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Psychostimulant-induced plasticity of GIRK channel signaling in ventral tegmental area

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biology

by

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The Dissertation of Michaelanne Berry Munoz is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Dedication

This thesis is dedicated to those who suffer from mental illness and the family and friends that support them. It is my hope that this work helps serve as testament that there are people in the world working to help.

Table of Contents

List of Figures

Chapter V

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ABSTRACT OF THE DISSERTATION

Psychostimulant-induced plasticity of GIRK channel signaling

in ventral tegmental area

by

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Natural rewards and addictive drugs increase the release of dopamine (DA) in the brain's reward circuit by augmenting the activity of dopaminergic neurons in the ventral tegmental area (VTA). Exposure to drugs of abuse produces persistent adaptations in neural signaling, both excitatory and inhibitory. G protein-gated inwardly rectifying potassium (GIRK or Kir3) channels activate a slow inhibitory postsynaptic current in dopamine neurons mediated by $GABA_B$ and D2 receptors and are involved in the response to multiple drugs of abuse. How the psychostimulants methamphetamine or cocaine affect GIRK channel signaling, however, remains unclear. This thesis describes novel mechanisms of GIRK channel plasticity and regulation in VTA dopamine and GABA neurons in response to methamphetamine. Activation of dopamine neurons induces a biphasic response of $GABA_B$R-GIRK signaling in VTA, with

short-term drug exposure down-regulating $GABA_BR$-GIRK signaling in GABA neurons, increasing VTA inhibition, while repeated methamphetamine exposure down-regulates $GABA_BR$-GIRK currents in DA neurons through a mechanism involving GIRK3-containing channels, dis-inhibiting DA neurons. SNX27 is an endosomal trafficking protein that regulates the surface expression and signaling of GIRK3 channels and is inducible by repeated exposure to psychostimulants. Through conditional deletion of SNX27, I describe the first neuronal function of a sorting nexin *in vivo*, which is to regulate $GABA_BR$-GIRK signaling and inhibitory control of VTA DA neurons. Mice lacking SNX27 in DA neurons display hyperlocomotion in a novel environment and an enhanced sensitivity to the locomotor-stimulant effects of cocaine, suggesting that SNX27 regulation of GIRK channel signaling is involved in addictive behaviors. The data presented in this thesis thus identify novel mechanisms of psychostimulants-induced plasticity in the VTA and highlight SNX27 and GIRK channels as promising therapeutic targets for addiction and other complex psychiatric disorders.

I. Introduction

In the mammalian brain, ion channels allow selective flow of ions through specific pores in the membrane of neurons. These channels are key to the generation of excitability that transforms electrical activity into cognition and behavior. Not only are we able to understand our environment, we can learn, form memories, and adapt to new situations. In other words, experiencing the world changes us, and changes our brains in a physical way. Plasticity refers to the ability of neurons to selectively modify synaptic strength in response to excitatory input, and is believed to be the biological correlate of behavioral adaptations such as learning and memory (Citri and Malenka, 2008; Kandel, 1997). For example, movement of ionotropic AMPA receptors into and out of the synapse is required for plasticity to potentiate or depress the strength of excitatory synapses (Malinow and Malenka, 2002).

Being able to quickly learn what is rewarding or aversive, and to retain that information for future use, is imperative to an individual's survival. A relatively small number of neurons located in the ventral midbrain of mammals produce the neurotransmitter dopamine (DA) and are implicated in a variety of behaviors related to salience processing and reward learning (Schultz, 2007; Tritsch and Sabatini, 2012; White, 1996; Wise, 2004). Particularly, the subset of dopamine neurons found in the ventral tegmental area (VTA) is critical for control of motivation and incentive salience (Schultz, 2007; Wise, 2004). Dysfunction of VTA dopamine neurons have been implicated in a variety of psychiatric

disorders, including drug addiction (Koob and Volkow, 2009; Lüscher and Malenka, 2011; Robinson and Berridge, 1993), depression (Krishnan and Nestler, 2008; Tye et al., 2012), anxiety (Zweifel et al., 2011), psychosis (Robinson and Becker, 1986), schizophrenia (Pankow et al., 2012), obsessive-compulsive disorder (Tritsch and Sabatini, 2012) and Tourette syndrome (Buse et al., 2012). Elucidating the mechanisms of how ion channel trafficking shapes the activity of dopamine neurons offers the opportunity to understand the biology of reward processing and psychiatric disorders and open up new targets for improved treatment.

VTA Dopamine Neurons

The mesocoriticolimbic dopamine circuit comprises the VTA and its projections and target regions. The VTA is composed primarily of dopamine neurons (~65%), as well as GABA (~30%) and glutamatergic (~5%) neurons (Bonci and Malenka, 1999; Hearing et al., 2012; Johnson and North, 1992). VTA dopamine neurons project primarily to the nucleus accumbens or prefrontal cortex, and also to hippocampus, amygdala, and lateral hypothalamus (Kauer and Malenka, 2007b; Koob & Volkow, 2009 Nestler, 2001; Swanson, 1982). Glutamatergic projections of the medial prefrontal cortex (mPFC) and lateral habenula form the primary excitatory synapses onto VTA neurons that increase activity, and inhibitory input comes from local interneurons and GABAergic projections from the nucleus accumbens, ventral pallidum, and rostromedial

tegmental nucleus (Hyman et al., 2006; Jhou et al., 2009; Lammel et al., 2008; Lammel et al., 2012; Sugita et al., 1992; Tan et al., 2012). Dopamine neurons are heterogeneous, with subsets receiving input from and projecting to specific brain regions, and these unique circuits mediate different aspects of dopamine-dependent behaviors (Lammel et al., 2008; Lammel et al., 2011; Lammel et al., 2012; Margolis et al., 2006).

Dopamine neurons in the VTA are basally active, irregularly firing action potentials at low frequency, and transition to either a hyperpolarized, silent state in response to aversive stimuli, or a phasic bursting state in response to unexpected reward or reward-predicting cues, characterized by high firing frequency of action potentials for short durations (Grace and Bunney, 1983, 1984a, b; Grace et al., 2007; Schultz, 2007). Burst firing leads to the release of dopamine in projection targets (Overton and Clark, 1997; Tsai et al., 2009) and requires both excitatory and inhibitory activity, through NMDA receptor activation and released GABA-mediated inhibition (Floresco et al., 2003; Grace and Bunney, 1985; Seutin et al., 1994; Zweifel et al., 2008).

Dopamine release is tightly controlled in the mammalian brain, with a wide range of time course and duration that is believed to aid in the ability of dopamine to differentiation a plethora of behavior (Schultz, 2007). Primary regulation is an auto-feedback loop that silences neuronal activity, mediated by dopamine D2 receptors on dopamine neurons that detect release from adjacent neurons (Beckstead et al., 2004; Ford et al., 2010). Additionally, $GABA_B$ receptor activation is able to silence dopamine neurons and prevent burst firing when

GABA release is increased in the VTA (Kalivas and Stewart, 1991; Lacey et al., 1988; Seutin et al., 1991). $GABA_BR$ agonists prevent acquisition of behavioral response to psychostimulants (Brebner et al., 1999; Broadbent and Harless, 1999; Kalivas and Stewart, 1991; Lhuillier et al., 2006), and is in clinical trials as an anti-craving compound, presumably from its action on VTA dopamine neurons (Roberts, 2005).

$GABA_B$ and dopamine D2 G protein-coupled receptors (GPCRs) activate pertussis toxin-sensitive $G_{i/o}$ proteins, which postsynaptically open a potassium conductance mediated by G protein-gated inwardly rectifying potassium (GIRK or Kir3) channels, inhibit adenylyl cyclase (AC), and presynaptically inhibit Ca_V channels. GIRK channel activation produces a slow inhibitory postsynaptic current (sIPSC) in dopamine neurons that hyperpolarizes dopamine neurons and prevents firing (Beckstead et al., 2004; Cruz et al., 2004; Koyrakh, 2005; Lacey et al., 1988).

Kir3 Potassium Channels

In the mammalian brain, there are 2 primary groups of potassium channels, voltage-gated (K_V) and inwardly rectifying (K_{IR}; (Jan and Jan, 1997; Luján, 2010). Inward rectification refers to the preferential conductance of potassium at hyperpolarized potentials, such that there is a large current below E_K and a much smaller current above, due to channel blockage by magnesium and polyamines (Lüscher and Slesinger, 2010; Nishida and MacKinnon, 2002).

The Kir family has 7 members, and of particular importance to neural physiology are the Kir3 subtype, or GIRK channels. Kir3 channels are unique to this family in that they are gated directly by the βγ subunit of G proteins, hence G protein-gated inwardly rectifying potassium (GIRK) channel (Hille, 1992; Lüscher and Slesinger, 2010). GIRK channels were initially described in the heart, where their activation by the muscarinic acetylcholine GPCR is involved in slowing pacemaker activity (Kubo et al., 1993; Pfaffinger et al., 1985). A number of different GPCRs signaling through pertussis toxin-sensitive $G_{i/o}$ proteins are capable of activating GIRK channels (Hille, 1992; Lüscher and Slesinger, 2010), including M2 muscarinic, 5HT1A serotonergic, A1-purinergic, alpha-2 adrenergic, mu- and delta-opioid, somatostatin, $GABA_B$, and dopamine D2 receptors (Lacey et al., 1988; North and Uchimura, 1989).

There are 4 mammalian GIRK channels subtypes, with GIRK1-3 expressed in the brain and GIRK4 primarily expressed in heart (Dascal, 1997; Krapivinsky et al., 1995; Lesage et al., 1995; Nishida and MacKinnon, 2002). Channels are generally heterotetramers composed of GIRK1/GIRK2 in brain and GIRK1/4 in heart (Krapivinsky et al., 1995; Liao et al., 1996; Luscher et al., 1997). GIRK1 and GIRK3 are both obligate heterotetramers, generally assembling with GIRK2, while GIRK2 can form homotetramers (Ma et al., 2002; Schoots et al., 1999). There are 3 splice variants described for GIRK2, GIRK2a-c, with unique expression patterns and physiology in brain (Dascal, 1997; Inanobe et al., 1999; Lesage et al., 1995).

Functionally, GIRK channels are involved in slowing heart rate (Pfaffinger et al., 1985), and in the brain play a key role in maintaining resting potential and regulating slow inhibitory postsynaptic currents (Koyrakh, 2005; Luscher et al., 1997). Fast inhibition in neurons is generated by $GABA_A$ receptors in the postsynaptic density; perisynaptic GIRK channels on dendritic shaft and spines mediate slow inhibition. A high degree of GABA release results in spillover from the synapse to activate $GABA_B$R-GIRK complexes around the synaptic contact and on neighboring glutamatergic spines (Lüscher and Slesinger, 2010).

The role of GIRK channels in behavior was initially established by the *weaver* mutant mouse, which is characterized by Parkinsonian-like symptoms and defects in cerebellar granule cells (Signorini et al., 1997). *Weaver* was discovered to be a functional mutation of the GIRK2 subunit that produces a loss of potassium selectivity (Slesinger et al., 1996). Mice lacking GIRK subunits have a wide variety of behavioral deficits, such as increased seizure susceptibility (Signorini et al., 1997), hyperactivity (Y et al., 2002), reduced anxiety (Blednov et al., 2001a; Pravetoni and Wickman, 2008), and altered responses to drugs of abuse (Arora et al., 2010; Blednov et al., 2001b; Cruz et al., 2008; Marker, 2004; Morgan et al., 2002). The specific neuronal populations that mediate these behaviors, however, have yet to be explored.

Initial descriptions of GIRK channels in the brain identified dopamine neurons of the substantia nigra and VTA as unique in channel composition (Inanobe et al., 1999; Karschin et al., 1996). While most neuronal GIRK channels are GIRK1/2 heterotetramers, dopamine neurons do not express GIRK1 subunits

and are instead composed primarily of GIRK2c in the substantia nigra and GIRK2c or GIRK2c/3 subunits in the VTA (Cruz et al., 2004; Inanobe et al., 1999; Koyrakh, 2005). Subunit-specific expression has implications in reward processing induced by $GABA_B$ receptor activation. The club drug γ-hydroxybutyrate (GHB), which is highly addictive, selectively activates GIRK1/2 channels in GABA neurons, while baclofen, also a $GABA_BR$ agonist but reported as anti-craving, activates GIRK2/3 subunits on DA neurons. Through differential coupling of GIRK subunits to RGS2, GHB preferentially inhibits GABA neurons, dis-inhibiting DA neurons, while baclofen, acting on DA and GABA neurons, inhibits dopamine activity and prevents reward sensation (Cruz et al., 2004; Labouèbe et al., 2007).

In addition to GHB, morphine and other opioids bind to opioid receptors and activate GIRK channels on GABA neurons, thus dis-inhibiting dopamine neurons to promote bursting (Fiorillo and Williams, 1998; Johnson and North, 1992). Studies have identified polymorphisms in the human GIRK2 gene as involved in opioid analgesia and addiction (Lötsch et al., 2010; Nishizawa et al., 2009). Ethanol binds directly to GIRK channels and increases their activation (Aryal et al., 2009), which, in addition to a number of other receptors, mediates some of its effects *in vivo* (Blednov et al., 2001b; Luscher and Ungless, 2006). Knockout studies have also identified a role of GIRK channels in psychostimulant response (Morgan et al., 2002), but the mechanism is unclear.

Kir3 Trafficking

Distinct protein motifs and phosphorylation sites on GIRK subunits regulate channel trafficking. GIRK2 contributes most prominently to efficient GIRK channel surface expression, as GIRK2a-c subunits have forward trafficking and ER export motifs, and a post-Golgi surface-promoting motif that is regulated by excitatory activity (Chung et al., 2008b; Ma et al., 2002). NMDA stimulation leads to de-phosphorylation of serine-9 by protein phosphatase-1 (PP1) on the GIRK2 subunits, leading to increased surface expression and currents of GIRK2-containing channels (Chung et al., 2008a). GIRK1 and GIRK3 lack forwarding motifs and reside exclusively in the ER when expressed alone, likely why both subunits are obligate heterotetramers with GIRK2 (Dascal, 1997; Ma et al., 2002). Negative regulation of GIRK channels is mediated by a strong lysosomal targeting signal in GIRK3 subunits and an internalization motif in GIRK2a-c (Lunn et al., 2007; Ma et al., 2002).

GIRK channel trafficking is additionally regulated by the presence of a class I PDZ-binding motif *–ESKV* on GIRK2c and GIRK3 subunits interacts with the PDZ domain of sorting nexin 27 (SNX27; (Balana et al., 2011; Inanobe et al., 1999; Lunn et al., 2007). SNX27 binding to GIRK3-containing channels causes decreased surface expression and reduced functional currents activated by $GABA_B$ or M2 muscarinic receptors. Diminished current correlates with a reduction of GIRK1 protein (Lunn et al., 2007). GIRK1 associates with GIRK3 but not with SNX27, suggesting that GIRK3-SNX27 interaction promotes lysosomal degradation of GIRK3-containing channels (Lunn et al., 2007; Nassirpour and

Slesinger, 2007). Protein motifs present on GIRK subunits are able to dramatically alter the regulation of functional currents in cultured neurons, though how trafficking is regulated *in vivo* remains largely unexplored.

Sorting nexins and SNX27

Sorting nexins are a family of endosomal trafficking proteins defined by the presence of a phox-homology (PX) domain that binds to phosphatidylinositol-3-monophosphate (PI3P) in early endosomes (EE) (Carlton et al., 2005; Teasdale and Collins, 2012; Worby and Dixon, 2002). An NCBI search identified 33 mammalian sorting nexins, a number that has increased as additional family members are identified (Cullen and Korswagen, 2011; Worby and Dixon, 2002). Sorting nexins were initially described as lysosomal sorting proteins (Kurten et al., 1996; Worby and Dixon, 2002), however, the recent discovery that many sorting nexins associate with the retromer complex has uncovered additional roles in mediating retrograde transport from endosomes to trans-Golgi network for recycling (Cullen and Korswagen, 2011; Temkin et al., 2011). Whether the same sorting nexin can mediate both recycling and degradation in the EE, or if specific protein-protein interactions on different sorting nexins determine their function, remains to be determined.

SNX27 is unique in its family in that it is the only sorting nexin to contain a PDZ domain (PDZ = post synaptic density (PSD)-95, discs large, zona occludens), which is critical for its sorting abilities (Lauffer et al., 2010; Lunn et

al., 2007). Additionally, SNX27 has a Ras-associated (RA) or FERM-like domain that associates with H-ras and can thus mediate the formation of signaling complexes in early endosomes (Ghai and Collins, 2011; Ghai et al., 2011). There are 2 splice variants of SNX27, a and b, that differ at the last 15 amino acids of the C-terminal and the 5'UTR, and it has been suggested that the splice variants represent constitutive and inducible forms, respectively (Fujiyama et al., 2003; Kajii et al., 2003).

SNX27 has been implicated in both recycling and degradation pathways. For example, SNX27 binds to the β2-adrenergic receptor through a PDZ interaction, and associates the receptor with retromer, directing membrane recycling from EE (Lauffer et al., 2010; Temkin et al., 2011). Interaction of SNX27 with GIRK3-containing channels, 5HT-4a serotonergic receptors, NMDA2c receptors, or multidrug resistance-associated protein 4 (MRP4), a cAMP-binding protein, results in increased lysosomal degradation (Cai et al., 2011; Hayashi et al., 2012; Joubert, 2004; Lunn et al., 2007a). Additional binding partners include diacylglycerol zeta in T cells (Rincon et al., 2011) and CASP in lymphocytes (MacNeil et al., 2007).

The role of sorting nexins *in vivo* is only just beginning to emerge. This is complicated by the ubiquitous expression of sorting nexins in mammalian cells and the critical role of endocytic regulation in many cellular processes, including development. Genetic deletion of sorting nexins tends to result in embryonic or early postnatal lethality (Cai et al., 2011; Schwarz, 2002; Zheng et al., 2006). Expression analysis in the nervous system reveals that sorting nexins are

expressed in both neurons and glia and are often developmentally regulated (Cai et al., 2011; Kajii et al., 2003; Lunn et al., 2007a; Mizutani et al., 2012; Rodal et al., 2011; Shin et al., 2007; Zhao et al., 2012). In addition, expression of SNX27 can be induced by drugs of abuse (Fujiyama et al., 2003; Kajii et al., 2003). However, the functional role of sorting nexins in neurons has not been elucidated. In this thesis, I will describe the role of SNX27 in regulating GABAergic inhibition in VTA dopamine neurons and addiction-mediated behaviors.

VTA Dopamine Neurons in Psychostimulant Addiction

Drugs of abuse induce persistent adaptations in VTA dopamine circuitry that is believed to represent a form of aberrant learning, leading to the development of addictive behaviors (Di Chiara, 1999; Di Chiara et al., 1999; Kauer and Malenka, 2007b; Schultz, 2011). Exposure to drugs of abuse produce a wide variety of cellular adaptations in regions of the mesocorticolimbic dopamine circuit, from epigenetic (Renthal and Nestler, 2008), neurotrophic growth factors, microRNAs (Jonkman and Kenny, 2012; Russo et al., 2009), and ion channel trafficking (Kalivas, 2009; Kauer and Malenka, 2007a). Indeed, dopamine neurons are highly adaptive; a single exposure to many drugs of abuse can induce LTP (Saal et al., 2003; Ungless et al., 2001). Insertion of GluR2-lacking AMPA receptors following cocaine administration is critical to the

induction of LTP in dopamine neurons (Kauer and Malenka, 2007; Mameli et al., 2011).

In addition to excitatory transmission, drug-induced adaptations occur in inhibitory regulation, either on GABA neurons or dopamine neurons in the mesocorticolimbic circuitry (Koob, 2004; Luo et al., 2010; Niehaus et al., 2010; Rahman et al., 2003). Psychostimulant exposure decreases $GABA_A$ receptor-induced fast IPSCs to facilitate LTP in dopamine neurons and social deprivation alters metabotropic glutamate receptors (mGluRs), inducing LTP and affecting conditioned place preference to amphetamine (Liu et al., 2005; Whitaker et al., 2013). Importantly, repeated psychostimulant administration weakens negative autocrine and long-loop feedback (Hearing et al., 2012; White, 1990; White et al., 1990), which suggest plasticity and dysregulation of inhibitory control by drugs of abuse.

$G_{i/o}$ signaling, that mediates much of the negative feedback in VTA, is implicated in drug-induced plasticity (Hearing et al., 2012). Impairing $G_{i/o}$ signaling via pertussis toxin impairs cocaine sensitization when injected into the VTA (Steketee and Kalivas, 1991). G protein activity is also implicated; levels of RGS9-2, a GTPase-activating protein, are increased in nucleus accumbens, while G protein regulator AGS3, which binds $Gi\alpha$-GDP, is up-regulated in prefrontal cortex following cocaine withdrawal (Bowers et al., 2004).

GIRK channels are the effectors of $G_{i/o}$ GPCR signaling and are involved in the response to a variety of different drugs of abuse. For instance, cocaine, which blocks dopamine re-uptake by binding to the dopamine transporter (DAT;

(Ritz et al., 1987), is self-administered less in animals lacking GIRK subunits (Morgan et al., 2002). GIRK channels in drug response have been implicated through the activation of stress hormones, which are critical for the transition from casual to compulsive drug use (Koob, 2008, 2010; Logrip et al., 2011). Corticotropin releasing factor (CRF), a stress-induced neuropeptide, acutely increases the sIPSC mediated by GIRK channels in dopamine neurons. Repeated exposure to stress or psychostimulants blunts the ability of CRF to enhance the sIPSC in dopamine neurons (Beckstead et al., 2009). However, the direct effects of psychostimulants on GIRK channel activity in the VTA and functional mechanisms of inhibitory plasticity involving $G_{i/o}$ GPCR signaling remain largely unexplored.

Interestingly, SNX27 was initially described as mrt1, *m*ethamphetamine *r*esponsive *t*ranscript 1, a developmentally regulated transcript (Fujiyama et al., 2003; Kajii et al., 2003). SNX27b splice variant is inducible following repeated methamphetamine or cocaine, increasing SNX27b expression rodent cortex (Kajii et al., 2003). If SNX27 expression is increased following psychostimulant exposure in the mesocorticolimbic dopamine system, we would hypothesize that trafficking of GIRK3-containing channels will be altered in these neurons, affecting inhibitory feedback. As VTA dopamine neurons contain only GIRK2c and GIRK3 subunits, both of which interact with SNX27 through its PDZ domain, altered SNX27 expression in dopaminergic neurons is likely to have dramatic effects on inhibitory control and behavioral response to drugs of abuse.

In this thesis, I present evidence for mechanisms of GIRK channel signaling plasticity in the VTA. Using a selective deletion of SNX27 from dopamine neurons, I found that SNX27 traffics GIRK3-containing channels to regulate the ability of dopamine neurons response to inhibitory input. Chapters 2 and 3 describe how methamphetamine treatment acutely down-regulates GIRK channels in VTA GABA neurons, while repeated exposure alters GIRK channels in dopamine neurons through a distinct mechanism requiring GIRK3 subunits and environmental novelty. In Chapter 4, I examine the effect of SNX27 deletion in dopamine neurons in addictive behaviors. Together, these findings expand the role of GIRK channel regulation and plasticity *in vivo*, and will hopefully advance the understanding of the neurobiology of behavior and development of new treatments for psychiatric disorders.

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II. Sorting Nexin 27 mediates slow GABAergic inhibition in VTA dopamine neurons

Abstract

Natural rewards and addictive drugs increase the release of dopamine (DA) in the reward circuit by augmenting the activity of dopaminergic neurons in the ventral tegmental area (VTA). Activation of G protein-gated inwardly rectifying potassium (GIRK or Kir3) channels produces a slow inhibitory postsynaptic current mediated by $GABA_B$ and dopamine D2 receptors that provides an essential inhibitory signal in the control of DA neuron excitability. Sorting nexin 27 (SNX27) regulates the surface expression and functional activity of GIRK channels through a PDZ-domain interaction. Dopamine neurons express only PDZ-binding GIRK subunits; thus we generated a conditional mutant with selective deletion of SNX27 in midbrain dopamine neurons. Here we report that SNX27-directed trafficking of GIRK channel regulates the ability of dopamine neurons to respond to GABA-mediated inhibition. A loss of slow inhibitory control occurs through a decreased number of functional GIRK channels gated by $GABA_B$ receptors, as auto-inhibitory D2 receptor-activated GIRK currents are spared. These data identify a novel pathway for inhibitory regulation involving SNX27 and GIRK channels in VTA DA neurons and highlight SNX27 as a

promising therapeutic target for addiction and other complex psychiatric disorders.

Introduction

Dopamine is a critical regulator of incentive salience, encoding both rewarding and aversive responses *in vivo*, and has been implicated in a variety of psychiatric disorders such as addiction, schizophrenia and depression (Lammel et al., 2012; Schultz, 2007, 2012; Tsai et al., 2009; Wise, 2004). Altered inhibitory signaling in dopamine neurons is sufficient to produce dramatic changes in neuronal activity and behavior (Barrot et al., 2012; Kalivas and Hu, 2006; Koob, 2004; Liu et al., 2005).

G-protein-gated inwardly rectifying potassium (GIRK) channels underlie a slow inhibitory postsynaptic current (sIPSC) in midbrain dopamine neurons activated by $GABA_B$ or dopamine D2 receptors that is sufficient to silence neuronal activity (Beckstead et al., 2004; Johnson and North, 1992; Lacey et al., 1988). GIRK channels in neurons are composed of GIRK1-3 subunits (Dascal, 1997; Lesage et al., 1995; Lüscher and Slesinger, 2010). Dopaminergic neurons specifically express only GIRK2 and -3 subunits (Cruz et al., 2004; Davila et al., 2003). Genetic deletion of the GIRK2 subunit leads to loss of sIPSC in dopamine neurons and altered reward-related behaviors (Beckstead et al., 2004; Morgan et al., 2002; Signorini et al., 1997), suggesting an important role for the GIRK channels in motivational processing in vivo.

GIRK channels are dynamically regulated in neurons in response to excitatory stimulation (Chung et al., 2008a; Chung et al., 2008b). The different GIRK subunits provide unique contributions to this trafficking (Ma et al., 2002).

GIRK2c and GIRK3 contain a class I PDZ-binding motif, which interacts directly with the early endosomal protein sorting nexin 27 (SNX27) (Lunn et al., 2007b). SNX27 regulates cell surface expression and receptor-activated GIRK currents of GIRK3-containing channels (Balana et al., 2011; Cruz et al., 2004). SNX27 is expressed in neurons (Balana et al., 2011; Cai et al., 2011), though its role in GIRK channel trafficking *in vivo* has yet to be elucidated.

Based on the specific expression of SNX27-binding GIRK channel subunits GIRK2c and GIRK3 in dopaminergic neurons, we hypothesized that GIRK channel trafficking by SNX27 would have a significant impact on dopamine neuron physiology and reward-related behavior. Here we report the generation of a conditional knockout of SNX27 specifically in dopaminergic neurons. Deletion of SNX27 led to a dramatic reduction of slow GABAergic signaling in ventral tegmental dopamine neurons and a concurrent increase in neuronal excitability, both of which can be restored by selective expression of a non-SNX27-interacting GIRK channel subunit. Behaviorally, reduced GABAergic inhibition led to altered novelty detection. Thus, SNX27 trafficking of GIRK channels represents a selective target of dopamine neuron excitability and may aid in elucidation of novel treatments for a number of different psychiatric disorders.

Results

SNX27b transcript is selectively increased in VTA following repeated methamphetamine administration

SNX27 expression in dopamine neurons was first confirmed by immunohistochemistry using a pan SNX27 antibody that detects both splice variants (**Fig. 2.1A**). To specifically investigate the psychostimulant-inducible SNX27b transcript, we extracted RNA from tissue punches of the mesocorticolimbic DA circuit: prefrontal cortex (PFC), hippocampus, nucleus accumbens (NAc) and VTA. SNX27b variant is basally expressed in all brain regions, with TH expression only in VTA, demonstrating specificity of punches (**Fig. 2.1B**).

We next examined change in SNX27b transcript expression in these brain regions following various methamphetamine-dosing regimens with qRT-PCR. Mice were treated either acutely or repeatedly with 2mg/kg methamphetamine and samples taken 24 hr after the last of administration. SNX27b expression is increased nearly 150% specifically in VTA after repeated exposure to methamphetamine with no change in other brain regions (**Fig. 2.1C**). This implicates SNX27 in drug-induced adaptations in VTA, potentially through GIRK channel trafficking.

Genetic deletion of SNX27 in vivo

We generated a pre-conditional allele of SNX27 ($SNX27^{fl/fl}$) from animals

obtained from the Knockout Mouse Project (www.komp.org) (Collins et al., 2007; Skarnes et al., 2011). SNX27 knockout-first constructs allow generation of either whole animal or pre-conditional mutants (Collins et al., 2007). Breeding knockout-first heterozygotes failed to generate any $SNX27^{-/-}$ animals, however, embryos at ~E15 were at the expected Mendelian ratios. Homozygous embryos were phenotypically indistinguishable from WT embryos, suggesting that development proceeds normally in the absence of SNX27 and observed lethality occurs peri-natally. This is in contrast to a previously described mouse line with a global deletion of SNX27 in a mixed S129/C57Bl6 background, where animals were born but failed to thrive and died prior to reaching adulthood (Cai et al., 2011). Our pre-conditional line was generated using B6 ES cells (Pettitt et al. 2009) and maintained on a B6 background, suggesting that genetic background affects survival rate of SNX27 mutants. The lack of homozygous knockout-first animals was confirmed by independent breeding of the same construct at the KOMP facility. These mutants underscore the critical role of SNX27 in basic cellular function and highlight the importance of conditional deletion for ubiquitously expressed genes.

SNX27 regulates GIRK currents in DA neurons

Dopamine-specific deletion of SNX27 protein was achieved by crossing $SNX27^{fl/fl}$ to a dopamine transporter (DAT, Slc6j) cre line (DAT-$cre^{+/-}$, Zhuang et al. 2005), as revealed by anti-tyrosine hydroxylase (TH) staining in knockout mice (**Fig. 2.2A-B**). SNX27 mutants ($SNX27^{dat-/-}$) are viable and healthy into

adulthood. Whole-cell patch clamp electrophysiology of acutely prepared midbrain-containing sections show that VTA dopamine neurons lacking SNX27 have normal capacitance, resting potential and size of I_H current responses (**Fig. 2.2C-E**). Thus, SNX27 does not lead to global changes in general membrane properties and excitability. Dopamine neurons were identified by location, presence of I_H, and slow firing frequency. Criteria were confirmed by cre-dependent YFP expression in $SNX27^{dat-/-}$ (**Fig. 2.5-2.6**)

Bath application of a maximally-activating concentration of the $GABA_BR$ agonist baclofen (300μM, (Cruz et al., 2004) show that $GABA_BR$-GIRK currents are significantly decreased compared to control neurons (**Fig. 2.2F**). Interestingly, this decrease is specific to $GABA_BR$-GIRK currents, as there is no significant change in maximal activation of D2R-GIRK currents using the D2R-like agonist quinpirole (30μM, **Fig. 2.2G**).

VTA dopamine neurons are under constant inhibitory control from local and projection GABA neurons (Floresco et al., 2003; Grace et al., 2007), and activation of $GABA_BR$-GIRK currents in VTA is sufficient to silence dopamine neurons (Kalivas and Stewart, 1991). Thus we would expect that reduced $GABA_BR$-GIRK signaling would lead to increased basal excitability with diminished inhibitory control of firing. Indeed, dopamine neurons from $SNX27^{dat-/-}$ mice were hyperactive in response to a series of depolarizing current steps (**Fig. 2.3A**). While baclofen activation was sufficient to significantly inhibit action potential firing at all current steps from wild-type cells (**Fig. 2.3B-C**), this inhibition

was completely abolished in SNX27$^{-/-}$ dopamine neurons (**Fig. 2.3D-E**), suggesting a loss of GABAergic inhibition mediated by $GABA_B$ receptors.

SNX27 mediates forward trafficking of GIRK channels

The decrease in GABAergic inhibition in SNX27$^{-/-}$ dopamine neurons, coupled to a lack of change in D2R-GIRK currents, suggests a specific change in $GABA_B$R-GIRK complexes. While it is known that SNX27 binds directly to GIRK channels via a PDZ domain interaction (Balana et al., 2011; Lunn et al., 2007b), it is possible that the observed decrease is an effect of altered surface expression of the $GABA_B$ receptor in dopamine neurons or changes in the coupling efficiency of $GABA_B$ receptors to GIRK channels (Cruz et al., 2004; Terunuma et al., 2010). Excitatory activation leads to phosphorylation-induced trafficking of the $GABA_B$ receptor (Terunuma et al., 2010), and $G_{\alpha q}$ activation of phopholipase C (PLC) can directly inhibit GIRK currents (Lei et al., 2001). Therefore, we wanted to distinguish possible changes in GIRK channel expression from defects in $GABA_B$R signaling. To investigate this, we used GTPγS, a non-hydrolyzable form of GTP, which bypasses GPCR activation and constitutively activates G proteins. GIRK channels are directly gated by the βγ subunit of G proteins (Hille, 1992; Lüscher and Slesinger, 2010). With GTPγS (100μM) included in the intracellular recording solution, we observed activation of an outward, Ba2+-sensitive current in WT dopamine neurons. By contrast, GTPγS-induced currents were significantly smaller in SNX27$^{dat-/-}$ dopamine

neurons, suggesting a reduced number of GIRK channels expressed on the plasma membrane (**Fig. 2.4A-C**).

SNX27 traffics GIRK channels through a PDZ domain interaction

In addition to GIRK2c/3, SNX27 is known to interact with a variety of other proteins (Cai et al., 2011; Joubert, 2004; Lauffer et al., 2010), raising the possibility that the reduced levels of GIRK channels are due to an indirect effect. To address this, we stereotaxically injected an AAV virus expressing a double-floxed inverted GIRK2a-YFP sequence (**Fig. 2.5A**), where YFP is bound to the C-terminus of GIRK2a. In this way, GIRK2a will be expressed only in Dat-cre+ neurons, eliminating any possibility of off-target effects due to virus expression in non-dopaminergic neurons (Tsai et al., 2009; Tye et al., 2012). GIRK2a lacks a PDZ-binding motif (Inanobe et al., 1999) and is not regulated by SNX27 (Lunn et al., 2007). Additionally, native GIRK2 subunits form functional homotetramers in dopamine neurons and are natively expressed in dopamine neurons (Inanobe et al., 1999).

12-20 days after injection of either GIRK2a-YFP or YFP AAV control, acute midbrain sections were prepared for imaging and electrophysiology. In both YFP and GIRK2a-YFP injected mice, green fluorescence was observed in VTA-containing slices (**Fig. 2.5A**). As shown previously with $SNX27^{dat-/-}$ neurons (**Fig. 2.2F**), $GABA_B$-GIRK currents from YFP-injected $SNX27^{dat-/-}$ mice were significantly reduced and activity was not uninhibited by baclofen (**Fig. 2.5B-C**).

By contrast, dopamine neurons from SNX27$^{dat-/-}$ mice injected with GIRK2a-YFP AAV exhibited $GABA_BR$-GIRK currents restored to levels similar to WT and significantly larger than YFP controls (**Fig. 2.5B**). These findings demonstrate that GIRK2a traffics to the plasma membrane surface in the absence of SNX27. Moreover, the baclofen-dependent inhibition of action potential firing in GIRK2a-infected dopamine neurons from knockout animals was restored to WT levels, suggesting a restoration of GABAergic inhibition through $GABA_B$ receptors (**Fig 2.5D**). Thus, loss of SNX27 affects trafficking of only GIRK channels containing a PDZ binding motif.

To investigate whether the reduction in $GABA_BR$-GIRK signaling was specific for the loss of SNX27 protein and not development defect, we attempted to rescue $GABA_BR$-GIRK currents by ectopically expressing SNX27b, using a double floxed inverted SNX27b followed by an IRES site and GFP (**Fig. 2.6A**). In SNX27$^{dat-/-}$ mice, SNX27b-ires-GFP AAV stereotaxically injected into the VTA led to GFP expression in dopamine neurons (**Fig. 2.6A**). Whole-cell patch clamp recordings revealed that $GABA_BR$-GIRK currents were restored to WT levels in knockout animals expressing SNX27b (**Fig. 2.6B**). Like GIRK2a, baclofen-induced inhibition of activity is also rescued in SNX27-infected dopamine neurons from SNX27$^{dat-/-}$ (**Fig. 2.6D**), suggesting that SNX27 forward trafficking of GIRK channels mediates $GABA_BR$-GIRK inhibition in dopamine neurons.

Previous studies in hippocampal neurons and transiently transfected HEK cells reveled that over-expression of SNX27 also leads to a reduced $GABA_BR$-GIRK as well as muscarinic M2R-GIRK currents (Balana et al., 2011; Lunn et al.,

2007). We therefore investigated the effect of over-expressing SNX27 in VTA dopamine neurons. We injected the double-floxed inverted SNX27b virus into the VTA of DAT-cre+ mice (**Fig. 2.7A**). Remarkably, over-expression of SNX27 in dopamine neurons led to significantly smaller GABABR-GIRK currents (**Fig. 2.7B**), similar to that observed in dopamine neurons of $SNX27^{dat-/-}$ mice. Baclofen inhibited action potential firing in SNX27 over-expressing neurons, though to a qualitatively smaller extent than control (**Fig. 2.7C-D**). Taken together, these results suggest that SNX27 plays an important role in regulating trafficking of GIRK channels, where too much or too little interferes with maintenance of basal levels of GIRK channels on the plasma membrane.

Discussion

Regulating the surface expression of signaling molecules, especially via receptors and ion channels, is a key mechanism by which cells are able to quickly alter function in response to external stimuli (von Zastrow and Williams, 2012). Here we have identified a key role of SNX27 in neuronal trafficking, where a PDZ-domain-mediated interaction with GIRK channels mediates the GABA-dependent signaling capacity of VTA dopamine neurons. GIRK channel trafficking is sufficient to regulate basal excitability of this neuronal population, which, together with diminished inhibitory response, alters novelty-induced exploratory activity.

VTA dopamine neurons are under constant inhibitory regulation (Grace et al., 2007) through local interneurons and projections from regions such as RMTg (Jhou et al., 2009), lateral habenula (Lammel et al., 2012) and nucleus accumbens (Kalivas, 2007). Altered VTA inhibitory control has been implicated in a number of psychiatric disorders, such as response to addictive drugs (Koob and Volkow, 2009; Lüscher and Malenka, 2011), aversive stimuli (Jhou et al., 2009; Lammel et al., 2012), and depression (Krishnan and Nestler, 2008; Luscher et al., 2010; Tye et al., 2012). Thus, the ability to regulate inhibitory input is critical to shaping behavioral responses in vivo, and represents a potential intervention point for novel therapeutics.

This is especially intriguing given the observation that SNX27 manipulation leads to dramatically altered, but not complete loss, of GIRK

currents in these cells, which could mitigate the severity of behavioral response to current manipulation. For example, a low dose of baclofen produces anti-craving properties (Roberts, 2005), but at higher doses is sedative (Cruz et al., 2004). Perhaps altering GIRK channel trafficking through manipulation of SNX27 could target specific behavioral responses without affecting others.

A potential source of selectivity could occur through the specificity of SNX27-directed GIRK channel trafficking. Genetic deletion of SNX27 significantly affects $GABA_BR$-GIRK inhibition without affecting auto-inhibitory D2R-GIRK currents. The SNX27 knockout is not a total loss of GIRK currents, so perhaps the remaining channels are sufficient to activate the relatively small D2R-mediated conductance (~50pA), while the much larger $GABA_BR$-GIRK current (~200pA) is more severely affected by altered trafficking (Beckstead et al., 2004; Lacey et al., 1988). Additionally, it is possible that D2 receptors selectively activate GIRK2 homotetramers, and SNX27 trafficking of GIRK3-containing channels would therefore not be affected by loss of GIRK2/3 heterotetramers, though this is purely speculation.

$GABA_B$ receptors are known to form macromolecular complexes with muscarinic receptors (Boyer et al., 2009), but it is not known if they complex with D2 or any other receptors in DA neurons, or if different receptors use a common pool of GIRK channels or independent complexes. GIRK channels form a macromolecular signaling complex with $GABA_BR$ and RGS proteins in dopamine neurons (Labouèbe et al., 2007; Lüscher and Slesinger, 2010; Padgett et al., 2012), but it has yet to be determined if other GPCRs are part of this complex. In

dopamine neurons, GIRK channels are located peri-synaptically along the dendritic shaft with $GABA_BR$ (Koyrakh, 2005). In the hippocampus $GABA_B$ receptors are both pre-synaptic, where they couple to Ca_V channels, and post-synaptic, coupling to GIRK channels (Kulik et al., 2003; Luscher et al., 1997; Lüscher and Slesinger, 2010), but in substantia nigra primarily colocalize with dendritic GIRK subunits (Koyrakh, 2005). D2 receptors in dopamine neurons are located on both dendritic shafts and axo-dendritic processes and couple to GIRK channels, and axonal, in nucleus accumbens terminals, coupled to Ca_V channels (Ford et al., 2010; Sesack et al., 1994). Our data offers the intriguing possibility that GIRK channels coupled to these receptors have differential composition and regulation *in vivo*.

Both over-expression and deletion in dopamine neurons lead to a functional decrease in maximally activated GIRK currents (**Fig. 2.2, 2.7**). Additionally, siRNA-mediated deletion of SNX27 in cultured cells leads to decreased surface expression of the β2-adrenoreceptor (Lauffer et al., 2010; Temkin et al., 2011; von Zastrow and Williams, 2012). Similar results have been observed with a number of different regulatory proteins (Cremona et al., 1999; Di Paolo et al., 2004; Kim et al., 2005; Lauffer et al., 2010), including sorting nexins, and it has been suggested that any abnormality in levels of key endocytic proteins impairs the homeostasis of protein networks and disrupts activity (Shin et al., 2007). SNX9, for example, is able to form dimers, and therefore increased SNX9 expression has been suggested to act as a dominant negative, leading to similar defects in recycling as protein knockdown (Shin et al., 2007). At this time,

the ability of SNX27 to dimerize remains unknown, though dimerization could represent an intriguing mechanism for fast alteration of GIRK channel regulation through altered SNX27 expression *in vivo*.

This study describes the first functional role of sorting nexins in neurons, and, to our knowledge, the first non-redundant sorting nexin knockout that survives to adulthood (Cai et al., 2011; Schwarz, 2002; Zheng et al., 2006), emphasizing the importance of cell-specific deletion to understand *in vivo* function of sorting nexin endocytic regulation. Several sorting nexins, in addition to SNX27, are expressed in neurons and are suggested to have functional roles, such as SNX3 and SNX18 in neural development (Mizutani et al., 2012; Nakazawa et al., 2011), SNX9 in synaptic vesicle endocytosis (Shin et al., 2007), and SNX12 binding β-site APP cleaving enzyme 1 or SNX17 binding APP to regulate Aβ levels in the pathogenesis of Alzheimer's Disease (Lee et al., 2008; Zhao et al., 2012). Here we have identified SNX27 forward trafficking of GIRK channels as a means by which dopamine neurons regulate response to inhibitory input. Thus, sorting nexins appear to have critical and specific roles through protein-protein interactions in the early endosome and represent an enticing area of research for neuromodulator regulation as potential therapeutic targets in a variety of neurological disorders.

Materials and Methods

Generation of SNX27-/-dat animals: Knockout-first promoter driven line Snx27$^{tm1a(KOMP)Wtsi}$ heterozygous animals were purchased from UCDavis KOMP Repository and either interbred to generate a homozygous whole animal knockout line or crossed to flp deleter line B6.SJL-Tg(ACTFLPe)9205Dym/J (JAX stock #005703) to remove selection cassette and create conditional allele (SNX27fl/fl).

SNX27$^{fl/fl}$ animals were crossed to the DAT-cre line (Zhuang et al. 2005), then SNX27$^{fl/+}$-dat-cre$^{+/-}$ intercrossed to generate SNX27$^{fl/fl}$-dat-cre$^{+/-}$ animals. Experimental animals and littermate controls were generated by crossing SNX27$^{fl/fl}$-dat-cre$^{+/-}$ (SNX27$^{dat-/-}$) to SNX27$^{fl/fl}$ (conditional line). Dat-cre$^{+/-}$ crossed to C57Bl/6J were maintained separately, and only cre$^{+/-}$ animals (dat-cre control) were included in experiments.

Genotyping: Tail biopsies were collected from animals at weaning (>P21) and PCR genotyping was performed using the following primers:

SNX_loxP_geno_F: 5'-AAAGGGCTGGGCGGTAGTGG-3'

SNX_loxP_geno2_R: 5'-CAGGGCCCAGATCATTCAACACTTC-3'

Flp_geno_F: 5'-ACCATAGGGTTGATGAGATGGCCAA-3'

Flp_geno_R: 5'-ACAGCATGACTTGGCTGACATACGTG-3'

Cre320 F: 5'-GAACC TGATG GACAT GTTCA GG-3'

Cre320 R: 5'-AGTGC GTTCG AACGC TAGAG CCTGT-3'

Cre_myo internal control F: 5'-TTACG TCCAT CGTGG ACAGC-3'

Cre_myo internal control R: 5'-TGGGC TGGGT GTTAG CCTTA-3'

Animal husbandry: Animals were housed under constant temperature and humidity on a 12-hr light-dark cycle (light 6 am–6 pm) with food and water available *ad libitum* (except during behavioral analysis). Mixed genotypes were housed together in groups of 2-5, separated by sex. All procedures were performed in the light cycle using IACUC-approved protocols for animal handling at the Salk Institute.

Electrophysiology: Male and female mice age P30-P50 were euthanized with isoflurane and horizontal slices from midbrain (200μm) were prepared in ice-cold artificial cerebral spinal fluid containing (in mM): NaCl 119, KCl 2.5, $MgCl_2$ 1.3, $CaCl_2$ 2.5, NaH_2PO_4 1.0, $NaHCO_3$ 26.2 and glucose 11, pH 7.2, bubbled with 95% O_2 and 5% CO_2. Slices are equilibrated for 42 min and transferred to a recording chamber equipped with constant perfusion of ACSF (2 ml/min).

Neurons were visualized on an Olympus scope (BX50 or BX51) and whole-cell patch-clamp recordings were made from neurons in the VTA, identified as the region medial to the medial terminal nucleus of the accessory optical tract. DA neurons were identified by presence of I_h current, large capacitance (20–50 pF) and slow spontaneous firing (1–3 Hz). Pitx3-GFP mice expressing GFP in DA neurons (Zhao et al., 2004) and viral expression in Dat-cre+ cells were used to confirm electrophysiological identification. The internal

solution contained (in mM) potassium gluconate 140, NaCl 4, $MgCl_2$ 2, EGTA 1.1, HEPES 5, Na_2ATP 2, sodium creatine phosphate 5 and Na_3GTP 0.6 (pH 7.3) with KOH. Whole-cell voltage-clamp recordings were used to measure GIRK currents. For agonist-induced currents, changes in holding currents (V_m = -35 mV with junction potential -15.7mV) in response to bath application of a saturating dose of baclofen (300 μM) or quinpirole (30 μM) were measured (at -50 mV every 10 sec). Currents were amplified (Axopatch 200B), filtered at 1 kHz and digitized at 5 kHz (Digidata). I_H current were monitored through a series of hyperpolarizing 200 ms voltage steps to –120 mV. Series resistance (Rs) was monitored throughout the experiment and recordings were excluded from analysis if the Rs varied by more than 20%. Clampex 9.0 software was used for data acquisition and analysis. GIRK basal current were Ba^{2+} (1mM), a selective inhibitor of inward rectifiers. For the GTPγS experiment, GTPγS (0.1 μM) was added to the internal solution in place of Na_3GTP.

Tissue extraction: 24 hours after last drug administration (day 6), animals were sacrificed using an overdose of pentobarbatol and brains extracted. Bilateral tissue punches were taken from 1mm coronal brain sections using a 15-gauge needle, as described previously (McClung and Nestler, 2003; Terwilliger et al., 1991). Punches were taken of the VTA, NAc, PFC and hippocampus. RNA was immediately extracted from fresh unfixed tissue.

RNA extraction and qRT-PCR: RNA was isolated using Trizol reagent (Invitrogen) for isolation and precipitation with RNeasy column (Qiagen) purification followed by DNase treatment (Invitrogen). RNA was reverse-transcribed (Superscript III, Invitrogen) and real-time PCR run using a Roche Lightcycler 480. Data were normalized to 18S rRNA expression and analyzed using the ddCt method (Nolan et al., 2006), and expressed as percent saline control.

Immunohistochemistry & imaging: Animals were anesthetized with 350mg/kg chloral hydrate i.p. and perfused with 4% paraformaldeyde. Brains were post fixed and cyroprotected in 20% sucrose in 0.1M phosphate buffer. 30μM frozen coronal sections were cut and stained with antibodies for SNX27 (1:1000, (Balana et al., 2011) and TH (1:1000, PelFreeze) followed by fluorophore-conjugated secondary. Sections were mounted and imaged on a confocal microscope.

Stereotaxic Surgery: Mice age P24-26 were anesthetized with ketamine/xylazine 10/100mg/kg i.p. and placed in a stereotaxic apparatus. 0.5-1.0μl adeno-associated virus was injected into VTA (x,y,z: +/-0.5mm, -2.4mm, -4.5mm from bregma). Post-operative analgesia was ibuprofen in drinking water for duration of recovery. Animals were allowed to recover at least 12 days prior to use in electrophysiology or behavior experiments.

Virus Production: pAAV-EF1a-double floxed-EYFP-WPRE-HGH pA (Addgene plasmid 20296) was made into AAV serotype 5 at >10^10 GC/ml by Salk Institute Viral Vector Core using standard protocols. GIRK2a-YFP and SNX27b-ires-GFP were sub-cloned into this vector in at Nhe1 and Asc1 sites and also generated into AAV serotype 5 virus.

Statistical analysis: Data were analyzed using Prism 5.0 software. ANOVA with Bonferroni post-test or Student's t-test were used as indicated. Significance was defined as $p<0.05$.

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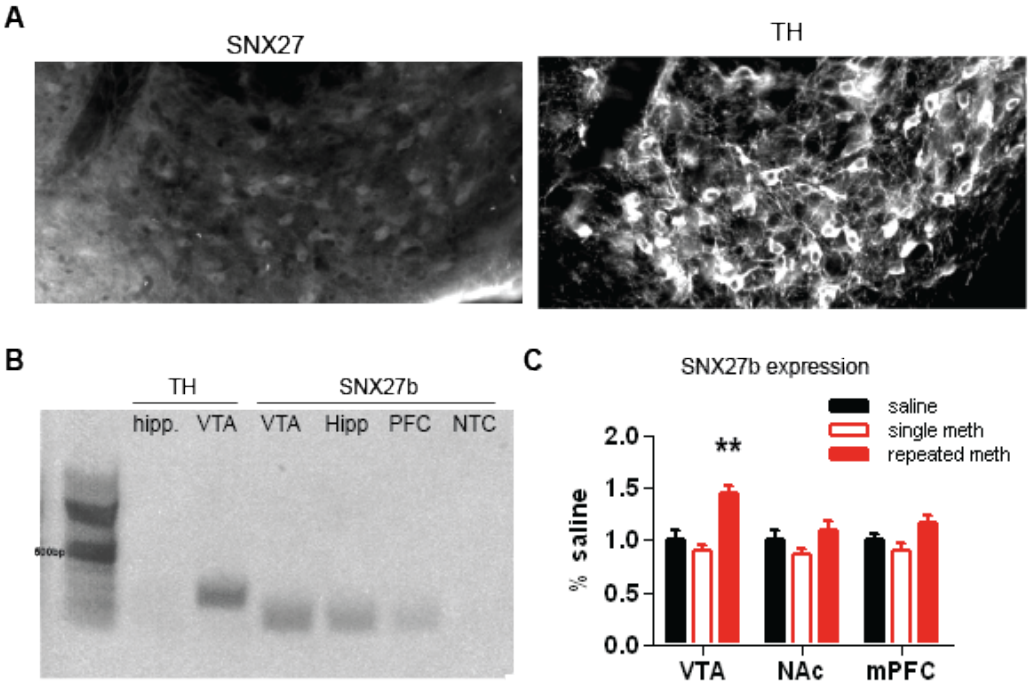
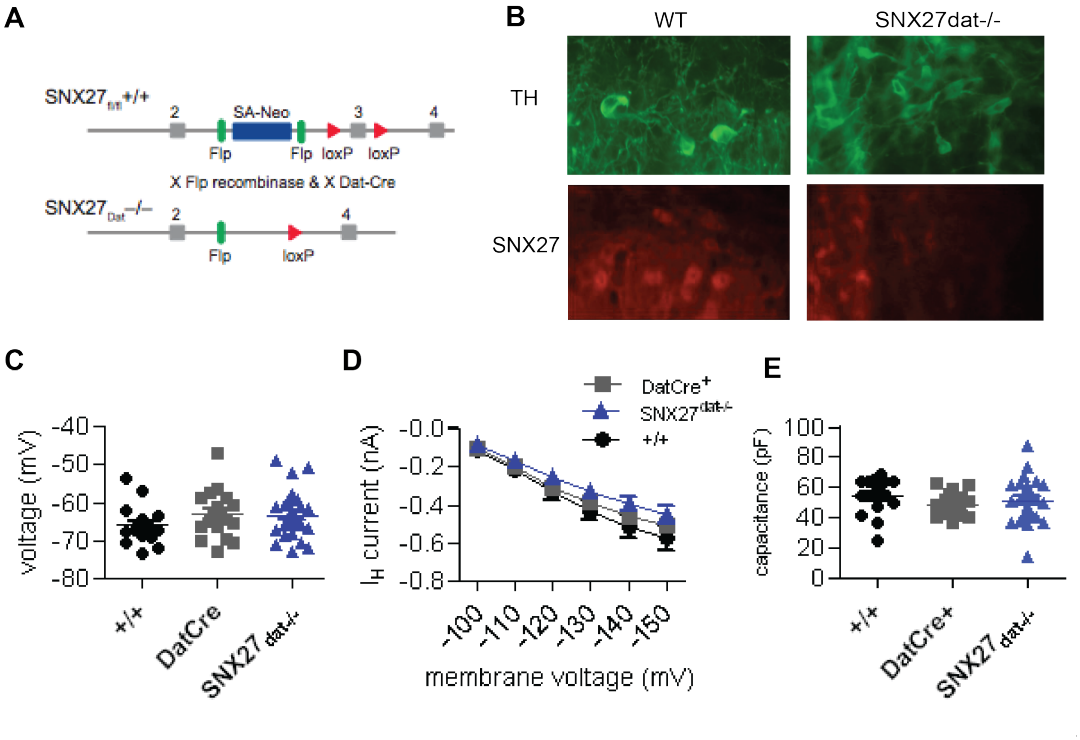
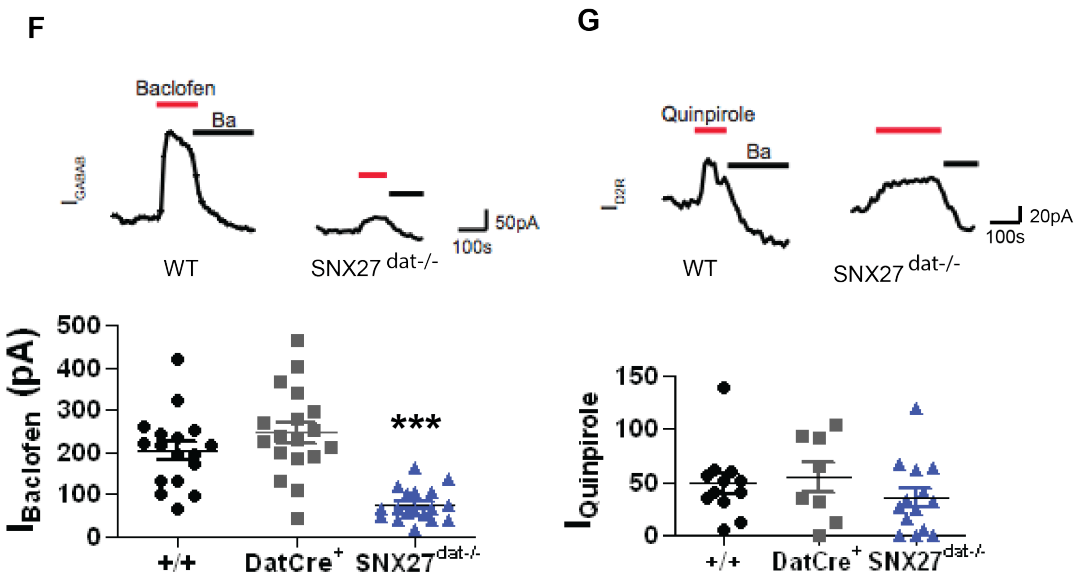


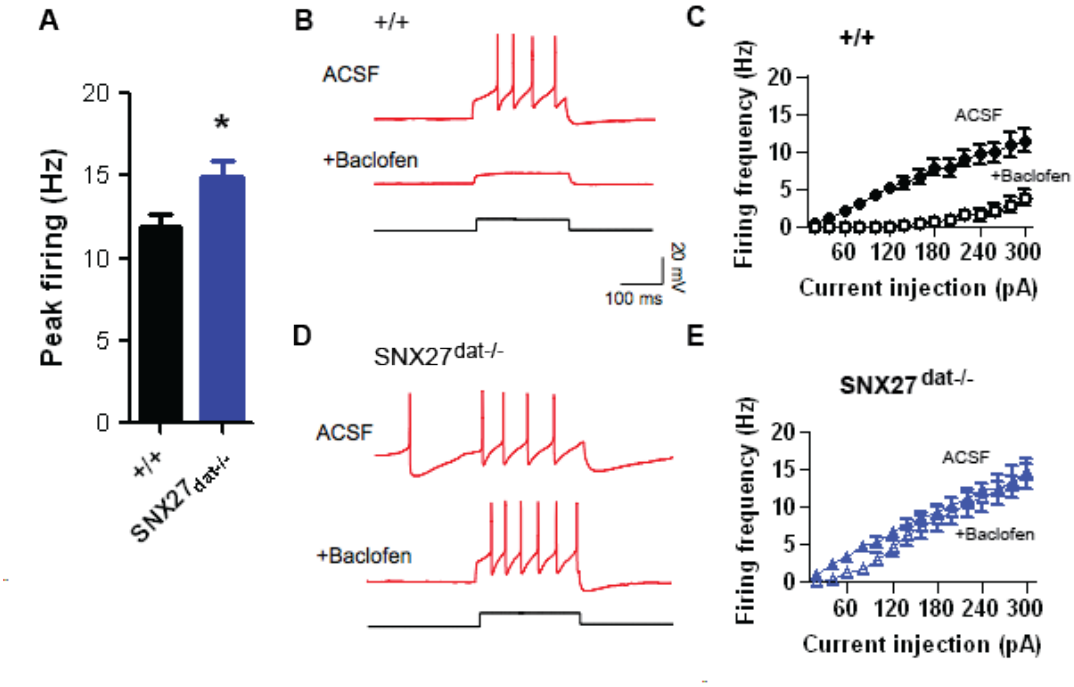
2.1 SNX27 expression in the mesolimbic dopamine circuit. (A) Serial coronal sections of VTA / substantia nigra stained for SNX27 (left) and tyrosine hydroxylase (TH, right). Images at 40x magnification. **(B)** RT-PCR for TH and SNX27b in tissue punches of VTA, hippocampus (Hipp), mPFC, NTC, no template control. **(C)** qRT-PCR for SNX27b expression normalized to 18S in tissue punches of VTA, NAc, mPFC from saline, acute or 5 days methamphetamine-injected mice are significantly enhanced in VTA of repeated meth treated (3-4 mice per group). (**, $p<0.01$ two-way ANOVA with Bonferroni posttest.)



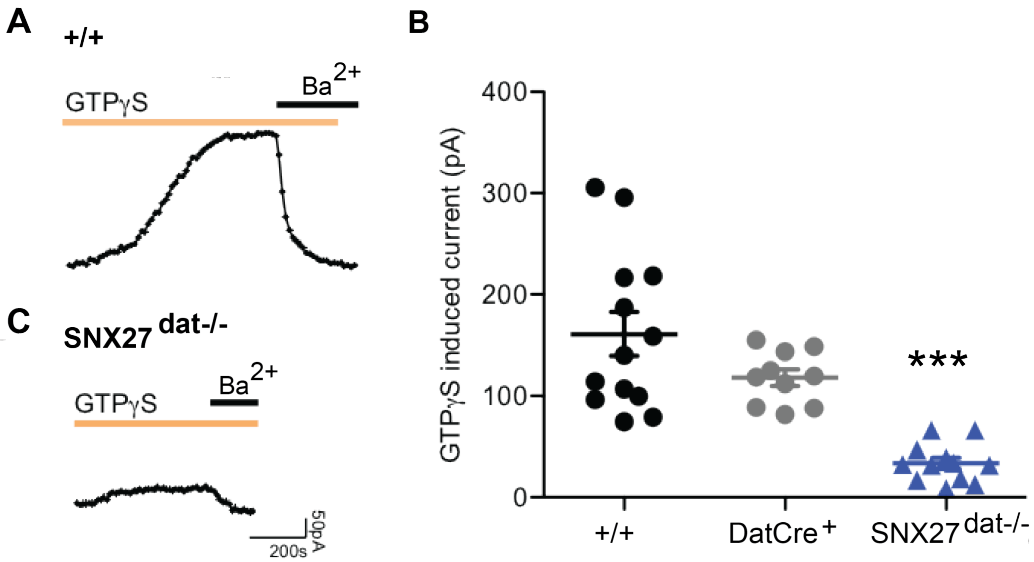
2.2 SNX27 regulates $GABA_BR$-GIRK signaling in VTA dopamine neurons. (A) Schematic of conditional deletion of SNX27 exon 3 in DAT-expressing cells. (Top) Knockout-first construct of SNX27 gene. Selection/stop cassette (SA-Neo) flanked by flp recognition sites between exon 2 and 3, exon 3 flanked by *loxP* sites. Animals were crossed to flp deleter line then DAT-cre line. (Bottom) SNX27 exon 3 is excised by Cre in DAT-expressing dopamine neurons in $SNX27^{dat-/-}$ mice. **(B)** Dual IF staining in VTA for SNX27 and TH in coronal sections of WT and $SNX27^{dat-/-}$. Images at 40x magnification. **(C-E)** Basic membrane properties and excitability of $SNX27^{dat-/-}$ dopamine neurons. Membrane voltage **(C)**, I_H current induced by a series of hyperpolarizing current steps **(D)**, and capacitance **(E)** are unchanged in $SNX27^{dat-/-}$ neurons (C-E n.s. one-way ANOVA).



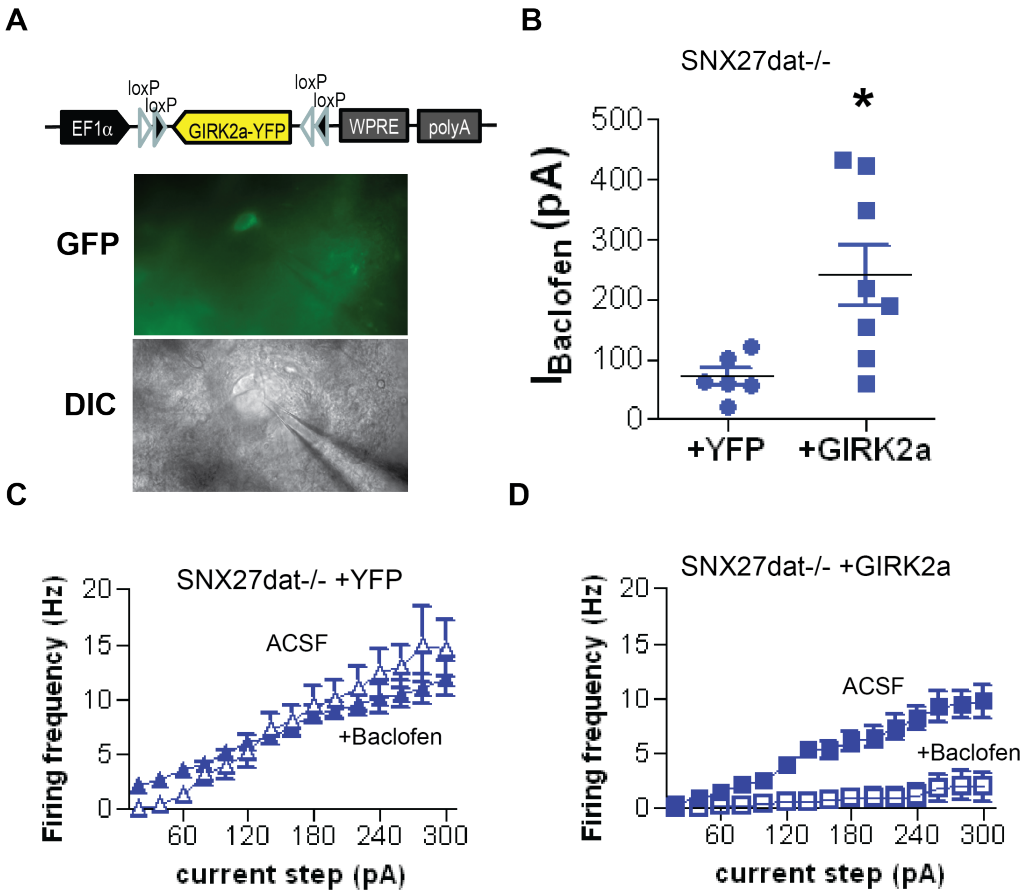
2.2 (continued) SNX27 regulates $GABA_BR$-GIRK signaling in VTA dopamine neurons. (F) Baclofen-activated GIRK current ($I_{Baclofen}$) from VTA dopamine neurons reversed by inward rectifier antagonist barium (Ba). (Top) Outward currents recorded at -50mV plotted as a function of time from WT (top left) and $SNX27^{dat-/-}$ (top right) neurons. (Bottom) Bar graph shows average $I_{Baclofen}$ is significantly reduced in $SNX27^{dat-/-}$ neurons. Scale bars at 50pA and 100s. (***p<0.0001, one-way ANOVA) **(G)** Quinpirole-induced GIRK current ($I_{Quinpirole}$) from dopamine neurons. (Top) Outward current. Scale bars at 20pA and 100s. (Bottom) Average currents are unchanged in $SNX27^{dat-/-}$ neurons (n.s., one-way ANOVA).



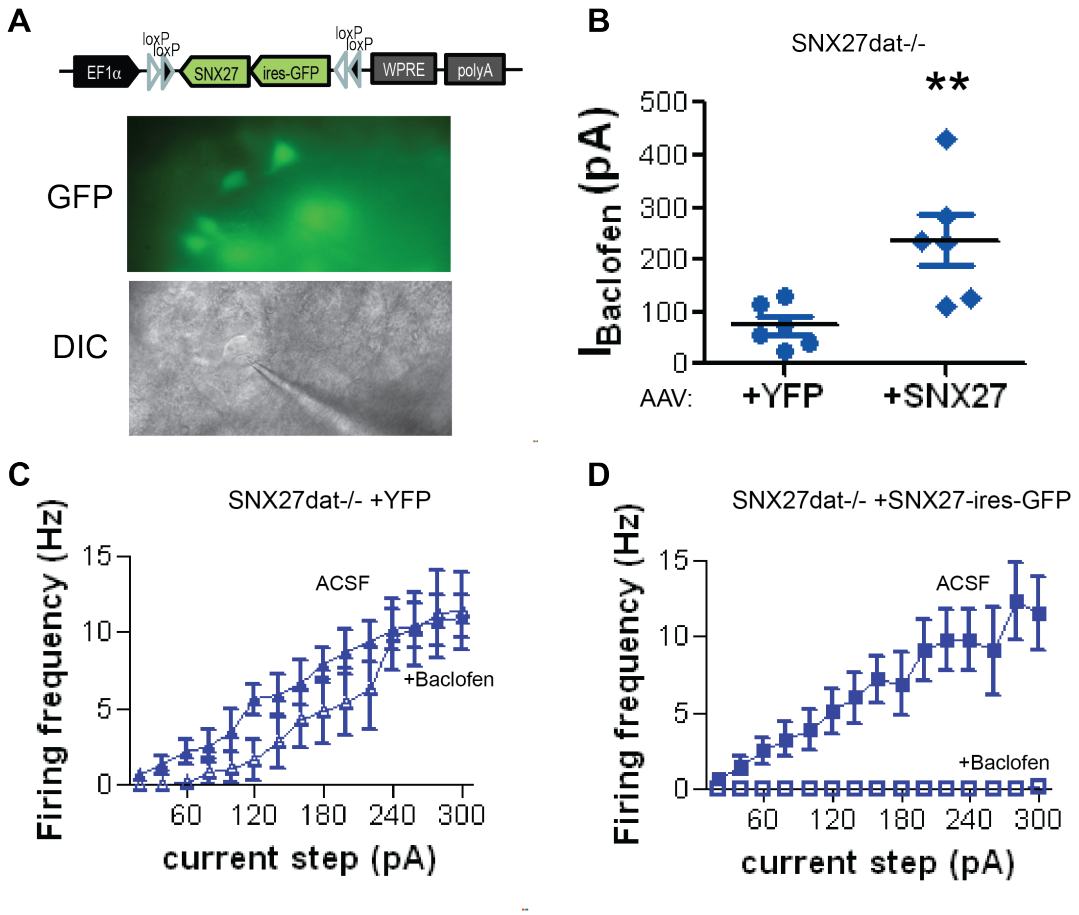
2.3 SNX27 regulates slow GABAergic inhibition in dopamine neurons. (A) Peak firing induced by 300pA current injection is significantly increased in SNX27$^{dat-/-}$ neurons. (n=43 +/+, 30 SNX27$^{dat-/-}$, *p<0.05, Student's t test) **(B, D)** Current clamp recordings show spiking of dopamine neurons elicited by 200pA current injection in ACSF (top trace) or 300μM baclofen (bottom trace) as a function of time in WT **(B)** or SNX27dat-/- neurons **(D)**. Scale bars are at 100ms and 20mV. **(C, E)** Input-output graph of firing frequency by current injection in presence of ACSF (filled circles/triangles) or baclofen in ACSF (open circles/triangles). Baclofen inhibits firing frequency in WT (n=16) **(C)** but not SNX27$^{dat-/-}$ **(E)** neurons (n=24).



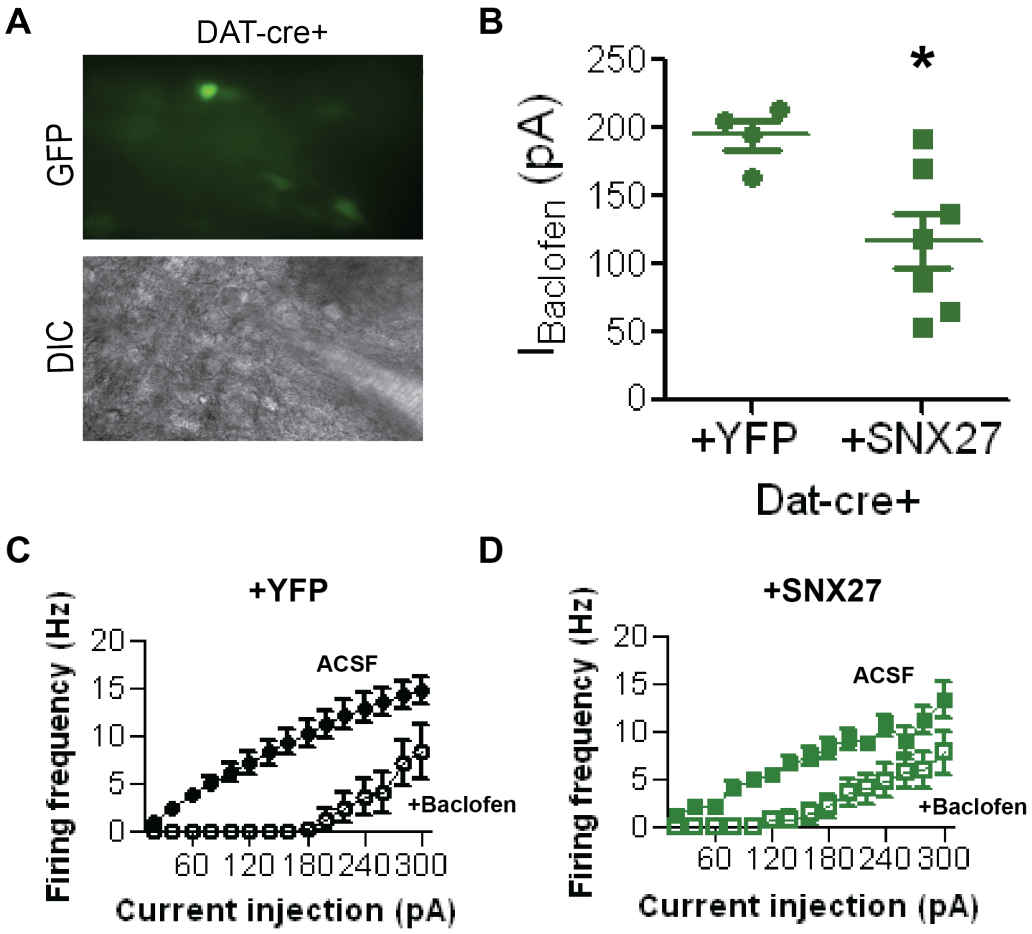
2.4 SNX27 regulates functional GIRK channels. (A,C) Outward currents activated by 100μM GTPγS included in the recording pipette from WT **(A)** and SNX27$^{dat-/-}$ **(C)** dopamine neurons. Scale bars at 50pA and 200s. **(B)** Bar graph shows activated current in significantly reduced in SNX27$^{dat-/-}$ neurons (***p<0.0001, one-way ANOVA).



2.5 SNX27 regulates GABAergic inhibition through a PDZ-domain interaction. (A) Schematic of double floxed inverted GIRK2a-YFP AAV construct (top). (Middle & bottom) Fluorescent and DIC image of YFP+ cell during recording from *ex vivo* midbrain section of SNX27$^{dat-/-}$ animal injected with GIRK2a AAV (20X). **(B)** Baclofen-induced currents of GIRK2a-YFP+ cells are significantly increased compared to YFP+ control (*p<0.05 t test). **(C)** Firing frequency as a function of current injection from cells infected with YFP **(C)** (n=6) or GIRK2a-YFP **(D)** (n=8). Baclofen inhibits activity in GIRK2a-YFP+ **(D)** but not from YFP+ neurons **(C)** of SNX27$^{dat-/-}$ mice.



2.6 Restoration of SNX27 to dopamine neurons rescues GABAergic inhibition. (A) Schematic of double floxed inverted SNX27-ires-GFP AAV construct (top). Fluorescent and DIC image (middle & bottom) of GFP+ cell selected for recording from *ex vivo* midbrain section of SNX27$^{dat-/-}$ animal injected with SNX27-ires-GFP AAV (20X). **(B)** Baclofen-induced currents of SNX27-ires-GFP+ cells are significantly increased from YFP+ (**p<0.001, t test). **(C)** Firing frequency as a function of current injection from cells infected with YFP **(C)** (n=6) or SNX27-ires-GFP **(D)** (n=6). **(C)** Baclofen inhibits activity in SNX27-ires-GFP+ **(D)** but not from YFP+ neurons **(C)** of SNX27$^{dat-/-}$ mice.



2.7 SNX27 over-expression in dopamine neurons. (A) GFP+ cell during recording from *ex vivo* midbrain section of Dat-cre+ animal injected with SNX27-ires-GFP AAV. Top, GFP fluorescence. Bottom, DIC image (20X). **(B)** Baclofen-induced currents of SNX27-ires-GFP+ neurons are reduced (*p<0.05 Student's t test). **(C-D)** Firing frequency as a function of current injection from current clamp recording of cells infected with YFP **(C)** (n=4) or SNX27-ires-GFP **(D)** (n=7). Steps from 100pA-300pA are significantly inhibited by baclofen in both treatment groups except 260pA is not significant in (D). 2-way repeated measures ANOVA with Bonferroni post-test.

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III. Methamphetamine-evoked depression of $GABA_BR$-GIRK signaling in VTA GABA neurons

Abstract

Psychostimulants induce neuroadaptations in excitatory and fast inhibitory transmission in the ventral tegmental area (VTA). Mechanisms underlying drug-evoked synaptic plasticity of slow inhibitory transmission mediated by $GABA_B$ receptors and G protein-gated inwardly rectifying potassium (GIRK/Kir_3) channels, however, are poorly understood. Here, we show that a single *in vivo* exposure to methamphetamine significantly down-regulates baclofen-activated $GABA_BR$-GIRK signaling in VTA GABA neurons though phosphorylation-dependent trafficking of $GABA_B$ receptors at Ser783 of $GABA_{B2}R$. Inhibition of protein phosphatases recovered $GABA_BR$-GIRK currents in VTA GABA neurons of METH-injected mice. Currents are also diminished in VTA DA neurons, but this effect does not last after 24 hours. This psychostimulant-evoked impairment in $GABA_BR$ signaling removes an intrinsic brake on GABA neuron spiking, which may augment GABA transmission in the mesocorticolimbic system in the initial response to drugs of abuse.

Introduction

Changes in the motivation for drugs and natural rewards are central to the development of addiction (Koob and Volkow, 2010). Addictive drugs highjack the mesocorticolimbic DA system, leading to dysregulation of DA in target tissues and changes in DA neuron excitability (Koob and Volkow, 2010; Luscher and Malenka, 2011). One of the key pathways for controlling excitability in the VTA is through activation of a slow GABA-dependent inhibitory current, mediated by $GABA_B$ receptors ($GABA_B$Rs) and G protein-gated inwardly rectifying potassium (GIRK/Kir_3) channels (Johnson and North, 1992; Cruz et al., 2004; Labouèbe et al., 2007), and through an autoinhibitory pathway mediated by D2 dopamine receptors (D2Rs) and GIRK channels (Johnson and North, 1992; Beckstead et al., 2004). *In vivo* exposure to psychostimulants leads to reduced sensitivity of D2R autoreceptors, and increased DA neuron excitability (White and Wang, 1984; Henry et al., 1989; White, 1996), implicating GIRK channels in the response to addictive drugs. Consistent with this, mice lacking GIRK channels self-administer less cocaine (Morgan et al., 2003) and show reduced withdrawal after chronic exposure to morphine (Cruz et al., 2008). In addition, the rewarding property of the club drug gamma hydroxybutyrate (GHB) disinhibits the reward circuit through selective activation of $GABA_B$R-GIRKs on VTA GABA neurons, which impinge on VTA DA neurons (Cruz et al., 2004; Labouèbe et al., 2007). Lastly, *Girk2* transcripts in the mesocorticolimbic dopamine pathway are increased in some human cocaine addicts (Lehrmann et al., 2003). The cellular

mechanisms underlying regulation of $GABA_B$-GIRK signaling in response to addictive drugs is poorly understood.

Accumulating evidence suggests that acquisition of addictive behaviors is learned and, similar to other learning and memory models, involves persistent changes in synaptic strength within the reward circuit and changes in DA neuron signaling (Koob and Volkow, 2010; Luscher and Malenka, 2011). Early drug-evoked neuroadaptations are thought to occur within the VTA and are critical for remodeling the reward circuit and facilitating the development of addiction (Roberts and Koob, 1982). Neuroadaptations have been described that occur 24h following exposure to addictive drugs *in vivo*. Systemic injection of a psychostimulant strengthens excitatory synapses in the VTA (White et al., 1995; Zhang et al., 1997; Ungless et al., 2001; Borgland et al., 2004; Argilli et al., 2008) through recruitment of GluA2-lacking AMPA receptors to the synapses (Mameli et al., 2011). Neuroadaptations in fast GABA transmission have also been reported; fast inhibitory currents mediated by $GABA_A$ receptors are impaired 24h after a single injection of morphine (Nugent et al., 2007) and the amplitudes of GABA-mediated synaptic currents are reduced in mice receiving several injections of cocaine (Liu et al., 2005). Chronic amphetamine enhances $GABA_B$ receptor transmission in the VTA during early withdrawal, but the cellular mechanism underlying this change is unknown (Giorgetti et al., 2002). Following chronic cocaine or morphine, D1R stimulation decreases $GABA_B$-GIRK currents in DA neurons but this occurs from a change in presynaptic GABA release (Bonci and Williams, 1996). In this study, we sought

to characterize the early modulation of $GABA_B$ signaling by a single exposure to psychostimulants. We discovered that ~24h following intraperitoneal injection of methamphetamine or cocaine, $GABA_B$ receptor signaling in VTA GABA neurons is strongly and persistently impaired. This drug-evoked depression of $GABA_BR$-GIRK signaling involves de-phosphorylation of the $GABA_B$ receptor and changes in $GABA_BR$ and GIRK channel trafficking. As a consequence, VTA GABA neuron firing is not affected by the $GABA_BR$ agonist baclofen, suggesting GABAergic function may be augmented in the VTA with psychostimulants.

Results

Psychostimulant-evoked plasticity of GABA VTA neurons

A single injection of psychostimulants enhances glutamatergic synaptic efficacy in the VTA 24h later (Ungless et al., 2001; Borgland et al., 2004; Argilli et al., 2008). We examined whether a single injection of psychostimulant alters $GABA_BR$-GIRK signaling in the VTA. To test this, we injected C57BL/6 mice with methamphetamine at 2 mg/kg, a dose that elicits locomotor sensitization when administered repeatedly (Shimosato et al., 2001; Fukushima et al., 2007; Scibelli et al., 2010), and examined $GABA_BR$-GIRK signaling in the VTA 24h later.

To investigate the effects of methamphetamine on the maximal activation of $GABA_BR$-GIRK currents, we bath applied baclofen to stimulate synaptic and extra-synaptic $GABA_B$ receptors. As described previously (Labouèbe et al., 2007), saturating doses of baclofen (300 mM for DA and 100 mM for GABA) elicited large and desensitizing $GABA_BR$-activated GIRK currents in DA neurons and small non-desensitizing currents in GABA neurons (**Figure 3.1A,C**). All baclofen-activated currents were inhibited with the inwardly rectifying K channel inhibitor Ba^{2+} or the $GABA_B$ receptor antagonist (CGP 54626 – not shown). In contrast to the sIPSC recordings (Padgett et al. 2012), there was a ~40% decrease in the $GABA_BR$-GIRK currents of DA neurons 24h following a methamphetamine injection **(Figure 3.1A-B**). However, this decrease in current was not apparent at 7d following methamphetamine injection. By contrast, the baclofen-activated GIRK ($I_{Baclofen}$) currents in GABA neurons were significantly

depressed by 53±10% 24h following a single methamphetamine injection and persisted for 7 days (**Figure 3.1C-D**).

We also examined whether methamphetamine altered $GABA_B$R-GIRK signaling in other brain regions of the mesocorticolimbic dopamine circuit. There was no change in baclofen-induced current in hippocampal CA1 pyramidal neurons or GABAergic neurons, or in prelimbic cortex pyramidal or GABAergic neurons. However, presynaptic $GABA_B$ receptors in VTA GABA neurons that couple to voltage-gated calcium channels were diminished (Padgett et al. 2012). Taken together, a single exposure to methamphetamine triggered a profound and long-lasting depression specifically in GABA neurons of the VTA.

Cellular mechanism underlying $GABA_B$R-GIRK depression in GABA neurons

Quantitative immunogold electron microscopy data suggested that METH treatment induced internalization of the receptor and channel (Padgett et al. 2012). The phosphorylation status of the $GABA_B$ receptor is important for regulating surface expression of the receptor (Fairfax et al., 2004; Koya et al., 2009; Guetg et al., 2010; Terunuma et al., 2010). We therefore asked whether phosphorylation of the $GABA_B$ receptor could play a role in mediating the methamphetamine-dependent depression. We examined the phosphorylation of Ser783 in $GABA_{B2}$ because dephosphorylation is associated with reduced surface expression of $GABA_B$ receptors in neurons (Terunuma et al., 2010). Protein isolated from tissue punches of the VTA, NAc, hippocampus or mPFC

from saline- and methamphetamine-injected mice (24h) were examined using a phospho-specific antibody for phosphorylated Ser783 in $GABA_{B2}$ (Dobi et al., 2010). Remarkably, phosphorylation of $GABA_{B2}$-S783 reduced by ~25% in the VTA but not in the NAc, mPFC or hippocampus from methamphetamine-injected mice (**Figure 3.2A-D**). There was no apparent change in total $GABA_{B2}$ receptor protein levels (**Figure 3.2F**), suggesting little degradation of internalized receptor. Examination of S892, another phosphorylation site on $GABA_{B2}$ (Fairfax et al., 2004), revealed no significant difference in phospho-specific labeling in METH-injected mice, indicating the effect of METH was unique to $GABA_{B2}$-S783 (**Figure 3.2E**).

Dephosphorylation of $GABA_{B2}$-S783 has previously been shown to be regulated by protein phosphatase 2A (PP2A) (Terunuma et al., 2010), raising the possibility that in vivo exposure to METH enhances the phosphatase activity in VTA GABA neurons. To address this, we examined the effect of acutely inhibiting PP1/PP2A phosphatases with okadaic acid (100nM, OA). In saline-injected mice, there was no significant difference in the amplitude of baclofen-induced current with OA in the pipet, suggesting basal activity of PP1/PP2A does not significantly regulate $GABA_B$R-GIRKs (**Figure 3.2G,J**). In methamphetamine-injected mice, however, intracellular application of OA promoted recovery of baclofen-induced current (**Figure 3.2H,J**). Note the slow time course of activation for $I_{Baclofen}$ in the presence of OA in METH injected mice. This increase could reflect insertion of $GABA_B$ receptors and GIRK channels on the plasma membrane or restoration of functional G protein coupling. For control, we examined the effect of PKC(19-36),

a peptide inhibitor of PKC (**Figure 3.2I,K**). Unlike OA, the presence of PKC inhibitor in the pipet did not restore baclofen-induced current, similar to the effect of methamphetamine alone. Taken together, these findings suggest that *in vivo* exposure to methamphetamine triggers a phosphatase-dependent down-regulation of $GABA_BRs$ and GIRK channels from the plasma membrane of GABA neurons, which results in reduced $GABA_BR$-GIRK signaling and accumulation of $GABA_B$ receptor complexes in intracellular compartments.

Loss of $GABA_BR$-dependent inhibition of VTA GABA neuron firing

To investigate the functional consequence of reduced $GABA_BR$-GIRK currents in GABA neurons of methamphetamine-injected mice, we examined the effect of baclofen on the induced firing rate of GABA neurons (**Figure 3.3**). We predicted that a loss of $GABA_BR$-GIRK signaling would attenuate GABA-mediated suppression of firing in GABA neurons. To test this, a series of current steps (20-100pA) were injected to elicit a train of action potentials in GABA neurons. In saline-injected mice and methamphetamine-injected mice, the input-output (I-O) plot shows a linear increase in firing rate with larger current injections (**Figure 3.3B**). As expected, baclofen (100 mM) significantly suppressed firing in GABA neurons of saline-injected mice, decreasing the slope of the I-O curve (**Figures 3.3A-B**). By contrast, a saturating dose of baclofen (100 mM) was unable to significantly change in the I-O curve in methamphetamine-injected mice (**Figures 3.3C-D**). These results demonstrate that a loss of $GABA_BR$-GIRK

currents in GABA neurons removes an important ‘brake’ on GABA neuron firing in the VTA.

Discussion

Drug-evoked synaptic plasticity can cause persistent modifications of neural circuits that eventually lead to addiction. We report here that a single dose of METH or cocaine is sufficient to significantly weaken the ability of $GABA_B$ receptors to control VTA GABA neuron firing when measured *ex vivo* 24h later. As such, this adaptive change is not likely sufficient to cause addiction, but rather represents a building block of the adaptations that underlie addictive behavior with repetitive exposure. Studying the effect of a single injection of drug enabled us to systematically probe the mechanism underlying the plasticity of the slow IPSC. We discovered the methamphetamine-induced loss of the slow IPSC arises from a reduction in the $GABA_B$R-GIRK currents, due to changes in protein trafficking, and is accompanied by a significant decrease in the sensitivity of presynaptic $GABA_B$ receptors in GABA neurons of the VTA. In contrast, GABA neurons of the hippocampus and prelimbic cortex did not show similar changes in $GABA_B$-GIRK signaling, suggesting the $GABA_B$Rs in the VTA are uniquely targeted by psychostimulants.

The psychostimulant-evoked reduction of $GABA_B$-GIRK currents in VTA GABA neurons could arise from a change in G protein coupling (Nestler et al., 1990; Labouèbe et al., 2007) or internalization of the receptor-channel (Gonzalez-Maeso et al., 2003; Fairfax et al., 2004; Guetg et al., 2010; Maier et al., 2010; Terunuma et al., 2010). In support of the latter possibility, quantitative immunogold electron microscopy revealed a significant reduction in

surface expression of $GABA_B$ receptors and GIRK channels in GABA neurons of METH-injected mice, coincident with a decrease in phosphorylation of $GABA_B$Rs. In cortical and hippocampal neurons, a balance of AMP-activated protein kinase (AMPK)-dependent phosphorylation of $GABA_{B2}$-S783 and PP2A-dependent dephosphorylation governs postendocytic sorting of $GABA_B$ receptors (Terunuma et al., 2010). The persistence of the $GABA_B$-GIRK depression and the rapid recovery with phosphatase inhibitors suggest the balance of surface and internalized $GABA_B$ receptors in GABA neurons might be controlled by a molecular switch in a phosphatase, perhaps akin to the autophosphorylation switch in CaMKII (Lucchesi et al., 2011), or through an endogenous regulator of protein phosphatase activity (Guo et al., 1993). It remains possible that other kinases are also involved; both PKA- and CaMKII-dependent phosphorylation have been implicated in stabilization of $GABA_{B1}$ on the plasma membrane (Couve et al., 2002; Guetg et al., 2010). Interestingly, total protein levels of $GABA_{B2}$ receptors levels were not significantly changed in METH-injected mice, suggesting the internalized pool of receptors was not redirected to a degradation pathway, in contrast to activity-dependent degradation of $GABA_B$ receptors observed in cortex (Terunuma et al., 2010).

If phosphorylation controls surface expression of $GABA_B$ receptors, then what controls the surface expression of GIRK channels? CaMKII-dependent phosphorylation of GIRK2 has been implicated in stabilizing GIRK2 channels on the plasma membrane of hippocampal neurons (Chung et al., 2009). In these neurons, protein phosphatase-1-mediated dephosphorylation promotes GIRK

channel recycling and increases surface expression (Chung et al., 2009); therefore, a phosphatase inhibitor would be expected to reduce GIRK expression on the plasma membrane. An alternative explanation is that GIRK channels internalize via association with $GABA_B$ receptors in a macromolecular signaling complex. Previous studies have shown that both GPCRs and GIRK channels are physically close (Lavine et al., 2002; Nobles et al., 2005; Riven et al., 2006; Fowler et al., 2007) and can traffic together through intracellular compartments (Clancy et al., 2007).

How may the psychostimulant-evoked depression in $GABA_B$-GIRK signaling in VTA GABA neurons alter the physiology of the VTA and contribute to addiction? DA neurons fire in two modes, tonic and phasic, with phasic firing leading to higher DA levels (Cooper, 2002). A balance of NMDAR activation and $GABA_B$R signaling controls tonic vs phasic firing, and activation of $GABA_B$ receptors plays an important role in reducing phasic firing in VTA DA neurons (Erhardt et al., 2002). The VTA GABA neurons provide a local source of GABA for controlling the firing of VTA DA neurons (Grace and Bunney, 1985; Johnson and North, 1992; Tan et al., 2010). Recent electron microscopy studies have confirmed synaptic contacts between local GABA and DA neurons within the VTA (Omelchenko and Sesack, 2009). The depression of $GABA_B$R-GIRK signaling in somatodendritic regions along with the reduced sensitivity of $GABA_B$Rs in presynaptic GABA terminals of VTA GABA neurons would markedly impair an intrinsic ‘brake’ on GABA release several days after a single injection of methamphetamine. Together, these pre and postsynaptic neuroadaptations are

predicted to increase GABA-mediated inhibition of VTA DA neurons. In line with this model, other groups have reported psychostimulant-evoked neuroadaptations in $GABA_BR$-signaling that lead to enhanced GABAergic transmission in the VTA (Giorgetti et al., 2002), the dorsolateral septal nucleus (Shoji et al., 1997) and the NAc (Xi et al., 2003). Similarly, chronic morphine increases the sensitivity of $GABA_B$ receptors on glutamatergic terminals in the VTA, which would further enhance the inhibition of DA neurons mediated by augmented GABA release (Manzoni and Williams, 1999).

The enhanced GABAergic inhibition of VTA DA neurons may represent an attempt to restore balance in activity of the VTA circuit and therefore $GABA_BR$-GIRK adaptation may be considered a form of synaptic scaling. Neuroadaptive changes in $GABA_BR$-GIRK signaling for reestablishing balance in neural circuits have been described in other model systems. In a mouse model of succinic semialdehyde dehydrogenease (SSADH) deficiency, an autosomal recessive disorder of GABA catabolism that leads to elevated synaptic GABA, $GABA_BR$-GIRK currents are significantly depressed in cortical neurons (Vardya et al., 2010). On the other hand, the $GABA_BR$-mediated IPSC in hippocampal pyramidal neurons is enhanced in response to potentiation of excitatory synaptic transmission (Huang et al., 2005). The level of inhibition mediated by $GABA_BR$-GIRK currents may be tightly tuned to changes in neuronal excitability.

The down-regulation of $GABA_B$ receptor signaling in VTA GABA neurons occurs in parallel with other plastic changes in VTA DA neurons, such as the redistribution of AMPAR and NMDARs (White et al., 1995; Zhang et al., 1997;

Ungless et al., 2001; Borgland et al., 2004; Argilli et al., 2008; Mameli et al., 2011) and alterations of fast GABAergic transmission (Nugent et al., 2007). As proposed above, the drug-evoked depression of $GABA_BR$ signaling in GABA neurons removes a “brake” on GABA neuron firing that may enhance GABA-mediated inhibition of DA neurons and potentially reduce reward perception (Koob and Volkow, 2010; Luscher and Malenka, 2011). However, repeated psychostimulant administration leads to increases in the firing rates of VTA DA neurons (White and Wang, 1984; Henry et al., 1989; White, 1996), partly through reduced sensitivity of D2 autoreceptors (White, 1996). Thus, the increase in GABA-mediated inhibition of VTA DA neurons, while efficient at first, may eventually be inadequate to suppress the potentiating effects of psychostimulants on VTA DA neurons. Clearly, additional experiments will be needed to better understand how the adaptation of $GABA_BR$-GIRK signaling affects VTA GABA neuron function and, more generally, the role of the slow $GABA_B$-mediated inhibition in drug-evoked remodeling of the mesocorticolimbic circuitry.

In conclusion, we have identified a novel, molecular switch in $GABA_B$ receptor signaling that occurs in response to a single *in vivo* exposure to psychostimulant – this depression of $GABA_BR$-GIRK signaling persists for days after the injection. This cellular memory trace of drug exposure is encoded in a phosphorylation-dependent depression of $GABA_B$ receptor signaling in VTA GABA neurons, which may augment GABA transmission in the mesocorticolimbic system.

Materials and Methods

Animals: C57BL/6 mice were purchased from Harlan laboratories or bred in-house, and housed under constant temperature and humidity on a 12h light-dark cycle (light 6am-6pm) with free access to food and water. Pitx3-GFP is a knock-in mouse that was kindly provided by Dr. M. Li. $GABA_{BR2}$ S783A mouse is a mutant mouse kindly provided by Dr. Steven Moss. All procedures were performed in the light cycle according to IACUC guidelines for animal handling at the Salk Institute.

Drug treatment: Male and female mice (P15-35) were injected intraperitoneally with 0.9% saline (control), 2 mg/kg methamphetamine (METH) using a 15 gauge insulin syringe and injection volume < 200ul to minimize stress. Experimental procedures were performed 24h-7d later. Methamphetamine was purchased from Sigma.

Electrophysiology in acute slices: Horizontal slices from midbrain (250 μm) were prepared 24h following injections in ice cold artificial cerebral spinal fluid (ACSF) containing (in mM) NaCl (119), KCl (2.5), $MgCl_2$ (1.3), $CaCl_2$ (2.5), NaH_2PO_4 (1), $NaHCO_3$ (26.2) and glucose (11), pH 7.3 continuously bubbled with 95/5% O_2/CO_2. Slices were warmed to 33^{o}C and incubated for 45 min in ACSF supplemented with myo-inositol (3), ascorbic acid (0.4) and sodium pyruvate (2) and then transferred to the recording chamber superfused with ACSF (2ml/min). Visualized whole-cell voltage clamp recording techniques were used to measure

currents within the VTA, identified as the region medial to the medial terminal nucleus of the accessory optical tract. GABA neurons were identified by the absence of I_h current, a small capacitance (<20pF) and a fast spontaneous firing rate (5-10Hz). In contrast DA neurons have an I_h current, large capacitance (20-50 pF) and slow spontaneous firing (1-3Hz). Pitx3-GFP mice expressing GFP in DA neurons and GAD67-GFP mice expressing GFP in GABA neurons were used to confirm electrophysiological identification. The internal solution for measuring baclofen-activated $GABA_B$ currents contained (in mM) potassium gluconate (140), NaCl (4), $MgCl_2$ (2), EGTA (1.1), HEPES (5), Na_2ATP (2), sodium creatine phosphate (5) and Na_3GTP (0.6), pH 7.3 with KOH.

Cell membrane resistance and approximate access resistance were measured with a 200ms 10mV hyperpolarizing step imposed to measure (Clampex 8). We did not observe any differences with wild-type mice and Pitx3-GFP or GAD67-GFP; therefore we have pooled the data. All electrophysiological chemicals for electrophysiology were purchased from Sigma; drugs purchased from Tocris. Data are expressed as mean ± s.e.m. and statistical significance determined by one-way ANOVA with Holm-Sidak post hoc test for significance ($P<0.05$).

Antibodies. A rabbit polyclonal antibody anti-Glutamate Decarboxylase 65 & 67 (AB1511, Millipore, Billerica, MA, USA), anti-phospho T172-AMPK (p-T172) (Cell Signaling Danvers, MA, USA), anti-AMPK (Cell Signaling, Danvers, MA, USA), anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho

S783-$GABA_{B2}$ (p-S783) (Terunuma et al., 2010), anti-phospho S892-GABAB2 (p-S892) (Couve et al., 2001) were used. A monoclonal antibody anti-GABAB1 (Clone N93A/49, NeuroMab, Davis, CA, USA) and anti-GABAB2 (Clone N81/37, NeuroMab, Davis, CA, USA) were used. A guinea-pig polyclonal antibody anti-GIRK2 (Aguado et al., 2008) was used.

Western blotting. Tissue punches from VTA, NAc and PFC obtained from saline and METH injected mice were lysed in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM NaF, 2 mM Na3VO4, 10 mM Na4P2O7, 10 µg/mL leupeptin, 1 µg/mL aprotinin, 10 µg/mL antipain and 250 µg/mL 4-(2-Aminoethl) benzenesulfonyl fluoride hydrochloride. Soluble material was then subjected to immunoblotting with anti-GABAB2, anti-phospho S783-GABAB2 (p-S783), anti-phospho S892-GABAB2 (p-S892), anti-AMPK, anti-phospho T172-AMPK (p-T172), anti-GAPDH, and detected by SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). The luminescence images were captured by Luminescent image analyzer (LAS3000, Fujifilm) and the intensity of bands was measured by Multi gauge (ver. 3, Fujifilm).

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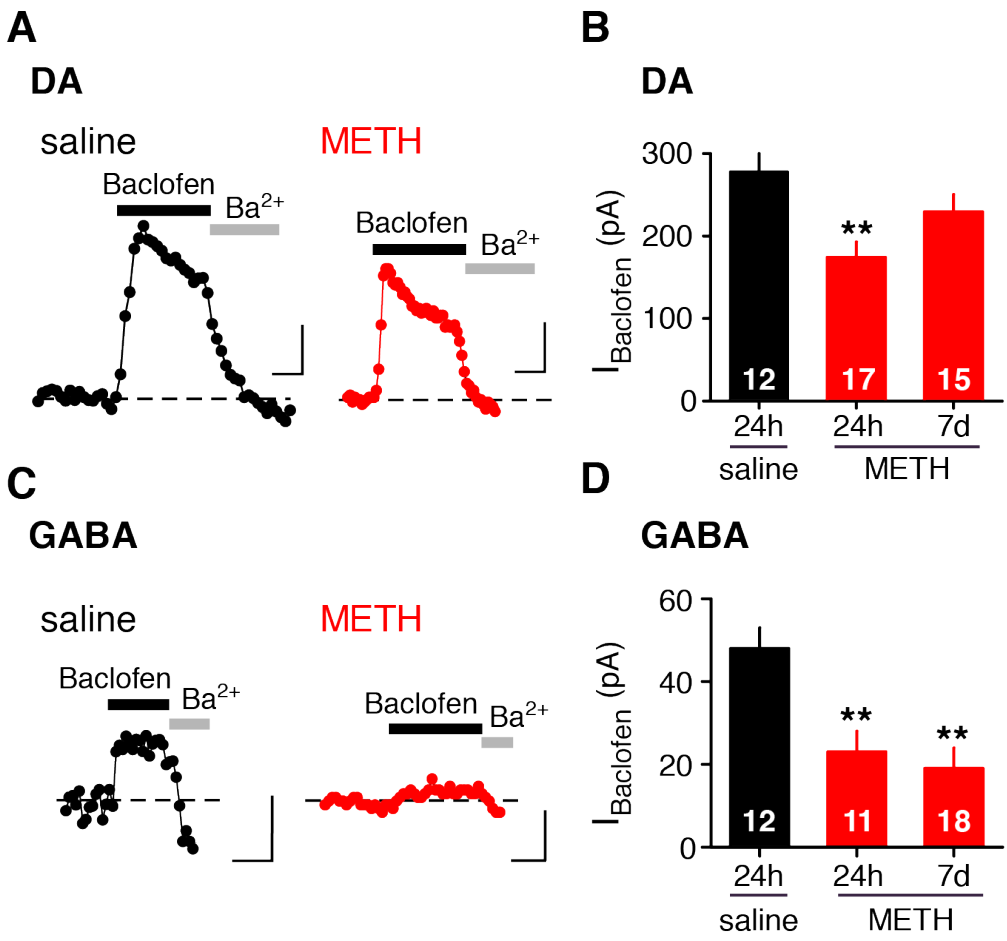
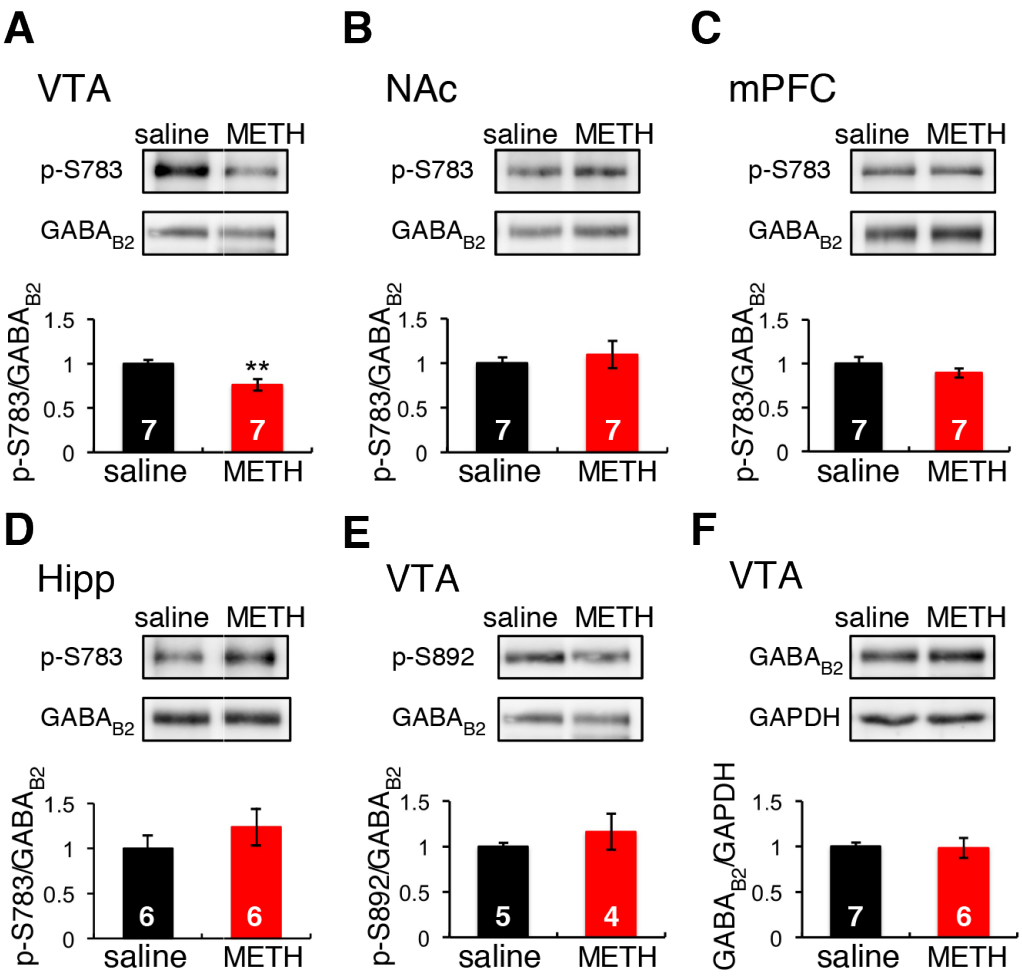
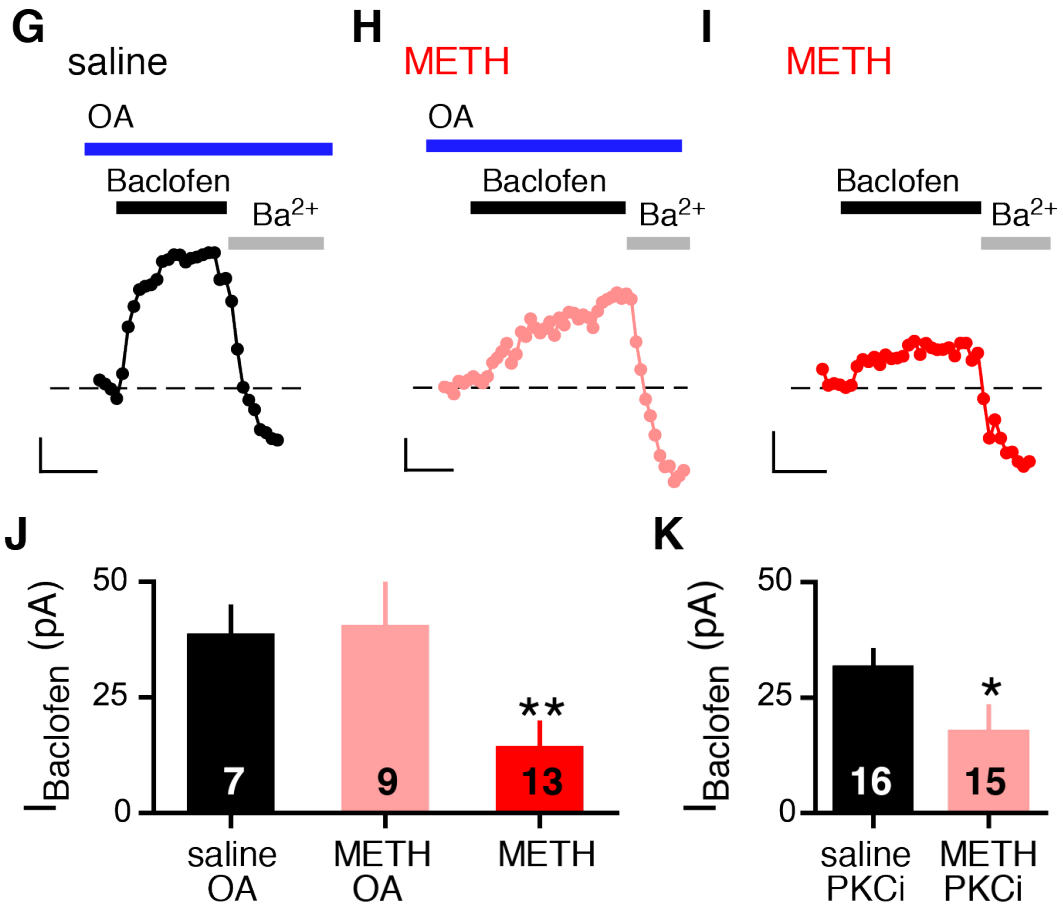


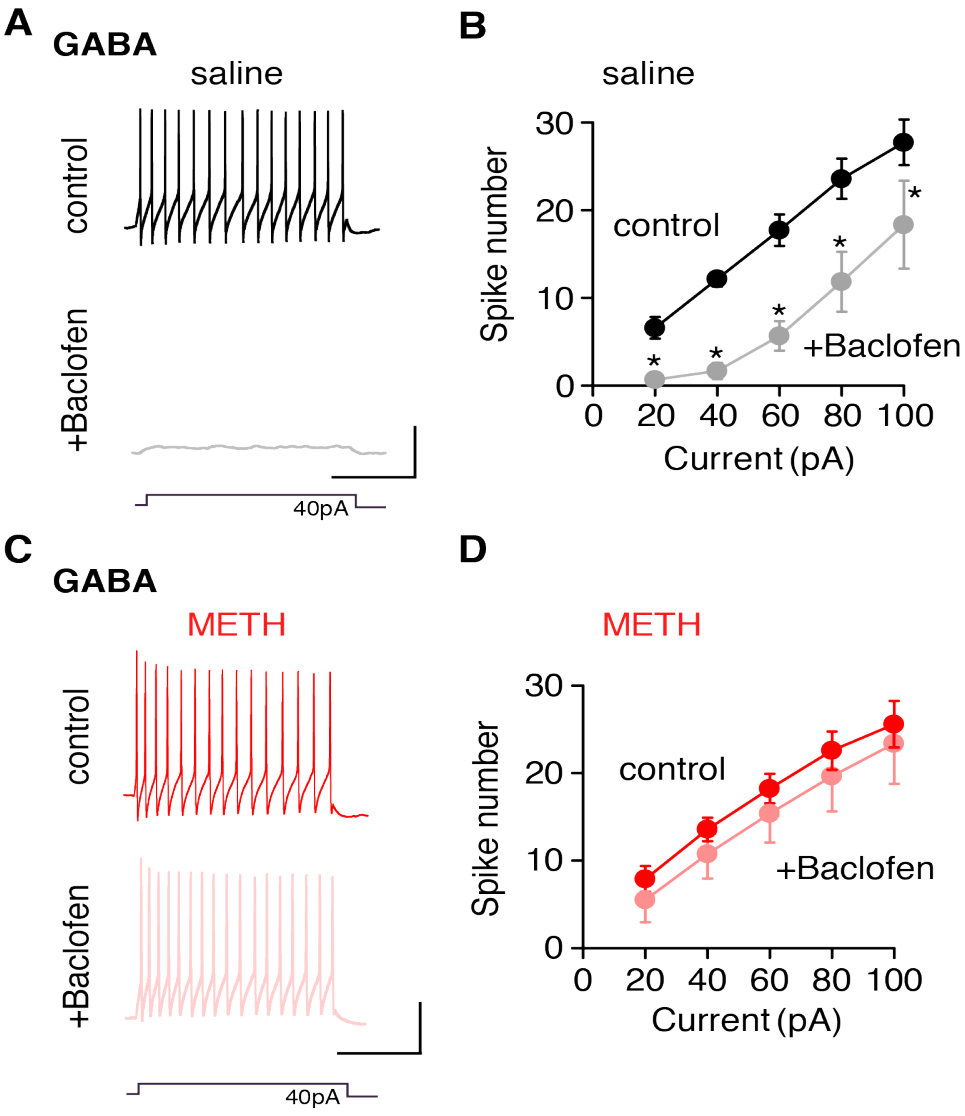
3.1 Reduced $GABA_BR$-GIRK currents in VTA GABA neurons 24h and 7d following *in vivo* meth exposure. The baclofen-activated GIRK currents ($I_{Baclofen}$) recorded from VTA DA **(A)** and GABA **(C)** neurons 24h following a saline (0.9%) or meth (2mg/kg) injection. Outward currents recorded at -50 mV are plotted as a function of time. $I_{Baclofen}$ is blocked by the inward rectifier inhibitor Ba^{2+} (1mM) or by the $GABA_BR$ antagonist CGP 54626 (data not shown). Scale bars: 100pA **(A)** 50 pA **(C)**, 100s. **B**, Average $I_{Baclofen}$ in DA neurons 24h following saline or 24h and 7d following meth injection. **D**, Bar graph shows average $I_{Baclofen}$ in GABA neurons 24h following saline injection or 24h and 7d following meth injection. Note significant decrease in $I_{Baclofen}$ in GABA neurons of meth-injected mice that persists for 7d. **p<0.05 One-way ANOVA



3.2 Role of de-phosphorylation of GABA$_B$Rs in meth-dependent depression of GABA$_B$R-GIRK currents in GABA neurons. (A-D) Western blots using phospho-specific antibody for p-S783 in GABA$_{B2}$ and total GABA$_{B2}$ in tissue punches of VTA, NAc, PFC and hippocampus from saline and meth injected mice (6-7 mice per group). Bar graphs show quantification of western blots normalized to GABA$_{B2}$ levels. Note significant decrease in p-S783 in VTA (**p<0.05 Student's t-test). **(E)** Western blot and quantification for p-S892 in GABA$_{B2}$ and total GABA$_{B2}$ in VTA. **(F)** Western blot and quantification for total GABA$_{B2}$ and GAPDH in VTA.



3.2 (continued) Role of de-phosphorylation of $GABA_BRs$ in meth-dependent depression of $GABA_BR$-GIRK currents in GABA neurons. (G-I) Intracellular application of OA but not PKC inhibitor recovered $I_{Baclofen}$ in meth-injected mice. Representative recordings of $I_{Baclofen}$ in GABA neurons from saline and meth-injected mice are shown with 100nM okadaic acid (OA) included in patch electrode (OA_{pipet}) (V_m = -50 mV). Scale bars: 10pA, 100s. **(J)** Bar graph shows average $I_{Baclofen}$ for saline-injected/OA_{pipet} (38.3 ± 6.3pA), meth-injected/OA_{pipet} (40.2 ± 9.4pA), and meth-injected (14.1 ± 5.6 pA). **p<0.05 vs saline using one-way ANOVA. **(K)** For control, a PKC inhibitor (PKC(33-69), 1 mM) included in the pipet ($PKCi_{pipet}$) did produce significant recovery of $I_{Baclofen}$ (saline+ $PKCi_{pipet}$: 31.5 ± 3.9 pA; meth+ $PKCi_{pipet}$: 17.5 ± 5.7pA; *p< 0.05 Student's t-test).



3.3. Methamphetamine-injected mice lack $GABA_BR$-dependent inhibition of VTA GABA neuron firing. **A,C** Current clamp recordings show spiking of VTA GABA neurons elicited by a current injection (40 pA) in saline (**A**) and meth (**C**) injected mice 24h later. Spiking recorded in the absence (top trace) and presence (bottom trace) of 100mM baclofen. **B,D** Input-output graphs show spike number in GABA neurons as a function of current injection for saline (**B,** N=7) and meth (**D,** N=7) injected mice. *P<0.05 Two Way Repeated Measure ANOVA.

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IV. Chronic effects of psychostimulants

Abstract

Repeated exposure to drugs of abuse leads to lasting changes in plasticity and signaling in the mescocorticolimbic dopamine circuit. Evidence suggests a role for plasticity of inhibitory regulation in the response to drugs of abuse. GIRK (Kir3) channels mediate a slow inhibitory postsynaptic current (sIPSC) in dopamine (DA) neurons. Acute exposure to methamphetamine down-regulates $GABA_BR$-GIRK signaling in GABA and DA neurons. Here we examined the effect of repeated methamphetamine on GIRK currents in VTA DA neurons. Similar to acute administration, repeated methamphetamine down-regulates $GABA_BR$-GIRK signaling in DA neurons. However, there is a requirement for GIRK3 subunits in repeated methamphetamine-induced down-regulation in DA neurons, suggesting distinct mechanisms of plasticity induced by acute or repeated drug exposure in GABA and DA neurons. Repeated psychostimulant exposure enhances the acquisition of behavioral sensitization when drug administration occurs outside of the home cage. Similarly, down-regulation of $GABA_BR$-GIRK currents following methamphetamine exposure requires environmental novelty, demonstrating a role for GIRK channels in the learning-evoked changes that occur in the VTA with psychostimulant-induced sensitization.

Introduction

Drug addiction is a psychiatric disorder characterized by continued use despite severe adverse life consequences, and poses a risk of relapse long after the last drug ingestion (Koob and Le Moal, 2006; Robinson and Berridge, 2003). Addiction is thought to occur through long-term adaptations in the mesolimbic dopamine circuit in response to repeated drug use (Kauer and Malenka, 2007; Renthal and Nestler, 2008). Thus it is necessary to examine molecular and physiological changes that occur in the brain following repeated drug administration for both the prevention of addiction development and improved treatment options.

Recurrent exposure to addictive compounds produces long lasting behavioral adaptations. For example, repeated psychostimulant administration leads to a progressive increase in the locomotor stimulating effects of these drugs in rodents, termed behavioral sensitization (Vanderschuren & Kalivas 2000). The development of sensitization requires increased activity in the mesocorticolimbic dopamine circuit, particularly in the VTA (Vezina & Stewart 1991), and induces plasticity, as sensitization to cocaine potentiates glutamatergic synapses onto VTA dopamine neurons (Ungless et al. 2001, Borgland et al. 2004).

Several lines of evidence suggest a role for inhibitory regulation in plasticity following repeated psychostimulant administration. Repeated cocaine exposure diminishes the ability of dopamine to auto-inhibit dopamine neurons

(Henry et al. 1995) and down-regulates $GABA_A$ receptor signaling (Liu & Poo 2005). Inhibition of $G_{i/o}$ signaling by injection of pertussis toxin into the VTA (Sketetee & Kalivas 1991) and antagonizing NMDAR on non-dopaminergic VTA neurons (Luo et al. 2010) prevents sensitization to psychostimulants. Acute cocaine and CRF enhance the D2R-GIRK slow IPSC in dopamine neurons; however, repeated cocaine or methamphetamine exposure eliminates the CRF enhancement (Beckstead et al. 2004, 2009). Lastly, animals lacking GIRK2 subunits have an enhance sensitization response to cocaine (Morgan et al. 2002). While these studies identify a role of inhibitory regulation in drug sensitization, the mechanisms of inhibitory drug-induced plasticity remain largely unexplored. Thus we sought to examine plasticity of GIRK channels on inhibitory regulation following repeated exposure to psychostimulants.

We have previously found that acute administration of psychostimulants induces a down-regulation of $GABA_BR$-GIRK currents in VTA GABA neurons through a mechanism requiring de-phosphorylation of S783 in the $GABA_BR2$ subunit by PP1/PP2A phosphatases (Chapter 3, Padgett et al. 2012). $GABA_BR$-GIRK currents in dopamine neurons are also depressed following acute methamphetamine, but we find that repeated administration does not down-regulate $GABA_BR$-GIRK currents unless methamphetamine is administered outside the home cage.

Additionally, the mechanism of drug-induced plasticity in dopamine neurons following repeated psychostimulant exposure differs from GABA neurons in a mechanism requiring GIRK3 subunits but not $GABA_BR2$ de-

phosphorylation. This suggests that slow inhibitory regulation undergo plasticity changes in response to drugs of abuse and highlights the role of GIRK channels in novelty detection and development of sensitized responses *in vivo*.

Results

$GABA_BR$-GIRK currents in VTA DA neurons following repeated drug exposure

Repeated administration of a number of different types of drugs with abuse potential induces persistent changes in physiology of dopamine neurons (Di Chiara & Imperato 1988, Kalivas & Stewart 1991; Borgland et al. 2004). To further characterize the effects of psychostimulants on slow inhibitory transmission in dopamine neurons, we examined maximally activated $GABA_B$-GIRK currents in DA neurons following repeated administration of methamphetamine.

Animals were injected with 2mg/kg methamphetamine (i.p.) for 5 days in the home cage. 24-48 hours after the last administration, VTA-containing sections were prepared for electrophysiology. Dopamine neurons were identified primarily by GFP fluorescence in Pitx3-$GFP^{+/-}$ (Labouebe et al 2007) mice that were used in these studies, and in mutant mice as previously described (Ch. 1 & 2). Unlike an acute *in vivo* exposure, repeated methamphetamine administration did not significantly affect baclofen-activated $GABA_BR$-GIRK signaling in VTA dopamine neurons (**Fig. 4.1A**).

Environmental context is required for $GABA_BR$-GIRK current depression

While sensitization of the locomotor stimulant effects of psychostimulants occurs with repeated administration in the home cage, the sensitized response is

much more robust when drug is administered in a separate, novel environment (Badiani et al. 1995, Badiani & Robinson 2004). Thus, it was possible that baclofen-induced currents from home cage-treated animals were not affected due to insufficient induction of methamphetamine sensitization.

We sought to create a novel environment for drug administration. To enhance the novelty cues available to our animals, we used an alternate type of cage wrapped in brightly colored wallpaper with different flooring material, and injections took place in a separate room from the home cage. To minimize stress effects, we kept cagemates together in each novel environment cage. Using this novel environment sensitization protocol, we find a significant decrease in maximally activated $GABA_BR$-GIRK currents in dopamine neurons of methamphetamine-treated animals (**Fig. 4.1B**). Reduced $GABA_BR$-GIRK signaling effect also occurs following 5 days cocaine administered outside the home cage (Aurora et al. 2011), suggesting that repeated psychostimulant exposure (either methamphetamine or cocaine) affects $GABA_BR$-GIRK signaling in dopamine neurons. We additionally quantified sensitization response to methamphetamine using locomotor activity chambers; however, exposure to these chambers produced a significant down-regulation of $GABA_BR$-GIRK currents in saline treated animals for reasons that are unclear (data not shown). Thus we focused our studies on the novel environmental context, which did not affect control measurements.

Methamphetamine-induced $GABA_BR$-GIRK current reduction requires dopamine signaling

Activation of dopamine D1-class receptors is required for locomotor sensitization (Kalivas and Stewart, 1991) and potentiation of glutamatergic synapses with psychostimulants (Argilli et al., 2008; Brown et al., 2010). Antagonizing D1 receptors is sufficient to block physiological adaptations and behavioral adaptations to drugs of abuse (Xu et al. 1994, Witkin et al. 1999, Wall et al. 2011). We sought to investigate the requirement of D1R signaling in the methamphetamine-induced $GABA_BR$-GIRK down-regulation by co-administering the D1 receptor antagonist SCH39166 (0.3mg/kg i.p.) with methamphetamine (2mg/kg). Co-administration of SCH39166 + methamphetamine in a novel environment prevented the decrease in $GABA_BR$-GIRK currents following repeated methamphetamine (**Fig. 4.1E**). Thus, down-regulation of $GABA_BR$-GIRK signaling in dopamine neurons following repeated exposure to psychostimulants requires both dopamine signaling and environmental novelty.

Repeated methamphetamine specifically reduces GABA-mediated inhibition of DA neurons

Concurrent with an observed decrease in maximally activated $GABA_BR$-GIRK currents in DA neurons, there is a reduction in the ability of baclofen to inhibit induced firing (**Fig. 4.1C**). Dopamine neurons from saline treated mice require at least 200pA of current to induce activity in the presence of baclofen, whereas in methamphetamine treated mice, neurons become active with 80-

100pA current (**Fig. 4.1C**). Thus, a down-regulation of $GABA_BR$-GIRK signaling limits the ability of dopamine neurons to regulate activity through slow GABAergic inhibition.

In addition to $GABA_B$ receptors, GIRK channels in dopamine neurons are activated by dopamine D2-type receptors, which are involved in auto-inhibition following dopamine release (Beckstead et al. 2004). To examine the effect of repeated methamphetamine treatment on D2R-GIRK currents, we used the D2-like receptor agonist quinpirole (30μM). Unlike $GABA_BR$-GIRK currents, repeated methamphetamine treatment in a novel environment had no effect on quinpirole-activated D2R-GIRK currents (**Fig. 4.2D**). Thus, methamphetamine treatment triggers a specific change in $GABA_BR$-GIRK signaling.

$GABA_BR$-GIRK current reduction in dopamine neurons occurs by a mechanism requiring GIRK3, not $GABA_BR2$

Acute exposure to methamphetamine induces de-phosphorylation of $GABA_BR2$ S783 (**Fig 3.2**). A serine-to-alanine mutation of the $GABA_BR2$ subunit at position 783 (S783A) prevents activity-induced cell surface removal of $GABA_BR$ in transfected cells (Terunuma et al. 2010). We obtained a de-phosphomimetic mutant mouse line of S783A $GABA_{BR2}$. S783A mutants were acutely or repeatedly exposed to methamphetamine (2mg/kg). Repeated exposure took place in the novel environment context. $GABA_BR$-GIRK signaling in GABA neurons of $GABA_B$ R2 S783A mutants were unaffected by an acute exposure to methamphetamine, consistent with a role of $GABA_B$ receptor

phosphorylation in acute drug-induced plasticity (**Fig. 4.2A**). Interestingly, in dopamine neurons, $GABA_BR$-GIRK signaling was reduced 24-48 hr following repeated (5 days) *in vivo* methamphetamine (**Fig. 4.1B**), suggesting that multiple drug exposures induce plasticity in dopamine neurons through distinct mechanisms.

SNX27 traffics GIRK3-containing channels and transcript expression is increased in VTA following repeated, but not acute, methamphetamine (Chapter 2). Thus, we sought to examine $GABA_BR$-GIRK signaling following repeated methamphetamine administration in a $GIRK3^{-/-}$ mutant mouse (Torrecilla et al. 2002). Repeated methamphetamine administration in a novel environment had no effect on maximal $GABA_BR$-GIRK currents in dopamine neurons of $GIRK3^{-/-}$ mutants (**Fig. 4.2C**), exposing a requirement for GIRK3-mediated trafficking in psychostimulant-induced plasticity following multiple drug exposure.

Discussion

A single exposure to drugs of abuse induces persistent drug-induced plasticity, which can set the stage for compulsive behavior (Ungless et al. 2001, Padgett et al. 2012). Continued drug ingestion, however, upsets that balance, inducing adaptations that promote further drug seeking (Mameli et al. 2009, Luscher & Malenka 2011). A single exposure to cocaine produces plasticity changes at excitatory synapses in VTA dopamine neurons (Ungless et al. 2001, Saal et al. 2003, Padgett et al. 2012). Excitatory LTP following acute exposure in dopamine neurons requires NMDAR activation and dopamine acting through D5 receptor, and is short lived, lasting about 1 week (Ungless et al. 2001, Schilstrom et al. 2006, Zweifel et al. 2008). Repeated administration leads to potentiation in both excitatory and inhibitory synapses, and is sufficient to induce downstream plasticity in the nucleus accumbens (Borgland et al. 2004, Liu & Poo 2005, Bellone & Luscher 2006). Moreover, this potentiation persists for months after last exposure, and only when drug was actively ingested or injected (Chen et al. 2008). It has therefore been suggested that acute psychostimulant-induced plasticity does not occlude additional potentiation that further drug exposure produces (Bellone & Luscher 2006, Mameli et al. 2009).

Metabotropic glutamate receptors were found to prevent the persistence of LTP, an adaptation that is overridden by repeated drug exposure (Mameli et al. 2009). mGluRs mediate a slow IPSC in dopamine neurons through SK channels (Fiorillo & Williams 1998), highlighting a role for slow inhibition in

persistence of plasticity. An acute administration of methamphetamine diminishes $GABA_BR$-GIRK signaling in GABA neurons, representing a potential brake on excitability (chapter 3, Padgett et al. 2012), while repeated exposure minimizes GABAergic inhibition in dopamine neurons through a distinct mechanism requiring GIRK3 subunits (**Fig. 4.1-4.2**). These changes likely work in concert with enhanced excitatory transmission and altered mGluR signaling to produce an overwhelming drive on dopaminergic activity in the VTA that can substantially change the motivation for continued drug seeking.

A weakening of $GABA_BR$-GIRK signaling of VTA DA neurons was recently described as induced by acute or repeated cocaine exposure (Arora et al., 2011), expanding our methamphetamine results to psychostimulants. Unlike what we have found by antagonizing D1R with repeated methamphetamine, Aurora et al. found down-regulation to be D2R- but not D1R-sensitive. However, this study made use of SCH23390, which, in addition to being an effective D1R antagonist, directly antagonizes GIRK currents (Kuzhikandathil & Oxford, 2002, Huang et al. 2005), confounding interpretation of the result. Our data suggest that antagonizing D1Rs is sufficient to block the meth-induced $GABA_BR$-GIRK current depression in both GABA (Padgett et al. 2012) and DA neurons (**Fig. 4.1E**), however, is known that D1R and D2R act in concert to influence acquisition of methamphetamine sensitization (Kelly et al., 2008).

How separate mechanisms of $GABA_BR$-GIRK down-regulation are achieved by drug exposure in GABA and dopamine neurons, and whether this difference is due to cell specificity or drug duration was not directly addressed in

these studies. GABA and DA neurons in the VTA have a number of different mechanisms, for example, at excitatory synapses, paired pulses stimuli produce facilitation or depression in GABA and dopamine neurons, respectively, and dopamine neurons undergo LTP following high frequency stimulation, while GABA neurons do not (Bonci & Malenka 1999). Dopamine D2 receptors are found on dopamine neurons, while GABA neurons express D1 receptors (Weiner et al. 1991, Beckstead et al. 2004, White 1996).

The methamphetamine-induced reduction of $GABA_BR$-GIRK signaling in dopamine neurons after repeated exposure requires GIRK channel trafficking (**Fig. 4.2C**). Reduced signaling could represent plasticity of GIRK channel trafficking, either through an increase in channel degradation or decrease in recycling. GIRK3 channels contain a strong lysosomal targeting sequence and, when complexed with other GIRK subunits, reduce maximal currents (Schoots et al. 1999, Ma et al. 2002). Any change in GIRK3 subunit trafficking is therefore likely to lead to dramatically altered GIRK signaling in neurons. SNX27 regulates trafficking of GIRK3-containing channels in dopamine neurons (chapter 2) and repeated, but not acute, methamphetamine exposure increases expression of SNX27b transcript in VTA (**Fig. 2.1C**). Together, these data suggest a novel mechanism of inhibitory regulation following repeated psychostimulant exposure involving plasticity of GIRK channel signaling in the VTA.

Materials and Methods

Drug administration: Animals were injected with 10ul/g methamphetamine (Sigma) at 2mg/kg (i.p.) or equivalent volume saline. For home cage injections, animals were weighed, injected, and returned to home cage. For novel environment, animals were transported to a new room, weighed, injected and placed in new cage with cagemates for 1 hour, then returned to home cage.

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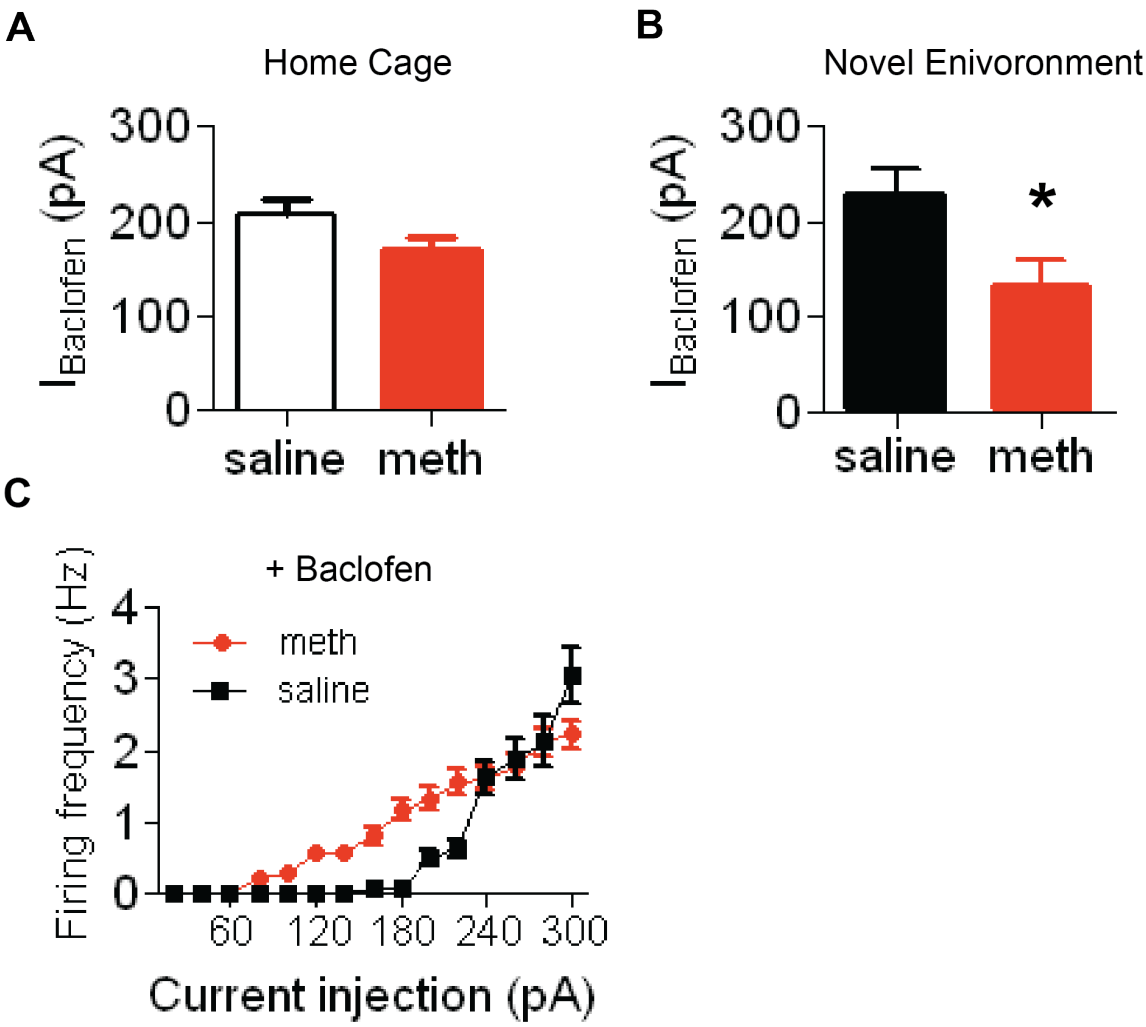
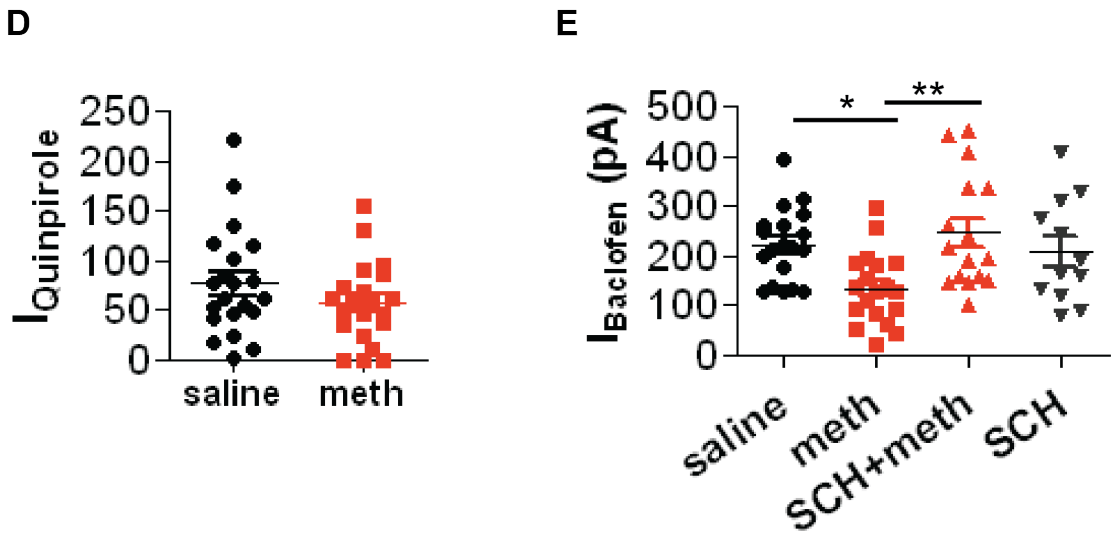
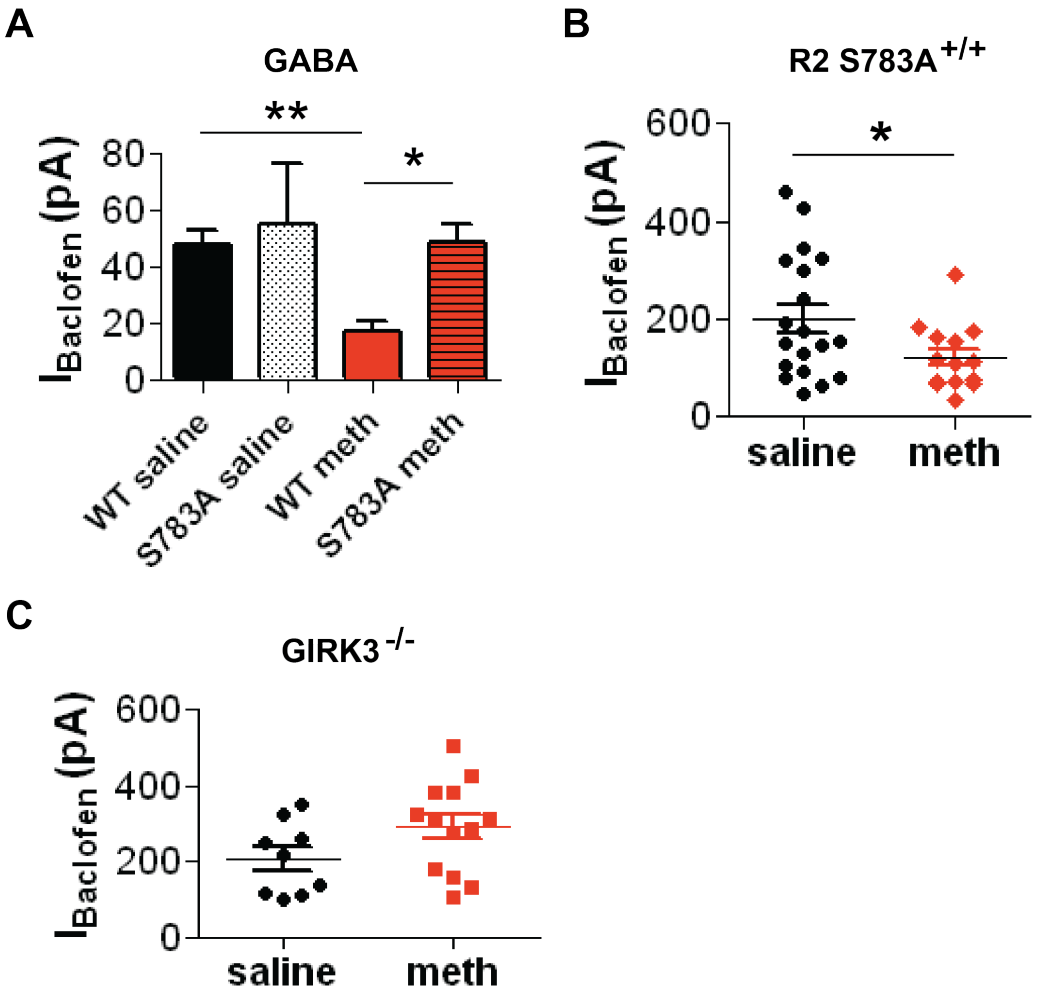


4.1 $GABA_BR$-GIRK signaling is down-regulated following repeated methamphetamine in a novel environment. (A) Baclofen-activated currents ($I_{Baclofen}$) recorded from VTA dopamine neurons 24-48 hr following 5 days injection of saline (0.9%, n=47) or methamphetamine (2 mg/kg, n=58) in the home cage (n= 47 saline, n=58 meth, p>0.05 Student's t test). **(B)** Baclofen-activated currents 24-48 hr following 5 days injection of saline (0.9%, n=10) or meth (2 mg/kg n=10) injected in novel environment (**p<0.0.01, Student's t test). **(C)** Firing frequency in the presence of baclofen.



4.1 (continued) $GABA_BR$-GIRK signaling is down-regulated following repeated methamphetamine in a novel environment. (D) Quinpirole-induced current ($I_{Quinpirole}$) recorded 24-48 hr following 5 days saline or methamphetamine (n.s. Student's t test) in novel environment. **(E)** Baclofen-induced currents 24-48 hr following 5 days injection of saline (0.9%), methamphetamine (2 mg/kg), D1R antagonist SCH39166 (0.3 mg/kg), or SCH39166+methamphetamine in novel environment. (Separate cohort from **(A)**). (*p<0.05, **p<0.01 one-way ANOVA.)



4.2 Methamphetamine-induced $GABA_BR$-GIRK signaling down-regulation requires GIRK3 subunits. (A) Baclofen-induced current in GABA neurons 24 hr after acute saline (0.9%) or methamphetamine (2mg/kg) in WT (n=23 saline, n=21 meth) or $GABA_BR2$ S783A homozygous knockin+ mouse (n=6 saline, n=7 meth). WT data is reproduced from Fig. 3.1. (**A**, courtesy of Dr. Claire Padgett). **(B-C)** Baclofen-induced current in DA neurons recorded 24-48 hr after 5 days injection saline (0.9%) or methamphetamine (2mg/kg) in a novel environment from $GABA_BR2$ S783 knockin **(B)** or $GIRK3^{-/-}$ mice **(C)**. (*p<0.05, **p<0.01 Student's t test or one-way ANOVA with Bonferroni posttest)

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V. Role of SNX27 in addiction

Abstract

G protein-gated inwardly rectifying potassium channels (GIRK or Kir3 channels) mediate a slow inhibitory postsynaptic current in dopamine neurons that can silence activity and are involved in behavioral and physiological responses to drugs of abuse, though how they contribute to stimulant response is not known. Sorting nexin 27 (SNX27) regulates GIRK channels surface expression and is up-regulated in the neocortex and VTA of rodents following methamphetamine sensitization. The effect of psychostimulant exposure on SNX27 trafficking of GIRK channels in the mesolimbic dopamine circuit and *in vivo* is unknown. Psychostimulant response was examined in animals lacking SNX27 specifically in dopamine neurons ($SNX27^{dat-/-}$). $SNX27^{dat-/-}$ animals are hyperactive in a novel environment, a phenotype correlated with addiction development, identifying a previously undescribed role for GIRK channels in novelty detection. $SNX27^{dat-/-}$ animals sensitize to methamphetamine, while cocaine exposure produces an enhanced response. Together, these data demonstrate that drug-induced plasticity affects GIRK channel trafficking by SNX27, opening up new treatment targets for addictive disorders.

Introduction:

Behavioral sensitization is a model of persistent neuroadaptation in addiction, based on the observation that locomotor activity is progressively augmented following repeated administration of drug (Post and Rose, 1976; Vanderschuren and Kalivas, 2000). Animals can be withdrawn from drug for periods ranging from a week to over a year, and upon challenge injection in a similar context, display the heightened response observed after multiple injections (Badiani et al., 1995; Post and Rose, 1976; Vanderschuren and Kalivas, 2000). Sensitization is thought to underlie various components of addiction, particularly drug craving or incentive salience of a drug and to be a measure of susceptibility to addiction in humans (Boileau et al., 2006; Kalivas et al., 1998; Robinson and Berridge, 2003).

Dopamine released from VTA neurons into projection targets of the mesolimbic dopamine circuit is required for reward learning behavior, such as conditioned place preference (Tsai et al., 2009) and behavioral sensitization (Heusner & Palmiter, 2005; Vanderschuren and Kalivas, 2000; Vezina & Queen, 2000). While much research has focused on changes in excitatory transmission in the VTA, less is known about adaptations in inhibitory signaling in these behavioral responses (Filip and Frankowska, 2008; Koob, 2004; Labouebe et al., 2007; Liu et al., 2005).

G protein inwardly rectifying (GIRK) channels mediate inhibitory postsynaptic effects in neurons and have been implicated in response to

psychostimulants (Labouebe et al., 2007; Luscher et al., 1997; Morgan et al., 2002). However, how GIRK channels directly contribute to psychostimulant response *in vivo* has yet to be demonstrated. Two independent observations have highlighted a potential mechanism by which stimulant drugs alter GIRK channel signaling in the VTA: Sorting nexin 27 (SNX27) traffics GIRK3-containing channels, decreasing $GABA_B$ receptor-induced GIRK currents (Lunn et al., 2007), and SNX27 expression is increased in animals sensitized to methamphetamine (Kajii et al., 2003). How changes in the expression of SNX27 affect behavioral response to psychostimulants is unknown.

There are currently few treatment options for addictive disorders and almost none for preventing relapse (Renthal and Nestler, 2008). Thus it is important to identify novel drug targets for the development of improved therapies for addiction and relapse. SNX27 regulation of GIRK channels would represent a novel mechanism of persistent neuroadaptation to psychostimulants and provide new targets for addiction therapy.

Results

Enhanced spontaneous activity in SNX27$^{dat-/-}$ animals

During the locomotor sensitization studies, we noted a consistent change in the acclimatization of SNX27$^{dat-/-}$ animals upon initial exposure to the activity chambers. Animals were therefore tested in an open field environment. SNX27$^{dat-/-}$ mice are hyperactive in a novel open field environment with delayed acclimatization (**Fig. 5.1A-B**). The increase in activity is restricted to the initial exposure to a novel environment (first 30min of 1 hr session, **Fig. 5.1B**), after which time SNX27$^{dat-/-}$ animals habituate to the chamber at wildtype level. All groups spent similar amounts of time in the center (**Fig. 5.1C**), eliminating altered thigmotaxis (wall-hugging) or anxiety levels as possible interpretations for enhanced activity levels, suggesting that response to novel environment is enhanced in animals lacking SNX27 in DA neurons.

Psychostimulant sensitization in SNX27$^{dat-/-}$ animals

Repeated exposure to psychostimulants results in a progressive increase in the locomotor-activating response to the drug, termed behavioral sensitization (Vanderschuren and Kalivas, 2000). SNX27$^{dat-/-}$ and control (SNX27$^{fl/fl}$ (+/+) and/or Dat-cre^{+}) mice undergo a similar enhancement of locomotor activity from baseline following an acute administration of methamphetamine at 2mg/kg (response on D0 vs. D1, **Fig. 5.2A**) following 2 days of saline injections. Sensitization is measured in response to baseline activity on the second day of

saline injection to control for activity changes upon introduction to activity chambers. Repeated administration of methamphetamine over a series of 5 days led to a significant enhancement of locomotor activity in all groups (D1 vs. D5), demonstrating acquisition of behavioral sensitization. Similarly, after 2 weeks of withdrawal, a challenge injection of methamphetamine led to a sensitized response in both genotypes (D1 vs. D19). Thus, SNX27$^{dat-/-}$ animals sensitize to 2mg/kg methamphetamine similar to control.

It is possible that 2mg/kg methamphetamine produced a ceiling effect in locomotor response to drug that would mask sensitivity changes to drug. 2mg/kg is a median dose of methamphetamine in mice (Fukushima et al., 2007; Scibelli et al., 2010; Shimosato et al., 2001). Therefore, we examined sensitization to a dose of drug at the basal level of detection, 0.5mg/kg methamphetamine (**Fig. 5.2B**). No animals of any genotype responded to an acute dose of 0.5mg/kg methamphetamine (D0 vs. D1), however, repeated administration produced a sensitized response in WT mice over 5 days. SNX27$^{dat-/-}$ mice and Dat-cre^{+} controls failed to sensitize to repeated administration of low-dose drug (D1 vs. D5). The lack of sensitization in Dat-cre^{+} mice suggests an occlusion of dopamine transporter function in the cre allele and therefore that the lack of sensitization in SNX27$^{dat-/-}$ mice is due to presence of cre. Thus the altered sensitization response in Dat-cre^{+} controls could be masking a phenotype in SNX27$^{dat-/-}$ mice, preventing determination of the role of SNX27 in methamphetamine response.

Mrt1 expression is increased with both methamphetamine and cocaine treatment (Kajii et al., 2003), therefore, we measured response to cocaine in $SNX27^{dat-/-}$ animals. At 20mg/kg (i.p.), cocaine increased locomotor activity in control Dat-cre+ animals by 2.8-fold after 2 days of saline injections, and in $SNX27^{dat-/-}$ animals, the response is enhanced, with activity increasing by 9.9-fold (**Fig. 5.3**). SNX27 in dopamine neurons affects the *in vivo* response to cocaine.

Discussion

The role of dopamine neurons in sensitization has only recently been resolved (Luo et al., 2010). Antagonizing NMDA receptors in the VTA is sufficient to prevent development of amphetamine sensitization (Vanderschuren and Kalivas, 2000; Vezina and Queen, 2000), however, selective deletion of NMDAR in dopamine neurons does not prevent psychostimulant sensitization (Beutler et al., 2011; Zweifel et al., 2008). In the same mutant lacking dopamine NMDAR, antagonizing NMDAR was still sufficient to prevent cocaine sensitization, demonstrating a key role for non-dopaminergic VTA neurons in sensitized responses (Luo et al., 2010).

Additionally, several studies have elegantly demonstrated that expression of sensitization requires excitatory transmission in D1R-expressing medium spiny neurons (MSNs) of the nucleus accumbens, using NMDAR-D1R mutant mice or optogenetic control of D1R-MSNs (Beutler et al., 2011; Heusner and Palmiter, 2005; Pascoli et al., 2011). LTP in D1R MSNs occurs on a similar time course to development of sensitization, and de-potentiation of LTP is sufficient to eliminate cocaine-induced sensitization (Pascoli et al., 2011). Interestingly, repeated administration of psychostimulants produces LTP in nucleus accumbens that requires LTP induction in dopamine neurons through mGluR1-dependent modulation (Mameli et al., 2009), thus demonstrating a link by which enhanced dopaminergic activity can induce sensitized behaviors that require D1R MSNs. It is therefore not unreasonable to postulate that dis-inhibition of dopamine neurons

through reduced GABAergic response enhances dopamine transmission to induce LTP and sensitization in D1R MSNs, that lead to the basal ganglia direct pathway stimulation of locomotion (Kravitz et al., 2010).

Amphetamines exert their action by both blocking reuptake of released dopamine and reversing the dopamine transporter, causing non-vesicular release (Fleckenstein et al., 2007; Sulzer, 2011), while cocaine binds to the DAT and only prevents reuptake to induce reward response (Chen et al., 2006). Cocaine produces ~75% increase in dopamine concentration in the nucleus accumbens after either acute or sensitized responses, while methamphetamine acutely enhances dopamine by ~130%, with sensitization producing a nearly 400% change in nucleus accumbens dopamine concentration (Zhang et al., 2001). The massive outpouring of dopamine by methamphetamine is likely to overpower the effect of enhanced activity and plasticity in dopamine neurons in nucleus accumbens LTP induction, which may explain why sensitization to a median range dose of methamphetamine (2mg/kg) (Fukushima et al., 2007; Scibelli et al., 2010; Shimosato et al., 2001), did not affect sensitization in SNX27$^{dat-/-}$ animals while cocaine and the natural reward of novelty produce a significant acute enhancement.

It should be noted that we observed a drug response phenotype in the dat-cre animals, which may mask some addiction phenotypes in SNX27 KO animals. Cyclic voltammetry measurement of dopamine signal duration in Dat-cre$^{+/-}$ mice shows slightly impaired dopamine re-uptake (Hernandez et al., 2012), which could explain the difference in drug detection observed in these animals.

However, laboratories that utilize the Dat-cre line for conditional gene deletion use of Dat-cre$^{+/-}$ as a control, ensuring that any difference in dopamine reuptake is controlled within the study (Beutler et al., 2011; Parker et al., 2011; Zweifel et al., 2008; Zweifel et al., 2011).

Enhanced novelty response is a strong indicator of addiction-like behavior in both humans and rodents (Belin et al., 2010; Marinelli and White, 2000). Dopamine neurons increase activity in response to novel stimuli and aid in hippocampal LTP to spatial novelty (Li et al., 2003; Schultz, 2007). GIRK2$^{-/-}$ animals are hyperactive in a novel environment (Blednov et al., 2001). Our study suggests that the source of the hyperactive phenotype in GIRK2$^{-/-}$ animals is altered $GABA_BR$-GIRK-mediated inhibition in dopamine neurons. Several other studies of genetic deletion specifically in dopamine neurons have identified a hyperactivity or novelty phenotype in their mutants, including DAT (Pogorelov et al., 2005), D2R (Bello et al., 2011), $GABA_BR1$ and R2 subunits (Mombereau et al., 2005; Schuler et al., 2001; Vacher et al., 2006), hypothalamic neurons sending GABAergic projections to VTA (Dietrich et al., 2012), and K_{ATP} channel in substantia nigra (Schiemann et al., 2012).

Interestingly, the common denominator across these studies is a change in slow inhibitory or auto-inhibitory input onto DA neurons. NMDAR dopamine neuron deletion either does not affect (Zweifel et al., 2008) or leads to decreased activity in a novel environment (Luo et al., 2010), and $GABA_AR$ dopamine deletion has no effect on locomotor activity (Parker et al., 2011). Therefore, it is not surprising that SNX27$^{dat-/-}$ animals, with decreased slow GABAergic inhibition,

show a similar enhanced response to novelty. This suggests that GIRK channels are key players in novelty detection, which has not been described previously.

Enhanced reaction to novelty in mutant mice often correlates with an augmentation of response to cocaine (Arora et al., 2010; Bello et al., 2011; Dietrich et al., 2012), similar to SNX27$^{dat-/-}$ animals that demonstrate both enhanced activity in a novel environment and cocaine supersensitivity, highlighting the importance of VTA dopamine neuron inhibitory regulation as critical for mediating reward response *in vivo*.

Materials and Methods

Open field test: All behavior experiments were conducted in the light phase from 1100-1500. Animals were brought into the testing room at least 30min prior to the start of behavioral experiments, then placed individually into locomotor activity chambers (Med Associates, St Albans, VT) for 1 hr. Total distance traveled was recorded in 5 min bins for 60 min. Zone analysis was conducted to determine time spent in center, defined as area of open field excluding peripheral 5 inches on each side of 16x16" square. The same procedure was repeated a second day to measure response to repeated exposure to novel environment.

Behavioral Sensitization: Male and female mice age P30-P70 underwent behavioral sensitization to methamphetamine (2mg/kg i.p.). Total distance traveled was measured using locomotor activity chambers (Med Associates, St Albans, VT). Animals were habituated to the testing chamber each test day for 30 minutes. Test day -1 and 0, all animals received saline. Days 1-5, animals received methamphetamine (2mg/kg i.p.), cocaine (20mg/kg), or equivalent volume of saline at 1ml/kg. Following injection, locomotor activity was monitored for 60 minutes. In some studies, animals were withdrawn from drug for 2 weeks. On test D19 (2 week withdrawal), animals received challenge injections of methamphetamine (2mg/kg i.p.) for validation of sensitized phenotype (with methamphetamine pre-treatment).

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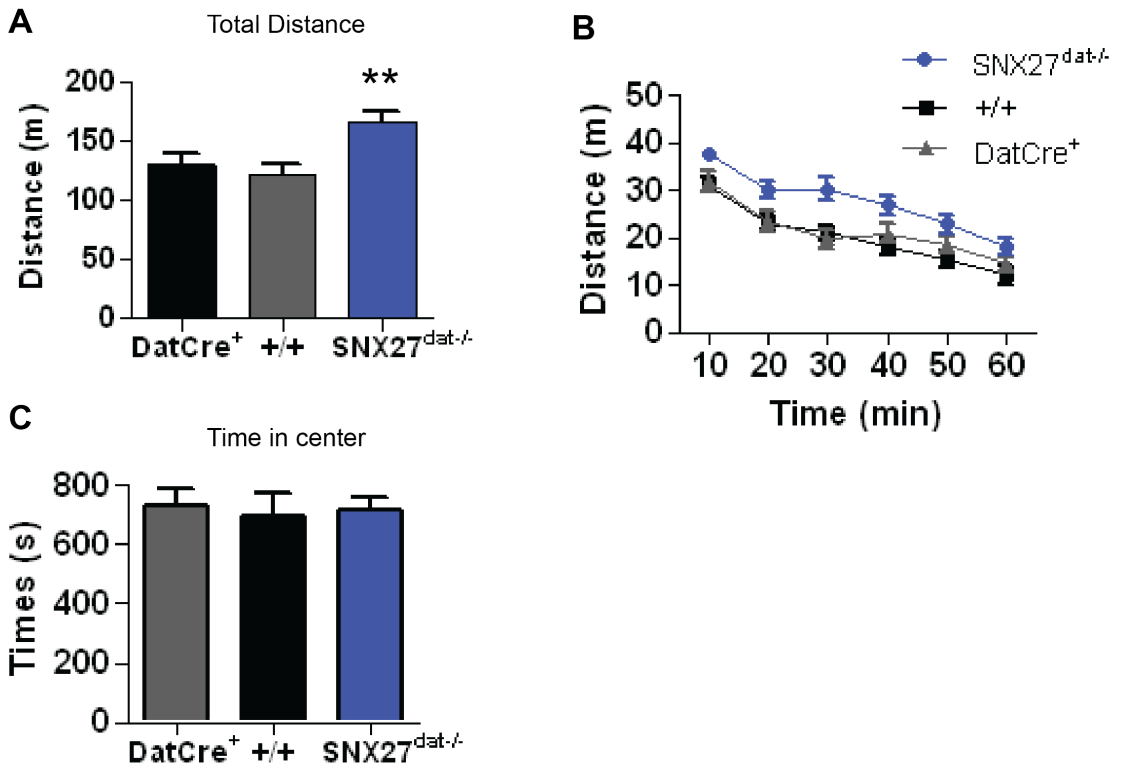
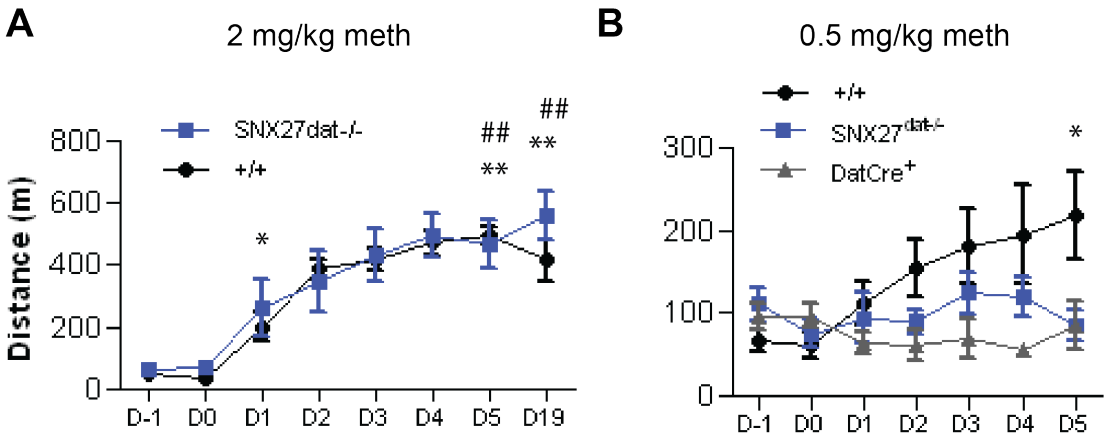
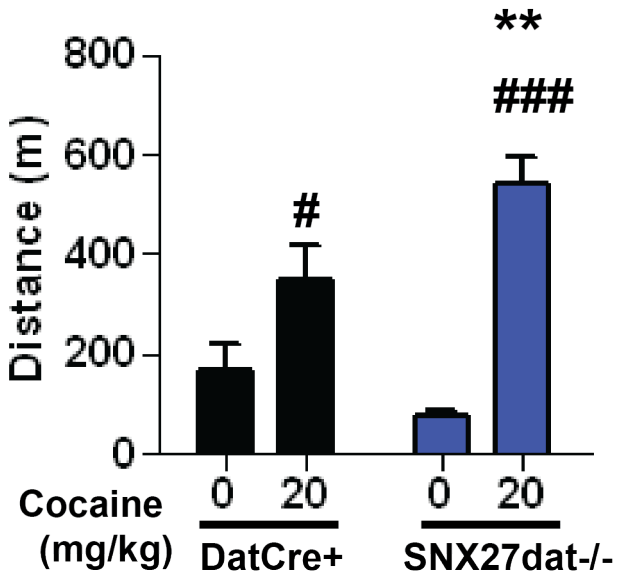


5.1 Spontaneous locomotor activity in SNX27$^{dat-/-}$ mice. (A) Total distance traveled in novel open field environment by Dat-cre (n=17), WT (n=18) and SNX27$^{dat-/-}$ (n=17) mice. (**p<0.01 vs WT, *p<0.05 vs Dat-cre, one-way ANOVA with Bonferroni posttests.) **(B)** Distance traveled in same open field separated into 10-minute bins. **(C)** Time spent in center of chamber (10in squared of 16in squared chamber, n.s. one-way ANOVA).



5.2 Methamphetamine sensitization in SNX27$^{dat-/-}$ mice. (A-B) Locomotor activity plotted as function of treatment day. D-1 and D0 all animals receive saline (0.9% i.p.). D1-D5 animals receive 2 mg/kg **(A)** or 0.5 mg/kg **(B)** methamphetamine. After 5 days injections, animals are withdrawn for 2 weeks and returned to chamber on day 19 (D19) for challenge injection of methamphetamine (2 mg/kg) **(A)**. (A, n=8/group, B, n=8 WT, n=8 SNX27$^{dat-/-}$, n=4 DatCre+; *p<0.05, **p<0.01 vs D0, $^{\#\#}$p<0.01 vs D1, two-way repeated measures ANOVA)



5.3 Cocaine supersensitivity in SNX27$^{dat-/-}$ mice. Locomotor activity in Dat-cre^{+} (n=6) or SNX27$^{dat-/-}$ (n=8) mice following injection of saline (0.9%) or cocaine (20mg/kg). ($^{\#}p<0.05$, $^{\#\#\#}p>0.0001$ drug $^{**}p<0.01$ interaction two-way ANOVA)

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VI. Discussion

Role of SNX27 and GIRK channels in novelty detection

VTA dopamine neurons respond to novel stimuli (Schultz 2007) in a functional circuit believed to involve hippocampal input onto ventral pallidum, whose GABAergic projections to VTA dopamine neurons preferentially activate $GABA_B$ receptors (Sugita et al. 1992, Lisman & Grace 2005). High preference for environmental novelty is a strong indicator of cocaine abuse in humans (Kreek et al. 2005) and high novelty-seeking rodents more quickly acquire sensitization and self-administration to psychostimulants (Hooks et al. 1991, Blanchard et al. 2009).

Interestingly, dopamine neurons from high novelty rats have increased basal activity and burst firing in VTA dopamine neurons (Marinelli & White 2000). $SNX27^{dat-/-}$ animals are high responders in a novel environment (**Fig. 5.1**) and have increased induced dopamine neuron activity (**Fig. 2.3**). Additionally, the ability of repeated methamphetamine to inhibit GIRK currents in VTA dopamine neurons requires environmental novelty (**Fig. 4.1**), much like sensitization response outside of the home cage (Badiani & Robinson 2004). These data therefore uncover a previously undescribed role of GIRK channels in dopamine neurons as molecular effectors of novelty processing.

Endocytic regulation by SNX27 in neurons

Sorting nexins direct trafficking of targets either for lysosomal degradation or cell surface recycling (Worby & Dixon 2002). How similar proteins can mediate such divergent responses is still being elucidated. It was recently postulated that the function of sorting nexins depend on the cocktail of different molecules encountered in the early endosome, including multiple sorting nexins or retromer complexes, that are able to differentially affect trafficking outcomes (Johannes & Wunder 2011, Robinson et al. 2012). SNX27, in addition to the PDZ binding motif that interacts with GIRK3 or β2AR, contains an RA / FERM-like domain which binds Ras GTPase (Lunn et al. 2007, Ghai et al. 2011), implicating SNX27 in an endosomal signaling hub, translating external activation to coordinated action in multiple downstream targets. GIRK3 contains a strong lysosomal targeting sequence (Ma et al. 2002), and its co-expression with any other GIRK subunit decreases GIRK current (Schoots et al. 1999), thus it is possible that endosomal retention of GIRK3-containing channels by SNX27 allows for the degradation signal to dominate trafficking.

The data presented in this thesis represent, to the best of my knowledge, the first description of a functional role for a sorting nexin in the brain. A number of sorting nexins and their binding partners have been identified in the brain and are often developmentally regulated (Kajii et al. 2003, Shin et al. 2009, Rodal et al. 2011, Zhou et al. 2012, Mizutani et al. 2012), and altered endocytic regulation has been implicated in a number of neuronal disorders, including Alzheimer's

and Huntington's disease (Lee et al. 2008, Li & DiFiglia 2012, Zhou et al. 2012). However, the role sorting nexins play in regulating neuronal activity is largely unknown. In chapter 1 I have demonstrated that SNX27 regulates forward trafficking of GIRK channels in dopamine neurons and the ability to respond to inhibitory input.

Both over-expression of SNX27 in hippocampal neurons (Lunn et al. 2007) and dopamine neurons (**Fig 2.7**) and deletion in dopamine neurons lead to a functional decrease in maximally activated GIRK currents. siRNA-mediated depletion of SNX27 in cultured cells leads to decreased surface expression of the beta(2)-adrenoreceptor (Temkin et al. 2011, Lauffer et al. 2010). Similar results have been observed with a number of different regulatory proteins, including sorting nexins, and it has been suggested that any abnormality in levels of key endocytic proteins impairs the homeostasis of protein networks and leads to disrupted activity (Shin et al. 2007).

For example, SNX9 is able to form dimers, and increased expression has been suggested to act as a dominant negative, leading to similar defects in recycling as protein knockdown (Shin et al. 2007). At this time, the ability of SNX27 to dimerize remains unknown, though dimerization could represent an intriguing mechanism for fast alteration of SNX27 regulation *in vivo*. While most dimerization of sorting nexins occurs through the BAR domain (van Weering et al. 2012), which is lacking in SNX27 (Teasdale & Collins 2012, Carlton et al. 2005), PDZ domains are capable of forming dimers (Chang et al. 2011), and

SNX27 PDZ domain could potentially be a part of this group, though additional experiments are required to determine an interaction.

Both increased or decreased levels of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2), which is regulated by SNX9, leads to postnatal lethality within several hours of birth, with no obvious developmental defects (Cremona et al. 1999, Di Paolo et al. 2004), much like what is observed with congenic deletion of SNX27 (Supplemental Fig 1, Cai et al. 2011). The lethality appears to be due to impaired synaptic transmission and vesicle recycling, leading to an inability to feed after birth (Di Paolo et al. 2004). SNX27 has been implicated in vesicle recycling in T cells, via a PDZ-domain interaction with diacylglycerol kinase zeta (DAG-zeta) (Rincon et al. 2007). DAG-zeta is expressed in neurons and its deletion, though not lethal, leads to reduced mini-EPSP frequency and diminished maintenance of dendritic spines (Kim et al. 2009). It is likely that compensatory mechanisms by other DAGs, such as DAG-alpha, which has highly overlapping function in T cells (Zhong et al. 2003), allow for vesicular trafficking and survival of the DAG-zeta$^{-/-}$ animal, unlike the SNX27 knockout. Future studies will be needed to confirm the role of SNX27 interaction with neuronal DAG-zeta in postnatal lethality of SNX27$^{-/-}$ mice.

GIRK channel plasticity in addiction

SNX27 in dopamine neurons selectively attenuates inhibitory $GABA_BR$-GIRK signaling (chapter 2). What does this mean for dopamine function and

psychostimulant-induced plasticity? $GABA_B$ receptors in dopamine neurons are selectively activated by GABAergic ventral pallidum projections to VTA (Sugita et al. 1992). Silencing ventral pallidum GABAergic afferents onto VTA increases the population activity of dopamine neurons and extra-synaptic dopamine release in nucleus accumbens (Floresco et al. 2003). It therefore seems likely that basal dopamine levels are affected by SNX27 trafficking, rather than dopamine release through burst firing. Changes in tonic dopamine levels through population activity are implicated in a number of behavioral processes, including working memory, sensory discrimination and planning (Schultz 2007), and has been suggested to underlie the memory deficits that occur in drug abusers (Schultz 2011) and the pathogenesis of psychiatric disorders such as Tourette syndrome (Buse et al. 2012). Thus, SNX27 regulation in dopamine neurons can potentially mediate a number of behaviors in humans, in addition to drug response.

Here I propose a model where acute psychostimulant exposure induces adaptations in VTA GABA neurons through phosphorylation-dependent trafficking of the $GABA_BR$ (chapter 3), while repeated exposure to psychostimulants induces additional adaptations in dopamine neurons. SNX27b expression is increased in the mesocorticolimbic dopamine regions of cortex and VTA after multiple drug exposure (chapter 1, Kajii et al. 2003) and increased SNX27b expression, potentially through dimerization or other protein-protein interaction, occludes signaling, disrupting forward trafficking of GIRK3-containing channels and targeting them for lysosomal degradation (chapter 3, Lunn et al. 2007). Altered SNX27 expression and altered GIRK subunit composition is likely

to occur in multiple neurons in the brain. We can postulate that in dopamine neurons, which only express GIRK2c and GIRK3 (Cruz et al. 2004), the effect of reduced GIRK3 forward trafficking produces a net decrease of $GABA_BR$-GIRK signaling that is more augmented in dopamine neurons than in other brain regions. Reduced $GABA_BR$-GIRK current would potentially increase the population activity of dopamine neurons, raising basal dopamine levels and promoting persistent LTP induction in VTA (Borgland et al. 2004, Mameli et al. 2009). Persistent LTP in dopamine neurons aids induction of LTP in D1R MSNs of the nucleus accumbens (Mameli et al. 2009), which promote locomotion through the basal ganglia direct pathway (Kreitzer et al. 2010), thus enhancing the locomotor response to cocaine (chapter 4). While much more work will need to be done to definitely prove this model, the work presented in this thesis establishes a solid framework for a novel mechanism psychostimulant-induced plasticity of GIRK channel signaling in neuronal regulation and psychostimulant response.

Future Perspective

The development of the knockout mouse has provided significant insight into the function and behavioral relevance of gene products *in vivo*. Moving forward from the traditional knockout, the need to control the specificity and timing of gene deletion in specific cell types led to the conditional knockout. Our generation of a SNX27 conditional mutant has started to elucidate the neuronal

function of SNX27 *in vivo*, but there is much more to be learned. Crossing to an ever-expanding list of cre-driver lines will allow more specific descriptions of SNX27 function, both macroscopic and microscopic.

For instance, deletion of SNX27 in hippocampal neurons, that are relatively easy to culture, offers the potential to probe further into the cell biology of SNX27 in endocytic regulation, and, on the opposite scope, the effect of inhibitory regulation on hippocampal-dependent behavior. Similarly, more complex behavioral assays are available to assess impaired neuronal inhibition in VTA using the $SNX27^{dat-/-}$ mutants, such as self-administration and *in vivo* physiology with fast-scan cyclic voltammetry to demonstrate how activity and dopamine release are affected by altered GIRK channel trafficking.

The importance of plasticity is becoming increasingly evident, not just in enhancing excitatory transmission, but in regulating the balance of excitation and inhibition in complex behavior. Additional studies will help to confirm the function of GIRK channel regulation in reward processing and role in addiction. It is my hope that many exciting new findings and potential treatment targets will stem from the work presented here.

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