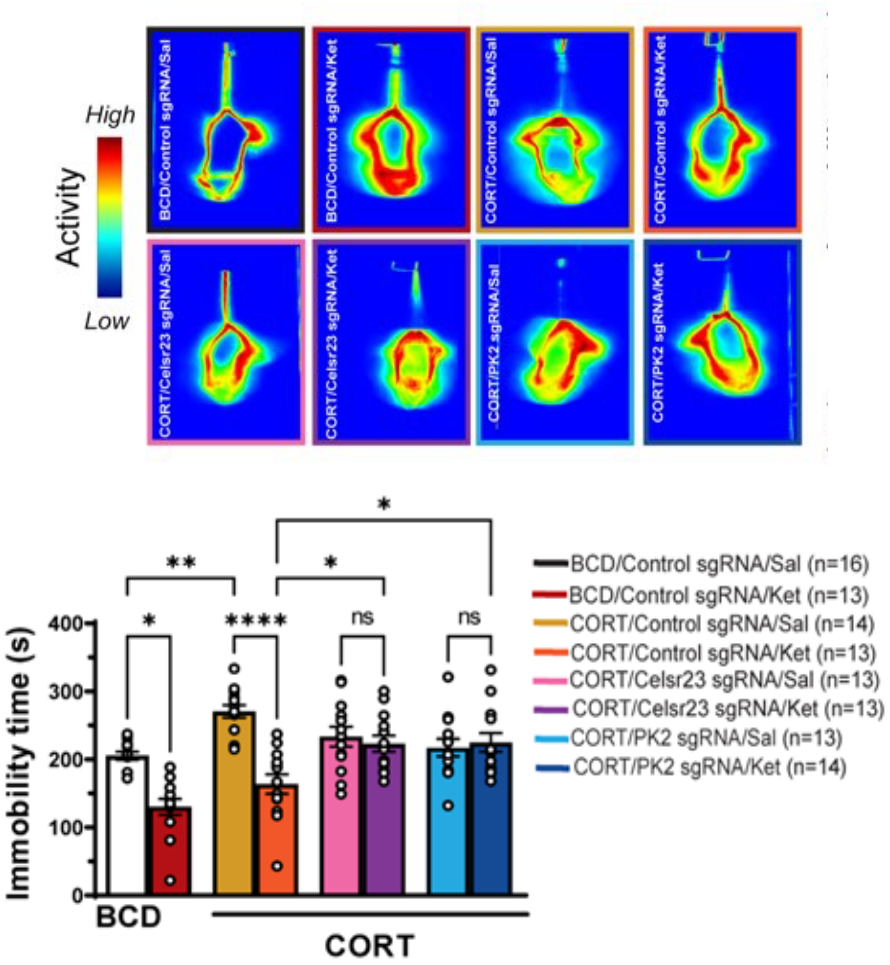

Figure 3.2. Celsr2/3 and PK2 are required for ketamine's restoration of motivational behavior following chronic stress. Representative heatmaps from the tail suspension test are shown to visually represent the differences in immobility among the eight groups. The immobility time raw data was collected and the mean +/- S.E.M. plotted in the graph. The immobility time mean data are represented in the graph in terms of seconds. Each bar represents the mean +/- S.E.M. of 13-16 mice. A two-way ANOVA was performed followed by Tukey's test to determine statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, comparisons indicated by each bar-to-bar bracket).

Beginning with Figure 3.2, analysis of immobility time data indicated that mice in the non-stressed condition (those exposed to β-Cyclodextrin in the water and no corticosterone) who were treated with ketamine showed significantly lower immobility time than mice treated with saline. When mice were chronically exposed to CORT in the drinking water, immobility time

increased significantly in the TST as compared to non-stressed mice treated with saline. When ketamine was administered to chronically stressed mice, immobility time was significantly reduced as compared to chronically stressed saline-treated mice. However, when Celsr2/3 or PK2 was knocked out in the IL PFC > BLA pathway, mice exposed to chronic stress and treated with ketamine showed no significant reduction in immobility time as compared to untreated (saline-injected) chronically stressed mice.

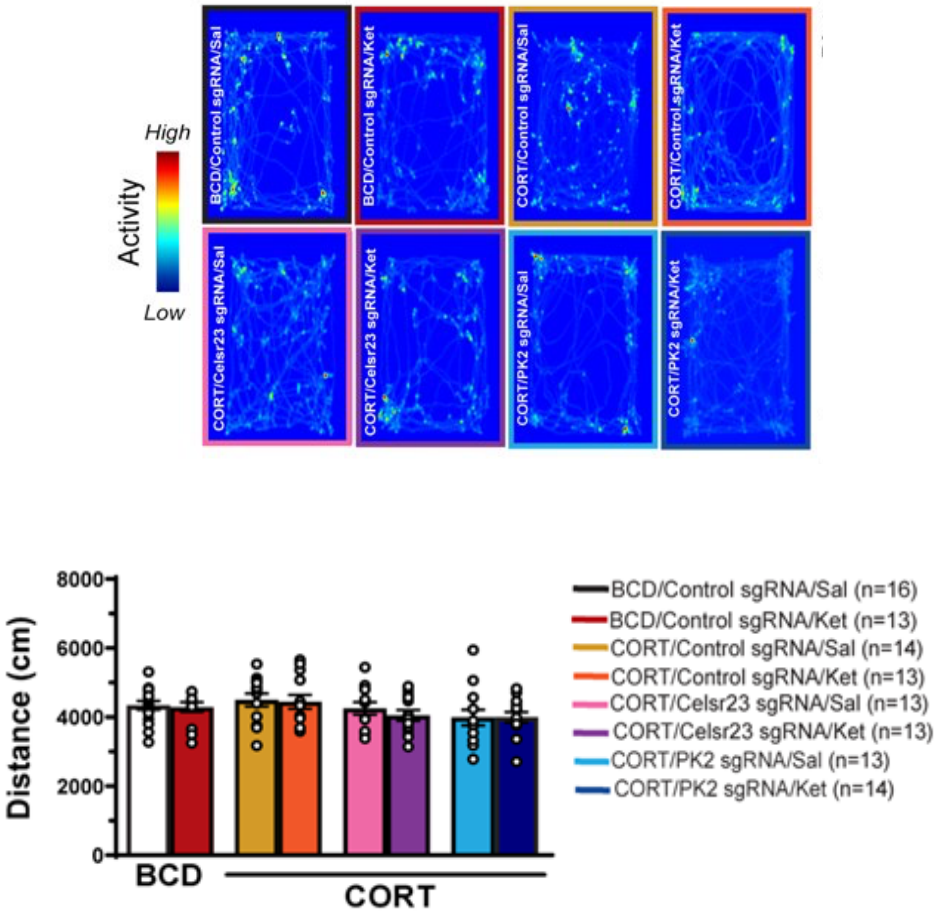

Figure 3.3. Locomotion is not affected by ketamine administration or chronic exposure to corticosterone. Representative heatmaps illustrate locomotion in each of the eight groups. The total distance traveled raw data was collected and the mean +/- S.E.M. plotted in the graph below. The total distance traveled mean data are shown in the graph in centimeters. Each bar represents the mean +/- S.E.M. of 13-16 mice. A two-way ANOVA was performed followed by Tukey's test for statistical analysis (no significance found among any of the groups).

Next, Figure 3.3 demonstrates the results of the open field test. As seen in the graph, the two-way ANOVA and Tukey's test indicated that there was no statistically significant difference in distance traveled among any of the experimental groups.

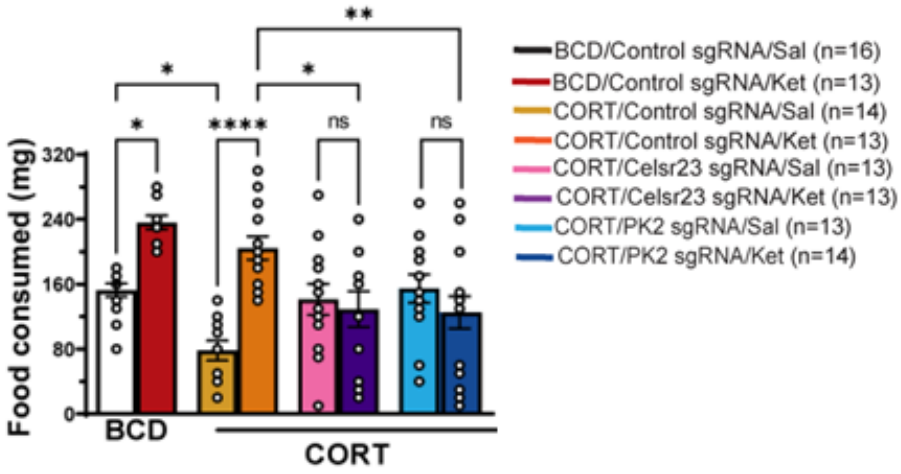

Figure 3.4. Celsr2/3 and PK2 are required for ketamine's restoration of food consumed following chronic stress. Shown is the mean amount of food consumed in milligrams in each of the groups. Each bar represents the mean +/- S.E.M. of 13-16 mice. A two-way ANOVA was performed followed by Tukey's test for statistical analysis (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, comparisons indicated by each bar-to-bar bracket).

Lastly, Figure 3.4 shows the results of the food preference test as an assessment for anhedonia (lack of pleasure). When non-stressed mice were treated with ketamine, they consumed a significantly greater amount of food compared to non-stressed saline-treated mice. When chronically exposed to CORT, mice experienced a significant reduction in the amount of food consumed compared to that of non-stressed, saline-treated mice. Mice under chronic stress and treated with ketamine, however, showed a significant increase in amount of food consumed as compared to those treated with saline. However, in chronically stressed mice when PCP proteins Celsr2/3 or PK2 were knocked out, ketamine did not exhibit the same effect as in control sgRNA mice, and food consumption amounts remained significantly low.

Discussion

In the corticosterone, ketamine, knockout behavioral experiments, it was observed that ketamine's sustained antidepressant-like effect in the various behavioral tests was prevented in the circuit-specific absence of PCP proteins. From the TST, it was observed that chronically stressed mice exhibited a decrease in motivational behavior, seen in their increased immobility time. This behavioral change was rescued by ketamine administration, which significantly reduced immobility time and thus restored motivational behavior. Importantly, ketamine administration was not effective in rescuing motivational behavior in either of the PCP knockout groups, seen as it was unable to reduce immobility time in chronically stressed mice. In other words, knocking out PCP proteins Celsr2/3 and PK2 prevented ketamine's restoration of motivational behavior in the chronic stress model, indicating that this antidepressant-like effect is dependent on expression of these proteins in IL PFC > BLA projecting neurons. Results of the open field test demonstrate no significant difference in locomotion among all groups, indicating that CORT's effect on behavior cannot be attributed to locomotor impairment and is truly due to a reduction in motivation due to chronic stress. Furthermore, ketamine's effect on motivational behavior as seen in the TST is not due to a psychostimulant effect and is thus due to a true behavioral effect on motivation. For this reason, we can confirm that the TST results are antidepressant specific. Lastly, the food preference test showed that although chronically stressed mice experienced a decrease in food consumption, ketamine rescued this anhedonia-like behavior and resulted in an increase in the amount of food consumed. Interestingly, ketamine treatment of mice in both Celsr2/3 and PK2 knockout groups was not able to reverse the decrease in food consumption, indicating that ketamine's restoration of anhedonia behavior is dependent on these proteins. Overall, these results indicate that ketamine's restoration of motivational and

pleasure behavior is dependent on circuit-specific expression of these planar cell polarity proteins, further elucidating their essential role in mediating ketamine's sustained antidepressant-like behavioral effect.

Figures 3.2, 3.3, and 3.4 are currently being prepared for submission for publication of the material. Freitas, A. E., Feng, B., Baker, C., Galli, S., Ban, Y., Zou, Y. (2022). *Ketamine reverses stress-induced behavior by restoring excitatory synapses in the basolateral amygdala-projecting infralimbic prefrontal cortex neurons.* (In preparation). The thesis author was a co-author of this material.

Chapter 4 – Celsr2/3 and PK2 were successfully knocked out in experimental mice

Methods

Andiara E. Freitas performed the immunostaining protocol as well as section mounting and imaging using Zeiss 880 confocal microscope, and Shae helped with section mounting. Both Shae and Andiara performed image analysis and quantification using Fiji ImageJ and collected the raw data, and Andiara carried out statistical analysis and graph generation using GraphPad Prism.

To confirm the knockout of PCP proteins Celsr2, Celsr3, and PK2, brain sections from mice in all experimental groups were stained in three separate staining protocols with primary antibodies against each of the three PCP proteins. Secondary antibodies AlexaFluor 647nm far red were used against the primary antibodies in each sample, therefore the red puncta seen in the images of Figure 4.1 and 4.2 are representative of each PCP protein. Since mice were injected with FLEX-EGFP in the prefrontal cortex and retrograde Cre in the basolateral amygdala, GFP is only expressed in those projecting neurons and thus the green fluorescence is indicative of dendrites of these circuit specific neurons. Sections were then imaged in layer 2/3 pyramidal neurons of the IL PFC using Zeiss 880 confocal microscope at 63x magnification, and their images were used to quantify protein expression in double blinded conditions. Images were analyzed using Fiji Image J to quantify the number of proteins stained (puncta).

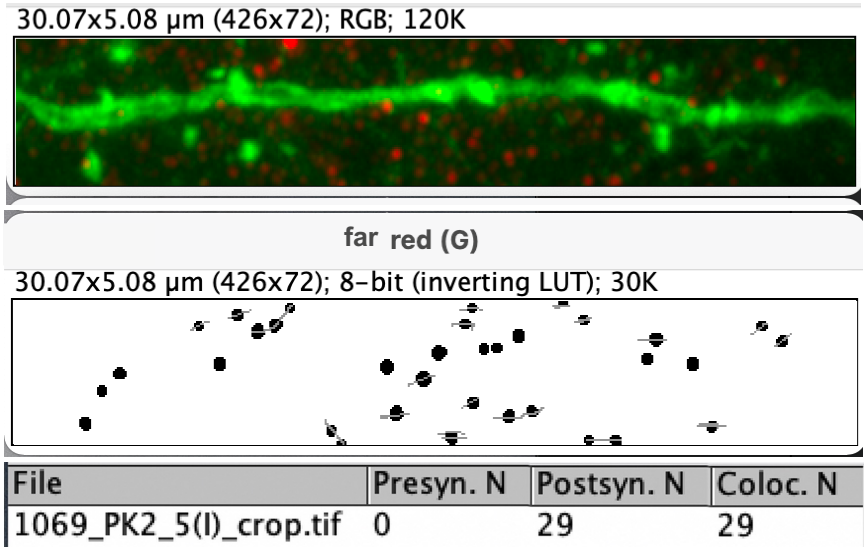

Figure 4.1. A representation of the output from synapse counter plugin Fiji ImageJ, used to identify and quantify PCP puncta. Far red indicates the far red channel in which PCP puncta are visible. The output given by synapse counter plugin indicates the total number of PCP puncta identified by the software, adjusted based on inclusion/exclusion criteria prior to quantification and statistical analysis.

The software gives an output of the far red fluorescent channel, allowing specific puncta to be counted or eliminated based on their anatomical location. Puncta were eliminated if they were not located on dendritic shaft or spines; those overlapping or adjacently touching a shaft or spine were included. Proximity to the dendrite was the primary consideration for including or eliminating puncta; any puncta located farther from the dendrite were quantified only if connected by the visible neck of a dendritic spine. Following identification, far red puncta were quantified. After these data were quantified, puncta numbers were normalized to 100% control and their mean +/- S.E.M. graphed. Significance was evaluated using an unpaired Student's t-test and their results were graphed in Figure 4.2 using GraphPad Prism (n=2 for each group).

Celsr2/3 and PK2 were successfully knocked out in experimental mice

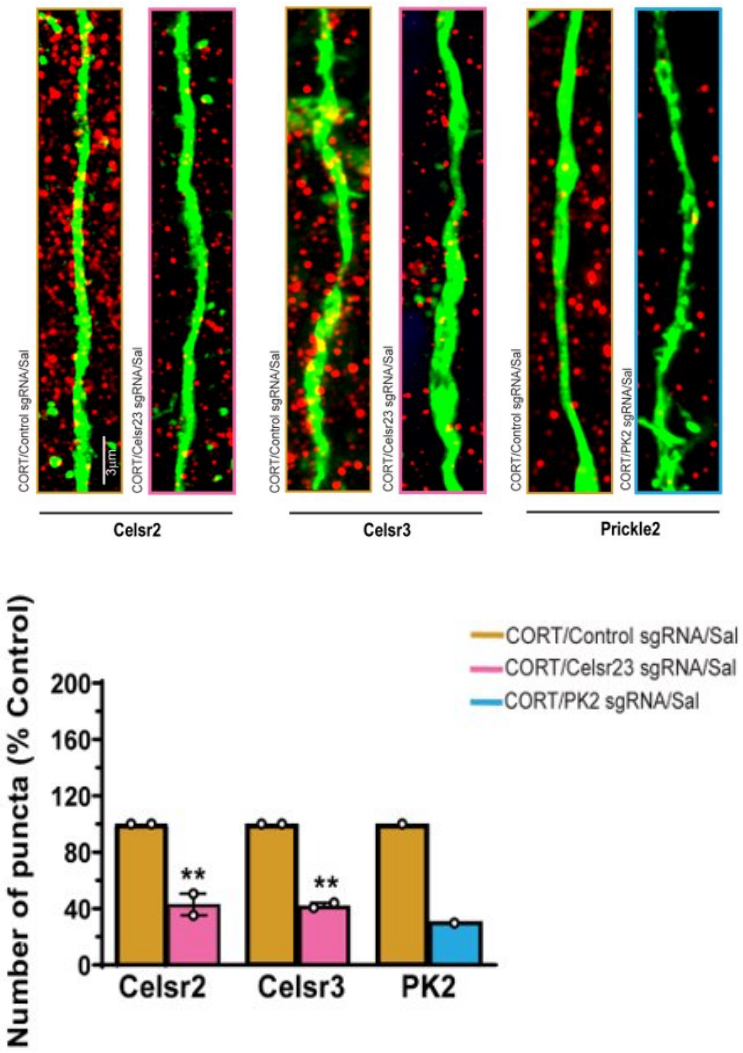

Figure 4.2. Celsr2/3 and PK2 were successfully knocked out in experimental mice. Representative images of the dendrites of IL PFC > BLA projecting neurons are shown for each experimental group, with puncta showing labeled PCP proteins Celsr2, Celsr3, and PK2 respectively. Dendrites are 30um in length and images are taken at 63x magnification of 30um brain slices. Figure 3b shows the quantification of puncta normalized to 100% of puncta number in control samples (no protein knockout). Each bar represents the mean +/- S.E.M. of 2 mice, and asterisks represent the p-value resulting from an unpaired t-test between the control and experimental groups for each staining. An unpaired Student's t-test was performed for statistical analysis (**p<0.01) (*Note: each PK2 bar represents 1 mouse, more samples to be included, work in progress*).

Shown in the graph of Figure 4.2 is the quantification of planar cell polarity (PCP) proteins Celsr2, Celsr3, and Prickle2 in dendrites of the IL PFC > BLA projecting neurons.

When compared to control sgRNA mice, Celsr2/3 knockout mice showed a statistically significant reduction in both Celsr2 and Celsr3 protein expression in the dendritic spines of IL PFC > BLA projecting neurons. Similarly, Prickle2 knockout mice showed a significant reduction in PK2 protein expression when compared to control sgRNA mice.

Discussion

The analysis results quantifying PCP puncta confirm that the Celsr2, Celsr3, and Prickle2 proteins were successfully and sufficiently knocked out in the IL PFC > BLA circuit of sgRNA knockout mice, validating the results of the excitatory synapse analysis and behavioral analysis performed in the study (Figure 4.2).

Figure 4.2 is currently being prepared for submission for publication of the material. Freitas, A. E., Feng, B., Baker, C., Galli, S., Ban, Y., Zou, Y. (2022). *Ketamine reverses stress-induced behavior by restoring excitatory synapses in the basolateral amygdala-projecting infralimbic prefrontal cortex neurons.* (In preparation). The thesis author was a co-author of this material.

Final Discussion/Conclusion

Modern day research shows that a single sub-anesthetic dose of ketamine increases dendritic spine branching and restores excitatory synaptic connectivity lost in mouse models of chronic stress and depression (Moda-Sava et al., 2019). The medial prefrontal cortex is the primary region in which these connections are restored, so investigating circuit-specific mechanisms of ketamine's effects at the synapse level will provide significant insight into depression and its potential treatments. There is currently a limited understanding of the molecular mechanisms involved in synapse formation and restoration resulting from rapid-acting antidepressant treatment, so we sought to investigate the role of planar cell polarity proteins in ketamine's sustained antidepressant mechanism.

Silencing of IL PFC > BLA projecting neurons implicated the role of this circuit in mediating emotional behavior. The tail suspension test demonstrated that when this circuit is silenced, mice experience increased immobility, and the food preference test showed that mice experienced a decrease in consumption of their normal food pellets. These results indicate that the IL PFC > BLA pathway plays a significant role in encoding motivation and pleasure, behaviors that are dysregulated in depression.

Synapse analysis of corticosterone-treated chronically stressed mice showed that ketamine increases excitatory synaptic connectivity in neurons projecting from the infralimbic prefrontal cortex to the basolateral amygdala, and behavioral tests showed that ketamine significantly reduces depressive-like behavior in the chronic stress mouse model. Furthermore, in the experimental mice with either Celsr2/3 or PK2 knocked out, ketamine's restoration of circuit-specific excitatory synapses lost was prevented, and it was unable to rescue depressive-like behavior, indicating ketamine's dependence on these planar cell polarity proteins.

Ketamine is known to increase the number of excitatory synapses in the prefrontal cortex, and this effect is also known to have an antidepressant-like effect in mice (Duman, 2014, Moda Sava et al., 2019). Our results show that PCP proteins Celsr2, Celsr3, and PK2 serve a critical role in the synapse formation and maintenance underlying ketamine's mechanism of action. Ketamine works by blocking NMDA receptors, but it correlates with an increase in excitatory synapse number and activity (Duman, 2014). The existing hypothesis for this mechanism is that ketamine blocks NMDA receptors on GABAergic interneurons, leading to overall disinhibition of excitatory pathways and thus enabling increased excitatory synapse activity (Gerhard et al., 2020). Prior research has shown that glutamatergic synapses are dependent on planar cell polarity proteins for their stability and maintenance (Thakar et al., 2017, Feng et al., 2021, Ban et al., 2021). This, combined with our findings, suggests that ketamine induces synapse formation through mechanisms dependent on Celsr2, Celsr3, and Prickle2.

Due to the multifaceted characteristics of depression, there is a need for more circuit-specific studies in projections between various implicated regions of the brain. In the future, investigating projections from the medial prefrontal cortex to other limbic and cortical structures may further elucidate mechanistic dysfunction in depression. This would allow development of more physiologically based treatments, greatly improving the direction of potential novel therapeutics. Furthermore, current research focuses mainly on the formation of synapses in excitatory neurons since these pathways are thought to be the main route by which depressive symptoms are alleviated. Future research, therefore, would benefit from the study of other neurons and non-neuronal cell types. For example, interneurons that modulate the activity of excitatory neurons are proposed to play a significant role in ketamine's mechanism and thus could serve as another therapeutic target (Gerhard et al., 2020).

References

Freitas, A. E., Feng, B., Baker, C., Galli, S., Ban, Y., Zou, Y. (2022). *Ketamine reverses stress-induced behavior by restoring excitatory synapses in the basolateral amygdala-projecting infralimbic prefrontal cortex neurons.* (In preparation).

World Health Organization. (2021). *Depression*. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/depression

Bains, N., & Abdijadid, S. (2022). Major Depressive Disorder. In *StatPearls*. StatPearls Publishing. https://pubmed.ncbi.nlm.nih.gov/32644504/

Duman R. S. (2014). Neurobiology of stress, depression, and rapid acting antidepressants: remodeling synaptic connections. *Depression and anxiety*, *31*(4), 291–296. https://doi.org/10.1002/da.22227

Hasselmann H. W. (2014). Ketamine as antidepressant? Current state and future perspectives. *Current neuropharmacology*, *12*(1), 57–70. https://doi.org/10.2174/1570159X113119990043

Sanacora, G., Treccani, G., & Popoli, M. (2012). Towards a glutamate hypothesis of depression: an emerging frontier of neuropsychopharmacology for mood disorders. *Neuropharmacology*, *62*(1), 63–77. https://doi.org/10.1016/j.neuropharm.2011.07.036

Bratsos, S., & Saleh, S. N. (2019). Clinical Efficacy of Ketamine for Treatment-resistant Depression. *Cureus*, *11*(7), e5189. https://doi.org/10.7759/cureus.5189

Harmer, C. J., Duman, R. S., & Cowen, P. J. (2017). How do antidepressants work? New perspectives for refining future treatment approaches. *The lancet. Psychiatry*, *4*(5), 409–418. https://doi.org/10.1016/S2215-0366(17)30015-9

Duman, R. S., & Aghajanian, G. K. (2012). Synaptic dysfunction in depression: potential therapeutic targets. *Science (New York, N.Y.)*, *338*(6103), 68–72. https://doi.org/10.1126/science.1222939

Moda-Sava, R. N., Murdock, M. H., Parekh, P. K., Fetcho, R. N., Huang, B. S., Huynh, T. N., Witztum, J., Shaver, D. C., Rosenthal, D. L., Alway, E. J., Lopez, K., Meng, Y., Nellissen, L., Grosenick, L., Milner, T. A., Deisseroth, K., Bito, H., Kasai, H., & Liston, C. (2019). Sustained rescue of prefrontal circuit dysfunction by antidepressant-induced spine formation. *Science (New York, N.Y.)*, *364*(6436), eaat8078. https://doi.org/10.1126/science.aat8078

Zou Y. (2020). Breaking symmetry - cell polarity signaling pathways in growth cone guidance and synapse formation. *Current opinion in neurobiology*, *63*, 77–86. https://doi.org/10.1016/j.conb.2020.03.010

Thakar, S., Wang, L., Yu, T., Ye, M., Onishi, K., Scott, J., Qi, J., Fernandes, C., Han, X., Yates, J. R., 3rd, Berg, D. K., & Zou, Y. (2017). Evidence for opposing roles of Celsr3 and Vangl2 in glutamatergic synapse formation. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(4), E610–E618. https://doi.org/10.1073/pnas.1612062114

Feng, B., Freitas, A. E., Gorodetski, L., Wang, J., Tian, R., Lee, Y. R., Grewal, A. S., & Zou, Y. (2021). Planar cell polarity signaling components are a direct target of β-amyloid-associated degeneration of glutamatergic synapses. *Science advances*, *7*(34), eabh2307. https://doi.org/10.1126/sciadv.abh2307

Ban, Y., Yu, T., Feng, B., Lorenz, C., Wang, X., Baker, C., & Zou, Y. (2021). Prickle promotes the formation and maintenance of glutamatergic synapses by stabilizing the intercellular planar cell polarity complex. *Science advances*, *7*(41), eabh2974. https://doi.org/10.1126/sciadv.abh2974

Gerhard, D. M., Pothula, S., Liu, R. J., Wu, M., Li, X. Y., Girgenti, M. J., Taylor, S. R., Duman, C. H., Delpire, E., Picciotto, M., Wohleb, E. S., & Duman, R. S. (2020). GABA interneurons are the cellular trigger for ketamine's rapid antidepressant actions. *The Journal of clinical investigation*, *130*(3), 1336–1349. https://doi.org/10.1172/JCI130808

Dieterich, A., Srivastava, P., Sharif, A., Stech, K., Floeder, J., Yohn, S. E., & Samuels, B. A. (2019). Chronic corticosterone administration induces negative valence and impairs positive valence behaviors in mice. *Translational psychiatry*, *9*(1), 337. https://doi.org/10.1038/s41398-019-0674-4

Steru, L., Chermat, R., Thierry, B., & Simon, P. (1985). The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology*, *85*(3), 367–370. https://doi.org/10.1007/BF00428203

Seibenhener, M. L., & Wooten, M. C. (2015). Use of the Open Field Maze to measure locomotor and anxiety-like behavior in mice. *Journal of visualized experiments : JoVE*, (96), e52434. https://doi.org/10.3791/52434

Jackson, K. L., Dayton, R. D., Deverman, B. E., & Klein, R. L. (2016). Better Targeting, Better Efficiency for Wide-Scale Neuronal Transduction with the Synapsin Promoter and AAV-PHP.B. *Frontiers in molecular neuroscience*, *9*, 116. https://doi.org/10.3389/fnmol.2016.00116

Roy, D. S., Zhang, Y., Aida, T., Choi, S., Chen, Q., Hou, Y., Lea, N. E., Skaggs, K. M., Quay, J. C., Liew, M., Maisano, H., Le, V., Jones, C., Xu, J., Kong, D., Sullivan, H. A., Saunders, A., McCarroll, S. A., Wickersham, I. R., & Feng, G. (2021). Anterior thalamic dysfunction underlies cognitive deficits in a subset of neuropsychiatric disease models. *Neuron*, *109*(16), 2590–2603.e13. https://doi.org/10.1016/j.neuron.2021.06.005

MacLaren, D. A., Browne, R. W., Shaw, J. K., Krishnan Radhakrishnan, S., Khare, P., España, R. A., & Clark, S. D. (2016). Clozapine N-Oxide Administration Produces Behavioral Effects in Long-Evans Rats: Implications for Designing DREADD Experiments. *eNeuro*, *3*(5), ENEURO.0219-16.2016. https://doi.org/10.1523/ENEURO.0219-16.2016

Juszczak, G. R., Sliwa, A. T., Wolak, P., Tymosiak-Zielinska, A., Lisowski, P., & Swiergiel, A. H. (2006). The usage of video analysis system for detection of immobility in the tail suspension test in mice. *Pharmacology, biochemistry, and behavior*, *85*(2), 332–338. https://doi.org/10.1016/j.pbb.2006.08.016