NOVEL ROLES FOR THE ACTIVITY-REGUALTED GENES *ARC* AND *NPAS4* IN

STRESS- AND COCAINE-INDUCED PLASTICITY

APPROVED BY SUPERVISORY COMMITTEE

Christopher W. Cowan, Ph.D.

Kimberly M. Huber, Ph.D., Chair

Eric N. Olson, Ph.D.

Andrew R. Zinn, M.D., Ph.D.

DEDICATION

There are a number of individuals to whom I owe a great deal of gratitude during the course of my graduate training. First and foremost, I thank my family – my mom, dad, my older sister Amy, and my younger brother Mannie. They have stuck by me through the multiple years I have spent thus far in graduate and medical school, never losing patience or faith in me, and constantly providing reassurance that hard work does pay off. In particular I have to thank my father, Dr. Devinder Kumar, who, for as long as I can remember, has been a role model for me. As a practicing anesthesiologist who treats patients suffering from chronic pain, he was the one who introduced me to the world of medicine and inspired me not just to enter the medical field, but also to enjoy what I do. Without him challenging me throughout the years and without his confidence in my capabilities, I wouldn't have arrived at this moment.

I also want to thank my scientific mentors who have been an inspiration and source of guidance for me these past several years. I would be remiss if I didn't thank Drs. Colleen McClung and Michael Lutter, who together introduced me to the field of molecular psychiatry, and who were pivotal in helping me discover and shape my passion for neuroscience research. Working alongside them, I was able to complete a project that amounted to a first-author publication, for which I am very grateful.

I must also thank Dr. Christopher Cowan, my eventual thesis advisor and graduate school mentor. Dr. Cowan was gracious enough to take me into his lab and grant me the opportunity to pursue projects with an independence and open mindedness that is a luxury to have in graduate school. His remarkable ability to conduct scientific inquiry with an unparalleled breadth and depth of analysis has been a sight to see. It has truly been my pleasure to learn from him how to think through science in the careful and insightful manner that he does.

I would also like to thank members of the Cowan lab for their support and camaraderie. Thank you to Makoto Taniguchi, Laura Smith, Rachel Penrod-Martin, Adam Harrington, Maria Carrerira, Jakub Jednyak, Yuhong Guo, Ben Zirlin, Carly Hale, and Michael Robichaux, not just for your technical guidance, but also for being there for me, as a colleague, as a friend, and as someone I can always call on for help, no matter the reason. Additionally, I must thank members of the UTSW community, past and present, who were instrumental in my training, including Dr. James Bibb, Dr. Shari Birnabum, Dr. Jeffrey Zigman, Dr. Lisa Monteggia, as well as countless other individuals who made graduate school the enjoyable experience that it was (Florian Plattner, Karine Pozo, Scott Philibin, Angela Walker, Melissa Bawa, Daniel Guzman, Angela Desmond, and my many MSTP colleagues). I must also thank members of the McLean Hospital community and other friends in the Boston area for support (James O'Neil, Darrick Balu, Magdalena Ardelt, Sarah Mauney, Matej Markota, and Rachel Donahue). I also can't forget to acknowledge Dr.

Bahareh Azizi, my freshman chemistry TA from college who was my very first research mentor, and who convinced me to get a PhD in the first place.

There is a seemingly endless supply of questions that can be asked about human emotion and cognition, and the excitement that such questions generate is what I credit with helping me wake up every morning over the past several years. In my lifetime, I hope to be able to witness the biological mysteries that guide human behavior and psychopathology finally solved. My work, both in this document and what I hope follows it, will undoubtedly contribute very little to the vast knowledge base of psychiatry that has been generated before my time, and that continues to grow at an exponential rate. Nonetheless, it has been an honor to have been given the time, funding, mentorship, and confidence to conduct basic research in psychiatry. And in a world where we now see substance abuse, depression, and autism increasing, not decreasing, in prevalence, it is incumbent upon us as scientists and clinicians to use our knowledge and skillsets to help alleviate the chronic suffering wrought by mental illness. It is my hope that in the future, my work will be guided by this basic principle.

NOVEL ROLES FOR THE ACTIVITY-REGULATED GENES *ARC* AND *NPAS4* IN STRESS- AND COCAINE-INDUCED PLASTICITY

by

JASWINDER SINGH KUMAR

DISSERTATION / THESIS

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

May, 2016

NOVEL ROLES FOR THE ACTIVITY-REGULATED GENES *ARC* AND *NPAS4* IN STRESS- AND COCAINE-INDUCED PLASTICITY

Jaswinder Kumar, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2016

Supervising Professor: Christopher W. Cowan, Ph.D.

Mood, anxiety, and substance abuse disorders are chronic medical illnesses that contribute significantly to morbidity and mortality worldwide. Currently, these conditions are treated symptomatically using pharmacological and psychotherapeutic approaches; however, the efficacy of these modalities is limited by the dearth of understanding of neurobiological mechanisms underlying mental illness. The high rate of mortality associated with mood, anxiety, and substance abuse disorders is compounded by their shared comorbidity, warranting an investigation into potential shared pathophysiological mechanisms. Studies from

human patients and rodent models suggest that these mechanisms may be attributed to disrupted structural and functional plasticity in brain regions involved in mood, reward, and motivation, including the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC). However, the molecules and signaling pathways within these structures that regulate these behaviors, and how they are dysregulated in pathological psychiatric conditions, have yet to be fully identified and characterized. Here, we focus on two key proteins that participate in activity-dependent synaptic plasticity, the neuronal Per Arnt Sim Domain protein 4 (NPAS4) and the activity-regulated cytoskeleton-associated protein (Arc). We utilize a series of ethologically relevant behavioral paradigms to identify Arc and NPAS4 as two important mediators of stress, anxiety, and addiction-related behaviors. *Npas4* and *Arc*, two activity-regulated genes, are robustly induced by stressful, anxiogenic stimuli. Loss of either gene confers an antidepressant and anxiolytic response in mice, and these behavioral phenotypes are mediated by local function of these two proteins in limbic forebrain regions. In a related study, we ask whether loss of Arc influences behavioral responses to cocaine administration. We find that *Arc* knockout (KO) animals exhibit increased sensitivity to the locomotor activating and rewarding effects of cocaine, and these two phenotypes are associated with a selective increase in synaptic strength in the NAc. Taken together, our results highlight a heretofore-unidentified role for Arc and NPAS4 in stress- and anxiety-like behaviors, as well as Arc in cocaine-related behavioral adaptations. We propose that these two

molecules play a vital role in regulating synaptic and behavioral plasticity evoked by exposure to stress and drugs of abuse, likely via experience-dependent synaptic remodeling.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Kumar, J., Chuang, J.C., Na, E.S., Kuperman, A., Gillman, A.G., Mukherjee, S., Zigman, J.M., McClung, C.A., and Lutter, M. (2013). Differential effects of chronic social stress and fluoxetine on meal patterns in mice. Appetite *64*, 81-88.

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

ΔΔCt – delta delta cycles threshold
ΔCt – delta cycles threshold
5-HT – serotonin
aCSF – artificial cerebrospinal fluid
AgRP – agouti-related peptide
AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR – AMPA receptor
ANOVA – analysis of variance
Arc – activity-regulated cytoskeleton-associated protein
ARC – arcuate nucleus of the hypothalamus
BDNF – brain-derived neurotrophic factor
BOLD – blood-oxygen-level dependent
BS^3 – bis(sulfosuccinImidyl) suberate
BSA – bovine serum albumin
C – Celsius
CA1 – cornu ammonis area 1
Ca^{2+} – calcium
CamK – calcium/calmodulin-dependent kinase
cAMP – cyclic adenosine monophosphate
CBP/p300 – CREB-binding protein
CBT – cognitive behavioral therapy
ChIP-seq – chromatin immunoprecipitation-deep sequencing
cm – centimeter
coc – cocaine
CPP – conditioned place preference
CPu – caudate putamen
Cre – cre recombinase
CREB – cAMP response element binding protein
CSDS – chronic social defeat stress
CUS – chronic unpredictable stress
d – day
D/L – dark/light exploration test
D1R – dopamine 1 receptor
DA – dopamine
DAG – diacylglycerol
DAergic – dopaminergic
DAPI – 4',6-diamidino-2-phenylindole

DMEM – Dulbecco's modified Eagle's medium
DNA – deoxyribonucleic acid
DSM – Diagnostic and Statistical Manual
DTT – dithiothreitol
E/I – excitatory/inhibitory
EDTA – ethylenediaminetetraacetic acid
eEF2 – eukaryotic elongation factor 2
EPM – elevated plus maze
ERK – extracellular-related kinase
Fig – figure
fMRI – functional magnetic resonance imaging
FST – forced swim test
g – grams
G protein – guanosine nucleotide-binding proteins
GABA – γ-aminobutyric acid
GFP – green fluorescent protein
GluA1 – glutamate receptor subunit A1
GluA2/3 – glutamate receptor subunit A2/A3
GPCR – G protein coupled receptor
Gria1 – glutamate receptor 1 gene
Hab – habituation
HAT – histone acetyltransferase
HCl – hydrochloric acid
HDAC – histone deacetylase
HEK – human embryonic kidney
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Het – heterozygous
hr – hour
HSV – herpes simplex virus
i.p. – intraperitoneal
Inj – injection
IP – immunoprecipitation
IP_3 – inositol triphosphate
kDa – kilodalton
kg – kilogram
KO – knockout
Loc – locomotor response to novelty
LTD – long-term depression
LTP – long-term potentiation

MAO – monoamine oxidase
MAO-I – monoamine oxidase inhibitor
MAPK – mitogen-activated protein kinase
MC4R – melanocortin-4-receptor
MDD – major depressive disorder
MEF2 – myocyte enhancer factor 2
mEPSC – miniature excitatory postsynaptic current
mg – milligram
min – minute
miRNA – microRNA
mL – milliliter
mm – millimeter
MNK – MAP kinase interacting kinase
mOsm – milliosmole
mPFC – medial prefrontal cortex
mRNA – messenger RNA
MSN – medium spiny neuron
mTOR – mammalian target of rapamycin
n.s. – not significant
NAc – nucleus accumbens
NaCl – sodium chloride
NaF – sodium fluoride
NaN_3 – sodium azide
NE – norepinephrine
NMDA – *N*-Methyl-D-aspartate
NPAS4 – neuronal Per Arnt Sim domain 4
NPY – neuropeptide Y
NTS – nucleus tractus solitarius
VTA – ventral tegmental area
ODN – oligodeoxynucleotide
OF – open field
P-S279 – phospho-serine 279
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PFA – paraformaldehyde
PFC – prefrontal cortex
PKA – protein kinase A
PLC – phospholipase C
PKC – protein kinase C

PMSF – phenylmethanesulfonylfluoride
POMC – pro-opiomelanocortin
Post – posttest
Pre – pretest
PTSD – posttraumatic stress disorder
PVDF – polyvinylidene fluoride
PVN – paraventricular nucleus of the hypothalamus
qRT-PCR – quantitative real-time polymerase chain reaction
racl – raclopride
RIPA – radioimmunoprecipitation assay
RMANOVA – repeated measures ANOVA
RNA – ribonucleic acid
S279 – serine-279
sal – saline
Scr – scramble
SDS – sodium dodecyl sulfate
SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis
shRNA – short hairpin RNA
SNRI – serotonin norepinephrine reuptake inhibitor
SRF – serum response factor
SSRI – serotonin specific reuptake inhibitor
TBS-T – tris-buffered saline with Tween20
TCA – tricylclic antidepressant
TrkB – tropomyosin-related kinase B
UBE3A – ubiquitin-protein ligase E3A
ug – microgram
uL – microliter
V – volt
VTA – ventral tegmental area
WT – wild-type
ZT – zeitgeber

CHAPTER ONE
Introduction

STRESS, DEPRESSION, AND ANXIETY

Stress-related disorders, including depression and anxiety, represent a complex, heterogeneous set of mental disorders characterized by psychological and physiological symptoms such as anhedonia, hopelessness, change of appetite, impaired cognitive function, disruption of circadian rhythms, and relentless fear and avoidance. Patients with depression and/or anxiety exhibit disturbances in anatomy and metabolic activity within specific brain regions, including the striatum, prefrontal cortex, hippocampus, and amygdala, suggesting dysfunction of corticolimbic reward circuitry. In cases where standard antidepressant and anxiolytic medications fail, deep brain stimulation of these regions has proven to be a successful treatment modality. Nonetheless, given the cursory understanding of disease mechanism and lack of rational therapeutics, these conditions still exert a staggering toll on society and are linked to significant morbidity and mortality. As such, a more thorough understanding of etiology and pathophysiology is warranted, and is the subject of this document. In this introduction, we provide an overview of stress-related disorders, including depression and anxiety disorders, focusing on clinical aspects of these illnesses alongside relevant neuro-pharmacology and -biology that are postulated to underlie the development and treatment of these conditions.

Stress-related disorders are a leading cause of morbidity and mortality

Major depressive disorder (MDD) is the most common of all psychiatric disorders. With a lifetime risk of 7-12% in men and 20-25% in women, MDD ranks among the top causes of disease burden and disability worldwide (Kessler et al., 2005). Despite the availability of moderately effective treatments, such as selective serotonin reuptake inhibitors (SSRIs) and psychotherapy, up to 20% of patients are unresponsive to these conventional interventions, while nearly 60% achieve only partial remission of symptoms (Keller et al., 1992). Just as common and debilitating are the anxiety disorders, which include panic disorder, post-traumatic stress disorder (PTSD), phobias, and generalized anxiety disorder. Together, they constitute a combined lifetime prevalence of over 28%, and patients show similar failure rates of response to standard therapies. (Greenberg et al., 1999).

Accumulating evidence points to shared psychological and neurobiological mechanisms of depression and anxiety disorders, and therefore, these two entities can be considered as one and the same, for several reasons. First, there is an extremely high comorbidity between the two conditions; studies indicate that 90% of patients with anxiety disorders experience MDD at some point in their lifetime (Gorman, 1996). Second, the symptom criteria, as defined by the Diagnostic and Statistical Manual of Mental Disorders (DSM), overlap extensively between the two disorders. Additionally, from a neuroimaging perspective, the neural circuits that are

thought to be dysregulated under both conditions are oftentimes difficult to distinguish. Finally, the most effective, albeit not fully adequate, therapies directed against both conditions are the same, including antidepressants such as SSRIs and cognitive-behavioral therapy (CBT) (Ressler and Mayberg, 2007).

Diagnostic criteria for stress-related disorders

The DSM sets out the criteria utilized to assign a diagnosis of MDD (American Psychiatric Association. and American Psychiatric Association. Task Force on DSM-IV., 1994). Generally, five or more of the following symptoms must be present during a continuous 2-week period. These symptoms include: (1) depressed mood most of the day, nearly every day, as indicated by either subjective report by the patient or observation made by others (such as the patient's family, or mental health professional); (2) markedly diminished interest or pleasure in all, or nearly all, activities that used to be once pleasurable, or what is commonly referred to as anhedonia; (3) significant weight loss when not dieting or significant weight gain, or a decrease or increase in appetite; (4) insomnia or hypersomnia nearly every day; (5) feelings of worthlessness or guilt nearly every day; (6) neurocognitive deficits, including decreased ability to think or concentrate, or indecisiveness; (7) frequent thoughts of death, suicidal ideation without a specific plan, or a suicide attempt or specific plan to commit suicide. For a diagnosis of MDD, at least one of the symptoms must be either depressed mood or anhedonia, and symptoms that are

secondary to a general medical condition like chronic terminal diseases, are not included in the diagnostic assessment. The same is true for symptoms induced by administration of a substance such as prescription drugs or illicit drugs of abuse.

There are additional details related to the 5-symptom classification that are relevant to a diagnosis of MDD. First, the symptoms do not meet criteria for a mixed, manic-depressive episode. Second, the symptoms themselves cause impairment in areas of functioning that are important to the life of the patient, such as social and occupational life. Third, the symptoms cannot be attributed to bereavement, that is, the loss of a loved one, and finally, the symptoms persist for longer than 2 months or are characterized by preoccupation with worthlessness, suicide, psychotic symptoms, or psychomotor retardation.

For anxiety disorders, the common thread amongst various presentations of the illness is a “marked, persistent, and excessive or unreasonable fear” that significantly interferes with everyday life (American Psychiatric Association. and American Psychiatric Association. Task Force on DSM-IV., 1994). Under conditions of elevated psychological stress, a momentary anxiety response to threats in the environment is an adaptive behavior that permits survival. However, in its pathological form, anxiety can severely interfere with normal life. This latter form of anxiety can be divided generally into two categories: innate anxiety, a measure of acute levels of anxiety, and learned anxiety, a long-term tendency of an individual to show anxiety in response to external events (Gross and Hen, 2004). Beneath these

two broad classifications, the DSM categorizes anxiety into six disorders: generalized anxiety disorder, social phobia, simple phobia, panic disorder, post-traumatic stress disorder (PTSD), and obsessive-compulsive disorder (OCD). The signs and symptoms associated with these various subdivisions of anxiety disorder include: (1) avoidance of places or people from which escape is difficult (agoraphobia and social phobia), (2) sudden, intense onset of fearfulness (panic attack), with physical signs such as increased respiration and heart rate, (3) re-experiencing a traumatic event, resulting in elevations in arousal and avoidance of contexts or cues associated with the event (PTSD), and (4) anxiety-provoking obsessions and anxiety-reducing compulsions (OCD) (American Psychiatric Association. and American Psychiatric Association. Task Force on DSM-IV., 1994). Despite the wide range of anxieties encompassed by these disorders, all appear to share similar behavioral and neurobiological characteristics, as most anxiety disorders respond positively to anxiolytic drugs that target specific neurotransmitter systems in the brain.

Current therapies used to treat stress-related disorders

The very first antidepressant and anxiolytic therapies were discovered in the 1950s, and to this day, commonly prescribed agents used to treat these illnesses are based on these same founder drugs. Their primary mode of action is to elevate synaptic levels of the monoamines serotonin and norepinephrine by modulating their

synthesis, degradation, release, or synaptic availability. Based on their initially reported efficacy in patients with MDD, these drugs, including the monoamine oxidase inhibitors (MAO-Is), tricyclic antidepressants (TCAs), and serotonin and norepinephrine-specific reuptake inhibitors (SSRIs and SNRIs), gave birth to the monoamine deficiency hypothesis of MDD and anxiety disorders. This theory postulates that the etiology and pathophysiology of these conditions stem from a deficiency in serotonin or norepinephrine neurotransmission. Monoaminergic neurotransmission is mediated by serotonin (5-HT) or norepinephrine (NE) released from terminals of presynaptic neurons. Both 5-HT and NE are stored in presynaptic vesicles and released into the synaptic cleft, where they bind to both pre- and postsynaptic receptors and exert physiological effects (see below). The downstream effects of neurotransmitter binding are terminated via two distinct routes: (1) reuptake through 5-HT and NE transporters, and (2) negative feedback control of vesicle release through the presynaptic 5-HT_{1A} and 5-HT_{1B} autoreceptors for 5-HT and the α_2-noradrenergic autoreceptors for NE (Belmaker and Agam, 2008). In addition, the enzyme MAO-A catabolizes monoamines in the presynaptic neuron, thereby regulating the availability of neurotransmitter for vesicular packaging.

Postsynaptically, both serotonin and norepinephrine bind two types of guanine nucleotide triphosphate-binding protein (G protein)-coupled receptors: G_s-coupled receptors, which activate adenylate cyclase to generate cyclic adenosine monophosphate (cAMP), and G_q-coupled receptors, which activate phospholipase C

(PLC). Upon cAMP generation, the cAMP-dependent protein kinase A (PKA) is activated; PLC generates inositol triphosphate (IP_3) and diacylglycerol (DAG), resulting in an intracellular rise in the second messenger Ca^{2+} and subsequent activation of protein kinase C (PKC). Together, both PKA and PKC function to regulate various cellular signaling pathways that ultimately converge on the nucleus to regulate gene expression and neuroplasticity within the central nervous system (Pittenger and Duman, 2008).

Treatment for anxiety has generally involved the use of SSRIs as well as drugs with sedating properties, including barbiturates, opiates, beta-blockers, and benzodiazepines (Nemeroff, 2003). Of these, the benzodiazepines are the most widely prescribed, owing to their enhanced specificity and efficacy. These drugs' primary mechanism of action is to increase the potency of GABA (γ-aminobutyric acid), the major inhibitory neurotransmitter of the brain, by increasing the function of $GABA_A$ receptors (Martin, 1987). On the basis of the effectiveness of GABA-enhancing drugs, it has been proposed that imbalance of excitatory vs. inhibitory neurotransmission is an important pathophysiological signature of stress-related disorders (Luscher et al., 2011). However, the precise neuroanatomy and biological mechanism underlying these presumed changes in circuit function are not known.

While SSRIs and benzodiazepines are used widely and oftentimes successfully to treat depression and anxiety disorders, these drugs are associated with a number of clinical challenges. First, not all patients achieve full remission, with

up to 50% of patients reporting little to no relief in response to combinations of SSRIs and CBT (Berton and Nestler, 2006). Second, the side effect profiles associated with long-term use of these drugs include sedation and dependence in the case of benzodiazepines, and obesity, diabetes, or in unfortunate scenarios, suicide, with SSRIs. Another important criticism of the use of the SSRIs is that, despite acutely elevating brain levels of neurotransmitter, these agents require weeks, if not months, of dosing before remission is actually achieved. In critical emergent settings, when treatment-resistant patients present with life-threatening symptoms, immediate relief is oftentimes necessary to prevent further suffering. For these reasons, the last few decades of research have focused on better understanding the neurobiology and pharmacology of stress disorders, so as to identify and characterize novel targets that may be used to develop more efficacious therapeutics.

Preclinical models of stress- and anxiety-related disorders

One of the major predisposing risk factors for MDD is exposure to trauma and psychological stress; accordingly, in psychological terms, this condition can be conceptualized as the inability to cope with stress. On that basis, many preclinical models and assays for measuring depression-related behavior involve exposure to stressful situations. Of these experimental procedures, the forced swim test (FST), a test of behavioral despair, is probably the most commonly utilized (Petit-Demouliere et al., 2005; Porsolt et al., 1978; Porsolt et al., 1977). The FST is based on a

predictable, highly replicable finding observed when rodents are placed in an enclosed, inescapable cylinder filled with water: the animal will initially mount a series of vigorous escape behaviors. Minutes thereafter, though, the animal will display immobility, with the number of bouts of immobility increasing with time. There are two major strengths of the FST as a test of depression-like behavior. First, it has high predictive validity; that is, administration of antidepressant drugs before the test, including SSRIs and TCAs, causes rodents to more actively and persistently exhibit escape-oriented behaviors for longer periods of time. As such, the FST has been widely accepted as a rapid screening assay to test the effects of novel antidepressants. Second, tests of behavioral despair, including the FST, have been shown to be sensitive to a number of different factors that can either precipitate or result from MDD in humans, such as genetic vulnerability, prior exposure to stress, changes in body weight homeostasis, sleep disturbances, and anhedonia induced by drug withdrawal (Cryan et al., 2005). As such, tests of despair can be employed to screen for depression-related phenotypes in mutant mice, with decreases in basal immobility (relative to non-mutant, wild-type control mice) indicating an antidepressant-like phenotype and, conversely, increased immobility interpreted as increased depression-related behavior in the mutant.

Another model based on exposure to repeated bouts of stress is chronic social defeat stress (CSDS), a rodent model of psychosocial stress that shares features with major depression and comorbid anxiety. In this model, inbred c57Bl/6

intruder mice are subjected to 5 min daily bouts of physical aggression by a larger, unfamiliar resident CD1 retired breeder mouse, followed by continuous protected sensory contact with the aggressor over the course of 10 days. At the end of this procedure, “defeated” mice exhibit a depressive-like syndrome, consisting of disruptions in sleep/wake cycles, reduced motivation to seek natural rewards, dysregulation of body weight homeostasis, and long-lasting deficits in social interaction with a novel target mouse (Krishnan et al., 2007). This latter finding has been shown to be responsive to the SSRI antidepressant fluoxetine, such that a chronic, but not an acute dose, can restore social interaction to control levels (Berton et al., 2006), a pattern that matches the clinical phenomenon of remission of symptoms in depressed patients only after several weeks of fluoxetine use. Collectively, these findings lend the CSDS paradigm a degree of predictive validity, allowing one to gain further insight into the important molecular substrates that facilitate depression-like behavior and antidepressant response (Krishnan and Nestler, 2008). Furthermore, this model provides an avenue for investigating mechanisms of “resilience,” or the maintenance of normal psychological functioning in the face of adversity, and how these mechanisms break down to result in long-lasting behavioral maladaptations resembling human psychiatric illnesses (Feder et al., 2009).

The CSDS model is effective in modeling certain aspects of stress-induced psychopathology, particularly the use of severe psychosocial trauma to elicit

depression- and anxiety-like responses, akin to PTSD. However, it does not necessarily recapitulate the steady, festering nature of stress, or what McEwen and colleagues have termed the body's "allostatic load," *i.e.* the steady wear and tear on the body elicited by daily life stress. To model this more subtle form of stress-induced pathology, some researchers employ the chronic unpredictable stress (CUS) model. In this assay, a variety of different stressors are administered daily to animals in an unpredictable order, over the course of days to weeks. The stressors are mostly physical in nature, such as restraint, food or water restriction, exposure to soiled or wet bedding, cage tilt, tail suspension, changing of cagemates, temperature fluctuations, and footshocks. In response to these stressors, animals exhibit anhedonia-like symptoms, deficits in grooming, and disturbances in hypothalamic-pituitary-adrenal axis function (Hill et al., 2012). Like the CSDS and FST assays, CUS also exhibits predictive validity and is responsive to antidepressant treatment. However, one drawback of this technique is that it is more suited for use in rats than mice, limiting its utility in transgenic models.

As mentioned previously, anxiety can be conceptualized as not just maladaptive and pathological, but also an adaptive response to stressors encountered in the environment. Working from the assumption that both forms of anxiety are rooted in similar biological mechanisms, many animal models of anxiety have therefore been designed into behavioral tasks that exploit the natural, normal behaviors of rodents (Rodgers et al., 1997). Most of these are based on exploratory

approach-avoidance tasks. Small rodents possess an innate aversion to exposed, well-lit spaces, a behavior that is thought to be due to selective pressure on defense against predation. Moreover, rodents are a naturally foraging, exploratory species, and tasks based on exploratory behavior tap into the conflicting tendency to approach versus avoid a potentially dangerous, yet novel area. This so-called aversive area is a commonly found component among the different tests of anxiety: open, elevated arms in the elevated plus-maze, a brightly lit compartment in the light/dark exploration test, or a small, central area of a brightly, open-faced box in the open field test (Belzung and Griebel, 2001; Holmes, 2001). During these tests, which usually take place over the duration of 5 or more min, WT control mice avoid the aversive areas and remain in the protected zones of the apparatus for the majority of the testing period. Because many anxiety disorders are characterized by avoidance of a feared object or situation, this pattern of behavior is thought to represent anxiety-like behavior with high face validity. Additionally, the ability of clinically efficacious benzodiazepines to reduce anxiety-like behaviors in these assays (Rodgers, 1997) – and conversely, the ability of drugs that induce anxiety in humans to similarly elicit increased anxiety in rodents – lends a degree of predictive, pharmacological validity to these measures.

Neural circuitry of stress-related disorders

Much of our insight into the neurocircuitry of stress-related disorders emerges from studies of drug addiction, a behavioral disorder characterized by disturbances in normal reward learning. The behavioral signs and symptoms of depression involve disturbances in emotional, motivational, and cognitive domains. A common thread linking these various spheres of depression symptomatology is a primary deficit in reward, defined by responses to positive emotional stimuli such as food, sex, and social interaction (Nestler and Carlezon, 2006). It is no surprise, then, that nearly 20% of individuals with a mood or anxiety disorder also abuse drugs, and, conversely, that 30-40% of individuals who suffer from substance abuse disorders also carry a diagnosis of MDD or anxiety disorder (Conway et al., 2006). These considerations suggest that a large degree of overlap exists among the brain regions, neural circuits, and molecules that are involved in addiction and stress-related pathologies. In this section, we will review key components of the mesocorticolimbic circuit, a series of interconnected brain regions that have been classically implicated in reward and motivation, and provide preclinical evidence to support an overarching hypothesis that disrupted plasticity of these regions plays an important role in the pathogenesis of these disorders.

The brain reward circuit comprises dopaminergic neurons in the ventral tegmental area (VTA) that project to the nucleus accumbens (NAc) found in the ventral aspect of the striatum. The NAc is composed predominately (95%) of GABAergic medium spiny neurons (MSNs), which project back to the VTA and to the

globus pallidus (Sesack and Grace, 2010). Several other forebrain structures receive dopaminergic input from the VTA, including the prefrontal cortex (PFC), amygdala, and hippocampus, as well as other regions. The NAc receives dense glutamatergic innervation from the PFC, amygdala, and hippocampus; and the PFC, amygdala, and hippocampus form reciprocal glutamatergic connections with one another. Within each of these regions, functional output is modified by the activity of local GABAergic interneurons and, in the NAc, by cholinergic interneurons as well. Moreover, serotonergic and noradrenergic fibers from midbrain raphe nuclei and the locus coeruleus, respectively, innervate these forebrain regions, highlighting their importance in antidepressant action (Krishnan and Nestler, 2010). There is also evidence suggesting that, in addition to dopamine, glutamate or GABA is released from VTA neurons, and that release or co-release of these neurotransmitters may contribute to the functional effects of dopamine (Hnasko et al., 2012; Tritsch et al., 2012).

A number of clinical and preclinical studies have begun to detail how different corticolimbic structures are affected by stress, depression, and anxiety. The PFC is a primary target of stress-induced psychopathology in humans. Patients with major depression have a smaller cortical volume, including reduced white matter volume in the orbitofrontal cortex and medial PFC (mPFC), two subregions within the PFC (Caetano et al., 2006; Drevets et al., 1997; Steffens et al., 2003). In addition, depressed subjects show smaller changes in blood-oxygen-level dependent (BOLD)

signal, as measured by functional magnetic resonance imaging (fMRI) during a learning task, in the ventrolateral and dorsomedial PFC (Taylor Tavares et al., 2008). While the precise mechanism for these changes in both size and activity of the PFC are still unknown, they are thought to result, in part, from the neuronal atrophy that is evident in post-mortem tissue, or from loss of glial cells in this region (Rajkowska, 2000; Uranova et al., 2004). These findings support the hypothesis that stress disorders are characterized in part by diminished PFC-mediated excitatory control over subcortical reward-related structures, such as the NAc and amygdala, resulting in inappropriate processing of reward-related as well as aversive events (Disner et al., 2011). In line with these findings, a recent study in postmortem tissue from depressed patients identified a loss of excitatory synapse number in the mPFC, lending further credence to the idea that decreased BOLD signal can be attributed to loss of excitatory drive in this brain region (Kang et al., 2012).

Rodent models corroborate the findings in human studies, implicating disrupted PFC function in depression. Neuronal atrophy, reduced synaptic density, and cell loss are commonly reported in animal models of depression and stress. Chronic unpredictable stress, which elicits anhedonia in rodents, causes a reduction in the length and branching of apical dendrites and decreases the number and function of spine synapses in layer V pyramidal neurons of the mPFC (Li et al., 2011). Chronic restraint stress, which induces anhedonic and anxiety-like behaviors in rodents, causes similar reductions in dendrite complexity and spine density in PFC

neurons (Liu and Aghajanian, 2008). These morphological deficits, along with the behavioral deficits produced by stress, can be reversed by antidepressants, enriched environment, and exercise (Li et al., 2010; Li et al., 2011; McEwen et al., 2012). These findings suggest that the structural integrity of the PFC, which is disrupted by stress in both humans and rodents, may serve as a key therapeutic target in treating stress-related disorders.

In both human populations and rodent models, the NAc also appears to be a neuroanatomical substrate of stress. While gross volume of this structure is unchanged in MDD, the activity of the ventral striatum, which encompasses the NAc, is reduced in depressed patients, as assessed by fMRI (Drevets et al., 1992; Mayberg et al., 2000), and deep brain stimulation of this region can promote remission of symptoms (Schlaepfer et al., 2008). These findings may support the idea that patients with MDD exhibit reduced motivation to seek rewards and can no longer experience pleasure from activities that were once pleasurable (anhedonia), and that antidepressant responses can be achieved via enhancement of ventral striatal function. In animal models of stress, NAc MSNs exhibit modifications in synapse structure and function. Stress has been shown to increase MSN spine density and dendritic length (Bessa et al., 2013; Christoffel et al., 2011; Muhammad et al., 2012), both reversible by antidepressants (Bessa et al., 2013). Not surprisingly, then, stress also increases the number of functional glutamatergic synapses in the NAc, as evidenced by an increased frequency of α-amino-3-

hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-mediated mini-excitatory postsynaptic currents (mEPSCs) (Campioni et al., 2009). These findings appear to contradict those in human studies demonstrating overall decreases in activity in this region. It may be the case that the observed increase in spine density and mEPSC frequency represents a homeostatic response induced by a decrease in afferent activity to NAc MSNs. As such, it remains to be clarified whether alterations in NAc function in stress-related disorders originate from pre- or postsynaptic alterations in MSN properties, and how such changes are relevant to development and reversal of stress-related behavior.

Molecular insights into stress-induced behavior: role of synaptic plasticity

Based on a review of the literature, a common theme emerges in the study of stress- and anxiety-related pathologies: deficits in these behavioral domains correlate with alterations in both brain activity as well as in neuronal structure and function. Thus, it is likely that genes, proteins, and small molecule second messengers that orchestrate synaptic connectivity and communication between neurons are mechanistically involved in stress, depression, and anxiety. Here, we will review the literature as it relates to synaptic plasticity and its implications for stress-related behavior, with a particular focus on activity-dependent signaling and glutamatergic neurotransmission.

Activity-dependent synaptic plasticity

In response to a salient experience, glutamate, the most common excitatory neurotransmitter in the central nervous system, is released at specific synapses, where it binds to postsynaptic receptors for AMPA and *N*-Methyl-D-aspartate (NMDA). These ionotropic receptors are capable of fluxing Na^+ and K^+, permitting depolarization of the cell membrane. AMPA receptors (AMPARs), which mediate fast excitatory neurotransmission, can then be dynamically inserted and removed from the synaptic membrane, thus regulating the extent of the postsynaptic response to synaptic glutamate. When the membrane is depolarized and glutamate binds to NMDARs, these receptors open and conduct Ca^{2+} current, resulting in the propagation of a number of calcium-dependent signaling pathways, including those that converge on calcium/calmodulin-dependent kinases (CaMKs), which stimulate transcription of genes in the nucleus that ultimately function to engage in synaptic remodeling (Bading, 2013). For instance, elevated calcium signaling results in CaMKII-mediated phosphorylation of AMPARs, which increases their conductivity, and alters actin cytoskeleton dynamics to regulate dendritic growth (Cohen and Greenberg, 2008). In addition, neuronal activity and Ca^{2+} release can elicit stable changes in the efficacy of synaptic transmission, processes termed long-term potentiation (LTP) and long-term depression (LTD) (Malenka and Bear, 2004). These two forms of plasticity are dependent on the insertion or removal of AMPARs into and from the synapse, resulting in synaptic strengthening or weakening.

Neuronal activity also regulates nuclear transcriptional programs that affect synaptic function. Neuronal activity-induced elevations in intracellular calcium lead to the activation of multiple signaling molecules, including CaMKII, PKA, and the calcium-dependent phosphatase calcineurin, to name a few. These kinases and phosphatases phosphorylate or dephosphorylate, respectively, transcription factors, coactivators, and other components of the transcriptional machinery within the nucleus. For instance, multiple stimuli, via cAMP and Ca^{2+} signaling, can promote phosphorylation of the cyclic AMP-dependent-responsive-element-binding protein (CREB) at serine-133, resulting in the recruitment of CREB-binding protein (CBP) to gene promoters. CBP, which has intrinsic acetylation activity, relaxes chromatin structure via acetylation of histones, thereby facilitating the recruitment of RNA polymerase II and the activation of gene transcription programs (Greer and Greenberg, 2008; Kornhauser et al., 2002). Neuronal activity also triggers the dephosphorylation of the transcription factor myocyte enhancer factor 2 (MEF2) by calcineurin, disrupting the association of MEF2 with histone deacetylases including HDAC4 and HDAC5, leading to elevated gene transcription (Flavell et al., 2006).

There exist multiple, distinct routes by which neuronal activity elicits transcriptional responses tied to synaptic function. Neuronal activity results in the recruitment of a number of activity-dependent transcription factors, including MEF2 (Flavell et al., 2006), Fos (Greenberg et al., 1986), and NPAS4 (Lin et al., 2008), all of which can induce the expression of genes that function directly at the synapse,

such as brain-derived neurotrophic factor (*Bdnf)*, *Arc,* and *Ube3A.* Activity-regulated MEF2, for example, suppresses excitatory synapses by inducing *Arc* (Flavell et al., 2006; Flavell et al., 2008); CREB and NPAS4, on the other hand, regulate inhibitory synapse formation onto excitatory neurons via *Bdnf* induction (Hong et al., 2008; Lin et al., 2008). Thus, because of their activity dependence, these transcription factors are thought to reside at an important node within active neural circuits, converting neuronal activity into enduring changes not only in synapse structure and function, but also into final endpoints such as cognition and behavior.

The role of glutamate in stress disorders

Traditionally, the catecholaminergic system, which comprises the neurotransmitters serotonin, norepinephrine, and dopamine, has received the greatest attention in the study of stress- and anxiety-related disorders. However, given the abundance of evidence implicating disrupted excitatory synaptic structure and function in these disorders, it is likely that the glutamatergic system plays a key role as well. Indeed, many reports have now highlighted alterations in glutamate signaling as well as changes in the expression of AMPA or NMDA receptor subunits in depression, although significant variations exist across brain regions, and the functional consequences of these changes are unclear (Feyissa et al., 2009; Karolewicz et al., 2009; Sanacora et al., 2008). In animal models of depression, stress tends to inhibit glutamate signaling and disrupt synaptic plasticity (Yuen et al.,

2012), whereas antidepressants promote such signaling by enhancing plasticity (Duman and Aghajanian, 2012). For example, the SSRI class of antidepressants, when administered chronically, enhances synaptic transmission and LTP (Bath et al., 2012). Specifically, fluoxetine reinstates ocular dominance plasticity in the adult mouse visual cortex and enhances the extinction of conditioned fear, an LTP-dependent phenomenon (Karpova et al., 2011; Maya Vetencourt et al., 2008). Additionally, chronic fluoxetine administration can increase spine density (Ampuero et al., 2010) or prevent the loss of dendrites and excitatory spine synapses induced by chronic stress (Bessa et al., 2009; Magarinos and McEwen, 1995).

Antagonism of NMDA receptors has recently emerged as a promising therapeutic strategy to treat depression. In patients who have failed to respond to SSRIs, a single intravenous infusion of the NMDAR antagonist, ketamine, ameliorates depressive symptoms within hours, an effect that persists for 7 to 10 days (Machado-Vieira et al., 2012). In rodents, single administration of a sub-anesthetic dose of this drug alleviates anxiety- and anhedonic-like symptoms produced by stress, and is correlated with the induction of synaptic proteins as well as rescue of dendritic spine pathology in layer V pyramidal neurons of the mPFC (Li et al., 2010). Mechanistically, ketamine is thought to exert its antidepressant effects via inhibition of tonically active GABAergic interneurons in the PFC, an effect that elicits glutamate release and resultant changes in activity-dependent signaling, requiring such activity-regulated molecules like BDNF and mammalian target of

rapamycin (mTOR) (Li et al., 2010; Li et al., 2011). Along these lines, it has been shown that activation of AMPA glutamate receptors plays an important role in facilitating the antidepressant effect of ketamine (Maeng et al., 2008), and animals lacking the AMPAR subunit GluA1 show depression-like behavior (Chourbaji et al., 2008). Thus, it appears that NMDAR- and AMPAR-mediated glutamate signaling can exert antidepressant and anxiolytic effects in preclinical models. It remains to be clarified the precise signaling networks and molecules associated with glutamate signaling that contribute to the development, expression, and reversal of stress- and anxiety-related behavior.

SUBSTANCE ABUSE AND THE NEUROBIOLOGY OF ADDICTION

Drug addiction is one of the nation's most serious health problems. Often unrecognized as a true medical disorder by the general public, inclusion of this condition into the lexicon of medical practice is nonetheless beginning to take hold, as the enormous societal and economic costs associated with this condition continue to accumulate. Despite decades of research describing the neuropharmacology and mechanisms of action of addictive drugs, a descriptive model detailing the brain circuits, genes, proteins, molecular and cellular mechanisms that guide both the initial use, continued abuse, and relapse into the seeking and taking of drugs has eluded the field. Much of our understanding concerning these many questions has been garnered from human, primate, and rodent models of addiction-related behaviors. These studies have guided our understanding of the neural circuitry and neurochemistry on which addictive substances act, and have additionally shed light on a collection of important molecules involved in mediating the behavioral consequences of drugs. Here, we discuss several key areas related to the human disorder of drug addiction and provide insight into the neurobiology underlying this devastating condition.

Substance abuse disorders are a leading cause of morbidity and mortality

Drug addiction is defined as a chronic, relapsing medical condition in which an individual engages in compulsive drug use despite serious negative

consequences (American Psychiatric Association. and American Psychiatric Association. Task Force on DSM-IV., 1994). As such, addiction is considered a behavioral disorder. The life of an addicted person becomes progressively focused on obtaining, using, and recovering from the effects of drugs, despite adverse consequences including comorbid illnesses, unstable relationships, and repeated encounters with the legal system. Studies estimate that, in the US, nearly 15.6% of the adult population will use illicit drugs at some time in their lives. Within this population of users, approximately 20% will then go on to develop dependence to drugs (Grant and Dawson, 1998). To date, no safe, efficacious treatments that prevent the development of addiction or relapse have emerged. Thus, a central goal of drug abuse research is to characterize the psychobiology, genetics, and molecular and cellular mechanisms that govern the transition from occasional or recreational drug use to compulsive drug seeking, drug taking, and relapse associated with loss of behavioral control over drug use.

Behavioral consequences of chronic drug use

Drugs of abuse exhibit two distinct, but related properties that contribute to their addictive characteristics: their rewarding and reinforcing properties (Hyman and Malenka, 2001). Psychostimulants, including cocaine and amphetamine, opiates, nicotine, ethanol, and cannabinoids, are rewarding in nature, and users readily learn cues and contexts in the environment that signal availability of these drugs. Once

these associations are learned, re-exposure to the cues or contexts can stimulate drug-seeking behaviors. In addition to their rewarding properties, drugs are also reinforcing: behaviors directed towards acquisition and use of drug tend to increase with experience, and with repeated exposures, users will oftentimes prioritize the seeking and use of drugs over more pressing goals. Over time, behaviors aimed at obtaining and using drugs become more and more susceptible to motivational pressure; that is, in the face of substantial obstacles, they tend to resist interruption and to proceed to completion. Once this series of behavioral outcomes is set in motion, they tend to follow a long-term, chronic course, involving cycles of abstinence to drug taking followed by relapse to drug seeking and/or taking (Koob and Volkow, 2010).

While many psychological theories can be used to explain first-time drug use, the mechanisms underlying the progression from casual drug use to abuse remain poorly understood. Initial drug use is often driven by a desire to experience euphoria, or “the high” elicited by drug; however, the pleasure produced by drugs often reaches a plateau, and higher and higher doses of drug are required to elicit the same effect (a phenomenon termed tolerance). The mechanism behind this transition from first-time use to escalating abuse has been proposed to be driven by a process known as “incentive-sensitization,” put forth by Robinson and Berridge in 1993. In this theory of addiction, it is hypothesized that neural circuits that encode incentive motivation, but not pleasure, are recruited during the course of addiction,

and become hypersensitive to drugs and drug-associated stimuli. This form of neuroplasticity is sensitized with repeated exposure to drug, increasing the incentive salience of drug. Thus, in addiction, as opposed to drug experimentation, the primary emotional response to drugs is no longer “liking”, but intense cravings, or “wanting”, and it is this urge that underlies the compulsive drug-taking and drug-seeking behaviors that are characteristic of continuous abstinence/relapse cycles (Robinson and Berridge, 1993).

As stated above, behaviors associated with compulsive drug use are subject to a chronic remitting/relapsing cycle, and understanding the behavioral and neurological mechanisms underlying this pattern has become a key focus of drug abuse research. One theory put forth to explain this behavior is that drugs of abuse are capable of inducing cue-mediated relapse to seeking and taking behaviors. In addicts, drug craving and subsequent conditioned responses can be initiated by environmental cues that have been previously associated with drug, such as people, places, or paraphernalia. After a period of abstinence, these cues are responsible for driving relapse (O'Brien et al., 1998). This idea has been tested in rodent models of drug administration, where it has been shown that drug-associated cues can elicit drug-seeking behaviors. In rats, for example, repeated daily injections of cocaine or amphetamine produce a progressive increase in locomotor activity in response to a fixed dose of the drug, a process termed behavioral sensitization. Sensitization has been shown to be dependent on context. For example, a rat can be removed from its

home cage and be given repeated cocaine or amphetamine injections in a novel test cage. The sensitized locomotor response to a challenge dose is much greater if the animal is challenged in the test cage rather than in the home cage or in a different environment (Badiani et al., 1995; Koya et al., 2009). Context-specific sensitized responses can also be long-lasting; it has been reported in rats that locomotor sensitization is preserved for over a year after termination of amphetamine administration (Paulson et al., 1991). This context- and chronic drug experience-dependent property of sensitization, along with its long-lived effect, has raised the possibility that it could be a model of relapse in humans. However, the relevance of locomotor activity to addiction is not yet clear. Nonetheless, it represents a behavioral adaptation induced by repeated drug administration that is very persistent even after long periods of drug withdrawal, much like human drug addiction.

Circuit mechanisms of addiction

The neurocircuitry of addiction is complex, involving a collection of different brain regions, each consisting of various afferent and efferent structures that communicate within and across the brain during various stages of addiction (Koob and Volkow, 2010). The specific neural circuits that orchestrate reward-related behavior in response to drug administration have been extensively characterized. They primarily consist of the mesolimbic dopaminergic reward circuit and glutamatergic afferent projections onto the NAc, a key reward-related brain region.

Approximately 95% of neurons in the NAc are GABAergic medium-sized spiny projection neurons (MSNs). These neurons receive excitatory glutamatergic projections from the prefrontal cortex, hippocampus, and amygdala, which synapse onto the heads of dendritic spines. Midbrain dopaminergic axons synapse onto the necks of spines, where dopamine neurotransmitter is released and modulates glutamatergic synaptic transmission (Robinson and Kolb, 1999).

A wealth of evidence implicates these dopaminergic neurons emanating from the VTA and its projections to the NAc, and to other forebrain sites including the dorsal striatum, as well as glutamatergic projections onto the NAc, as the two major substrates of reward and reinforcement for addictive drugs (Di Chiara and Imperato, 1988; Hyman et al., 2006). The NAc is responsible for orchestrating responses to the motivational significance of drug, and the dorsal striatum is involved in the learning of habits that permit acquisition of drug. Levels of synaptic dopamine are increased by addictive drugs in the NAc; moreover, studies using dopamine receptor antagonists or lesion studies demonstrate that dopamine is required for reward and reinforcement (Koob and Bloom, 1988). Human studies corroborate many of these findings; using fMRI imaging, it has been shown drug infusions activate this reward circuit (Breiter et al., 1997). Thus, it appears that dopamine is a neurochemical correlate of reward, such that levels of this neurotransmitter may be associated with the “liking” of drug.

Additional studies have gone on to further characterize the precise role of

dopamine neurotransmission in reward. Seminal studies performed in primates have demonstrated that dopamine not only signals reward, but also primes the organism to learn rules and conditions associated with the administration of reward (Schultz et al., 1993; Waelti et al., 2001). An unexpected reward elicits transient firing of dopamine neurons in monkeys, but as the monkey is subjected to an increasing number of signals that precede a reward (for example, a light flash or test noise), the physiological response to presentation of the reward habituates and, instead, dopamine neuron activity is evoked by the signals that predict reward. Moreover, if a predicted reward is omitted, basal levels of firing decrease, and if the reward exceeds expectation, firing is increased above basal levels (Waelti et al., 2001). These important experiments led to the formation of a model which postulates that dopamine release functions as an error-detection or learning signal as it relates to reward, and that this neurotransmitter is critically involved in the formation of rules that are directed towards the acquisition of reward. Along these lines, if behaviors that guide the acquisition of reward are to be learned, then it is plausible that dopamine released into the striatum encodes information related to various aspects of the reward, including its context and salience. Indeed, excitatory neurotransmission, carried by projection neurons from the PFC, hippocampus, and amygdala, and which is modulated by dopaminergic input, encodes detailed information related to drug exposure, including context (hippocampus), valence or saliency of drug (amygdala), and executive function (PFC) (Pascoli et al., 2014). In

this sense, the striatum, particularly the ventral, NAc-containing region, serves as a "hub" for the integration of multiple drug-related signals; it is in this region that drug-induced synaptic plasticity regulates addiction-like responses by consolidating behaviors that guide drug-liking, drug-wanting, and drug-seeking (Hyman et al., 2006).

Cellular mechanisms of addiction

Increasing evidence suggests that, in response to drug exposure, excitatory neurotransmission in the NAc increases, resulting in the strengthening of accumbal synapses onto MSNs. Moreover, this plasticity may represent a cellular mechanism by which drug exposure elicits persistent changes in addiction-related behaviors. Changes in synaptic strength can occur via a number of different routes, including changes in glutamate release from presynaptic projection neurons, or increased trafficking of neurotransmitter receptors to the postsynaptic membrane. The most well described mechanisms for synapse-specific plasticity, discussed previously, are long-term potentiation (LTP) and long-term depression (LTD), which involve the insertion or removal of AMPARs into or out of the synaptic membrane, respectively. Recently, it has been hypothesized that these mechanisms underlie the drug-induced remodeling of neural circuitry that occurs during addiction (Wolf and Ferrario, 2010)

Increasing evidence also suggests that enhanced glutamatergic signaling via AMPARs in the NAc plays an essential role in cocaine-related behaviors. AMPAR antagonists injected locally into the NAc block the expression of sensitization after withdrawal, and conversely, intra-NAc injections of the glutamate receptor agonist AMPA potentiate the locomotor response of sensitized rats 3 weeks after the last cocaine injection (Li et al., 1997; Pierce et al., 1996). In a more translational model of addiction, microinjection of AMPA into the NAc is capable of producing reinstatement in an animal model of relapse, and this effect is blocked by intra-NAc infusion of an AMPAR antagonist (Cornish and Kalivas, 2000).

In line with these findings, there is abundant evidence that NAc MSNs undergo a series of glutamate-dependent neuroadaptations as a consequence of drug administration, and that these are important in the development of addiction (Wolf, 2010). Most of this literature pertains to changes that ensue following withdrawal from chronic drug exposure. In this setting, a series of different neuroadaptations occurs, all of which enhance plasticity of NAc MSNs. First, in response to long-term withdrawal from drug administration, AMPARs are upregulated at NAc synapses, resulting in an increase in reactivity of MSNs to glutamate afferents. Interestingly, this increased reactivity may be more pronounced after prolonged withdrawal from extended-access cocaine self-administration, a time point at which it has been shown that higher-conductance calcium-permeable AMPARs are incorporated at the synapse (Conrad et al., 2008). Second, withdrawal

from chronic cocaine dampens the activity of glial cells in the NAc; specifically, the function of the cysteine-glutamate transporter within these cells is decreased after cocaine (Baker et al., 2003), resulting in reduced levels of extracellular, extrasynaptic glutamate in NAc. Extracellular glutamate normally exerts inhibition on presynaptic terminals in the NAc, putting a “brake” on glutamate release into the NAc by these afferents. A reduction in this pool of neurotransmitter, then, would result in disinhibition of synaptic glutamate release. Third, NAc MSNs from dosed and withdrawn animals exhibit reductions in intrinsic membrane excitability (Hu, 2007), a parameter that determines whether and how many action potentials are fired upon membrane depolarization. The decrease in NAc MSN excitability is thought to be due to a complex mechanism involving decreased Na^+ and Ca^{2+} conductance, as well as increased K^+ conductance. It has been proposed that this adaptation paradoxically enhances NAc output by enhancing the signal to noise ratio of NAc input; MSNs become more selective in their response to input, particularly as it relates to incoming action potentials that encode salient information (Kalivas and Hu, 2006), thereby enhancing behavioral output. These three independent neuroadaptations that ensue following withdrawal from sensitizing regimens of cocaine are all correlated with not just enhanced MSN function, but also greater addiction-related behavior (Wolf, 2010). What has yet to be characterized are the molecular signaling mechanisms by which cocaine induces multiple synaptic and

cellular changes in brain reward circuitry, and how these contribute to drug-related behaviors.

OVERVIEW OF RELEVANT MOLECULES

An extensive literature describing the circuitry of addiction and stress-related neuropsychiatric pathology has been well-documented, as noted in the previous sections. However, a thorough investigation into the genes, proteins, and other pertinent molecules that participate in the development, expression, and reversal of stress- and addiction-like behaviors is a task that requires more careful examination, and is the subject of this document. Here, we have chosen to focus on the role of epigenetics and neuronal plasticity in mediating behavioral responses to both stress and drug of abuse. Specifically, we focus on the molecules histone deacetylase (HDAC) 5 and activity-regulated cytoskeleton-associated protein (Arc). In the following sections, we provide a brief overview of these two molecules, outlining their roles in normal cellular function as well as their potential links to neuropsychiatric illness.

Epigenetics and gene regulation – role of HDAC enzymes

Epigenetics is defined as changes in gene expression that do not involve alterations in the DNA sequence. Broadly speaking, it can also be conceptualized as the biological events leading to long-lasting changes in chromatin structure that permit regulation of gene transcription (Jaenisch and Bird, 2003). Chromatin is comprised of DNA that is wrapped around the histone octamer that contains two copies each of the core histones H2A, H2B, H3, and H4 (Borrelli et al., 2008). Both

histone acetyltransferases (HATs) and histone deacetylases (HDACs) are capable of modulating gene activity by inducing either an active or inactive chromatin state, respectively. Histone acetyltransferases catalyze the acetylation of lysine residues of the histone tail, causing the chromatin to adopt a relaxed conformation and permit access by transcriptional machinery. Histone deacetylases catalyze the removal of acetyl groups from these residues, resulting in the tightening of chromatin structure and reduction in transcription. Together, these two enzymes collaborate to regulate the chromatin state by influencing histone acetylation and impacting gene expression. A number of other posttranslational modifications occur on histone residues that modulate gene expression, including, but not limited to, phosphorylation, sumoylation, methylation, and ubiquitination. Because these epigenetic mechanisms occur in postmitotic neurons, it is thought that these processes may play a vital role in regulating adult brain function.

The histone deacetylases (HDACs) play a crucial role in regulating gene activity by inducing modifications of the chromatin structure. There are 11 mammalian HDACs classified into four distinct HDAC families, class I, IIa, IIb, and IV, which are grouped according to sequence homology, subcellular localization, and expression patterns (Bolden et al., 2006). The class IIa HDACs have garnered substantial attention in recent years, for a variety of reasons. These consist of the molecules HDAC4, 5, 7, and 9, and are restricted in their expression to muscle and brain tissue, suggestive of perhaps a unique, tissue-specific function for these

HDACs. Unlike the predominant class I HDACs, which solely function in the nucleus, the class IIa molecules are distributed between nucleus and cytoplasm, and their relative compartmentalization between these two spaces is dependent on their phosphorylation state at a series of critical serine residues (Chawla et al., 2003; McKinsey et al., 2000a). Phosphorylation at these sites renders these HDACs susceptible to binding by the adaptor protein 14-3-3, facilitating its export out of the nucleus (McKinsey et al., 2000b). Because the HDACs function to suppress transcription, regulation of their subcellular localization provides a means by which the cell can regulate gene transcription. In response to elevated calcium signaling, the serine sites are preferentially phosphorylated, and nuclear export ensues (McKinsey et al., 2000a). In contrast, it has been recently shown that elevated cAMP signaling promotes the opposite effect, namely, the dephosphorylation of these sites (at least with respect to HDAC5), which results in nuclear accumulation and presumably, repression of HDAC5 target gene expression (Taniguchi et al., 2012). Thus, the class IIa HDACs can shuttle in and out of the nucleus depending on second messenger signaling, providing the cell with a means by which to translate extracellular signaling into dynamic changes in gene expression and downstream function.

HDAC5 in neuropsychiatric illness

The class IIa HDACs, particularly HDAC5, have been extensively studied in the context of cocaine-induced behavioral and neural adaptations (Kumar et al., 2005; Renthal et al., 2007; Taniguchi et al., 2012). *Hdac5* knockout (KO) mice exhibit potentiated reward learning after being pretreated for 1 week with a sensitizing regimen of cocaine (Renthal et al., 2007). Conversely, mice in which *Hdac5* has been overexpressed in the NAc show reduced reward learning, and this phenotype returns to WT levels with intra-NAc infusions of the HDAC inhibitor trichostatin A, suggesting that HDAC5 can bidirectionally regulate cocaine reward-learning behavior (Renthal et al., 2007). Based on these findings, it was hypothesized that perhaps cocaine regulates HDAC5 function, and that this regulation may be important in controlling behavioral responses to drug. Accordingly, in a follow up study, it was found that both cocaine and dopamine D1 receptor agonism stimulates the dephosphorylation of a serine residue, Ser-279, located near the nuclear localization sequence of HDAC5. This cAMP-dependent dephosphorylation is accompanied by nuclear accumulation of this HDAC 4 hr after drug exposure, but not 24 hr later (Taniguchi et al., 2012). The dynamic, transient nature of this dopamine-mediated regulation of HDAC5 exerts a functionally relevant role. When animals received intra-NAc infusions of HDAC5 S279A, a mutant form of HDAC5 which mimics dephosphorylation, cocaine reward learning was markedly attenuated compared to animals receiving WT HDAC5 or S279E HDAC5 (a phospho-mimic mutant). These findings suggest that cocaine- and dopamine-

dependent dephosphorylation and nuclear import of HDAC5 serve as an endogenous mechanism, recruited in the NAc by cocaine-mediated signaling, to antagonize the rewarding effects of cocaine.

In addition to its ability to suppress cocaine reward, HDAC5 also may be involved in mediating behavioral responses to chronic stress. As discussed previously, chronic social defeat stress (CSDS), a preclinical model of stress-induced psychopathology, can elicit long-term changes in depression- and anxiety-related behavior that can be reversed with a chronic, but not acute dose, of antidepressant drug. It was previously found that *Hdac5* KO mice exhibit exaggerated social avoidance behavior and anhedonic behavior in response to CSDS compared to WT mice subjected to the same stress protocol, a difference that did not exist basally, in a stress-free condition. Moreover, administration of a chronic, but not acute dose, of the tricyclic antidepressant imipramine increased mRNA levels of *Hdac5* in the NAc; conversely, CSDS, but not acute stress, dampened its expression (Renthal et al., 2007). Taken together, these data highlight an essential role for *Hdac5* in behavioral responses to chronic stress, and suggest that this molecule may be involved in gating responses to a chronic, but not acute, salient stimulus, irrespective of valence.

In a study related to Renthal et al., the role of HDAC5 in antidepressant responses to CSDS was examined. Here, it was found that in the hippocampus, repressive histone methylation at the promoters of *Bdnf* splice variants was

increased in response to CSDS, resulting in reduced expression of this gene. Moreover, chronic imipramine treatment not only reversed CSDS-induced behaviors, but also restored expression of *Bdnf* and increased histone acetylation at the promoter. This increase in acetylation, as well as antidepressant-mediated effects on CSDS behavior, was blocked by overexpression of HDAC5 in the hippocampus (Tsankova et al., 2006). Therefore, it appears that, in addition to mediating responses to CSDS, HDAC5 plays an additional role in mediating antidepressant efficacy.

The activity-regulated cytoskeleton associated protein: regulation

Arc is in immediate-early gene that was originally identified as a transcript rapidly upregulated in the hippocampus in response to seizure (Link et al., 1995; Lyford et al., 1995). Since then, a number of different studies have further characterized the signaling pathways and neuroanatomy in which Arc is induced. Arc exhibits a postsynaptic expression pattern, found primarily in dendrites, but not axons or presynaptic terminals. Its expression is high in glutamatergic neurons in cortical and hippocampal regions; however, there is evidence to suggest that it can be detected in subcortical brain regions within other cell types (Daberkow et al., 2007). Under basal conditions, in the absence of synaptic activity, Arc is transcribed at a low level. Its transcription is upregulated many-fold by activating BDNF-TrkB signaling (Ying et al., 2002), group 1 metabotropic glutamate receptors (Park et al.,

2008; Waung et al., 2008), muscarinic acetylcholine receptors (Teber et al., 2004) and NMDARs (Steward and Worley, 2001). Transcription of Arc depends on neuronal activity; accordingly, a number of activity-dependent transcription factors, including MEF2, serum response factor (SRF), and CREB, bind to response elements within Arc's activity-responsive enhancer, all of which are required for its expression (Kawashima et al., 2009). Thirty minutes after synaptic activity-induced *Arc* transcription, the mRNA transcript is exported from the nucleus to the cytoplasm and, by 1 hour, can be measured in distal dendrites, where it predominately resides (Rao et al., 2006).

Similar to its transcription, *Arc* translation also seems to be tightly regulated by activity-dependent signaling. Activation of NMDARs and G_S-coupled receptors increases Arc translation in a PKA-dependent manner (Bloomer et al., 2008). There is also evidence that LTP-induced translation of *Arc* requires extracellular-related kinase (ERK) signaling downstream of MAP kinase-interacting kinase (MNK) (Panja et al., 2009). Induction of metabotropic glutamate receptor-mediated LTD can also result in immediate, local translation of pre-existing *Arc* mRNA, and this appears to depend on phosphorylation of eukaryoytc elongation factor 2 (eEF2) (Park et al., 2008). Once translated, stability of the protein is controlled by binding to ubiquitin-protein ligase E3A (UBE3A). Nearly six hours following synaptic stimulation, UBE3A is synthesized and ubiquitinates Arc, which targets it for proteasomal degradation (Greer et al., 2010). Thus, neuronal activity not only provides the mechanism by

which Arc is induced, but it is also tied to mechanisms that function in parallel to return Arc levels to baseline after prolonged activity.

Arc in structural and functional synaptic plasticity

Synaptic plasticity, or the strengthening and weakening of synaptic connections in response to neuronal activity, is manifested by changes not just in synapse function, but also in its microscopic structure, and Arc appears to play a role in dictating both forms of plasticity. In hippocampal neurons, overexpression of Arc increases spine density *in vitro*, whereas loss of Arc (*Arc* KO) reduces spines in the hippocampus *in vivo* (Peebles et al., 2010). In addition, Arc modulates spine morphology; specifically, overexpression of Arc in hippocampal neurons *in vitro* increases the proportion of thin spines, which are more plastic and thought to be involved in learning and memory mechanisms (Holtmaat and Svoboda, 2009). This change is accompanied by a corresponding decrease in the proportion of more stable stubby spines (Peebles et al., 2010).

Functionally, Arc is required for long-term depression, or weakening of synaptic connections, in response to activity. Low-frequency stimulation of CA1 pyramidal neurons derived from *Arc* KO slices induces only a transient and smaller LTD than what is normally observed in WT controls (Plath et al., 2006). The mechanism behind this Arc-mediated LTD likely involves its role in regulating AMPAR endocytosis. Arc is capable of decreasing the amplitude of AMPAR-

mediated currents by binding to endophilin 1 and dynamin 2 and facilitating AMPAR endocytosis (Chowdhury et al., 2006; Rial Verde et al., 2006). At the level of transcription, Arc also exerts a negative regulatory effect on AMPARs. In response to activity, Arc is imported into the nucleus, where it associates with various transcriptional proteins, most notably CBP, to repress GluA1 transcription and reduce AMPAR-mediated current (Korb et al., 2013).

Paradoxically, in addition to its role in LTD, Arc appears to be important for long-term potentiation. In response to a high-frequency stimulus protocol that normally induces LTP, *Arc* KO mice, while initially showing an enhanced response, exhibit a steady decline of potentiation, reflecting a deficit in LTP maintenance (Plath et al., 2006). This deficit in LTP can be recapitulated with transient, postnatal knockdown of *Arc* levels. Infusion of *Arc* antisense oligodeoxynucleotides (ODNs) into the hippocampus 1.5 hours before LTP induction blocks the maintenance of the late phase of LTP (Guzowski et al., 2000). When *Arc* expression is acutely ablated 2 hours after stimulation, the same effect is achieved (Messaoudi et al., 2007). Taken together, these studies suggest that Arc may be involved in regulating early-phase LTP and strongly support an essential role for Arc in the maintenance of late-phase LTP. To fully explain how Arc can mediate both LTP and LTD, additional research is needed to better understand the cellular functions of Arc and how they are regulated by various stimuli.

Arc participates in Hebbian forms of plasticity, including LTP and LTD, and appears to also be required for a non-Hebbian form of plasticity termed homeostatic scaling. In this form of plasticity, a neuron responds to long-term increases or decreases in activity by scaling its response to activity down or up, respectively, to maintain the same average firing rate (Turrigiano and Nelson, 2004). This cell-wide form of plasticity, operating at multiple synapses, has been shown to be dependent on levels of Arc. In neurons derived from *Arc* KO animals, synaptic scaling mechanisms, which are normally recruited by manipulating neuronal activity *in vitro*, are absent; neurons in which Arc is overexpressed exhibit similar deficits (Korb et al., 2013; Shepherd et al., 2006). Arc also appears to be required for single synapse-specific scaling, in which the strength of a synapse is increased in response to a reduction in presynaptic firing rate (Beique et al., 2011).

Arc in behavioral plasticity

Arc's role in maintaining structural and functional synaptic plasticity implies that it is likely to play an important role in determining behavioral responses to environmental stimuli. In line with the notion that LTP represents a cellular mechanism of learning, *Arc* KO mice indeed exhibit deficits in this behavioral domain; these mice can learn a new behavioral task but are unable to consolidate memory of the task (Plath et al., 2006), reflecting a deficit in long-term, but not short-term memory. This finding was demonstrated using classic tests of learning and

memory, including the Morris water maze test for spatial learning, cue- and context-dependent fear conditioning, conditioned taste aversion, and novel object recognition (Plath et al., 2006). In the postnatal, adult brain, Arc appears to be required for consolidation of memories, because infusion of antisense ODNs against *Arc* into the mouse brain prior to or immediately following training in the task is sufficient to elicit similar deficits in memory consolidation (Czerniawski et al., 2011; Guzowski et al., 2000; Holloway and McIntyre, 2011; Ploski et al., 2008). Furthermore, knockdown of *Arc* after the conditioning phase of a fear conditioning protocol and during reactivation of the fear memory is also sufficient to drive loss of memory (Maddox and Schafe, 2011). Taken together, these findings underscore two important points related to Arc function in learning and memory: (1) that the deficits observed in the *Arc* KO are likely not due to development deficits, and (2) that Arc is required for both the development and the expression of memories in the adult.

In other behavioral domains, Arc exerts important functional control. Loss of Arc results in increased network excitability in the hippocampus and increased seizure susceptibility (Peebles et al., 2010), as might be expected in the absence of mechanisms to control homeostatic plasticity. In subcortical limbic brain regions that are not traditionally associated with learning and memory, Arc also functions to regulate emotional behaviors, particularly as they relate to addiction. Acute, postnatal knockdown of *Arc* in the rat amygdala increases alcohol drinking-behaviors (Pandey et al., 2008), suggesting that Arc limits addiction-like behaviors. In

agreement with this relationship, knockdown of *Arc* in the dorsal stratum in rats that have been trained to self-administer cocaine interrupts their ability to extinguish this behavior (Hearing et al., 2011). The mechanism underlying these related findings has not been fully explored, but it is interesting to point out that a variety of different drugs of abuse have been shown to induce *Arc* expression in corticolimbic circuitry (Chase et al., 2007; Freeman et al., 2008; Marie-Claire et al., 2004). It may be the case, then, that Arc functions to counteract changes in appetitive behavior evoked by drug exposure by strengthening or weakening particular synaptic connections. Much more detailed analysis will be required to fully address this possibility. In all likelihood, Arc's involvement in a variety of unrelated behavioral outcomes reflects the multiple cellular functions it can take on, including its ability to bidirectionally regulate synaptic plasticity, neuronal morphology, and transcription.

CHAPTER TWO
Materials and Methods

Animals and Housing

Animals were housed in either the UT Southwestern vivarium or McLean Hospital Animal Care Facility in a temperature-controlled environment on a 12 hr light/dark cycle (lights on 0700) and maintained on regular chow (4% fat diet #7001, Harlan-Teklad, Madison, WI) *ad libitum.* For the feeding and metabolism studies, mice in which a transcriptional/translational blocking cassette flanked by lox-P sites was inserted into the MC4R gene (MC4R-TB) to create a functionally null allele were generated and characterized as reported previously (Balthasar et al., 2005). For the HDAC5 studies, *Hdac5* KO mice were generated from het x het crosses as described previously (Chang et al., 2004; Zhang et al., 2002). For all studies in which *Arc* KO mice were used, animals were bred from het x het breeding crosses as described previously (Wang et al., 2006). Unless otherwise noted, animals were group-housed with animals of the same sex with no more than 4 other cage-mates.

Drugs

Cocaine-HCl (Mallinckrodt), D1-class dopamine receptor antagonist SCH23390 (Tocris), and the D2-class dopamine receptor antagonist raclopride (Tocris) were dissolved in 0.9% sterile saline. For all injection studies, a volume of 0.1 mL/10 g body weight was intraperitoneally (i.p.) injected.

Chronic social defeat stress and antidepressant treatment

For all CSDS studies performed in this document, except those shown in Fig 6, a recently reported paradigm was utilized (Golden et al., 2011). Eight-week-old male c57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME) were group-housed and acclimated to the vivarium 1 week prior to the commencement of social defeat. Mice were exposed to a different CD1 strain aggressor mouse (Charles River Laboratories, Wilmington, MA) each day prior to lights out (1900) for 5 min over a total of 10 days. After 5 min of physical contact, test mice were separated from the aggressor and placed across a Plexiglas separator with holes, where they remained in sensory contact with the CD1 aggressor for the remainder of the 24 hours, but physical contact was prevented. Control mice were handled daily in the palm of the hand for 30 seconds and housed in equivalent cages with members of the same strain. After the last defeat, all mice were housed individually and a social interaction task was performed to measure the behavioral consequences of chronic defeat stress.

The social interaction task involved placing mice in a new arena (44 cm x 44 cm) with a small animal cage (5 cm x 10 cm) at one end. Their movements were tracked for 2.5 min in the absence of another mouse, followed by 2.5 min in the presence of a caged, unfamiliar CD1 mouse. Social interaction was quantified by comparing the amount of time the test mouse spent in the interaction zone near the small animal cage in the presence vs. in the absence of the target CD1 mouse, as

determined using Ethovision 3.0 software (Noldus, Leesburg, VA). For experiments examining antidepressant response, twenty-four hours following social interaction testing, test mice were implanted with either a fluoxetine or placebo pellet in the dorsal interscapular region under brief isoflurane anesthesia. Custom-made continuous release pellets (Innovative Research of America, Sarasota, FL) were used to deliver a chronic dose of fluoxetine (15 mg/kg/d) for 20 d. Previous studies showed that pellets prepared in this manner produce physiologically relevant concentrations of fluoxetine 20 d after implantation (Covington et al., 2009)

For CSDS studies in *Npas4* cKO mice, a modified version of the protocol described above was employed. Prior to stereotaxic surgery and defeat, animals were tested for social interaction, as described above, with a novel CD1 mouse in a "pretest" trial. Following surgery, animals were kept in home cages conditions for 2 weeks to allow viral expression to commence, after which point all surgerized animals were subjected to 5 d of social defeat stress. On Day 6, animals were again put through social interaction with a novel mouse, and interaction scores obtained from this additional test were normalized to the animal's pretest score, to obtain a relative interaction score. An additional 5 d of social defeat stress were administered, and a sucrose preference test was performed 24 hr following the last bout of stress.

Metabolic Studies

Experiments were conducted in the UT Southwestern Metabolic Phenotyping Core using metabolic cages (TSE Systems, Chesterfield, MO). Animals were pair matched for body weight and lean body mass to correct for presumed body composition-related differences in food intake. These mice were transferred to the metabolic phenotyping core for 4 d of acclimation and then placed in metabolic cages for 4 d with access to regular chow. Meal analysis over a 48-hr period was assessed using Lab Master Software (TSE Systems, Chesterfield, MO).

Serum hormone levels

Blood samples were collected across the circadian cycle every four hours starting one hour after lights on. Animals from each time point were fasted four hours prior to sacrifice by cervical dislocation in order to control for the effects of food intake on leptin and ghrelin levels. Trunk blood was immediately collected in EDTA-coated tubes (BD Biosciences, San Jose, CA) and was further processed by the addition of 4-(hydroxymercuri)benzoic acid sodium salt (#55540, Sigma Aldrich, St. Louis, MO) to preserve neuropeptide hormones. A fraction of blood sample was set aside and acidified by the addition of 0.1 N HCl in order to stabilize acyl-ghrelin. Serum levels of leptin and corticosterone were measured as previously described (Chuang et al., 2010a; Chuang et al., 2010b), with intra- and inter-assay variabilities of 5% and 12% for leptin, and 3% and 5% for corticosterone, respectively. Levels of acyl-ghrelin were quantified using a commercially available enzyme immunoassay kit

(#10006307, Cayman Chemical, Ann Arbor, MI), with an intra- and inter-assay variability of 4% and 9%, respectively. All hormone levels were analyzed in duplicate.

Sample preparation from socially defeated mice

c57Bl/6J mice (Jackson Laboratories), 8 weeks old, were either chronically stressed via CSDS or subjected to control handling conditions for 10 d, as described above. Animals were then singly housed for 24 hr, at which point a social interaction test was performed. Animals were then exposed again to an unfamiliar CD1 aggressor mouse for 5 min, where they were subjected to physical defeat. A subset of control (no CSDS) animals were placed behind a Plexiglas divider, preventing physical contact with the aggressor, while the remaining “no CSDS” animals were subjected to defeat stress without the protective Plexiglas. All chronically defeated mice were exposed to defeat stress following social interaction testing. Animals were then sacrificed at specified timepoints, and whole brains were mounted in a mouse brain matrix (Braintree Scientific) for sectioning into 1 mm slices. Nucleus accumbens and amygdala tissue were obtained by punch dissection using a 15-gauge needle, and dorsal striatum, prefrontal cortex, and hippocampus were microdissected, as described previously (Smith et al., 2014), and stored at -80°C until further processing.

For analysis of HDAC5 phosphorylation and subcellular localization, a previously published protocol was employed (Taniguchi et al., 2012). For phosphorylation analysis, tissue was dissolved in a 1% sodium dodecyl sulfate (SDS) tissue lysis buffer containing protease and phosphatase inhibitors and diluted 9-fold in a modified radioimmunoprecipitation assay (RIPA) buffer. An aliquot of lysate was collected as a total cell lysate fraction and used for analysis of other non-HDAC proteins. HDAC5 was immunoprecipitated from the total cell lysate using an anti-HDAC5 monoclonal antibody (Abcam, #50001) and an anti-HDAC5 polyclonal antibody (Cell Signaling, #2082) with Protein-G agarose beads (Roche). A commercially available NE-PER nuclear and cytoplasmic extraction kit (Pierce) was utilized for biochemical fractionation of whole striatum. For nuclear extracts, signal intensity of HDAC5 was normalized to that of the nuclear protein Lamin A/C.

Quantitative real-time PCR

Messenger RNA and miRNAs were isolated according to instructions provided by a commercially available kit (Qiagen). Complementary DNA synthesis and quantitative real-time PCR was performed as previously described (Hale et al., 2011). Gene expression levels were normalized to GAPDH and expressed as fold change using the ΔΔCt method reported previously (LaPlant et al., 2009). Primer sequences used include: *Npas4 5'-*AGCATTCCAGGCTCATCTGAA-*3'* (forward), *5'-*GGCGAAGTAAGTCTTGGTAGGATT-3' (reverse); *cFos 5'-*

GTCGACCTAGGGAGGACCTTAC-3' (forward), 5'-CATCTCTGGAAGAGGTGAGGAC-3' (reverse); *Gria1* (or *GluA1*) 5'-CACGATCCCAGCCAGAATCA-3' (forward), 5'-CTCCGCCATCACCTTCACAC-3' (reverse); *Gapdh* 5'-AGGTCGGTGTGAACGGATTTG-3' (forward), 5'-TGTAGACCATGTAGTTGAGGTCA-3' (reverse); and *Arc* 5'-AGCAGCAGACCTGACATCCT-3' (forward), 5'-GGCTTGTCTTCACCTTCAGC-3' (reverse).

Quantitative Western blotting

Protein concentrations were assessed by a modified Lowry-based assay using the DC protein assay kit (Biorad). Protein samples were loaded onto SDS-PAGE gels (6-8%) and separated by electrophoresis. For HDAC5 phosphorylation analysis, 15 uL of immunoprecipitated sample were loaded into each lane. For Arc and AMPAR subunit analysis, 40 and 20 ug of protein were loaded, respectively. Proteins were transferred overnight at 4°C, 40 V onto PVDF membranes. For analysis via ECL chemiluminescence, membranes were blocked with 10% nonfat milk in 1X TBS-T (0.05%), rinsed 3X in 1X TBS-T for 5, 5, and 15 min, and incubated with shaking overnight at 4°C with primary antibodies diluted in 5% BSA supplemented with 0.02% NaN_3: anti-HDAC5 (1:500, Abcam, #50001), anti-P-S279 HDAC5 (1:50), anti-PS259 HDAC5 (1:500, from Dr. Timothy A. McKinsey), anti-ß-tubulin (1:10,000, Millipore #05-661), anti-Lamin A/C (1:500, Cell Signaling #2032S),

anti-Arc (1:100, Santa Cruz Biotechnology, #C-7), anti-GluR1 (1:1000, Pierce PA1-37776), and anti-GluR2/3 (1:1000, Millipore #AB1506). For analysis via Odyssey infrared imaging technology, primary antibodies were supplemented with 0.1% Tween20. Following primary antibody incubation, membranes were rinsed in 1X TBS-T for 5, 5, and 15 min, and then incubated in the appropriate species-specific secondary antibody, protected from light: HRP-conjugated anti-mouse or anti-rabbit (1:10,000, Calbiochem) for ECL-chemiluminescence; for Odyssey infrared imaging: IRDye 680RD goat anti-rabbit (1:10,000, Li-Cor) or IRDye 800CW goat anti-mouse (1:10,000, LiCor). Membranes were then washed in 1X TBS-T and imaged using either ECL Prime reagents (GE Healthcare Amersham) with X-ray film or Odyssey Infrared imaging (LiCor). Signal intensities were quantified using ImageJ software (NIH) for ECL analysis or ImageStudio v3.1.4 (LiCor).

Cell-surface protein crosslinking

Isolation of cell surface proteins in brain tissue was conducted using a crosslinking assay described previously (Boudreau et al., 2012), with slight modifications. Briefly, mice were decapitated, brains rapidly removed, and 1 mm slices prepared using a chilled brain matrix. Relevant brain regions were microdissected as described above, and tissue samples were added to prechilled artificial cerebrospinal fluid (aCSF) spiked with 2 mM BS^3 crosslinker (Pierce). Samples were then incubated with rotation at 4ºC for 30 min, after which they were

quenched with the addition of 100 mM glycine and rotated again 4ºC for 10 min. Samples were then pelleted by centrifugation at 20,000 x *g* for 2 min at 4ºC and resuspended in freshly made ice-cold tissue lysis buffer containing the following: 25 mM HEPES, pH 7.4, 500 mM NaCl, 2 mM EDTA, 1 mM DTT, 1 mM PMSF, 20 mM NaF, 1X protease inhibitor cocktail tablet (Roche), 0.1% NP-40, 1 uM okadaic acid, 1 uM microcystin-LF, and 1 mM sodium orthovanadate. Tissue was homogenized by sonication at 30% amplitude for 5 s, or until tissue was completely dissolved in lysis buffer. After a brief centrifugation step to pellet debris, supernatant was collected and processed for Western blotting using standard methods, as described above.

Stereotaxic Surgery

Stereotaxic surgery was performed as described previously (Smith et al., 2014). Animals were anesthetized with a ketamine/xylazine cocktail (100 mg/kg:10 mg/kg) and mounted in a stereotaxic frame (Kopf Instruments), where the skull surface was exposed with local lidocaine anesthesia. Hamilton syringes fitted with bilateral 33-gauge needles were used to deliver 1 uL of herpes simplex virus (HSV)-Arc or adeno-associated virus (AAV)-NPAS4 short hairpin RNA (shRNA) into the mouse brain at a rate of 0.1 uL/min for 10 min. Coordinates (relative to bregma at skull surface) used for NAc were A/P +1.6, M/L +1.5, D/V -4.4, with the manipulator set at 10º. For mPFC, 0.5 uL of virus was infused over the course of 5 min with needles positioned at A/P +1.75, M/L +0.75, D/V -2.65, manipulator set at 15º. The

needles were then raised by 0.25 mm, whereupon the remaining 0.5 uL of virus was infused into mPFC over the course of 5 min. In this manner, virus was delivered to both prelimbic and infralimbic cortices comprising the mPFC, as has been previously reported (Covington et al., 2010). Needles were retracted 10 min following infusion of total virus volume, and incisions were closed using 7 mm stainless steel wound clips. Animals were allowed to recover under low heat and returned to regular housing conditions (described above) until the commencement of experimental testing. For AAV experiments, behavioral testing began 14 d following surgery. For HSV experiments, testing commenced 48 hr post-surgery.

To confirm proper anatomical placement of virus, animals were rapidly decapitated, and brains were isolated and drop-fixed in 4% PFA for incubation with shaking at 4ºC. Twenty-four hours later, brains were cryoprotected in 30% sucrose solution in 1X PBS containing 0.1% NaN_3 for incubation at 4ºC with shaking for 24 hr. Brains were then sectioned into 50 uM slices on a sliding microtome (Leica Instruments) and further processed using standard immunohistochemical techniques as described previously (Smith et al., 2014). AAV placement was visualized using an anti-GFP antibody (1:1000, Aves) with donkey anti-chicken 488 secondary antibody (1:200, Jackson ImmunoResearch). For HSV placements, native mCherry signal was visualized. All sections were counterstained with DAPI (1:1000) for nuclear visualization. Sections that contained GFP or mCherry immunoreactivity outside the

region of interest, or little to no staining within the relevant regions, were removed from further analysis.

Behavioral Testing

Sucrose Preference Testing

Preference for a 1% sucrose solution was assessed as described previously (Krishnan et al., 2007). Animals were singly housed for the duration of the 8-day testing period. For the first 4 days, animals were given access to two 50 mL conical vials fitted with ball-point sippers (Ancare) containing either both tap water or both 1% sucrose on alternating days. During the latter 4 test days, animals were given access to two tubes containing either water or sucrose, and volume of consumption was noted at approximately 1600 hr each day. The positions of each bottle were swapped on each day of the testing period. Preferences were calculated as follows: preference = (volume sucrose consumed)/(volume sucrose consumed + volume water consumed) x 100%. Data from the first two days of testing were used in the calculation of sucrose preference.

Anxiety-related behaviors

Open field, elevated plus maze, and dark/light exploration testing was conducted as previously described (Mukherjee et al., 2010). Briefly, for open field testing, animals were placed in a 44 cm x 44 cm brightly lit open field box

constructed with Plexiglas. The animal's movements were tracked inside and outside a predefined center zone. For elevated plus maze, animals were individually placed in the center of a Plexiglas cross-maze, each arm 30 cm long and 5 cm wide, with two opposite arms closed by 25 cm high walls, elevated 96 cm from the ground. Activity in both open and closed arms was recorded for 5 min. For dark/light exploration testing, animals were placed individually in a black Plexiglas chamber (25 cm x 26 cm) and allowed to explore for 2 min. After this habituation period, a small door is opened, allowing access to the light side of the apparatus (25 cm x 26 cm, lit to approximately 1700 lux) for 10 min. The location of the animal was monitored by photobeams.

Porsolt (Forced) Swimming Test

FST was performed as described previously (Mukherjee et al., 2010). Test mice were placed in a 4-liter beaker of water at 21-25ºC for 6 min, and their movements were recorded for 6 min using a video camera. Fore- and hindlimb movements were noted during the latter 4 min of the test. An animal was scored as "immobile" when only minor movements strictly necessary to maintain the animal's head above water were made. The total time the animal remained immobile was recorded by an experimenter blind to genotype.

Cocaine conditioned place preference

Cocaine conditioned place preference (CPP) was carried out in a manner described previously (Smith et al., 2014). Three-chambered conditioning apparatuses were utilized (MedAssociates), with opposite sides differing by wall color (black vs white) and floor pattern (grid vs bar), connected by a smaller distinct chamber. Following a 20 min pretest session (Day 1), in which animals had access to all 3 chambers of the box, experimental groups were balanced such that pre-test scores (pretest time on cocaine-paired side – time on saline-paired side) were not statistically different from one another. On conditioning days (Days 2-5), animals were given an i.p. injection of either 0.9% saline or cocaine and confined to either of the two opposing sides of the box for 30 min. Cocaine was administered on days 2 and 4, while saline was administered on days 3 and 5. On Day 6, animals were given unobstructed access to all three chambers, and time spent in each of the three chambers was collected by photobeams. CPP scores were calculated by the following formula: (time spent in cocaine-paired side on test day) – (time spent in cocaine-paired side on pretest day). Animals showing a >30% pre-test side bias (> 360 s spent on either side of the chamber on Day 1) were excluded from analysis.

Context-dependent locomotor sensitization

To accommodate context-dependent sensitization, a previous protocol was employed (Smith et al., 2014), with modifications. Clear rectangular hamster cages (26.7 cm x 48.3 cm x 15.2 cm) were divided into two distinct chambers by a black

Plexiglas divider. On one side, alpha chip bedding (Lab Products) was used along with a plain, nonstriped cage pattern. On the opposite side, aspen chip bedding (Lab Products) was used with a striped wall pattern surrounding the chamber. On days 1-3, animals were confined to one context in the AM and another in the PM, and i.p. saline injections, spaced apart by at least 6 hr, were administered to all animals. Activity patterns were recorded by photobeams for at least 1 hr. Prior to these injections, habituation activity was recorded for 1 hr. On days 4-10, animals were confined to one context and given AM i.p. saline injections after a 1 hr habituation trial, and activity was similarly recorded. In the PM session, animals were confined to the opposite context and given i.p. cocaine (15 mg/kg) after a 1 hr habituation trial, and their activity was recorded. Treatment groups were randomized such that a specific context was just as likely to be associated with cocaine as with saline. Animals were housed in the ante-room of the testing room in between AM and PM sessions for days 1-10 of the experiment. Following the last day of sensitization, animals were returned to housing rooms in the main animal colony. Seven days later, animals were returned to the testing chambers between 1600 and 1900, and their activity pattern over the course of 1 hr was recorded on the cocaine-paired side. The following day, animals were again tested for locomotor activity for 1 hr in their saline-paired side between 0700 and 1000.

Virus preparation

For HSV-Arc preparation, a pcDNA3.1 vector containing the open reading frame of the *Arc* gene (a gift from Dr. Kimberly Huber) was modified using PCR to allow subcloning into a p1005 vector, In this vector, *Arc* is constitutively expressed under the control of an IE 4/5 promoter, with co-expression of mCherry driven by a CMV promoter. Plasmid was packaged into HSV particles with the assistance of the McGovern Institute for Brain Research Viral Core Facility at MIT, using proprietary protocols. A viral titer of 3-5 x 10^8 transducing units/mL was achieved.

AAV preparation and purification was accomplished using previously published protocols (Hommel et al., 2003; Zolotukhin et al., 1999). HEK 293 cells were grown in ten 150 × 25 mm cell culture dishes containing 1X DMEM supplemented with high glucose and 10% penicillin-streptomycin. Once cells reached 80% confluency, they were transfected with either pAAV-NPAS4 shRNA (a gift from Dr. Yingxi Lin) (Ramamoorthi et al., 2011) or pAAV-NPAS4 Scramble (a nontargeting vector), pHelper, and pAAV-RC plasmids (Stratagene), using a standard calcium phosphate method. After 72 hours, cells were pelleted, resuspended in freezing buffer (0.15 M NaCl and 50 mM Tris, pH 8.0), and then subjected to three successive freeze-thaw cycles to lyse the cells. In order to digest DNA and RNA, benzoase (50U/mL) was added to the lysate and incubated at 37°C for 30 min. Viral supernatant was collected by centrifugation at 3700 × *g* for 20 min. The lysate was added to a centrifuge tube containing a 15%, 25%, 40%, and 60% iodixanol step gradient. After a 350,000 × *g* spin for 2 hr min at 10 °C, the 40%

fraction was collected, added to a heparin affinity column, washed with 0.1 M NaCl, and eluted with 0.4 M NaCl. In order to concentrate the virus, elution buffer was exchanged with 1X PBS using an Amicon BioMax centrifugal filter unit concentrator (Millipore). High-titer, concentrated virus was stored at -80°C until use in stereotaxic surgery experiments.

Electrophysiology

Miniature excitatory postsynaptic currents (mEPSCs) were measured as described previously (Smith et al., 2014). Mice were rapidly decapitated, and coronal slices containing the NAc shell were prepared as previously described (Thomas et al., 2001). Slices recovered for 30 min in aCSF solution saturated with 95% O_2/5% CO_2 containing (in mM) 119 NaCl, 2.5 KCl, 1.0 NaH_2PO_4, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 26.2 $NaHCO_3$ and 11 glucose. Picrotoxin (100 uM) and lidocaine (0.7 mM) were added to aCSF to block GABAergic neurotransmission and prevent action potentials. Cells were visualized using infrared-differential contrast microscopy, and MSNs were identified by their morphology and typical hyperpolarized resting potential (-70 to -80mV). Using an Axon Instruments Multiclamp 700A, MSNs were voltage clamped at -80 mV using electrodes (3-5 MΩ) containing (in mM) 117 cesium gluconate, 2.8 NaCl, 20 HEPES, 0.4 EGTA, 5 TEA-Cl, 2 MgATP, and 0.3 MgGTP, pH 7.2-7.4 (265-275 mOsm). Data were filtered at 2 kHz by Axonclamp amplifier and digitized at 10 kHz via custom Igor Pro software (Wavemetrics, Lake Oswego, OR). At the

beginning of each sweep, a depolarizing step (4 mV for 100 ms) was generated to monitor series (10-40 MΩ) and input resistance (>400 MΩ). Data were collected in series of traces until >300 events were recorded. Synaptic events were detected via custom parameters in MiniAnalysis software (Synaptosoft, Decatur, GA) and subsequently confirmed by observer. For each event, amplitude and frequency was measured and used to determine average mean and construct probability plots.

Statistical analyses

Data are reported as mean±SEM. Statistical analyses were performed using Student's *t*-test, repeated measures analysis of variance, or two way ANOVA followed by Bonferroni, Tukey, or Sidak *post hoc* tests. All statistical analyses were performed using Prism (v 6.0, GraphPad Software Inc., San Diego, CA) software. Statistical significance was defined as $p < 0.05$.

CHAPTER THREE

THE ROLE AND REGULATION OF HDAC5 IN STRESS-RELATED BEHAVIORS

Jaswinder Kumar[1,2], Makoto Taniguchi[2], and Christopher W. Cowan[1,2]

[1]Department of Psychiatry, UT Southwestern Medical Center, Dallas, TX, USA

[2]Department of Psychiatry, Harvard Medical School, McLean Hospital, Belmont, MA, USA

Author Contributions

JK, MT, and CWC designed experiments. JK and MT conducted experiments. MT conducted additional experiments (identification of NPAS4 as an HDAC5 target gene, preparation of AAV-NPAS4 shRNA).

Abstract

Major depressive disorder and anxiety disorders like PTSD share the common feature of being precipitated in part by psychological stress. How stress modifies the nervous system to elicit long-lasting changes in mood and anxiety are questions that remain unanswered in the field. It was recently reported that mice lacking HDAC5 are hypersensitive to the aversive effects of chronic social stress, but the mechanism underlying this phenotype has not yet been fully investigated. Our laboratory recently characterized a novel cAMP-dependent mechanism through which dopamine receptor activation regulates the dephosphorylation of HDAC5 at S-279 and its subsequent nuclear import. Chronic social stress results in elevations in dopamine neuron activity; thus, we hypothesized that HDAC5 is dephosphorylated and imported into the nucleus in response to stress, and that this regulatory mechanism may underlie the development of depression-like behavior. In our analysis, we found no significant regulation of HDAC5 in the striatum. Additionally, we were unable to establish that DA signaling is a causative factor in the development of CSDS-related behaviors. In the course of our studies, however, we identified an HDAC5 target gene, *Npas4*, which is elevated in mice lacking HDAC5 and is required for stress-induced depression-like behavior. Taken together, our results do not implicate a particular mechanism directly involving HDAC5 in the regulation of stress-related behavior; nonetheless, we identify a downstream target of HDAC5 that plays a key role in stress-induced behaviors.

Introduction

Both genetic factors and environmental stress influence the development of psychiatric disorders such as major depression and anxiety (Kendler, 2001). However, the underlying biological mechanisms through which experience interacts with the genome to impair nervous system function and induce sustained disturbances in mood are incompletely understood phenomena. Gene regulation by histone deacetylases (HDACs) represents a potential mechanism by which environmental stimuli are transduced at the molecular level to changes in gene transcription. These enzymes catalyze the removal of acetyl groups from histone and non-histone proteins, resulting in chromatin condensation and repression of gene transcription. The class IIa HDACs (HDAC4, 5, 7, and 9) are of particular interest due to their selective expression in brain and muscle as well as their ability to shuttle between the nucleus and cytoplasm in response to various extracellular signals (Chawla et al., 2003; Taniguchi et al., 2012). For these reasons, the class IIa HDACs represent attractive candidates for studying the mechanisms governing transcriptional responses to experience within neurons. However, the role and regulation of HDACs and their gene targets in depression and anxiety-related disorders have yet to be thoroughly characterized in the literature.

Rodent models of addiction-related behaviors have shed light on key mechanisms involved in stress-related mood disorders. This is due to the fact that many of the behaviors elicited by drugs of abuse, including reward, motivation, and

craving, are orchestrated by neural circuits that are dysregulated in major depression (Koob and Kreek, 2007; Nestler and Carlezon, 2006). Indeed, the literature is replete with studies demonstrating a cross-sensitization of psychostimulant and stress responses in rodents, such that stress facilitates the subsequent taking of drug (Miczek et al., 2011; Schindler et al., 2010), while administration of drugs of abuse confers enhanced vulnerability to stress (Covington et al., 2011).

A number of studies have demonstrated that chromatin remodeling enzymes like HDACs are important mediators of the behavioral responses to both stress and drugs of abuse. Overexpression of the class IIa HDACs 4 and 5 in the NAc of mice individually reduces reward behavior in response to cocaine (Kumar et al., 2005; Renthal et al., 2007). Interestingly, loss of HDAC5 confers an enhanced vulnerability to social stress, as *Hdac5* KO mice display reduced levels of social interaction and increased anhedonic behavior following exposure to CSDS compared to WT littermates (Renthal et al., 2007). These data suggest that HDAC5 may be involved in suppressing the perceived salience of an environmental stimulus, irrespective of valence. The underlying mechanism through which HDAC5 mediates this effect, however, has yet to be fully described.

Recently, our lab sought to understand the cellular mechanisms governing the control of cocaine-related behavior by HDAC5. We injected WT animals with either a nuclear (S279A) or cytoplasmic (S279E) form of HDAC5 in the NAc and measured cocaine reward behavior. We found that animals expressing nuclear HDAC5 showed

attenuated reward behavior compared to those expressing the cytoplasmic form, suggesting that the dephosphorylation of HDAC5 at S-279 and its nuclear accumulation in striatal neurons contributes to the ability of HDAC5 to limit this behavior. This mechanism is thought to exert functional control over reward behavior within an acute setting, as the administration of a single dose of cocaine to naïve animals results in the dephosphorylation and nuclear import of HDAC5 in the dorsal striatum *in vivo* that persists up to 4 hr following injection, but disappears 24 hr later. Taken together, these findings corroborate previous work demonstrating the importance of HDAC5 in the regulation of behavioral responses to cocaine, and suggest that the mechanism of action through which this takes place involves the dynamic regulation of gene transcription by HDAC5. In the current study, we speculated that this mechanism is critical for the HDAC5-mediated regulation of stress-induced social avoidance behavior. We hypothesized that stress promotes the dephosphorylation and delayed nuclear import of HDAC5, and that this mechanism is recruited in response to stress to limit the expression of genes involved in the development of depression-like behavior.

Results

Regulation of HDAC5 phosphorylation and subcellular localization by stress

It was previously reported that loss of HDAC5 results in exaggerated CSDS-induced social avoidance behavior (Renthal et al., 2007). This finding implicates

HDAC5 in mediating behavioral responses to stress. As a first step in understanding this relationship, we sought to characterize the regulation of HDAC5 by CSDS. We decided to focus on two brain regions: the striatum, which includes its dorsal (caudate-putamen) and ventral (NAc) aspects, and the prefrontal cortex (PFC), two regions that are known to be important in determining mood, motivation, and reward (Alexander et al., 2010; Mayberg et al., 2005). We assembled a cohort of c57Bl/6 mice and subjected them to 10 d CSDS or control handling conditions (Fig 1A). Upon confirming that the CSDS protocol elicited robust social avoidance behavior (Fig 1B), all CSDS animals were subjected to an 11th bout of social stress for 5 min. They were then sacrificed to obtain tissue either 2 or 4 hr after the acute stress, timepoints at which significant regulation of the cAMP-responsive S-279 site on the HDAC5 protein has been previously noted (Taniguchi et al., 2012). A subset of previously unstressed mice was similarly subjected to an acute stress, such that three groups emerged from this experiment: a control (no stress) group, acute stress, and chronic stress.

In the NAc, no change in HDAC5 phosphorylation was observed 2 hr after stress (Fig 2A). At the 4 hr timepoint, slight trends for both dephosphorylation of HDAC5 at S-279 and nuclear localization were noted, but none of these trends reached statistical significance (Figs 2B and C). In the PFC, stress did not alter levels of phosphorylated S-279 HDAC5 4 hr after stress (Fig 2B). Therefore, we

concluded that neither acute nor chronic social stress regulates HDAC5 phosphorylation or localization in the NAc or PFC.

Role of dopamine receptor antagonists in stress-induced depression-related behavior

A number of lines of evidence suggest that disruptions in monoaminergic signaling underlie the pathogenesis of mood and anxiety disorders, including major depression and PTSD (Wong and Licinio, 2004). Monoamines including norepinephrine, serotonin, and dopamine, serve as ligands for G_s-coupled GPCRs, which, upon activation, results in elevations in intracellular cAMP. Previous work has identified cAMP as a key regulator of HDAC5 phosphorylation status at the S-279 site. Specifically, elevations in cAMP, elicited by systemic administration of the D1R agonist SKF81297 *in vivo*, promote the dephosphorylation of HDAC5 at this residue (Taniguchi et al., 2012). Given the classic importance of monoamines in both clinical depression and antidepressant function, and the previously established role of HDAC5 in mediating such behaviors in rodents, we wanted to ask whether pharmacological manipulation of neurotransmitter signaling could prevent the onset of depression-related behavior. To answer this question, we focused on dopamine signaling, because a number of recent studies have implicated VTA DAergic activity in the development and expression of stress-induced depression-related behavior (Krishnan et al., 2007; Lammel et al., 2011; Valenti et al., 2011). We tested the effects of pretreatment of dopamine receptor antagonists SCH23390, which

specifically blocks G_s-coupled, D1-type receptors, and raclopride, which blocks $G_{i/o}$-coupled D2-type receptors, on CSDS-induced social avoidance behavior. Animals were subjected to the standard CSDS 10 d paradigm or control handling conditions, but prior to each defeat episode, mice were i.p. injected with either D1 (0.02 mg/kg), D2 (0.2 mg/kg), or a combination of D1+D2 receptor antagonists. After this procedure, social avoidance behavior with a novel mouse was assessed.

Animals that were pretreated with saline prior to defeat showed robust social avoidance compared to nonstressed, saline-treated animals, as expected (Fig 3A). In all drug-treated animals, social avoidance was observed compared to nonstressed, drug-treated animals, regardless of drug. In nonstressed animals, dopamine receptor blockade had no effect on basal levels of social interaction. Therefore, these data suggest that blockade of dopamine receptors does not influence the development of social avoidance behavior following CSDS.

The doses chosen for the study summarized in Fig 3A were based on previous reports that showed that at 0.02 mg/kg and 0.2 mg/kg of SCH23390 and raclopride, respectively, cocaine-induced locomotor activity, a dopamine-dependent phenomenon, could be blocked (Cabib et al., 1991; Ralph et al., 2001). However, it may be possible that at these doses, stress-related behaviors are not affected. To rule out this possibility more thoroughly, we repeated the experiment shown in Fig 3A, looking at only the effects of SCH23390 on CSDS-induced social avoidance behavior, but at a 25-fold higher dose. Two-way ANOVA revealed a significant effect

of defeat on interaction score. However, post-hoc analysis did not indicate a significant difference in social interaction score between defeated animals receiving saline and defeated animals receiving the antagonist (Fig 3B). Taken together, these data strongly suggest that, within the parameters of our experimental design, dopamine receptor blockade prior to defeat does not influence the development of depression-related behavior.

Regulation of the HDAC5 target gene Npas4 by stress

The HDAC molecules are thought to repress expression of their target genes via modification of local chromatin structure, either resulting in a more or less permissive structure that is accessible to transcriptional machinery (Kouzarides, 2007). During the course of the above-mentioned experiments, we conducted an experiment to identify HDAC5 target genes *in vitro* by ChIP-Seq. The transcription factor NPAS4 was identified as a gene bound by HDAC5 upstream of its coding region. This finding led us to speculate that HDAC5 negatively regulates *Npas4* expression levels. In order to test this hypothesis, we collected brain tissue from WT and *Hdac5* KO mice and measured transcript levels of *Npas4*. We found that in both the NAc and PFC, basal NPAS4 levels were elevated in KO mice compared to WT littermates (Fig 4). Given the previously established role of HDAC5 in CSDS behavior, we further hypothesized that *Npas4* expression is regulated by social defeat stress, and we embarked on a series of experiments testing the effect of

stress on *Npas4* expression in brain regions known to play important roles in depression-related behaviors. Similar to the experimental design outlined above, we assembled a cohort of c57Bl/6 mice and divided them into nonstressed, acutely stressed, and chronically stressed groups and measured levels of mRNA for *Npas4* and *cFos*, another activity-regulated gene, at various timepoints following the cessation of stress (Fig 5A).

We found a prominent role for stress in the regulation of *Npas4*. In both the NAc and PFC, we observed robust induction of *Npas4* 15 min after cessation of stress. This regulation exhibited a time dependence, as *Npas4* expression returned to baseline levels 60 min following stress (Fig 5B). In line with its role as a transcription factor (Lin et al., 2008), we additionally observed induction of genes reported to be downstream of NPAS4 (Ramamoorthi et al., 2011), such as *cFos* and *Arc* (Fig 5B and 7A). Interestingly, we also noted an attenuated induction of *Npas4* in the PFC of chronically (10 d) stressed animals as compared to those which were acutely (1 d) stressed, suggesting the presence of certain adaptations to repeated stress which blunt inducible *Npas4* expression. This pattern of attenuated expression was also observed with *cFos* at both the 15 min and 60 min timepoint, while it was absent in the NAc at 15 min, but apparent at 60 min.

Because *Npas4* is an HDAC5 target gene induced by stress, we next sought to determine whether in mice lacking HDAC5, a negative regulator of *Npas4*, stress-induced induction is potentiated compared to WT animals in which HDAC5-mediated

repression of *Npas4* expression presumably occurs. We subjected WT and KO animals to a 5 min of social defeat stress or control conditions and measured NPAS4 levels 1 hr later. Because both stress and the absence of HDAC5 result in increases in *Npas4* expression, we reasoned that at the 1 hr timepoint, perhaps residual induction of this activity-regulated gene would still be apparent. Contrary to our hypothesis, we did not note any observable difference in *Npas4* expression between WT and KO in either the NAc or PFC (Fig 4), suggesting that HDAC5 may only regulate basal, not inducible, NPAS4 levels.

Npas4 in the mPFC is required for the establishment of stress-induced depression-like behavior

We found that *Npas4* is rapidly induced by social defeat stress, an anxiogenic and pro-depressant stimulus, in corticolimbic circuitry (Fig 5B). In particular, we noted a reduction in stress-induced *Npas4* expression in the PFC, but not NAc, among chronically stressed animals that exhibit depression-like behaviors following CSDS compared to acutely stressed animals that show no overt depression-like phenotype. This brain-region specific phenomenon of blunted *Npas4* induction prompted us to question what potential neuroadaptations ensue within the PFC that block the full induction of this gene.

In order to begin answering this question, we decided to determine what role, if any, *Npas4* in the PFC plays in stress-related behaviors. Given that *Hdac5* KO

mice are hypersensitive to CSDS (Renthal et al., 2007), we hypothesized that loss of an HDAC5 target gene would produce an antidepressant-like phenotype in mice subjected to CSDS. In order to test this prediction, we utilized an shRNA construct (Ramamoorthi et al., 2011) packaged in AAV to locally knock down *Npas4* expression in the mPFC of c57Bl/6 mice. Surgerized animals were subjected to CSDS with subsequent testing of avoidance behavior and sucrose preference (Fig 6A). We found that Scr shRNA animals showed a trend towards modest social avoidance, while NPAS4 shRNA-injected animals showed no difference in this behavior compared to a nonstressed control group (Fig 6B), suggesting that loss of NPAS4 may block the development of social avoidance behavior. A similar pattern was observed in sucrose preference. Animals that had received a scramble virus in the mPFC showed an expected statistically significant reduction in sucrose preference compared to nonstressed animals (Fig 6C). However, animals lacking *Npas4* in the mPFC showed greater sucrose preference compared to the Scr condition. No difference in preference between nonstressed animals and stressed NPAS4 shRNA animals was noted, indicating that loss of *Npas4* blocks the establishment of CSDS-induced anhedonia behavior.

Discussion

In recent years, a number of key signaling pathways, downstream effector molecules, and behaviorally-relevant genes and gene networks have been identified

as potential molecular mechanisms linking exposure to psychosocial stress to the development of depressed mood (Krishnan and Nestler, 2010). In the current study, we investigated the role of HDAC5 in behavioral responses to chronic social stress. Because it had been previously reported that *Hdac5* KO mice show exaggerated social avoidance behavior in response to CSDS, we hypothesized that stress may in fact regulate HDAC5 function, and that this regulatory mechanism may be involved in the development of stress-induced depression-like behavior. Our findings give rise to several important observations regarding the biology of HDAC5 function, as well as the underlying molecular, cellular, and circuit mechanisms of stress-induced behavior.

HDAC5 regulation by social defeat stress

Loss of HDAC5 is associated with the development of exaggerated depression-like behavior following CSDS and increased reward behavior in response to chronic cocaine experience (Renthal et al., 2007). Our group went on to characterize the S-279 residue of HDAC5 as an important cocaine- and cAMP-regulated phosphorylation site that controls HDAC5 nuclear localization and reward responses to cocaine (Taniguchi et al., 2012). Based on a number of studies pointing to shared signaling mechanisms underlying stress and drug-related behaviors, particularly the role of dopamine and cAMP signaling in both addiction and depression models (Martin-Soelch, 2009; Nestler et al., 2002), we speculated

that the cAMP-sensitive S-279 site would be regulated by stress, resulting in changes in the subcellular localization of HDAC5, and perhaps behavior.

At a 2 hr timepoint following the cessation of social stress, there does not appear to be any obvious regulation of HDAC5 phosphorylation (Fig 2A and C) in either the dorsal or ventral striatum; however, a trend of decreased phosphorylation at this site in the NAc ($p = 0.09$, control vs. chronic, *t* test), along with an increase in nuclear-localized HDAC5 in whole striatum ($p = 0.10$, control vs chronic, *t* test), were apparent 4 hr after stress (Fig 2A and C). These changes are suggestive, but not conclusive, of the idea that defeat stress can induce dephosphorylation and nuclear import of HDAC5 in the striatum. In line with these findings, we noted a similar trend in phosphorylation of the cAMP- and PKA-sensitive phospho-serine 845 residue on the GluA1 protein (Fig 2D), suggesting that at this timepoint, cAMP signaling may be elevated, and that this increase may contribute to stress-induced dephosphorylation of HDAC5.

In our hands, significant regulation of HDAC5 in response to stress may be difficult to detect for a number of reasons. First, in the striatum, while ~95% of the cells in this region are GABAergic, medium spiny neurons, they are functionally distinct in terms of their innervation and anatomy. It may be the case that D1R-expressing NAc neurons are particularly susceptible to stress- and cAMP-induced dephophorylation of HDAC5, and that in the tissue samples we prepared, these effects are masked by the presence of non-D1R-expressing neurons (such as D2R-

expressing neurons) in which HDAC5 is not as strongly regulated by stress. In the future, it would be interesting to probe for levels of phosphorylated S-279 and examine HDAC5 localization solely in MSNs in which D1Rs are highly expressed, as dopamine binding to its G_s-coupled receptor on these cells would more robustly elicit cAMP elevations. Second, while it has been reported that social stress increases dopaminergic cell activity of NAc-projecting VTA neurons, there are likely other, non-dopaminergic circuits activated by stress, including excitatory afferents from the basolateral amygdala, prefrontal cortex, and hippocampus (Perrotti et al., 2004). These glutamatergic inputs would promote the phosphorylation of HDAC5 at the calcium-sensitive S-259 and S-498 sites, resulting in nuclear export rather than import. It is also possible that, while in the case of cocaine, maximal dephosphorylation and nuclear localization is apparent 4 hr after drug administration, in the case of stress, these events occur over a longer timescale. Perhaps at a more terminal timepoint, the trends we observe would become more apparent. Social defeat stress and cocaine administration likely differ in their abilities to elicit dopamine cell firing in the VTA-NAc circuit. For example, it is unclear whether one 20 mg/kg injection of cocaine results in the same amount of synaptic dopamine release as one 5-min episode of social stress, and if so, if these dopaminergic projections actually terminate in the striatum and not in another projection site such as the PFC. Until questions like these are fully addressed, it is difficult to predict

whether CSDS increases synaptic dopamine to promote dephosphorylation of HDAC5.

Dopamine signaling in CSDS

Our examination of HDAC5 phosphorylation and nuclear localization provided clues that perhaps the S-279 site was selectively dephosphorylated in the NAc with concomitant nuclear import, two events that are cAMP-dependent. These findings would be expected based on published findings of a number of groups, indicating that chronic social stress results in elevations in VTA dopaminergic neuronal firing and presumably, elevations in dopamine signaling. To assess the behavioral consequences of this elevated dopamine cell activity, we decided to pharmacologically block dopamine signaling and assess its effect on CSDS-induced social avoidance behavior. Our results do not provide evidence that dopamine signaling, either via D1Rs or D2Rs, is necessary for the establishment of social avoidance behavior following CSDS. During the course of these studies, a separate group reported similar findings, using the same dosing regimen and behavioral testing schedule as employed in Fig 3A. Administration of the D1R antagonist SCH23390 (0.02 mg/kg) did not elicit any change in avoidance behavior in WT mice (Tanaka et al., 2012). Therefore, it appears that dopamine receptor signaling is dispensable for the development of CSDS behaviors.

The role of dopaminergic cell firing has been extensively documented, by at least two independent groups, in stress-induced behaviors and antidepressant responses (Cao et al., 2010; Lammel et al., 2011). However, in these studies, it was not investigated whether downstream dopamine signaling per se was necessary for the development of stress-induced depression-related behaviors. Our findings provide additional information to the existing model of dopamine function in CSDS, namely, that dopamine cell firing may be uncoupled from its potential downstream effects on signaling. Perhaps the co-release of another neurotransmitter or neurotrophin, such as BDNF (Krishnan et al., 2007), by VTA DA terminals in response to stress triggers certain signaling events in NAc neurons that drive social avoidance behavior. Alternatively, it may be that certain NAc-projecting VTA neurons, when activated, promote depression-like behaviors, while mPFC-projecting neurons promote resilience to stress, as suggested by a recent study (Chaudhury et al., 2013), and our assay is incapable of selectively modulating these two (or more) populations.

The caveats associated with the findings referenced above parallel the general lack of consensus in the literature on how exactly dopamine cell firing influences stress, anxiety, and despair-like behaviors. In one study, optogenetic induction of phasic VTA cell firing achieves an antidepressant effect after chronic stress (Tye et al., 2013), whereas in a separate study, the same stimulus protocol results in social avoidance following submaximal social defeat (Chaudhury et al.,

2013), suggesting a pro-depressant effect. In a transgenic mouse model exhibiting increased exploratory and hedonic drive, increased phasic VTA dopamine cell firing is also observed, and both the electrophysiological and behavioral changes are restored to control levels by administration of a mood-stabilizing agent (Coque et al., 2011; Roybal et al., 2007). These studies collectively raise the question of how mechanistically dopamine cell firing contributes to the development and expression of mood-related behaviors. Even after these complex circuit mechanisms are further parsed out by other investigators, the question of how at the molecular level, downstream D1-type and D2-type receptor-mediated signaling events control stress- and anxiety-like behaviors will still warrant intensive study.

It should be noted that in this study, only 2 doses of the D1R antagonist were examined. We did not go on to provide evidence that either dose results in effective blockade of dopamine signaling. The 0.02 mg/kg dose was chosen because it is at this dose that cocaine-induced hyperlocomotion, a dopamine-dependent phenomenon, is blocked when administered 30 min prior to injection (Cabib et al., 1991; Ralph et al., 2001). In our analysis, we did not observe any significant effects of a 25-fold higher dose (Fig 3B), however, a very slight trend for increased social interaction was noted in the SCH23390 defeat group compared to the saline-defeat group (32.46 ± 10.83 s for SCH-defeat vs. 24.09 ± 6.94 s for Sal-defeat). It may be the case that increasing the dose by many orders of magnitude will not elicit any additional behavioral response, but rather, delivering the drug locally to the NAc, for

example, and not systemically, will result in more profound effects on behavior. Such a follow-up study would then implicate dopamine signaling specifically within the NAc as a critical mediator of CSDS-related behaviors. It is also possible that post-, rather than pre-treatment, with the DA antagonists, after the experience of social stress, would modulate depression-related behavior. In support of this idea, it was recently shown that if defeated animals are treated with the D2R agonist quinpirole, which attenuates cAMP signaling, just prior to social interaction testing, the expression of social avoidance is effectively blocked (Barik et al., 2013). Accordingly, one might predict that administration of a D1R antagonist, which also acts to pharmacologically reduce cAMP signaling, would ameliorate the social avoidance behavior if administered just prior to interaction testing.

Npas4 regulates behavioral responses to chronic stress

Here we identify the activity-regulated gene and HDAC5 binding target (Makoto Taniguchi, unpublished observations) *Npas4* as a stress-regulated and behaviorally-relevant molecule. We confirm that in *Hdac5* KO mice *Npas4* mRNA levels are elevated in the PFC and NAc, which suggests that HDAC5 limits its expression in these brain regions. We also find that acute social defeat stress induces its expression in WT mice, but in *Hdac5* KO mice, where NPAS4 levels are basally elevated, no additional elevation in transcript levels is noted. Interestingly,

the stress-induced induction of *Npas4* is blunted among animals subjected to 10 d, but not 1 d, of social defeat stress in the PFC, but not NAc.

Our data indicate that in two important limbic structures, NPAS4 is elevated in the context of *Hdac5* KO, but the mechanism remains unclear. It may be the case that in the absence of the transcriptional repressive function of HDAC5, NPAS4 levels remain elevated compared to WT in response to stress, in line with HDAC5's role as a deacetylase that limits gene expression via local remodeling of chromatin structure. Currently, our data do not support a model in which HDAC5 plays a role in acutely inactivating stress-induced *Npas4* increases (Fig 4, right). Rather, HDAC5 may be more involved in controlling the homeostatic set point of basal NPAS4 levels.

These data also provide reason to speculate that NPAS4 may be involved in the regulation of stress-related behaviors (as well as other behaviors that have been attributed to *Hdac5* KO – see (Renthal et al., 2007). *Hdac5* KOs exhibit exaggerated depression-like behaviors in response to CSDS; therefore, the elevation in basal *Npas4* expression we observe may be associated with this phenotype. Indeed, loss of *Npas4* (local, conditional knockdown) in WT mice appears to prevent the development of CSDS-induced anhedonia (Fig 6C), lending credence to this argument. In the future, it would be interesting to test the hypothesis that restoration of NPAS4 levels to baseline in the *Hdac5* KO mouse ameliorates the depression-like phenotype that emerges from CSDS. Such a finding would more directly implicate

the repressive function of HDAC5 on NPAS4 in the development of stress-induced behaviors.

What might be the functional implications of elevated NPAS4 levels? The basal increase in NPAS4 may drive the development of the anhedonic phenotype that is observed after CSDS. This is a plausible hypothesis, given the known functions of NPAS4. A recent report identified NPAS4 as an activity-dependent transcriptional regulator of genes that promote the development of GABAergic, inhibitory synapses in primary neuronal culture systems (Lin et al., 2008). Both in rodent models of chronic stress as well as in postmortem brain tissue from patients diagnosed with MDD, a consistently reported finding is the loss of glutamatergic spine synapses in the PFC (Kang et al., 2012), which is reversed by antidepressant drugs (Li et al., 2011). Thus, in MDD, there is a loss of excitatory input to the PFC, implicating an excitatory/inhibitory imbalance as an important neural mechanism underlying this illness (Luscher et al., 2011). Thus, it is possible that elevation of NPAS4 function *in vivo* might phenocopy the behavioral consequences of chronic stress, namely, the shift in E/I imbalance towards less excitation and more inhibition, and conversely, that loss of NPAS4 mimics the antidepressant response, prevents the formation of GABAergic synapses, and shifts the balance of neuronal activity towards excitatory output. In the future, it will be important to test the hypothesis that in response to CSDS, NPAS4 drives the development of inhibitory synapses onto

regions such as the mPFC, a region that we implicate here in the development CSDS-induced anhedonia.

Another important function attributed to NPAS4 is its role as a transcription factor. It has been reported that NPAS4 is required for full recruitment of the transcriptional machinery to specific neuronal promoter and enhancer regions of the genome (Ramamoorthi et al., 2011). Notably, it was found that NPAS4 is required for activity-induced expression of the BDNF gene. This finding is of particular interest, as BDNF has been identified in both clinical and preclinical studies as an important candidate in the development of stress-induced psychopathology (Duman and Monteggia, 2006). In the CSDS model, stress increases BDNF protein and downstream signaling in the NAc, and local infusion of BDNF into the NAc potentiates the behavioral effects of social defeat stress (Krishnan et al., 2007). Conversely, local knockdown of BDNF from the VTA results in decreased BDNF protein levels in the NAc and resistance to the behavioral effects of CSDS (Berton et al., 2006; Krishnan et al., 2007). Thus, there is a positive correlation between BDNF levels in the NAc and depressive behaviors following defeat, and it is therefore possible that local knockdown of upstream regulators of BDNF, such as NPAS4, would ameliorate the effects of chronic stress, as we have observed in our study.

In a previous study, it was postulated that anterograde transport of BDNF from VTA dopamine neurons to the NAc enhances depressive-like phenotypes after CSDS (Krishnan et al., 2007). However, it was not fully established what contribution

other projection sites play in anterograde transport of BDNF to the NAc. Cortical afferents have been shown to be a source of anterogradely-transported BDNF to its projection sites, including the NAc (Altar et al., 1997). It is interesting to consider the idea that NPAS4-driven expression of BDNF in the mPFC contributes to local accumulation of BDNF in the NAc, and that knockdown of BDNF in the mPFC would phenocopy the behavioral effects we observe with local knockdown of NPAS4 in the mPFC.

In our initial characterization of NPAS4 regulation by stress, we noted the differential regulation of this gene in PFC vs NAc in stress-sensitive vs stress-naïve groups (Fig 5B). The attenuated induction of NPAS4 in the PFC, along with other activity-regulated genes such a cFos and Arc (Fig 7A), may indicate that CSDS selectively engages certain prefrontal cortical mechanisms that suppress the normal activity-inducible expression of these genes specifically in this brain region. One hypothesis explaining this finding is that chronic stress promotes the selective loss of glutamatergic input onto mPFC neurons, as discussed above. This would result in a decrease in activity-dependent signaling via voltage-sensitive calcium channels and ionotropic glutamate receptors, manifested by a reduction in inducible immediate-early gene expression. In line with these findings, Covington and colleagues reported that basally, 48 hr after the cessation of CSDS, stress-susceptible animals show a reduction in Arc and zif268 levels in the mPFC (Covington et al., 2010). These findings support a model in which chronic stress decreases excitatory input

onto PFC neurons, resulting in reduced activity of this brain region. This hypothesis could be tested in the future by experimentally enhancing the activity of the PFC after social defeat, using either optogenetic or other manipulations to stimulate neuronal activity, and observing whether this manipulation rescues the full inducible expression of NPAS4 and other activity-regulated genes.

Figure 1. Timeline and experimental design to investigate HDAC5 regulation by social defeat stress

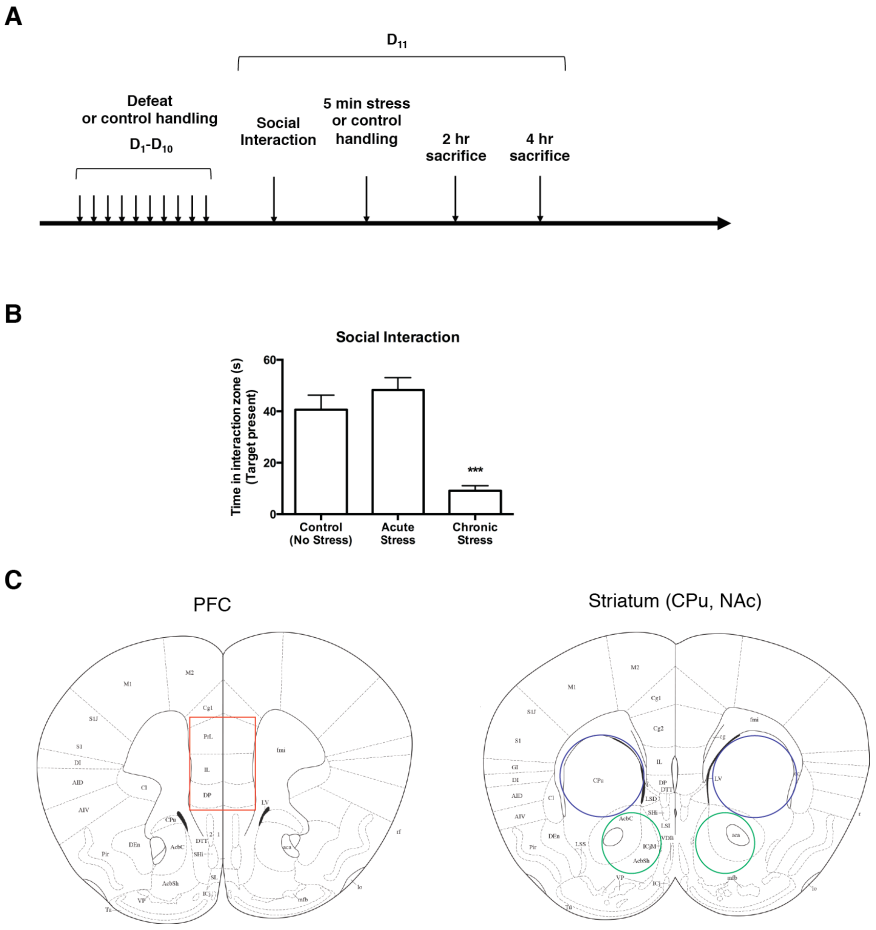

Figure 1. Experimental design and behavioral profile of animals utilized for investigation of HDAC5 regulation by social defeat stress. **A.** Timeline for CSDS, social interaction testing, and brain dissection of animals for analysis of HDAC5 regulation. **B.** Social interaction scores following CSDS or control handling conditions. CSDS decreases time spent interacting with a novel mouse ($F_{2,89}$ = 31.39). **C.** Brain regions dissected for analysis of HDAC5 phosphorylation and subcellular localization. Red indicates outline of PFC dissection. Blue and green indicate boundaries for CPu and NAc dissection, respectively. n=24-29 per group. For **B,** statistical analysis by one-way ANOVA, Bonferroni post-hoc comparison, *** $p < 0.001$ compared to control condition.

Figure 2. Regulation of HDAC5 phosphorylation and subcellular localization by social defeat stress

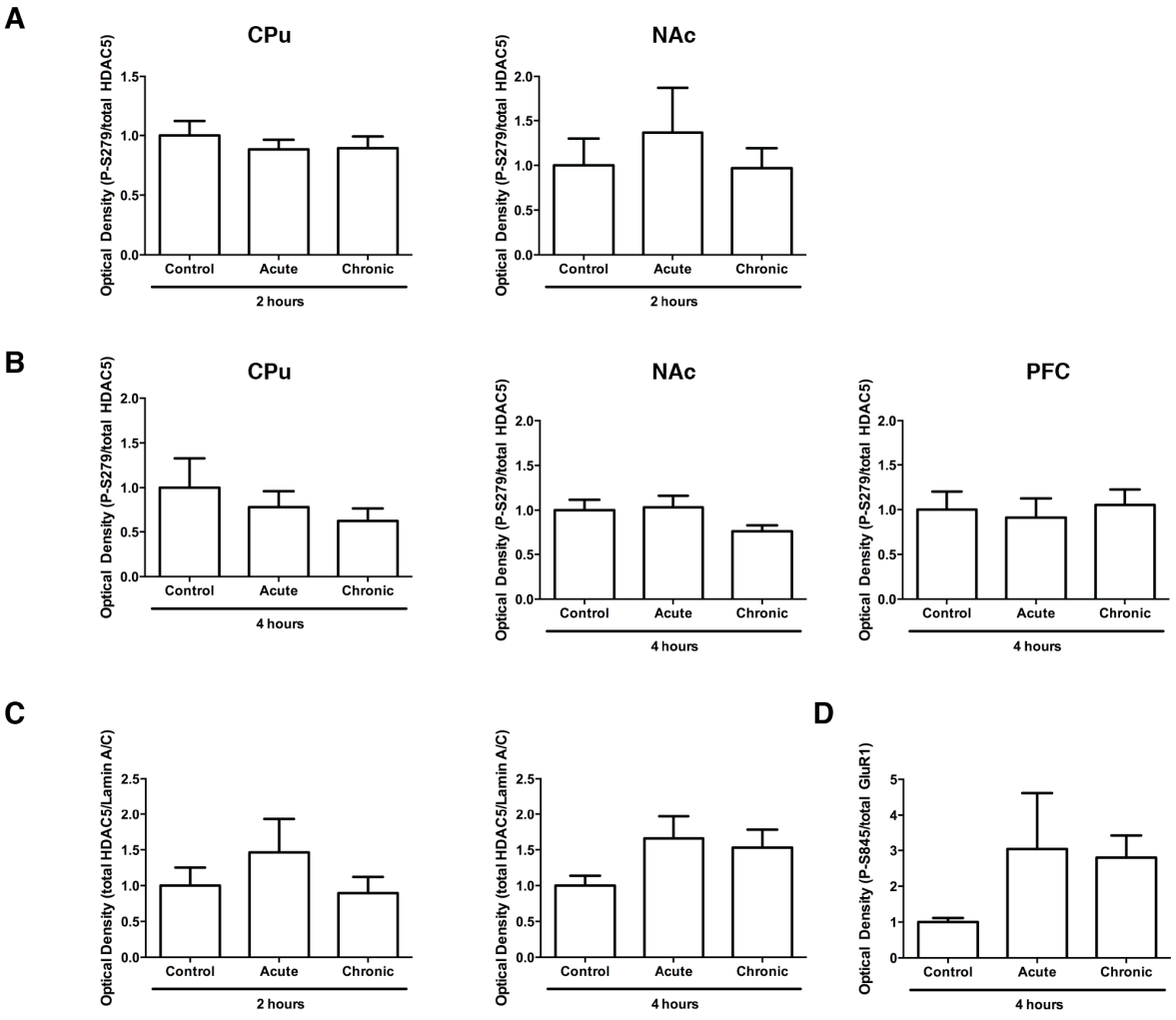

Figure 2. Regulation of HDAC5 phosphorylation and subcellular localization by social defeat stress. Wild-type c57Bl/6 mice were subjected to either control handling, acute (1 d) stress, or CSDS followed by an acute stress bout either 2 or 4 hr prior to sacrifice. Both CPu and NAc were microdissected, as well as whole striatum, from all animals. Total HDAC5 was isolated from protein lysate by IP, and levels of P-S279 HDAC5 were assessed by quantitative western blot in CPu, NAc, and PFC **(A)** 2 hr and **(B)** 4 hr following the cessation of acute stress. **C.** Nuclear-localized HDAC5 was assessed by biochemical fractionation of whole striatum at 2 hr (left) and 4 hr (right) post-stress. n=5-10/group for (A-B), 15-21/group for (C)**. D.** Levels of phosphorylated GluR1 (P-845 GluR1) were assessed by quantitative western blot 4 hr after stress in PFC. n = 5-10/group.

Figure 3. Effect of dopamine receptor antagonists on CSDS-induced depression-related behavior

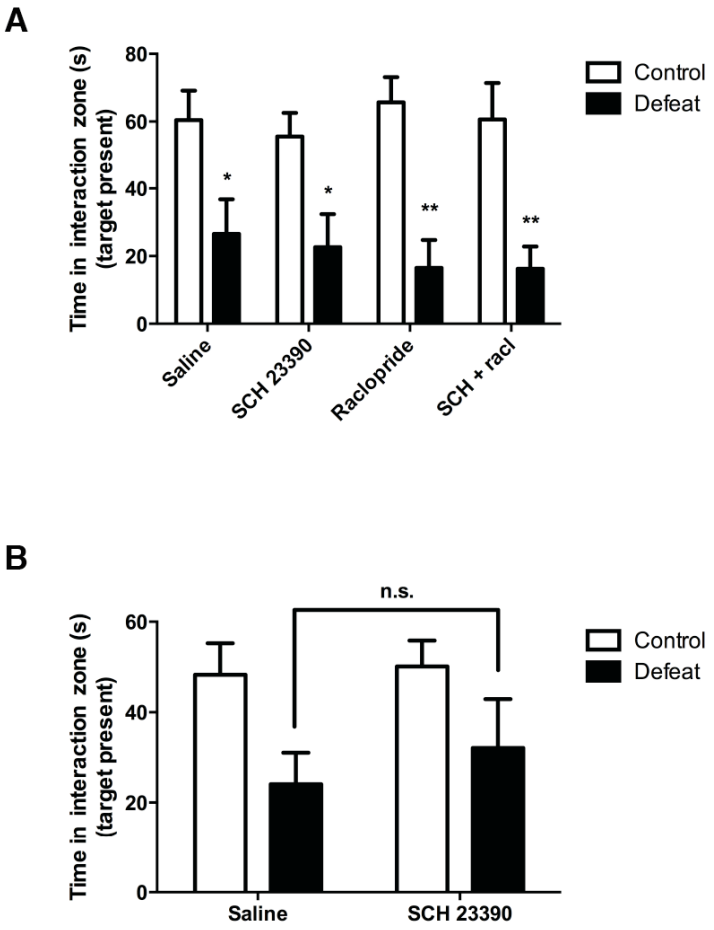

Figure 3. Effect of dopamine receptor antagonists on CSDS-induced social avoidance behavior. **A.** A cohort of c57Bl/6 mice was treated with either i.p. saline, SCH23390 (D1 class receptor antagonist, 0.02 mg/kg), raclopride (D2 class receptor antagonist, 0.2 mg/kg), or SCH23390+raclopride (0.02 mg/kg and 0.2 mg/kg, respectively) 30 min prior to the onset of social defeat stress for 10 days. Social interaction with a novel CD1 mouse was recorded 24 hr after the last bout of stress (significant effect of *defeat*, $F_{1,62} = 37.84$). n = 6-10/group. **B.** A separate cohort of c57Bl/6 mice was treated with either i.p. saline or SCH23390 (0.5 mg/kg) 30 min prior to the onset of social defeat stress for 10 days. Social interaction with a novel CD1 mouse was then recorded 24 hr after the last bout of stress (significant effect of *defeat*, $F_{1,33} = 7.30$). n = 8-10/group. * $p < 0.05$, ** $p < 0.01$

Figure 4. *Npas4* mRNA levels are elevated in the *Hdac5* KO mouse

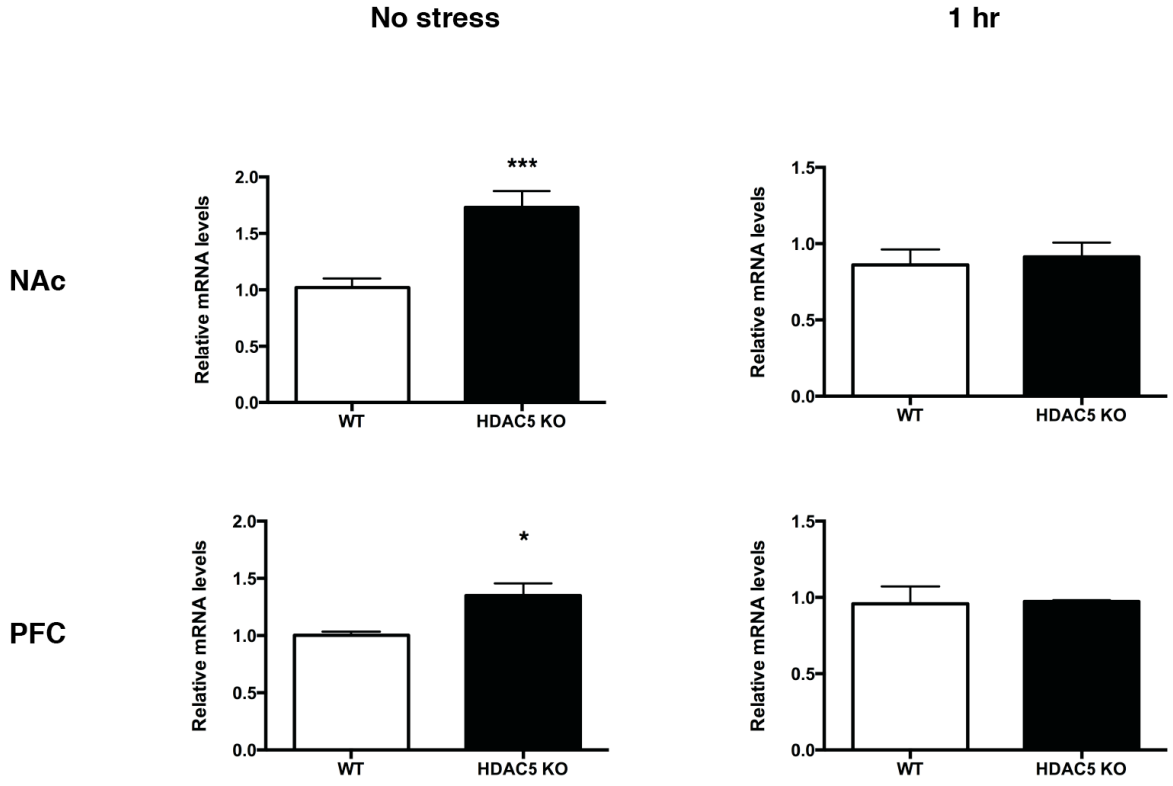

Figure 4. NPAS4 mRNA levels are elevated in the *Hdac5 KO* mouse. Tissue samples from NAc and PFC were dissected from singly housed WT and *Hdac5* KO littermates, and total mRNA levels of *Npas4* either in the absence of prior stress (left) or 1 hr after social defeat stress (right) were quantified by qRT-PCR. Fold change calculated as $2^{-\Delta\Delta Ct}$. Ct values normalized to GAPDH. n = 5-10/group. * $p < 0.05$, *** $p < 0.001$ compared to WT, *t* test.

Figure 5. Regulation of the HDAC5 target gene *Npas4* by social defeat stress

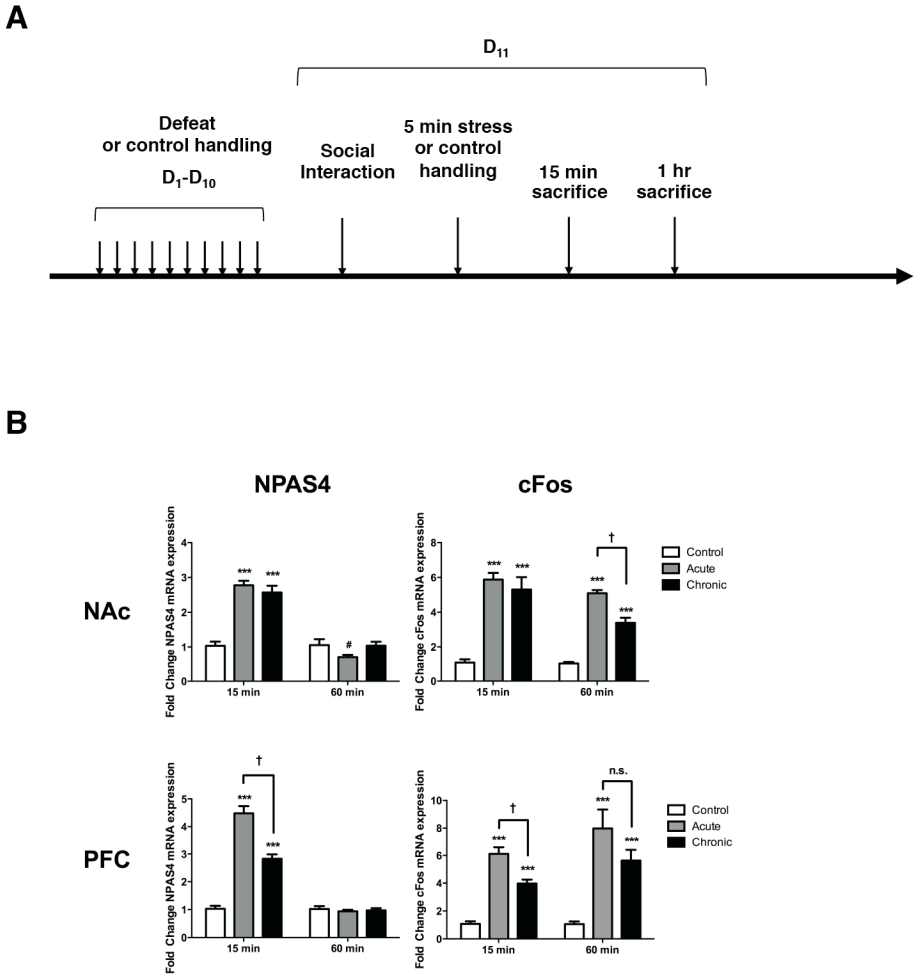

Figure 5. Regulation of the HDAC5 target gene *Npas4* by social defeat stress. **A.** Timeline for CSDS and brain dissection of animals destined for analysis of mRNA. **B.** Quantification of *Npas4* and *cFos* mRNA in the NAc and PFC of defeated mice. Both acute and chronic social defeat stress induce NPAS4 and cFos 15 min and 60 min after stress. Fold change calculated as $2^{-\Delta\Delta Ct}$. Ct values normalized to GAPDH. n=5-10/group. *** $p < 0.001$, # $p < 0.10$ compared to control condition, *t* test. † $p < 0.001$ between acute and chronic conditions, *t* test.

Figure 6. NPAS4 expression in the mPFC is required for the establishment of stress-induced depression-like behavior

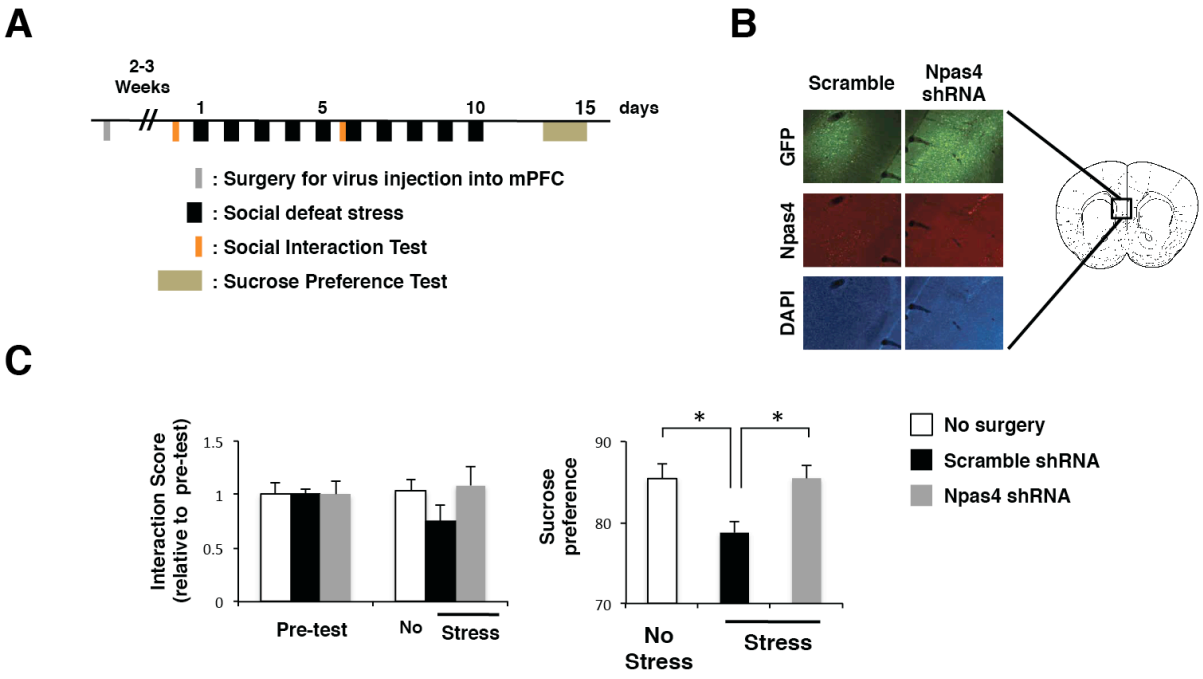

Figure 6. NPAS4 expression in the mPFC is required for the establishment of stress-induced depression-like behavior. **A.** Timeline of CSDS followed by social avoidance and sucrose preference testing. **B.** Representative images of AAV-NPAS4-shRNA expression in mPFC. **C.** Social interaction (left) and sucrose preference (right) following exposure to CSDS in mice injected with AAV-scramble and AAV-NPAS4-shRNA. n=8-10/group, * $p < 0.05$, *t* test

CHAPTER FOUR

ESSENTIAL ROLE FOR THE ACTIVITY-REGULATED CYTOSKELETON-ASSOCIATED PROTEIN (ARC) IN STRESS- AND ANXIETY-RELATED BEHAVIORS

Jaswinder Kumar[1,2], Makoto Taniguchi[2], Rachel D. Penrod[2], Rachael L. Neve[3], and Christopher W. Cowan[1,2]

[1]Department of Psychiatry, UT Southwestern Medical Center, Dallas, TX, USA

[2]Department of Psychiatry, Harvard Medical School, McLean Hospital, Belmont, MA, USA

[3]McGovern Institute for Brain Research, Massachusetts Institute of Technology, Cambridge, MA, USA

Author Contributions

JK and CWC designed experiments. JK conducted experiments. MT and RDP assisted with stereotaxic surgeries. RLN provided HSV constructs.

Abstract

Stress and anxiety disorders are chronic, debilitating conditions for which few effective treatment options exist. To date, little progress has been made in describing the molecular and cellular events that culminate in the expression of their associated symptoms. Studies from human patients and rodent models suggest that the underlying etiologies of stress-related disorders arise from disrupted structural and functional plasticity in brain regions involved in mood, reward, and motivation, including the NAc and mPFC. In the current study, we utilize a series of ethologically relevant behavioral paradigms to identify Arc as an important mediator of these behaviors in mice. Using the *Arc* KO mouse, we find that loss of Arc results in enhanced exploratory drive and resilience to both acute and chronic stress, suggesting an anxiolytic and antidepressant-like effect of Arc deletion. To determine if Arc can bidirectionally regulate anxiety-like behavior, we then tested the effects of HSV-mediated overexpression of Arc in the NAc or mPFC of mice. Overexpression of Arc in the NAc does not significantly alter anxiety-like behavior. However, when expressed in the mPFC, this manipulation results in an anxiogenic-like phenotype. Taken together, our results highlight a heretofore unidentified role for Arc in stress- and anxiety-like behaviors. We present the *Arc* KO mouse as a model that can be used to investigate synaptic and circuit mechanisms in anxiety and depressive disorders. These findings may aid in the development of therapeutic plasticity-modifying agents for these related conditions.

Introduction

One of the most consistently reported findings in patients with stress and anxiety disorders is disrupted functional connectivity between regions of the brain involved in emotion regulation (Treadway and Pizzagalli, 2014). The underlying biological mechanisms by which stress, depression, and anxiety interfere with the activity of neural circuitry to elicit changes in mood and anxiety remain unknown. Recently, a number of clinical studies have demonstrated how stress and depression modify the structure and function of corticolimbic circuitry. Gross morphological and cellular alterations in postmortem PFC and hippocampal tissue samples derived from depressed patients have been well documented. These include an overall decrease in the size of these regions, along with decreases in both dendritic arborization and spine synapse number (Kang et al., 2012; Rajkowska et al., 1999). Supporting these findings is a similar decrease in the expression of neuronal markers of activity in affected regions (Covington et al., 2010). Antidepressant modalities used to treat these illnesses appear to promote neuroplastic modifications that oppose those evoked by stress (Castren and Hen, 2013). In cases where standard pharmacotherapies fail to elicit improvements in mood, deep brain stimulation of these affected regions, including the anterior cingulate cortex (Mayberg et al., 2005) and ventral striatum (Schlaepfer et al., 2008) has proven to be an effective alternative.

Rodent models of chronic stress support these clinical findings implicating

circuit dysfunction in stress and anxiety. Exposure to chronic restraint stress, a rodent model of stress-induced anhedonia, results in dendritic atrophy of pyramidal neuronal dendrites in the hippocampus (Magarinos and McEwen, 1995). Pyramidal neurons within the PFC are similarly susceptible to stress. In response to chronic restraint stress, both the length and arbor complexity of apical dendrites of layer V mPFC neurons are diminished (Liu and Aghajanian, 2008). Interestingly, these morphological changes are reversed upon administration of the fast-acting antidepressant ketamine (Li et al., 2011), suggesting that these maladaptations may be mechanistically involved in the development of depression-like behavior. Along these lines, traditional antidepressant medications, such as fluoxetine, have been shown to promote another form of plasticity – hippocampal neurogenesis (Malberg et al., 2000; Santarelli et al., 2003), and this plasticity appears to be required for its antidepressant effects (Airan et al., 2007; Santarelli et al., 2003).

Chronic stress and clinical depression appear to share the common outcome of disrupted structural plasticity, and antidepressant drugs can reverse these changes. It is likely, then, that genes, molecules, and the signaling pathways responsible for synaptic plasticity play a role in the development and reversal of stress-related behaviors. One important mediator of structural and functional plasticity in the nervous system is the activity-regulated cytoskeleton-associated protein, Arc. Arc is expressed predominantly in neuronal dendrites and soma, and in the former, it functions to maintain homeostatic levels of surface AMPARs by

facilitating their endocytosis in response to neuronal activity. As an activity-regulated, immediate-early gene, Arc is ideally positioned to exert control over a cell's responsiveness to activity, promoting a depotentiation of synapses under conditions of enhanced network activity, while under conditions of reduced activity, Arc levels remain low, and synaptic scaling mechanisms are no longer active (Shepherd et al., 2006). Recently, it was also reported that Arc can exert control over homeostatic plasticity via a transcriptional mechanism. In response to synaptic activity, Arc is imported into the nucleus, where it suppresses the activity of the transcription factor CREB, thereby reducing levels of the CREB target gene and AMPAR subunit *Gria1* (or GluA1). In this manner, Arc can be induced by high levels of activity to downscale surface AMPAR number and maintain optimal circuit activity, both via transcriptional and post-transcriptional regulation of receptor subunits. These functional roles of Arc in regulating surface AMPARs appear to have consequences for structural plasticity as well. Arc is required for the proper formation of spine synapses in the hippocampus, as evidenced by an overall decrease in dendritic spine density in the *Arc* KO mouse (Peebles et al., 2010). Conversely, overexpression of Arc in dissociated neurons promotes the formation of spines, whereas a mutant form of Arc that is incapable of facilitating AMPAR endocytosis fails to alter spine density (Peebles et al., 2010).

Given the known association between stress and disrupted structural and functional plasticity, we were interested in asking what role if any might Arc have in

stress- and anxiety-related behaviors. To date, no study has been published that identifies Arc as a candidate risk gene for MDD or related anxiety disorders (Fromer et al., 2014); however, there is evidence to suggest that changes in Arc expression levels may contribute to stress-related psychopathology. It was found recently that in anterior cingulate cortical tissue obtained from postmortem brain samples of depressed patients, levels of *Arc* mRNA are reduced, along with other immediate-early genes including *zif268* and *cFos* (Covington et al., 2010). A similar reduction in *Arc* was noted in the mPFC of chronically stressed animals 48 hr after stress. These tonic reductions in an activity-regulated gene provided us with reason to hypothesize that loss of Arc *in vivo* increases anxiety- and depression-related behavior and impairs structural and functional plasticity in mood- and anxiety-related brain regions.

Results

Arc expression is regulated by stress

Expression of the immediate early gene *Arc* is coupled to changes in synaptic activity. A variety of different behavioral paradigms have been previously shown to induce *Arc* expression throughout corticolimbic circuitry, including Pavlovian learning (Ramamoorthi et al., 2011), seizure induction (Guzowski et al., 1999), and exposure to drugs of abuse (Tan et al., 2000). We therefore wanted to ask whether Arc is regulated by a salient stimulus that can induce stress- and anxiety-like responses in mice. We focused on social defeat stress as a model of stress-induced depression

and anxiety-related behavior, because a number of studies have now shown that CSDS can elicit activation of depression- and anxiety-related neuronal circuits, as evidenced by increased expression of the neuronal activity marker cFos (Berton et al., 2006; Yu et al., 2011). Moreover, a previous study had found that CSDS reduces expression of *Arc* in the mPFC long after the cessation of stress, perhaps implicating Arc in behavioral responses to stress (Covington et al., 2010). Therefore, as was carried out in our previous study (Fig 5), a cohort of c57Bl/6 mice was subjected to no stress, acute stress, or chronic stress, and tissue was collected over a number of timepoints to assess *Arc* mRNA transcript levels. As early as 15 min after exposure to social defeat stress, *Arc* mRNA is increased above baseline control levels in both the PFC and NAc, a pattern that is sustained at 60 min post-defeat. However, by 24 hrs after the last stress exposure, *Arc* mRNA levels returned to baseline in the NAc, suggesting that social stress transiently induces *Arc* expression in this brain region (Fig 7A).

Once *Arc* mRNA is transcribed, its protein product is synthesized and remains stable, hours after the mRNA has been degraded (Greer et al., 2010). We wanted to assess whether the regulation of transcript levels observed in Fig 7A was mirrored by changes in Arc protein levels. In a separate cohort of c57Bl/6 mice, we administered an acute bout of social defeat stress or subjected the animals to control handling conditions, and collected both NAc and PFC tissue 2 and 4 hr later to quantify levels of Arc protein by Western blot. At the 2 hr timepoint, no changes in

Arc protein in either the NAc or PFC were observed. At the 4 hr timepoint, however, we observed an ~80% increase in Arc protein levels in the NAc but not PFC (Fig 7B). These findings demonstrate that Arc message and protein can be regulated by a stressful, anxiogenic stimulus in a temporal and region-dependent manner. Based on these data, we hypothesized that Arc might play a role in the NAc in mediating stress-induced behavioral responses.

Arc KO mice show reduced anxiety-like behaviors

Our data thus far show that Arc can be induced by a behavioral paradigm that is known to elicit depression- and anxiety-like behaviors in rodents. We were therefore motivated to ask whether Arc is an essential mediator of stress- and anxiety-related behaviors. We decided to answer this question by utilizing standard behavioral assays that model anxiety and depression with face, construct, and predictive validity (Cryan and Holmes, 2005). A large cohort of WT, Het, and *Arc* KO littermates was assembled and tested in a behavioral battery, starting first with tests of anxiety, then ending with tests of despair- and stress-related behavior (Fig 8A).

In the anxiety-related behavioral battery, we observed that Arc-deficient mice showed an overall decrease in anxiety-like behavior. In the elevated plus maze, compared to WT and Het littermates, *Arc* KO mice entered the open arms more frequently than WT and Het mice, and spent more time during the 5 min test on the open arms than their corresponding littermates (Fig 8B). In the open field task, *Arc*

KO animals spent significantly less time in the periphery of the chamber and more of it in the center, suggestive of a lower anxiety-like state. In addition, the latency to enter the center zone was decreased compared to littermates (Fig 8C). In the dark/light exploration task, when animals were given the choice to spend time in a dark protected chamber vs. a brightly-lit open chamber, *Arc* KO, WT, and Het littermates spent the majority of their time on the dark side; however, *Arc* KO mice spent more time than littermates on the light side. Their latency to cross over into the light side was also markedly diminished compared to littermates (Fig 8D). Taken together, these data, acquired from 3 independent tests of anxiety, strongly suggest that *Arc* KO mice display an overall anxiolytic-like behavioral profile.

A number of behavioral tasks used to measure stress- and anxiety-like responses in rodents rely on the animal's ability to locomote, and therefore, the results of such tests can be difficult to interpret when an animal shows basal differences in locomotor activity. To rule out the possibility that the phenotypes that we observed could be attributed to changes in overall activity, we subjected *Arc* KO, Het, and WT littermates to a locomotor response to novelty test, in which the animals are placed in an unfamiliar cage, and their movements tracked for 2 hr by photobeam. The KO mice showed no difference compared to WT in total activity over this 2 hr time period. However, the Het mice showed a modest, but statistically significant, increase in cumulative activity (Fig 8E). These data provide support to the hypothesis that locomotor activity does not influence the behaviors we are

measuring for two reasons. First, the KO mice displayed no differences in activity when compared to WT littermates, yet the KO mice showed significant and robust decreases in anxiety-related behavior. Second, the Het mice, which show slight hyperactivity, are indistinguishable from WT mice in all of the tests of anxiety. If in fact locomotor activity influences behavioral outcomes in these tasks, one might expect the Het mice to display an overt phenotype, which is not the case. Therefore, we concluded that the anxiolytic behaviors observed in the KO are not secondary to changes in locomotor activity.

Arc KO mice show reduced depression-like behaviors

The data in Fig 8 indicate that *Arc* KO mice show reductions in anxiety-like behavior. The assays used to arrive at this conclusion assess the animal's basal, innate anxiety, rather than their behavioral response to an anxiogenic stimulus. In order to assess whether *Arc* KO mice display differences in behavioral adaptations to repeated stress, we subjected the same cohort of mice used in Fig 8 to CSDS. Animals were exposed to a novel CD1 aggressor mouse for 10 days and assessed for social interaction 24 hr after the last stress episode. Wild-type mice showed the expected social avoidance phenotype compared to nonstressed animals, and Het mice displayed a strong trend towards avoidance. *Arc* KO mice subjected to CSDS, however, showed no difference in social interaction compared to nonstressed KOs (Fig 9A), suggesting that loss of Arc is protective against the behavioral

consequences of CSDS. To analyze the mice in a different model of depression-like behavior, we tested a new cohort of *Arc* KO, WT, and Het mice to a 1-day Porsolt forced swimming test and measured the time the animals spent immobile during the task, a correlate of "despair"-related behavior that is reversible by administration of antidepressant drugs (Porsolt et al., 1977). In this test, KO mice spent more time swimming than WT mice, although this antidepressant-like effect in the KO mice was only a statistical trend ($p = 0.06$) (Fig 9B). Together with the CSDS findings, these data suggest that loss of Arc renders an animal resilient to the effects of both acute and chronic stress, and that possessing even a single copy of the Arc gene (Het condition) is sufficient to support the expression of normal anxiety and depression-like behaviors.

Overexpression of Arc in the mPFC, but not the NAc, increases anxiety-like behavior

The results from the behavioral phenotyping of *Arc* KO mice indicate that these animals are both profoundly anxiolytic and resistant to developing both behavioral despair and stress-induced depression-like behavior, suggesting that Arc is required for these behaviors. In order to dissect potential mechanisms explaining these findings, we employed mouse stereotaxic brain surgery to overexpress Arc in the adult brain and determine if and how this manipulation can modify anxiety-related behaviors. We focused on two important depression and anxiety-related brain regions: the NAc and mPFC, for a number of reasons. First, based on the initial

characterization of Arc expression, the NAc appeared to be a likely candidate brain region in which stress-mediated regulation of Arc may play an important role in behavioral outcomes (Fig 7B). The mPFC also appeared to be a likely locus in which these behaviors may be modified, in light of previous reports showing that chronic stress and clinical depression can dampen the expression of *Arc* in this brain region (Covington et al., 2010). Towards this end, we designed an HSV virus in which an *Arc* overexpression plasmid was packaged, and this virus was then delivered into either the NAc or mPFC of WT mice (Fig 10A). This strategy results in robust Arc protein expression that is detectable over native Arc levels 48 hr after infection (Fig 10A). We found that overexpression of Arc in the NAc did not alter anxiety-like behavior. However, when expressed in the mPFC, this manipulation results in an anxiogenic-like phenotype as measured in the open field assay (Fig 10C). These results suggest that Arc in the mPFC, and not the NAc, may be important in regulating anxiety, and that perhaps certain molecular, cellular, and synaptic perturbations within the mPFC of the *Arc* KO mouse may contribute to the expression of the anxiolytic phenotype that we observe.

Analysis of CREB target genes in the PFC of the Arc KO mouse

The *Arc* KO mice show a strong antidepressant and anxiolytic-like phenotype, yet at this stage the mechanism through which loss of Arc results in these behaviors is unclear. Our investigation into the brain region-specific effect of Arc

overexpression on anxiety-related behavior provided some evidence that perhaps prefrontal cortical Arc may play an important role in maintaining normal levels of anxiety. To investigate this possibility further, we decided to explore whether downstream targets of Arc are dysregulated in the PFC of the *Arc* KO mouse. The Arc protein is required for homeostatic synaptic plasticity, via its regulation of surface AMPARs, and recent evidence indicates that this form of plasticity is achieved via activity-dependent transcriptional repression of *GluA1* in the nucleus (Korb et al., 2013). Moreover, Arc in the nucleus appears to be involved in the repression of other genes via an indirect interaction with CBP/p300, ultimately suppressing the activity of the transcription factor CREB (Korb et al., 2013). Therefore, it is possible that in the absence of Arc, the expression of CREB target genes is elevated, and that this alteration may contribute to biochemical and behavioral abnormalities in the *Arc* KO mouse.

Given the known association between Arc and CREB, as well as the abundance of literature implicating decreased CREB function in the expression of stress- and anxiety-like behaviors (Barrot et al., 2002; Covington et al., 2011; Muschamp et al., 2011; Pliakas et al., 2001), we hypothesized that in the *Arc* KO mouse, we would observe increased CREB function, which would manifest as increased expression of its target genes. We therefore measured the expression of 4 such genes: *cFos, GluA1, Bdnf, and Npy*. The latter three were particularly interesting to investigate, as they have been shown to be downregulated by stress

and upregulated by antidepressants (Duman and Monteggia, 2006; Primeaux et al., 2005; Vialou et al., 2010). Evidence from a study published in 2010 also supported this hypothesis; in this paper, it was reported that in the hippocampus of *Arc* KO mice, NPY protein levels were elevated basally compared to WT littermates (Peebles et al., 2010). Therefore, it is plausible that in the PFC, a region in which Arc may be required for anxiety-related behavior (Fig 10C), there is an increase in CREB target gene expression in *Arc* KO mice, consistent with an anxiolytic profile (Barrot et al., 2005). Analysis of gene expression in this brain region, however, did not seem to support this hypothesis. There did not appear to be any statistically significant differences in any of the CREB target genes examined in the PFC (Fig 11). Therefore, we concluded that, at least within the PFC of the *Arc* KO mouse, Arc is dispensable for the normal expression of several CREB target genes, and that modulation of CREB function in this region does not likely contribute to the anxiolytic-like phenotype we observe.

Discussion

In this study, we hypothesized that loss of Arc would result in enhanced anxiety- and depression-related behavior. This prediction is based on findings from a recent study in which it was shown that in response to CSDS or clinical depression, levels of *Arc* in the mPFC or anterior cingulate cortex, respectively, are basally decreased (Covington et al., 2010). Because stress tonically reduces *Arc* levels, we

speculated that loss of Arc would mimic a behavioral state of enhanced anxiety- and depression-like behavior. Instead, what we observe is an anxiolytic and antidepressant effect of Arc deletion in the *Arc* KO. Using a conditional Arc overexpression strategy, we then found that an anxiogenic phenotype emerged by increasing Arc levels in the mPFC of WT mice. Our results provide preliminary evidence to support a model through which Arc gates the transition from acute stress responses to depressive-like behaviors (Fig 12), and give rise to a number of different conclusions clarifying the role of Arc function in stress and anxiety.

First, it is possible that the tonic reduction of *Arc* observed after CSDS is dissociable from the expression of the CSDS phenotype. That is, the reduction in *Arc* levels may have no bearing on the behavioral consequences of stress and may simply reflect a parallel phenomenon occurring in tandem with the development of stress-related behavior. In agreement with this hypothesis, it was found that optogenetic stimulation of mPFC reverses the depression-related phenotypes, but does not rescue the Arc expression deficits that emerge after CSDS, suggesting that Arc induction is not necessary to promote an antidepressant response (Covington et al., 2010). The so-called “hypofrontality” reported in the aforementioned study may be accounted for by the cortical atrophy that is widely reported in postmortem tissue analyses in depressed patients or in preclinical stress models (Kang et al., 2012). These structural changes would be accompanied by loss of dendritic spine synapses and proteins that are localized to these structures, such as Arc. It remains to be

clarified whether the tonic reduction in Arc observed in human patients or preclinical models has any functional consequences for behavior.

Our data highlight a second, important point regarding the role of Arc in anxiety-related behaviors. Even though the transgenic line we utilize in these studies is a constitutive, developmental knockout, it is likely that the behavioral phenotype we observe is due to a postnatal role for Arc in maintaining normal levels of anxiety rather than a developmental deficit. This is because we are able to show that in a WT background, overexpression of Arc can effectively mimic an anxiogenic state. Perhaps, then, in the *Arc* KO mouse itself, viral-mediated gene transfer of Arc would similarly reduce the anxiolytic behavior to WT levels. This prediction is supported by a number of other related studies in which other phenotypes attributed to *Arc* knockout, including deficits in memory consolidation and LTP maintenance (Plath et al., 2006), can be reproduced in the adult with acute knockdown of *Arc* (Czerniawski et al., 2011; Guzowski et al., 2000; Holloway and McIntyre, 2011; Ploski et al., 2008), suggesting that synaptic and behavioral changes can be modulated in the adult brain via acute manipulation of this gene.

The mechanism by which acute overexpression of Arc induces anxiety is still unclear, but it likely involves changes in synaptic plasticity of brain regions implicated in stress and anxiety (Fig 12). It is possible that, given its role in maintaining homeostatic levels of surface AMPARs, Arc can modulate anxiety by increasing or decreasing the responsiveness of certain neuronal populations to

activity. In the mPFC, where overexpression of Arc is anxiogenic, this manipulation may exert a silencing effect on the activity of this brain region by promoting the endocytosis of AMPARs and the subsequent weakening of synaptic connections onto cortical neurons. This reduced responsiveness would then have inhibitory-like effects on downstream, subcortical structures that have been heavily implicated in anxiety, such as the NAc and amygdala. Indeed, reduced activity of these postsynaptic sites has been documented by others to be sufficient to drive anxiety and depression-like responses to stress (Tye et al., 2011; Wallace et al., 2009).

In contrast to the effects we observe with mPFC-specific gene delivery, overexpression of Arc in the NAc does not produce the same pro-anxiety phenotype. It is plausible that in the NAc, surface AMPARs are already at a relatively low level, and that introduction of Arc would not be expected to produce a strong physiological response in this brain region. Alternatively, Arc in amygdala-projecting mPFC neurons may contribute more to exploratory drive than the NAc, given the noted role of mPFC-originating amygdalar afferents in controlling anxiety- and fear-related behaviors (Milad et al., 2004). Using electrophysiological techniques, it would be informative to dissect the circuitry of deep layer mPFC pyramidal projection neurons and test whether and how introduction of Arc into specific subregions of the mPFC (infralimbic vs. prelimbic, for example) affects evoked postsynaptic responses in the NAc, amygdala, and other projection sites, and if these responses correlate with anxiety-like behaviors.

Conversely, how loss of Arc in the context of a constitutive KO reduces anxiety- and depression-like behavior is a question that also warrants further study. The biochemical studies conducted here and elsewhere provide some initial informative clues. We originally hypothesized that CREB activity would be heightened in the absence of the transcriptional repressive function reported to be exerted by Arc (Korb et al., 2013). Our results suggest that in the absence of Arc, CREB mediated gene expression is normal. It is possible that in the *in vivo* condition, there are compensatory mechanisms that drive the expression of CREB target genes, such as activity from transcription factors like MEF2, which have many overlapping gene targets with CREB (Flavell et al., 2008). The 2013 study by Korb and colleagues relied primarily on an embryonic neuronal culture system, where such compensatory mechanisms would naturally be absent.

We have shown that in the *Arc* KO, there is selective upregulation of surface AMPARs in the NAc, but not PFC, amygdala, or hippocampus (see Chapter 5, Fig 13). As one might expect, these changes occur in concert with an increase in synaptic strength of NAc MSNs, manifested by increased amplitude of miniature excitatory postsynaptic current (mESPC) in this region (see Chapter 5, Fig 14). These data would suggest that, in the context of the *Arc* KO, NAc neurons receive stronger synaptic input from afferent projects. Given the importance of the ventral striatum in controlling mood, motivation, and reward-related behaviors, it may be the case that Arc-mediated synaptic strengthening and weakening in the NAc dictates

levels of stress- and anxiety-like behaviors. A conditional knockdown strategy utilizing acute, postnatal knockdown specifically in the NAc would help to strengthen this argument. Based on our current findings, we hypothesize that such a manipulation would not only increase surface AMPARs, but also phenocopy the behavioral responses we have observed with respect to stress and anxiety.

Finally, it is worth pointing out that in a previous report by Plath and colleagues, no differences in anxiety-related behavior could be detected in the elevated zero maze (a variant of the EPM), dark/light exploration test, and open field test (Plath et al., 2006), which contrasts with the findings presented here. In this study, a different *Arc* KO line was utilized than the one we have characterized. The mouse line used in our studies contains a GFP allele substituted into the open reading frame of *Arc*, such that GFP is expressed under the control of the *Arc* promoter (Wang et al., 2006), whereas the mouse line used in the Plath 2006 study was generated by targeted deletion of the *Arc* open reading frame. In both scenarios, Western blot analysis of brain lysate reveals the absence of a 55 kDa band, corresponding to Arc protein; thus, functionally speaking, these two different mouse lines are similar. However, it is possible that strain-genotype interactions vary per mouse line, such that differential effects of genotype emerge based on genetic background. For example, in the mouse line we use here, it was reported that in the hippocampus there is a decrease in dendritic spine density in CA1 (Peebles et al., 2010), whereas in the 2006 study, no change in spine density in this region was

reported (Plath et al., 2006). In order to reconcile the anxiety findings reported here with those reported previously, it would be informative to show that the phenotypes we observe can be restored to WT levels via introduction of full-length *Arc* into the KO, thereby implicating the loss of Arc in the expression of anxiety-like behavior.

Figure 7. Regulation of Arc mRNA and protein by social defeat stress

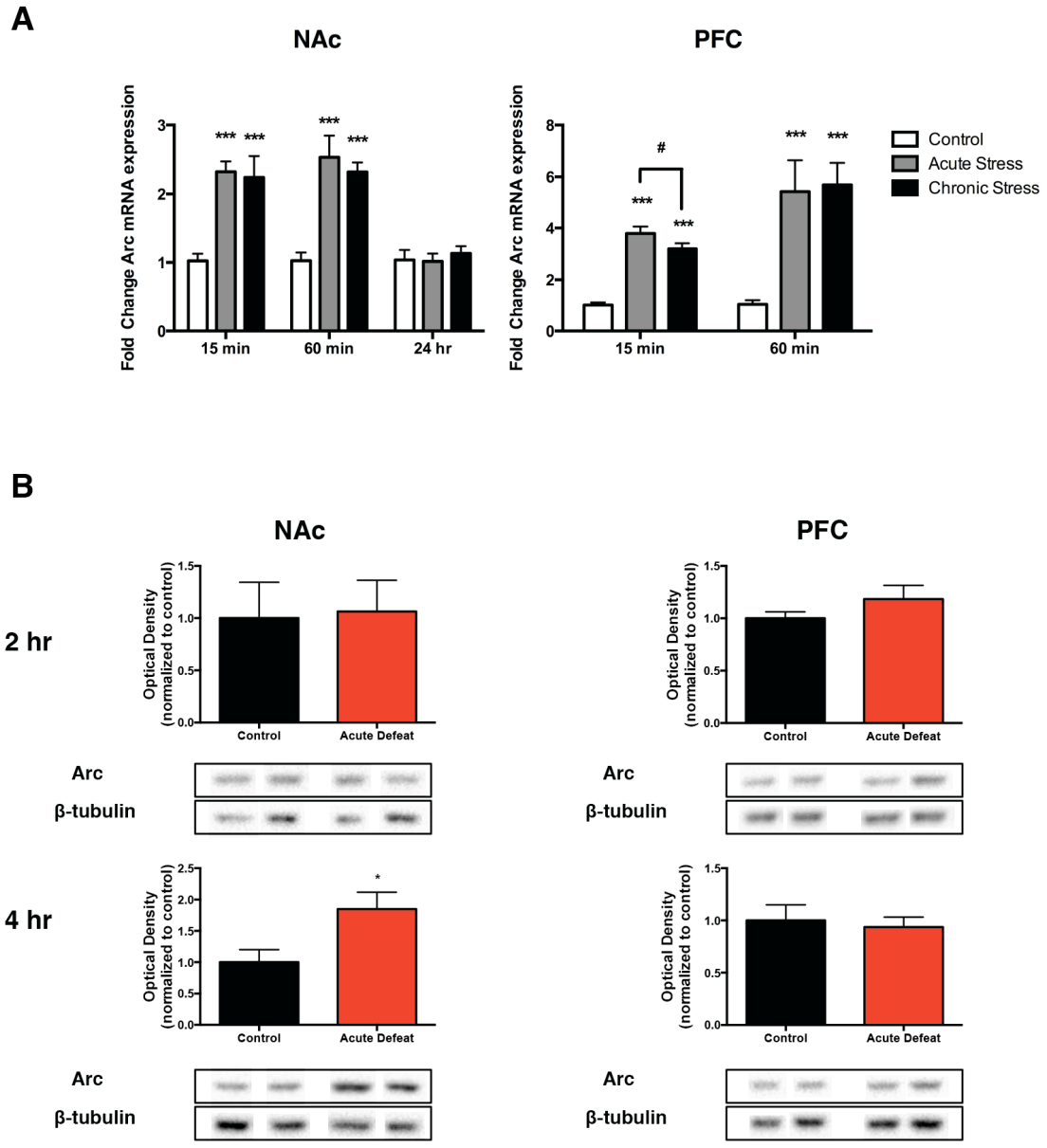

Figure 7. Regulation of Arc mRNA and protein by social defeat stress. **A.** Relative mRNA levels of Arc at 15 min, 1 hr, and 24 hr after acute (1 day) or chronic (10 days) social defeat stress in the NAc and PFC. Fold change calculated as $2^{-\Delta\Delta Ct}$. Ct values normalized to GAPDH. n=5-10/group. *** $p < 0.001$, compared to control condition, *t* test. # $p < 0.10$ between acute and chronic conditions, *t* test. **B.** Relative levels of Arc protein as assessed by quantitative Western blotting in the NAc and PFC 2 hr and 4 hr after 1 episode of social defeat stress. n=5-9/group, * $p < 0.05$ compared to control, *t* test.

Figure 8. *Arc* KO mice show reduced anxiety-like behaviors

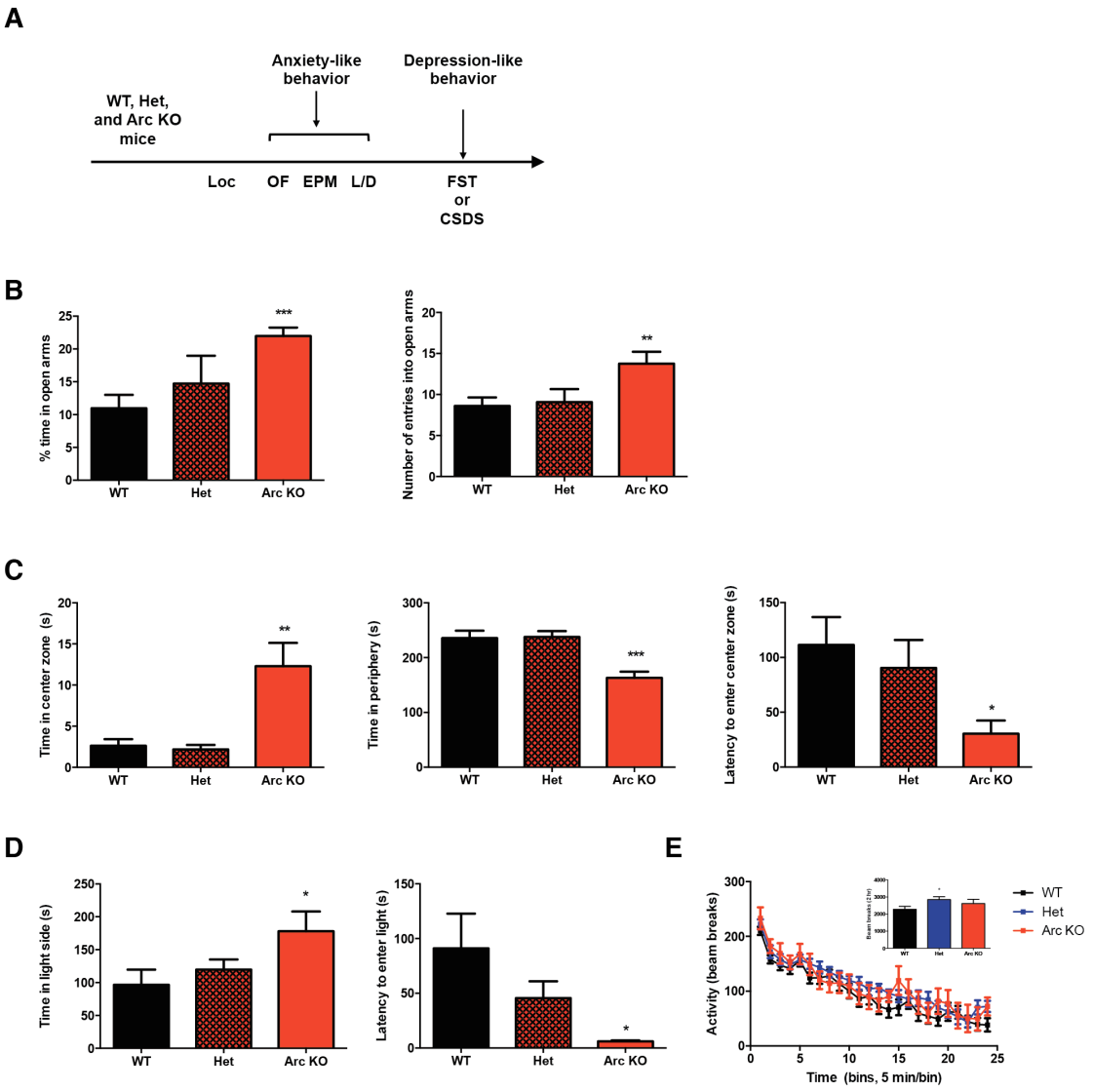

Figure 8. *Arc* KO mice show reduced anxiety-like behaviors. **A.** Timeline for behavioral phenotyping of WT, Het, and *Arc* KO littermates. **B.** Time spent (left) and number of entries (right) into the open arms of the EPM. **C.** Time spent in the center (left) and periphery (middle) zones, and latency to enter the center zone (right), during exposure to a novel open field. **D.** Time spent on light side (left) and latency to enter light side (right) in the light/dark exploration test. **E.** Locomotor response to a novel home cage. Inset: total locomotion, assessed by beam breaks, over a 2 hr time course. n=10-16/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to WT, *t* test.

Figure 9. *Arc* KO mice show reduced depression-like behaviors

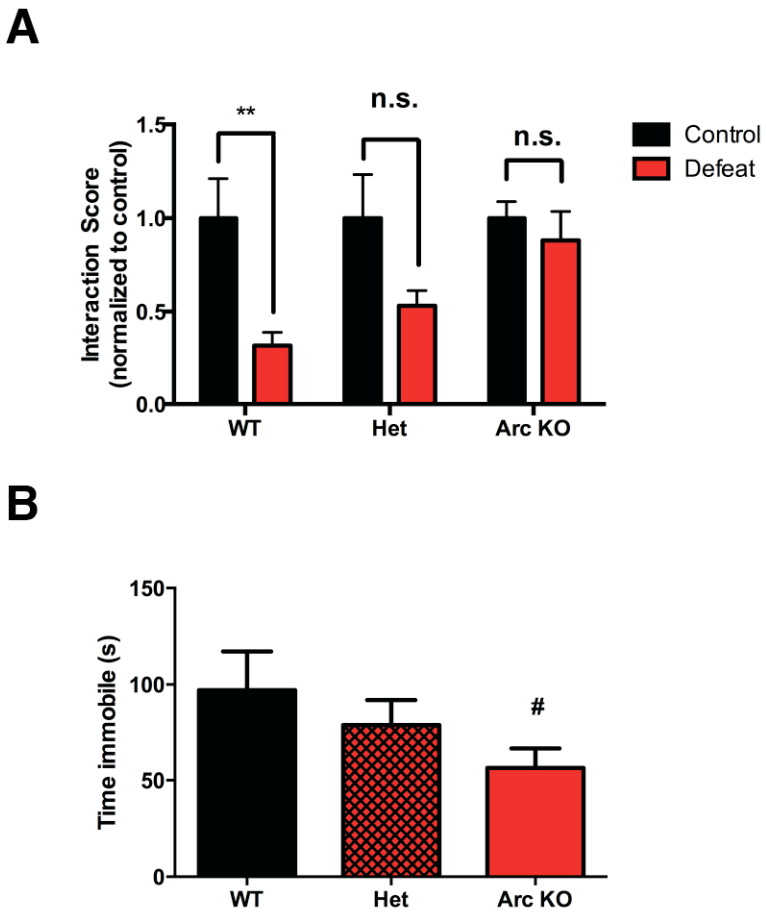

Figure 9. *Arc* KO mice show reduced depression-like behaviors. **A.** Social interaction scores (calculated as time in interaction zone in the presence vs. absence of target mouse, normalized to corresponding control condition) for WT, Het, and *Arc* KO littermates after 10 days of social defeat stress. n=8-15/group. ** $p < 0.01$ compared to WT control, *t* test. **B.** Time spent immobile in the Porsolt forced swimming test. n=10-16/group. # $p < 0.10$ compared to WT, *t* test.

Figure 10. Overexpression of Arc in the mPFC, but not the NAc, increases anxiety-like behavior

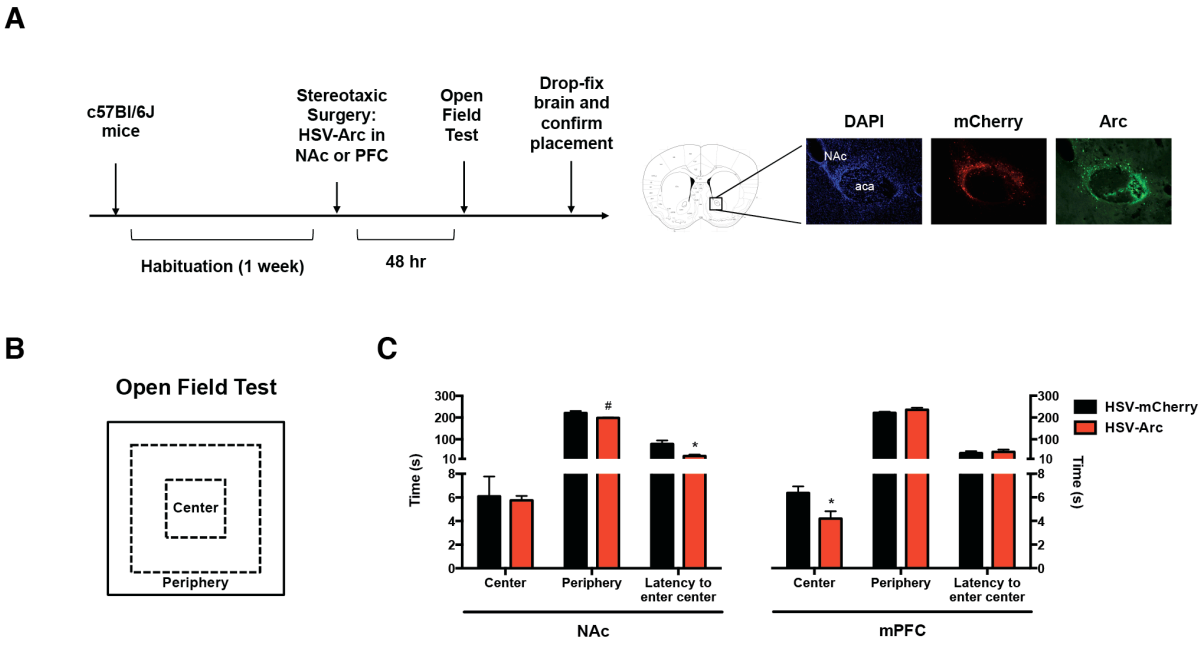

Figure 10. Overexpression of Arc in the mPFC, but not the NAc, increases anxiety-like behavior. **A.** Timeline for behavioral testing of mice following bilateral, viral-mediated delivery of Arc into the mouse brain. Right, representative fixed coronal section demonstrating infection of NAc with HSV-Arc. **B.** Schematic of open field used for behavioral testing. **C.** Anxiety-like behavior in a novel open field following overexpression of Arc in the NAc or mPFC of c57Bl/6 mice. n=4-5/group (NAc); n=7-11/group (mPFC). * $p < 0.05$, $^{\#}$ $p < 0.10$ compared to HSV-mCherry condition, *t* test.

Figure 11. Analysis of CREB target genes in the PFC of the *Arc* KO mouse

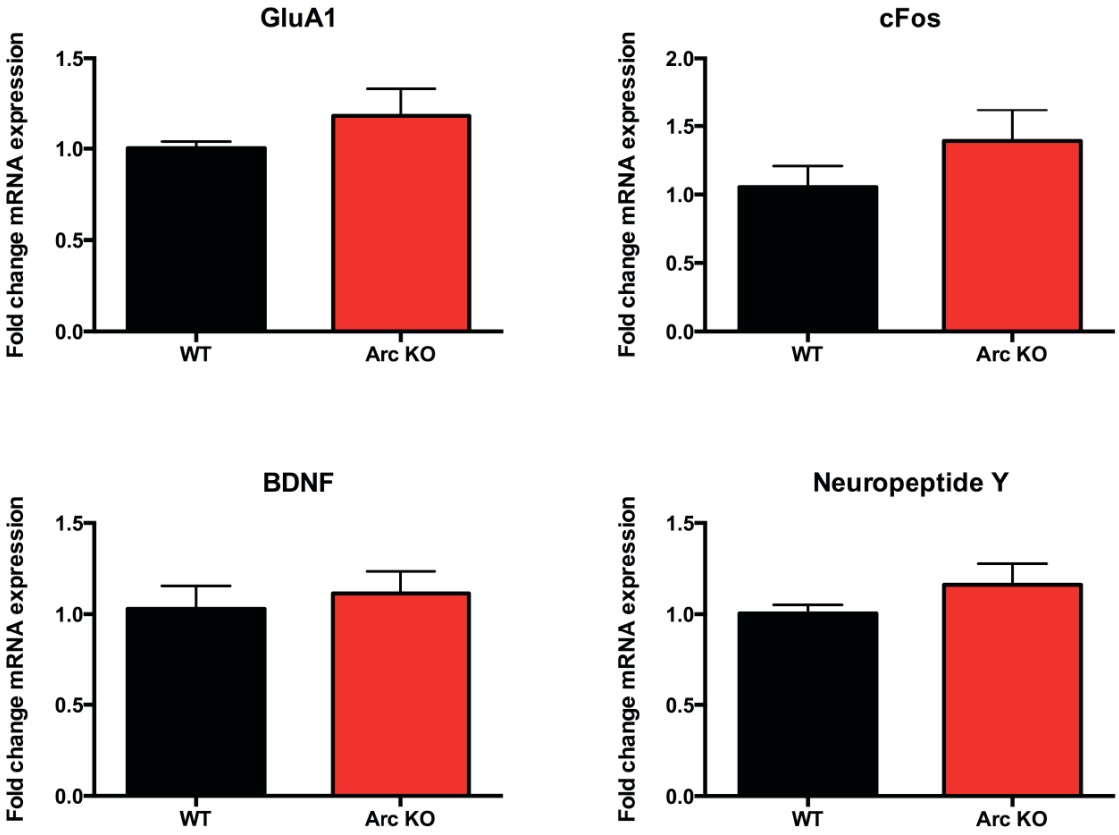

Figure 11. Analysis of CREB target genes in the PFC of the *Arc* KO mouse. Tissue samples from PFC were dissected from WT and *Arc* KO littermates, and total mRNA levels of CREB target genes were quantified by qRT-PCR. Fold change calculated as $2^{-\Delta\Delta Ct}$. Ct values normalized to GAPDH. n = 5-6/group.

Figure 12. Proposed model of Arc regulation of stress- and anxiety-related behavior

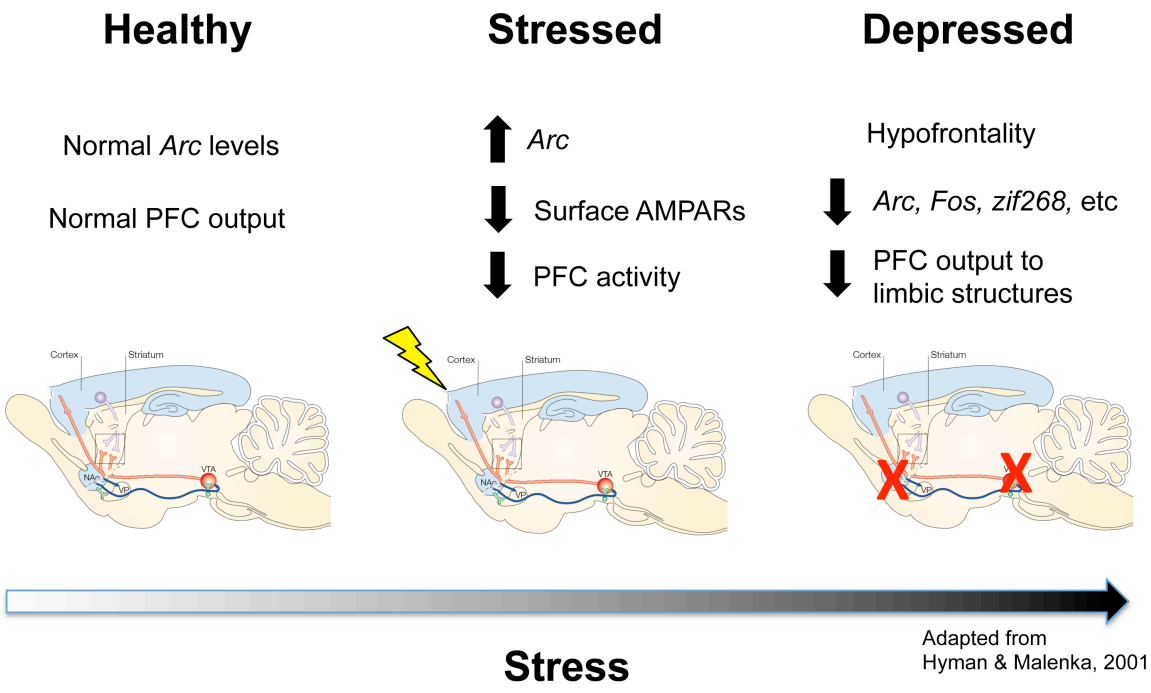

Figure 12. Proposed model of Arc regulation of stress- and anxiety-related behavior. Acute stress transiently induces Arc in limbic brain regions, resulting in a reduction in neuronal activity. Under conditions of chronic stress, both activity-regulated gene expression and excitatory output to subcortical limbic structures from PFC are reduced. These changes are accompanied by an increase in anxiety- and depression-related behavior. The transition from acute stress to depression is dependent on Arc; thus, *Arc* KO mice exhibit resistance to the behavioral consequences of chronic stress.

CHAPTER FIVE

ACTIVITY-REGULATED CYTOSKELETON-ASSOCIATED PROTEIN REGULATES MULTIPLE BEHAVIORS INDUCED BY REPEATED COCAINE

Jaswinder Kumar[1,2], Laura N. Smith[2], Jakub P. Jedynak[2], and Christopher W. Cowan[1,2]

[1]Department of Psychiatry, UT Southwestern Medical Center, Dallas, TX, USA

[2]Department of Psychiatry, Harvard Medical School, McLean Hospital, Belmont, MA, USA

Author Contributions

JK, LNS, and CWC designed experiments. JK conducted biochemistry and CPP experiments. LNS conducted locomotor sensitization experiments. JPJ conducted electrophysiological experiments.

Abstract

Cocaine dependence is often characterized by short cycles of abuse, abstinence, and relapse, the latter of which is thought to result from time-dependent incubation of craving and re-exposure to cocaine-conditioned cues. Previous studies indicate that acute cocaine exposure transiently upregulates mRNA and protein expression of the immediate early gene, activity-regulated cytoskeleton-associated protein (Arc), in reward-related brain regions of rodents, an effect that becomes more persistent after multiple exposures. We show here that mice lacking Arc develop altered psychomotor sensitization compared to WT littermates in response to moderate doses of cocaine, a difference that is evident beginning very early in exposure. Additionally, *Arc* KO mice demonstrate enhanced cocaine reward learning following cocaine preexposure. As may be expected given Arc's known cellular functions, we observe a selective basal increase in AMPA receptor surface expression in the NAc of the *Arc* KO mouse as well as increased synaptic strength, which potentially contributes to the altered cocaine behavioral responses observed in these mice. Together, our results demonstrate that mice lacking Arc have enhancements in both cocaine-induced behavioral plasticity and AMPAR function in the NAc. Future studies addressing the role of this protein in behaviors that are thought drive continued abuse and addiction, such as cue-induced craving and relapse, are warranted.

Introduction

One of the most challenging aspects in treating patients suffering from substance abuse disorders is the prevention of relapse, or the act of resuming drug-taking behaviors after a period of time during which the user has remained abstinent. A number of human and preclinical studies suggest that during this withdrawal period, a set of neurobiological events are set in motion that promote the expression of relapse-like behaviors, including drug craving. This so-called “incubation of craving” (Gawin and Kleber, 1986) intensifies over long periods of withdrawal, a phenomenon that has been demonstrated in both humans and non-human experimental models. Accordingly, a major goal in the treatment of human addicts is understanding the psychological and neurobiological processes that drive both the development of craving during abstinence as well as the expression of such craving, or what is conceptualized as “drug-seeking behaviors.”

Much of the neural circuitry involved in the rewarding and incentive-motivating properties of drugs has been described in rodent models of psychostimulant addiction. Both cortical and subcortical brain regions, including the amygdala, hippocampus, and prefrontal cortex, are thought to each encode various aspects of drug exposure, such as salience of drug, interoceptive state produced by drug, and context in which drug is administered. These brain regions send afferent projections encoding this information to the nucleus accumbens (NAc), where glutamate released from presynaptic terminals binds to iontropic AMPA receptors found in NAc

medium spiny neurons. The NAc then transmits information through MSN projections to motor regions that ultimately subserve the completion of subsequent addiction-like behaviors (Groenewegen et al., 1999; Pascoli et al., 2014). Thus, the underlying mechanisms through which drugs of abuse increase NAc MSN function, and the molecules and signaling pathways that are recruited and required for AMPAR-dependent glutamatergic signaling in the NAc, have become a focus of intense study.

One mechanism by which excitatory neurotransmission in the NAc can be enhanced is through the trafficking of AMPARs into synapses. As it relates to drug exposure, a number of studies now indicate that surface AMPARs are elevated in response to withdrawal from psychostimulants. In rats given repeated injections of cocaine, biochemical analysis revealed an increase in surface levels of AMPAR subunits GluA1 and GluA2/3 at 21 d of withdrawal, and this was not accompanied by changes in total protein levels of these AMPARs. In contrast, after repeated cocaine injections and 1 day of withdrawal, surface AMPARs were unchanged, suggesting an extended withdrawal-dependent redistribution of AMPARs in response to cocaine (Boudreau and Wolf, 2005). These findings were replicated in a cocaine self-administration study, where it was found that 45 d after the last session of cocaine, surface AMPARs were increased compared to a 1 d withdrawal time point. Interestingly, at this 45 d withdrawal time point, elevations in GluA1, but not GluA2, were noted, suggesting that long withdrawal times selectively increase GluA2-

lacking, Ca^{2+} permeable AMPARs. Such alterations in AMPAR composition would result in higher current conductance, further elevating NAc output (Conrad et al., 2008). This particular finding was corroborated by electrophysiological analysis of NAc MSNs after protracted withdrawal from cocaine self-administration (McCutcheon et al., 2011), in which inward rectification of AMPAR EPSCs in NAc MSNs was demonstrated. Therefore, it appears that during withdrawal, there is a progressive strengthening of accumbal synapses mediated by enhanced trafficking of subunit-specific AMPARs to and from the synapse.

Because drugs of abuse modulate the strength of excitatory synapses onto NAc neurons, and because AMPAR-mediated synaptic transmission is required for the development and expression of many cocaine-induced behaviors (Li et al., 1997), we were interested in asking whether and how manipulations in surface AMPAR availability contribute to drug-related behaviors. One important mediator of AMPAR trafficking is the activity-regulated cytoskeleton-associated protein, Arc. Arc is enriched in neuronal dendrites, where it facilitates activity-dependent AMPAR endocytosis at glutamatergic synapses by binding endophilin-2 and dynamin-3, two proteins required for vesicle-mediated endocytosis (Chowdhury et al., 2006). Arc is induced by synaptic activity, and a number of studies have now demonstrated that acute exposure to drugs of abuse can transiently induce Arc specifically in corticolimbic circuitry (Fosnaugh et al., 1995; Fumagalli et al., 2009; Klebaur et al., 2002; Tan et al., 2000). Interestingly, this effect becomes more persistent with

repeated exposures (Fumagalli et al., 2006), suggesting a potential role for Arc in the synaptic and behavioral adaptations that ensue during abstinence, long after drug was last administered. Here, we sought to characterize the role of Arc in these events by utilizing an *Arc* KO mouse. Because Arc participates in the removal of AMPARs from the synapse, we hypothesized that in the *Arc* KO mouse, surface AMPARs would be elevated in reward-related brain regions, and that as a consequence, these animals would exhibit addiction-related phenotypes across a spectrum of behavioral assays that capture various facets of addiction, including cocaine psychomotor sensitization and reward.

Results

Surface AMPARs are selectively upregulated in the NAc of Arc KO mice

A number of studies have examined the effect of Arc overexpression on surface AMPARs, either via biochemical, immunohistochemical, or electrophysiological means. However, to date it has not been established whether constitutive knockout of Arc alters surface AMPAR levels. To answer this question, we collected brain tissue from *Arc* KO and WT littermates and conducted a surface protein crosslinking assay, allowing us to segregate cytoplasmic and surface populations of proteins from a single tissue sample (Boudreau et al., 2012). We then conducted quantitative western blotting of AMPAR subunits, including GluA1, GluA2 and GluA3. We found no difference between WT and KO in levels of internal,

cytoplasmic GluA1, 2, or 3, in the prefrontal cortex, hippocampus, and amygdala (Fig 13A-B). However, in the NAc, we observed a significant reduction in the internal pool of GluA1, 2, and 3 (Fig 13A-B), without any changes in mRNA levels in GluA1 (Fig 13C), nor in protein levels (J. Kumar, unpublished observations). If we assume that the internal, protected pool of GluA1, 2, and 3 subunits decreases in concert with a proportionate increase in surface levels, then these results indicate that in the *Arc* KO, surface AMPARs, as detected by the protein cross-linking assay, are increased only in the NAc, without any change in total GluA1. These data suggest, then, that normal AMPAR trafficking or surface retention is altered in the NAc.

The biochemical analysis of surface GluA1 levels provides insight into the cellular distribution of AMPARs, but does not aid in discerning whether these receptors are localized to the synapse, and therefore, functionally relevant to glutamatergic neurotransmission. To answer this question, we conducted whole-cell patch clamp recording of NAc shell MSNs in WT and *Arc* KO mice and analyzed miniature excitatory postsynaptic currents (EPSCs) (Fig 14A). In our analysis, we observed that, compared to the WT condition, MSNs from the NAc of naïve KO mice exhibit greater mEPSC amplitude, suggestive of an increase in postsynaptic function via elevations in synaptic AMPARs (Fig 14B). Next, we asked whether loss of Arc affects the absolute number of mEPSC events, manifested by a change in mEPSC frequency. We found a basal increase in frequency in KO neurons compared to WT, indicative of either an overall increase in number of glutamatergic synapses in the

NAc, or enhanced presynaptic release of neurotransmitter onto NAc MSNs (Fig 14C). Thus, in the *Arc* KO mouse, our electrophysiological analysis suggests the presence of a basal increase in both the strength and possibly number of excitatory synapses present in the NAc.

Loss of Arc enhances cocaine locomotor sensitization

Previous reports demonstrate that Arc levels are elevated in the NAc following exposure to cocaine. In addition, it has been shown that an increase in AMPAR-mediated neurotransmission is apparent in NAc MSNs after protracted withdrawal from chronic cocaine administration (Conrad et al., 2008; McCutcheon et al., 2011). Our biochemical and electrophysiological analysis thus far indicates that the *Arc* KO mouse exhibits a synaptic profile reminiscent of a cocaine-experienced animal subjected to protracted withdrawal. For these reasons, we were curious to investigate whether Arc was functionally important in determining behavioral responses to drug exposure. Because Arc is required for AMPAR-dependent synaptic plasticity (Rial Verde et al., 2006), we hypothesized that loss of Arc would influence behavioral plasticity to repeated cocaine. To address this question, we decided to examine behavioral sensitization to cocaine, an assay used to measure the locomotor activating effects of drugs of abuse. In this procedure, animals given repeated intraperitoneal (i.p.) injections of cocaine develop a progressive enhancement in locomotor activity that occurs during repeated administration and

persists long after withdrawal from drug. This model is thought to represent the intensification of drug craving that takes place during periods of abstinence, or the process of drug "wanting" (Robinson and Berridge, 1993, 2000); however, the relevance of locomotor sensitization to drug addiction-related behaviors is debated in the field.

Here, we gave mice daily injections of cocaine (15 mg/kg) for 7 consecutive days. One hour before and one hour after each injection, locomotor activity was measured (Fig 15A). We found that, as expected, WT and Het mice sensitize to cocaine, with a greater locomotor response to cocaine on each day compared to previous days. This behavior was long-lasting, as these animals respond with high levels of activity to various challenge doses following withdrawal (Fig 15C). The *Arc* KO animals also sensitize to cocaine; however, the level of activity during the one hour following cocaine injection was significantly greater than WT mice on all days of cocaine administration (Fig 15C). The sensitized locomotion was also preserved at multiple withdrawal timepoints and at multiple cocaine challenge doses across all genotypes, but *Arc* KO mice displayed a significantly higher level of locomotion than littermate controls at all tested doses and withdrawal time points (Fig. 15C). Interestingly, during the habituation phase of the test (Figure 15B), which occurs each day 1 hr prior to cocaine injection, *Arc* KO mice exhibit a hyperactivity phenotype that is readily apparent at withdrawal timepoints, as evidenced by increased activity 1 hour prior to the challenge injection (Fig 15B). Taken together,

these data indicate that animals lacking Arc sensitize to cocaine to a greater extent than their WT littermates, and that loss of Arc may render an animal more susceptible to the locomotor-enhancing effects of psychostimulants such as cocaine.

Arc KO mice develop pre-injection hyperactivity in a context- and drug-independent manner

Our results thus far indicate that *Arc* KO mice show potentiated locomotor sensitization to cocaine. This phenotype is accompanied by pre-injection rises in locomotion that develop after withdrawal, a phenomenon suggestive of, and that may be conceptualized as, “anticipation” to the receipt of drug (Koob and Volkow, 2010). We were curious to understand the nature of this anticipatory activity. Specifically, does the increase in locomotor activity during the first hour of the assay occur only in response to cocaine, or this is a general novelty-induced hyperactivity phenotype? To begin answering this question, we devised an experiment in which we could administer cocaine to animals in one unique context, and saline in another distinct context. In line with previous studies looking at context-dependent cocaine behaviors (Koya et al., 2009), we constructed two different contexts consisting of two sides of a large mouse cage physically separated by a Plexiglas divider and differing only in the type of bedding used (tactile cues) and the wall patterns (visual cues) (Fig 16A). Using these chambers, we designed an experiment in which chronic saline would only be administered in one context, while in the other context animals would

receive a chronic, sensitizing dose of cocaine. We hypothesized that *Arc* KO animals would exhibit sensitized locomotion in the cocaine-paired side, but not in the saline-paired side. Furthermore, we predicted that, following 7 d withdrawal from drug, only in the cocaine-paired side would the KO mice display enhanced activity, and that this behavior would be absent in the saline-paired side. We found, as we saw previously, that the KO mice showed potentiated sensitization to cocaine in the cocaine-paired context (Fig 16B), while in the saline-paired context, no differences in post-injection locomotion emerged between WT and KO. Much to our surprise, though, we found that at a 7 d withdrawal timepoint, where we previously noted enhanced pre-injection activity in the KO (Fig 15B), the *Arc* KO mice showed anticipatory-like behavior in both contexts (Fig 16B). Therefore, it appears that while behavioral sensitization to drug is maintained only in response to cocaine, the anticipatory activity that we previously observed was not specific to a cocaine-paired context.

There could be a number of reasons for *Arc* KO mice showing a generalized, pre-injection hyperactivity in both a drug paired and non-paired environment. The animals may be unable to effectively make associations between drug and context; alternatively, it is possible that *Arc* KOs simply exhibit cocaine experience-independent hyperactivity to the locomotor chamber, possibly as a reversal of prior habituation to the chamber that occurs after a week of separation from the locomotor chambers. To rule out this latter possibility, we repeated the experiment conducted in Fig 16A, with minor modifications. We dosed *Arc* KO and WT littermates with

either saline or cocaine (15 mg/kg x 1 injection/day) for 7 consecutive days in a standard novel mouse cage and measured sensitized locomotor responses. After a 7 d home cage withdrawal period, we brought animals back to the locomotor boxes and measured activity in an injection-free setting (Fig 16C). As we observed previously, *Arc* KO mice showed increased activity in the first 15 min of testing; however, this potentiated locomotion was observed in both treatment groups, such that both the KO-sal and KO-coc groups exhibited higher locomotion than their WT counterparts (Fig 16D). Taken together, these experiments strongly suggest that, after withdrawal, *Arc* KO mice display a hyperactivity phenotype that is independent of cocaine administration.

Arc KO mice show enhanced cocaine reward learning

Studies in human addicts have pinpointed specific neural circuits underlying the craving of drugs of abuse, a term that can be associated, albeit loosely, with drug "liking" and drug "wanting" (Berridge et al., 2009). Our data indicate that, insofar as psychomotor sensitization is a model of incubation of drug craving, loss of Arc renders an animal more susceptible to the "wanting" of drug. We were next motivated to ask whether the interoceptive state of the animal in response to cocaine – the "liking" component of drug craving – is altered in the *Arc* KO mouse. Given the extensive circuit, cellular, and molecular overlap between processes involving drug "liking" and drug "wanting" (Koob and Volkow, 2010), we hypothesized that animals

showing increased locomotor responses would also exhibit increased reward-related behavior in response to cocaine. To answer this question, we took *Arc* KO and WT littermates that had been sensitized to cocaine and conducted a conditioned place preference assay after ~one week of drug withdrawal. We conditioned these animals to associate the administration of 5 mg/kg cocaine with a novel, specific context, while also receiving saline in a separate context (Fig 17A). On test day, following the conditioning sessions, we assayed the animal's preference to spend time in the cocaine- or saline-paired contexts. We observed an increase in cocaine CPP score in the *Arc* KOs (Fig 17B-C), suggesting that the absence of Arc results in cocaine experience-dependent sensitization of cocaine reward.

A number of groups have reported that pre-exposure to drugs in genetically identical adult rodents sensitizes reward-related behaviors (Deroche et al., 1999; Meririnne et al., 2001; Russo et al., 2009), whereas other groups have reported no change (Nocjar and Panksepp, 2002). In our hands, we observed no preference for cocaine after previous cocaine experience in WT mice (Fig 17C). One possible interpretation of this data is that drug pre-exposure induces a tolerance to its rewarding effects, as has been demonstrated by another group (Zachariou et al., 2001). In order to determine whether the reward behavior observed in *Arc* KO mice represents an enhancement in the rewarding effects of drug or a lack of tolerance-like effect from prior cocaine exposure, we repeated the experiment in Fig 17, with modifications. We dosed *Arc* KO and WT littermates with either saline or cocaine (15

mg/kg x 1 injection/day), and following ~one week of home cage withdrawal, assessed their place preference for cocaine (Fig 18A). We found that in chronic saline-injected animals (sal), there was no difference in cocaine reward behavior in *Arc* KO mice compared to WT littermates (Fig 18B). In cocaine-experienced mice (coc), *Arc* KOs showed a more robust increase in preference for a cocaine-paired side compared to WT, cocaine-sensitized mice (Fig 18B). Two-way ANOVA revealed a trend for a significant interaction effect between genotype and prior cocaine exposure ($P = 0.08$). *Post-hoc*, multiple comparison analysis of the data revealed a significant increase in cocaine CPP in *Arc* KOs compared to WT. While this study is ongoing (*i.e.* additional mice are being tested currently to complete the study), the preliminary findings suggest that the absence of Arc results in cocaine experience-dependent sensitization of cocaine reward, and that loss of Arc in drug-naïve mice does not alter the sensitivity for cocaine reward. Surprisingly, prior chronic cocaine exposure in WT mice under these conditions did not result in sensitized cocaine reward, suggesting that cocaine-induced elevation of Arc levels in the NAc serves to antagonize the development and/or expression of cocaine reward sensitization.

Discussion

Accumulating evidence points to regulation of glutamate receptor trafficking by drugs of abuse in reward-related brain regions, yet the role of these events in mediating addiction-related behaviors has yet to be fully described. In the current

study, we explored whether Arc, a known mediator of neuronal activity-dependent AMPAR endocytosis, influences cocaine-related behaviors, and identified a unique role for this gene in dictating behavioral plasticity to repeated cocaine administration. Our results implicate Arc in the development of psychomotor sensitization, reward learning, and AMPAR plasticity in the NAc.

Arc, behavioral sensitization, and cocaine reward

Arc KO mice display augmented acute locomotor responses to 15 mg/kg cocaine and sensitize to drug at similar rates as WT controls, but to different levels proportional to their respective Day 1 starting point (Fig 15). Given Arc's known cellular function in regulating AMPAR plasticity, it is possible that the enhancement in sensitization is due to elevated surface AMPARs in the NAc (Fig 13 and 14). This is a plausible explanation, in light of several studies that have shown AMPAR function in the NAc to be important for the expression of sensitized responses to psychostimulants. For example, AMPAR antagonists injected locally into the NAc block the expression of sensitization after withdrawal, and conversely, intra-NAc injections of AMPA potentiate the locomotor response of sensitized rats 3 weeks after the last cocaine injection (Li et al., 1997; Pierce et al., 1996), a timepoint at which surface AMPARs are likely to be elevated. In the absence of Arc, we observe an increase in surface AMPARs (Fig 13 and 14) basally in the NAc, which presumably renders MSNs more reactive to glutamate release that accompanies

cocaine administration (Wolf and Ferrario, 2010). Thus, an injection of cocaine might stimulate enhanced NAc glutamatergic synaptic transmission compared to WT controls, likely resulting in increased locomotion. In this sense, the *Arc* KO mouse might be conceptualized as a partially "pre-sensitized" model of cocaine-induced plasticity; that is, it displays the synaptic and behavioral profile of a cocaine-experienced animal after long-term withdrawal (2-3 weeks or more).

On the other hand, it is conceivable that the mechanism underlying enhanced behavioral sensitization in the KO has little to do with AMPARs. This is based on a number of reports demonstrating a dissociation of surface AMPARs from the expression of sensitized locomotion. In response to chronic regimens of cocaine, animals develop locomotor sensitization; however, sensitization develops as early as 24 hr after the last injection of cocaine, long before any changes in surface AMPARs are apparent (Boudreau and Wolf, 2005). Additionally, when animals are given a challenge dose of cocaine during withdrawal, AMPARs in the NAc have been reported to undergo internalization, reflecting an LTD-like phenomenon, even though the sensitized response persists (Thomas et al., 2001). These two observations suggest that elevated AMPARs are not necessary for the expression of behavioral sensitization. Nevertheless, our data may support a model in which elevated surface AMPARs are sufficient to enhance the locomotor response in a naïve mouse, and that a behavioral sensitization process proportionally increases locomotion in the *Arc* KO mouse.

The sensitized cocaine reward behavior in the *Arc* KO is potentially reminiscent of a cocaine-experienced animal after long-term withdrawal, and as such, the *Arc* KO effect might represent a precocious development of reward sensitization (or incubation of craving or drug seeking that develops after long-term withdrawal). It might also represent an abnormal change in animal behavior that is not normally expressed in WT mice due to an Arc-dependent function that blocks sensitized reward behavior. To test these hypotheses, it would be informative to locally ablate Arc function in the NAc and demonstrate that this manipulation not only enhances surface AMPARs, but also augments sensitization. Alternatively, by restoring Arc expression using viral-mediated gene transfer, for example, into the NAc of the *Arc* KO mouse, perhaps the increases in sensitization and synaptic strength that we observe would be normalized to WT levels. Such a finding would lend further credence to the idea that Arc in the NAc functions to limit sensitization behavior via trafficking of AMPARs out of the synapse, thereby reducing synaptic strength and output of NAc MSNs.

One interpretation from this study is that the process of behavioral sensitization occurs normally in the *Arc* KO mice, but naïve cocaine locomotion response is basally elevated. This would also be consistent with our observation of a basal increase in surface and synaptic AMPARs in the NAc of *Arc* KO mice. It is intriguing to consider the possibility that the apparent increase in cocaine reward sensitivity in drug-experienced, but perhaps not drug-naïve, mice is a result of

enhanced or precocious further elevation of synaptic AMPARs in the NAc of *Arc* KO mouse. In the future, it will be important to study the effects of cocaine on synaptic AMPARs after repeated cocaine exposure and after different withdrawal periods. Since re-exposure to cocaine after withdrawal normalizes the elevated AMPA/NMDA ratio observed after 10-14 d of withdrawal, we might also predict that Arc is important for this process since Arc is known to induce endocytosis of AMPARs. Along these lines, it would be useful to test whether experimental upregulation of surface AMPARs, either by locally knocking down Arc expression, or by infusion of a peptide that blocks AMPAR endocytosis, for example (Brebner et al., 2005), alone is sufficient to produce enhanced behavioral sensitization.

Analysis of locomotor sensitization in the *Arc* KO also reveals the presence of a pre-injection hyperactivity phenotype. We hypothesized that this behavior may represent a form of “anticipation” to the administration of cocaine, a terminology borrowed from the clinical literature, in which addicts who are abstinent for long periods of time develop an enhanced sensitivity to conditioned contexts and cues. In the presence of such cues, a latent preoccupation, or craving, for drug is unmasked, which can manifest as drug-seeking behavior (Koob and Volkow, 2010). We were unable to demonstrate that the KO mice discriminate between a drug-paired and non drug-paired context, as the animals exhibit enhanced activity on test day in both contexts (Fig 16). Our follow up analysis revealed that KO animals that had been dosed chronically with saline also exhibited this pre-injection hyperactivity. Thus, this

behavior is uncoupled from previous cocaine experience, and may simply be an effect of Arc deletion. It may be the case that over the course of the 10 d of exposure, WT mice habituate to the chambers, such that re-exposure after 7 d withdrawal results in similar levels of activity. In the case of the *Arc* KO, though, perhaps these mice show enhanced sensitivity to novelty, owing to an inability to consolidate the experience of 10 d of habituation over a 7 d withdrawal time period. This explanation is supported by the current consensus regarding the role of Arc in memory formation, namely, that these animals exhibit deficits in long-term memory formation, while short-term memory mechanisms remain intact (Peebles et al., 2010; Plath et al., 2006)

The role of Arc in cocaine reward learning behavior

A number of important observations emerge from our finding of enhanced cocaine reward learning in cocaine-experienced *Arc* KO animals. First, it is interesting that in the *Arc* KO mouse, which is purported to exhibit deficits in long-term memory formation (Plath et al., 2006), normal cocaine reward learning is intact, as evidenced by positive CPP scores (in either KO-sal or KO-coc groups) that are expressed 24 hr after conditioning (Fig 17-18). In a series of studies following up on the findings of Plath and colleagues, a number of groups have dissected potential mechanisms by which Arc regulates long-term memory. They utilized antisense ODNs to acutely knock down *Arc* and examine its effects on fear conditioning

behavior; it was found that both the dorsal hippocampus (Czerniawski et al., 2011) and basolateral amygdala (Ploski et al., 2008) were critical brain regions in which Arc is required for long-term context- and cue-dependent fear memories, respectively. In a similar vein, lesion studies demonstrate that both of these brain regions alone are required for the development of cocaine conditioned place preference (Fuchs et al., 2002; Meyers et al., 2003). Based on these studies, it is not unreasonable to hypothesize, then, that Arc in either the amygdala or hippocampus controls the formation of long-term memories related to drug exposure, such as those assayed by the CPP procedure. If this is in fact the case, then it may be possible that the enhanced place preference we observe in the *Arc* KO, in which Arc is absent in both of these critical brain regions, is being driven by Arc functioning directly or indirectly in other brain regions. Additional studies are needed here to sufficiently answer a complex behavioral question such as that. At this stage, though, we propose a model, based on our understanding of the role of Arc in regulating AMPAR plasticity, in which activity of the VTA-NAc circuit is selectively enhanced in the *Arc* KO, resulting in greater reward learning, which we detail below.

Cocaine reward learning relies heavily on dopaminergic transmission from the VTA to the NAc (Koob and Volkow, 2010). Psychostimulants like cocaine induce phasic dopamine (DA) cell firing, resulting in the release of supraphysiologic levels of DA into the synapse. It is thought that at these high levels, DA attributes motivational salience to cocaine and encodes the heightened arousal, conditioning learning, and

reward that become associated with the drug (Bromberg-Martin et al., 2010; Volkow et al., 2004). Indeed, the role of this neurotransmitter in the rewarding aspects of drugs of abuse has been demonstrated in pharmacological studies. Administration of the D1-R antagonist SCH23390 prior to conditioning sessions blocked the development of place preference to cocaine in rats (Nazarian et al., 2004). Moreover, place preference was similarly abolished in animals in which the NAc shell was subjected to 6-hydroxydopamine lesions, a manipulation that effectively ablates dopamine terminals in this region (Sellings et al., 2006).

Our biochemical and electrophysiological findings point to the presence of increased glutamatergic signaling in NAc MSNs, due to increased surface AMPARs (Figs 13-14). This finding alone would suggest alterations in NAc-dependent behaviors, such as reward (Koob and Volkow, 2010). Another anatomical substrate of drugs of abuse, unexplored in our study, which could contribute to enhanced reward learning, and whose activity is a critical determinant of NAc function, is the VTA. In response to *in vivo* cocaine exposure, dopaminergic neurons of the VTA are known to undergo LTP at excitatory synapses, involving the insertion of new AMPARs to the cell surface (Ungless et al., 2001). This synaptic adaptation is thought to increase levels or patterns of DAergic cell firing in downstream structures like the NAc, where dopamine-dependent behavioral outcomes, such as reward learning, are enhanced (Kauer and Malenka, 2007). Thus, in the *Arc* KO mouse, we would predict that, much like the NAc, there is an elevation in surface AMPARs on

DAergic cells, owing to the absence of endocytosis-promoting function of Arc. With more AMPARs present at the cell surface, glutamatergic drive onto VTA DAergic cells is increased, and as a consequence, perhaps dopamine cell firing is elevated in the KO. These circuit adaptations would then contribute to increased reward learning in response to cocaine. Supporting this hypothesis, a previous study found that in mice constitutively lacking the AMPAR subunit GluA1, a single exposure to cocaine failed to elicit LTP at VTA excitatory synapses. Moreover, these animals, while capable of expressing behavioral sensitization to cocaine, failed to exhibit place preference (Dong et al., 2004). These findings support a model in which increased plasticity at VTA DA neurons is required to elicit reward learning, and may provide a testable hypothesis to explain how loss of Arc enhances reward behavior, even in the absence of proper learning and memory mechanisms.

Arc regulates functional AMPAR plasticity in the NAc

Our initial biochemical findings suggested a role for Arc in regulating surface levels of AMPARs solely in the NAc. We then analyzed mEPSCs in NAc MSNs of the *Arc* KO and found an elevation in mEPSC amplitude, suggesting an increase in AMPAR-mediated synaptic strength. Interestingly, we also noted an elevation in mEPSC frequency in the KO, which is suggestive of one of two possibilities: (1) in the absence of Arc, there is an overall increase in functional synapse number in the NAc, or (2) loss of Arc renders presynaptic afferents in the NAc more susceptible to

glutamate release. Additional studies, investigating presynaptic release probability of various NAc afferents, as well as analysis of dendritic spine density of NAc MSNs, would help to clarify this question.

It is interesting to speculate that loss of Arc increases synapse number in the NAc, based on a number of other studies implicating Arc in the maintenance of synaptic plasticity in other brain regions. Previously, it was demonstrated that Arc is required for activity-dependent synapse elimination in cerebellar Purkinje cells (Mikuni et al., 2013) as well as in hippocampal CA1 neurons (Wilkerson et al., 2014). Thus, without Arc present throughout development, one might predict that experience-dependent synaptic pruning mechanisms that require Arc are no longer functional, resulting in an increase in structural and functional synapses. However, in a separate study, another group noted that in areas CA1 and the dentate gyrus of the hippocampus, there is an overall decrease in dendritic spine density in the *Arc* KO (Peebles et al., 2010). While this finding does not support the observations noted by the more recent studies, it is worth pointing out that Peebles and colleagues conducted their morphological analysis in adult brain slices, whereas the studies by both Mikuni et al. and Wilkerson et al. relied on either embryonic or early postnatal culture or slice preparations. If in fact our data suggest an increase in synapse number in the NAc, then it may be the case that Arc differentially regulates synapse number in a development- and brain-region-dependent manner. Future studies will

be needed to clarify what other mechanisms are at play, either in parallel or in convergence with Arc, to dictate synapse number.

Figure 13. Surface AMPARs are selectively increased in the NAc of the *Arc* KO mouse

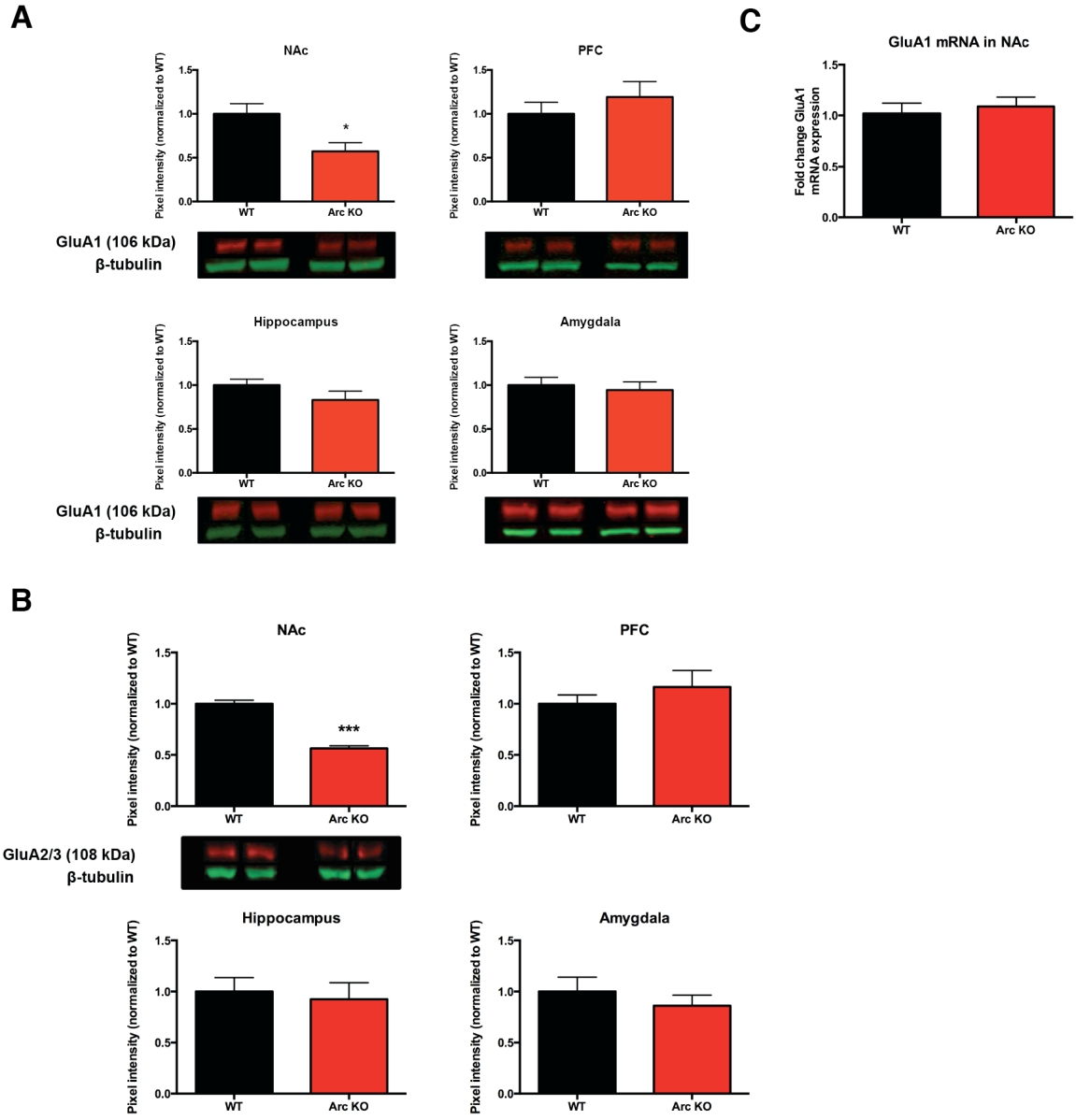

Figure 13. Surface AMPARs are selectively increased in the NAc of the *Arc* KO mouse. **A.** Levels of internal GluA1 protein, as assessed by quantitative Western blot, in lysates from WT and *Arc* KO tissue subjected to cell surface cross-linking with membrane-impermeable BS^3. n=4/group. **B.** Levels of internal GluA2/3 protein, measured from the same samples as in **A.**, using an antibody that detects both GluA2 and GluA3. **C.** Levels of GluA1 mRNA in the NAc, measured from a separate cohort of mice as **(A) and (B)**. * $p < 0.05$, *** $p < 0.001$, compared to WT, *t* test.

Figure 14. Arc regulates synaptic strength in the NAc

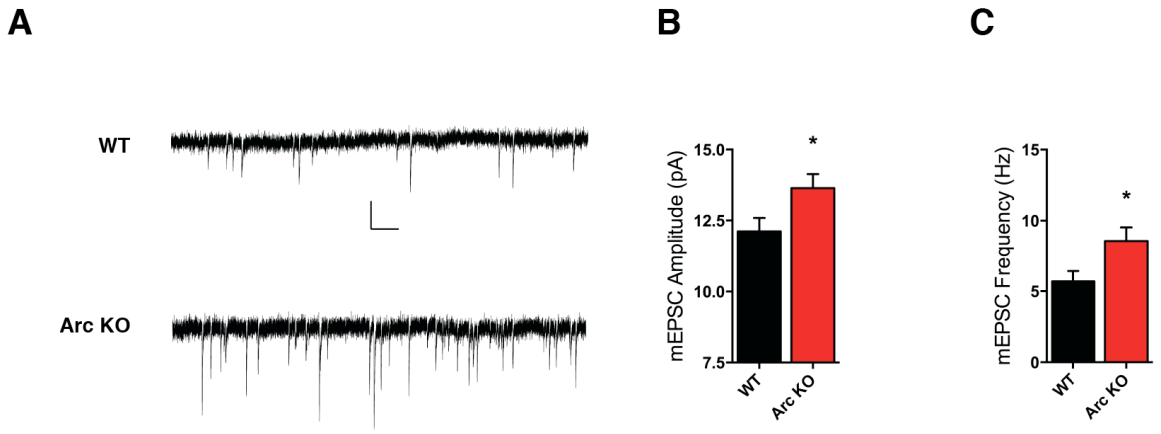

Figure 14. Arc regulates synaptic strength in the NAc. *Arc* KO and WT NAc slices were obtained, and whole-cell patch clamp physiology was conducted to measure miniature EPSCs in MSNs. **A.** Representative traces of mEPSCs from WT (top) and *Arc* KO (bottom) littermates. The average amplitude of mEPSC events **(B)** as well as total number of events **(C)** was measured. * $p < 0.05$, compared to WT, *t* test. n = 10-15 cells/group. Scale bar, 10 pA/200 ms.

Figure 15. *Arc* KO mice display augmented sensitization to cocaine administration

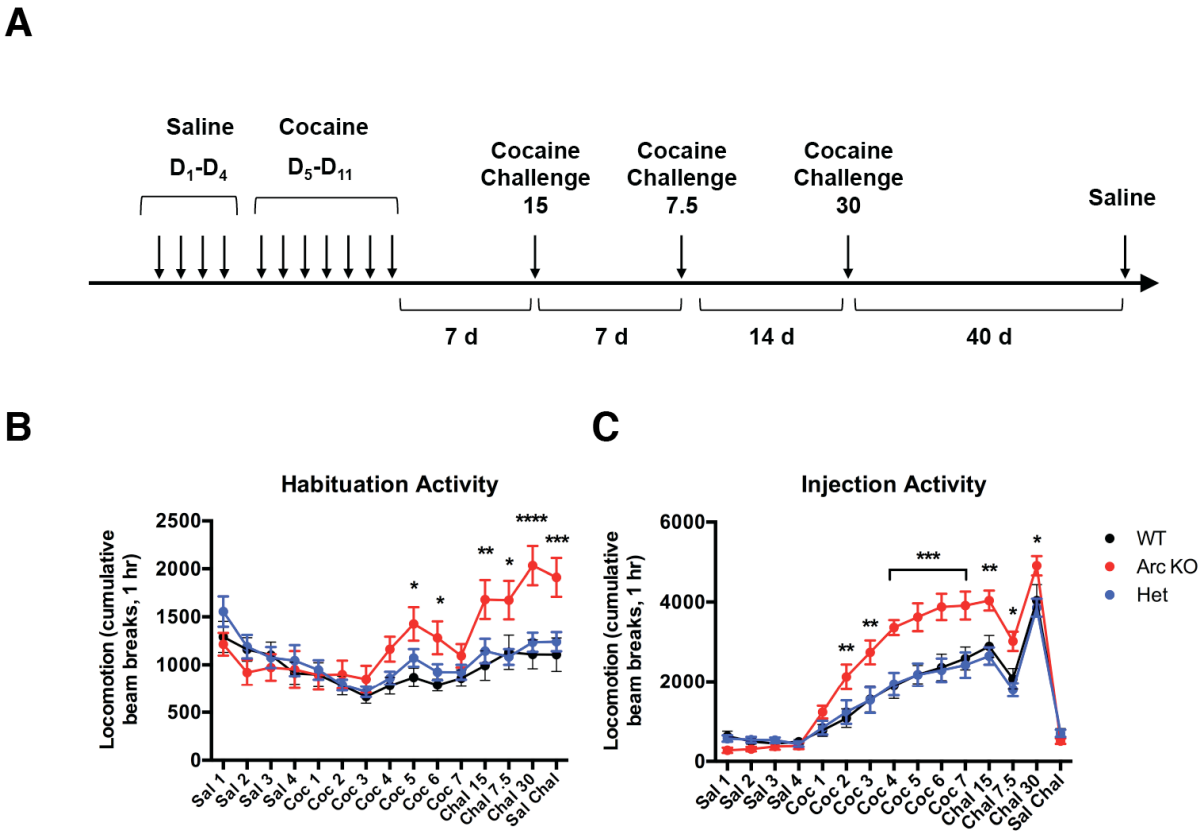

Figure 15. *Arc* KO mice display augmented sensitization to cocaine administration. **A.** Timeline of experiment. *Arc* KO, WT, and Het littermates were exposed to a novel cage for 1 hour, during which time total activity counts were measured by beam breaks **(B)**. Animals were then injected i.p. with either 0.9% saline (first 4 days) or cocaine (15 mg/kg, 7 d), and activity counts for an additional hour following injection were measured for each day **(C)**. After 7 d cocaine administration, animals were withdrawn from drug and given challenge doses (at the indicated doses, mg/kg), spaced apart by at least 7 days. n=10-16/group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (WT vs KO), Tukey post-hoc analysis following RMANOVA

Figure 16. Context- and drug-dependent locomotor sensitization in the *Arc* KO mouse

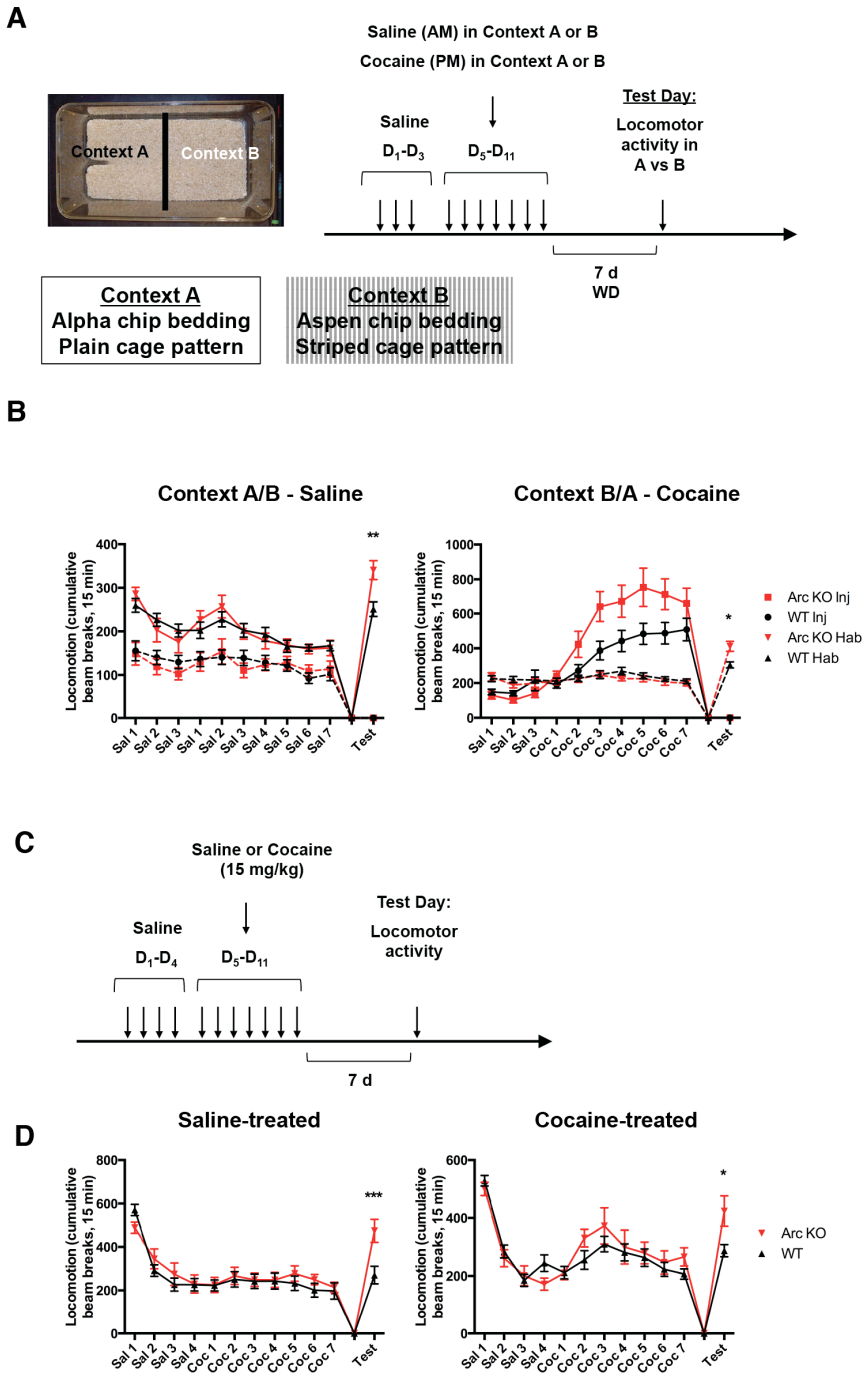

Figure 16. Context- and drug-dependent locomotor sensitization in the *Arc* KO mouse. **A.** Animals were tested for locomotor behavior in response to cocaine in two-chambered cages consisting of a different tactile and visual environment in each compartment (left). Animals were given daily injections of either saline in the AM or cocaine in the PM in different contexts (right). **B.** Locomotor activity in response to saline in context A or B (left) or cocaine in context B or A (right), and following 7 d after the last injection in both contexts. n=18/group. * $p < 0.05$, ** $p < 0.01$, compared to WT, Sidak post-hoc analysis following RMANOVA. **C.** Animals were tested for locomotor behavior in response to either cocaine or saline in a novel home cage devoid of contextual cues, withdrawn from injection for 7 d, and then tested again for activity. **D.** Locomotor activity during the hour prior to repeated administration of either saline (left) or cocaine (right). * $p < 0.05$, * $p < 0.001$, Sidak post-hoc analysis following RMANOVA.

Figure 17. Prior cocaine experience enhances cocaine reward learning in the *Arc* KO

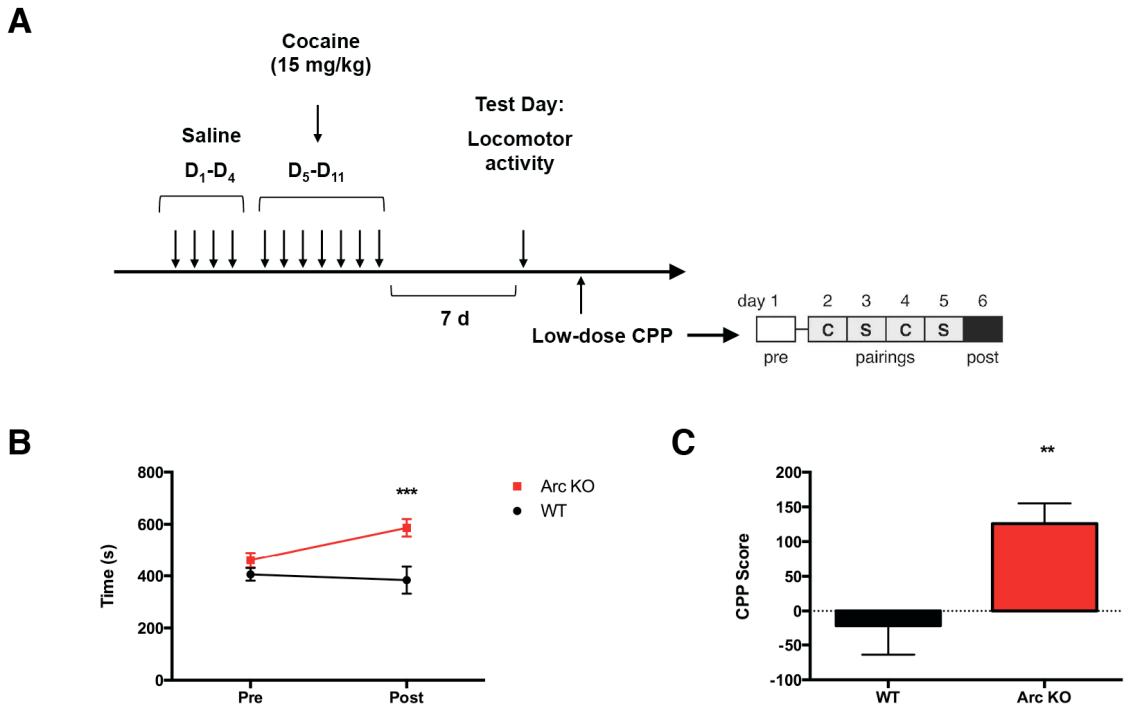

Figure 17. Prior cocaine experience enhances cocaine reward learning in the *Arc* KO. **A.** Following a sensitizing dose of cocaine and 7 d of withdrawal (left), animals were subjected to a 5 mg/kg, 6 day CPP assay consisting of 4 pairings of cocaine or saline to a specific context (right, taken from (Smith et al., 2014)). **B.** Line plot of time spent in cocaine-paired side among WT and *Arc* KO animals before and after conditioning. **C.** Data from (**B)** calculated as a CPP score (time in cocaine-paired side post-conditioning day 6 minus corresponding time at pretest day 1). n=12-15/group, ** $p<0.05$, *** $p<0.001$ compared to WT condition, Sidak post-hoc analysis following RMANOVA **(B)** or *t*-test **(C)**.

Figure 18. Reward learning is sensitized by cocaine experience in *Arc* KO, but not WT mice

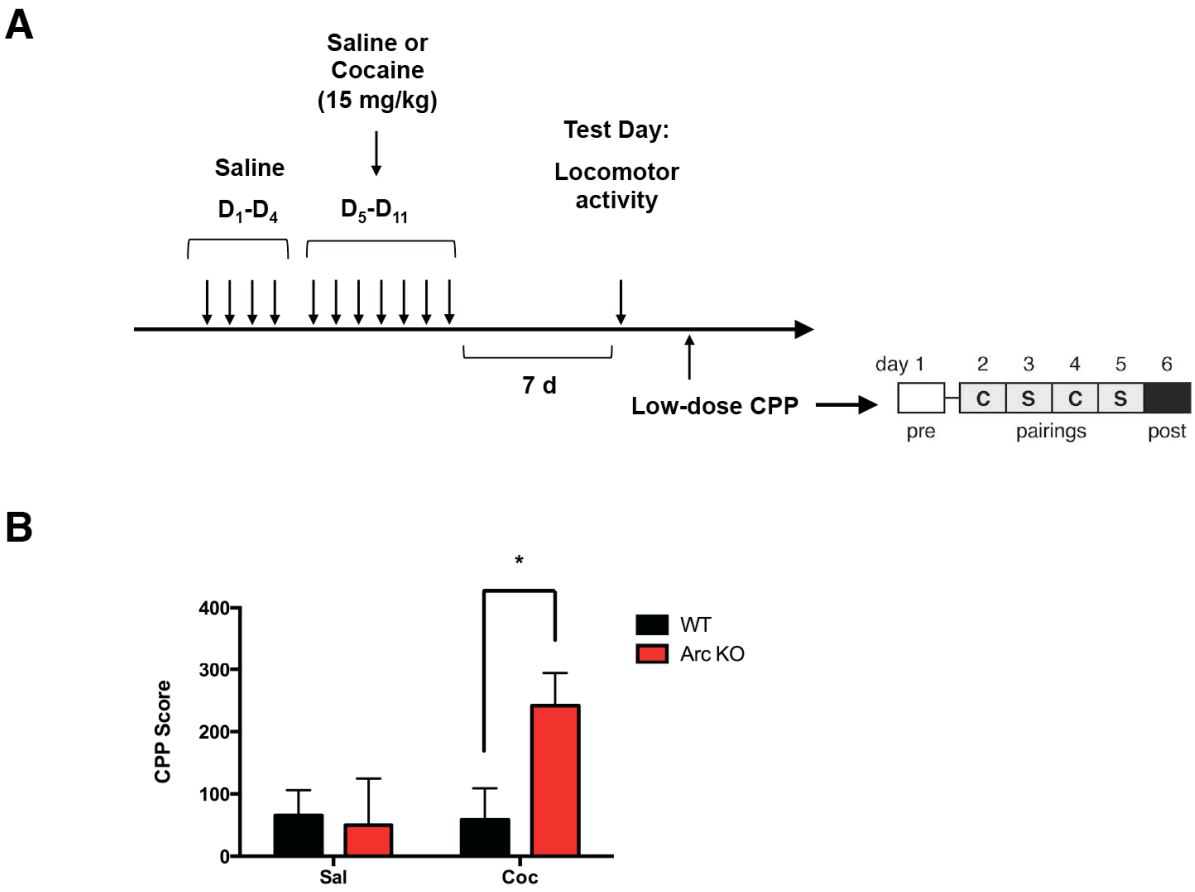

Figure 18. Reward learning is sensitized by cocaine experience in *Arc* KO, but not WT mice. **A.** Following a sensitizing dose of cocaine or saline and 7 d of withdrawal, animals were subjected to a 5 mg/kg CPP assay **B.** CPP score (time in cocaine-paired side post-conditioning day 6 minus corresponding time at pretest day 1). * $p < 0.05$, compared to WT-Coc condition, Sidak post-hoc analysis following 2-way ANOVA. n=8-10/group.

CHAPTER SIX
Conclusion and Future Directions

In the studies conducted here, we implicate a number of different neuronal proteins in stress- and addiction-related behaviors in rodents. By demonstrating that they play important roles in preclinical animal models, we begin to assign new translational significance to these molecules and thus reveal potential new avenues of research in the study of stress, depression, and anxiety, as well as in substance abuse. Based on our findings, a number of different questions arise regarding the roles of HDAC5 and associated target genes, as well as Arc and its downstream targets, in synaptic and behavioral plasticity to stress and drugs of abuse.

The histone deacetylase 5 had been previously implicated in behavioral responses to chronic social stress and antidepressants (Renthal et al., 2007; Tsankova et al., 2006). Despite the well-characterized role of this HDAC in stress responses, knowledge of the downstream gene targets of HDAC5 has been heretofore limited. Here, we identified a novel HDAC5 target gene, NPAS4, which is robustly induced by social stress and is required for anhedonic like behavior in the mPFC. Our work does not go so far as to implicate the regulation of NPAS4 by HDAC5 per se in the development of anhedonia. However, if we suppose that the basal increase in *Npas4* transcript levels seen in the *Hdac5* KO drives the development of depression-like behavior, then it is reasonable to hypothesize that restoration of the repressive function of HDAC5 would not only attenuate NPAS4 levels, but also function in a pro-depressant manner. In these studies, we did not

directly test whether *Hdac5* KOs are hypersensitive to CSDS; we would propose to test the effects of mPFC-specific knockdown of NPAS4 in the KO mouse on depression-like behaviors, with the expectation that hypersensitivity to stress would be normalized to WT levels.

If it is indeed the case that HDAC5-mediated regulation of NPAS4 is a critical signaling node that determines stress reactivity, a next question to consider is what transcriptional mechanisms are recruited by HDAC5 to limit NPAS4 induction, and whether or not these accessory molecules are also relevant to stress-induced pathologies. One of the best-characterized binding partners of HDAC5 is the transcription factor MEF2 (Lu et al., 2000). Interestingly, preliminary analysis indicates that the promoter region of NPAS4 contains 4 consensus MEF2 response elements. We have gone on to demonstrate that a constitutively-active form of MEF2 can induce NPAS4 in cultured cells, and that HDAC5 attenuates this induction (M. Taniguchi, unpublished observations). Based on these initial results, it is interesting to speculate that MEF2 might be required for the stress-induced increases in NPAS4 and/or the basal elevation in NPAS4 that we observe in the *Hdac5* KO mice. If MEF2 is required for the activation of NPAS4 by stress *in vivo*, we would hypothesize that in a *Mef2*-null background, not only would this induction be dampened, but the development of depression-like behavior after chronic stress might also be attenuated. In agreement with this hypothesis, we have found that conditional ablation of the *Mef2c* isoform from the mouse cortex causes a profound reduction in

anxiety-like behavior (Cowan Lab, unpublished observations). Taken together, our results, which implicate NPAS4 in stress-related behavior, support a model in which a MEF2-HDAC5-NPAS4 pathway may operate within the mPFC to control mood and anxiety.

With respect to Arc, we identify this protein for the first time as a critical mediator of stress- and anxiety-related behavior. Because most of the behaviors we test here are based on the animal's innate, and not learned, anxiety, it is likely that Arc regulates anxiety states via a mechanism independent of its established effects on learning and memory. Previous reports identify the hippocampus and amygdala as two critical anatomical substrates in which Arc functions to regulate long-term memory (Guzowski et al., 2000; Ploski et al., 2008). Our studies implicate the mPFC, and potentially the NAc, in anxiety phenotypes. In the future, it would be useful to genetically ablate Arc within distinct neural circuits, perhaps via viral Cre-mediated recombination, resulting in excision of a floxed Arc allele, or by use of a viral RNAi-based strategy. Using these transgenic animals, we could then assess the relative contributions of different anatomical sites to learning vs. anxiety.

Our data do not ascribe a particular mechanism to Arc's regulation of anxiety. A number of upstream factors regulate Arc function, including activity-dependent transcription factors such as CREB, MEF2, and SRF. As implied from the discussion above, our data would lend further credence to a model in which Arc, as a result of enhanced MEF2 function, enhances anxiety, whereas loss of MEF2 is anxiolytic, due

to reduced levels of Arc. While it has not been fully examined whether Arc levels are reduced in a *Mef2*-null background *in vivo*, activity-inducible levels of Arc are blunted in hippocampal neurons that lack Mef2A and Mef2D (Flavell et al., 2006). It is therefore interesting to speculate whether, in the absence of MEF2, differences in anxiety emerge due to differential expression of Arc. Another, similar mechanistic explanation for the regulation of stress- and anxiety-like behavior by Arc involves a potential role for NPAS4. A previous report identified NPAS4 as required for the full activity-inducible expression of immediate-early genes such as *cFos, zif268, and Arc* (Ramamoorthi et al., 2011). In our hands, when Arc is overexpressed in the mPFC, we observed increased anxiety behavior. When NPAS4 is deleted from the very same brain region, we observe the opposite effect. A model in which NPAS4 in the mPFC regulates stress-induced behaviors via regulation of Arc can rationalize these data. It would be informative to first establish that loss of Arc in the mPFC recapitulates the behavioral phenotype of the NPAS4 cKO. If this were in fact true, then we would propose that overexpression of Arc into the mPFC of an NPAS4 KO mouse would reverse the behavioral deficits that ensue as a result of NPAS4 deletion.

The relationship between Arc and anxiety outlined in this thesis updates and informs our understanding of stress, anxiety, and plasticity. Deep brain stimulation of the cingulate cortex and the nucleus accumbens has been shown to ameliorate symptoms in treatment-refractory patients. This treatment modality elevates

neuronal activity within these structures, and it is interesting to speculate what if any role Arc plays in facilitating DBS-induced remission of symptoms. In the *Arc* KO mouse, a commonly observed phenotype is increased seizure susceptibility, due to increased excitability of hippocampal circuitry (Peebles et al., 2010). In clinical populations, patients presenting with refractory depression often undergo electroconvulsive therapy (ECT), a treatment modality in which electric current is artificially administered to the brain, mimicking seizure. Our behavioral findings here provide one of the first examples in the literature of a mouse model in which reduced depression- and anxiety-like behavior is accompanied by seizure, and provides preclinical validation for the use of, and a potential model to understand the neurobiology of ECT.

In the *Arc* KO mouse, we describe a second novel phenotype: enhanced cocaine-induced behavioral plasticity. Our findings of enhanced psychomotor sensitization and reward learning in the *Arc* KO suggest that Arc is involved in antagonizing the effects of repeated cocaine, such that in the absence of Arc, these effects remain unopposed, and enhanced behavioral plasticity develops in the form of exaggerated sensitization and reward. Cocaine abuse is capable of eliciting long-lasting changes in the structure and function of brain reward regions, and such changes are thought to drive continued abuse and relapse among addicts. Our behavioral models of choice restrict our ability to fully assess whether chronic cocaine elicits changes in withdrawal-induced behaviors, such as incubation of

craving and cue-mediated relapse. The behavioral tests we have employed here, locomotor sensitization and cocaine CPP, are at best approximations of true addiction-related phenomena; they only serve to model a uniquely human illness involving volitional, motivated consumption of drug. In the future, we hope to assess the role of Arc in addiction-related behavior via rodent intravenous self-administration. Doing so will provide useful information regarding the relationship between drug reward, psychomotor sensitization, and drug craving.

At baseline, *Arc* KO animals exhibit normal reward learning; however, in response to cocaine experience, these animals show exaggerated reward behavior. Thus, loss of Arc in and of itself does not render an animal more susceptible to the rewarding effects of cocaine. Rather, prior cocaine experience is required to unmask the latent reward hypersensitivity phenotype observed in the KO. Our findings highlight a previously unidentified candidate genetic risk factor that might confer increased addiction liability in humans. Moreover, they provide support for a model of addiction akin to diathesis-stress models; individuals with a genetic predisposition go on to develop addiction in response to cocaine, while healthy individuals exposed to drug do not (Goldman et al., 2005).

What are the potential mechanisms facilitating the emergence of this behavior? In a wild-type animal, prior cocaine experience primes the NAc towards the development of enhanced plasticity during withdrawal. Naïve *Arc* KOs show basal enhanced AMPAR plasticity in the NAc, reminiscent of this withdrawal-like

state. We speculate that this increase in AMPARs drives the susceptibility towards heightened reward. There are a number of different avenues through which heightened AMPAR function may influence reward. First, in a KO background, it may be the case that the addition of even more AMPARs into NAc synapses during withdrawal potentiates reward learning, and that in a WT background, AMPAR number fails to reach a critical threshold wherein cocaine experience can potentiate reward. According to this hypothesis, one would predict that reward is simply a linear function of surface AMPAR number. Alternatively, perhaps the withdrawal-induced upregulation of AMPARs is a process that requires Arc. It may be the case that during withdrawal, mechanisms controlling exocytosis of AMPAR-containing vesicles are counterbalanced and fine-tuned by mechanisms of vesicle-mediated AMPAR endocytosis, to achieve an appropriate number of surface AMPARs (Anggono and Huganir, 2012). With this latter arm of the cycle dysfunctional, owing to Arc deletion, perhaps in the KO, the normal complement of AMPARs that are trafficked to the synapse during withdrawal is inappropriately elevated, resulting in even greater NAc synaptic strength. Finally, there may be AMPAR subtype differences between WT and KO conditions that affect reward. Withdrawal may induce upregulation of AMPARs in the NAc in both genetic backgrounds; in the KO, though, GluA2-lacking, Ca^{2+} permeable AMPARs may be preferentially trafficked to the synapse, resulting in greater rectification and potentiated NAc output.

Mechanisms aside, our data provide novel insight into how genetics and cocaine experience interact to affect subsequent addiction-like behaviors, and may provide a window through which to understand how environmental factors predispose individuals towards developing addiction. We present the *Arc* KO mouse as a model system by which to understand the relevant brain regions, circuitry, cell types, and molecules through which cocaine functions to elevate reward. Other predisposing genes and environmental factors have been shown to elicit differences in reward function. It would be interesting to examine AMPAR physiology in such model systems, and whether and how Arc is involved in regulating the function of reward circuits under these conditions.

In conclusion, the work presented in this document establishes important functional roles for HDAC5- and Arc-mediated regulation of stress- and addiction-related psychopathology. Specifically, we show that an HDAC5 target gene, Npas4, influences behavioral responses to chronic stress. We extend these findings to the Arc protein, demonstrating its necessity in mood- and anxiety-related behaviors. Additionally, this molecule appears to influence behavioral responses to cocaine and as such represents a potential therapeutic target in the treatment of both stress and addiction-related behavioral disorders. While a number of questions still remain regarding the mechanisms underlying these findings, our results nonetheless add to our current understanding of the neurobiology of depression, anxiety, and addiction,

and may aid in the discovery of more targeted, efficacious treatments for these related conditions.

APPENDIX

EFFECTS OF CHRONIC STRESS AND ANTIDEPRESSANT DRUGS ON FEEDING AND METABOLISM

Jaswinder Kumar[1,2], Jen-Chieh Chuang[3], Elisa Na[1], Anna Kuperman[3], Andrea G Gillman[5], Shibani Mukherjee[1], Colleen A. McClung[5], Jeffrey M. Zigman[1,3 4], and Michael Lutter[6]

[1]Department of Psychiatry, UT Southwestern Medical Center, Dallas, TX, USA

[2]Department of Psychiatry, Harvard Medical School, McLean Hospital, Belmont, MA, USA

[3]Division of Hypothalamic Research, Department of Internal Medicine, UT Southwestern Medical Center, Dallas, TX, USA

[4]Division of Endocrinology and Metabolism, Department of Internal Medicine, UT Southwestern Medical Center, Dallas, TX, USA

[5]Department of Psychiatry, University of Pittsburgh Medical School, Pittsburgh, PA, USA

[6]Department of Psychiatry, University of Iowa Carver College of Medicine, Iowa City, IA, USA

Author Contributions

JK, CAM, and ML designed the experiments. JK conducted the experiments and analyzed the data. J-CC, EN, AK, AGG, and SM assisted with experiments. JMZ provided protocols and reagents for serum analysis.

Abstract

Both chronic stress and antidepressant medications have been associated with increased weight gain. However, it is not known how stress and antidepressant medications interact to affect body weight homeostasis. In the current study, we investigate mechanisms by which stress and antidepressant medications interact to affect food intake and body weight. A group of mice was subjected to the CSDS model of major depression followed by fluoxetine treatment and was subsequently analyzed for food intake and energy expenditure using metabolic cages. CSDS and fluoxetine both significantly increased body weight. CSDS increased food intake by specifically increasing meal size during the dark phase, an effect that was reversed by fluoxetine treatment. In contrast, fluoxetine reduced metabolic rate in both CSDS and control groups. CSDS induced a peak in acyl-ghrelin levels just prior to lights off, which was prevented by fluoxetine treatment. Taken together, these data suggest that CSDS and fluoxetine induce weight gain by distinct mechanisms. Fluoxetine reverses CSDS-induced changes in meal patterns but at the expense of reduced metabolic rate.

Introduction

Multiple studies now indicate that certain mental disorders such as major depressive disorder (Anderson et al., 2007; Beydoun and Wang, 2010; Cizza, 2011; de Wit et al., 2010; Gavin et al., 2010; Merikangas et al., 2011; Papakostas et al., 2005; Rivera et al., 2011) and post-traumatic stress disorder (Chwastiak et al., 2011; Coughlin, 2011; Coughlin et al., 2011; Dedert et al., 2010; Perkonigg et al., 2009; Scott et al., 2008; Vieweg et al., 2006; Vieweg et al., 2007; Wilson, 2010) significantly increase the risk of developing obesity. Conversely, several medications used to treat these psychiatric disorders are also associated with weight gain, including antidepressant medications (Serretti and Mandelli, 2010). Despite the clear association between psychiatric disorders, antidepressants, and weight gain, relatively little is known about the underlying mechanisms that mediate this risk.

Rodent models of major depression have confirmed the importance of chronic stress in the development of obesity (Tamashiro et al., 2005). The visible burrow system, one of the best-understood models of stress, induces changes in food intake and body composition (Tamashiro et al., 2007a; Tamashiro et al., 2006; Tamashiro et al., 2004; Tamashiro et al., 2007b). In this model, grouped housed rats form a dominance hierarchy. While dominant male rats maintain normal body weight and food intake, subordinate males significantly reduce their food intake and lose weight during the test period. Interestingly, during the recovery phase after removal from the visible burrow system, subordinate male rats regain their lost weight mostly as

adipose tissue and develop obesity and elevated leptin levels (Tamashiro et al., 2007b). A follow-up study revealed that subordinate males reduced food intake primarily via decreased meal frequency and meal size during the stressful hierarchy-formation phase. In contrast, during the recovery phase, subordinate males increased food intake by increased meal size (Melhorn et al., 2010).

Using the chronic social defeat stress (CSDS) model in mice, which shares features with both post-traumatic stress disorder and major depression with co-morbid anxiety (Avgustinovich et al., 1997; Krishnan et al., 2008), our group has also analyzed the effect of chronic stress on body weight regulation. In this model, mice subjected to repeated social aggression develop behavioral deficits similar to symptoms observed in human mental illness including social avoidance. Mice exposed to CSDS display multiple metabolic abnormalities, including body weight gain, redistribution of body fat into internal organs, and insulin and leptin resistance (Chuang et al., 2010a; Perello et al., 2010). The circulating hormone acyl-ghrelin appears to be an important mediator of certain CSDS-induced metabolic disturbances. CSDS increases acyl-ghrelin levels, and mice lacking acyl-ghrelin receptors exhibit exaggerated social avoidance behavior after CSDS (Lutter et al., 2008). More recently we have shown that following CSDS, wild-type mice, but not acyl-ghrelin receptor-null littermates, display an increased preference for high fat diet. Importantly restoration of acyl-ghrelin receptor expression specifically in catecholaminergic neurons reduces social isolation, but at the expense of also

restoring preference for high fat diet (Chuang et al., 2011). These findings suggest that acyl-ghrelin might be an important mediator linking changes in appetite and mood symptoms.

While a link between mental disorders and feeding has long been appreciated, less is known about the neurobiological mechanisms that underlie the association of antidepressant treatment and weight gain. A large meta-analysis of 116 studies in humans demonstrated that several antidepressants including amitriptyline, mirtazapine, and paroxetine were associated with increased weight gain (Serretti and Mandelli, 2010). In a study of female rats subjected to chronic variable stress, fluoxetine reduced intake of sweet food and leptin levels, however no interaction with stress was noted (Gamaro et al., 2008). Finally, using a model in which rats are exposed to recurrent restraint stress and then given access to a high fat diet, it was demonstrated that a brief 7-day exposure to antidepressant medications resulted in increased calorie intake and body weight up to 22 weeks after discontinuation of the antidepressant (Mastronardi et al., 2011). These studies demonstrate the highly heterogenous relationship between stress and antidepressant exposure with regards to body weight. What remains unknown is the mechanism by which stress and antidepressant exposure interact to affect body weight homeostasis. In the current study, we exposed a large cohort of mice to CSDS or control handling conditions. After verifying that CSDS induced social avoidance, the mice were randomized into two equal groups receiving pellets that

released fluoxetine or placebo. Multiple metabolic parameters were then measured, including body weight, meal pattern analysis, energy expenditure, and circulating levels of serum hormones.

Results

Effects of chronic social defeat and fluoxetine on food intake and energy expenditure

Eight-week-old male c57Bl/6J mice were subjected to either 10 days of CSDS or control handling conditions (Fig 19A). After this 10-day period, defeated mice that displayed social avoidance ("CSDS" mice, Fig 19B) and nonstressed controls were randomized into two equal groups that received a subcutaneous pellet releasing a 20-day supply of either fluoxetine or placebo. This protocol resulted in four groups of mice with a large number: control-placebo (n = 41), control-fluoxetine (n = 40), CSDS-placebo (n = 39), and CSDS-fluoxetine (n = 37). As previously reported (Berton et al., 2006), chronic fluoxetine treatment reversed social avoidance 29 days after the last bout of social stress (Figure 19B). At the completion of the study, two-way ANOVA revealed significant effects of both CSDS and fluoxetine on body weight (Fig 19C).

Next, we sought to determine if changes in food intake or energy expenditure caused the increased body weight. However, there has been much uncertainty on how to correct measures of food intake and energy expenditure for differences in body composition (Butler and Kozak, 2010; Tschop et al., 2012). Therefore to

rigorously answer this question, a sub-set of mice from all four groups were pair-matched for body weight and lean body mass, and food intake and energy expenditure were measured over a 4-day period using metabolic cages.

As shown in Fig 20, there were no differences noted in total chow consumed, meal number, meal size, or satiety ratio among groups during the light phase, when mice are typically inactive. In contrast, mice exposed to CSDS plus placebo demonstrated an increase in total chow consumed during the dark phase (Fig 20A). Meal pattern analysis revealed that this increase was the result of fewer meals (Fig 20B) and increased meal size (Fig 20C). The satiety ratio (g of food per meal/intermeal interval) was the same in all groups, indicating that CSDS increases food intake primarily by impairing satiation (the process of intrameal termination) during the dark phase without affecting satiety (the length of appetite suppression after a meal). The effects of fluoxetine on meal patterning were restricted to the CSDS-exposed mice, for which fluoxetine normalized CSDS-induced changes in total chow consumed, meal number, and meal size (Fig 20A-C).

Because fluoxetine normalized CSDS-induced changes in total chow consumed, meal number, and meal size, we were interested in determining how fluoxetine increased body weight. Therefore, we next analyzed measures of energy expenditure. Whole body oxygen consumption was reduced in both control and CSDS groups receiving fluoxetine (Fig 21A). No differences were noted in carbon dioxide production between groups (Fig 21B). The combination of lower oxygen

consumption and no change in carbon dioxide production resulted in a significantly increased respiratory exchange ratio in both fluoxetine groups (Fig 21C). Because lipid requires more oxygen than carbohydrate to metabolize, respiratory exchange ratio is a relative measure of fuel preference, with lower numbers indicating primary lipid usage and higher numbers signifying a preference for carbohydrate. Therefore, the elevated respiratory exchange ratio suggests that fluoxetine exposure spares lipid utilization.

Regulation of serum feeding-related peptides by CSDS and fluoxetine

The metabolic cage data indicate that CSDS and fluoxetine increase body weight by distinct pathways. Furthermore, CSDS appears to increase food intake only during the dark phase, suggesting a diurnal effect, and this increase is reversible by fluoxetine. For further mechanistic insight into the effects of CSDS and fluoxetine on meal patterning, we next analyzed levels of circulating hormones after CSDS. Acyl-ghrelin, leptin, and corticosterone levels have previously been demonstrated to be disturbed in mice following CSDS (Chuang et al., 2010a; Krishnan et al., 2007; Lutter et al., 2008). However, the measurements in these previous studies were made at a single time point, and thus likely were not reflective of time-of-day differences in appetitive behavior, as detected in the current study. Because the current study was designed to collect samples over a 24-hour period,

we were therefore able to measure serum hormone levels across the circadian cycle.

Consistent with previous observations (Lutter et al., 2008), acyl-ghrelin levels are increased in susceptible mice exposed to CSDS in the late light phase just prior to lights off (Fig 22A, ZT9), with trends towards increased levels in this group during the preceding two timepoints. Fluoxetine treatment normalizes acyl-ghrelin levels after CSDS at ZT9. A significant peak of acyl-ghrelin expression at ZT17 was noted only in the CSDS-fluoxetine group. We next analyzed leptin levels, which have been previously shown to be reduced after CSDS (Chuang et al., 2010a). We found significant reductions in leptin in both CSDS groups at ZT5, ZT9, and ZT13. Fluoxetine treatment did not significantly alter leptin levels in either control or CSDS groups (Fig 22B). Chronic social stress reduced morning levels of corticosterone in both CSDS groups (Fig 22C), consistent with previous observations (Krishnan et al., 2007). Fluoxetine also caused a significant reduction of corticosterone levels at ZT21 in both control and CSDS groups. No other effects of CSDS or fluoxetine were noted in corticosterone levels at other time points.

A role for the melanocortin-4 receptor in the regulation of satiation

Both leptin and acyl-ghrelin mediate feeding-related behaviors via direct action on the brain (Abizaid et al., 2006; Chuang et al., 2011; Fulton et al., 2006; Hommel et al., 2006), as well as indirectly via action on the melanocortin system

(Cone, 2006; Williams and Schwartz, 2005). Additionally, previous reports have demonstrated a role for decreases in melancortin 4 receptor (MC4R) in mediating CSDS-induced hyperphagia (Chuang et al., 2010b). Mice lacking MC4R (Balthasar et al., 2005) were therefore subjected to the same metabolic cage monitoring as above, and meal patterns were analyzed. Consistent with CSDS-induced changes in meal patterns (Fig 20), mice lacking MC4R demonstrated an increase in meal size specifically during the dark phase (Fig 23A), suggesting that impairment in satiation following CSDS may be due to decreased MC4R signaling secondary to alterations in leptin and acyl-ghrelin levels.

Discussion

Though the link between stress and body weight change has long been appreciated, less is known about the neurobiological mechanisms that underlie the effect of antidepressant treatment on body weight changes. We hypothesized that fluoxetine treatment would reverse the hyperphagia observed in mice following CSDS. In the current study, we report several novel observations on the association of stress, antidepressants, food intake, and body weight homeostasis among stress-susceptible animals.

Stress and satiation

It is a common belief that stress is an important regulator of feeding behaviors and body weight homeostasis. Furthermore, antidepressant treatments are also thought to affect feeding and body weight, although it is not clear if this is a direct effect of antidepressant exposure or secondary to treatment of depressive symptoms. In this study, we report several novel observations on the association of stress, antidepressants, and body weight regulation. We confirm that stress increases food intake primarily by increasing meal size, suggesting a specific deficit in satiation or the process that terminates a meal. This finding is consistent with previous work in the visible burrow system in which subordinate male rats increased food intake during the recovery phase by increasing meal size (Melhorn et al., 2010). In contrast, other stress models lacking a social component, such as the chronic unpredictable stress and chronic restraint stress models, result in a decrease in food intake in response to stress (Chiba et al., 2012; Varga et al., 2011). It may be the case that psychosocial stress, rather than more neutral environmental stressors, uniquely contributes to the development of hyperphagia.

This observation has several potential clinical implications. First, it indicates that behavioral techniques aimed at improving satiation and not satiety should be the focus of treatment of obesity in patients with mental disorders (Blundell and MacDiarmid, 1997; Green et al., 1997). Secondly, it begins to delineate the neural circuits that control different aspects of food intake following exposure to stress. Ghrelin signaling directly on catecholaminergic neurons affects mood symptoms and

conditioned place preference for high fat diet following CSDS (Chuang et al., 2011). In contrast, other brain regions, such as the nucleus tractus solitarius and paraventricular nucleus of the hypothalamus, which may be affected by stress, play a more important role in maintaining satiety (Fig. 23C). Satiety is regulated by the coordinated action of the vagus nerve and gut hormones, which serve as peripheral sensors of energy intake to control meal size (Berthoud, 2008). Importantly, previous work has demonstrated that MC4R regulates vagal nerve activity. Melanocortin 4 receptor signaling modulates synaptic transmission on glutamatergic neurons in the nucleus tractus solitarius, thereby decreasing food intake primarily via enhancement of vagal afferent satiation signals from the gastrointestinal tract (Wan et al., 2008). Additional work has implicated MC4R signaling in neurons of the paraventricular nucleus of the hypothalamus in regulating satiation (Balthasar et al., 2005; Blevins et al., 2009). These studies lend further support to previous findings that α-melanocyte stimulating hormone, the ligand for MC4R, is decreased after CSDS and that defeated mice are extremely sensitive to the appetite suppressing effect of the melanocortin 3/4 receptor agonist melanotan II (Chuang et al., 2010b). It is also consistent with our data demonstrating that MC4R-null mice have increased meal size specifically during the dark phase.

Fluoxetine and satiation

Our observation that meal size is restored to control levels in CSDS animals treated with chronic fluoxetine carries several implications for the role of serotonin in satiation mechanisms. Serotonin has been demonstrated to be an important regulator of feeding in rodents. Administration of serotonin receptor agonists activates POMC-expressing and inhibits AgRP/NPY-expressing neurons in the arcuate nucleus of the hypothalamus, resulting in a suppression of food intake (Heisler et al., 2006; Lam et al., 2008). Additionally, animals lacking MC4R, as well as transgenic mice overexpressing the MC4R antagonist agouti, are unresponsive to the anorectic effects of augmented serotonin signaling (Heisler et al., 2006; Lam et al., 2008), which suggests an essential role for melanocortin signaling in the regulation of food intake by serotonin. These studies provide a general framework through which to interpret our finding that fluoxetine suppresses meal size; however, it is unclear why we do not observe a similar reduction among control mice. In nonstressed rats, it was also reported that daily food intake in the terminal phase of a chronic fluoxetine regimen was unaltered by the drug (Gamaro et al., 2008). The mechanism explaining this discrepancy between control and CSDS animals may involve important neural adaptations to repeated social stress that sensitize feeding circuits to the appetite-suppressing effects of fluoxetine. This is a plausible hypothesis in the context of CSDS, which is known to induce long-lasting disturbances in limbic circuitry responsible for orchestrating both emotional and appetitive behaviors (Berton et al., 2006; Tsankova et al., 2006). As an example,

various models of both acute and chronic stress, including CSDS, result in increased activity of mesocorticolimbic dopaminergic circuitry (Krishnan et al., 2007; Lammel et al., 2011; Valenti et al., 2011), a circuit which is thought to coordinate motivated behaviors associated with feeding (Palmiter, 2007). Moreover, in the CSDS model, hyperactivity of VTA dopaminergic neurons is restored to control, nonstressed levels by a chronic fluoxetine regimen similar to the one we utilize here (Cao et al., 2010). Of note is the fact that dopamine neuron firing among nonstressed animals is unaffected by fluoxetine (Cao et al., 2010), again pointing to a phenotype-specific effect of this antidepressant. Interestingly, ghrelin receptors expressed on presynaptic dopaminergic neurons of the VTA are known to provide excitatory input to this neuronal population (Abizaid et al., 2006), and infusion of ghrelin directly into the VTA results in increased food intake (Naleid et al., 2005). It is possible that, in the CSDS model, fluoxetine normalizes the stress-induced decrease in satiation by reducing acyl-ghrelin levels, which in turn function to reverse the neuronal adaptations associated with CSDS. While this possibility has yet to be fully examined, we speculate that CSDS alters the basic function of satiation circuits in such a way as to render them plastic to modulation by fluoxetine.

Regulation of ghrelin and leptin levels by CSDS and fluoxetine

Ghrelin and leptin levels have previously been shown to be increased and decreased, respectively, in rodent models of stress including chronic unpredictable

stress and CSDS (Asakawa et al., 2001; Chuang et al., 2010a; Chuang et al., 2011; Garza et al., 2011; Kristenssson et al., 2006; Lu et al., 2006; Lutter et al., 2008; Ochi et al., 2008; Patterson et al., 2010; Rouach et al., 2007). Here, our findings indicate that acyl-ghrelin, but not leptin, levels are altered after fluoxetine exposure following CSDS (Fig 22A and 22B). It should be noted that serum hormone levels were measured from the overall pool of mice and not the sub-group selected for metabolic cage monitoring, although similar levels of hyperphagia were observed in both groups, suggesting that the groups are likely comparable.

Fluoxetine treatment after CSDS is associated with reduced ghrelin levels at ZT9, but with the appearance of a peak at ZT17. One possible mechanism for this action was reported by the Inui Laboratory, who demonstrated that selective serotonin reuptake inhibitors, including fluoxetine, decrease acyl-ghrelin levels via central activation of the 5-HT_{2c} receptor (Fujitsuka et al., 2009). Our data suggest that 5-HT_{2c} receptor signaling may be important for the timing of the acyl-ghrelin peak and that fluoxetine treatment shifts the peak until late in the dark phase (the potential implications of this shift on feeding behaviors will be discussed below). It has been previously shown that ghrelin receptor signaling is required for the increased food intake and conditioned place preference for high fat diet induced by CSDS (Chuang et al., 2011; Lutter et al., 2008). Further studies will be necessary to determine if fluoxetine normalizes satiation after CSDS via changes in ghrelin signaling or through a distinct mechanism.

Regulation of meal patterns by CSDS-induced changes in leptin and ghrelin

We also show that changes in leptin and acyl-ghrelin levels only occur at specific times of the day. After CSDS, leptin levels are significantly lower than in control mice during the late light/early dark phase. Meanwhile, CSDS induces a peak in ghrelin levels in late light phase at ZT9. This well-timed increase in the relative ratio of ghrelin/leptin may explain why feeding differences are only observed in the dark phase. Recent work from the Sternson Laboratory identified a “flip-flop” memory circuit in AgRP/NPY neurons in the arcuate nucleus of the hypothalamus (Yang et al., 2011). In their model, acyl-ghrelin acts on pre-synaptic neurons to increase glutamate release onto AgRP/NPY neurons in a feed-forward loop. This excitatory neurotransmission increases feeding responses for hours, even after withdrawal of ghrelin, until leptin terminates feeding by acting on POMC-expressing neurons to inhibit AgRP/NPY neuronal activity via opioid release. Our data suggest that CSDS reduces leptin levels from ZT5 to ZT17, creating a 12-hour “window” in which hyperphagia can occur. However, feeding does not happen until late in the light phase when acyl-ghrelin levels peak. The feeding bout would then continue through the early dark phase until leptin levels rise at ZT17 in the late dark phase. This model would also explain why a shift in the peak of acyl-ghrelin levels in the CSDS-fluoxetine group from ZT9 to ZT17 is not associated with increased food intake

because leptin levels have returned to baseline and no longer permit activation of AgRP/NPY neurons.

Conclusions

Together, these observations support a model in which stress pathways increase acyl-ghrelin/leptin ratios to inhibit MC4R signaling and increase meal size. Antidepressant treatment reduces acyl-ghrelin levels to normalize food intake, but at the expense of reduced energy expenditure. These insights may yield new treatments for stress-induced obesity.

Figure 19. Social avoidance behavior and body weight changes induced by chronic social defeat stress

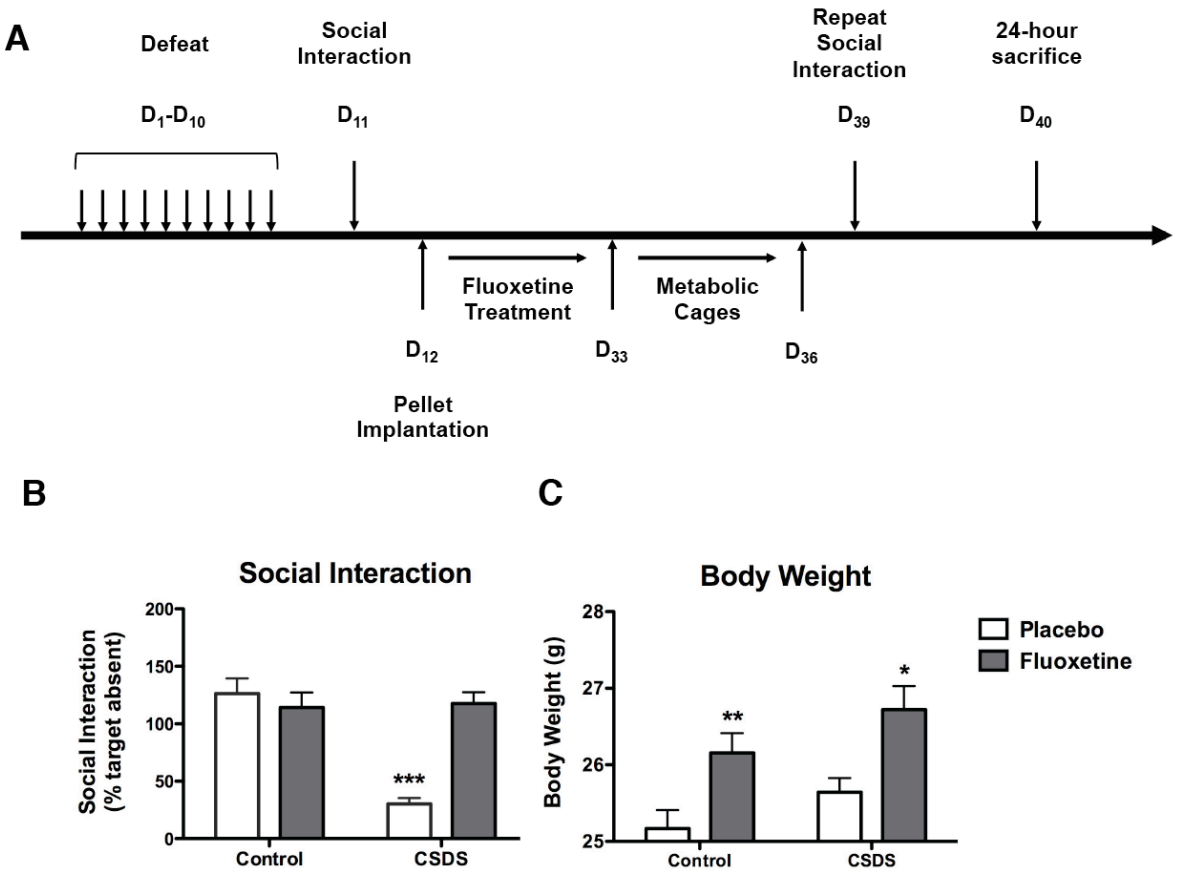

Figure 19. Social avoidance behavior and body weight changes induced by chronic social defeat stress. **A.** Eight-week-old male c57Bl/6J mice were subjected to ten days of CSDS and tested for social interaction. Animals displaying social avoidance along with nonstressed controls showing a social preference were randomized into two equal groups that received pellets releasing a 20-day supply of fluoxetine or placebo. A subset of mice from each experimental group was pair-matched for body weight and monitored in metabolic cages 4 days after social interaction testing (days 33-36). Meal patterns were analyzed over 48 hours on days 34-36. **B.** Day 39 social interaction results of control and CSDS mice following chronic placebo or fluoxetine treatment (significant *defeat X fluoxetine* interaction, $F_{1,140} = 21.33$). **C.** Day 39 body weight (significant effect of *defeat,* $F_{1,153} = 4.31$ and significant effect of *fluoxetine,* $F_{1,153} = 17.04$). n=37-41/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 20. Effects of chronic social stress and fluoxetine on meal patterning

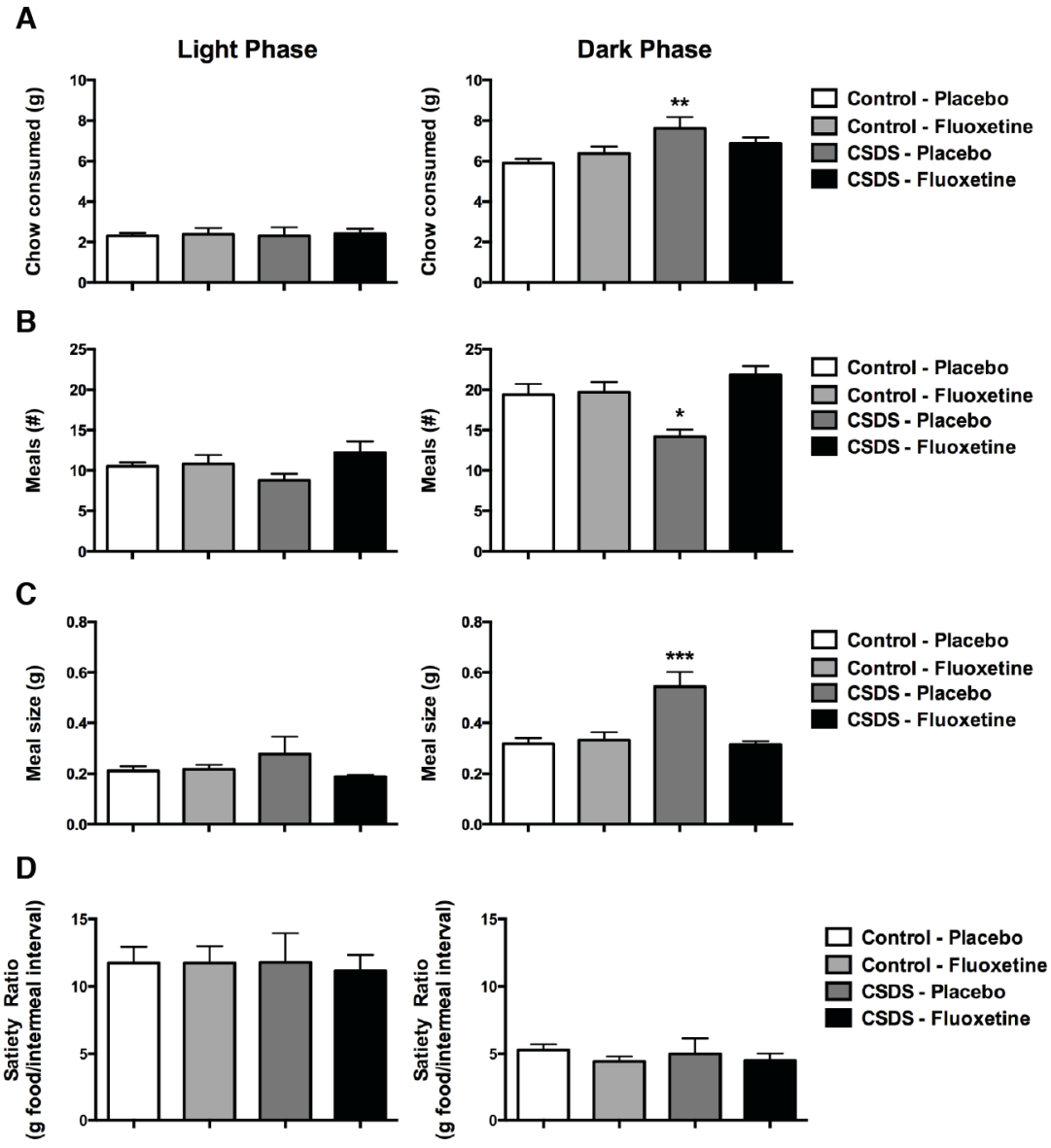

Figure 20. Effects of chronic social stress and fluoxetine on meal patterning. **A.** Total chow consumed in the light phase (no effect) and dark phase (significant effect of *defeat*, $F_{1,18} = 9.41$). **B.** Meal number in the light phase (no effect) and dark phase (significant *defeat X fluoxetine* interaction, $F_{1,18} = 9.11$). **C.** Meal size in the light phase (no effect) and dark phase (significant *defeat X fluoxetine* interaction, $F_{1,18} = 12.49$). **D.** Satiety ratio calculated during the light phase (no effect) and dark phase (no effect). n = 5-6/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 21. Effects of chronic social stress and fluoxetine on metabolic rate

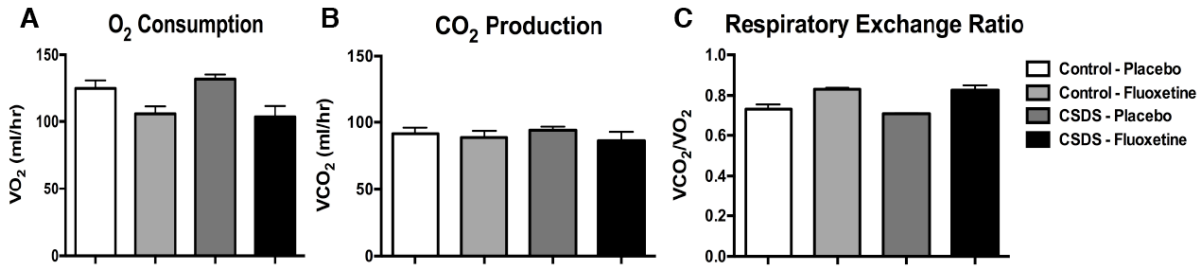

Figure 21. Effects of chronic social stress and fluoxetine on metabolic rate. Mice from Figure 20 were also monitored for **A.** oxygen consumption (significant effect of *fluoxetine,* $F_{1,17} = 16.55$), **B.** carbon dioxide production (no effect), and **C.** respiratory exchange ratio (significant effect of *fluoxetine,* $F_{1,17} = 46.55$). n = 5-6/group.

Figure 22. Effects of chronic social stress and fluoxetine on serum feeding-related hormones

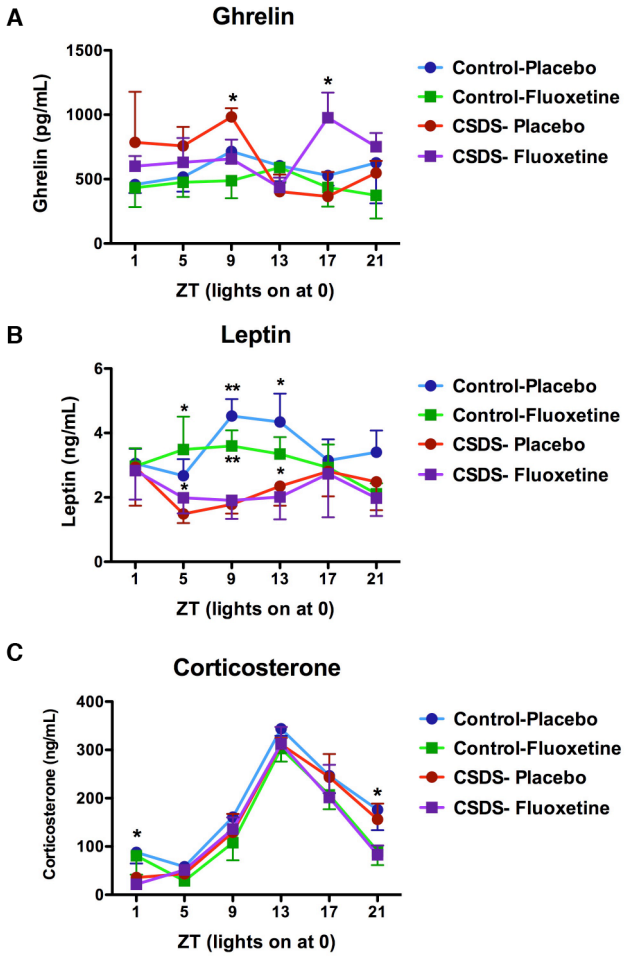

Figure 22. Effect of chronic social stress and fluoxetine on serum feeding-related hormones. Twenty-four hours after social interaction re-testing (Day 40), mice were fasted for 4 hours and sacrificed every 4 hours over a 24-hour period (ZT0 = lights on). Serum was collected and tested for **A.** acyl-ghrelin (no primary effect of *group* or *time of day*; significant effect of *fluoxetine,* $F_{1,16} = 5.94$ at ZT9; and significant *defeat X fluoxetine* interaction, $F_{1,16} = 4.54$ at ZT17). **B.** leptin (primary effect of *group* $F_{3,109} = 4.85$; significant effect of *defeat,* $F_{1,23} = 4.91$ at ZT5; significant effect of *defeat,* $F_{1,17} = 18.79$ at ZT9; and significant effect of *defeat,* $F_{1,17} = 5.37$ at ZT13) and **C.** corticosterone (primary effect of *time of day* $F_{5,124} = 48.06$; significant effect of *defeat,* $F_{1,21} = 5.05$ at ZT1; and significant effect of *fluoxetine,* $F_{1,20} = 6.17$ at ZT21). n = 6-8/group/timepoint. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 23. Meal pattern analysis in MC4R$^{-/-}$ mice

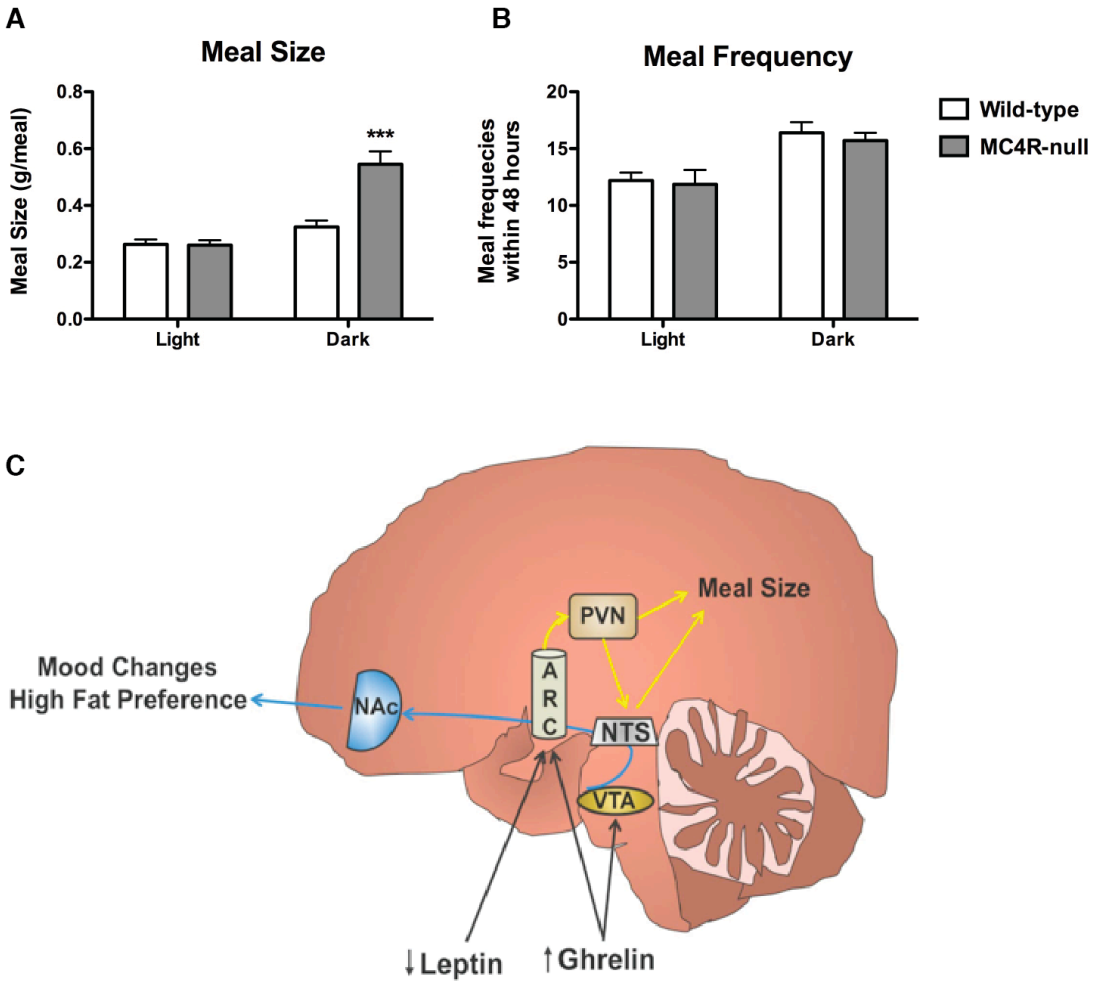

Figure 23. Meal pattern analysis in MC4R$^{-/-}$ mice. Seven-week-old male MC4R-null mice and WT littermates were monitored for 4 days in metabolic chambers. **A.** Meal size (significant *genotype X diurnal phase* interaction, $F_{1,30} = 43.65$) and **B.** Meal frequency (significant effect of *diurnal phase,* $F_{1,30} = 19.44$). n = 7-10/group. **C.** Model of leptin and acyl-ghrelin action on the brain after CSDS. Acyl-ghrelin directly acts on the VTA to influence mood symptoms and preference for high fat diet (blue). Leptin and acyl-ghrelin act on AgRP and POMC neurons in the arcuate nucleus to diminish melanocortin signaling, which increases meal size by decreasing satiation. *** $p < 0.001$, two-way ANOVA, Bonferroni post-hoc comparison.

BIBLIOGRAPHY

Abizaid, A., Liu, Z.W., Andrews, Z.B., Shanabrough, M., Borok, E., Elsworth, J.D., Roth, R.H., Sleeman, M.W., Picciotto, M.R., Tschop, M.H.*, et al.* (2006). Ghrelin modulates the activity and synaptic input organization of midbrain dopamine neurons while promoting appetite. The Journal of clinical investigation *116*, 3229-3239.

Airan, R.D., Meltzer, L.A., Roy, M., Gong, Y., Chen, H., and Deisseroth, K. (2007). High-speed imaging reveals neurophysiological links to behavior in an animal model of depression. Science *317*, 819-823.

Alexander, B., Warner-Schmidt, J., Eriksson, T., Tamminga, C., Arango-Lievano, M., Ghose, S., Vernov, M., Stavarache, M., Musatov, S., Flajolet, M.*, et al.* (2010). Reversal of depressed behaviors in mice by p11 gene therapy in the nucleus accumbens. Science translational medicine *2*, 54ra76.

Altar, C.A., Cai, N., Bliven, T., Juhasz, M., Conner, J.M., Acheson, A.L., Lindsay, R.M., and Wiegand, S.J. (1997). Anterograde transport of brain-derived neurotrophic factor and its role in the brain. Nature *389*, 856-860.

American Psychiatric Association., and American Psychiatric Association. Task Force on DSM-IV. (1994). Diagnostic and statistical manual of mental disorders : DSM-IV, 4th edn (Washington, DC: American Psychiatric Association).

Ampuero, E., Rubio, F.J., Falcon, R., Sandoval, M., Diaz-Veliz, G., Gonzalez, R.E., Earle, N., Dagnino-Subiabre, A., Aboitiz, F., Orrego, F.*, et al.* (2010). Chronic fluoxetine treatment induces structural plasticity and selective changes in glutamate receptor subunits in the rat cerebral cortex. Neuroscience *169*, 98-108.

Anderson, S.E., Cohen, P., Naumova, E.N., Jacques, P.F., and Must, A. (2007). Adolescent obesity and risk for subsequent major depressive disorder and anxiety disorder: prospective evidence. Psychosom Med *69*, 740-747.

Anggono, V., and Huganir, R.L. (2012). Regulation of AMPA receptor trafficking and synaptic plasticity. Current opinion in neurobiology *22*, 461-469.

Asakawa, A., Inui, A., Kaga, T., Yuzuriha, H., Nagata, T., Fujimiya, M., Katsuura, G., Makino, S., Fujino, M.A., and Kasuga, M. (2001). A role of ghrelin in neuroendocrine and behavioral responses to stress in mice. Neuroendocrinology *74*, 143-147.

Avgustinovich, D.F., Gorbach, O.V., and Kudryavtseva, N.N. (1997). Comparative analysis of anxiety-like behavior in partition and plus-maze tests after agonistic interactions in mice. Physiol Behav *61*, 37-43.

Badiani, A., Browman, K.E., and Robinson, T.E. (1995). Influence of novel versus home environments on sensitization to the psychomotor stimulant effects of cocaine and amphetamine. Brain research *674*, 291-298.

Bading, H. (2013). Nuclear calcium signalling in the regulation of brain function. Nature reviews Neuroscience *14*, 593-608.

Baker, D.A., McFarland, K., Lake, R.W., Shen, H., Tang, X.C., Toda, S., and Kalivas, P.W. (2003). Neuroadaptations in cystine-glutamate exchange underlie cocaine relapse. Nature neuroscience *6*, 743-749.

Balthasar, N., Dalgaard, L.T., Lee, C.E., Yu, J., Funahashi, H., Williams, T., Ferreira, M., Tang, V., McGovern, R.A., Kenny, C.D.*, et al.* (2005). Divergence of melanocortin pathways in the control of food intake and energy expenditure. Cell *123*, 493-505.

Barik, J., Marti, F., Morel, C., Fernandez, S.P., Lanteri, C., Godeheu, G., Tassin, J.P., Mombereau, C., Faure, P., and Tronche, F. (2013). Chronic stress triggers social aversion via glucocorticoid receptor in dopaminoceptive neurons. Science *339*, 332-335.

Barrot, M., Olivier, J.D., Perrotti, L.I., DiLeone, R.J., Berton, O., Eisch, A.J., Impey, S., Storm, D.R., Neve, R.L., Yin, J.C.*, et al.* (2002). CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. Proceedings of the National Academy of Sciences of the United States of America *99*, 11435-11440.

Barrot, M., Wallace, D.L., Bolanos, C.A., Graham, D.L., Perrotti, L.I., Neve, R.L., Chambliss, H., Yin, J.C., and Nestler, E.J. (2005). Regulation of anxiety and initiation of sexual behavior by CREB in the nucleus accumbens. Proceedings of the National Academy of Sciences of the United States of America *102*, 8357-8362.

Bath, K.G., Jing, D.Q., Dincheva, I., Neeb, C.C., Pattwell, S.S., Chao, M.V., Lee, F.S., and Ninan, I. (2012). BDNF Val66Met impairs fluoxetine-induced enhancement of adult hippocampus plasticity. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *37*, 1297-1304.

Beique, J.C., Na, Y., Kuhl, D., Worley, P.F., and Huganir, R.L. (2011). Arc-dependent synapse-specific homeostatic plasticity. Proceedings of the National Academy of Sciences of the United States of America *108*, 816-821.

Belmaker, R.H., and Agam, G. (2008). Major depressive disorder. The New England journal of medicine *358*, 55-68.

Belzung, C., and Griebel, G. (2001). Measuring normal and pathological anxiety-like behaviour in mice: a review. Behavioural brain research *125*, 141-149.

Berridge, K.C., Robinson, T.E., and Aldridge, J.W. (2009). Dissecting components of reward: 'liking', 'wanting', and learning. Current opinion in pharmacology *9*, 65-73.

Berthoud, H.R. (2008). Vagal and hormonal gut-brain communication: from satiation to satisfaction. Neurogastroenterol Motil *20 Suppl 1*, 64-72.

Berton, O., McClung, C.A., Dileone, R.J., Krishnan, V., Renthal, W., Russo, S.J., Graham, D., Tsankova, N.M., Bolanos, C.A., Rios, M.*, et al.* (2006). Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. Science *311*, 864-868.

Berton, O., and Nestler, E.J. (2006). New approaches to antidepressant drug discovery: beyond monoamines. Nature reviews Neuroscience *7*, 137-151.

Bessa, J.M., Ferreira, D., Melo, I., Marques, F., Cerqueira, J.J., Palha, J.A., Almeida, O.F., and Sousa, N. (2009). The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. Molecular psychiatry *14*, 764-773, 739.

Bessa, J.M., Morais, M., Marques, F., Pinto, L., Palha, J.A., Almeida, O.F., and Sousa, N. (2013). Stress-induced anhedonia is associated with hypertrophy of medium spiny neurons of the nucleus accumbens. Translational psychiatry *3*, e266.

Beydoun, M.A., and Wang, Y. (2010). Pathways linking socioeconomic status to obesity through depression and lifestyle factors among young US adults. J Affect Disord *123*, 52-63.

Blevins, J.E., Morton, G.J., Williams, D.L., Caldwell, D.W., Bastian, L.S., Wisse, B.E., Schwartz, M.W., and Baskin, D.G. (2009). Forebrain melanocortin signaling enhances the hindbrain satiety response to CCK-8. Am J Physiol Regul Integr Comp Physiol *296*, R476-484.

Bloomer, W.A., VanDongen, H.M., and VanDongen, A.M. (2008). Arc/Arg3.1 translation is controlled by convergent N-methyl-D-aspartate and Gs-coupled receptor signaling pathways. The Journal of biological chemistry *283*, 582-592.

Blundell, J.E., and MacDiarmid, J.I. (1997). Fat as a risk factor for overconsumption: satiation, satiety, and patterns of eating. J Am Diet Assoc *97*, S63-69.

Bolden, J.E., Peart, M.J., and Johnstone, R.W. (2006). Anticancer activities of histone deacetylase inhibitors. Nature reviews Drug discovery *5*, 769-784.

Borrelli, E., Nestler, E.J., Allis, C.D., and Sassone-Corsi, P. (2008). Decoding the epigenetic language of neuronal plasticity. Neuron *60*, 961-974.

Boudreau, A.C., Milovanovic, M., Conrad, K.L., Nelson, C., Ferrario, C.R., and Wolf, M.E. (2012). A protein cross-linking assay for measuring cell surface expression of glutamate receptor subunits in the rodent brain after in vivo treatments. Current protocols in neuroscience / editorial board, Jacqueline N Crawley [et al] *Chapter 5*, Unit 5 30 31-19.

Boudreau, A.C., and Wolf, M.E. (2005). Behavioral sensitization to cocaine is associated with increased AMPA receptor surface expression in the nucleus accumbens. The Journal of neuroscience : the official journal of the Society for Neuroscience *25*, 9144-9151.

Brebner, K., Wong, T.P., Liu, L., Liu, Y., Campsall, P., Gray, S., Phelps, L., Phillips, A.G., and Wang, Y.T. (2005). Nucleus accumbens long-term depression and the expression of behavioral sensitization. Science *310*, 1340-1343.

Breiter, H.C., Gollub, R.L., Weisskoff, R.M., Kennedy, D.N., Makris, N., Berke, J.D., Goodman, J.M., Kantor, H.L., Gastfriend, D.R., Riorden, J.P.*, et al.* (1997). Acute effects of cocaine on human brain activity and emotion. Neuron *19*, 591-611.

Bromberg-Martin, E.S., Matsumoto, M., and Hikosaka, O. (2010). Dopamine in motivational control: rewarding, aversive, and alerting. Neuron *68*, 815-834.

Butler, A.A., and Kozak, L.P. (2010). A recurring problem with the analysis of energy expenditure in genetic models expressing lean and obese phenotypes. Diabetes *59*, 323-329.

Cabib, S., Castellano, C., Cestari, V., Filibeck, U., and Puglisi-Allegra, S. (1991). D1 and D2 receptor antagonists differently affect cocaine-induced locomotor hyperactivity in the mouse. Psychopharmacology *105*, 335-339.

Caetano, S.C., Kaur, S., Brambilla, P., Nicoletti, M., Hatch, J.P., Sassi, R.B., Mallinger, A.G., Keshavan, M.S., Kupfer, D.J., Frank, E.*, et al.* (2006). Smaller cingulate volumes in unipolar depressed patients. Biological psychiatry *59*, 702-706.

Campioni, M.R., Xu, M., and McGehee, D.S. (2009). Stress-induced changes in nucleus accumbens glutamate synaptic plasticity. Journal of neurophysiology *101*, 3192-3198.

Cao, J.L., Covington, H.E., 3rd, Friedman, A.K., Wilkinson, M.B., Walsh, J.J., Cooper, D.C., Nestler, E.J., and Han, M.H. (2010). Mesolimbic dopamine neurons in the brain reward circuit mediate susceptibility to social defeat and antidepressant action. The Journal of neuroscience : the official journal of the Society for Neuroscience *30*, 16453-16458.

Castren, E., and Hen, R. (2013). Neuronal plasticity and antidepressant actions. Trends in neurosciences *36*, 259-267.

Chang, S., McKinsey, T.A., Zhang, C.L., Richardson, J.A., Hill, J.A., and Olson, E.N. (2004). Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development. Molecular and cellular biology *24*, 8467-8476.

Chase, T., Carrey, N., Soo, E., and Wilkinson, M. (2007). Methylphenidate regulates activity regulated cytoskeletal associated but not brain-derived neurotrophic factor gene expression in the developing rat striatum. Neuroscience *144*, 969-984.

Chaudhury, D., Walsh, J.J., Friedman, A.K., Juarez, B., Ku, S.M., Koo, J.W., Ferguson, D., Tsai, H.C., Pomeranz, L., Christoffel, D.J.*, et al.* (2013). Rapid regulation of depression-related behaviours by control of midbrain dopamine neurons. Nature *493*, 532-536.

Chawla, S., Vanhoutte, P., Arnold, F.J., Huang, C.L., and Bading, H. (2003). Neuronal activity-dependent nucleocytoplasmic shuttling of HDAC4 and HDAC5. Journal of neurochemistry *85*, 151-159.

Chiba, S., Numakawa, T., Ninomiya, M., Richards, M.C., Wakabayashi, C., and Kunugi, H. (2012). Chronic restraint stress causes anxiety- and depression-like behaviors, downregulates glucocorticoid receptor expression, and attenuates glutamate release induced by brain-derived neurotrophic factor in the prefrontal cortex. Progress in neuro-psychopharmacology & biological psychiatry *39*, 112-119.

Chourbaji, S., Vogt, M.A., Fumagalli, F., Sohr, R., Frasca, A., Brandwein, C., Hortnagl, H., Riva, M.A., Sprengel, R., and Gass, P. (2008). AMPA receptor subunit 1 (GluR-A) knockout mice model the glutamate hypothesis of depression. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *22*, 3129-3134.

Chowdhury, S., Shepherd, J.D., Okuno, H., Lyford, G., Petralia, R.S., Plath, N., Kuhl, D., Huganir, R.L., and Worley, P.F. (2006). Arc/Arg3.1 interacts with the endocytic machinery to regulate AMPA receptor trafficking. Neuron *52*, 445-459.

Christoffel, D.J., Golden, S.A., Dumitriu, D., Robison, A.J., Janssen, W.G., Ahn, H.F., Krishnan, V., Reyes, C.M., Han, M.H., Ables, J.L.*, et al.* (2011). IkappaB kinase regulates social defeat stress-induced synaptic and behavioral plasticity. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 314-321.

Chuang, J.C., Cui, H., Mason, B.L., Mahgoub, M., Bookout, A.L., Yu, H.G., Perello, M., Elmquist, J.K., Repa, J.J., Zigman, J.M.*, et al.* (2010a). Chronic social defeat stress disrupts regulation of lipid synthesis. Journal of lipid research *51*, 1344-1353.

Chuang, J.C., Krishnan, V., Yu, H.G., Mason, B., Cui, H., Wilkinson, M.B., Zigman, J.M., Elmquist, J.K., Nestler, E.J., and Lutter, M. (2010b). A beta3-adrenergic-leptin-melanocortin circuit regulates behavioral and metabolic changes induced by chronic stress. Biological psychiatry *67*, 1075-1082.

Chuang, J.C., Perello, M., Sakata, I., Osborne-Lawrence, S., Savitt, J.M., Lutter, M., and Zigman, J.M. (2011). Ghrelin mediates stress-induced food-reward behavior in mice. J Clin Invest *121*, 2684-2692.

Chwastiak, L.A., Rosenheck, R.A., and Kazis, L.E. (2011). Association of psychiatric illness and obesity, physical inactivity, and smoking among a national sample of veterans. Psychosomatics *52*, 230-236.

Cizza, G. (2011). Major depressive disorder is a risk factor for low bone mass, central obesity, and other medical conditions. Dialogues Clin Neurosci *13*, 73-87.

Cohen, S., and Greenberg, M.E. (2008). Communication between the synapse and the nucleus in neuronal development, plasticity, and disease. Annual review of cell and developmental biology *24*, 183-209.

Cone, R.D. (2006). Studies on the physiological functions of the melanocortin system. Endocrine reviews *27*, 736-749.

Conrad, K.L., Tseng, K.Y., Uejima, J.L., Reimers, J.M., Heng, L.J., Shaham, Y., Marinelli, M., and Wolf, M.E. (2008). Formation of accumbens GluR2-lacking AMPA receptors mediates incubation of cocaine craving. Nature *454*, 118-121.

Conway, K.P., Compton, W., Stinson, F.S., and Grant, B.F. (2006). Lifetime comorbidity of DSM-IV mood and anxiety disorders and specific drug use disorders: results from the National Epidemiologic Survey on Alcohol and Related Conditions. The Journal of clinical psychiatry *67*, 247-257.

Coque, L., Mukherjee, S., Cao, J.L., Spencer, S., Marvin, M., Falcon, E., Sidor, M.M., Birnbaum, S.G., Graham, A., Neve, R.L.*, et al.* (2011). Specific role of VTA dopamine neuronal firing rates and morphology in the reversal of anxiety-related, but not depression-related behavior in the ClockDelta19 mouse model of mania. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *36*, 1478-1488.

Cornish, J.L., and Kalivas, P.W. (2000). Glutamate transmission in the nucleus accumbens mediates relapse in cocaine addiction. The Journal of neuroscience : the official journal of the Society for Neuroscience *20*, RC89.

Coughlin, S.S. (2011). Post-traumatic Stress Disorder and Cardiovascular Disease. Open Cardiovasc Med J *5*, 164-170.

Coughlin, S.S., Kang, H.K., and Mahan, C.M. (2011). Selected Health Conditions Among Overweight, Obese, and Non-Obese Veterans of the 1991 Gulf War: Results from a Survey Conducted in 2003-2005. Open Epidemiol J *4*, 140-146.

Covington, H.E., 3rd, Lobo, M.K., Maze, I., Vialou, V., Hyman, J.M., Zaman, S., LaPlant, Q., Mouzon, E., Ghose, S., Tamminga, C.A.*, et al.* (2010). Antidepressant effect of optogenetic stimulation of the medial prefrontal cortex. The Journal of neuroscience : the official journal of the Society for Neuroscience *30*, 16082-16090.

Covington, H.E., 3rd, Maze, I., LaPlant, Q.C., Vialou, V.F., Ohnishi, Y.N., Berton, O., Fass, D.M., Renthal, W., Rush, A.J., 3rd, Wu, E.Y.*, et al.* (2009). Antidepressant actions of histone deacetylase inhibitors. The Journal of neuroscience : the official journal of the Society for Neuroscience *29*, 11451-11460.

Covington, H.E., 3rd, Maze, I., Sun, H., Bomze, H.M., DeMaio, K.D., Wu, E.Y., Dietz, D.M., Lobo, M.K., Ghose, S., Mouzon, E.*, et al.* (2011). A role for repressive histone methylation in cocaine-induced vulnerability to stress. Neuron *71*, 656-670.

Cryan, J.F., and Holmes, A. (2005). The ascent of mouse: advances in modelling human depression and anxiety. Nature reviews Drug discovery *4*, 775-790.

Cryan, J.F., Valentino, R.J., and Lucki, I. (2005). Assessing substrates underlying the behavioral effects of antidepressants using the modified rat forced swimming test. Neuroscience and biobehavioral reviews *29*, 547-569.

Czerniawski, J., Ree, F., Chia, C., Ramamoorthi, K., Kumata, Y., and Otto, T.A. (2011). The importance of having Arc: expression of the immediate-early gene Arc is required for hippocampus-dependent fear conditioning and blocked by NMDA receptor antagonism. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 11200-11207.

Daberkow, D.P., Riedy, M.D., Kesner, R.P., and Keefe, K.A. (2007). Arc mRNA induction in striatal efferent neurons associated with response learning. The European journal of neuroscience *26*, 228-241.

de Wit, L.M., Fokkema, M., van Straten, A., Lamers, F., Cuijpers, P., and Penninx, B.W. (2010). Depressive and anxiety disorders and the association with obesity, physical, and social activities. Depress Anxiety *27*, 1057-1065.

Dedert, E.A., Becker, M.E., Fuemmeler, B.F., Braxton, L.E., Calhoun, P.S., and Beckham, J.C. (2010). Childhood traumatic stress and obesity in women: The intervening effects of PTSD and MDD. J Trauma Stress.

Deroche, V., Le Moal, M., and Piazza, P.V. (1999). Cocaine self-administration increases the incentive motivational properties of the drug in rats. The European journal of neuroscience *11*, 2731-2736.

Di Chiara, G., and Imperato, A. (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proceedings of the National Academy of Sciences of the United States of America *85*, 5274-5278.

Disner, S.G., Beevers, C.G., Haigh, E.A., and Beck, A.T. (2011). Neural mechanisms of the cognitive model of depression. Nature reviews Neuroscience *12*, 467-477.

Dong, Y., Saal, D., Thomas, M., Faust, R., Bonci, A., Robinson, T., and Malenka, R.C. (2004). Cocaine-induced potentiation of synaptic strength in dopamine neurons: behavioral correlates in GluRA(-/-) mice. Proceedings of the National Academy of Sciences of the United States of America *101*, 14282-14287.

Drevets, W.C., Price, J.L., Simpson, J.R., Jr., Todd, R.D., Reich, T., Vannier, M., and Raichle, M.E. (1997). Subgenual prefrontal cortex abnormalities in mood disorders. Nature *386*, 824-827.

Drevets, W.C., Videen, T.O., Price, J.L., Preskorn, S.H., Carmichael, S.T., and Raichle, M.E. (1992). A functional anatomical study of unipolar depression. The Journal of neuroscience : the official journal of the Society for Neuroscience *12*, 3628-3641.

Duman, R.S., and Aghajanian, G.K. (2012). Synaptic dysfunction in depression: potential therapeutic targets. Science *338*, 68-72.

Duman, R.S., and Monteggia, L.M. (2006). A neurotrophic model for stress-related mood disorders. Biological psychiatry *59*, 1116-1127.

Feder, A., Nestler, E.J., and Charney, D.S. (2009). Psychobiology and molecular genetics of resilience. Nature reviews Neuroscience *10*, 446-457.

Feyissa, A.M., Chandran, A., Stockmeier, C.A., and Karolewicz, B. (2009). Reduced levels of NR2A and NR2B subunits of NMDA receptor and PSD-95 in the prefrontal cortex in major depression. Progress in neuro-psychopharmacology & biological psychiatry *33*, 70-75.

Flavell, S.W., Cowan, C.W., Kim, T.K., Greer, P.L., Lin, Y., Paradis, S., Griffith, E.C., Hu, L.S., Chen, C., and Greenberg, M.E. (2006). Activity-dependent regulation of MEF2 transcription factors suppresses excitatory synapse number. Science *311*, 1008-1012.

Flavell, S.W., Kim, T.K., Gray, J.M., Harmin, D.A., Hemberg, M., Hong, E.J., Markenscoff-Papadimitriou, E., Bear, D.M., and Greenberg, M.E. (2008). Genome-wide analysis of MEF2 transcriptional program reveals synaptic target genes and neuronal activity-dependent polyadenylation site selection. Neuron *60*, 1022-1038.

Fosnaugh, J.S., Bhat, R.V., Yamagata, K., Worley, P.F., and Baraban, J.M. (1995). Activation of arc, a putative "effector" immediate early gene, by cocaine in rat brain. Journal of neurochemistry *64*, 2377-2380.

Freeman, W.M., Patel, K.M., Brucklacher, R.M., Lull, M.E., Erwin, M., Morgan, D., Roberts, D.C., and Vrana, K.E. (2008). Persistent alterations in mesolimbic gene expression with abstinence from cocaine self-administration. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *33*, 1807-1817.

Fromer, M., Pocklington, A.J., Kavanagh, D.H., Williams, H.J., Dwyer, S., Gormley, P., Georgieva, L., Rees, E., Palta, P., Ruderfer, D.M.*, et al.* (2014). De novo mutations in schizophrenia implicate synaptic networks. Nature *506*, 179-184.

Fuchs, R.A., Weber, S.M., Rice, H.J., and Neisewander, J.L. (2002). Effects of excitotoxic lesions of the basolateral amygdala on cocaine-seeking behavior and cocaine conditioned place preference in rats. Brain research *929*, 15-25.

Fujitsuka, N., Asakawa, A., Hayashi, M., Sameshima, M., Amitani, H., Kojima, S., Fujimiya, M., and Inui, A. (2009). Selective serotonin reuptake inhibitors modify physiological gastrointestinal motor activities via 5-HT2c receptor and acyl ghrelin. Biol Psychiatry *65*, 748-759.

Fulton, S., Pissios, P., Manchon, R.P., Stiles, L., Frank, L., Pothos, E.N., Maratos-Flier, E., and Flier, J.S. (2006). Leptin regulation of the mesoaccumbens dopamine pathway. Neuron *51*, 811-822.

Fumagalli, F., Bedogni, F., Frasca, A., Di Pasquale, L., Racagni, G., and Riva, M.A. (2006). Corticostriatal up-regulation of activity-regulated cytoskeletal-associated protein expression after repeated exposure to cocaine. Molecular pharmacology *70*, 1726-1734.

Fumagalli, F., Franchi, C., Caffino, L., Racagni, G., Riva, M.A., and Cervo, L. (2009). Single session of cocaine intravenous self-administration shapes goal-oriented behaviours and up-regulates Arc mRNA levels in rat medial prefrontal cortex. Int J Neuropsychopharmacol *12*, 423-429.

Gamaro, G.D., Prediger, M.E., Lopes, J., Bassani, M.G., and Dalmaz, C. (2008). Fluoxetine alters feeding behavior and leptin levels in chronically-stressed rats. Pharmacology, biochemistry, and behavior *90*, 312-317.

Garza, J.C., Guo, M., Zhang, W., and Lu, X.Y. (2011). Leptin restores adult hippocampal neurogenesis in a chronic unpredictable stress model of depression and reverses glucocorticoid-induced inhibition of GSK-3beta/beta-catenin signaling. Mol Psychiatry.

Gavin, A.R., Rue, T., and Takeuchi, D. (2010). Racial/ethnic differences in the association between obesity and major depressive disorder: findings from the Comprehensive Psychiatric Epidemiology Surveys. Public Health Rep *125*, 698-708.

Gawin, F.H., and Kleber, H.D. (1986). Abstinence symptomatology and psychiatric diagnosis in cocaine abusers. Clinical observations. Archives of general psychiatry *43*, 107-113.

Golden, S.A., Covington, H.E., 3rd, Berton, O., and Russo, S.J. (2011). A standardized protocol for repeated social defeat stress in mice. Nature protocols *6*, 1183-1191.

Goldman, D., Oroszi, G., and Ducci, F. (2005). The genetics of addictions: uncovering the genes. Nature reviews Genetics *6*, 521-532.

Gorman, J.M. (1996). Comorbid depression and anxiety spectrum disorders. Depression and anxiety *4*, 160-168.

Grant, B.F., and Dawson, D.A. (1998). Age of onset of drug use and its association with DSM-IV drug abuse and dependence: results from the National Longitudinal Alcohol Epidemiologic Survey. Journal of substance abuse *10*, 163-173.

Green, S.M., Delargy, H.J., Joanes, D., and Blundell, J.E. (1997). A satiety quotient: a formulation to assess the satiating effect of food. Appetite *29*, 291-304.

Greenberg, M.E., Ziff, E.B., and Greene, L.A. (1986). Stimulation of neuronal acetylcholine receptors induces rapid gene transcription. Science *234*, 80-83.

Greenberg, P.E., Sisitsky, T., Kessler, R.C., Finkelstein, S.N., Berndt, E.R., Davidson, J.R., Ballenger, J.C., and Fyer, A.J. (1999). The economic burden of anxiety disorders in the 1990s. The Journal of clinical psychiatry *60*, 427-435.

Greer, P.L., and Greenberg, M.E. (2008). From synapse to nucleus: calcium-dependent gene transcription in the control of synapse development and function. Neuron *59*, 846-860.

Greer, P.L., Hanayama, R., Bloodgood, B.L., Mardinly, A.R., Lipton, D.M., Flavell, S.W., Kim, T.K., Griffith, E.C., Waldon, Z., Maehr, R.*, et al.* (2010). The Angelman Syndrome protein Ube3A regulates synapse development by ubiquitinating arc. Cell *140*, 704-716.

Groenewegen, H.J., Wright, C.I., Beijer, A.V., and Voorn, P. (1999). Convergence and segregation of ventral striatal inputs and outputs. Annals of the New York Academy of Sciences *877*, 49-63.

Gross, C., and Hen, R. (2004). The developmental origins of anxiety. Nature reviews Neuroscience *5*, 545-552.

Guzowski, J.F., Lyford, G.L., Stevenson, G.D., Houston, F.P., McGaugh, J.L., Worley, P.F., and Barnes, C.A. (2000). Inhibition of activity-dependent arc protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory. The Journal of neuroscience : the official journal of the Society for Neuroscience *20*, 3993-4001.

Guzowski, J.F., McNaughton, B.L., Barnes, C.A., and Worley, P.F. (1999). Environment-specific expression of the immediate-early gene Arc in hippocampal neuronal ensembles. Nature neuroscience *2*, 1120-1124.

Hale, C.F., Dietz, K.C., Varela, J.A., Wood, C.B., Zirlin, B.C., Leverich, L.S., Greene, R.W., and Cowan, C.W. (2011). Essential role for vav Guanine nucleotide exchange factors in brain-derived neurotrophic factor-induced dendritic spine growth and synapse plasticity. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 12426-12436.

Hearing, M.C., Schwendt, M., and McGinty, J.F. (2011). Suppression of activity-regulated cytoskeleton-associated gene expression in the dorsal striatum attenuates extinction of cocaine-seeking. Int J Neuropsychopharmacol *14*, 784-795.

Heisler, L.K., Jobst, E.E., Sutton, G.M., Zhou, L., Borok, E., Thornton-Jones, Z., Liu, H.Y., Zigman, J.M., Balthasar, N., Kishi, T.*, et al.* (2006). Serotonin reciprocally regulates melanocortin neurons to modulate food intake. Neuron *51*, 239-249.

Hill, M.N., Hellemans, K.G., Verma, P., Gorzalka, B.B., and Weinberg, J. (2012). Neurobiology of chronic mild stress: parallels to major depression. Neuroscience and biobehavioral reviews *36*, 2085-2117.

Hnasko, T.S., Hjelmstad, G.O., Fields, H.L., and Edwards, R.H. (2012). Ventral tegmental area glutamate neurons: electrophysiological properties and projections. The Journal of neuroscience : the official journal of the Society for Neuroscience *32*, 15076-15085.

Holloway, C.M., and McIntyre, C.K. (2011). Post-training disruption of Arc protein expression in the anterior cingulate cortex impairs long-term memory for inhibitory avoidance training. Neurobiology of learning and memory *95*, 425-432.

Holmes, A. (2001). Targeted gene mutation approaches to the study of anxiety-like behavior in mice. Neuroscience and biobehavioral reviews *25*, 261-273.

Holtmaat, A., and Svoboda, K. (2009). Experience-dependent structural synaptic plasticity in the mammalian brain. Nature reviews Neuroscience *10*, 647-658.

Hommel, J.D., Sears, R.M., Georgescu, D., Simmons, D.L., and DiLeone, R.J. (2003). Local gene knockdown in the brain using viral-mediated RNA interference. Nature medicine *9*, 1539-1544.

Hommel, J.D., Trinko, R., Sears, R.M., Georgescu, D., Liu, Z.W., Gao, X.B., Thurmon, J.J., Marinelli, M., and DiLeone, R.J. (2006). Leptin receptor signaling in midbrain dopamine neurons regulates feeding. Neuron *51*, 801-810.

Hong, E.J., McCord, A.E., and Greenberg, M.E. (2008). A biological function for the neuronal activity-dependent component of Bdnf transcription in the development of cortical inhibition. Neuron *60*, 610-624.

Hu, X.T. (2007). Cocaine withdrawal and neuro-adaptations in ion channel function. Molecular neurobiology *35*, 95-112.

Hyman, S.E., and Malenka, R.C. (2001). Addiction and the brain: the neurobiology of compulsion and its persistence. Nature reviews Neuroscience *2*, 695-703.

Hyman, S.E., Malenka, R.C., and Nestler, E.J. (2006). Neural mechanisms of addiction: the role of reward-related learning and memory. Annual review of neuroscience *29*, 565-598.

Jaenisch, R., and Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nature genetics *33 Suppl*, 245-254.

Kalivas, P.W., and Hu, X.T. (2006). Exciting inhibition in psychostimulant addiction. Trends in neurosciences *29*, 610-616.

Kang, H.J., Voleti, B., Hajszan, T., Rajkowska, G., Stockmeier, C.A., Licznerski, P., Lepack, A., Majik, M.S., Jeong, L.S., Banasr, M.*, et al.* (2012). Decreased expression of synapse-related genes and loss of synapses in major depressive disorder. Nature medicine *18*, 1413-1417.

Karolewicz, B., Szebeni, K., Gilmore, T., Maciag, D., Stockmeier, C.A., and Ordway, G.A. (2009). Elevated levels of NR2A and PSD-95 in the lateral amygdala in depression. Int J Neuropsychopharmacol *12*, 143-153.

Karpova, N.N., Pickenhagen, A., Lindholm, J., Tiraboschi, E., Kulesskaya, N., Agustsdottir, A., Antila, H., Popova, D., Akamine, Y., Bahi, A.*, et al.* (2011). Fear erasure in mice requires synergy between antidepressant drugs and extinction training. Science *334*, 1731-1734.

Kauer, J.A., and Malenka, R.C. (2007). Synaptic plasticity and addiction. Nature reviews Neuroscience *8*, 844-858.

Kawashima, T., Okuno, H., Nonaka, M., Adachi-Morishima, A., Kyo, N., Okamura, M., Takemoto-Kimura, S., Worley, P.F., and Bito, H. (2009). Synaptic activity-responsive element in the Arc/Arg3.1 promoter essential for synapse-to-nucleus signaling in activated neurons. Proceedings of the National Academy of Sciences of the United States of America *106*, 316-321.

Keller, M.B., Lavori, P.W., Mueller, T.I., Endicott, J., Coryell, W., Hirschfeld, R.M., and Shea, T. (1992). Time to recovery, chronicity, and levels of psychopathology in major depression. A 5-year prospective follow-up of 431 subjects. Archives of general psychiatry *49*, 809-816.

Kendler, K.S. (2001). Twin studies of psychiatric illness: an update. Arch Gen Psychiatry *58*, 1005-1014.

Kessler, R.C., Chiu, W.T., Demler, O., Merikangas, K.R., and Walters, E.E. (2005). Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. Archives of general psychiatry *62*, 617-627.

Klebaur, J.E., Ostrander, M.M., Norton, C.S., Watson, S.J., Akil, H., and Robinson, T.E. (2002). The ability of amphetamine to evoke arc (Arg 3.1) mRNA expression in the caudate, nucleus accumbens and neocortex is modulated by environmental context. Brain research *930*, 30-36.

Koob, G., and Kreek, M.J. (2007). Stress, dysregulation of drug reward pathways, and the transition to drug dependence. Am J Psychiatry *164*, 1149-1159.

Koob, G.F., and Bloom, F.E. (1988). Cellular and molecular mechanisms of drug dependence. Science *242*, 715-723.

Koob, G.F., and Volkow, N.D. (2010). Neurocircuitry of addiction. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *35*, 217-238.

Korb, E., Wilkinson, C.L., Delgado, R.N., Lovero, K.L., and Finkbeiner, S. (2013). Arc in the nucleus regulates PML-dependent GluA1 transcription and homeostatic plasticity. Nature neuroscience.

Kornhauser, J.M., Cowan, C.W., Shaywitz, A.J., Dolmetsch, R.E., Griffith, E.C., Hu, L.S., Haddad, C., Xia, Z., and Greenberg, M.E. (2002). CREB transcriptional activity in neurons is regulated by multiple, calcium-specific phosphorylation events. Neuron *34*, 221-233.

Kouzarides, T. (2007). SnapShot: Histone-modifying enzymes. Cell *128*, 802.

Koya, E., Golden, S.A., Harvey, B.K., Guez-Barber, D.H., Berkow, A., Simmons, D.E., Bossert, J.M., Nair, S.G., Uejima, J.L., Marin, M.T.*, et al.* (2009). Targeted disruption of cocaine-activated nucleus accumbens neurons prevents context-specific sensitization. Nature neuroscience *12*, 1069-1073.

Krishnan, V., Berton, O., and Nestler, E. (2008). The use of animal models in psychiatric research and treatment. Am J Psychiatry *165*, 1109.

Krishnan, V., Han, M.H., Graham, D.L., Berton, O., Renthal, W., Russo, S.J., Laplant, Q., Graham, A., Lutter, M., Lagace, D.C.*, et al.* (2007). Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. Cell *131*, 391-404.

Krishnan, V., and Nestler, E.J. (2008). The molecular neurobiology of depression. Nature *455*, 894-902.

Krishnan, V., and Nestler, E.J. (2010). Linking molecules to mood: new insight into the biology of depression. The American journal of psychiatry *167*, 1305-1320.

Kristenssson, E., Sundqvist, M., Astin, M., Kjerling, M., Mattsson, H., Dornonville de la Cour, C., Hakanson, R., and Lindstrom, E. (2006). Acute psychological stress raises plasma ghrelin in the rat. Regulatory peptides *134*, 114-117.

Kumar, A., Choi, K.H., Renthal, W., Tsankova, N.M., Theobald, D.E., Truong, H.T., Russo, S.J., Laplant, Q., Sasaki, T.S., Whistler, K.N.*, et al.* (2005). Chromatin remodeling is a key mechanism underlying cocaine-induced plasticity in striatum. Neuron *48*, 303-314.

Lam, D.D., Przydzial, M.J., Ridley, S.H., Yeo, G.S., Rochford, J.J., O'Rahilly, S., and Heisler, L.K. (2008). Serotonin 5-HT2C receptor agonist promotes hypophagia via downstream activation of melanocortin 4 receptors. Endocrinology *149*, 1323-1328.

Lammel, S., Ion, D.I., Roeper, J., and Malenka, R.C. (2011). Projection-specific modulation of dopamine neuron synapses by aversive and rewarding stimuli. Neuron *70*, 855-862.

LaPlant, Q., Chakravarty, S., Vialou, V., Mukherjee, S., Koo, J.W., Kalahasti, G., Bradbury, K.R., Taylor, S.V., Maze, I., Kumar, A.*, et al.* (2009). Role of nuclear factor kappaB in ovarian hormone-mediated stress hypersensitivity in female mice. Biological psychiatry *65*, 874-880.

Li, N., Lee, B., Liu, R.J., Banasr, M., Dwyer, J.M., Iwata, M., Li, X.Y., Aghajanian, G., and Duman, R.S. (2010). mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. Science *329*, 959-964.

Li, N., Liu, R.J., Dwyer, J.M., Banasr, M., Lee, B., Son, H., Li, X.Y., Aghajanian, G., and Duman, R.S. (2011). Glutamate N-methyl-D-aspartate receptor antagonists rapidly reverse behavioral and synaptic deficits caused by chronic stress exposure. Biological psychiatry *69*, 754-761.

Li, Y., Vartanian, A.J., White, F.J., Xue, C.J., and Wolf, M.E. (1997). Effects of the AMPA receptor antagonist NBQX on the development and expression of behavioral sensitization to cocaine and amphetamine. Psychopharmacology *134*, 266-276.

Lin, Y., Bloodgood, B.L., Hauser, J.L., Lapan, A.D., Koon, A.C., Kim, T.K., Hu, L.S., Malik, A.N., and Greenberg, M.E. (2008). Activity-dependent regulation of inhibitory synapse development by Npas4. Nature *455*, 1198-1204.

Link, W., Konietzko, U., Kauselmann, G., Krug, M., Schwanke, B., Frey, U., and Kuhl, D. (1995). Somatodendritic expression of an immediate early gene is regulated by synaptic activity. Proceedings of the National Academy of Sciences of the United States of America *92*, 5734-5738.

Liu, R.J., and Aghajanian, G.K. (2008). Stress blunts serotonin- and hypocretin-evoked EPSCs in prefrontal cortex: role of corticosterone-mediated apical dendritic atrophy. Proceedings of the National Academy of Sciences of the United States of America *105*, 359-364.

Lu, J., McKinsey, T.A., Nicol, R.L., and Olson, E.N. (2000). Signal-dependent activation of the MEF2 transcription factor by dissociation from histone deacetylases. Proceedings of the National Academy of Sciences of the United States of America *97*, 4070-4075.

Lu, X.Y., Kim, C.S., Frazer, A., and Zhang, W. (2006). Leptin: a potential novel antidepressant. Proc Natl Acad Sci U S A *103*, 1593-1598.

Luscher, B., Shen, Q., and Sahir, N. (2011). The GABAergic deficit hypothesis of major depressive disorder. Molecular psychiatry *16*, 383-406.

Lutter, M., Sakata, I., Osborne-Lawrence, S., Rovinsky, S.A., Anderson, J.G., Jung, S., Birnbaum, S., Yanagisawa, M., Elmquist, J.K., Nestler, E.J.*, et al.* (2008). The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress. Nat Neurosci *11*, 752-753.

Lyford, G.L., Yamagata, K., Kaufmann, W.E., Barnes, C.A., Sanders, L.K., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Lanahan, A.A., and Worley, P.F. (1995). Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. Neuron *14*, 433-445.

Machado-Vieira, R., Ibrahim, L., Henter, I.D., and Zarate, C.A., Jr. (2012). Novel glutamatergic agents for major depressive disorder and bipolar disorder. Pharmacology, biochemistry, and behavior *100*, 678-687.

Maddox, S.A., and Schafe, G.E. (2011). The activity-regulated cytoskeletal-associated protein (Arc/Arg3.1) is required for reconsolidation of a Pavlovian fear memory. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 7073-7082.

Maeng, S., Zarate, C.A., Jr., Du, J., Schloesser, R.J., McCammon, J., Chen, G., and Manji, H.K. (2008). Cellular mechanisms underlying the antidepressant effects of ketamine: role of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors. Biological psychiatry *63*, 349-352.

Magarinos, A.M., and McEwen, B.S. (1995). Stress-induced atrophy of apical dendrites of hippocampal CA3c neurons: involvement of glucocorticoid secretion and excitatory amino acid receptors. Neuroscience *69*, 89-98.

Malberg, J.E., Eisch, A.J., Nestler, E.J., and Duman, R.S. (2000). Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. The Journal of neuroscience : the official journal of the Society for Neuroscience *20*, 9104-9110.

Malenka, R.C., and Bear, M.F. (2004). LTP and LTD: an embarrassment of riches. Neuron *44*, 5-21.

Marie-Claire, C., Courtin, C., Roques, B.P., and Noble, F. (2004). Cytoskeletal genes regulation by chronic morphine treatment in rat striatum. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *29*, 2208-2215.

Martin, I.L. (1987). The benzodiazepines and their receptors: 25 years of progress. Neuropharmacology *26*, 957-970.

Martin-Soelch, C. (2009). Is depression associated with dysfunction of the central reward system? Biochemical Society transactions *37*, 313-317.

Mastronardi, C., Paz-Filho, G.J., Valdez, E., Maestre-Mesa, J., Licinio, J., and Wong, M.L. (2011). Long-term body weight outcomes of antidepressant-environment interactions. Mol Psychiatry *16*, 265-272.

Maya Vetencourt, J.F., Sale, A., Viegi, A., Baroncelli, L., De Pasquale, R., O'Leary, O.F., Castren, E., and Maffei, L. (2008). The antidepressant fluoxetine restores plasticity in the adult visual cortex. Science *320*, 385-388.

Mayberg, H.S., Brannan, S.K., Tekell, J.L., Silva, J.A., Mahurin, R.K., McGinnis, S., and Jerabek, P.A. (2000). Regional metabolic effects of fluoxetine in major depression: serial changes and relationship to clinical response. Biological psychiatry *48*, 830-843.

Mayberg, H.S., Lozano, A.M., Voon, V., McNeely, H.E., Seminowicz, D., Hamani, C., Schwalb, J.M., and Kennedy, S.H. (2005). Deep brain stimulation for treatment-resistant depression. Neuron *45*, 651-660.

McCutcheon, J.E., Wang, X., Tseng, K.Y., Wolf, M.E., and Marinelli, M. (2011). Calcium-permeable AMPA receptors are present in nucleus accumbens synapses after prolonged withdrawal from cocaine self-administration but not experimenter-administered cocaine. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 5737-5743.

McEwen, B.S., Eiland, L., Hunter, R.G., and Miller, M.M. (2012). Stress and anxiety: structural plasticity and epigenetic regulation as a consequence of stress. Neuropharmacology *62*, 3-12.

McKinsey, T.A., Zhang, C.L., Lu, J., and Olson, E.N. (2000a). Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. Nature *408*, 106-111.

McKinsey, T.A., Zhang, C.L., and Olson, E.N. (2000b). Activation of the myocyte enhancer factor-2 transcription factor by calcium/calmodulin-dependent protein

kinase-stimulated binding of 14-3-3 to histone deacetylase 5. Proceedings of the National Academy of Sciences of the United States of America *97*, 14400-14405.

Melhorn, S.J., Krause, E.G., Scott, K.A., Mooney, M.R., Johnson, J.D., Woods, S.C., and Sakai, R.R. (2010). Meal patterns and hypothalamic NPY expression during chronic social stress and recovery. American journal of physiology Regulatory, integrative and comparative physiology *299*, R813-822.

Merikangas, A.K., Mendola, P., Pastor, P.N., Reuben, C.A., and Cleary, S.D. (2011). The association between major depressive disorder and obesity in US adolescents: results from the 2001-2004 National Health and Nutrition Examination Survey. J Behav Med.

Meririnne, E., Kankaanpaa, A., and Seppala, T. (2001). Rewarding properties of methylphenidate: sensitization by prior exposure to the drug and effects of dopamine D1- and D2-receptor antagonists. The Journal of pharmacology and experimental therapeutics *298*, 539-550.

Messaoudi, E., Kanhema, T., Soule, J., Tiron, A., Dagyte, G., da Silva, B., and Bramham, C.R. (2007). Sustained Arc/Arg3.1 synthesis controls long-term potentiation consolidation through regulation of local actin polymerization in the dentate gyrus in vivo. The Journal of neuroscience : the official journal of the Society for Neuroscience *27*, 10445-10455.

Meyers, R.A., Zavala, A.R., and Neisewander, J.L. (2003). Dorsal, but not ventral, hippocampal lesions disrupt cocaine place conditioning. Neuroreport *14*, 2127-2131.

Miczek, K.A., Nikulina, E.M., Shimamoto, A., and Covington, H.E., 3rd (2011). Escalated or suppressed cocaine reward, tegmental BDNF, and accumbal dopamine caused by episodic versus continuous social stress in rats. J Neurosci *31*, 9848-9857.

Mikuni, T., Uesaka, N., Okuno, H., Hirai, H., Deisseroth, K., Bito, H., and Kano, M. (2013). Arc/Arg3.1 is a postsynaptic mediator of activity-dependent synapse elimination in the developing cerebellum. Neuron *78*, 1024-1035.

Milad, M.R., Vidal-Gonzalez, I., and Quirk, G.J. (2004). Electrical stimulation of medial prefrontal cortex reduces conditioned fear in a temporally specific manner. Behavioral neuroscience *118*, 389-394.

Muhammad, A., Carroll, C., and Kolb, B. (2012). Stress during development alters dendritic morphology in the nucleus accumbens and prefrontal cortex. Neuroscience *216*, 103-109.

Mukherjee, S., Coque, L., Cao, J.L., Kumar, J., Chakravarty, S., Asaithamby, A., Graham, A., Gordon, E., Enwright, J.F., 3rd, DiLeone, R.J.*, et al.* (2010). Knockdown of Clock in the ventral tegmental area through RNA interference results in a mixed state of mania and depression-like behavior. Biological psychiatry *68*, 503-511.

Muschamp, J.W., Van't Veer, A., Parsegian, A., Gallo, M.S., Chen, M., Neve, R.L., Meloni, E.G., and Carlezon, W.A., Jr. (2011). Activation of CREB in the nucleus accumbens shell produces anhedonia and resistance to extinction of fear in rats. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 3095-3103.

Naleid, A.M., Grace, M.K., Cummings, D.E., and Levine, A.S. (2005). Ghrelin induces feeding in the mesolimbic reward pathway between the ventral tegmental area and the nucleus accumbens. Peptides *26*, 2274-2279.

Nazarian, A., Russo, S.J., Festa, E.D., Kraish, M., and Quinones-Jenab, V. (2004). The role of D1 and D2 receptors in the cocaine conditioned place preference of male and female rats. Brain research bulletin *63*, 295-299.

Nemeroff, C.B. (2003). Anxiolytics: past, present, and future agents. The Journal of clinical psychiatry *64 Suppl 3*, 3-6.

Nestler, E.J., Barrot, M., DiLeone, R.J., Eisch, A.J., Gold, S.J., and Monteggia, L.M. (2002). Neurobiology of depression. Neuron *34*, 13-25.

Nestler, E.J., and Carlezon, W.A., Jr. (2006). The mesolimbic dopamine reward circuit in depression. Biological psychiatry *59*, 1151-1159.

Nocjar, C., and Panksepp, J. (2002). Chronic intermittent amphetamine pretreatment enhances future appetitive behavior for drug- and natural-reward: interaction with environmental variables. Behavioural brain research *128*, 189-203.

O'Brien, C.P., Childress, A.R., Ehrman, R., and Robbins, S.J. (1998). Conditioning factors in drug abuse: can they explain compulsion? Journal of psychopharmacology *12*, 15-22.

Ochi, M., Tominaga, K., Tanaka, F., Tanigawa, T., Shiba, M., Watanabe, T., Fujiwara, Y., Oshitani, N., Higuchi, K., and Arakawa, T. (2008). Effect of chronic

stress on gastric emptying and plasma ghrelin levels in rats. Life sciences *82*, 862-868.

Palmiter, R.D. (2007). Is dopamine a physiologically relevant mediator of feeding behavior? Trends in neurosciences *30*, 375-381.

Pandey, S.C., Zhang, H., Ugale, R., Prakash, A., Xu, T., and Misra, K. (2008). Effector immediate-early gene arc in the amygdala plays a critical role in alcoholism. The Journal of neuroscience : the official journal of the Society for Neuroscience *28*, 2589-2600.

Panja, D., Dagyte, G., Bidinosti, M., Wibrand, K., Kristiansen, A.M., Sonenberg, N., and Bramham, C.R. (2009). Novel translational control in Arc-dependent long term potentiation consolidation in vivo. The Journal of biological chemistry *284*, 31498-31511.

Papakostas, G.I., Petersen, T., Iosifescu, D.V., Burns, A.M., Nierenberg, A.A., Alpert, J.E., Rosenbaum, J.F., and Fava, M. (2005). Obesity among outpatients with major depressive disorder. Int J Neuropsychopharmacol *8*, 59-63.

Park, S., Park, J.M., Kim, S., Kim, J.A., Shepherd, J.D., Smith-Hicks, C.L., Chowdhury, S., Kaufmann, W., Kuhl, D., Ryazanov, A.G.*, et al.* (2008). Elongation factor 2 and fragile X mental retardation protein control the dynamic translation of Arc/Arg3.1 essential for mGluR-LTD. Neuron *59*, 70-83.

Pascoli, V., Terrier, J., Espallergues, J., Valjent, E., O'Connor, E.C., and Luscher, C. (2014). Contrasting forms of cocaine-evoked plasticity control components of relapse. Nature *509*, 459-464.

Patterson, Z.R., Ducharme, R., Anisman, H., and Abizaid, A. (2010). Altered metabolic and neurochemical responses to chronic unpredictable stressors in ghrelin receptor-deficient mice. The European journal of neuroscience *32*, 632-639.

Paulson, P.E., Camp, D.M., and Robinson, T.E. (1991). Time course of transient behavioral depression and persistent behavioral sensitization in relation to regional brain monoamine concentrations during amphetamine withdrawal in rats. Psychopharmacology *103*, 480-492.

Peebles, C.L., Yoo, J., Thwin, M.T., Palop, J.J., Noebels, J.L., and Finkbeiner, S. (2010). Arc regulates spine morphology and maintains network stability in vivo. Proceedings of the National Academy of Sciences of the United States of America *107*, 18173-18178.

Perello, M., Chuang, J.C., Scott, M.M., and Lutter, M. (2010). Translational neuroscience approaches to hyperphagia. The Journal of neuroscience : the official journal of the Society for Neuroscience *30*, 11549-11554.

Perkonigg, A., Owashi, T., Stein, M.B., Kirschbaum, C., and Wittchen, H.U. (2009). Posttraumatic stress disorder and obesity: evidence for a risk association. Am J Prev Med *36*, 1-8.

Perrotti, L.I., Hadeishi, Y., Ulery, P.G., Barrot, M., Monteggia, L., Duman, R.S., and Nestler, E.J. (2004). Induction of deltaFosB in reward-related brain structures after chronic stress. The Journal of neuroscience : the official journal of the Society for Neuroscience *24*, 10594-10602.

Petit-Demouliere, B., Chenu, F., and Bourin, M. (2005). Forced swimming test in mice: a review of antidepressant activity. Psychopharmacology *177*, 245-255.

Pierce, R.C., Bell, K., Duffy, P., and Kalivas, P.W. (1996). Repeated cocaine augments excitatory amino acid transmission in the nucleus accumbens only in rats having developed behavioral sensitization. The Journal of neuroscience : the official journal of the Society for Neuroscience *16*, 1550-1560.

Pittenger, C., and Duman, R.S. (2008). Stress, depression, and neuroplasticity: a convergence of mechanisms. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *33*, 88-109.

Plath, N., Ohana, O., Dammermann, B., Errington, M.L., Schmitz, D., Gross, C., Mao, X., Engelsberg, A., Mahlke, C., Welzl, H.*, et al.* (2006). Arc/Arg3.1 is essential for the consolidation of synaptic plasticity and memories. Neuron *52*, 437-444.

Pliakas, A.M., Carlson, R.R., Neve, R.L., Konradi, C., Nestler, E.J., and Carlezon, W.A., Jr. (2001). Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens. The Journal of neuroscience : the official journal of the Society for Neuroscience *21*, 7397-7403.

Ploski, J.E., Pierre, V.J., Smucny, J., Park, K., Monsey, M.S., Overeem, K.A., and Schafe, G.E. (2008). The activity-regulated cytoskeletal-associated protein (Arc/Arg3.1) is required for memory consolidation of pavlovian fear conditioning in the lateral amygdala. The Journal of neuroscience : the official journal of the Society for Neuroscience *28*, 12383-12395.

Porsolt, R.D., Anton, G., Blavet, N., and Jalfre, M. (1978). Behavioural despair in rats: a new model sensitive to antidepressant treatments. European journal of pharmacology *47*, 379-391.

Porsolt, R.D., Le Pichon, M., and Jalfre, M. (1977). Depression: a new animal model sensitive to antidepressant treatments. Nature *266*, 730-732.

Primeaux, S.D., Wilson, S.P., Cusick, M.C., York, D.A., and Wilson, M.A. (2005). Effects of altered amygdalar neuropeptide Y expression on anxiety-related behaviors. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *30*, 1589-1597.

Rajkowska, G. (2000). Postmortem studies in mood disorders indicate altered numbers of neurons and glial cells. Biological psychiatry *48*, 766-777.

Rajkowska, G., Miguel-Hidalgo, J.J., Wei, J., Dilley, G., Pittman, S.D., Meltzer, H.Y., Overholser, J.C., Roth, B.L., and Stockmeier, C.A. (1999). Morphometric evidence for neuronal and glial prefrontal cell pathology in major depression. Biological psychiatry *45*, 1085-1098.

Ralph, R.J., Paulus, M.P., Fumagalli, F., Caron, M.G., and Geyer, M.A. (2001). Prepulse inhibition deficits and perseverative motor patterns in dopamine transporter knock-out mice: differential effects of D1 and D2 receptor antagonists. The Journal of neuroscience : the official journal of the Society for Neuroscience *21*, 305-313.

Ramamoorthi, K., Fropf, R., Belfort, G.M., Fitzmaurice, H.L., McKinney, R.M., Neve, R.L., Otto, T., and Lin, Y. (2011). Npas4 regulates a transcriptional program in CA3 required for contextual memory formation. Science *334*, 1669-1675.

Rao, V.R., Pintchovski, S.A., Chin, J., Peebles, C.L., Mitra, S., and Finkbeiner, S. (2006). AMPA receptors regulate transcription of the plasticity-related immediate-early gene Arc. Nature neuroscience *9*, 887-895.

Renthal, W., Maze, I., Krishnan, V., Covington, H.E., 3rd, Xiao, G., Kumar, A., Russo, S.J., Graham, A., Tsankova, N., Kippin, T.E.*, et al.* (2007). Histone deacetylase 5 epigenetically controls behavioral adaptations to chronic emotional stimuli. Neuron *56*, 517-529.

Ressler, K.J., and Mayberg, H.S. (2007). Targeting abnormal neural circuits in mood and anxiety disorders: from the laboratory to the clinic. Nature neuroscience *10*, 1116-1124.

Rial Verde, E.M., Lee-Osbourne, J., Worley, P.F., Malinow, R., and Cline, H.T. (2006). Increased expression of the immediate-early gene arc/arg3.1 reduces AMPA receptor-mediated synaptic transmission. Neuron *52*, 461-474.

Rivera, M., Cohen-Woods, S., Kapur, K., Breen, G., Ng, M.Y., Butler, A.W., Craddock, N., Gill, M., Korszun, A., Maier, W.*, et al.* (2011). Depressive disorder moderates the effect of the FTO gene on body mass index. Mol Psychiatry.

Robinson, T.E., and Berridge, K.C. (1993). The neural basis of drug craving: an incentive-sensitization theory of addiction. Brain research Brain research reviews *18*, 247-291.

Robinson, T.E., and Berridge, K.C. (2000). The psychology and neurobiology of addiction: an incentive-sensitization view. Addiction *95 Suppl 2*, S91-117.

Robinson, T.E., and Kolb, B. (1999). Alterations in the morphology of dendrites and dendritic spines in the nucleus accumbens and prefrontal cortex following repeated treatment with amphetamine or cocaine. The European journal of neuroscience *11*, 1598-1604.

Rodgers, R.J. (1997). Animal models of 'anxiety': where next? Behavioural pharmacology *8*, 477-496; discussion 497-504.

Rodgers, R.J., Cao, B.J., Dalvi, A., and Holmes, A. (1997). Animal models of anxiety: an ethological perspective. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica [et al] *30*, 289-304.

Rouach, V., Bloch, M., Rosenberg, N., Gilad, S., Limor, R., Stern, N., and Greenman, Y. (2007). The acute ghrelin response to a psychological stress challenge does not predict the post-stress urge to eat. Psychoneuroendocrinology *32*, 693-702.

Roybal, K., Theobold, D., Graham, A., DiNieri, J.A., Russo, S.J., Krishnan, V., Chakravarty, S., Peevey, J., Oehrlein, N., Birnbaum, S.*, et al.* (2007). Mania-like behavior induced by disruption of CLOCK. Proceedings of the National Academy of Sciences of the United States of America *104*, 6406-6411.

Russo, S.J., Wilkinson, M.B., Mazei-Robison, M.S., Dietz, D.M., Maze, I., Krishnan, V., Renthal, W., Graham, A., Birnbaum, S.G., Green, T.A.*, et al.* (2009). Nuclear factor kappa B signaling regulates neuronal morphology and cocaine reward. The Journal of neuroscience : the official journal of the Society for Neuroscience *29*, 3529-3537.

Sanacora, G., Zarate, C.A., Krystal, J.H., and Manji, H.K. (2008). Targeting the glutamatergic system to develop novel, improved therapeutics for mood disorders. Nature reviews Drug discovery *7*, 426-437.

Santarelli, L., Saxe, M., Gross, C., Surget, A., Battaglia, F., Dulawa, S., Weisstaub, N., Lee, J., Duman, R., Arancio, O*., et al.* (2003). Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science *301*, 805-809.

Schindler, A.G., Li, S., and Chavkin, C. (2010). Behavioral stress may increase the rewarding valence of cocaine-associated cues through a dynorphin/kappa-opioid receptor-mediated mechanism without affecting associative learning or memory retrieval mechanisms. Neuropsychopharmacology *35*, 1932-1942.

Schlaepfer, T.E., Cohen, M.X., Frick, C., Kosel, M., Brodesser, D., Axmacher, N., Joe, A.Y., Kreft, M., Lenartz, D., and Sturm, V. (2008). Deep brain stimulation to reward circuitry alleviates anhedonia in refractory major depression. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *33*, 368-377.

Schultz, W., Apicella, P., and Ljungberg, T. (1993). Responses of monkey dopamine neurons to reward and conditioned stimuli during successive steps of learning a delayed response task. The Journal of neuroscience : the official journal of the Society for Neuroscience *13*, 900-913.

Scott, K.M., McGee, M.A., Wells, J.E., and Oakley Browne, M.A. (2008). Obesity and mental disorders in the adult general population. J Psychosom Res *64*, 97-105.

Sellings, L.H., McQuade, L.E., and Clarke, P.B. (2006). Evidence for multiple sites within rat ventral striatum mediating cocaine-conditioned place preference and locomotor activation. The Journal of pharmacology and experimental therapeutics *317*, 1178-1187.

Serretti, A., and Mandelli, L. (2010). Antidepressants and body weight: a comprehensive review and meta-analysis. J Clin Psychiatry *71*, 1259-1272.

Sesack, S.R., and Grace, A.A. (2010). Cortico-Basal Ganglia reward network: microcircuitry. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *35*, 27-47.

Shepherd, J.D., Rumbaugh, G., Wu, J., Chowdhury, S., Plath, N., Kuhl, D., Huganir, R.L., and Worley, P.F. (2006). Arc/Arg3.1 mediates homeostatic synaptic scaling of AMPA receptors. Neuron *52*, 475-484.

Smith, L.N., Jedynak, J.P., Fontenot, M.R., Hale, C.F., Dietz, K.C., Taniguchi, M., Thomas, F.S., Zirlin, B.C., Birnbaum, S.G., Huber, K.M.*, et al.* (2014). Fragile x mental retardation protein regulates synaptic and behavioral plasticity to repeated cocaine administration. Neuron *82*, 645-658.

Steffens, D.C., McQuoid, D.R., Welsh-Bohmer, K.A., and Krishnan, K.R. (2003). Left orbital frontal cortex volume and performance on the benton visual retention test in older depressives and controls. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *28*, 2179-2183.

Steward, O., and Worley, P.F. (2001). Selective targeting of newly synthesized Arc mRNA to active synapses requires NMDA receptor activation. Neuron *30*, 227-240.

Tamashiro, K.L., Hegeman, M.A., Nguyen, M.M., Melhorn, S.J., Ma, L.Y., Woods, S.C., and Sakai, R.R. (2007a). Dynamic body weight and body composition changes in response to subordination stress. Physiology & behavior *91*, 440-448.

Tamashiro, K.L., Hegeman, M.A., and Sakai, R.R. (2006). Chronic social stress in a changing dietary environment. Physiology & behavior *89*, 536-542.

Tamashiro, K.L., Nguyen, M.M., Fujikawa, T., Xu, T., Yun Ma, L., Woods, S.C., and Sakai, R.R. (2004). Metabolic and endocrine consequences of social stress in a visible burrow system. Physiology & behavior *80*, 683-693.

Tamashiro, K.L., Nguyen, M.M., Ostrander, M.M., Gardner, S.R., Ma, L.Y., Woods, S.C., and Sakai, R.R. (2007b). Social stress and recovery: Implications for body weight and body composition. American journal of physiology.

Tamashiro, K.L., Nguyen, M.M., and Sakai, R.R. (2005). Social stress: from rodents to primates. Frontiers in neuroendocrinology *26*, 27-40.

Tan, A., Moratalla, R., Lyford, G.L., Worley, P., and Graybiel, A.M. (2000). The activity-regulated cytoskeletal-associated protein arc is expressed in different striosome-matrix patterns following exposure to amphetamine and cocaine. Journal of neurochemistry *74*, 2074-2078.

Tanaka, K., Furuyashiki, T., Kitaoka, S., Senzai, Y., Imoto, Y., Segi-Nishida, E., Deguchi, Y., Breyer, R.M., Breyer, M.D., and Narumiya, S. (2012). Prostaglandin

E2-mediated attenuation of mesocortical dopaminergic pathway is critical for susceptibility to repeated social defeat stress in mice. The Journal of neuroscience : the official journal of the Society for Neuroscience *32*, 4319-4329.

Taniguchi, M., Carreira, M.B., Smith, L.N., Zirlin, B.C., Neve, R.L., and Cowan, C.W. (2012). Histone deacetylase 5 limits cocaine reward through cAMP-induced nuclear import. Neuron *73*, 108-120.

Taylor Tavares, J.V., Clark, L., Furey, M.L., Williams, G.B., Sahakian, B.J., and Drevets, W.C. (2008). Neural basis of abnormal response to negative feedback in unmedicated mood disorders. NeuroImage *42*, 1118-1126.

Teber, I., Kohling, R., Speckmann, E.J., Barnekow, A., and Kremerskothen, J. (2004). Muscarinic acetylcholine receptor stimulation induces expression of the activity-regulated cytoskeleton-associated gene (ARC). Brain research Molecular brain research *121*, 131-136.

Thomas, M.J., Beurrier, C., Bonci, A., and Malenka, R.C. (2001). Long-term depression in the nucleus accumbens: a neural correlate of behavioral sensitization to cocaine. Nature neuroscience *4*, 1217-1223.

Treadway, M.T., and Pizzagalli, D.A. (2014). Imaging the pathophysiology of major depressive disorder - from localist models to circuit-based analysis. Biology of mood & anxiety disorders *4*, 5.

Tritsch, N.X., Ding, J.B., and Sabatini, B.L. (2012). Dopaminergic neurons inhibit striatal output through non-canonical release of GABA. Nature *490*, 262-266.

Tsankova, N.M., Berton, O., Renthal, W., Kumar, A., Neve, R.L., and Nestler, E.J. (2006). Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. Nature neuroscience *9*, 519-525.

Tschop, M.H., Speakman, J.R., Arch, J.R., Auwerx, J., Bruning, J.C., Chan, L., Eckel, R.H., Farese, R.V., Jr., Galgani, J.E., Hambly, C.*, et al.* (2012). A guide to analysis of mouse energy metabolism. Nature methods *9*, 57-63.

Turrigiano, G.G., and Nelson, S.B. (2004). Homeostatic plasticity in the developing nervous system. Nature reviews Neuroscience *5*, 97-107.

Tye, K.M., Mirzabekov, J.J., Warden, M.R., Ferenczi, E.A., Tsai, H.C., Finkelstein, J., Kim, S.Y., Adhikari, A., Thompson, K.R., Andalman, A.S.*, et al.* (2013). Dopamine neurons modulate neural encoding and expression of depression-related behaviour. Nature *493*, 537-541.

Tye, K.M., Prakash, R., Kim, S.Y., Fenno, L.E., Grosenick, L., Zarabi, H., Thompson, K.R., Gradinaru, V., Ramakrishnan, C., and Deisseroth, K. (2011). Amygdala circuitry mediating reversible and bidirectional control of anxiety. Nature *471*, 358-362.

Ungless, M.A., Whistler, J.L., Malenka, R.C., and Bonci, A. (2001). Single cocaine exposure in vivo induces long-term potentiation in dopamine neurons. Nature *411*, 583-587.

Uranova, N.A., Vostrikov, V.M., Orlovskaya, D.D., and Rachmanova, V.I. (2004). Oligodendroglial density in the prefrontal cortex in schizophrenia and mood disorders: a study from the Stanley Neuropathology Consortium. Schizophrenia research *67*, 269-275.

Valenti, O., Lodge, D.J., and Grace, A.A. (2011). Aversive stimuli alter ventral tegmental area dopamine neuron activity via a common action in the ventral hippocampus. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 4280-4289.

Varga, J., Domokos, A., Barna, I., Jankord, R., Bagdy, G., and Zelena, D. (2011). Lack of vasopressin does not prevent the behavioural and endocrine changes induced by chronic unpredictable stress. Brain research bulletin *84*, 45-52.

Vialou, V., Robison, A.J., Laplant, Q.C., Covington, H.E., 3rd, Dietz, D.M., Ohnishi, Y.N., Mouzon, E., Rush, A.J., 3rd, Watts, E.L., Wallace, D.L.*, et al.* (2010). DeltaFosB in brain reward circuits mediates resilience to stress and antidepressant responses. Nature neuroscience *13*, 745-752.

Vieweg, W.V., Fernandez, A., Julius, D.A., Satterwhite, L., Benesek, J., Feuer, S.J., Oldham, R., and Pandurangi, A.K. (2006). Body mass index relates to males with posttraumatic stress disorder. J Natl Med Assoc *98*, 580-586.

Vieweg, W.V., Julius, D.A., Bates, J., Quinn, J.F., 3rd, Fernandez, A., Hasnain, M., and Pandurangi, A.K. (2007). Posttraumatic stress disorder as a risk factor for obesity among male military veterans. Acta Psychiatr Scand *116*, 483-487.

Volkow, N.D., Fowler, J.S., Wang, G.J., and Swanson, J.M. (2004). Dopamine in drug abuse and addiction: results from imaging studies and treatment implications. Molecular psychiatry *9*, 557-569.

Waelti, P., Dickinson, A., and Schultz, W. (2001). Dopamine responses comply with basic assumptions of formal learning theory. Nature *412*, 43-48.

Wallace, D.L., Han, M.H., Graham, D.L., Green, T.A., Vialou, V., Iniguez, S.D., Cao, J.L., Kirk, A., Chakravarty, S., Kumar, A.*, et al.* (2009). CREB regulation of nucleus accumbens excitability mediates social isolation-induced behavioral deficits. Nature neuroscience *12*, 200-209.

Wan, S., Browning, K.N., Coleman, F.H., Sutton, G., Zheng, H., Butler, A., Berthoud, H.R., and Travagli, R.A. (2008). Presynaptic melanocortin-4 receptors on vagal afferent fibers modulate the excitability of rat nucleus tractus solitarius neurons. J Neurosci *28*, 4957-4966.

Wang, K.H., Majewska, A., Schummers, J., Farley, B., Hu, C., Sur, M., and Tonegawa, S. (2006). In vivo two-photon imaging reveals a role of arc in enhancing orientation specificity in visual cortex. Cell *126*, 389-402.

Waung, M.W., Pfeiffer, B.E., Nosyreva, E.D., Ronesi, J.A., and Huber, K.M. (2008). Rapid translation of Arc/Arg3.1 selectively mediates mGluR-dependent LTD through persistent increases in AMPAR endocytosis rate. Neuron *59*, 84-97.

Wilkerson, J.R., Tsai, N.P., Maksimova, M.A., Wu, H., Cabalo, N.P., Loerwald, K.W., Dictenberg, J.B., Gibson, J.R., and Huber, K.M. (2014). A Role for Dendritic mGluR5-Mediated Local Translation of Arc/Arg3.1 in MEF2-Dependent Synapse Elimination. Cell reports *7*, 1589-1600.

Williams, D.L., and Schwartz, M.W. (2005). The melanocortin system as a central integrator of direct and indirect controls of food intake. American journal of physiology Regulatory, integrative and comparative physiology *289*, R2-3.

Wilson, D.R. (2010). Health consequences of childhood sexual abuse. Perspect Psychiatr Care *46*, 56-64.

Wolf, M.E. (2010). The Bermuda Triangle of cocaine-induced neuroadaptations. Trends in neurosciences *33*, 391-398.

Wolf, M.E., and Ferrario, C.R. (2010). AMPA receptor plasticity in the nucleus accumbens after repeated exposure to cocaine. Neuroscience and biobehavioral reviews *35*, 185-211.

Wong, M.L., and Licinio, J. (2004). From monoamines to genomic targets: a paradigm shift for drug discovery in depression. Nature reviews Drug discovery *3*, 136-151.

Yang, Y., Atasoy, D., Su, H.H., and Sternson, S.M. (2011). Hunger states switch a flip-flop memory circuit via a synaptic AMPK-dependent positive feedback loop. Cell *146*, 992-1003.

Ying, S.W., Futter, M., Rosenblum, K., Webber, M.J., Hunt, S.P., Bliss, T.V., and Bramham, C.R. (2002). Brain-derived neurotrophic factor induces long-term potentiation in intact adult hippocampus: requirement for ERK activation coupled to CREB and upregulation of Arc synthesis. The Journal of neuroscience : the official journal of the Society for Neuroscience *22*, 1532-1540.

Yu, T., Guo, M., Garza, J., Rendon, S., Sun, X.L., Zhang, W., and Lu, X.Y. (2011). Cognitive and neural correlates of depression-like behaviour in socially defeated mice: an animal model of depression with cognitive dysfunction. Int J Neuropsychopharmacol *14*, 303-317.

Yuen, E.Y., Wei, J., Liu, W., Zhong, P., Li, X., and Yan, Z. (2012). Repeated stress causes cognitive impairment by suppressing glutamate receptor expression and function in prefrontal cortex. Neuron *73*, 962-977.

Zachariou, V., Caldarone, B.J., Weathers-Lowin, A., George, T.P., Elsworth, J.D., Roth, R.H., Changeux, J.P., and Picciotto, M.R. (2001). Nicotine receptor inactivation decreases sensitivity to cocaine. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *24*, 576-589.

Zhang, C.L., McKinsey, T.A., Chang, S., Antos, C.L., Hill, J.A., and Olson, E.N. (2002). Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. Cell *110*, 479-488.

Zolotukhin, S., Byrne, B.J., Mason, E., Zolotukhin, I., Potter, M., Chesnut, K., Summerford, C., Samulski, R.J., and Muzyczka, N. (1999). Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. Gene therapy *6*, 973-985.