INVESTIGATING THE ROLE OF VENTRAL TEGMENTAL AREA TORC2 IN STRESS AND STRESS-INDUCED CHANGES IN OPIATE REWARD

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfilment of the requirements
for the degree of

Pharmacology and Toxicology-Environmental Toxicology-Doctor of Philosophy

2017

ABSTRACT

INVESTIGATING THE ROLE OF VENTRAL TEGMENTAL AREA TORC2 IN STRESS AND STRESS-INDUCED CHANGES IN OPIATE REWARD

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Depression and opiate addiction are two prevalent neuropsychiatric diseases that produce a significant societal burden in terms of health and economic costs. Despite the substantial prevalence of depression and addiction both in the United States and worldwide, our understanding of the underlying neurobiological mechanisms remains incomplete, and elucidation of these neuroadaptations is necessary to develop more effective pharmacological therapies.

Depression and addiction are often co-morbid, suggesting that similar neuroadaptations may underlie both diseases. The ventral tegmental area (VTA) is an important brain region in the reward circuit with a well-established role in the effects of drugs of abuse and development of addiction. Interestingly, increasing evidence suggests that dysfunction of the reward pathway may also contribute to depression. Similar biochemical changes, specifically the phosphorylation of AKT, a serine/threonine kinase, at S473, occurs in the VTA in response to chronic social defeat stress (CSDS), a pre-clinical model of depression, and chronic morphine treatment. Importantly, manipulation of AKT S473 phosphorylation alters both depressive- and addictive-like behaviors. Thus, regulation of AKT S473 phosphorylation may be a critical determinant in the development of depression and addiction.

The protein complex responsible for phosphorylation of AKT S473 is mammalian (or mechanistic) Target of Rapamycin Complex 2 (TORC2). Previous work has demonstrated a critical role for VTA TORC2 in mediating morphine reward, but its role in stress had not been

examined. Thus, this dissertation investigated whether VTA TORC2 signaling mediated susceptibility to CSDS. Furthermore, it has been established that CSDS alters the rewarding effects of drugs of abuse, including morphine. Given that both CSDS and chronic morphine similarly decrease AKT S473 phosphorylation, these studies also determined whether TORC2 signaling was necessary for stress-induced changes in morphine reward. Finally, one mechanism by which TORC2 signaling is thought to mediate long-term adaptations underlying behavioral changes is the alteration of VTA DA neuronal morphology. Therefore, these studies also sought to determine whether TORC2 promotes cytoskeletal remodeling in the VTA via regulation of Rac1 signaling. Through viral and genetic manipulation of Rictor, an essential protein for TORC2 activity, this dissertation investigated the central hypothesis that *alteration of TORC2 signaling in the VTA contributes to changes in stress-induced morphine reward and CSDS susceptibility through modulation of specific downstream signaling molecules such as Rac1*.

Overall, the results of these studies reveal novel information and significance regarding the physiological role of VTA TORC2. While decreased TORC2 signaling in the VTA, or dopamine neurons, is not sufficient to increase susceptibility to CSDS or stress-induced drug reward, we identified a novel role for VTA TORC2 signaling in general consummatory behavior. Moreover, the data from our studies suggest that catecholaminergic TORC2 signaling might regulate behavior in a sex-specific matter, presenting novel opportunities for future studies. Finally, these data indicate that VTA TORC2 does not alter Rac1-PAK-Cofilin signaling and thus, further studies are needed to elucidate the mechanism by which TORC2 facilitates morphine-induced changes in VTA DA morphology.

To my family.

ACKNOWLEDGEMENTS

The past five years at Michigan State University have been an incredible journey that I could not have gone through alone. I have an army of mentors and friends, both professionally and personally, to thank for my development as a scientist.

First, I want to thank my advisor, Dr. Michelle Mazei-Robison. I know she wasn't expecting to take on a rotation student so soon after starting at MSU, especially one whose only knowledge of the brain is where it's located, but I'm glad she did. I really want to thank Michelle for taking a chance on me and allowing me to join her lab. It has been quite an adventure for both of us. Her passion for research and ability to juggle endless amounts of responsibility has been truly motivating and inspiring. She really cares about her students and somehow always seems to make sure that we're her top priority (well, at least it feels like that.) I'm also very thankful for Michelle's patience with me throughout the past four years to help guide me into the scientist that I am.

I would also like to like to whole-heartedly thank my dissertation committee for their guidance and support throughout the years. Dr. Jim Galligan, Dr. Bryan Copple, and Dr. Colleen Hegg always asked thought-provoking questions and provided insightful feedback at my committee meetings. They have also helped me think about my projects from different perspectives and think critically about my research.

The Department of Pharmacology and Toxicology at MSU has provided me with an incredible amount of support and such an enriching environment to help with my development as a scientist. Thank you to all to of the faculty, students, and staff that I've had the privilege of

getting to know throughout the years. Diane, Michelle, Becky, Steve, Beverly, Brad, and Julie have been a joy to work with and I'd like to thank them for all of their help to make this process as stress-free as possible. I would also especially like to thank Dr. Anne Dorrance for her support as our Graduate Program Director. She was always my go-to person whenever I had questions, problems, needed someone to talk things through, or just needed a place to cry. I will miss all of the social events the department hosts each year. The holiday parties, chili cook-offs, picnics, speed data seminars, and much needed happy hours all produced wonderful memories that I will leave with.

The Environmental and Integrative Toxicological Sciences program and Institute for Integrative Toxicology have been enormously supportive of my training and development as a graduate student. Thank you to the staff members of EITS and the IIT for all of their help throughout my career at MSU. I also have really appreciated the opportunities to present my research each year at the Research Evenings, the opportunities to bring in guest speakers through the EITS seminar series, and the travel awards to attend various meetings throughout the years. I have gotten to know a number of faculty and students through this program and will miss everyone and these opportunities.

Most of my graduate career has been supported by funding opportunities outside of the laboratory, for which I am very grateful for. First, I'd like to thank the BioMolecular Sciences program at MSU for their support for my first year of graduate school while I took classes and went through my research rotations. I would also like to thank the Integrative Pharmacological Sciences Training Program at MSU for their training and support. This opportunity provided me with the guidance that I needed to apply for my own fellowship funding. Lastly, I would like to thank the PhRMA Foundation for giving me the opportunity to be supported by the

Pharmacology/Toxicology Pre-Doctoral Fellowship. The independence that comes with having my own funding source allowed me to focus on my research and training as a scientist, which was especially important as I was finishing up the last part of my graduate career.

The pharmacology training and support I have received through the American Society of Pharmacology and Experimental Therapeutics (ASPET) has been a magnificent part of my graduate career. I am very thankful for all of the professional and networking opportunities ASPET has given me. From being awarded a travel award for my first ASPET meeting as a graduate student, to participating in the Mentoring Network, Young Scientist Committee, and Washington Fellows program, I have gained invaluable experiences and have met countless mentors and staff members who have been nothing but supportive in my graduate career.

Even though he has already been mentioned once, I would like to thank Dr. Bryan Copple, again, for his support throughout the years. The experience I gained as a Research Assistant in Bryan's lab helped me solidify my decision to pursue a graduate degree. From working on a number of projects in the lab, going to departmental seminars, to attending a national meeting, I was able to thoroughly explore the field of pharmacology. Those experiences helped me realize that pharmacology is a field that I truly enjoy. I can't thank Bryan enough for the opportunities he gave me in his lab, for all of the recommendation letters he has written me for both graduate school and for fellowships I applied for in graduate school, for his guidance as one of my committee members and also as one of my professors, and for the basketball bantering. (I will always argue that the Jayhawks are better at basketball than the Spartans.) In all seriousness, I have to thank Bryan for his guidance and support in helping me begin my career.

Finding a balance between school and having a life outside of school was always a struggle for me. It was not until I became part of a supportive fitness family at CrossFit Facta that I truly learned how to make myself a priority. Without the support of the coaches and fellow athletes at Facta, I don't know how I would have gotten through my last year of graduate school. They have been my cheerleaders both inside and outside of the box. Thank you for all of the constant reminders to take care of myself throughout this process. I will miss the Saturday morning workouts and hero WODs that I've been fortunate enough to do with my Facta Family. I'd especially like to thank Mike Romigh, Dan Romigh, Kathleeen Louis, Kathi and Skip Shipley, Eric Ozanich, and Jamie Latimer for their support, positivity, and words of encouragement, both related to CrossFit and regarding my studies as well.

Throughout my time at MSU, I've also been fortunate enough to build an amazing network of friends that have gotten me through the roller coaster ride of graduate school. I have had two exceptional lab mates who I consider to be wonderful friends. Thank you to Marie Doyle and Vedrana Bali for being wonderful co-workers and friends. I'm glad I have friends who understood and shared my love of coffee, macaroons, and El Oasis burritos. They would take time out their busy schedules to sneak out with me for coffee and snacks to celebrate even the smallest accomplishments. I also value the friendship of Alison Singer, with whom I knew I could always grab coffee with for off-campus work dates and who would happily share her two adorable dogs with me since I don't have any pets of my own. I also could not have imagined getting through graduate school without the support of the women who started this program with me. From studying together for class, helping each other prepare for our comprehensive exams, complaining about and troubleshooting experiments, to rooming together for conferences, and

exploring new cities during our down time at meetings, I will be forever grateful to have the friendships of Isola Brown, Brittany Winner, Juliette Brown, and Teri Lansdell.

Finally, I would like to thank my family for their endless support and love. From helping me move to Michigan from Kansas to always making sure I enjoy my time at home over the holidays, they have gone above and beyond to do what they can to help me succeed.

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KEY TO ABBREVIATIONS

AAV	Adeno-associated virus
ABP	Actin-binding proteins
ACTH	Adrenocorticotrophic hormone
ADP	Adenosine diphosphate
AGC	Named after cAMP-dependent protein kinase 1, cGMP-dependent protein kinase, and protein kinase C
AKT	Thymoma viral proto-oncogene
AMPH	Amphetamine
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
Cas9	CRISPR associated protein 9
CMS	Chronic mild stress
CON	Control
CPP	Conditioned place preference
CRE	Cre recombinase

CRF	Corticotrophin-releasing factor
CRISPR	Clustered regularly interspaced short palindromic repeats
CSDS	Chronic social defeat stress
CUMS	Chronic unpredictable mild stress
CUS	Chronic unpredictable stress
DA	Dopamine
DAT	Dopamine transporter
DEPTOR	DEP domain containing mTOR-interacting protein
DNA	Deoxyribonucleic acid
DSM-V	Diagnostic and Statistical Manual-V
EPM	Elevated plus maze
ERK1,2	Extracellular signal-regulated kinase 1,2
fMRI	Functional Magnetic Resonance Imaging
FST	Forced swim test
GABA	Gamma-aminobutyric acid
GAP	GTPase-activating protein
GAP-43	Growth-associated protein-43

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEF	Guanine exchange factor
GFP	Green fluorescence protein
GPCR	G-protein coupled receptor
GSK-3β	Glycogen synthase kinase-3 beta
HET	Heterozygous
HFD	High-fat diet
HOMO	Homozygous
HPA	Hypothalamic-pituitary-adrenal
HSV	Herpes simplex virus
IRS2	Insulin receptor substrate 2
KO	Knockout
LHPP	phospholysine phosphohistidine inorganic pyrophosphate phosphatase
LIMK	LIM kinase
LTM	Long-term memory
MAOI	Monoamine oxidase inhibitor
MARCKS	Myristolyated alanine-rich protein kinase C substrate

MDD	Major depressive disorder
MEF	Mouse embryonic fibroblasts
mLST8	Mammaliam lethal with sec-13 protein 8
MRI	Magnetic Resonance Imaging
mTOR	Mammalian/mechanistic Target of rapamycin
mTORC1	Mammalian/mechanistic Target of rapamycin complex 1
mTORC2	Mammalian/mechanistic Target of rapamycin complex 2
NAc	Nucleus accumbens
NE	Norepinephrine
NET	Norepinephrine transporter
NDRI	Norephinephrine/dopamine reuptake inhibitor
NDRG	N-myc downstream regulated gene
NMDA	N-methyl-D-aspartate
OF	Open field
PAK	p21-activated kinase
PAG	Periaqueductal grey
PBS	Phosphate buffered saline

PFC	Prefrontal cortex
PKC	Protein kinase C
PRAS40	Proline-rich AKT substrate 40 kDa
PVN	Paraventricular nucleus
Raptor	Regulatory-associated protein of mammalian target of rapamycin
Rictor	Rapamycin-insensitive companion of mTOR
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
ROCK	Rho-associated protein kinase
SI	Social interaction
SIRT1	Sirtuin
SGK	Serum- and glucocorticoid-induced protein kinase
SNRI	Serotonin-norepinephrine reuptake inhibitor
SSRI	Serotonin-specific (or selective serotonin) reuptake inhibitor
TCA	Tricyclic antidepressant
TESK	Testis-specific kinase

TH	Tyrosine hydroxylase
TORC2	Target of rapamycin complex 2
TST	Tail suspension test
VGAT	Vesicular GABA transporter
VGLUT	Vesicular glutamate transporter
VTA	Ventral tegmental area

CHAPTER 1

Introduction

Prevalence and costs of addiction and depression

Depression and drug addiction are two common neuropsychiatric diseases that pose a significant health and economic burden on society, as well as a considerable emotional cost for both patients and their families. Depression, also known as major depressive disorder (MDD), is a prevalent mood disorder characterized by persistent sadness lasting at least two weeks or more [1] and affects more than 300 million people worldwide [2]. Drug addiction, on the other hand, is a neurobiological disease characterized by continued drug seeking behavior and compulsive drug use despite adverse consequences [3]. While drug abuse and depression are prevalent individually, the diseases are also highly co-morbid, meaning a subset of patients are diagnosed with both disorders. Importantly, suffering from drug addiction does not dictate that one will also be diagnosed with depression, but it does increase the chance of being diagnosed with depression. In fact, being diagnosed with a substance use disorder increases one's chances for developing depression by a factor of 4.7 [4]. In 2012, 20.7 million adults were diagnosed with a substance use disorder and of those, 8.4 million, or approximately 40% were also diagnosed with a mental illness [5]. Similarly, illicit drug use is significantly higher in those who have experienced a major depressive episode in the past year compared to those who did not have a major depressive episode [5]. Of the 8.4 million patients who are diagnosed with this co-morbidity, 4.3% receive treatment for their substance use disorder, 34% receive mental health treatment and only 7.9% receive treatment for both their mental health and substance use disorders [5]. This means that over half of the 8.4 million patients don't receive any treatment and emphasizes the critical need for efficacious therapies for these disorders. Currently, depression and addiction also exert a significant financial toll in the United States, with estimated costs of $83.1 billion for depression [6] and $193 billion on issues related to illicit

drug use [7]. Opiate usage, especially in the United States, has been on the rise, as Americans use 80% of opioid drugs worldwide, and 99% of the world's hydrocodone supply [8]. The costs of these disorders are high, which is partly due to their high prevalence, but is also because they are chronic disorders without cures. While current therapies help manage symptoms in some patients, a large number of patients are refractive to current treatments. Thus, there is a critical need for improved therapies for depression and addiction. To improve treatment, we need a better understanding of the neuroadaptations underlying these disorders, including whether there are common molecular mechanisms that contribute to the development of both addiction and depression, as these might serve as especially valuable targets for therapeutic intervention.

Comorbidity of depression and addiction

There are a range of diseases that are comorbid with depression that vary from neuropsychiatric disorders such as anxiety, substance abuse, to neurodegenerative diseases like Alzheimer's, to diseases that are not necessarily connected to the central nervous system such as cancer. It is suggested that the comorbidity of depression and substance abuse could be due to the overlap of etiological factors such as stress and the alteration of neuronal signaling [9]. Stress is an important, well-established environmental factor in the development of addiction. Traumatic incidents, deaths of loved ones, and interpersonal conflicts are just a few examples of stressors that can increase a person's vulnerability to substance abuse. This is also true of early life stress, as physical abuse, emotional abuse, divorce of parents, or loss of a parent can increase a child or adolescent's vulnerability to drug abuse [10]. Importantly, stressful life experiences also increase the likelihood of developing depression. For example, emotional stress and abuse at an early age can sensitize individuals to stressful situations in adulthood. As a result, these individuals tend to experience more symptoms of depression when confronted with a life stressor

compared to those who were initially categorized as having low levels of childhood emotional abuse [11]. Critically, animal models of depression have been used to investigate stress effects in the central nervous system. Chronic stress, for instance, is used to induce a depressive-like state (described in detail in **Animal models of depression** below) and these models also induce alterations in drug reward and drug reinstatement, addictive-like phenotypes, suggesting a common link between the two disorders [12].

Biological bases of depression

The Diagnostic and Statistical Manual (DSM-V) of Mental Disorders defines a major depressive disorder as having five or more of the following symptoms on a nearly daily basis for at least two weeks: depressed mood or feelings of sadness or emptiness, loss of interest in activities that were once pleasurable, weight loss or weight gain of more than 5% of body weight or a change in appetite, insomnia or hypersomnia, changes in activity such as psychomotor agitation or retardation, fatigue, feelings of worthlessness, inability to concentrate or think, and thoughts of suicide or an attempt at suicide [13]. Importantly, depression is diagnosed by a health care provider such as a psychologist or psychiatrist, who helps determine the best course of treatment for the patient.

Understanding how depression develops can be helpful for the course of treatment. While the exact cause of depression is unknown, it is suspected to be due to a combination of biological and environmental factors. Epidemiological studies of twins and families indicate that depression is heritable [14, 15]. By performing a meta-analysis on both adoption and twin studies of depression, the likelihood for heritability was determined to be 31-42%, with environmental factors having a smaller influence (5%) [15]. Despite the observed heritability of depression,

there are no common genetic variants linked to the development of depression. A number of studies have attempted to associate polymorphisms in candidate genes to depression, but have failed to identify a direct cause-and-effect relationship [16]. However, recently, two loci located on chromosome 10 near the sirtuin 1 *(SIRT1)* and phospholysine phosphohistidine inorganic pyrophosphate phosphatase *(LHPP)* genes, were identified through whole-genome sequencing as risk factors for depression [17]. In this study, over 11,000 Han Chinese women were studied from 58 hospitals in China in which only women with repeated cases of depression were examined. This study is unique because it identifies polymorphisms in a specific population of patients, contrary to the belief that depression develops from overlapping disorders and from environmental factors. These chromosomal variants were found to be rarer in European cohorts, highlighting a caveat of the study, as a population with a similar genetic background would increase the likelihood of finding significant genetic effects. However, it could also suggest potential susceptibility to depression based upon one's racial background. The study could also be expanded to explore multiple subtypes of depression to more accurately search for genetic variants. Interestingly, work from animal models is consistent with changes in SIRT function contributing to depression, as alteration of SIRT expression increased susceptibility to social defeat stress [18]. Overall, while there is clear evidence that depression is heritable, little is known about the genetic variation that contributes to the phenotype, thus depression is likely to continue to be the subject of large whole genome sequencing studies.

Environmental stress has been linked to the development of depression, suggesting that the body's ability to handle stress through the hypothalamic-pituitary-adrenal (HPA) axis could play a role. The HPA axis utilizes hormones induced by stress to communicate between the hypothalamus, pituitary gland, and adrenal gland. Stress induces the release of corticotrophin-

releasing factor (CRF) from the paraventricular nucleus (PVN) of the hypothalamus. This causes the pituitary gland to release adrenocorticotrophic hormone (ACTH) and ultimately leads to the secretion of the stress hormones known as glucocorticoids (e.g. cortisol in humans or corticosterone in mice) [19, 20]. Glucocorticoids can then bind to glucocorticoid and mineralocorticoid receptors, throughout the body to initiate responses to stress. Importantly, the HPA axis has feedback mechanisms for tight regulation of glucocorticoid action. The PVN contains glucocorticoid receptors that respond to circulating levels of glucocorticoids by decreasing neuronal activity and subsequently decrease ACTH release from the pituitary gland [19]. Depressed patients have significantly increased cerebral spinal fluid CRF compared to controls, suggesting dysfunction of the HPA axis in depressed patients [21]. Interestingly, early life stress alters the stress response within the HPA axis. A small study followed young children who were exposed to maternal stress, which consisted of stressors such as having depression symptoms, parenting stress, and financial stress. These children had the tendency to have increased cortisol levels compared to children who were not exposed to early life maternal stressors. Importantly, those children exhibited abnormal internalization behaviors in their transition into a school setting [22], which provides evidence for stress being able to negatively alter HPA function. Larger studies and meta-analyses should be performed to strengthen the clinical data and to further investigate how early life stress can alter stress responses. In agreeance with clinical findings, rodent models find that reduced maternal care results in elevated stress hormones such as ACTH and corticosterone following an acute stress in adulthood [23]. These data suggest that early life stress produces long-lasting changes in the responsiveness of the HPA axis that could increase susceptibility to develop depression. This is supported by data in the human literature where a number of studies have reported an increase in

HPA axis activity in those who have undergone early life stress [20]. Overall, these findings highlight how various external factors can alter the stress responses and demonstrate the importance of the HPA axis in mediating these hormonal and behavioral changes.

In addition to environmental factors, there are also several biological factors that can influence the development of depression and other related mood disorders. One of these factors involves changes in brain chemistry. In the 1950's, study of the drug reserpine, which depleted monoamines from the brain and led to feelings of depression, led to the formation of what is currently known as the "monoamine hypothesis" [24]. In this hypothesis, depression is the result of monoamine depletion. Monoamines include both catecholamines (dopamine, norepinephrine) and tryptamines (serotonin), that act as neurotransmitters in the central and peripheral nervous systems to perpetuate a number of physiological tasks such as motor function, reward, and cardiac function [25]. Given the role of these neurotransmitters in the monoamine hypothesis, the next logical step to treating depression would be to restore these neurotransmitters to normal levels.

Pharmacological treatment of depression

Utilizing the idea of restoring monoamine levels back to normal in the synapse to treat depression, the overarching mechanism of action of antidepressants is to increase the presence of monoamines available for neurotransmission [26]. Monoamine oxidase inhibitors (MAOIs) were first discovered in the 1950's for the treatment of depression [27]. MAOIs function by preventing monoamine oxidase from degrading monoamines, which increases the availability of monoamines in the synapse [1]. Tricyclic antidepressants (TCAs) are another class of antidepressant compounds and they inhibit neurotransmitter reuptake, which also increases

monoamine availability [28]. Due to their narrow therapeutic window and their nonselective nature, TCAs also produce unwanted side effects [27]. Thus, TCAs are not commonly used for treating depression. Newer classes of antidepressants have more tolerable side effects, and are therefore more promising for chronic use. Serotonin-specific (or selective serotonin) reuptake inhibitors (SSRIs) were developed in the 1970's with the goal of reducing adverse effects by targeting specific proteins [27]. As the name implies, SSRIs block serotonin transporter function to prevent the reuptake of serotonin and thereby prolong serotonin binding to receptors on the postsynaptic neuron. While SSRIs are widely prescribed for the treatment of depression, some of the side effects can make the drug less tolerable to patients, such as sexual dysfunction, weight gain, and altered sleep [26]. However, they are still favored over TCAs since SSRIs are less likely to cause seizures [29]. Even with the unwanted side effects, TCAs and SSRIs have been found to be more effective compared to other classes of antidepressants [30]. Other classes of antidepressants target combinations of monoamine to help ameliorate symptoms of depression. For instance, serotonin/norepinephrine reuptake inhibitors (SNRIs) were developed to target serotonin and norepinephrine transporters and norepinephrine/dopamine reuptake inhibitors (NDRIs) target NET and DAT [26], to block reuptake of their corresponding neurotransmitters and prolong their signaling in the synapse. While traditional antidepressants require a patient to be on these drugs for several weeks to see therapeutic effects, another drug, ketamine, is undergoing large-scale clinical trials [31] as a fast-acting treatment for depression. Ketamine is a dissociative anesthetic drug that antagonizes the NMDA receptor [32] to stop neuronal communication. Several clinical trials have demonstrated the rapid effects of one dose of ketamine treatment for depression, but few have been able to demonstrate long-lasting effects. A consensus statement released by physicians in the Journal of the American Medical Association

suggests that if ketamine is to be used to treat depression, that the patient remain under vigilant care of the physician to avoid potential side effects such as cognitive impairment and drug abuse [33]. Overall, failure of these antidepressants to treat a significant fraction of depressed patients, combined with the development of therapeutics such as ketamine that rely on a different mechanism of action, suggest that monoamine dysregulation is not the sole cause of depression.

Physiological changes of the brain in depression

It is important to understand the physiology of the brain structures affected by depression in order to find more effective therapies to help manage this neuropsychiatric disease. A number of critical brain regions have been studied in humans using non-invasive techniques. For instance, the left nucleus accumbens (NAc), a structure involved in mediating natural and drug reward, was found to be less active in depressed patients compared to non-depressed controls, through functional magnetic resonance imaging (fMRI) [34]. Another study measured the blood flow in the left prefrontal cortex (PFC) and amygdala of depressed patients. The authors found that both the left PFC and amygdala of depressed patients had increased activity compared to controls. Furthermore, even after remission, the patients that had been diagnosed with depression still had increased amygdala activity [35], emphasizing the long-lasting effect of depression. Activity of key brain regions is not the only aspect of the brain that is altered by depression. Importantly, correlative studies in humans have looked at brain region size and the diagnosis of depression. Further investigation of the amygdala, a region known for processing emotions, revealed amygdala size in decreased in depressed females but not males [36]. In this same study, no significant changes in PFC or hippocampus volume were observed in either sex [36], but other studies have observed differences. For instance, one study reported a decrease in hippocampal volume in depressed patients through MRI [37]. Even though there are conflicting

data reported for brain region volume or size changes in depressed subjects, these findings are of importance and should be further investigated, as differences could be explained by variables such as age, severity of depression, potential influence of antidepressants, and gender. Collectively, these examples illuminate the numerous neuroadaptations that occur in human brains and demonstrate the need for further characterization.

Animal models of depression

The previous section illustrates that while some risk factors for depression have been identified, such as in alterations of monoaminergic signaling and HPA function, much remains unknown about external contributions to the development of this neuropsychiatric disease. Moreover, teasing apart how these changes produce depressive-like behaviors is difficult in clinical populations. Thus, animal models of depression are necessary to advance the understanding of the molecular mechanisms underlying this disease. Animal models do have limitations. For example, emotions such as sadness cannot be quantified in animals in a manner analogous to humans. However, animal models of depression have been developed that have both face validity, in which behavioral changes resemble depressive-like symptoms, and pharmacological validity, in which the behavioral response responds to antidepressants [38]. For example, the forced swim test (FST) and tail suspension test (TST), both have pharmacological validity. Specifically, drugs that have antidepressant actions in humans will increase the time animals spend struggling in the FST and TST, which both measure helplessness. Thus, these measures serve as good predictive tools for drugs with antidepressant qualities, but are not models of depression per se since they do not induce depressive-like symptoms [39]. To generate animal models of depression that recapitulate behaviors analogous to human symptoms such as

anhedonia or altered circadian rhythm, i.e. face validity, and pharmacological validity, investigators often use some form of chronic stress exposure.

The chronic mild stress (CMS) (also referred to as chronic unpredictable stress (CUS), or chronic unpredictable mild stress (CUMS) [40]) paradigm utilizes daily exposure to a mild stress for a varied period of time, usually for 2-4 weeks. A variety of stressors can be used; food depravation, water depravation, altered light cycle, cage tilting, or housing in a soiled (wet) cage are some common examples [41]. Chronic exposure to these mild stressors is sufficient to induce anhedonia, as measured by decreased consumption of sucrose-sweetened water, [41] and treatment with chronic antidepressants restores sucrose preference to normal levels [42]. Moreover, chronic mild stress also induces altered sleep patterns in rodents [43]. Physiological changes are also induced in rodents in CMS that are similar to humans, such as a decrease in hippocampal volume [44] and an increase in the stress hormone, corticosterone [45]. The caveat with CMS is that the stressors used are not ethologically valid. This is because mice do not normally experience soiled bedding or cage tilting. While this model does provide a means for collecting critical information regarding stress and allows for the evaluation of stress in both sexes, there still remains some concern regarding the degree by which the information gained can translate to humans.

To address the caveat of ethological validity, a paradigm that utilizes social subordination, a more ethologically relevant stress, can be utilized. This model is called chronic social defeat stress (CSDS). In mouse CSDS, an experimental mouse is subjected to a daily physical encounter with a large, aggressive, territorial mouse [46, 47]. CSDS is sufficient to induce depressive-like symptoms such as anhedonia and social avoidance [47], similar to symptoms expressed by humans. Importantly, CSDS has pharmacological validity, meaning that

chronic, but not acute, treatment with SSRIs will reverse the depression-like phenotype [46]. This model displays phenotypic heterogeneity; meaning that like humans, not all mice that undergo this stress will display depressive-like symptoms. The mice in this model are classified as either susceptible or resilient based upon their stress-induced behavioral phenotype, such as the degree to which they are willing to socially interact with a novel mouse [46]. Additionally, similar to humans, this alteration in social interaction is long-lasting. Identification of the molecular mechanisms underlying both susceptibility and resilience to CSDS are the topic of many current studies. For example, early studies described increases in brain-derived neurotrophic factor (BDNF), as well as phosphorylation of downstream targets such as AKT, GSK-3β, and ERK1,2 in the nucleus accumbens of susceptible mice [47]. In fact, a variety of studies have indicated that dysregulation of the brain reward circuit, and more specifically the ventral tegmental area (VTA), plays a critical role in the development of depressive-like behaviors following CSDS [47-49].

Ventral tegmental area and CSDS

Activity of the ventral tegmental area, a critical component of the mesocorticolimbic pathway, serves to mediate the rewarding aspects of drugs of abuse such as morphine and cocaine, in addition to natural rewards such as food and sex [50, 51]. The VTA is a heterogeneous region comprised of dopaminergic (~70%), GABAergic (~30%), and glutamatergic (~2-3%) neurons [52, 53]. Dopamine neurons (DA) are identified by their expression of tyrosine hydroxylase (TH), the rate limiting enzyme of dopamine synthesis [54]. The VTA sends many projections to other brain regions to mediate various behaviors. For instance, the lateral VTA DA neurons that project to the lateral shell of the nucleus accumbens (NAc) mediate reward [54, 55] (Figure 1). Medial VTA DA neurons, on the other hand, project

to the prefrontal cortex to mediate aversive responses [55] (Figure 1). Outside of the reward circuit, VTA DA neurons also project to other regions such as the amygdala, hippocampus, ventral pallidum, periaqueductal grey, and locus coeruleus [54]. The input onto VTA DA neurons is also very diverse. For example, stimulation of glutamatergic and cholinergic inputs from the laterodorsal tegmentum to the lateral VTA increases dopaminergic output to the lateral shell of the nucleus accumbens to increase reward [55]. Aversion, on the other hand, is largely mediated by glutamatergic input from the lateral habenula onto the rostromedial tegmental nucleus, to inhibit the lateral and medial VTA [55]. In addition, GABAergic neurons from the lateral hypothalamus also project to the VTA and serve to disinhibit VTA DA neurons by synapsing onto VTA GABAergic interneurons in motivated behaviors [56]. Overall, the inputs and outputs to and from the VTA are very complex and understanding their physiological roles will help with elucidating the dysfunctional pathways involved in the development of depression.

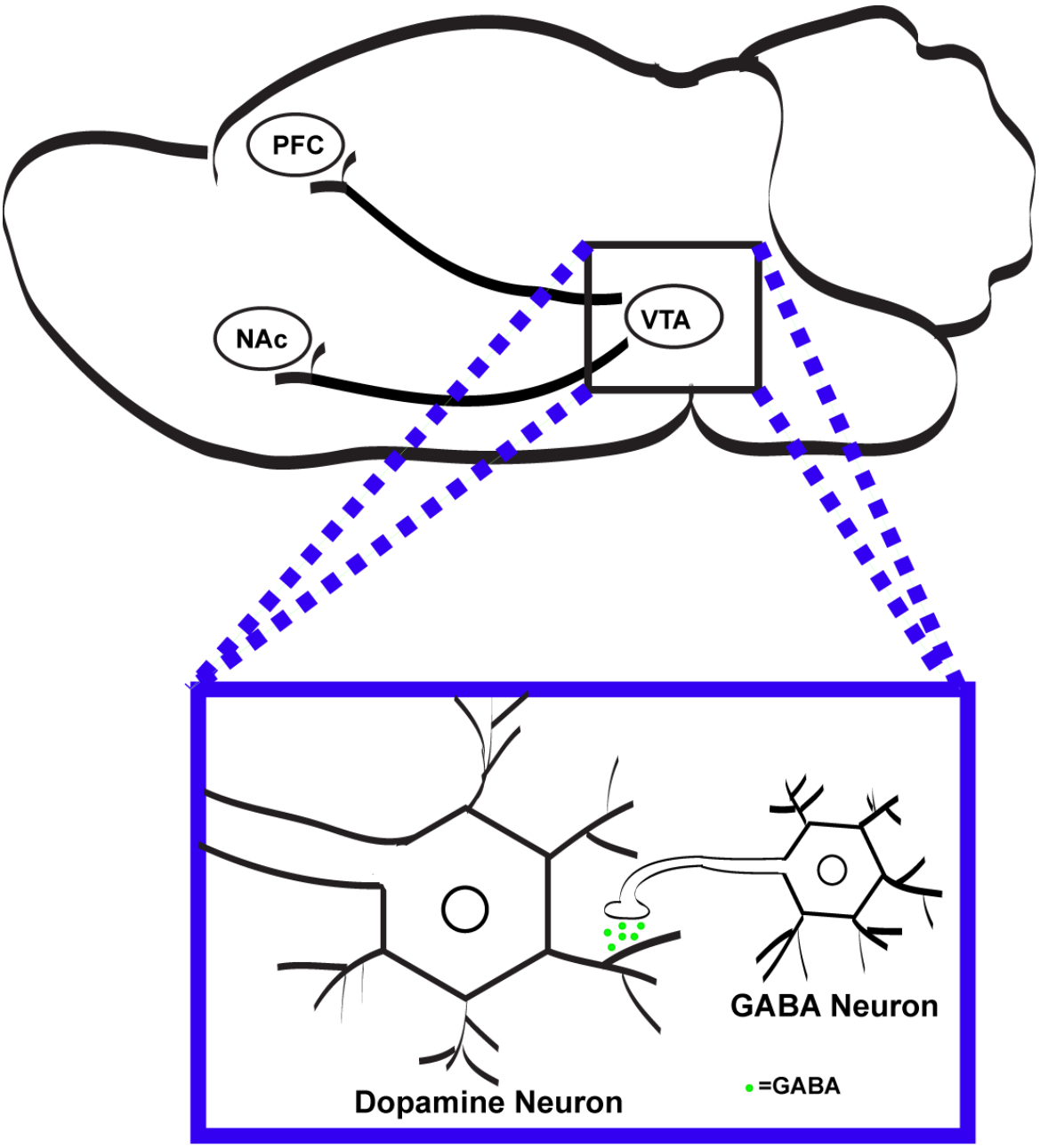

Figure 1: The reward pathway. Ventral tegmental area (VTA) dopamine neurons project to the nucleus accumbens (NAc) and prefrontal cortex (PFC). The VTA is largely composed of dopamine and GABAergic neurons. VTA GABAergic neurons synapse onto dopamine neurons, thereby influencing VTA dopamine neuron excitability and dopamine output to structures such as the NAc and PFC.

The aforementioned pathways, such as the projections from the VTA to NAc to mediate reward, can be altered in neuropsychiatric diseases like addiction and depression. Animal models of depression have been used to identify neuroadaptations that occur in these pathways. For example, CSDS induces changes in the VTA in both susceptible and resilient mice. Susceptible mice have a significant increase in neuronal firing compared to non-stressed controls and resilient mice [47]. Further electrophysiological studies revealed that resilient mice actually have a greater increase in VTA DA neuronal firing through the upregulation of an excitatory current [49]. Results from a DNA microarray found that resilient mice have an increase in potassium channel gene expression [47], which indeed, led to an increase in VTA DA potassium channel current in resilient mice [49]. Furthermore, when potassium channel openers are infused into the VTA of susceptible mice from CSDS, their depressive-like phenotypes are reversed [57]. Together, these studies reveal that CSDS induces neuroadaptations in all mice that go through physical stress. Importantly, the resilient mice have a compensatory mechanism to counter the increase in VTA DA firing. In addition to electrophysiological changes, biochemical changes are also induced in the VTA after CSDS. Because there was also an increase in brain-derived neurotrophic factor (BDNF) release in the nucleus accumbens [47], one of the subsequent studies also investigated neurotrophic factor signaling in the VTA. Interestingly, the phosphorylation of AKT, a protein downstream of BDNF, was decreased in susceptible mice but not in resilient mice [48]. Overall, these results present a strong role for the VTA in the pathogenesis of depression in CSDS and necessitate further investigation.

Addiction

In addition to depression, the VTA also plays a critical role in addiction, as it mediates the rewarding responses to drugs of abuse [58]. The Diagnostic and Statistical Manual (DSM-V)

of Mental Disorders defines addiction as having constant drug-seeking behavior combined with the lack of control to regulate drug intake in order to avoid withdrawal [13]. Drugs with abuse liability issues all increase dopamine signaling in the nucleus accumbens to produce the "high" that drug users feel [59]. This is typically achieved by increasing dopamine release from the ventral tegmental area neurons that project to the nucleus accumbens or by blocking dopamine transporters in the nucleus accumbens to prevent reuptake of dopamine. The exact causes of drug addiction remain largely undefined. This is due to a number of reasons, such as the number of drugs of abuse available, their varying mechanisms of action, and different factors that contribute to the addictive phenotype. Furthermore, this can be complicated by poly-drug use, the use of multiple drugs of abuse [60]. Importantly, one time exposure to a drug does not necessarily lead to addiction, nor does repeated use. In addition, genetics may play an important role in determining susceptibility to addiction, as it is estimated that genetics contribute to 40-60% of addiction-related vulnerabilities [3]. Lastly, the different stages of drug abuse, such as initial exposure to drug, changes in response that lead to tolerance and sensitization, progression, and withdrawal can also be affected by genetic variability in one or a combination of genes [61].

Due to the complexity of addiction, there are challenges presented in finding treatments for addiction. Some of these challenges are not even related to the neurobiology of the disease. For instance, the pharmaceutical industry is often responsible for driving the market with the types of drugs produced and sold on the market. Out of fear of a potentially low return on investment and small target populations, the pharmaceutical industry lacks interest on finding treatments for substance use disorders [62]. Combined with the lack of understanding on the neuroadaptations induced by drugs of abuse and continued research on the mechanisms of action of each drug of abuse, the ability to find effective pharmacological treatments is difficult and

highlights the need for furthering our knowledge on the molecular changes that occur in addiction, and also to increase the incentive for pharmaceutical companies to drive interest in developing life-saving therapies.

Opioid pharmacology

With the prescription of opioids rapidly increasing by 402% from 1997 to 2007 [8], it is important to understand their mechanism of action in order to understand how opioid addiction begins. Opioids are a class of analgesic drugs that provide pain relief through their interaction with opioid receptors [63-65]. Opiates were initially derived from the opium poppy and have been used for pain management for centuries. Interestingly, the active compound from the opium poppy, morphine, was isolated in 1806 by Sertürner, and has been used since then. Research on opioids, including the synthesis of agonists and antagonists of the opioid receptors accelerated in the 20^{th} century [66]. Examples of opioids that have been used for pain management include morphine, oxycodone, codeine, and hydrocodone [67]. In order to function as analgesics, opioids act as agonists of opioid receptors, in which there are three types: μ, κ, and δ. All opioid receptors are G-protein coupled receptors (GPCRs). Specifically, activation of the μ-opioid receptors produces two general signaling effects. First, G-protein signaling through G_i or G_o G-proteins produces analgesic effects [68, 69]. Second, β-arrestin can bind to μ-opioid receptors after they are phosphorylated by GPCR kinases [70] to produce unwanted side effects such as tolerance, increased drug liability, and respiratory depression [71] and ultimately lead to desensitization of the μ-opioid receptor itself [70]. In fact, deletion of β-arrestin 2 in mice increased analgesic efficacy of morphine [70], highlighting the importance of biased agonism signaling of opioid receptors. Antagonism of opioid receptors can be accomplished with compounds such as naloxone and naltrexone, which are used to treat opioid overdoses [72].

Knowledge of the pharmacokinetics and pharmacodynamics of opioids is critical for the understanding of analgesia and abuse potential. When administered orally, morphine rapidly undergoes first pass metabolism [68]. Metabolism of morphine occurs in the liver to produce morphine-3-glucuronide, the major metabolite, and morphine-6-glucuronide, the minor metabolite. Interestingly, morphine-6-glucoronide has greater analgesic activity than morphine itself [73]. To bypass first pass metabolism, opioids can be administered intravenously for rapid analgesic effects or through transdermal patches [68]. Morphine binds to μ-opioid receptors in the periaqueductal grey (PAG) to open voltage-dependent K+ channels, and in turn, disinhibits descending pain pathways to produce analgesia [65]. The VTA also contains opioid receptors. Here, the mechanism of action for morphine involves binding to μ-opioid receptors on GABA neurons, which inhibits their activity. This results in disinhibition of VTA DA neurons and increases release of DA into the nucleus accumbens [74].

Finally, it is important to note that the body does not produce opioid receptors to respond to exogenous opioid substances such as morphine or heroin, but for responses to endogenous opioid peptides. Endogenous opioid peptides such as enkephalin help modulate the body's response to pain, particularly by binding to opioid receptors in the periaqueductal grey (PAG) of the midbrain [75]. Similar to exogenous opioids, naloxone can also be used to block opioid peptide function, enforcing the fact that these peptides do indeed function through opioid receptors [75].

Animal models of addiction

In order to investigate mechanisms that contribute to addiction and reward, animal models can be used to evaluate the neuroadaptations that occur at the behavioral and molecular

level. One common paradigm used to evaluate reward for drugs of abuse such as morphine, is called conditioned place preference (CPP). In CPP, rodents are administered drug in one context and vehicle, as a control, in a different context. On test day, the rodent is allowed to explore the entire CPP chamber and the time spent in each chamber is recorded. If a drug is rewarding, the animal will associate the context with the drug, and spend more time in that chamber indicating preference [76]. CPP is an excellent behavioral tool to help elucidate mechanisms of opiate reward. For instance, viral overexpression of constitutively active proteins or dominant negative proteins can be used to determine the role of that protein [77]. In addition, mice can also be genetically altered to evaluate drug reward. For instance, in opiate reward, receptors can be knocked out to determine how that receptor contributes to the development of this behavior [78]. One caveat of CPP is that drugs are administered by the investigator, and thus, rodents do not have a choice to take the drug or not. This distinction is important for understanding drug addiction, as the increased motivation to consume and/or obtain drug is one of the hallmark characteristics of substance abuse compared to substance use. To address the caveats of CPP, operant conditioning is the gold standard paradigm that has been used for decades for studying addiction [79], particularly in rats [80]. While this behavior is very robust in rats, very few self-administration studies have been performed using opiates in mice. Thus, for opiate reward, an alternative paradigm to assess voluntary morphine intake is through a morphine drinking assay. Early work demonstrated that mice can be persuaded to voluntarily consume morphine in drinking water, especially when the water is slightly sweetened to lessen the impact of the bitter taste associated with alkaloids like morphine. Morphine preference can be assessed by allowing mice access to two water bottles in their home cage, one containing morphine, and the other containing quinine, which serves as a bitter taste control [81], both dissolved in slightly

sweetened water. Interestingly, there are strain differences in the oral consumption of morphine as it was initially observed that C57BL/6J mice exhibited greater morphine preference compared to DBA/2J mice [82]. In fact, behavioral work in the early 1990's investigated voluntary morphine consumption across 15 strains of mice and found that C57BL/6J mice are the most willing to voluntarily consume morphine [81]. Furthermore, C57BL/6J mice were found to become physically dependent via oral morphine consumption when high doses were used. Use of naloxone, an opioid receptor antagonist, was sufficient to induce withdrawal symptoms such as wet dog shakes, diarrhea, and hypothermia, following morphine dependence [83]. These studies emphasize the suitability of the C57BL/6J strain for evaluating voluntary morphine reward and in addition, by allowing rodents to voluntarily consume more morphine, this paradigm more closely mimics the decision making process of humans.

Neuroadaptations induced by chronic opiate exposure

The development of dependence to opiates can be evaluated by examining the changes that are induced after chronic exposure. One neuroadaptation observed after chronic morphine treatment is a decrease in VTA dopamine neuron soma size in both mice and rats [74, 77, 84-86]. The time course for these neuroadaptations is interesting, as this decrease in soma size is maintained for 14 days after the last morphine treatment and is then restored 30 days post-morphine exposure in rats. During the 14-day withdrawal period, rats experience decreased morphine CPP at both 1 and 14 days post-morphine exposure [77], demonstrating that the decrease in soma size correlates to decreased morphine reward. Importantly, VTA DA neuron soma size is also decreased in post-mortem human heroin addict brain samples [74], highlighting the clinical relevance of this morphological change. In addition to altering soma size, morphine also alters the physiological function of VTA DA neurons. Exposure to chronic morphine

increases VTA DA neuron firing while decreasing DA release to the NAc [74]. Moreover, overexpression of a dominant-negative K+ channel, which disrupts the activity of the wild-type K+ channel, was sufficient to decrease VTA DA soma size, while overexpression of a K+ channel prevented the morphine-induced decrease in soma size, linking the change in activity to the morphology. Similarly, decreased VTA DA neuron soma size in a mouse model of mania also yields an increase in firing rate that can also be rescued by the overexpression of K+ channels [87], demonstrating similar regulation by a variety of stimuli.

Given the potential translational relevance of decreased soma size observed in rodents and importantly, human heroin addicts, it is critical to understand the molecular mechanisms that underlie these changes. Early work investigating the decrease in VTA DA neuron soma size after chronic morphine treatment demonstrated that BDNF was able to rescue the decrease in soma size, [84] with the exact mechanism unknown at the time. Further studies investigated the downstream signaling of BDNF, namely the IRS2 (Insulin-receptor substrate 2)-AKT pathway. Specifically, chronic morphine decreased activity of this pathway and viral overexpression of IRS2 and AKT was sufficient to rescue the change in morphology and morphine reward [77]. Similarly to opiates, endogenous opioid peptides released after sexual experience also decrease VTA DA neuron soma size in mice. The decrease in size was noted at days one and seven following sexual experience, with the soma returning back to normal after 30 days. Treatment with naloxone prior to sexual experience blocked the decrease in soma size, demonstrating the role of opioid receptor signaling in this neuroadaptation [88]. It is important to note that this decrease in VTA DA neuron soma size has only been observed with opioids [74, 77, 84], as self-administration of cocaine, ethanol, or nicotine does not seem to alter VTA DA neuron soma size [89].

Molecular mechanisms underlying behavioral and morphological changes in response to stress and opiates

Given the co-morbid nature of depression and addiction, several groups are attempting to elucidate the molecular mechanisms of stress-induced drug reward. Early work investigating social defeat stress in rats has demonstrated that these rats will acquire self-administration of cocaine faster than their non-stressed controls [90]. After sensitization to social defeat stress, rats also had a significant increase in cocaine "binge" activity [91]. Chronic social defeat stress also increases cocaine CPP in susceptible mice [47]. Other forms of stress, such as repeated maternal separation, also increased behavioral sensitization to cocaine in male and female mice [92]. Importantly, the exact mechanisms that underlie these changes in stress-induced drug reward are still under investigation, especially for studies involving opiates, which highlights the need for more studies specifically on stress-induced opiate reward.

In review, chronic exposure to both stress and opiates, independently, induces neuroadaptations in the VTA that mediate depressive-like and addictive-like behaviors. Given the similarity of the changes in response to stress and opiates, such as the increase in firing of VTA DA neurons [47, 74] and decreases in phosphorylation of AKT [48, 77], this suggests a common molecular mechanism induced by both stimuli. Importantly, this demonstrates a need to further investigate what is mediating these similar neuroadaptations in the VTA, which could potentially be mTORC2.

TOR and its complexes: TORC1 and TORC2

TOR, or the mammalian/mechanistic Target of Rapamycin, is a serine/threonine kinase that associates with a number of proteins to form two distinct complexes, TORC1 and TORC2,

which have different cellular functions [93, 94]. Both TORC1 and TORC2 are comprised of TOR, DEPTOR (DEP domain containing TOR-interacting protein), and LST8 (lethal with sec-13 protein 8). Furthermore, TORC1 also includes Raptor (regulatory-associated protein of mammalian target of rapaymin), and PRAS40 (proline-rich AKT substrate 40 kDa) [94, 95] (Figure 2). TORC2, on the other hand, is differentiated from TORC1 through its association with Protor, SIN1, and Rictor (Rapamycin-insensitive companion of TOR) [96] [95] (Figure 2). As the name suggests, these two complexes have different sensitivities to rapamycin, an immunosuppressive compound that aided in the discovery of TOR in yeast *Saccharomyces cerevisiae [97]*. In *S. cerevisiae***,** TORC1 was discovered to be inhibited by rapamycin while TORC2 was found to not be affected by rapamycin treatment [98].

The components of TORC2 assist in distinguishing the functional differences between the two TOR complexes. TORC2 contains two critical proteins that are essential for its function. First, Rictor, also known as the mammalian analog of AVO3 from *S. cerevisiae*, was identified more recently in 2004 by two groups who were simultaneously working to understand TOR signaling. In an attempt to purify raptor-TOR associated proteins in HEK293T cells, a serendipitous discovery of a 200 kDa protein was made. This protein was eventually named rapamycin-insensitive companion of TOR, or Rictor [96]. Another group (Jacinto et. al.) was investigating the mammalian genome to identify a sequence with 25% similarity to AVO3, in which they named it mAVO3 [99]. Jacinto et. al. identified that TOR could be co-immunoprecipitated with either raptor or mAVO3, but not both, leading to the identification of two different TOR complexes [99]. Together, these two groups discovered a novel protein that is essential to form what we know now as TORC2. Further work to study the function of TORC2 identified SIN1 as another protein necessary for TORC2 function. Mutation at a highly

conserved site, G934, was sufficient to disrupt Rictor's interaction with SIN1, which abolishes the assembly of TORC2 [100]. Overall, TOR's assembly with these two components differentiate TORC2 from TORC1 and suggests that these two TOR complexes have unique functions.

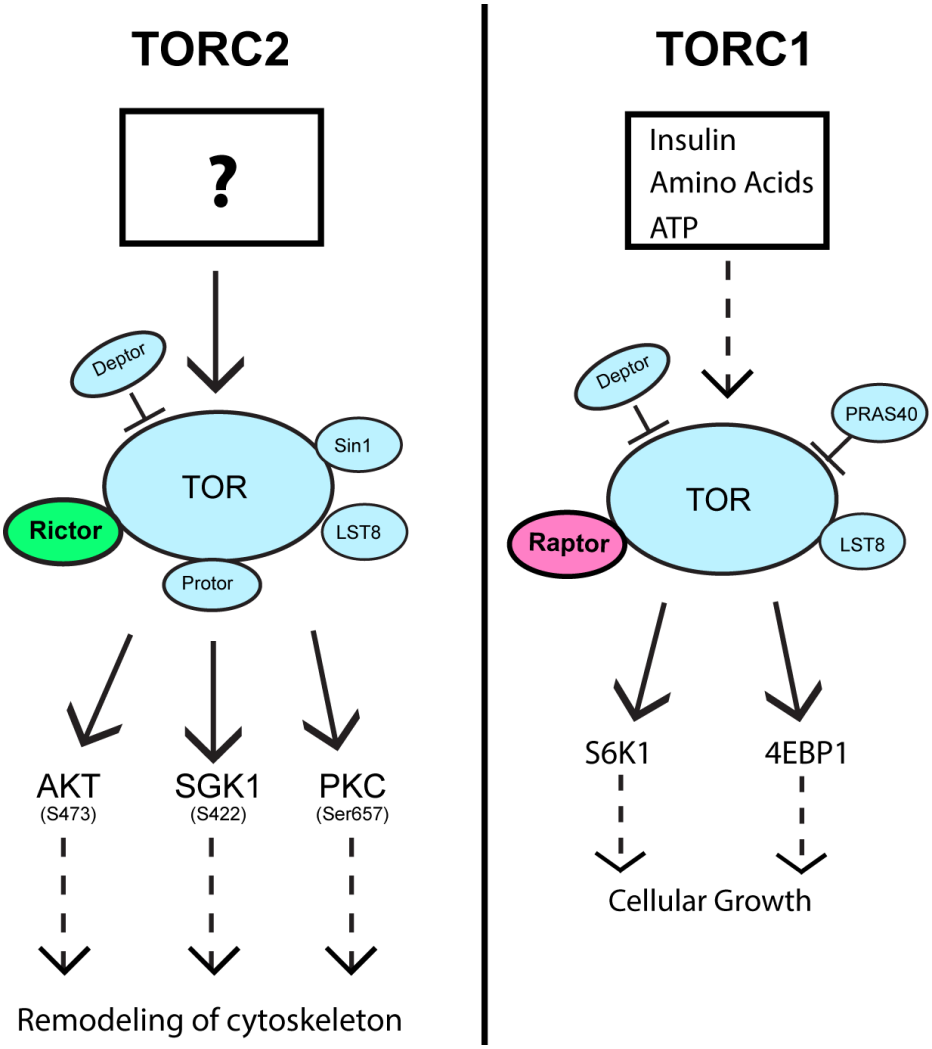

Figure 2: TORC2 vs. TORC1. While both complexes contain TOR, Deptor, and LST8, they also contain distinct proteins such as Rictor and Raptor. This difference in complex assembly likely contributes to the differences in TORC1 and TORC2 upstream and downstream signaling.

The functions and downstream and upstream signaling pathways of these two complexes are different. mTORC1 is localized to the lysosome and endoplasmic reticulum [101] and is regulated by insulin, amino acids, and adenosine triphosphate (ATP) to promote cellular growth, proliferation, and cell cycle progression [95, 101, 102]. mTORC1's known targets include S6K1 and 4E-BP, in which activation ultimately regulates the translation and synthesis of new proteins and lipids [102]. Because of the various stimuli that can induce mTORC1 activity, the pathway has several feedback mechanisms that control its function [103]. Dysregulation of mTORC1 signaling has been implicated in several disease states such as diabetes, neurodegeneration, and most notably, cancer [94]. Sharing the same core TOR kinase, TORC2 has been investigated both in the yeast line, *S. cerevisiae,* and also in mammalian cells. Similar to TORC1, TORC2 is localized to the endoplasmic reticulum [104] and when active, is associated with the ribosome [105]. On the other hand, yeast TORC2 is localized to the plasma membrane [106]. Interestingly, in endothelial cells, TORC2 has been shown to also localize in membrane rafts through its association with syndecan-4, a transmembrane protein that acts as a scaffold for signaling complexes. Importantly, knockout of syndecan-4 significantly decreased endothelial cell size, linking TORC2 to control of cell size [107]. Downstream, TORC2 phosphorylates the AGC family of kinases, which includes AKT (at Ser473), PKC (at Ser657), and SGK1 (at Ser422). Functionally, TORC2 signaling has been identified as a regulator of actin cytoskeleton reorganization and cell proliferation [102]. Unlike TORC1, not much is known about what regulates TORC2 activity. There is some evidence to suggest that Rac1, a Rho family GTPase, controls the activity of TORC2, as RNA interference in Hela cells abolishes phosphorylation at AKT S473 [108].

While there are studies that investigate TORC2 in yeast or cell culture, few have elucidated the role of TORC2 *in vivo*, due in part to the lack of the availability of a pharmacological inhibitor for TORC2. Fortunately, because Rictor is critical for TORC2 function, knockout of Rictor provides a strategy for investigating TORC2 function. Despite the fact that deletion of *rictor* in mice is embryonic lethal [109], Cre-lox technology can be used to investigate TORC2 signaling by allowing deletion of *rictor* in specific cell types. In addition, because no pharmacological inhibitors are available to inhibit TORC2, genetic manipulation of Rictor can be used to specifically investigate TORC2 signaling while leaving TORC1 intact [74, 110-114]. Dysfunction of TORC2 signaling has been implicated in schizophrenia [111] and opiate reward [74] due to evidence of dysregulation of AKT signaling, specifically altered phosphorylation at S473 [111]. Because TORC2 is known to regulate phosphorylation at that site, Rictor was deleted from forebrain neurons using Cre-lox technology, to investigate schizophrenia-like behaviors in mice. Deletion of Rictor led to a decrease in pAKT at S473 with no change in total AKT expression. This loss in pAKT signaling led to sensory motor dysfunction measured by prepusle inhibition, cortical hypodopaminergia, and an increase in norepinephrine transporter expression and function [111]. Similarly in opiate reward, manipulation of TORC2 signaling in the VTA, which controls the phosphorylation of pAKT at S473, increases and decreases preferences for morphine through overexpression and knockout of Rictor, respectively [74]. More recent work has explored TORC2 signaling in mediating reward in catecholaminergic neurons. Because food is considered to be a natural reward, feeding behavior was investigated in mice deficient of Rictor in catecholaminergic cells through the use of TH-Cre. Male Rictor KO mice exhibit a significant increase in mass, driven by an increase in lean mass, with no differences in standard chow intake or feed efficiency compared to controls.

Interestingly, an increase was seen in the consumption of high-fat diet (HFD) in Rictor KO mice in the first six days of consumption with no change in feed efficiency, or change in normal chow consumption. DA neurotransmission is also altered in Rictor KO mice, as they have a significant increase in novelty-induced locomotion and amphetamine-induced locomotion, while having a decrease in DA content in the nucleus accumbens [112]. Overall, while multiple studies have elucidated downstream mechanisms and functions for TORC2 in yeast and mammalian cells, much less has been determined in the brain. Thus, further investigation is needed to identify the role/s of brain TORC2 signaling.

Actin cytoskeleton remodeling and Rho GTPase signaling

Primary studies in yeast established a role for TORC2 in regulating the actin cytoskeleton [96]. The cytoskeleton of a mammalian cell consists of microtubules, intermediate filaments, and actin [115] to help maintain cellular shape and function. Microtubules form networks throughout the cell to help regulate intracellular trafficking [115]. Intermediate filaments provide support to cells under mechanical stress and work by interacting with microtubules and actin filaments [115]. Actin, a 43 kDa protein [116] that exists as G-actin monomer, assembles to form F-actin. F-actin is a polymer of G-actin monomers that assemble as a helical strand to provide structure for the cell [117] and is constantly turned over in a process called treadmilling. In this process, actin-ADP (adenosine diphosphate) monomers disassemble at the minus end while other actin-ATP monomers assemble at the plus end [118]. A number of proteins called actin-binding proteins (ABP) assist in the actin turnover and assembly to control the cytoskeletal remodeling process [117]. Profilin promotes actin polymerization by binding to and attaching actin monomers to actin filaments [119]. Cofilin, on the other hand, promotes actin depolymerization by binding to ADP-bound F-actin to destabilize actin filaments for severing [118]. These

proteins are regulated through multiple upstream mediators and complex signaling pathways to maintain the actin cytoskeleton. Accordingly, it is important to gain understanding of the actin cytoskeleton itself and identify the dynamic changes that occur when homeostasis is disrupted.

The actin cytoskeleton consists of a dynamic set of proteins that are constantly turning over and remodeling in order for the cell to maintain its shape and also respond to environmental cues though alteration in cell motility, division, and trafficking [117]. More importantly, the actin cytoskeleton is also critical for morphological changes in neurons, such as in alterations in spine density [120]. These changes in the actin cytoskeleton are mediated, in part, by the Rho family of GTPases. There are approximately 20 members of the Rho GTPase family, in which activation of these GTPases help cells respond to external stimuli [121]. For instance, in neurons, dendritic spine remodeling involves reorganization of the actin cytoskeleton in response to drugs [58, 122, 123] or a physiological stimulus [124]. Many studies have demonstrated a role for the Rho family of GTPases, such as Rac1, Cdc42, and Rho A, in spine remodeling [120]. Rac1 and Cdc42 function similarly by increasing spine density, while Rho A acts oppositely to decrease spine density.

Knowing how these Rho GTPases work is critical for understanding how the cytoskeleton functions. These GTPases begin in an active state, bound to GTP. Once the tertiary phosphate is hydrolyzed on GTP, the GTPase returns to an inactive, GDP-bound state. GTPase activating proteins (GAPs) can bind to active GTPases and increase the rate at which GTP is hydrolyzed, thus terminating the activity of the GTPase. Conversely, guanine exchange factors (GEFs) increase the activation of GTPases by increasing the rate by which GDP is released from GTPases to allow GTP to bind [120]. Rac1 initiates a kinase cascade by activating p21-activated kinase (PAK) to promote phosphorylation of LIM kinase (LIMK). LIMK then phosphorylates

proteins in the ADP/cofilin family, such as cofilin, specifically at S3 [121] (Figure 3). Phosphorylation of cofilin and other members of its family inhibit the actin-severing function of these proteins [117, 121]. Thus, through this cascade, Rac1 activation ultimately leads to changes in the actin cytoskeleton. Importantly, signaling through Rac1 is not linear in the sense that a lot of cross-talk occurs with the proteins in the Rac1-PAK-LIMK-Cofilin pathway. Testis-specific kinase (TESK) can also control cofilin phosphorylation, but the mechanism by which TESK is regulated remains to be elucidated [121]. Rho-associated protein kinase (ROCK) can also phosphorylate LIMK to promote Cofilin inactivation [121].

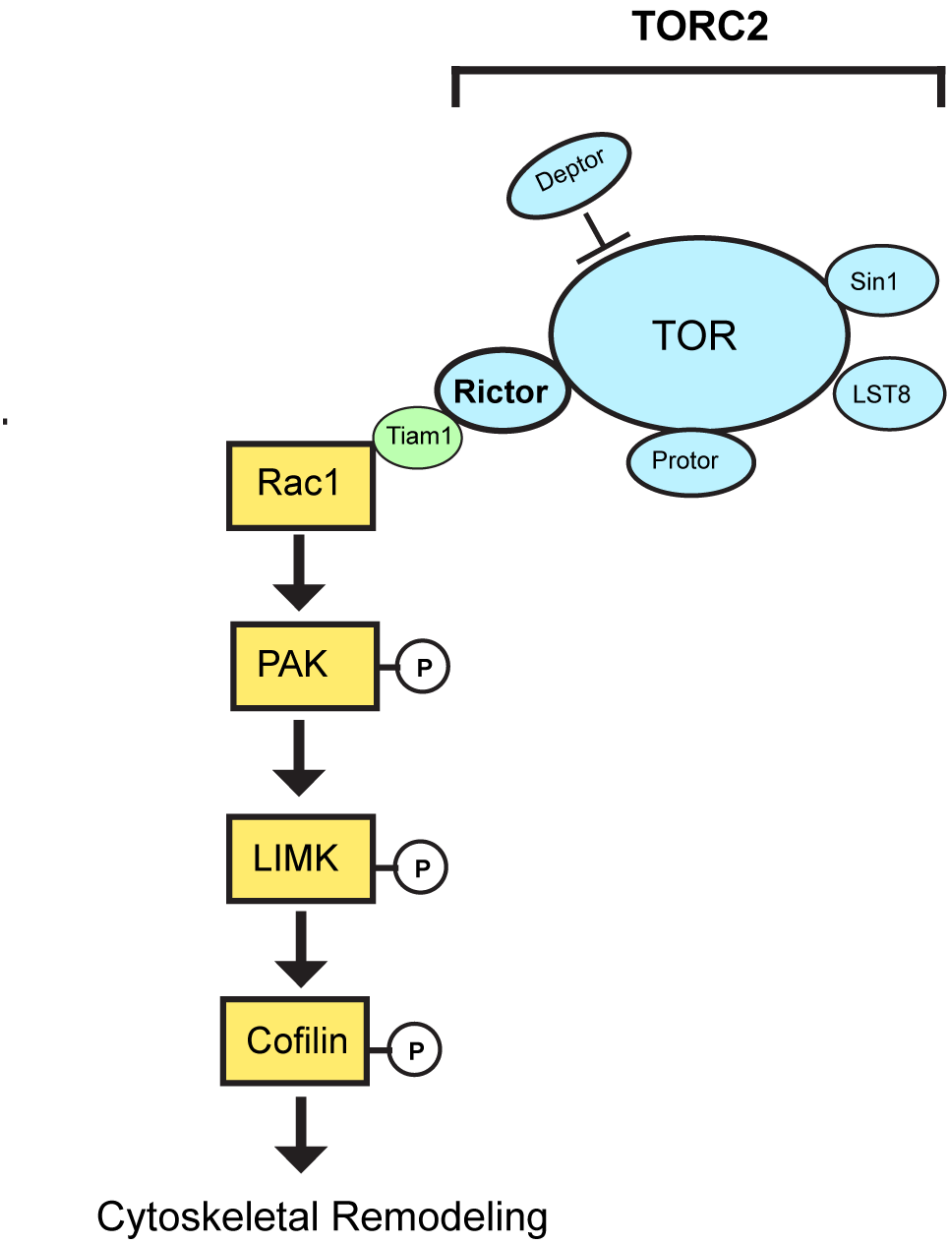

Figure 3: Proposed mechanism by which TORC2 alters the actin cytoskeleton. Rictor interacts with Tiam1 which activates Rac1. Upon activation, Rac1 initiates a cascade of phosphorylation reactions to ultimately promote cytoskeletal remodeling.

Manipulation of Rac1 in the brain has been implicated in drug reward and susceptibility to stress. In mice, repeated cocaine administration reduced Rac1 activity in the nucleus accumbens (NAc), and also decreased phosphorylated Cofilin and Tiam1 ((T-cell lymphoma invasion and metastasis-1, a guanine exchange factor of Rac1) expression [125]. Furthermore, overexpression of dominant-negative Rac1 in the NAc significantly increased total spine density and increased cocaine CPP at a relatively low dose [125], demonstrating the importance of cytoskeletal remodeling in drug reward. Similarly, CSDS significantly decreased Rac1 mRNA and protein in the NAc of susceptible, but not resilient, mice [126]. Susceptible mice also have a significant increase in stubby spines compared to their non-stressed controls, and this increase in spines can be rescued with the overexpression of a constitutively active Rac1 in the NAc [126], demonstrating the important role of Rac1 in cytoskeletal remodeling.

More importantly, Rac1 activity has been directly tied to TORC2 in the brain in regions such as the hippocampus. TORC2 signaling has been implicated in long-term memory consolidation in the hippocampus. Deletion of Rictor was driven by the expression of camk2a-Cre in floxed Rictor mice. Rictor KO mice displayed impaired long-term memory (LTM) as measured by contextual fear conditioning, and also displayed impaired spatial LTM as measured by the Morris water maze. As actin cytoskeleton dynamics are altered in long-term memory formation in the hippocampus [114], the role of TORC2 signaling in mediating cytoskeletal changes was investigated. Rac1 and Cdc42, two members of the Rho-GTPase family, were investigated. A significant decrease in Rac1 activity but not Cdc42 activity was observed. Consistent with decreased Rac1 activity, significant decreases in pPAK and pCofilin in the dorsal hippocampus were observed in Rictor KO mice. A decrease in the number of dendritic spines of CA1 pyramidal neurons in Rictor KO mice was also observed, linking TORC2-

dependent decreases in Rac1 signaling to cytoskeletal remodeling. Co-immunoprecipitation studies found that the GEF Tiam1 directly interacts with Rictor, defining the mechanism of TORC2 control of Rac1 signaling in hippocampus. Rictor KO has also been found to alter cellular size. Ablation of Rictor by nestin-Cre, which deletes neuronal Rictor throughout the brain, significantly decreased brain weight compared to littermate controls [85]. Examination of neurons in the hippocampus of these Rictor KO mice revealed a significant decrease in neuron soma size compared to their controls [85].

However, deletion of Rictor can also alter the cytoskeleton through mechanisms that do not utilize GTPases. For instance, in cerebellar Purkinje cells, not only did Rictor deletion significantly decrease soma size, but it also decreased the total dendritic length. These morphological changes were linked with functional changes, as frequency of both miniature excitatory and inhibitory postsynaptic currents of the Purkinje cells were decreased [85]. Biochemical analysis demonstrated that deletion of Rictor in the cerebellum decreases the phosphorylation of GAP-42 and pMARCKS, two proteins downstream of PKCα that are involved in actin turnover [85, 127]. This suggests that TORC2 can alter the actin cytoskeleton through multiple mechanisms and that this may be specific to different brain regions.

Conclusion

Depression and addiction are devastating comorbid disorders that result, in part, through altered signaling of the brain reward circuit. While current therapies help many patients, they are inadequate for others, and improved treatment is dependent on a better understanding of the underlying molecular mechanisms that contribute to altered circuit activity. In particular, our understanding of the mechanisms that contribute to changes in the VTA in response to chronic

stress and opiates is limited. Excitingly, evidence suggests that chronic stress and opiates induce similar changes in the VTA, suggesting there might be common molecular mechanisms underlying these disorders. For instance, the decrease in phosphorylation of AKT at S473 is observed in both chronic morphine treatment [74, 77] and CSDS [47]. More importantly, modulation of AKT signaling directly corresponds to changes in both morphine reward and susceptibility to stress [47, 77]. Upstream regulation of AKT at S473 is mediated by mTORC2 [102] and we know that modulating mTORC2 through the expression of Rictor, a critical component of TORC2, modulates morphine reward and VTA DA neuron soma size [74]. A direct connection between TORC2 and susceptibility to chronic social defeat stress has not been established, nor has the field investigated the potential role of TORC2 in stress-induced drug reward. Thus, for Aims 1 and 2, *we hypothesize that susceptibility to chronic social defeat stress and stress-induced changes in morphine reward are mediated by TORC2*. Given that VTA DA neuron soma size correlates with changes in VTA DA neuronal activity and morphine reward, and is observed across multiple species, including mice [74], rats [84], and humans [74], understanding the mechanisms of these neuroadaptation is essential for identifying novel targets for therapeutic intervention. Furthermore, neuronal size and morphology is tied to changes in the actin cytoskeleton and TORC2 has been demonstrated to be involved in mediating the actin cytoskeleton in yeast [98] and mammalian cells [99], both in the brain and in the periphery. Moreover, recent data find that TORC2-dependent changes in Rac1 signaling are sufficient to alter hippocampal neuronal morphology [114]. Thus, we hypothesize in Aim 3 that, *TORC2 regulates Rac1 signaling in the VTA and that morphine-induced decreases in TORC2 signaling leads to decreased Rac1 signaling*. Overall, this dissertation seeks to answer the central hypothesis that alteration of TORC2 signaling in the VTA contributes to changes in stress-

induced morphine reward and CSDS susceptibility through modulation of specific downstream signaling molecules such as Rac1.

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CHAPTER 2

Deletion of Rictor in Catecholaminergic Neurons Alters Locomotor Activity and Ingestive Behavior

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Neuropharmacology, 2017, 117 (158-170)

Abstract

While the etiology of depression is not fully understood, increasing evidence from animal models suggests a role for the ventral tegmental area (VTA) in pathogenesis. In this paper, we investigate the potential role of VTA mechanistic target of rapamycin 2 (TORC2) signaling in mediating susceptibility to chronic social defeat stress (CSDS), a well-established mouse model of depression. Utilizing genetic and viral knockout of Rictor (rapamycin-insensitive companion of target of rapamycin), a requisite component of TORC2, we demonstrate that decreasing Rictor-dependent TORC2 signaling in catecholaminergic neurons, or within the VTA specifically, does not alter susceptibility to CSDS. Opiate abuse and mood disorders are often comorbid, and previous data demonstrate a role for VTA TORC2 in mediating opiate reward. Thus, we also investigated its potential role in mediating changes in opiate reward following CSDS. Catecholaminergic deletion of Rictor increases water, sucrose, and morphine intake but not preference in a two-bottle choice assay in stress-naïve mice, and these effects are maintained after stress. VTA-specific knockout of Rictor increases water and sucrose intake after physical CSDS, but does not alter consummatory behavior in the absence of stress. These findings suggest a novel role for TORC2 in mediating stress-induced changes in consummatory behaviors that may contribute to some aspects of mood disorders.

Introduction

Depression is a serious mental illness that induces a significant societal burden as a leading cause of disability [1] and is highly co-morbid with other disorders such as drug addiction [2, 3]. While the exact causes of depression remain elusive, a combination of factors, including stressful life events, are known to increase the likelihood of developing a major mood disorder [4]. Increasing evidence suggests a role for the dopamine reward circuit, and specifically activity of ventral tegmental area (VTA) dopamine neurons, in mediating susceptibility to chronic social defeat stress (CSDS), a rodent model of depression [5]. Moreover, CSDS also induces biochemical changes in the VTA, including decreased phosphorylation of AKT at Ser473 (pAKT), and preventing or mimicking this biochemical event is sufficient to rescue or induce CSDS susceptibility, suggesting changes in VTA AKT activity are behaviorally relevant [6]. Interestingly, VTA pAKT is also decreased in rats and mice treated chronically with morphine, and modulation of VTA AKT activity is sufficient to alter opiate reward, as measured by conditioned place preference (CPP) [7]. Together, these data suggest that alteration of VTA AKT phosphorylation plays a critical role in both mood disorders and drug reward.

AKT is phosphorylated at Ser473 by the mechanistic target of rapamycin complex 2 (TORC2) [8], and we have recently shown that altering TORC2 activity in the VTA is sufficient to induce changes in morphine reward [9]. Given the lack of a selective pharmacological inhibitor, we altered VTA TORC2 signaling via genetic deletion and viral-mediated overexpression of rapamycin-insensitive companion of TOR (Rictor), as this protein is a necessary component for TORC2 kinase activity. Global deletion of TORC2 is embryonically lethal [10], and thus floxed-Rictor mice have been developed and used in combination with Cre driver lines [11-14] or stereotaxic infusion of AAV-Cre in adult mice [9] to produce cell-type or

brain-region specific Rictor KO mice to allow examination of the role of TORC2 signaling *in vivo*. Recently, floxed-Rictor mice have been crossed to the tyrosine hydroxylase (TH)-Cre reporter line to KO TORC2 signaling specifically from catecholaminergic neurons (TH-Rictor) [13]. TH-Rictor KO mice display an increase in novelty-induced locomotion compared to their wild-type controls, as well as an increase in lean and overall body mass, but with no reported difference in fat mass [13].

Given our data that VTA KO of Rictor was sufficient to modulate morphine reward, we sought to determine whether KO of Rictor in the VTA or in TH neurons would increase susceptibility to CSDS, as predicted by pAKT results. Further, we sought to determine whether VTA- or TH-Rictor KO mice would have altered morphine reward following CSDS. While data on morphine reward following CSDS is limited [15, 16], susceptible mice have increased cocaine CPP following CSDS [5] and social defeat stress also increases cocaine self-administration in both rats [15] and mice [17]. Thus, we assessed voluntary intake and preference for morphine using a two-bottle choice test. We found that while decreasing TORC2 signaling in either TH neurons or in the VTA does not increase susceptibility to CSDS, there were changes in consummatory behavior between Rictor KO mice and controls. Interestingly, whereas there were differences between TH- and VTA-Rictor KO prior to CSDS, all Rictor KO mice exhibited a similar phenotype post-CSDS. These data suggest that additional TORC2 substrates may exhibit competing effects to those of AKT in stress susceptibility and that behavioral outputs of TORC2 signaling may be dependent on subsets of catecholaminergic neurons.

Materials and Methods

Mice

All mice were housed at 22-25° C on a 12 hour light/dark cycle with food and water available *ad libitum*. Experiments utilized adult male and female mice (8-15 weeks). Homozygous floxed Rictor mice were generated as previously described [9-11], and were also crossed with heterozygous tyrosine hydroxylase (TH)-Cre mice (Jackson Laboratories, 008601) to generate developmental Rictor knock-out (KO) mice (Dadalko et al., 2015a); all mice were fully backcrossed to the c57Bl/6 background. Mouse genotypes were verified at 21-28 days using standard procedures. Published primers to assess floxed-Rictor (5'- CCT GAG CAG TGC CCG ACT TCT CTA G-3' and 5'- CCT TTC GCA TCG CCA CTG CA-3') and TH-Cre (5'- GAT CTC CGG TAT TGA AAC TCC AGC-3' and 5'- GCT AAA CAT GCT TCA TCG TCG G-3') status were used. Of note, Cre-mediated deletion of Rictor using this floxed-Rictor line (via either AAV-Cre infusion or cross with Cre-driver line) has been shown to be sufficient to disrupt TORC2-mediated kinase activity as assessed by phosphorylation of AKT at Ser473 (Dadalko et al., 2015b; Mazei-Robison et al., 2011; Siuta et al., 2010). For social defeat stress studies, retired CD-1 male breeders (Charles River) were purchased and screened for aggressive behavior as described previously [18]. All experiments were approved by the Institutional Animal Care and Use Committee at Michigan State University.

Drugs

Morphine sulfate (generously provided by the NIDA Drug Supply Program) and quinine sulfate (Sigma) were dissolved in water for drinking studies.

Viral-mediated gene transfer

Stereotaxic surgeries were completed as previously described [9]. Briefly, mice were anesthetized (100 mg/kg ketamine, 10 mg/kg xylazine) and bilateral infusions (0.5 μl) of AAV-GFP or AAV-Cre-GFP (UNC Vector Core) were targeted to the VTA (from bregma: -3.2 mm AP, +1.0 mm ML, and -4.6 mm DV, 7° angle). Mice were allowed to recover for ≥14 days prior to behavioral testing to allow for Cre-mediated gene silencing and the degradation of all remaining Rictor in target cells.

Validation of Rictor deletion

Viral targeting: Following experimental testing, mice were perfused with 4% paraformaldehyde-PBS and brains were cryo-preserved in 30% sucrose-PBS. Brains were sectioned (30 μm) and bilateral VTA targeting was confirmed by GFP expression. The representative VTA targeting and viral expression shown in Fig 4A was generated using standard immunohistochemistry techniques to label GFP- (Life Technologies A11122, 1:3000) and TH-positive (Sigma, T1299, 1:3000) cells in the VTA [9]. Mice with GFP expression outside the VTA were not included in analyses.

Quantitative Real-Time (RT)-PCR: Rictor deletion was verified by RT-PCR using published procedures [9]. Briefly, VTA was microdissected from mice and stored at -80° C until processing. RNA was isolated and purified from VTA using Trizol and RNeasy microcolumns (Qiagen). Following reverse-transcription (Applied Biosystems), RNA levels were quantified by RT-PCR using the ΔΔCt method and GAPDH as a normalization control, and all analyses were performed in triplicate. All primers were previously validated: Rictor: 5'-ATG GCG GCG ATC GGC CGC G-3' and 5'-GAT ACT CCT TGC AAT TTG GCC ACA-3'; GAPDH: 5'- AGG TCG GTG TGA ACG GAT TTG-3' and 5'- TGT AGA CCA TGT AGT TGA GGT CA-3'; Cre:

5’- CCC GGC AAA ACA GGT AGT TA-3’ and 5’- GAA CGA AAA CGC TGG TTA GC-3’ [9, 19].

Chronic social defeat stress (CSDS)

CSDS was performed as previously described [18]. Briefly, male control and Rictor KO mice were subjected to a brief daily physical encounter in the home-cage of an aggressive CD-1 retired breeder followed by sensory contact for the following 24 hours via a perforated plexiglass partition. Non-stress controls were handled and housed across from a novel c57Bl6 mouse daily. Following the 10^{th} defeat episode, mice were singly housed. A variant of CSDS that utilizes a purely psychological stressor, witness or emotional stress, was also performed as previously described [20]. Emotional CSDS was performed as described above, with the exception that a second experimental mouse was placed on the opposite side of the plexiglass partition during the physical encounter allowing this mouse to witness physical social defeat stress.

Behavioral overview

Behavioral characterization was completed ≥14 days following surgery to allow time for Cre-mediated gene silencing. Mice were 8-14 weeks of age and were weighed before, during, and following behavioral testing. All tests were completed during the light cycle under red light illumination and video-tracking software (Clever Systems Top Scan) was used for quantification. For cohorts of mice that underwent the entire battery of baseline behaviors, the order of testing was open field, elevated plus maze, then social interaction testing to minimize exposure effects; each completed on a different day.

Social interaction: Social interaction was assessed as previously described [18]. Mice were placed in a 38 cm x 38 cm arena for two-2.5 min. test sessions, with a CD-1 target mouse absent, then present in a plexiglass and mesh cylinder. Time spent in the interaction zone (7.5 cm

surrounding the cylinder) and corners was measured, along with distance traveled. The social interaction (SI) ratio was calculated as time spent in the interaction zone with target present/time spent with target absent *100. Susceptibility to CSDS was defined as an SI ratio <100 [5].

Open Field: Mice were placed in a 38cm x 38cm arena and distance traveled and time spent in the center and periphery in the 10 min. test session were assessed.

Elevated plus maze: Mice were placed in the center of a 5cm x 35cm plus maze. Distance traveled, time spent in open and closed arms, and entries into open and closed arms were assessed during the 5 min. test session.

Two-bottle choice voluntary intake: Mice were singly housed and had access to two 50 ml conical tubes with sipper tops in their home cage. Throughout the experiment, bottles were weighed at the same time each morning, bottle location was switched daily, and consumption was determined by the volume of fluid intake. Prior to sucrose or morphine preference, baseline water intake was measured for 2-3 days. For sucrose preference and consumption, mice had access to bottles containing water and 1% sucrose for 4 days. For morphine preference and consumption, morphine sulfate or quinine sulfate (taste control for morphine) were dissolved in 0.2% sucrose based on prior studies [21-23]. Bottles for male mice contained 0.06 mg/ml quinine and 0.3 mg/ml morphine and bottles for female mice contained 0.01 mg/ml quinine and 0.05 mg/ml morphine.

Statistics

All values are reported as mean +/- SEM. Graph Pad Prism was used to perform all statistical analyses. Unpaired t-tests were used to compare groups of two. One way analysis of variance (ANOVA) was used to compare groups of three, followed by a Tukey post-hoc test, when appropriate. Two way ANOVA was used to compare groups with two independent variables

(factors), followed by Tukey post-hoc test when appropriate. Differences were considered significant when $p<0.05$.

Results

TH-Rictor-KO mice do not exhibit baseline differences in anxiety- and depressive-like behavior

To determine whether developmental KO of Rictor in TH cells influences anxiety-like behavior, we first assessed performance in the elevated plus maze (EPM). Heterozygous or homozygous deletion of Rictor in male mice did not affect their time spent in the open arm compared to littermate controls (Fig 4A). Similarly, there was no significant difference in open arm time observed between female TH-Rictor-KO mice and littermate controls. We next completed open field (OF) testing and determined the time spent in the center as a second measure of anxiety-like behavior (Fig 4B). There were no significant differences in center time between male TH-Rictor KO mice and their littermate controls, nor were there differences in female mice. Together, these data suggest that deletion of Rictor in TH cells does not affect baseline anxiety-like behavior.

During OF testing, we also assessed general locomotor activity (Fig 4C). We found that the total distance traveled was significantly increased in male homozygous TH-Rictor KO mice compared to heterozygous TH-Rictor KO mice and littermate controls ($F(2,75)=5.1$, $p=0.008$, Tukey's post-hoc test, $p<0.05$), however this effect was not observed in female mice. This difference in activity may be partially explained by the fact that the total activity of female mice, regardless of genotype, was greater than that of males (Two-way ANOVA, Sex factor $F(1,81)=17.9$, $p<0.0001$). The increase in baseline locomotor activity in male homozygous Rictor-TH KO mice is consistent with published data (Dadalko et al., 2015a), however female mice were not examined in the previous study.

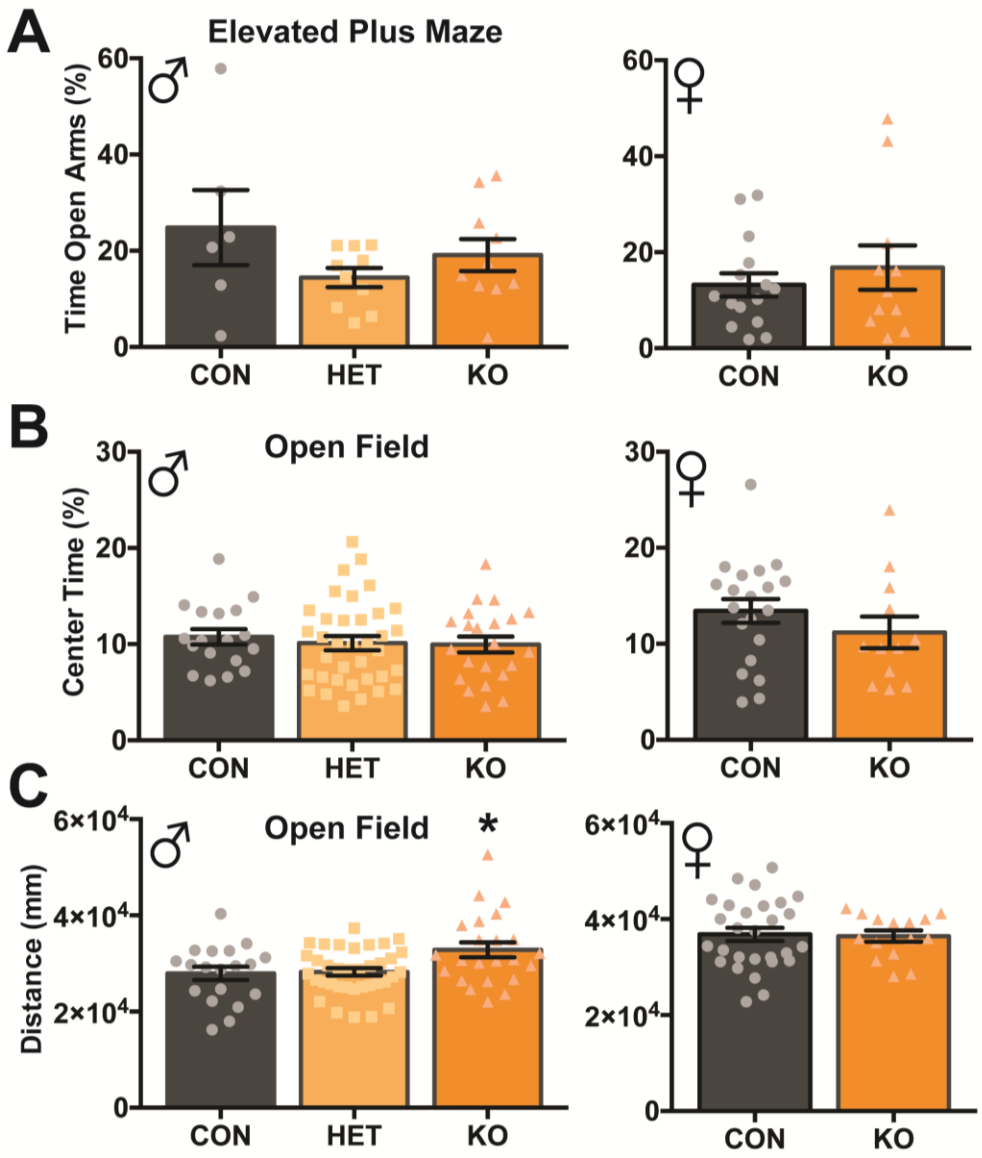

Figure 4: **Evaluation of anxiety-like baseline behaviors in stress naïve TH-Rictor-KO mice.** There were no statistical differences in anxiety-like behaviors including time spent in the open arms of the EPM test (A: male: CON=6, HET=10, KO=10; female: CON=15, KO=11) or center time in the OF test (B: male: CON=18, HET=36, KO=22; female: CON=20, KO=12) between control, heterozygous, or homozygous male (left) or female (right) TH-Rictor-KO mice. Homozygous male TH-Rictor-KO mice exhibited increased locomotor activity in the OF test compared to controls (CON=19, HET=36, KO=23), but this difference was not observed in female Rictor-KO-mice (CON=28, KO=15) (C), *$p<0.05$, n=6-36 mice/group, individual data points shown.

Given that modulation of VTA AKT activity, a TORC2/Rictor substrate, alters social interaction (SI) following CSDS, we next sought to determine whether TH-Rictor KO mice had any baseline differences in social interaction (Fig 5A). We found that SI scores did not differ between either male or female TH-Rictor KO mice and their control littermates. Additionally, we found no differences in locomotor activity during SI testing (males: Con: 6729 +/- 735, Het: 5842 +/- 352, Homo: 7397 +/- 731, $p>0.05$ and females Con: 9640 +/- 519, KO: 10,559 +/- 427, $p>0.05$), suggesting that the hyperlocomotion of male TH-Rictor mice may be abrogated in the presence of environmental stimuli, in this case an empty wire mesh enclosure.

As another indicator of depressive-like behavior, we examined sucrose preference using a two-bottle choice test (Fig 5B). Neither male nor female TH-Rictor mice exhibited significant differences in preference for a 1% sucrose solution compared to control mice, suggesting TH-Rictor KO does not induce anhedonia in the absence of stress. However, while there were no significant differences in sucrose preference, we found that TH-Rictor KO mice drank a greater volume of the sucrose solution (Fig 5C). This effect was significant for homozygous male TH-Rictor KO mice compared to heterozygous and wild-type littermates ($F(2,31)=5.96$, $p<0.01$, Tukey's post-hoc test, $p<0.05$) and a similar non-significant trend was observed in female TH-Rictor KO mice ($t(19)=1.8$, $p=0.08$). This drove a significant increase in the total volume of liquid consumed (sucrose + water) by TH-Rictor KO mice (Con: 7.27 +/- 0.23, Het: 7.83 +/- 0.19, Homo: 9.50 +/- 0.59; $F(2,31)=8.94$, $p<0.001$, Tukey's post-hoc test, $p<0.01$), and it appears that sucrose preference was not altered because water intake was also significantly increased (Con: 1.08 +/- 0.09, Het: 1.60 +/- 0.15, Homo: 1.59 +/- 0.10; $F(2,31)=4.83$, $p<0.05$, Tukey's post-hoc test, $p<0.05$ from Controls, Het not significantly different from Homo). Together, these data suggest that while TH-Rictor KO mice do not exhibit baseline depressive-like behavior,

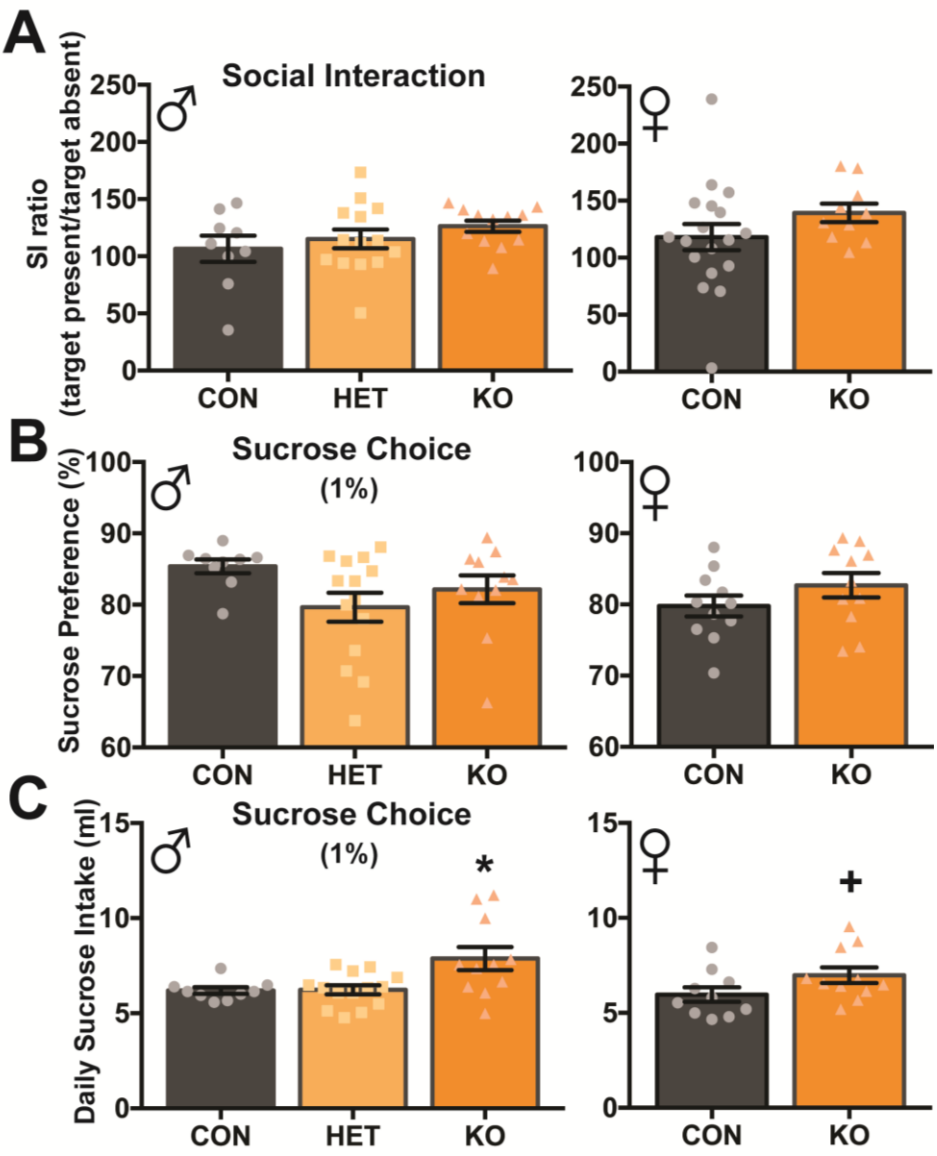

Figure 5: Evaluation of depressive-related behaviors in stress naïve TH-Rictor-KO mice. There were no statistical differences in social interaction (A: male: CON=9, HET=14, KO=12; female: CON=18, KO=10) or sucrose preference (B: male: CON=9, HET=14, KO=11; female: CON=11, KO=12) between control, heterozygous, or homozygous male (left) or female (right) TH-Rictor-KO mice. Homozygous male TH-Rictor-KO mice drank significantly more sucrose solution than heterozygous TH-Rictor-KO mice and controls (CON=9, HET=14, KO=11), with a similar non-significant trend observed in female TH-Rictor-KO mice (CON=10, KO=11) (C), *p<0.05, +p=0.08, n=9-18 mice/group, individual data points shown.

they do display sex-specific differences in general locomotor behavior and voluntary fluid intake.

TH-Rictor-KO mice have higher levels of voluntary fluid intake

To further explore this difference in voluntary fluid intake, we examined water intake in a two-bottle choice test where both bottles contain water. Consistent with our sucrose data, we found that male homozygous TH-Rictor KO mice drank significantly more water than heterozygous and wild-type littermates (Fig 6A, $F(2,40)=4.725$, $p=0.0144$, Tukey's post-hoc test, $p<0.05$). Interestingly, when we examined female TH-Rictor KO, we did not observe any differences in water intake from controls (Fig. 6A, $p>0.05$), in contrast to increased water intake in male TH-Rictor-KO mice, and the trend for increased sucrose intake in female TH-Rictor KO mice.

Given that Rictor KO in the VTA via AAV-Cre infusion is sufficient to decrease morphine reward as measured by CPP [9], we next decided to measure voluntary morphine consumption and preference using the two-bottle choice test in TH-Rictor KO mice. Similar to the sucrose preference results, we found that both male and female TH-Rictor KO mice exhibit similar morphine preference to littermate controls (Fig 6B). However, when we examined morphine intake in these mice, we found that both male and female TH-Rictor-KO mice had significantly elevated intake compared to controls (Figure 6C, Male: $F(2,40)=5.31$, $p=0.009$; Female: $t(19)=2.74$, $p=0.013$, Tukey's post-hoc test, $p<0.05$). Quinine solution intake was not significantly different between TH-Rictor KO and control mice (Male: Con: 0.89 +/- 0.10, Het: 0.95 +/- 0.10, Homo: 0.98 +/- 0.10; Female: Con: 1.69 +/- 0.24, KO: 1.91 +/- 0.18), which likely contributed to the lack of an overall effect on morphine preference. Together, these data suggest

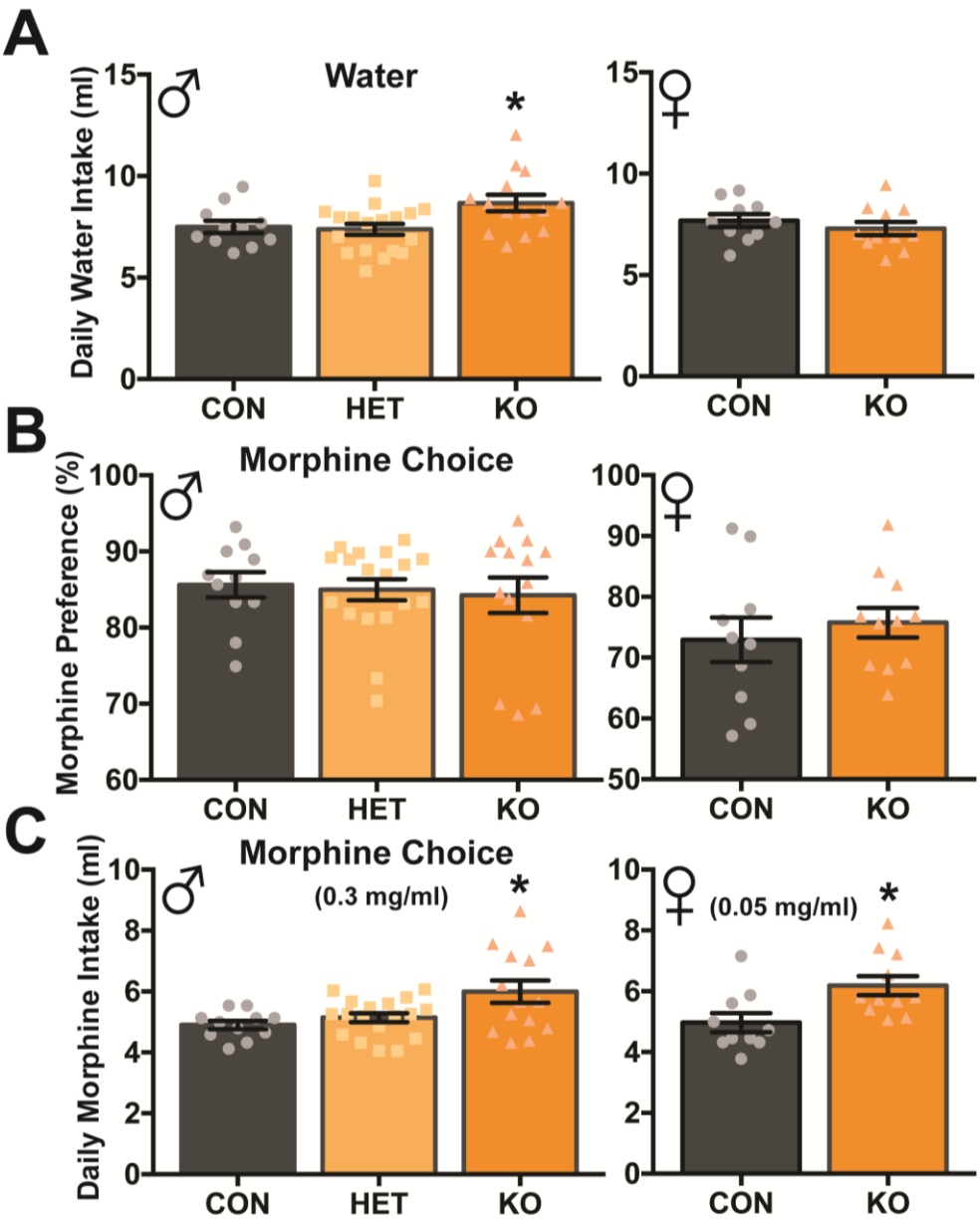

Figure 6: Evaluation of water and morphine consumption in stress naïve TH-Cre mice. Male TH-Rictor-KO mice drank more water (A: CON=11, HET=18, KO=14) and morphine solution (C: CON=11, HET=18, KO=14) compared to controls, with no difference in morphine preference (B: CON=11, HET=18, KO=14). Female TH-Rictor-KO mice did not drink more water than controls (A: CON=10, KO=11) but did drink more morphine solution (C: CON=10, KO=11), with no difference morphine preference (B; CON=10, KO=11), *p<0.05, n=10-18 mice/group, individual data points shown.

that TH-Rictor-KO mice have increased voluntary fluid intake, and that this effect is more pronounced in male mice, which exhibit significantly increased intake of water, sucrose, and morphine solutions.

VTA-Rictor-KO mice do not exhibit any differences in baseline behaviors

In order to determine whether the differences we observed in locomotor activity and fluid consumption in TH-Rictor-KO mice were consistent with changes in TORC2 signaling in the VTA, we generated VTA-specific KO mice (VTA-Rictor-KO) via AAV-Cre infusion into the VTA of floxed-Rictor mice. These mice have significantly decreased Rictor mRNA expression in VTA compared to AAV-GFP infused controls (Fig 7A, $t(9)=3.65$, $p=0.005$), however this effect is not genetically limited to TH-positive cells in the VTA, and Rictor KO may thus occur in any transduced VTA cells, including GABAergic, dopaminergic, or others.

We first examined VTA-Rictor-KO mice using the same measures of anxiety- and depressive-like behaviors used for TH-Rictor-KO mice. Similar to TH-Rictor-KO mice, we found no significant difference between VTA-Rictor-KO mice and their GFP controls during EPM testing (Fig 7B). Center time in the OF testing was also similar between VTA-Rictor-KO mice and controls (Fig 7C), suggesting that Rictor KO in VTA does not alter baseline anxiety. In contrast to male TH-Rictor-KO mice, we found that male VTA-Rictor-KO mice did not display increased locomotor activity compared to controls (Fig 7D). Similarly, there were no differences in locomotor activity between female VTA-Rictor-KO mice and their GFP controls (Fig 7D). These data suggest that changes in locomotor activity in male TH-Rictor-KO mice may be driven by brain regions other than VTA, such as substantia nigra, whose dopaminergic cells project to and modulate motor-oriented dorsal striatum neurons.

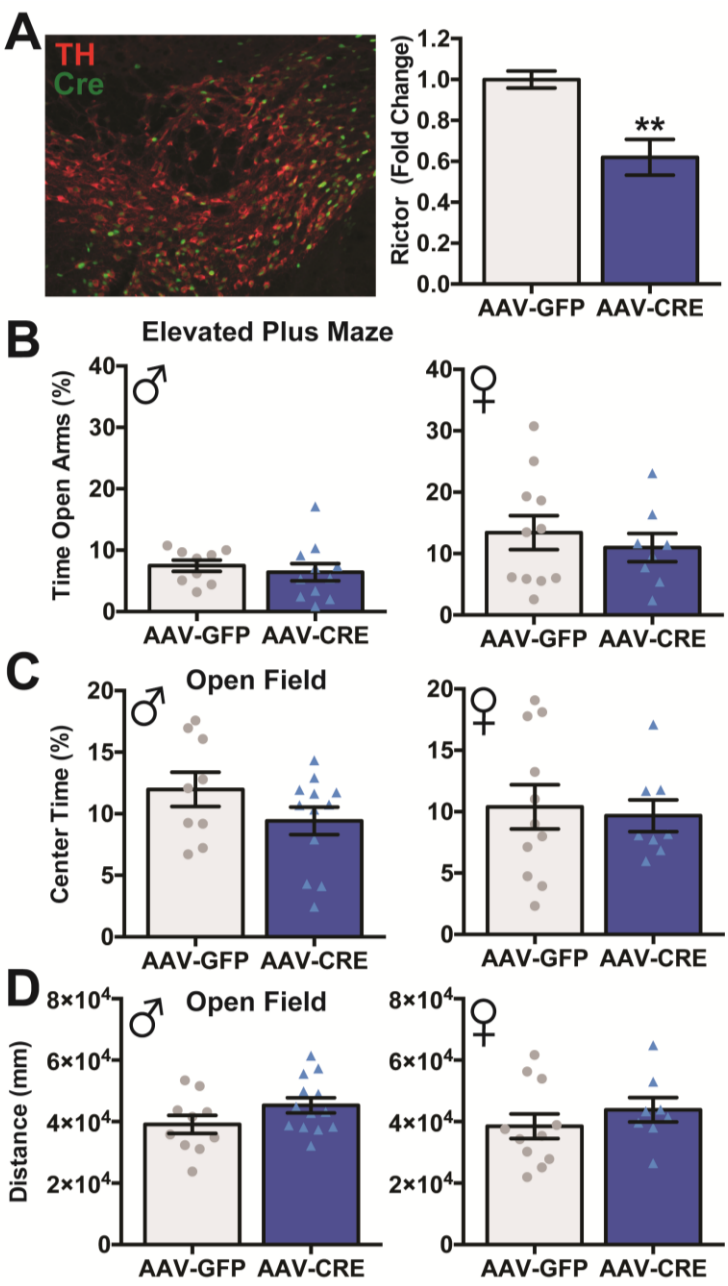

Figure 7: Evaluation of baseline behaviors in stress-naïve AAV-Rictor-KO mice. AAV-Cre significantly decreased VTA Rictor expression (A). Knockdown of Rictor using AAV-Cre did not alter anxiety-like behavior in EPM (B: male: GFP=9, CRE=11; female: GFP=11, CRE=8) or OF (C: male: GFP=9, CRE=12; female: GFP=11, CRE=8) testing in either male (left) or female (right) mice. Locomotor activity in the OF test was also similar between AAV-Rictor-KO mice and controls (D: male: GFP=10, CRE= 12; female: GFP=11, CRE=8), **p<0.01, n=8-12 mice/group, individual data points shown.

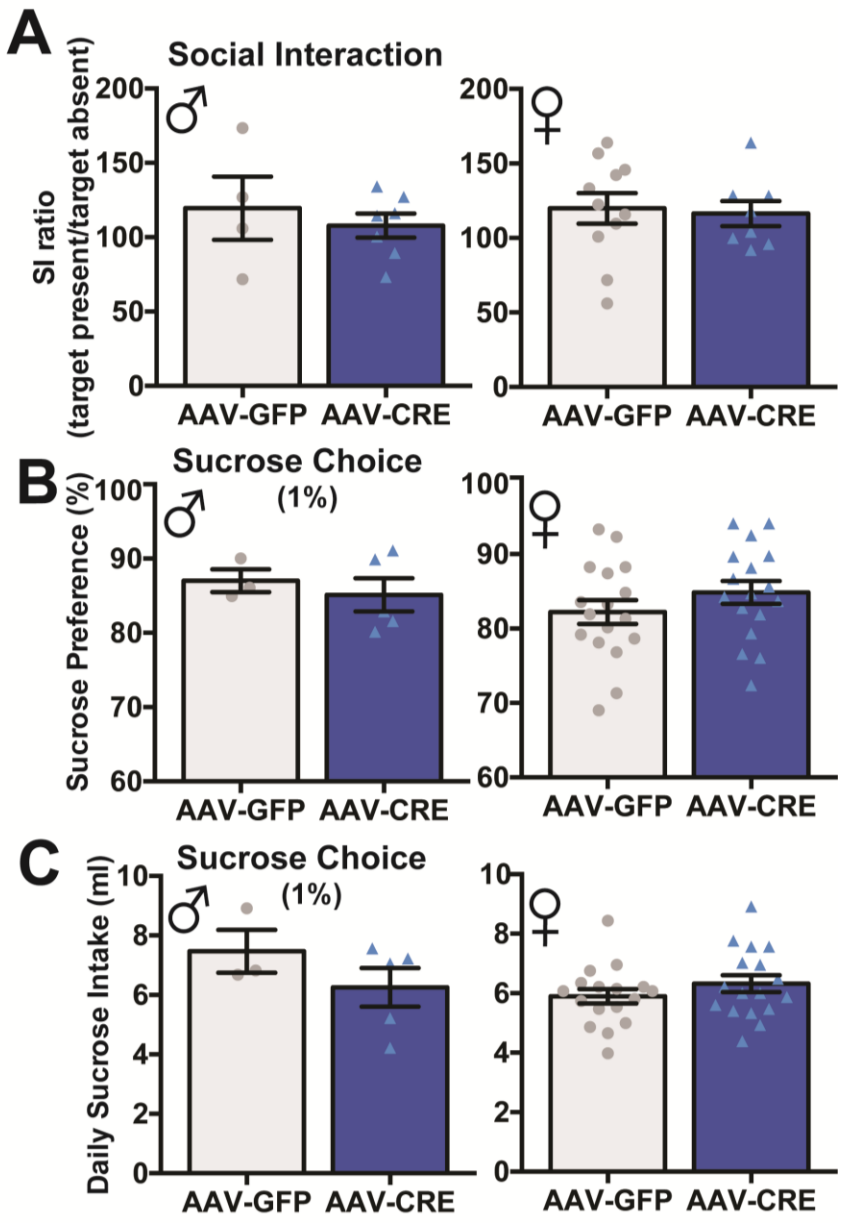

Figure 8: Evaluation of depressive-related behaviors in stress naïve AAV-Rictor-KO mice. There were no significant differences in social interaction (A: male: GFP=4, CRE=7; female: GFP=11, CRE=9), sucrose preference (B: male: GFP=3, CRE=5; female: GFP=17, CRE=17), or volume of sucrose intake (C: male: GFP=3, CRE=5; female: GFP=17, CRE=17) between male (left) or female (right) AAV-Rictor-KO mice and GFP controls, n=3-17 mice/group, individual data points shown.

We next examined social interaction and found no significant differences between VTA-Rictor KO mice and GFP controls (Fig 8A). Sucrose preference was also similar between VTA-Rictor KO mice and GFP controls (Fig 8B), consistent with data from TH-Rictor-KO mice that baseline depressive-like behaviors are unaltered by Rictor KO.

VTA-Rictor-KO mice do not exhibit any differences in voluntary fluid intake

In contrast to TH-Rictor-KO mice, there was no significant difference in the volume of sucrose consumed for either male or female VTA-Rictor-KO mice compared to controls (Fig 8C). Additionally, VTA-Rictor-KO mice do not exhibit increased water intake from GFP controls (Fig 9A). Finally, we found no differences in morphine preference (Fig 9B), volume of morphine consumed (Fig 9C), or quinine consumed (data not shown), in male or female VTA-Rictor-KO mice. Together, these data suggest that VTA-Rictor-KO is not sufficient to alter voluntary fluid intake

TH-Rictor-KO mice are not more susceptible to physical or emotional CSDS

Given previous data that decreasing VTA AKT activity increased susceptibility to physical CSDS [6], we sought to determine whether decreasing TORC2 signaling, thereby decreasing AKT phosphorylation and activity, would also increase susceptibility to CSDS. We exposed male TH-Rictor-KO mice and littermate controls to standard 10-day physical and emotional CSDS [18, 24]. As expected, male mice that underwent physical CSDS had significantly reduced SI scores compared to controls (Fig 10A). However, while TH-Rictor-KO mice and their wild type controls subjected to physical CSDS had SI scores significantly lower than their non-stressed controls (Fig 10A: $F(5,48)=7.18$, $p=0.0001$), there was no significant difference in SI scores between physical CSDS groups, nor was there an increase in the number of mice susceptible to stress in the KO group (SI scores <100, wt = 5/7, KO = 7/12). We also

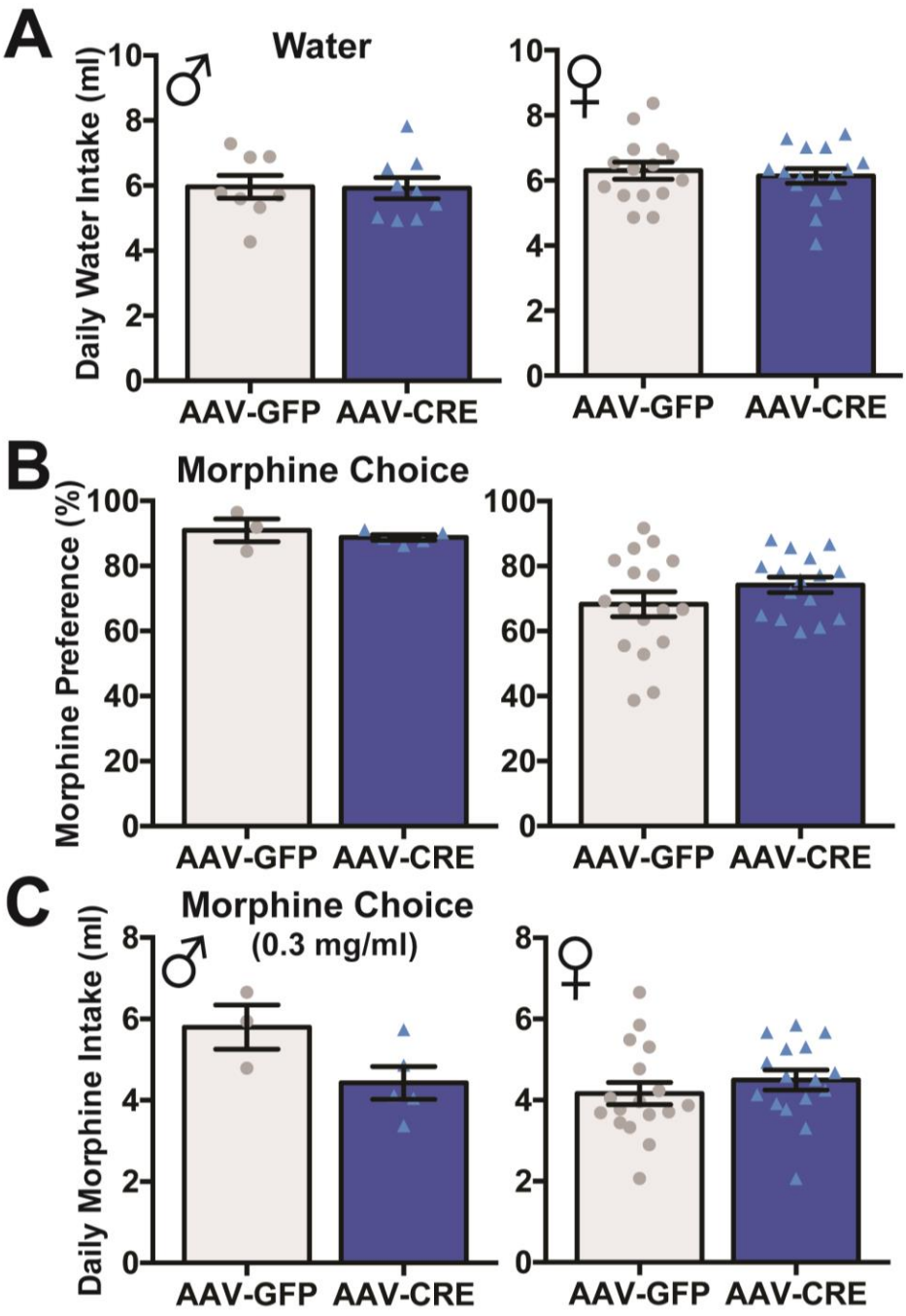

Figure 9: Evaluation of water and morphine consumption in stress naïve AAV-Rictor-KO mice. Knockdown of VTA TORC2 signaling did not alter water intake (A: male: GFP=8, CRE=9; female: GFP=17, CRE=17), morphine preference (B: male: GFP=3, CRE=5; female: GFP=17, CRE=16) or morphine intake (C: male: GFP=3, CRE=5; female: GFP=17, CRE=16) in either male or female mice compared to GFP controls, n=3-17 mice/group, individual data points shown.

assessed the impact of emotional CSDS, as this model of chronic stress induces a more subtle phenotype on day 11, allowing us to uncover even a weak effect of TH-Rictor-KO on susceptibility to stress. Mice exposed to emotional CSDS had SI ratios closer to 100, but there were no differences between TH-Rictor-KO mice and wild type controls (Fig 10A). These data suggest that decreasing TORC2 signaling in TH neurons does not increase susceptibility to physical and emotional CSDS.

VTA-Rictor-KO mice are not more susceptible to physical CSDS

To allow direct comparison to the previous VTA AKT study [6], we also subjected VTA-Rictor-KO mice and GFP controls to physical CSDS. While mice that underwent physical CSDS had decreased SI scores compared to non-stressed controls, there was no difference between VTA-Rictor-KO mice and GFP controls (Fig 11A), again suggesting that decreasing TORC2 signaling in VTA neurons does not increase susceptibility to physical CSDS.

Physical CSDS increases voluntary fluid intake of TH-Rictor-KO and VTA-Rictor-KO mice

Following SI testing, two-bottle choice testing was completed to assess water, sucrose, and morphine intake. Male TH-Rictor-KO mice that underwent physical CSDS exhibited a significant increase in water consumption compared to both unstressed controls and physical CSDS wild type mice (Fig 10B, $F(5,47)=5.09$, $p=0.0008$, one-way ANOVA, Tukey's post-hoc test, $p<0.05$). However, this effect was not evident in TH-Rictor-KO mice exposed to emotional CSDS (Fig 10B). Similarly, TH-Rictor-KO mice that underwent physical CSDS exhibited increased sucrose intake compared to wild-type controls (Fig 10C, $F(5,45)=4.17$, $p=0.003$, one-way ANOVA, Tukey's post-hoc test, $p<0.05$). Finally, morphine consumption was significantly increased in TH-Rictor-KO mice exposed to physical, but not emotional CSDS (Fig 10D, $F(5,45)=9.35$, $p=0.0001$, one-way ANOVA, Tukey's post-hoc test, $p<0.05$).

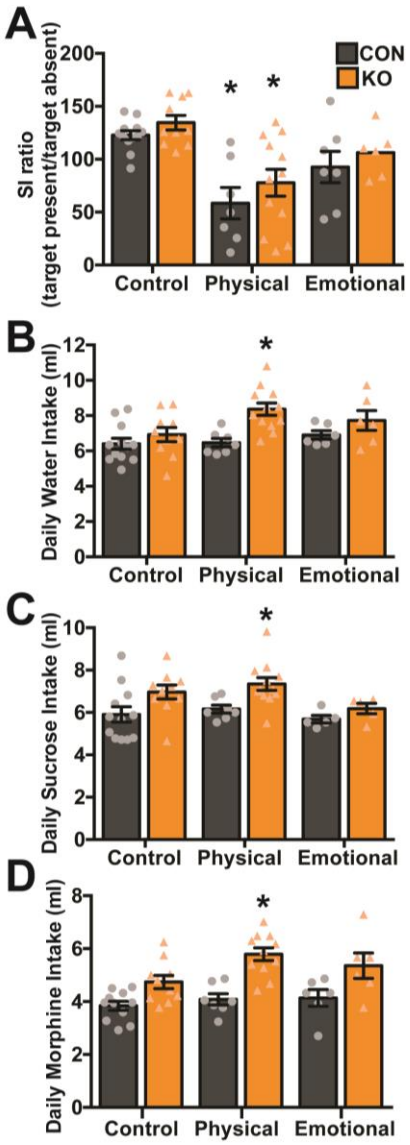

Figure 10: Evaluation of susceptibility of TH-Rictor-KO mice to physical and emotional CSDS and post-stress consumption of water, sucrose, and morphine solutions. Physical CSDS significantly decreased social interaction (SI) of both TH-Rictor-KO (n=12) and control mice (n=7), but there was no difference between genotypes (A; non-stressed controls: CON=12, KO=10). Exposure to emotional stress did not significantly alter SI (CON=7, KO=6). TH-Rictor-KO mice exposed to physical CSDS had significantly increased water (B: Non-stressed controls: CON=12, KO=10; Physical: CON=7, KO=12; Emotional: CON=6, KO=6), sucrose (C: Non-stressed controls: CON=12, KO=10; Physical: CON=7, KO=12; Emotional: CON=5, KO=5), and morphine (D: Non-stressed controls: CON=11, KO=10; Physical: CON=7, KO=11; Emotional: CON=5, KO=5) intake compared to control mice exposed to physical CSDS and non-stressed controls. No significant differences in consumption were observed in TH-Rictor-KO mice exposed to emotional CSDS, *$p<0.05$, n=6-12 mice/group, individual data points shown.

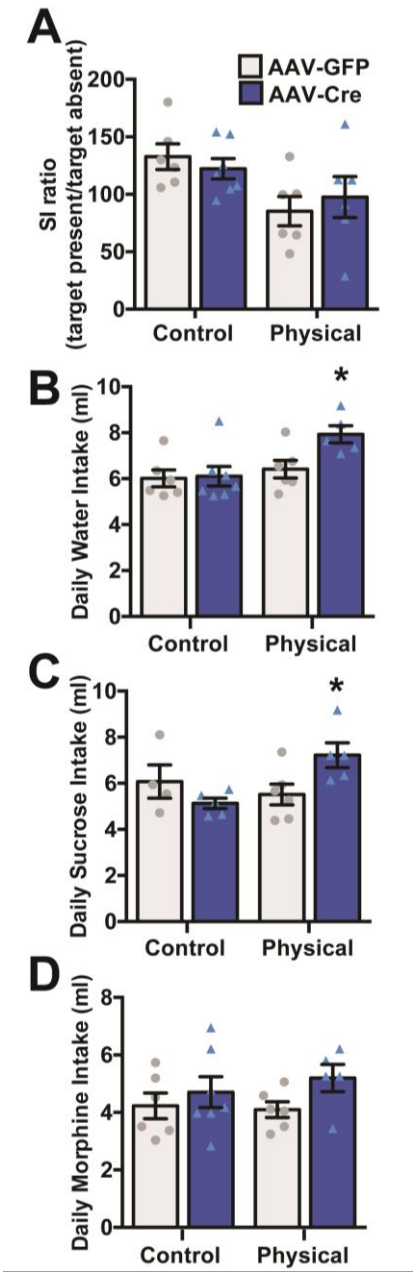

Figure 11: Evaluation of susceptibility of AAV-Rictor-KO mice to physical CSDS and post-stress consumption of water, sucrose, and morphine solutions. Physical CSDS generally decreased SI score but did not result in a significant differences between AAV-Rictor-KO mice and their GFP controls (A: Non-stressed controls: CON=6, KO=7; Physical Stress: CON=6, KO=7). Exposure to physical CSDS increased water (B: Non-stressed controls: CON=6, KO=7; Physical Stress: CON=6, KO=5) and sucrose (C: Non-stressed controls: CON=4, KO=5; Physical Stress: CON=6, KO=5) intake in AAV-Rictor-KO mice compared to AAV-GFP controls, while morphine intake was not significantly increased (D: Non-stressed controls: CON=6, KO=7; Physical Stress: CON=6, KO=5), *$p<0.05$, n=4-7 mice/group, individual data points shown.

We also examined the drinking behavior of VTA-Rictor-KO mice after exposure to physical CSDS. While there was no difference in water intake between non-stressed VTA-Rictor-KO mice and GFP controls, VTA-Rictor-KO mice exposed to physical CSDS drank significantly more water than physically stressed GFP controls (Fig 11B, $F(3,20)=4.46$, $p=0.01$, one-way ANOVA, Tukey post-hoc test). Similarly, VTA-Rictor-KO mice exposed to physical stress consumed more sucrose than non-stressed mice (Fig 11C, $F(3,16)=3.54$, $p=0.04$, one-way ANOVA, Tukey's post-hoc test, $p<0.05$). Finally, a similar trend for increased morphine consumption by VTA-Rictor-KO mice exposed to physical CSDS was observed (Fig 11D), but this did not reach statistical significance ($p>0.05$).

Discussion

Molecular and behavioral studies have identified VTA AKT activity as a mediator of susceptibility to stress, as overexpression of catalytically inactive AKT was shown to increase susceptibility to sub-chronic stress and increasing expression rescued CSDS-induced social avoidance [6]. Since TORC2 modulates AKT via Ser473 phosphorylation, and abrogation of phosphorylation at this site is sufficient to decrease AKT's ability to phosphorylate substrates such as Fox01/3a [25, 26], our goal was to determine if decreasing VTA TORC2 signaling was sufficient to increase susceptibility to CSDS. Because similar decreases in VTA AKT activity are observed following chronic opiate exposure and CSDS, we also investigated morphine intake after CSDS to determine the effect of decreasing TORC2 signaling on morphine reward and ingestive behavior.

We found that decreasing VTA TORC2 activity, either in catecholaminergic cells or specifically in the VTA, does not increase susceptibility to physical or emotional CSDS. One possible reason for the difference in the current findings from those that investigated AKT directly is that decreasing phosphorylation of AKT at Ser473 is not the same as overexpressing an AKT mutant form (K174M) that eliminates all catalytic activity. For example, in the current studies even though AKT is not phosphorylated at Ser473, it can be phosphorylated at other sites (such as Thr308, by PDK [11]), as well as interact with binding partners. Although studies indicate that AKT phosphorylation at Ser473 is necessary for full catalytic activation [8], it is possible that in our studies residual AKT activity is sufficient to mediate normal behavioral responses. For example, Rictor- or Sin1-deficient murine embryonic fibroblasts (MEFs) display a loss of phosphorylation of AKT Ser473 while retaining some phosphorylation of AKT Thr308, and while *in vitro* AKT kinase activity is decreased to 10-15% of control cells, phosphorylation

of the AKT substrate FOXO3 is significantly decreased while phosphorylation of GSK3B and TSC2 are not, suggesting AKT Ser473 and Thr308 phosphorylation might differentially impact substrate phosphorylation [25, 26]. Moreover, such substrate-selective effects may underlie different neuropsychiatric disorders, as Ser473 phosphorylation is linked to schizophrenia-associated symptoms in mouse models and human lymphocytes [11, 27, 28]. Additionally, while TORC2 controls the phosphorylation of AKT Ser473, it also phosphorylates other substrates, including other AGC kinases [29]. Thus, KO of TORC2 activity could be influencing activity of other substrates whose actions normally oppose those of AKT.

While Rictor KO mice were not more susceptible to CSDS, they did exhibit some baseline differences in behavior and these differed between the two Rictor KO models we employed. In our developmental model, Rictor expression is eliminated from neurons that express tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis. While this does decrease TORC2 signaling in the VTA, a brain region with a high concentration of TH-positive dopamine neurons, one caveat of this model is that it also decreases TORC2 signaling in other brain regions with a high proportion of DA neurons such as the substantia nigra, as well as those containing noradrenergic neurons such as the locus coeruleus. Thus, we also used Cre-expressing viral vectors to specifically target the VTA, but in this model TORC2 signaling is likely decreased in all subtypes of VTA neurons (GABAergic, glutamatergic and dopaminergic). Additionally, while Rictor expression is significantly decreased in this model (~50%, Fig 4A) and is sufficient to induce morphological and behavioral effects in mice [9], there still exists a population of cells with intact TORC2 signaling that could impact results.

We found that male developmental Rictor knockouts (TH-Rictor-KO) had increased locomotor activity compared to control littermates, consistent with previous data [13].

Interestingly, we did not observe any difference in locomotor activity in VTA-Rictor-KO mice. This difference could suggest that decreased TORC2 signaling in a catecholaminergic region outside the VTA, such as the substantia nigra, drives increased locomotor activity. However, a similar increase in novelty-induced locomotor activity has been observed in forebrain-specific Rictor KO mice (Nestin-Cre x flox-Rictor), which suggests TORC2 signaling in neurons that influence locomotor output centers such as the striatum is also sufficient to alter locomotor activity [12]. It is also possible that the altered locomotor response is developmentally regulated, as locomotor results have been consistently noted in crosses with various Cre lines, but Rictor KO in VTA of adult mice was not sufficient to alter morphine-induced locomotor activity [9]. However, local Rictor KO in striatum was sufficient to increase AMPH-induced locomotor activity [12], suggesting that alteration of TORC2 activity in adult mice, and its associated changes in dopaminergic signaling, can be sufficient to change locomotor behavior. Interestingly, we found that the increase in locomotor activity was limited to male TH-Rictor-KO mice, as female TH-Rictor-KO mice did not differ from their controls. This was surprising, but given that all prior studies utilized only male mice, the possibility of sex differences in TORC2-related behaviors was unexplored. One possibility is that we failed to see an increase in locomotor activity because females had higher overall rates of activity than males (Fig. 4C). However, given that higher rates of locomotor activity than those we observed are possible for female mice, as in psychostimulant studies, it is unlikely that a ceiling effect is the sole cause of this difference. Our sex-specific difference in locomotor activity could inform other behaviors, as one method to investigate individual differences in neuropsychiatric-related behaviors is to categorize rodents as “high” or “low” responders based on locomotor activity in a novel environment. This novelty-induced locomotion has been found to correlate with a variety of

traits including learning, anxiety, and drug reward [30-32]. However, most of the studies to date have only examined the correlation of novelty-induced locomotor response with other behaviors in male mice, so little is known about whether differences in novelty-induced locomotor activity are similarly predictive in females. A recent study attempted to address this question in mice, and while they didn't find any differences in the median locomotor activity or center time between males and females, they found by principal component analysis that male behavior on a battery of tests was explained by locomotion-related variables while anxiety- and depressive-like behaviors accounted for more of the variance in the females [31]. Thus, TORC2-dependent changes in locomotor activity may be expected to alter other behavioral phenotypes in a sex-dependent manner. We did not observe any sex-specific differences in the other baseline behaviors (EPM, FST), but future studies that examine differences in male vs. female TH-Rictor-KO responses to acute or chronic stress, or fear-related learning, may offer additional insights into whether changes in locomotor activity predict sex-specific phenotypes. Our findings suggest that there is a critical need to explore behavioral traits and the impact of altered neuronal signaling in both male and female mice, as the predictions made between assays may not correlate between the sexes. Future studies should delve further into this locomotor phenotype, including whether this difference is also apparent in the home cage or whether it is restricted to novel environments, and investigate the interplay between neuroendocrine and TORC2 signaling.

We also observed differences between male TH-Rictor-KO mice and VTA-Rictor KO mice in fluid consumption. At baseline, male TH-Rictor-KO mice consume more fluids (water, sucrose, or morphine) than their control littermates, while VTA-Rictor-KO mice are similar to GFP controls. The changes in ingestive behavior appeared more prominent when rewarding

substances like sucrose and morphine were available, for example TH-Rictor KO mice exhibited 116% water intake of wild-type controls, but 127% and 122% of sucrose and morphine, respectively. However, there is not a concomitant increase in sucrose or morphine preference, suggesting that the increased consumption is not driven by a difference in reward or palatability. One possibility is that the increased fluid intake is a result of the increased locomotor activity, given that both the activity and intake increases are found in the male TH-Rictor-KO mice, but not the VTA-Rictor-KO mice. However, we also found that female TH-Rictor-KO mice consumed more fluid than controls in sucrose and morphine choice experiments, and these mice did not exhibit increased locomotor activity. Moreover, while it was not formally investigated in this study, we did not observe any differences in consumption of normal chow in either male or female TH-Rictor-KO mice (data not shown), consistent with prior data from male TH-Rictor-KO mice [13], suggesting that there is not a global change in consummatory behavior. It is also possible that the mice are not actually ingesting the fluid, but exhibit increased interactions/manipulation of the sipper tops that results in increased fluid leaking from the bottles. We think this is unlikely based on our observations that the proportions of water and rewarding substance consumed are consistent from day to day, even as the bottle location is alternated, suggesting that animals are making a similar choice/activity at bottles throughout the experiment. Thus, the mechanism underlying increased fluid consumption in TH-Rictor-KO mice remains obscure. It is possible that the effect could be driven by signaling effects in noradrenergic cells (either central or peripheral), a hypothesis that could be tested by crossing the floxed-Rictor mice to norepinephrine transporter (NET)-Cre mice. However, given that we see a similar increase in fluid intake in VTA-Rictor-KO mice following stress, a region that lacks NE neurons, it suggests that there is also a role for VTA DA neuronal effects.

While neither TH-Rictor-KO nor VTA-Rictor-KO exhibited increased susceptibility to stress, exposure to physical stress increased fluid intake in both models. Since physical stress exposure induced increased intake in VTA-Rictor-KO mice that were unaffected at baseline, this suggests that Rictor KO mice do have altered responses to stress, albeit without changes in social avoidance. Similar to the baseline TH-Rictor-KO mice data, the stress-induced change in fluid intake was not accompanied by a concomitant increase in preference, suggesting changes in reward were not responsible. These data were somewhat surprising as we have previously found that VTA-Rictor-KO mice exhibited decreased morphine reward as assessed by conditioned place preference assay [9]. Thus, we predicted that in the baseline state, Rictor KO mice would exhibit a decrease in morphine preference in the two-bottle choice assay. Given the differences in the route of morphine administration between the two studies (voluntary vs. experimenter), it may be that KO of Rictor in VTA is sufficient to alter association of the relatively high dose of morphine in CPP studies to a context, but is not sufficient to alter the motivation to obtain a lower morphine dose in the two-bottle task. In contrast to exposure to physical stress, emotional stress did not alter fluid intake in Rictor KO mice. This difference might be due to the magnitude of the stress exposure, as the social avoidance phenotype incubates in emotional stress mice, with a small effect 1 day post-stress that then becomes indistinguishable from physical stress mice 28 days later [20].

Overall, these studies reveal that disruption of TORC2 signaling, either in catecholaminergic neurons or specifically within the VTA, does not influence susceptibility to CSDS-induced social avoidance. Instead, these experiments reveal a novel role for TORC2 signaling in mediating changes in activity and fluid intake that appear linked to stress responses. Further, given differences between the two models, our work demonstrates that it will be critical

in future studies to evaluate the specific functions of TORC2 signaling within DA versus NE neurons, their potential developmental contributions, and the role of sex in both baseline and stress-induced behaviors.

Acknowledgements, Funding, and Disclosure

We would like to thank Ken Moon for excellent technical assistance and AJ Robison and Andrew Eagle for helpful discussion. The authors have no financial interests or conflicts of interest to disclose. This work was funded by the National Institute on Drug Abuse (R01 DA039895 and R03 DA037426, MMR) and graduate fellowships from the PhRMA foundation and the National Institute of General Medical Sciences (T32 GM092715, SK), with no direct input on the design, conduct, analysis or publication of the studies. We would also like to thank the NIDA Drug Supply Program for generously providing morphine.

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CHAPTER 3

TORC2 Does Not Regulate Cytoskeletal Changes in the VTA Through Rac1-mediated Signaling

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(In preparation for submission.)

Abstract

Although previous work has demonstrated a role for the mammalian/mechanistic target of rapamycin complex 2 (TORC2) in controlling ventral tegmental area (VTA) DA neuron soma size after chronic morphine administration, the downstream mechanism by which TORC2 modulates this morphology change, such as reorganization of the actin cytoskeleton, remains unknown. In addition, while the connection between TORC2 signaling and cytoskeletal remodeling has been investigated in brain regions such as the hippocampus and cerebellum, it has not yet been investigated in the VTA. Given the fact that the activity of the Rac1-PAK-Cofilin cytoskeletal pathway is modulated by TORC2 signaling in the hippocampus, we investigated activity of this same pathway in the VTA following chronic morphine administration. We also utilized viral vectors and Cre-Lox technology to decrease VTA TORC2 signaling via knock-out of the TORC2 constituent protein Rictor, to determine if activity of Rac1 and its downstream effectors is impacted by TORC2 KO. Biochemical analysis of VTA tissue revealed that the Rac1-PAK-Cofilin signaling is not altered by chronic morphine exposure. Additionally, in contrast to the hippocampus, decreased TORC2 signaling does not alter Rac1-PAK-Cofilin signaling in the VTA. Contrary to our hypothesis, this suggests that the TORC2-dependent decrease in VTA DA soma size induced by morphine is not due to changes in Rac1-related signaling but is mediated by other cytoskeletal signaling pathways. Overall, this work highlights the diversity in signaling responses between brain regions and the need for further characterization of TORC2 targets in the VTA.

Introduction

The ventral tegmental area (VTA) is a critical component of the mesolimbic dopamine pathway that mediates opiate reward. Specifically, the soma size of VTA dopamine (DA) neurons decreases in response to chronic morphine treatment [1-3]. This decrease in size correlates to a decrease in morphine reward [2, 3]. The changes in morphology are dependent upon brain derived neurotrophic factor (BDNF), as infusion of BDNF into the VTA of rats rescues the soma size change induced by chronic morphine treatment [1]. More recent work has demonstrated that IRS2-AKT signaling modulates this change in soma size [2] downstream of BDNF. More recently, mammalian/mechanistic Target of Rapamycin Complex 2 (TORC2), a protein complex upstream of AKT, has been shown to mediate VTA DA neuron soma size and morphine reward [3].

TORC2 is a protein complex that consists of TOR, a serine/threonine kinase. TOR associates with Protor, Deptor, LST8, SIN1 [4] and is typically characterized by its association with rapamycin-insensitive companion of TOR (Rictor) [5]. Functionally, TORC2 serves to phosphorylate members of the AGC family of kinases, such as Serum- and glucocorticoid-inducible protein kinase (SGK1) at Ser422, Protein kinase C (PKC) at Ser657, and AKT at Ser473. Phosphorylation of these targets leads to changes in actin cytoskeleton dynamics [4] and turnover in both yeast [6, 7] and mammalian cells [8]. Importantly we know that altering TORC2 signaling modulates behaviors such as morphine reward. Increasing TORC2 signaling in the VTA increases morphine reward as measured by conditioned place preference (CPP). Conversely, decreasing TORC2 signaling in the VTA decreases morphine CPP [3]. Overall, the change in VTA DA neuron morphology affects behavior and demonstrates the need to investigate TORC2-mediated signaling in the VTA.

While there are no pharmacological inhibitors that specifically target TORC2, viral [3] and genetic [9-13] approaches can be used to control TORC2 signaling. Notably, deletion of the TORC2 constituent protein Rictor eliminates TORC2 catalytic activity. While deletion of Rictor is embryonically lethal [14], Cre-Lox approaches have been used to conditionally delete Rictor expression allowing control of TORC2 signaling in adult mice. In the brain, deletion of Rictor decreases overall brain weight and size, decreases pyramidal neuron soma size in the hippocampus, and also reduces Purkinje neuron soma size and dendrite length [12]. Further investigation in the hippocampus reveals that TORC2 also controls dendritic spine morphology, as deletion of Rictor decreases spine density of CA1 pyramidal neurons [13]. Specifically in the reward pathway, TORC2 has also been demonstrated to regulate VTA DA neuron soma size [3]. Increasing TORC2 signaling rescues chronic morphine-induced reduction of VTA DA neuron soma size [3], emphasizing TORC2's role in modulating the actin cytoskeleton. Little is known specifically about how TORC2 regulates these morphological changes in the VTA.

The actin cytoskeleton is a dynamic system that provides shape and stability within a cell and rapidly responds to physiological stimuli such as vascular distress [15] and metastasis [16]. Additionally, cytoskeletal reorganization is critical for memory formation and learning [13]. This process requires a number of proteins to control actin turnover. Investigation of downstream signaling of TORC2 in the hippocampus revealed the involvement of Rac1, a member of the Rho family of GTPases. Rac1 controls the phosphorylation of p21-activated kinase (PAK) and cofilin, which ultimately controls changes in dendritic spine density [13]. GTPases are controlled by two main types of proteins, GTPase-activating protein (GAP) and guanine exchange factor protein (GEF) [17]. More specifically, co-immunoprecipitation revealed that TORC2 interacts with Tiam1, a guanine exchange factor (GEF), through Rictor, to potentially direct changes in

the actin cytoskeleton [13]. As a GEF, Tiam1 functions to exchange GDP to GTP for Rac1, which activates Rac1 [18].

Changes in Rac1-dependent dendritic spine morphology have also been observed in the nucleus accumbens (NAc) in response to stimuli such as cocaine and stress. Rac1 GTPase activity was decreased after repeated cocaine treatment and importantly, viral-mediated control of Rac1 led to changes in dendritic spine morphology in the NAc [19]. Chronic social defeat stress also promotes changes in dendritic spine remodeling, and viral overexpression of constitutively active Rac1 decreases stubby spine density in the NAc of susceptible mice. Overall, work in other brain regions highlight the critical role of Rac1 in altering the actin cytoskeleton and thus, both expression level and activity of Rac1 can be investigated when examining changes in actin turnover.

Given the established relationships between TORC2, morphine reward, and VTA DA neuron soma size changes [3], and TORC2 and the cytoskeleton in the brain [13], this paper seeks to address two critical knowledge gaps regarding the lack of understanding of TORC2 signaling in the VTA. First, we test the hypothesis that *chronic morphine, which decreases TORC2 signaling, alters Rac1 signaling in the VTA*. Secondly, we will determine whether *altering TORC2 signaling in the VTA is sufficient to alter Rac1 signaling*. More specifically, we hypothesize that *decreasing VTA TORC2 signaling will decrease phosphorylation of cytoskeletal proteins through Rac1*. Through these studies, we hope to further our knowledge on TORC2-mediated cytoskeletal changes in the VTA and how TORC2 is functioning to mediate changes in soma size in response to morphine.

Materials and Methods

Mice

All mice were housed on a 12 hr light/dark cycle at 22-25° C with food and water available *ad libitum*. Adult male and female mice (8-10 weeks) were used for surgeries and tissue collection. Developmental homozygous floxed Rictor mice were generated as previously described [3, 9, 14]. Briefly, tyrosine hydroxylase (TH-Cre) mice (Jackson Laboratories, 008601) were initially crossed with floxed-Rictor mice to knock-out Rictor. The floxed-Rictor, Cre negative mice were then bred together to generate homozygous floxed Rictor, Cre negative mice for surgery. Genotypes were validated at 21-28 days using standard procedures and previously published PCR conditions [20]. Adult (8 weeks old) male C57BL/6J (Jackson Laboratories) mice were used for chronic morphine surgeries. All procedures were approved by the Michigan State University's Institutional Animal Care and Use Committee (IACUC) and adhere to the NIH's Guide for the Care and Use of Laboratory Animals.

Morphine

Subcutaneous morphine pellets were implanted in mice for chronic treatment of morphine in order to induce morphological and biochemical changes that have been previously established [1-3]. Mice were anesthetized with isoflurane and a small incision was made for subcutaneous implantation of either a morphine (25 mg) or sham pellet. Mice received a pellet on day one and a second pellet on day 3, mice were then sacrificed on day 5, as previously described [3]. Morphine and sham pellets were generously supplied by the NIDA Drug Supply Program.

Viral-mediated gene transfer

Targeted surgery for VTA infusion was completed as previously described [3, 20]. Briefly, mice were anesthetized with ketamine (100 mg/kg) and xylazine (100 mg/kg) and underwent stereotaxic surgery. Bilateral infusions (0.5 μl) of AAV-Cre or AAV-Cre-GFP (UNC Vector Core) were injected into homozygous floxed Rictor mice at the following coordinates to target the VTA: from bregma: -3.2 mm AP, +1.0 mm ML, and -4.6 mm DV, 7° angle. Mice were given at least 14 days to recover from surgery before VTA tissue was collected.

Western blot

VTA tissue was microdissected as described [3]. Briefly, unanesthetized mice were cervically dislocated and decapitated. Brains were removed and placed in cold phosphate buffered saline (PBS). Brains were sectioned using a 1 mm brain matrix and VTA was microdisseted using a 14-gauge punch. VTA tissue was then frozen immediately on dry ice and then transferred to -80 °C until processing. VTA tissue was processed in RIPA buffer (10 mM Trizma base, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% Na Deoxycholate, pH 7.4) containing protease inhibitor cocktail I (Sigma) and phosphatase inhibitor cocktails I and II (Sigma). For Western blot analysis, 20 ug protein per sample was run on 4-15% gradient gels (Biorad) for electrophoresis. Proteins were transferred to PVDF membranes (Millipore), blocked in 5% non-fat dry milk in PBS containing 1% Tween, and probed with the primary antibodies listed. Secondary antibodies were used at 1:40000 and are also listed in the table. Immunoblots were developed using chemiluminescence (Thermo Scientific) and images were obtained using autoradiography film or Omega Lum G Imaging System (Aplegen). Image J was used to perform densitometry for quantification of expression and all protein levels were normalized to GAPDH expression.

Antibody	Company	Product Number	Dilution
Rictor	Cell Signaling	2114	1:1000-2000
pAKT	Cell Signaling	9271	1:1000
AKT	Cell Signaling	9272	1:5000
pPKC	Cell Signaling	9375	1:1000
PKC	Cell Signaling	2056	1:1000
pNDRG	Cell Signaling	3217	1:3000
NDRG	Cell Signaling	5667	1:2000
Rac1	Cytoskeleton	ARC03	1:500
pPAK	Cell Signaling	2605	1:1000-3000
PAK	Cell Signaling	2602	1:1000
pCofilin	Cell Signaling	3311	1:1000
Cofilin	Cell Signaling	5175	1:10000
GAPDH	Cell Signaling	2118	1:10000-20000
TH	Sigma	T1299	1:2000-3000
GFP	Life Technologies	A11122	1:3000
HRP Goat Anti-Rabbit IgG Antibody	Vector Laboratories	PI-1000	1:40000
HRP Horse Anti-Mouse IgG Antibody	Vector Laboratories	PI-2000	1:40000

Table 1: List of antibodies used, product information, and usage information.

Rac1 GTPase Assay

The Rac1 GTPase assay was completed following the vendor protocol (New England Biosciences; Cat. No. 80501). Briefly, samples were sonicated in the provided assay/lysis buffer containing protease and phosphatase inhibitors. Samples underwent centrifugation at 4° C for 15 min. at 20000g. Supernatant was transferred to a new microcentrifuge tube. Anti-active Rac monoclonal antibody (from kit) and agarose beads (from kit) were added for incubation at 4°C for 1 hour. Beads were pelleted with centrifugation and washed with assay/lysis buffer. Bead pellet was resuspended in 2x SDS-PAGE sample buffer and boiled for 5 min. Samples underwent centrifugation prior to gel electrophoresis for Western blot analysis (protocol listed above.)

Statistics

All values are reported as mean +/- SEM. GraphPad Prism was used to calculate unpaired t-tests, which were performed in GTPase assay and for each protein in Western blot analysis. Significance is defined as *$p<0.05$ and **$p<0.001$.

Results

Chronic morphine does not alter Rac1 signaling in the VTA

We know that chronic morphine treatment decreases the phosphorylation of TORC2 targets [3]. We also know that when Rictor expression is decreased, which decreases TORC2 signaling, the cytoskeleton is altered [13]. Given this information, we sought to investigate the molecular changes that occur to the cytoskeleton in response to chronic morphine. We microdissected VTA from mice following subcutaneous implantation of morphine or sham control pellets, a well-established procedure that produces dependence and changes in VTA biochemical signaling and cellular morphology.

The change in VTA DA neuron morphology after exposure to chronic morphine suggests rapid actin turnover in order to respond to the external stimulus. Additionally, given that TORC2 has been observed to mediate cytoskeletal changes in yeast and mammalian cells, and that TORC2 specifically modulates spine morphology in mouse hippocampus through Rac1, we wanted to determine if TORC2 similarly modulates the cytoskeleton in the VTA through Rac1. Thus, we first examined the downstream targets in the Rac1 pathway. No significant changes in Rac1 expression, nor phosphorylation of its downstream targets PAK and Cofilin, were observed via western blot analyses of VTA samples from sham and morphine treated (Figure 12).

In order to confirm that morphine-induced biochemical changes were detectable in our sample set, we next examined phospho-NDRG, as levels of VTA phospho-NDRG are robustly increased by chronic morphine administration [21]. In agreeance with the aforementioned study, our chronic morphine treated samples displayed a significant increase in pNDRG expression

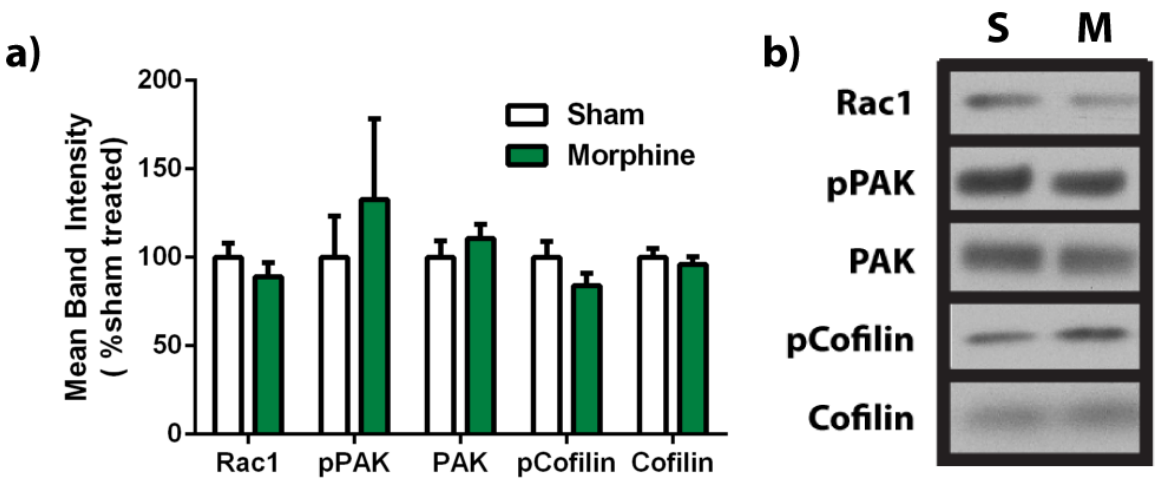

Figure 12: Evaluation of Rac1-PAK-Cofilin pathway proteins in the VTA after chronic morphine treatment. a) There were no significant changes in protein expression between sham and morphine treated animals. n=11-12 per group. b) Representative Western blot images.

(sham= 100.0 +/- 17.7, morphine = 262.2 +/-36.8 , Figure 13a), indicating that our morphine administration was sufficient to reproduce known signaling changes, and that our lack of Rac1 signaling changes was not due to issues with drug delivery. We also examined phosphorylation of known TORC2 targets in the AGC family of kinases. There was not a significant change in phosphorylation of AKT (sham = 100.0 +/- 4.9, morphine = 96.1 +/- 4.1, n=12 per group) or PKC (sham = 100.0 +/- 11.1, morphine = 105.1 +/- 8.1, n= 12 per group) between sham and morphine treated mice (Figure 13a). In contrast to the reported changes in phospho-NDRG which are robust (200 – 300%), changes in phospho-AKT are more modest, with ~20-25% decrease reported in both mice and rats [2, 3]. Thus, our inability to detect a significant change could be due in part to our lower sample size in this study. We also investigated the Rictor expression levels in the VTA. Chronic morphine treatment did not alter Rictor expression levels (sham = 100.0 +/- 11.6, morphine = 114.3 +/- 7.4, n=12 per group) in the VTA (Figure 13a) consistent with previous findings.

Chronic morphine does not alter Rac1 activity in the VTA

Although we did not detect changes in the total level of Rac1, this did not exclude the possibility that Rac1 activity could be altered. As a GTPase, Rac1's activity can be controlled by the availability of GTP and rate of exchange of GDP to GTP. Thus, we used a Rac1 GTPase assay to assess Rac1 activity after chronic morphine treatment. This assay utilizes a monoclonal antibody to recognize Rac1 only when it is in its "active" state, which is when Rac1 is bound to GTP. Immunoprecipitation followed by immunoblotting was used to examine the amount of Rac1-GTP obtained from the sham and morphine VTA samples. Results from the Western blot analysis do not demonstrate a change in Rac1 activity after morphine treatment (Figure 14, sham = 100.0 +/- 13.5, morphine = 96.6 +/- 8.6, n=9 per group).

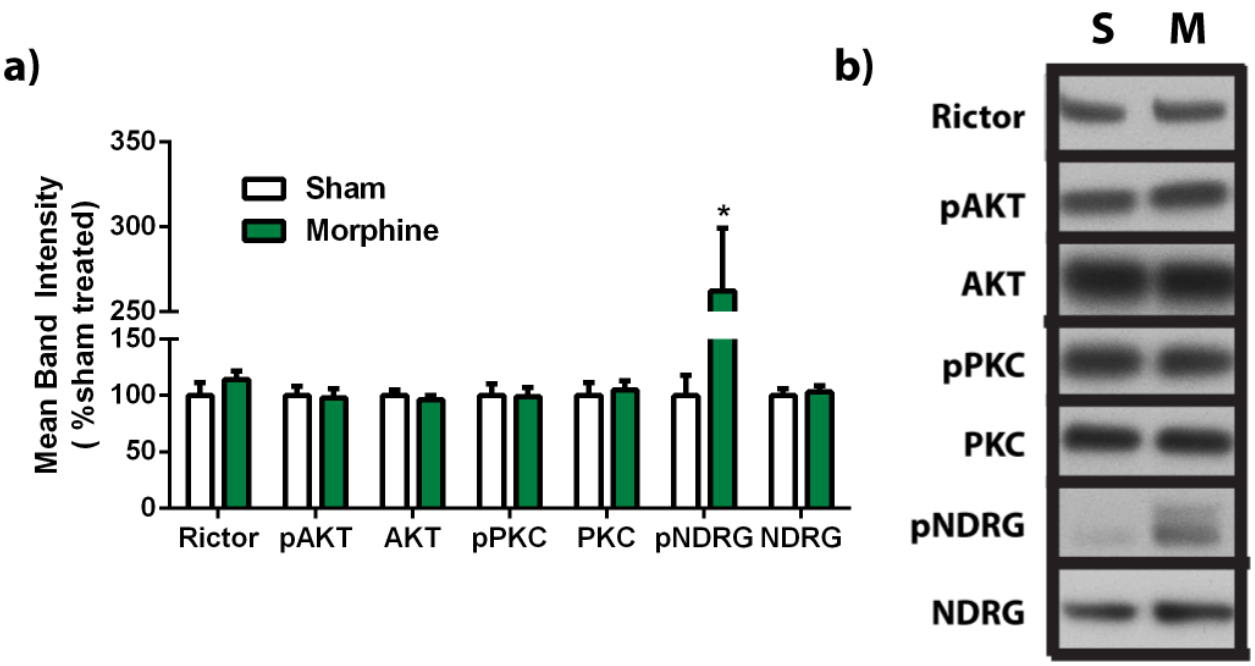

Figure 13: Evaluation of TORC2-related proteins in the VTA after chronic morphine treatment. a) There were no significant changes in Rictor expression or TORC2 targets such as pAKT and pPKC following morphine treatment. A significant increase in pNDRG was observed following morphine treatment. n=12 per group. *$p<0.001$, unpaired Student's t-test. b) Representative Western blot images.

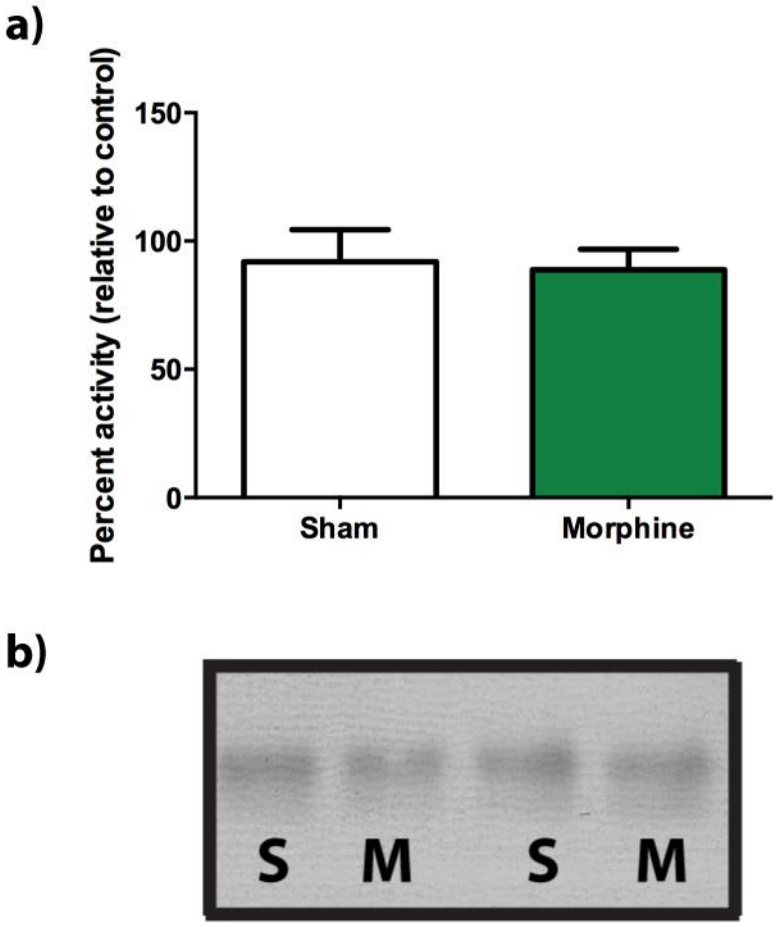

Figure 14: VTA Rac1 GTPase activity was not altered by chronic morphine treatment. a) There was no significant difference in Rac1-GTP between sham and morphine treated mice. n=9 per group. b) Representative Western blot images from GTPase assay.

Knockdown of TORC2 signaling in the VTA does not alter Rac1 signaling

Previous work indicates that Rictor KO in the VTA is sufficient to decrease soma size, suggesting a role for TORC2 signaling in cytoskeletal remodeling [3]. Since changes in the cytoskeleton through the Rac1 pathway were observed in the dorsal hippocampus of Rictor knockout mice [13], we hypothesized that the cytoskeleton would be mediated by TORC2 in a similar fashion in the VTA. Utilizing Cre-Lox technology to decrease TORC2 signaling, we performed stereotaxic surgery to infuse either an AAV-Cre-GFP or AAV-GFP virus into the VTA of floxed Rictor mice. We then investigated changes in Rac1 signaling (Figure 15). There was not a significant change in Rac1 expression in the VTA of Rictor-KO mice (AAV-GFP = 100.0 +/- 17.5, AAV-CRE-GFP = 88.2 +/- 11.4, n=8-9 per group). Interestingly, a significant decrease in PAK was observed (AAV-GFP = 100.0 +/- 13.5, AAV-CRE-GFP = 64.1 +/- 5.1, n=9 per group, p=0.02) , however a similar trend was also observed for pPAK (AAV-GFP = 100.0 +/- 14.49, AAV-CRE-GFP = 78.7 +/- 9.0, n=9 per group), resulting in no change in the ratio of pPAK/PAK (AAV-GFP = 100.0 +/- 14.5, AAV-CRE-GFP = 128.5 +/- 23.8, n=9 per group). The change in PAK is unexpected, as the literature to date has not implicated TORC2 signaling in changes in total protein expression. We next looked at Cofilin expression as it is downstream of PAK. We did not observe any changes in the phosphorylation of Cofilin (AAV-GFP = 100.0 +/- 13.5, AAV-CRE-GFP = 89.53 +/- 8.0, n=8 per group), nor did we observe changes in total Cofilin expression (AAV-GFP = 100.0 +/- 7.7, AAV-CRE-GFP = 105.2 +/- 11.6, n=8-9 per group).

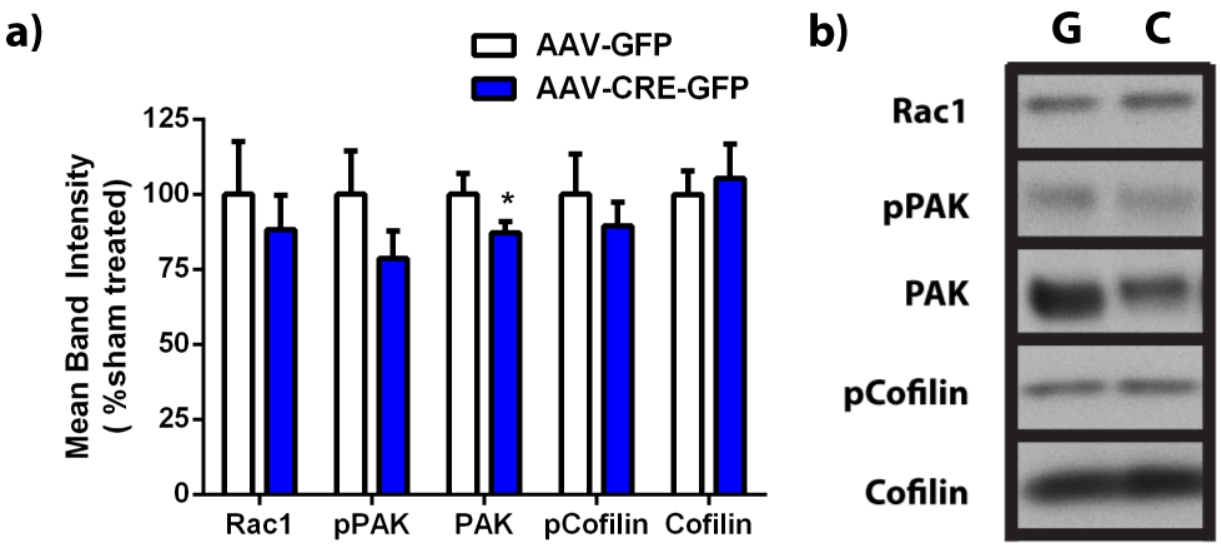

Figure 15: Evaluation of Rac1-PAK-Cofilin pathway proteins in the VTA after viral knockdown of Rictor. a) Rictor KO significantly decreased VTA PAK expression but did not change expression of other proteins in this pathway. n=8-9 per group *p<0.05; unpaired Student's t-test. b) Representative Western blot images.

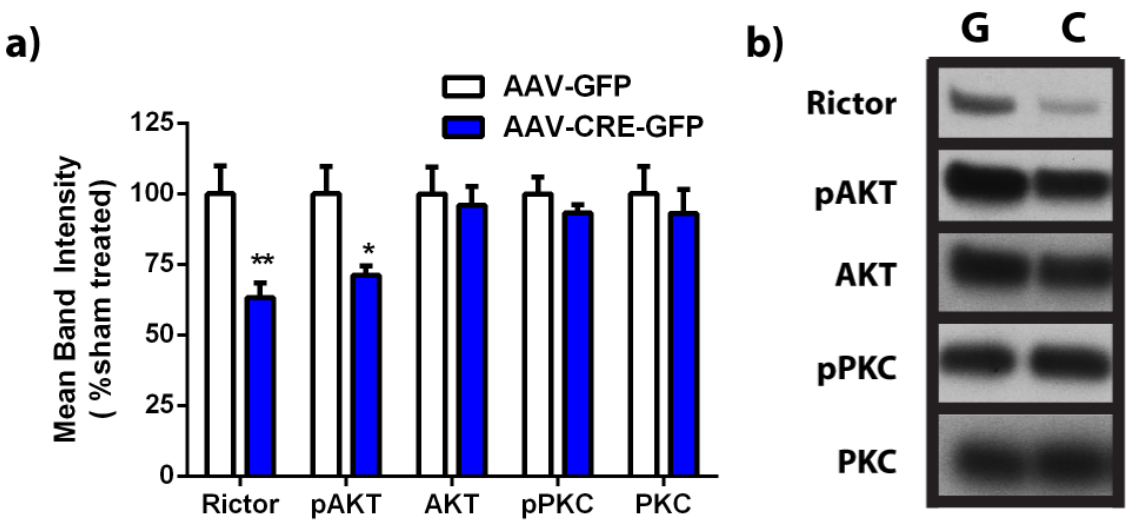

Figure 16: Evaluation of TORC2-related proteins in the VTA after viral knockdown of Rictor. a) AAV-CRE-GFP significantly decreased Rictor and pAKT expression in the VTA but did not alter pPKC, nor total AKT and PKC. n=8-9 per group. *$p<0.05$; **$p<0.01$; unpaired Student's t-test. b) Representative Western blot images.

Lastly, we performed Western blot analysis to confirm viral knockdown of Rictor expression (Figure 16). We demonstrate a significant decrease in Rictor expression (AAV-GFP = 100.0 +/- 9.9, AAV-CRE-GFP = 63.1 +/-5.4, p=0.004), demonstrating successful targeting of the AAV viruses and KO of Rictor. We proceeded to confirm changes in downstream signaling of TORC2's known targets (Figure 16). As expected, a significant decrease in pAKT was observed in AAV-Cre samples (AAV-GFP = 100.0 +/- 9.7, AAV-CRE-GFP = 71.2 +/- 3.3, n=8-9 per group), as well as a non-significant trend toward decreased pPKC (AAV-GFP = 100.0 +/- 6.9, AAV-CRE-GFP = 87.3 +/- 3.8, n=9 per group).

Overexpression of HSV-Rictor in the VTA

We know from both experiments in cell culture and also *in vivo*, that deletion of Rictor alters actin cytoskeleton signaling. What remains to be explored is whether increasing TORC2 signaling via Rictor overexpression can also alter the cytoskeleton-related signaling. We know that while overexpression of Rictor in the VTA will rescue the morphine-induced decreases in soma size [3], it does not alter soma size in the absence of drug treatment. Nonetheless, we wanted to increase VTA TORC2 signaling by overexpressing Rictor to determine whether it could affect Rac1 signaling. Unfortunately, we did not detect an increase in Rictor protein in HSV-Rictor-GFP mice (data not shown), despite a 249-fold increase in Rictor mRNA via qPCR analysis in separate cohort of mice that received infusion of the same batch of virus. The attempt to assess targeting of these viruses was assessed through Western blot analysis, but was not successful. This could possibly be due to technical Western blot issues, or it is also possible that the targeting of the virus was not accurate and/or the VTA tissue collection was not accurate. Future studies could incorporate the use of a fluorescent dissecting scope to identify the location of the virus and to determine which brain slice contains the greatest amount of virus in the region

that looks most like the VTA. Lastly, it is important to note that previous studies [3] have been able to validate increases in Rictor overexpression and subsequently, increases in phosphorylation of downstream TORC2 targets such as pAKT. Thus, this may suggest that a new viral prep is necessary for the HSV-Rictor-GFP construct.

Discussion

The goal of these studies was to determine how TORC2 regulates actin cytoskeleton remodeling in the VTA. TORC2 signaling has been demonstrated to influence basal Purkinje soma size changes in the cerebellum [12] and basal and morphine-induced adaptations in VTA DA neurons [3]. TORC2 signaling has also been shown to regulate dendritic spine morphology in the hippocampus [13], suggesting TORC2 signaling is involved in multiple forms of structural plasticity. Importantly, this TORC2 effect in the hippocampus was dependent on changes in Rac1 signaling and Rac1 has also been shown to regulate dendritic spine morphology in the NAc in response to cocaine [19] and stress [22], indicative of the importance of Rac1 signaling in the reward circuit. Thus, we hypothesized that VTA TORC2 modulates the actin cytoskeleton through the Rac1 pathway and VTA Rictor KO would decrease Rac1 signaling. Given the decrease VTA TORC2 signaling in chronic morphine treated rodents [1-3] that is responsible for the decrease in VTA DA soma size, we wanted to determine if the actin cytoskeleton is altered in chronic morphine treatment through decreased VTA TORC2 signaling through the Rac1 pathway.

We determined that morphine does not regulate activity of the Rac1-PAK-Cofilin pathway in the VTA, as there were no changes in expression of Rac1, nor were there changes in the phosphorylation of PAK and Cofilin. This suggests that alterations in the actin cytoskeleton in response to morphine are not driven via Rac1 signaling. While morphine decreases TORC2 activity in the VTA, we observed no changes in Rac1 signaling. This is in contrast to data from the hippocampus, where decreased TORC2 resulted in a decrease or Rac1 GTPase activity [13]. Thus, this raises the question of how morphine can alter TORC2 signaling and also, how it might alter Rac1 signaling. Additionally, TORC2-mediated modulation of Rac1 signaling can be

investigated by looking at the role of Tiam1. A stimulus, such as repeat administration of cocaine, decreased Tiam1 expression in the NAc, which correlates with decreased Rac1 GTPase activity [19]. Similarly, cocaine self-administration in mice also significantly decreases Tiam1 mRNA and protein expression in the nucleus accumbens [23]. Given the effect of cocaine on Tiam1 expression, the effect of morphine on Tiam1 expression and how that regulates TORC2-mediated Rac1 GTPase activity, or the activity of other GTPases, can also be investigated.

In parallel to the hippocampus, we also wanted to determine whether TORC2 is directly coupled to Rac1 signaling in the VTA. In our investigation, we decreased the expression of Rictor, a constituent protein of the TORC2 complex, by injecting AAV-Cre into the VTA of floxed Rictor mice. While we saw a significant decrease in PAK expression, when pPAK was normalized to PAK, there was significant difference between groups. A change in total PAK was not expected, especially when no difference in the phosphorylated version of PAK was observed. It could be possible that different isoforms of PAK are regulated differently by TORC2. Protein expression or phosphorylation of Rac1 or Cofilin in the Rac1-PAK-Cofilin pathway was not altered, suggesting that viral deletion of Rictor to decrease TORC2 signaling may not be sufficient to affect the Rac1 pathway. One possibility for this lack of an effect could be due to the inability of AAV-Cre to sufficiently decrease TORC2 signaling. For example, studies in the hippocampus used a developmental Rictor knockout [13], which likely affects a greater percentage of cells containing Rictor compared to a viral-mediated knockout model. Thus, future work using a developmental VTA Rictor knockout, such as the aforementioned TH-Rictor-KO model [20], would be informative to determine more conclusively whether Rictor deletion is incapable of altering the Rac1-PAK-Cofilin signaling in the VTA. In addition, AAV-Cre is not cell-type specific, thus Rictor KO should occur in infected DA, GABA, and glutamatergic

neurons. If alteration of the cytoskeleton through TORC2 is cell-type specific, further investigations are necessary to tease apart the potential differences in cytoskeletal remodeling. Lastly, while there were no changes in protein expression or phosphorylation, we cannot exclude the possibility that Rac1 activity is altered following VTA Rictor KO. Investigation of Rac1 activity, via GTPase assay, after knockout of Rictor signaling, both AAV-mediated and TH-Cre mediated will yield insight into the regulation of the actin cytoskeleton.

This evidence demonstrates that the Rac1-PAK-Cofilin pathway is not regulated through TORC2 in the VTA. However, this does not eliminate a role for TORC2 itself in mediating cytoskeletal signaling. Thus far, several studies have implicated a role for BDNF signaling in mediating VTA DA neuron soma size [1] as well as the role of the IRS2-AKT pathway [2]. Given that AKT is only one member of the AGC family of kinases that TORC2 phosphorylates, other kinases within that family can be investigated. For example, effects observed in Purkinje cells identified myristolyated alanine-rich protein kinase C substrate (MARCKS) and growth-associated protein-43 (GAP-43) as two proteins involved in dendritic branching and axon growth, respectively, that had significant decreases in phosphorylation as a result of Rictor knockout [12]. Thus, future investigations for novel VTA TORC2 targets should include MARCKS and GAP-43.

Collectively, these data suggest that VTA TORC2 does not mediate cytoskeletal changes through the Rac1 pathway, both basally and in response to morphine. Future identification of pathways involved in TORC2's regulation of the actin cytoskeleton will be critical for understanding cytoskeletal remodeling in the VTA and in understanding the neuroadaptations that occur in response to chronic morphine treatment for the treatment of addiction and neuropsychiatric diseases.

Acknowledgements, Funding, and Disclosure

We would like to thank Ken Moon for excellent technical assistance. This work was funded by the National Institute on Drug Abuse (R01 DA039895 and R03 DA037426, MMR) and graduate fellowships from the PhRMA foundation and the National Institute of General Medical Sciences (T32 GM092715, SK). We would also like to thank the NIDA Drug Supply Program for generously providing morphine. Finally, we would like to thank Dr. Rachael Neve at the MIT Viral Vector Core for production of the HSV constructs.

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CHAPTER 4

Significance, Summary, and Future Directions

Significance of dissertation

While research on depression and opiate addiction have been ongoing for decades and significant contributions have been made to understand the physiological and behavioral changes that occur, there still remain critical gaps in knowledge that need to be addressed in order to provide effective therapies for patients diagnosed with these diseases. This dissertation investigated the molecular mechanisms by which susceptibility to CSDS and stress-induced morphine reward are potentially mediated by TORC2 in the VTA, and whether previously observed morphological changes in the VTA are modulated by TORC2 through a Rac1-dependent cytoskeletal pathway. At a broader level, work in this dissertation contributed novel information to the field on the role of VTA TORC2 in mediating consummatory behaviors. Furthermore, the work in this dissertation also furthered investigation of downstream signaling mechanisms of TORC2 in mediating actin cytoskeleton changes in a critical part of the reward pathway. While VTA TORC2 was not implicated in mediating susceptibility to chronic social defeat stress, nor was there a change in stress-induced morphine reward measured by the two-bottle choice paradigm, we did identify a novel role for VTA TORC2 signaling in mediating consummatory behaviors of both water and rewarding substances such as morphine and sucrose (Figure 17). Given that VTA TORC2 signaling has been directly linked to morphine-induced alterations in structural plasticity, the mechanisms by which actin cytoskeleton remodeling occurs remains of interest. Despite the literature that has suggested that TORC2-mediated morphological changes could be dependent on Rac1 signaling, we demonstrated that cytoskeletal changes in the VTA are not modulated through Rac1. Excitingly, this paves the way for novel cytoskeletal protein pathways to be explored with respect to TORC2 signaling which will be discussed below. Overall, novel insight was gained from this dissertation on the important role of

TORC2 in the VTA on the rewarding effects of opiates such as morphine, but does not seem to affect the opposite valence of decreasing drug reward or in depression, despite both stimuli inducing a similar overall effect on VTA DA output. Additionally, work presented in this dissertation suggests that the downstream signaling of TORC2 may differ between brain regions.

Summary of findings

Susceptibility to CSDS and stress-induced morphine reward

Previous work demonstrated a role for AKT in mediating susceptibility to CSDS. Specifically, susceptible mice had a decrease in VTA pAKT, and overexpression of a constitutively active AKT in the VTA of susceptible mice rescued social avoidance [1]. Meanwhile, overexpression of a dominant negative AKT increased susceptibility to microdefeat, a modified version of CSDS used to identify critical mediators of susceptibility to stress [1]. Given this information on bi-directional signaling of AKT, we tested the hypothesis that TORC2, which is known to phosphorylate AKT at S473, mediates susceptibility to CSDS. Two models of Rictor knockouts were used for these studies. First, we used a developmental knockout in which Rictor is deleted from all TH-expressing cells in the VTA, but also deletes Rictor in other TH-containing cells in the body as well. To target the VTA more specifically, we injected AAV-Cre into the VTA of floxed Rictor mice, which addresses the aforementioned caveats of using a developmental knockout and also of knocking-out Rictor in other cells. Unfortunately, the caveat with the AAV knockout model is that Rictor is knocked-out in multiple cell types in the VTA, like GABA neurons, instead of only targeting VTA DA neurons. Using both of these knockout models, we determined that both catecholaminergic and VTA-specific knockdown of Rictor, despite decreasing TORC2 signaling and decreasing pAKT, do not increase susceptibility to

CSDS. Importantly, one caveat that can address this finding is that in the Krishnan et al. AKT studies [1], the infection of the HSV is not limited to one neuron type. Multiple neuron types, such as the DA and GABA cells, can both be affected. Thus, this could possibly suggest a role for TORC2-mediated AKT signaling in GABA cells. In addition, we also tested the hypothesis that increasing Rictor expression in the VTA, which increases TORC2 signaling and the phosphorylation of AKT, would rescue susceptibility to CSDS. We followed the experimental design set by Krishnan et al. [1] to overexpress HSV-Rictor into the VTA of susceptible mice. Interestingly, increasing TORC2 signaling in the VTA does not rescue susceptibility to CSDS (data not shown). While increasing TORC2 signaling would increase the phosphorylation of AKT in the VTA, it would also increase the phosphorylation of other TORC2 targets as well and thus, may have conflicting effects on mediating susceptibility to stress. Additionally, it was possible that our social defeat stress was too severe and that rescuing social interaction would not be possible. Thus, we took an alternate approach to determine if VTA TORC2 overexpression *during stress* could have a protective effect on susceptibility to CSDS. This was the case in a study investigating GSK3 signaling in the nucleus accumbens, as they found that viral-mediated alteration of GSK3 activity had to be present during their stress protocol to produce an antidepressant effect [2]. With this in mind, we tested the hypothesis that TORC2 signaling must be increased during stress to prevent a decrease in SI. To do this, we overexpressed Rictor in the VTA of C57BL/6 mice and subjected them to a compressed defeat. In a compressed defeat, mice are subjected to two physical defeats, once in the morning and once in the afternoon, for four consecutive days. This shortened defeat was used due to the short expression period of the HSV used to overexpress Rictor. Surprisingly, overexpression of TORC2 during a defeat was not sufficient to prevent a stress-induced decrease in social interaction. All together, these data

suggest that bi-directional modulation of VTA TORC2 signaling does not mediate susceptibility to CSDS.

We also wanted to determine if VTA TORC2 signaling affected morphine reward following stress. Unpublished data from our lab demonstrate that CSDS mice voluntarily consume more morphine in a two-bottle choice task than non-stressed controls, and this is true for mice exposed to both physical and emotional stress, despite differences in the magnitude of depressive-like behavior at this time point (data not shown). Given this information, we evaluated morphine reward after CSDS and found that knockdown of TORC2 signaling did not affect morphine preference. Because TORC2 phosphorylates a family of kinases and not solely AKT, this suggests that phosphorylation of a different kinase or a combination of kinases are involved with determining susceptibility to CSDS. Moreover, there could be compensatory changes in signaling in response to the increase in TORC2 signaling that could override the effect of AKT on mediating susceptibility to stress. It is also possible that TORC2 could have effects on small GTPases, such as the Rho family of GTPases (discussed in Chapter 3), that can potentially alter behavior as well. Collectively, the results of these experiments suggest that downstream targets of AKT should be investigated and that TORC2 itself would not be an optimal target for pharmaceutical treatment.

Novel role of VTA TORC2 signaling

Unrelated to our hypothesis, we discovered a novel role for VTA TORC2 in mediating consummatory behaviors both in naïve mice and mice that underwent CSDS (Figure 17). Briefly, in stress-naïve mice, we discovered that male TH-Rictor-KO mice consumed more water, sucrose, and morphine compared to their littermate controls with no alteration in preference for

sucrose and morphine [3]. Interestingly in female TH-Rictor-KO mice, an increase in drinking was only observed with morphine without a change in preference for morphine [3]. These findings suggest that sex plays a role in the consumption of water and rewarding substances such as sucrose, which was unexpected. Interestingly, we also discovered that female TH-Rictor-KO mice do not have the increase in novelty-induced locomotor activity that has been observed in males [4]. Again, this could suggest that sex plays a role in modulating motivation to explore a novel environment or in locomotor activity itself. The VTA-Rictor-KO model also contributed novel insight into TORC2's role in modulating consummatory behavior. While there were no differences in fluid intake volume between Cre knockdowns and GFP controls, a stress-induced increase in drinking was seen in the male VTA-Rictor-KO mice after physical CSDS. This change in drinking suggests that TORC2 signaling does contribute to stress responses and can affect post-stress behaviors independently of social interaction.

Collectively, these data suggest that the VTA may be an area of interest in understanding the physiological basis of liquid consumption and future studies should consider looking at the dysregulation of VTA TORC2 in disorders such as polydipsia, in which patients have an increase in thirst.

Importantly, this work also has implications for evaluating sex differences in studies involving TORC2. In addition to looking at liquid consumption, we also briefly investigated food consumption in our TH-Rictor-KO mice. Given that the dopaminergic-rich VTA region is a key component of the reward system, Dadalko et al. investigated the role of TORC2 signaling on the consumption of high fat diet (HFD), a palatable food, in male TH-Rictor-KO mice [4]. The decrease in TORC2 signaling in catecholaminergic cells increased HFD intake within the first six days of the study when normalized to low fat diet and overall, consumption of HFD over an

eight week span was increased significantly compared to littermate controls. Because this study was only performed in males, we wanted to determine if there were any differences in HFD consumption between sexes. Thus, we performed a one week study comparing male and female TH-Rictor-KO mice on HFD. We found that when the HFD amount was normalized to body weight, there were no significant differences in consumption of HFD between male and female KO and wildtype mice. In our studies, we had evaluated baseline food consumption with standard rodent chow, while Dadalko et al. study compared their food changes to low fat diet [4], which could explain why our studies did not find a difference in consumption between KO and wildtype mice. Overall, our food consumption studies suggest that there may not be a difference in HFD consumption between KO and wildtype female mice.

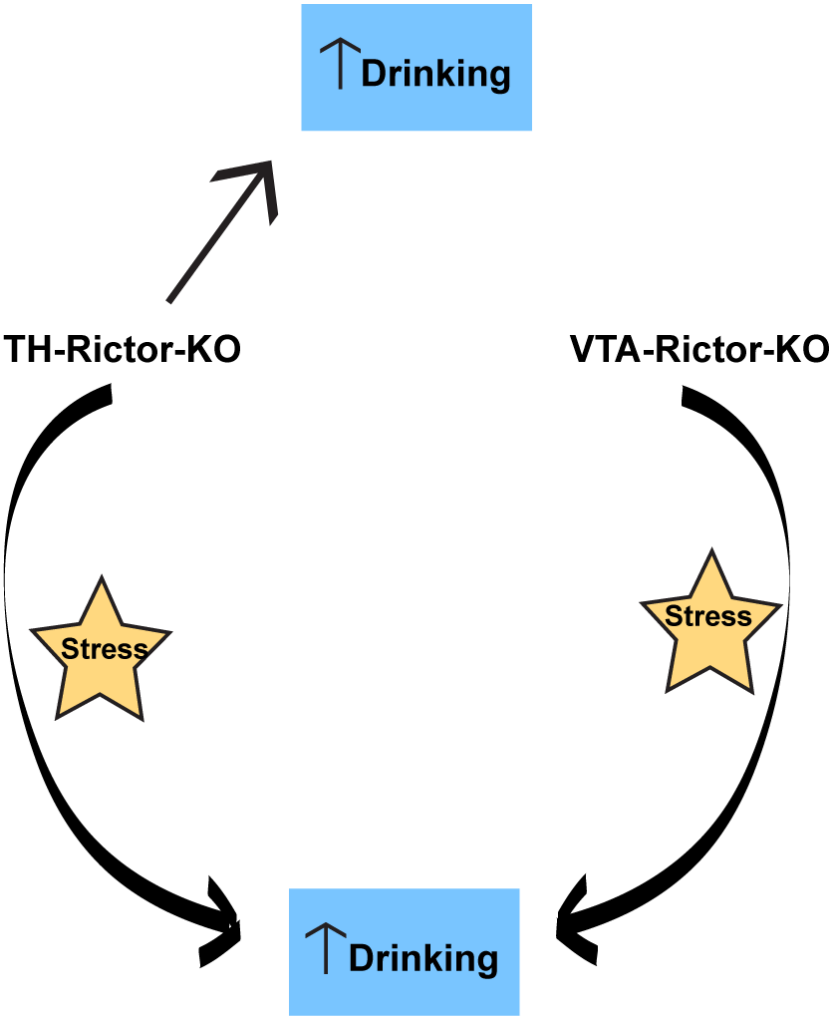

Figure 17: Novel role for VTA TORC2 in mediating consummatory behaviors. Catecholaminergic deletion of Rictor (TH-Rictor-KO) increases drinking of water and rewarding substances, such as sucrose and morphine, both in the absence and presence of stress. VTA-specific knockdown of Rictor (VTA-Rictor-KO), on the other hand, affected consummatory behaviors only in the presence of stress.

Modulation of actin cytoskeleton in VTA by TORC2

Neurons respond to stimuli and undergo remodeling of dendritic spines, dendritic branches, or can even change morphology through a process called structural plasticity [5, 6]. The changes in dendritic spines can then proceed to alter connectivity between neurons and alter their function [5]. Importantly, TORC2 signaling has been implicated in mediating changes in structural plasticity, but the mechanisms underlying these changes appear to differ between brain regions. Specific mechanisms for TORC2's role in cytoskeletal reorganization have been established in the hippocampus [7] and the cerebellum [8], but through two different mechanisms, highlighting the necessity to investigate TORC2 signaling in the VTA. While modulation of Rictor expression in the VTA modulates VTA DA neuron soma size [6], the cytoskeletal proteins downstream of TORC2 that are involved in mediating these changes have not been investigated specifically in the VTA. Thus, we sought to investigate VTA TORC2-mediated Rac1 regulation of the actin cytoskeleton, both basally and in response to chronic morphine. Even though decreasing TORC2 signaling in the VTA using viral vectors did not alter Rac1 expression or the phosphorylation of its downstream targets, future studies will be able to rule out the Rac1-PAK-Cofilin pathway in mediating these changes (Figure 18). Given the changes in structural plasticity observed in the cerebellum, future studies could determine if VTA TORC2 mediates these changes through PKC to alter MARCKS and GAP-43 phosphorylation (Figure 18). While it is unknown whether TORC2 alters dendritic spine morphology, it may still be interesting to investigate if VTA TORC2 modulates the actin cytoskeleton through MARCKS, as it is critical for mediating dendritic branching in rat primary hippocampal neurons [9]. Regulation of the cytoskeleton through GAP-43 may be less likely, as it usually plays a role in axonal pathfinding during development and is also known to play a role

in the development of new synapses [10]. Overall, the results of this chapter demonstrate that regulation of the cytoskeleton in the VTA through TORC2 signaling is not similar to the hippocampus, which will allow future studies to focus on other pathways.

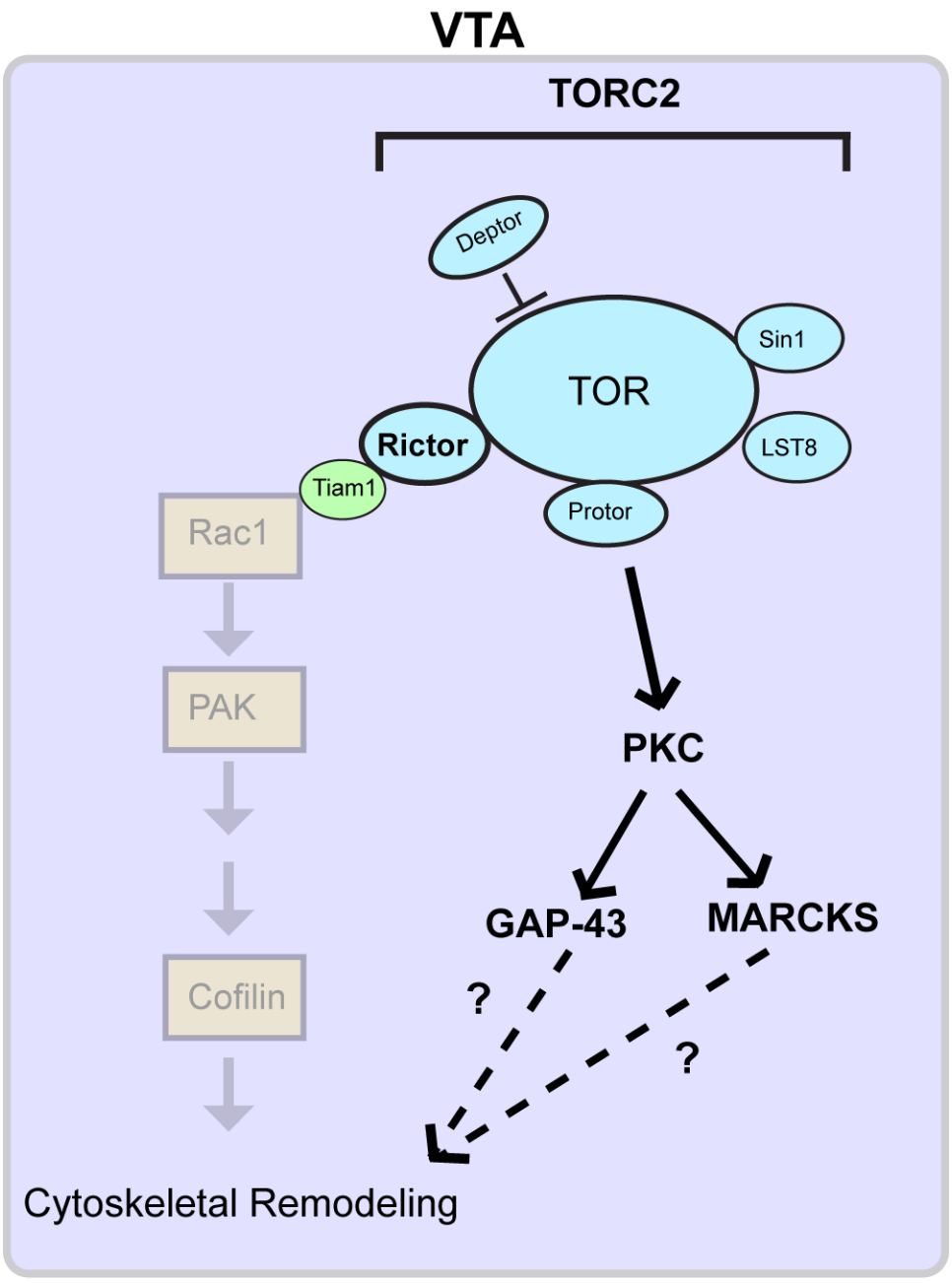

Figure 18: VTA TORC2 does not modulate the actin cytoskeleton through a Rac1-dependent manner. TORC2-mediated cytoskeleton remodeling pathways that were investigated in other brain regions can be explored in the VTA.

Future directions

Investigating VTA TORC2 function in consummatory behaviors

We unexpectedly discovered a novel role for VTA TORC2 in mediating consummatory behaviors, which presents an opportunity for a wide range of future studies. First, in the absence of stress, the increase in drinking volume is driven by deletion of Rictor in catecholaminergic neurons but not VTA neurons specifically. Because we did not observe changes in consummatory behavior in VTA- specific knockout mice, this suggests that TH-containing cells in other areas of the brain and/or body could be mediating these changes. Importantly, since TH is the rate-limiting enzyme for catecholamine synthesis of both dopamine and norepinephrine, these changes could be driven by TORC2 signaling in either noradrenergic or dopaminergic neurons. To determine if these changes in drinking are due to dopamine or norepinephrine, dopamine transporter (DAT)-Cre or norepinephrine transporter (NET)- Cre mice, respectively, can be crossed with the floxed Rictor line for molecular and behavioral studies. As most of these changes were detected in developmental knockout mice, additional studies could also be performed by injecting AAV-Cre into other brain regions of interest that contain TH to determine the role of TORC signaling in specific brain regions in modulating consummatory behavior.

In addition to their altered consummatory behavior, TH-Rictor-KO mice also have altered behavioral activity. Specifically, male TH-Rictor-KO mice have increased novelty-induced locomotor activity [4], but what remains unknown is whether or not their baseline locomotor activity is altered. Furthermore, the overall metabolic activity of TH-Rictor-KO animals can be investigated to determine if the increase in liquid consumption that we observed is linked to their

baseline locomotor activity. Additionally, because there is a significant increase in fluid consumption in the TH-Rictor-KO mice, future studies could investigate how their bodily fluid balance is maintained by measuring urine output through metabolic cages. This would yield critical knowledge on how catecholamines may regulate liquid intake, urine output, and overall homeostatic regulation of bodily fluids. Together, results from these studies may have clinical implications in disorders where fluid dysregulation occurs.

While VTA-Rictor-KO did not affect baseline drinking, consumption of water and sucrose increased following CSDS compared to control mice, suggesting that even though VTA TORC2 does not mediate susceptibility to CSDS, it is still involved in stress-induced consummatory behavior. Because injection of AAV-Cre-GFP into the VTA can knock-down Rictor expression in multiple cell types, future studies can determine which neurons, whether they are dopaminergic, GABAergic, or glutamatergic, are driving these changes in stress-induced consumption. In order to determine which neurons are involved, Cre-dependent CRISPR/Cas 9 viruses can be injected into the VTA of mice expressing Cre for each cell type (TH or dopamine transporter (DAT) for dopaminergic cells, vesicular GABA transporter (VGAT) for GABAergic cells, and vesicular glutamate transporter (VGLUT) for glutamatergic cells) with a guide RNA that targets Rictor for knockout. In addition, it would be interesting to see how decreasing TORC2 signaling through a decrease in Rictor affects communication between brain regions such as the lateral hypothalamus, which coordinates behavioral cues to facilitate eating and drinking, for example, to maintain proper energy balance [11]. Because physical CSDS was the only type of stress that was investigated in VTA-Rictor-KO mice, future studies can also investigate the effects of emotional stress in VTA-Rictor-KO mice. Since VTA-Rictor-KO mice only had altered drinking after exposure to stress, the hypothesis would be that emotionally

stressed VTA-Rictor-KO mice would also experience similar increases in consummatory behaviors.

Additionally, we wanted to investigate whether or not sex is a biological factor in our behavioral studies. Chapter two is the first publication that has explored sex as a biological factor in the characterization of floxed Rictor mice. The significant increase in drinking was observed in stress-naïve male TH-Rictor-KO mice but not females, and deserves additional investigation. It would be interesting to investigate if sex-related hormones are involved in TORC2-mediated consummatory behavior. For example, ovariectomies can be used to stop estrogen production [12] in order to determine if estrogen drives TORC2-mediated drinking behavior. Similarly, castration can be used to interrupt testosterone production in males. In addition, the degree or sensitivity of which floxed Rictor mice prefer rewarding substances would also be interesting to investigate, as female mice were more sensitive to morphine compared to males. In our two-bottle choice assay, the morphine to quinine ratios were adjusted to yield a 70% preference in wildtype, stress-naïve mice. Our female mice had a strong preference for morphine as a 0.1 mg/mL concentration initially gave an ~80% preference. Thus, we performed a dose response experiment and determined that 0.05 mg/mL morphine was the optimal dose to obtain a 70% preference. Lastly, one limitation of the CSDS paradigm is that only male mice can be used as experimental mice. This is because the aggressors used are male retired breeders, who would attempt to mate with female experimental mice instead of physically attacking them. To overcome this technical difficulty, alternate models of stress, such as subchronic variable stress, utilize varied physical stressors to induce depressive-like symptoms in both male and female rodents [13, 14]. Other models are currently being adapted and validated in order to be able to add female cohorts to studies. In fact, vicarious social defeat stress has been optimized for

female mice. This allows female mice to experience stress from witnessing physical stressors and allows for direct comparison with male witnesses without risking an attack from a male aggressor [15]. Another laboratory has also been investigating methods to induce aggressive behavior in male mice such that they will physically attack female mice in order to adapt and expand physical CSDS to female mice (unpublished). As paradigms are either modified or created to incorporate female mice into CSDS studies, the stress-induced drinking studies can also be repeated for both the TH-Rictor-KO mice and the VTA-Rictor-KO mice to look at the effects of physical and emotional stress on consummatory behaviors in order to investigate and address sex as a biological factor in stress and stress-induced consummatory behavior.

Identifying novel targets of VTA TORC2 in mediating actin cytoskeleton changes

While changes in the actin cytoskeleton are mediated by TORC2 signaling through the Rac1-PAK-Cofilin pathway in the hippocampus [7], work in this dissertation demonstrated that TORC2 does not utilize the same Rac1-mediated pathway in the VTA. This is not completely unexpected, as TORC2 was shown to mediate changes in Purkinje cells of the cerebellum through the PKC pathway by decreasing phosphorylation of MARCKS and GAP43 [8], which did suggest the possibility of VTA TORC2 mediating the cytoskeleton through a pathway unrelated to Rac1. Similar to what was investigated in the cerebellum, a potential future study could be to determine if MARCKS and/or GAP43 phosphorylation are decreased in the VTA when TORC2 signaling is decreased. Preliminary investigations from our lab suggest that the phosphorylation of MARCKS is not altered by chronic morphine, but a larger study must be conducted to be able to draw a firm conclusion.

The actin cytoskeleton consists of a complex network of proteins in which cross-talk between downstream proteins and pathways can occur. Even though our data demonstrates that VTA TORC2 signaling does not function through Rac1, there are other cytoskeletal GTPases that TORC2 may regulate. While Rac1, Rho A, and Cdc42 are the most characterized GTPases, there are a total of 18 mammalian GTPases that have been discovered so far [16] that TORC2 could be interacting with to alter the actin cytoskeleton. *In vitro* studies have identified Rap1 as a small GTPase that binds to SIN 1 [17], another constituent of TORC2, that may be of interest for investigation. Future studies would need to first determine if Rap1 is expressed in the VTA, and if so, co-immunoprecipitation studies could be completed to determine if Rap1 also binds to SIN1 in the VTA. If those interactions hold true in the VTA, future studies could then focus on downstream targets of Rap1.

Considering that there was no change in Rac1 activity in response to chronic morphine, another avenue of potential research is to investigate whether the cellular localization of TORC2 is altered by chronic morphine. To briefly recapitulate, TORC2 is localized to the endoplasmic reticulum [18] and then associates with the ribosome when active [19]. TORC2 can also be localized to membrane rafts through a scaffolding complex called synecan-4, a process shown to control endothelial cell size [20]. It would be interesting to see if chronic morphine affects the expression of syndecan-4, which would alter TORC2's ability to locate near the plasma membrane. Given the complexity of the actin cytoskeleton, multiple avenues will need be investigated in future studies to determine how VTA TORC2 regulates the actin cytoskeleton such as other AGC kinases that are downstream of TORC2 and their targets, different GTPases, or even the localization of TORC2 itself.

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