THE ROLES OF EXTRA-SYNAPTIC SEROTONIN RECEPTORS IN NEURONAL SIGNALING AND BEHAVIOR

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Abstract

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Serotonin signaling is implicated in a variety of psychiatric conditions and regulates behaviors through both synaptic and extra-synaptic neurotransmission. Two serotonin receptors, the 5-HT_{1B} and 5-HT_6 receptors, are noted for their mediation of extra-synaptic serotonin signaling and unique localization. 5-HT_{1B} receptors localize to axon terminals and inhibit the release of neurotransmitters. 5-HT_6 receptors localize to neuronal primary cilia where they serve as excitatory receptors and are particularly abundant in the direct and indirect pathway neurons of the striatum. While many aspects of the serotonergic system have been well studied, the ways in which these extra-synaptic serotonin receptors modulate neuronal signaling and circuit-specific behaviors are poorly understood. We first describe the creation of neuronal cell lines with stable expression of 5-HT_{1B} receptors and loss of either isoform of β-arrestin in

order to elucidate the intracellular signaling mechanisms of 5-HT_{1B} receptors. We next use these cell lines to show that 5-HT_{1B} receptors activate ERK1/2 signaling in a $G\alpha_s$- and β-arrestin-dependent manner. Finally, we demonstrate that 5-HT_6 receptors modulate cocaine reinforcement in a pathway-specific manner; specifically, increased 5-HT_6 receptor signaling in the indirect pathway of the nucleus accumbens reduces cocaine taking by increasing the reinforcing properties of the drug. Together, these findings increase our understanding of extra-synaptic serotonin receptors and the mechanisms by which they influence intracellular signaling cascades and circuit-specific behaviors.

DEDICATION

This dissertation is dedicated to my incredibly supportive mother, Lorie, and husband, Ted. Mom, thank you for nurturing my passion for science from an early age, for your unconditional love, and for teaching me what it means to be resilient in the face of adversity. Ted, thank you for being my partner throughout graduate school, and for all the encouragement, help, and love you have given me during this journey.

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LIST OF ABBREVIATIONS

5-HT: 5-hydroxytryptamine (serotonin)

AAV: adeno-associated virus

AC: adenylyl cyclase

ANOVA: analysis of variance

cAMP: cyclic adenosine monophosphate

CNS: central nervous system

CPP: conditioned place preference

CRISPR: clustered regularly interspaced palindromic repeats

DRN: dorsal raphe nucleus

Dyn: dynorphin

Enk: enkephalin

ERK: extracellular signal-related kinase

FR: fixed ratio

GDP: guanosine diphosphate

GFP: green fluorescent protein

GTP: guanosine triphosphate

GIRK: G protein-coupled inwardly-rectifying potassium channel

GPCR: G protein-coupled receptor

GSK3β: glycogen synthase kinase-3β

HA: hemagglutinin

HIV: human immunodeficiency virus

HSV: herpes simplex virus

JNK: c-Jun N-terminal kinase

KO: knockout

MAPK: mitogen-activated protein kinase

MEK: MAPK/ERK Kinase

MSN: medium spiny neuron

N2A: Neuro2A

NAc: nucleus accumbens

NAcSh: nucleus accumbens shell

PI3K: phosphoinositide 3-kinase

PR: progressive ratio

SA: self-administration

SERT: serotonin transporter

sgRNA: single guide RNA

shRNA: short hairpin RNA

SSRI: selective serotonin reuptake inhibitor

TPH2: tryptophan 5-hydroxylase 2

VLP: virus-like particle

VSV: vesicular stomatitis virus

CHAPTER 1

Introduction

Serotonin Neurotransmission in the Brain

Serotonin was first characterized in 1948 by Rapport and colleagues when it was identified as a chemical in blood serum capable of modulating vascular tone (Rapport et al., 1948a; Rapport et al., 1948b). In 1953 serotonin was found in the mammalian brain and the abbreviation of the chemical name (5-hydroxytryptamine, 5-HT) became standard in the field of pharmacology (Feldberg & Toh, 1953; Twarog & Page, 1953). In the years following its discovery, 5-HT has been elucidated as a neurotransmitter with a diversity of functions ranging from regulation of cellular activity to modulation of complex behaviors. 5-HT neurotransmission has a dramatic influence on mood and emotion, with dysfunction of the serotonergic system contributing to a variety of psychiatric conditions including depression, anxiety, and panic disorders (Mahar et al., 2014). First-line treatment for these disorders often involves a selective serotonin reuptake inhibitor (SSRI) that increases 5-HT availability in the brain by acting on the serotonin transporter (SERT) (Mahar et al., 2014). 5-HT directly engages fear and anxiety circuits, most notably in the extended amygdala, and increased 5-HT release is associated with acute stress responses (Marcinkiewcz et al., 2016). On the other hand, depression and chronic stress are linked to decreased serotonergic activity. Interestingly, the rapid rise in 5-HT levels following SSRI administration is not immediately sufficient for an antidepressant effect and often takes weeks for a therapeutic effect (Mahar et al., 2014). This indicates more long-term processes are likely involved in the regulation of depression by 5-HT.

Only 0.1% of neurons in the mammalian brain synthesize and release 5-HT (approximately 450,000 neurons in humans and 20,000-26,000 in rats and mice), and these neurons innervate almost every region of the brain as well as a large portion of the spinal cord (Jacobs & Azmitia, 1992; Okaty et al., 2019). The vast majority of forebrain serotonergic innervation comes from the dorsal raphe nucleus (DRN) in the brainstem, from which subpopulations of neurons project to distinct brain regions and regulate different behaviors (Lowry et al., 2008). Serotonergic neurons express specific proteins for the generation, packaging, release, and reuptake of 5-HT. The expression of tryptophan 5-hydroxylase 2 (TPH2) is necessary to convert the amino acid tryptophan into 5-hydroxytryptophan, which is then converted to 5-hydroxytryptamine by aromatic L-amino-acid decarboxylase (AADC). The vesicular monoamine transporter 2 (VMAT2) enables the packaging of 5-HT into synaptic vesicles for release, after which it can be taken back up by presynaptic SERT and repackaged or broken down by monoamine oxidase A (MAOA) or MAOB (Okaty et al., 2019). In the central nervous system (CNS), TPH2 is the only protein in this pathway specifically expressed by serotonergic neurons and thus serves as a marker for analyzing this population (Okaty et al., 2019; Walther et al., 2003).

Quantal release of 5-HT occurs via vesicular exocytosis (bulk transport) and is dependent on calcium influx following membrane depolarization (De-Miguel & Trueta, 2005). The mode of 5-HT release from axons varies depending on brain region and morphology of axon fibers. In some brain regions the vast majority of serotonergic axon terminals form direct synaptic connections with dendrites (e.g. the substantia nigra pars reticulata and visual cortex), while in others much of the serotonergic neurotransmission

occurs via paracrine signaling in which 5-HT acts as a volume neurotransmitter (e.g. the nucleus accumbens, basolateral amygdala, substantia nigra pars compacta, and spinal cord). In the latter scenario, 5-HT can be released from the soma, varicosities along the axon fiber, or terminal boutons and can diffuse many micrometers in the extracellular space (Bunin & Wightman, 1998; Bunin & Wightman, 1999; Fuxe et al., 2007; Moukhles et al., 1997; Okaty et al., 2019; Steinbusch, 1981; Steinbusch et al., 1978). Synaptic 5-HT is released from both clear and dense core vesicles at active zones following depolarization. 5-HT released outside of the synapse has been shown to come from clusters of vesicles in the absence of active zones and depends on stimulation frequency (De-Miguel & Trueta, 2005; Del-Bel & De-Miguel, 2018).

While volume transmission enables the extracellular diffusion of 5-HT, diffusion can also occur in cases of synaptic transmission. Evidence suggests that the distribution of transporters for serotonin reuptake predominantly determines the extent to which 5-HT diffuses in the extracellular space. Certain brain regions contain lower SERT levels, allowing for more 5-HT diffusion at both synaptic and non-synaptic sites of release (Eulenburg & Gomeza, 2010). Interestingly, experiments using SERT knockout mice demonstrate that while SERT mediates the majority of 5-HT uptake at low concentrations (less than 100 nM), non-SERT uptake of 5-HT is substantial at moderate to high concentrations and implicates other transporters in the regulation of 5-HT diffusion (Hagan et al., 2011). As a result of both 5-HT diffusion and reuptake, extra-synaptic 5-HT receptors are often exposed to lower concentrations of 5-HT compared to synaptic receptors which allows them to serve a more modulatory role. Thus, temporal activation and deactivation of these receptors also differs from synaptic receptors and

occurs over a slower timescale (Bunin & Wightman, 1998; Bunin & Wightman, 1999; Okaty et al., 2019).

There are 14 known receptors that mediate the effects of 5-HT neurotransmission. Thirteen of these 5-HT receptors are G protein-coupled receptors (GPCRs), the exception being the 5-HT_3 receptor which is an excitatory cation channel. Receptors from the 5-HT_1 and 5-HT_5 families are inhibitory G_i-coupled receptors, and 5-HT_2, 5-HT_4, 5-HT_6, and 5-HT_7 are excitatory G_s-coupled receptors. 5-HT receptors also differ based on their cellular localization and expression in both specific cell populations and brain regions (Nichols & Nichols, 2008). In particular, two receptors are noted for their unique cellular and extra-synaptic localization: the 5-HT_{1B} and 5-HT_6 receptors. These receptors are capable of sensing 5-HT in the extracellular space and can distinctively influence the cellular dynamics of neurons that respond to 5-HT. Both 5-HT_{1B} and 5-HT_6 receptors have relatively high affinities for 5-HT compared to other receptors such as those in the 5-HT_2 family. Consequentially, they are well suited to serve as extra-synaptic receptors due to their activation at lower 5-HT concentrations (Barnes & Sharp, 1999; Marazziti et al., 2013; McDevitt & Neumaier, 2011).

5-HT_{1B} Receptor Signaling and Behavioral Implications

5-HT_{1B} receptors are G_i-coupled receptors capable of inhibiting neuronal cAMP signaling. This receptor was characterized in 1991 and was subsequently found to localize to axons and near nerve terminals outside of the synapse (Boschert et al., 1994; Jin et al., 1992; Maroteaux et al., 1992; Voigt et al., 1991). Interestingly, these receptors can act as both heteroreceptors on non-serotonergic cells as well as

autoreceptors on serotonergic axons (Hen, 1992). 5-HT_{1B} autoreceptors play a critical role in regulating 5-HT neurotransmission throughout the brain by providing a negative feedback mechanism to reduce 5-HT release and increase reuptake in a rapid and region-specific manner. This regulation of 5-HT release occurs via alterations to the pathways and proteins responsible for 5-HT synthesis, release, and reuptake, all of which can be affected by 5-HT_{1B} receptor activation (Daws et al., 2000; Hagan et al., 2012; Hjorth et al., 1995; Middlemiss & Hutson, 1990). Due to their localization to axon terminals and distribution throughout the entire brain, 5-HT_{1B} receptors are well suited for the rapid detection and response to 5-HT levels in a specific area. This is particularly important in the case of 5-HT_{1B} autoreceptors which enable rapid, real-time feedback for serotonergic neurons.

Although the characteristics of the 5-HT_{1B} receptor as a GPCR have been well described, a detailed understanding of the mechanisms by which these receptors influence cellular dynamics has yet to be achieved. These receptors specifically couple to $G\alpha_{i/o}$ proteins and inhibit adenylyl cyclase, which in turn inhibits cAMP production and reduces neuronal signaling. Additionally, 5-HT_{1B} receptors promote hyperpolarization through activation of G protein-coupled inwardly-rectifying potassium channels (GIRKs) (Ghavami et al., 1997; Nichols & Nichols, 2008). 5-HT_{1B} receptor interaction with other intracellular kinases have been noted but the mechanisms of these signaling pathways are poorly understood. These receptors can engage both extracellular signal-related kinase 2 (ERK2) and p70 S6 kinase in a Gα- and phosphoinositide 3-kinase (PI3K)-dependent manner (Mendez et al., 1999; Pullarkat et al., 1998), and stimulation of 5-HT_{1B} receptors is capable of indirectly activating Akt1 via MEK1/2 (Hsu et al., 2001).

Coupling of 5-HT_{1B} receptors and glycogen synthase kinase-3β (GSK3β) is important for receptor activation and internalization following agonist binding, and coupling with p11 enhances receptor function and expression at the cell surface (Chen et al., 2009; Li & Jope, 2010; Svenningsson et al., 2006; Zhou et al., 2012). Although these studies shed light onto the potential mechanisms of 5-HT_{1B} receptor action, the majority of them relied on inconsistent and non-neuronal cell lines that confound their applicability to endogenous receptor function. Additional studies in neurologically relevant cell lines are needed to further elucidate the signaling mechanisms of these receptors. Finally, structural analyses of the 5-HT_{1B} receptor indicate a lack of functional selectivity for G protein and β-arrestin signaling pathways with numerous ligands, unlike other 5-HT receptors (Wacker et al., 2013).

5-HT_{1B} receptors, particularly autoreceptors, have been implicated in the regulation of fear, anxiety, stress, and depression-like behaviors. Numerous studies have demonstrated alterations to these behaviors following manipulation of 5-HT_{1B} receptor expression or function. Viral-mediated overexpression of 5-HT_{1B} receptors in the midrostrocaudal DRN of rats reduces anxiety and fear behaviors without an effect on mobility in the forced swim test, while overexpression in the caudal DRN results in increased mobility during forced swim testing but no effect on anxiety behaviors during open field testing. 5-HT_{1B} overexpression also reduces fear expression but not acquisition during contextual fear conditioning experiments (Clark et al., 2002; Clark et al., 2004; Liu et al., 2015; McDevitt et al., 2011). These results highlight the importance of 5-HT_{1B} receptors for the regulation of fear and anxiety and indicate that different

emotional behaviors are likely mediated by 5-HT_{1B} autoreceptors on different subpopulations of serotonergic neurons.

The expression of endogenous 5-HT_{1B} receptors can vary with stress, anxiety, fear, and even SSRI use. Daily social stress increases 5-HT_{1B} mRNA in the nucleus accumbens (NAc), while acute stress does not alter expression in this brain region (Furay et al., 2011). In the DRN, 5-HT_{1B} mRNA levels are inversely correlated with anxiety as measured by open field and elevated plus maze tests, and stress itself increases 5-HT_{1B} mRNA levels (Hiroi & Neumaier, 2009; Kaiyala et al., 2003; McDevitt & Neumaier, 2011). Finally, chronic SSRI use downregulates 5-HT_{1B} autoreceptors but not heteroreceptors. Blockage of 5-HT_{1B} receptors with antagonist augments SSRI function, and 5-HT_{1B} knockout mice show no change in mobility in the forced swim test following SSRI administration (Tiger et al., 2018). All of this evidence supports the hypothesis that 5-HT_{1B} autoreceptors are neuroprotective during periods of fear, anxiety, and stress. Of note, 5-HT_{1B} receptors become desensitized following stress but their expression remains unaltered, and 5-HT_{1B} overexpression cannot rescue this desensitization (Bolanos-Jimenez et al., 1995; McDevitt & Neumaier, 2011). This suggests that the consequences of 5-HT_{1B} receptor activation may involve regulation of downstream events as well. 5-HT_{1B} receptors are clearly important for SSRI function, and it is possible that 5-HT_{1B} receptor desensitization and decreased expression contributes to the delayed action of SSRIs.

As previously mentioned, ERK1/2 signaling has been implicated in 5-HT_{1B} signal transduction and could play a key role in modulating the intracellular and behavioral effects of these receptors. ERK1 and ERK2 are mitogen-activated protein kinases

(MAPKs), a family of serine-threonine kinases that are conserved across evolution, ubiquitously expressed, and activated by a variety of receptors. Upon receptor stimulation, signals converge on upstream MAPK kinases (MAPKKs) that phosphorylate and activate MAPKs, which can in turn activate a variety of intracellular targets and regulate the expression of numerous genes. In the case of ERK1/2, activation occurs via phosphorylation by MAPK/ERK Kinase 1 and 2 (MEK1/2), enzymes that have also been implicated in 5-HT$_{1B}$ signaling (Keshet & Seger, 2010). While 5-HT$_{1B}$ autoreceptors are desensitized following stress (but maintain expression levels), levels of activated ERK1/2 increase with stress (Mahar et al., 2014). Additionally, fear behaviors are also influenced by levels of ERK1/2 activation in the brain. Chronic stress results in increased ERK1/2 phosphorylation, and ERK signaling is required for the retention of fear memories (Villarreal & Barea-Rodriguez, 2006). As such, the relationship between 5-HT$_{1B}$ receptors and ERK1/2 activation warrants further investigation.

5-HT$_6$ Receptor Signaling and Behavioral Implications

The 5-HT$_6$ receptor was originally cloned in 1993 from the rat, and from humans shortly thereafter in 1996 (Kohen et al., 1996; Monsma et al., 1993; Ruat et al., 1993). An early immunolabeling study suggested that 5-HT$_6$ receptors localize to dendrites (Gerard et al., 1997); however, subsequent studies revealed that these receptors localize to neuronal primary cilia, making it the only serotonin receptor to do so (Berbari et al., 2008; Brailov et al., 2000; Hamon et al., 1999). Primary cilia are present in most non-dividing mammalian cells, originate from the centriole, and are comprised of a

central axoneme enclosed in a membrane that forms a signaling compartment separate from the rest of the cytosol. A diffusion barrier at the base of the cilium limits movement into and out of the ciliary compartment, yet these structures contain a high density of receptors that detect extracellular signals (Sterpka & Chen, 2018). In the CNS, both neurons and astrocytes display a single non-motile primary cilium, and these structures regulate numerous aspects of brain function including cognition, mood, and metabolism (Sterpka & Chen, 2018). Ciliary localization was thought to be due to a specific amino acid sequence in the third intracellular loop of the 5-HT_6 receptor (Berbari et al., 2008). However, trafficking of 5-HT_6 receptors to primary cilia is reduced but not entirely prevented following deletion of this consensus sequence (Brodsky et al., 2017), suggesting the presence of additional mechanisms for cilia localization.

Several studies have examined the expression profile of 5-HT_6 receptors in the brain using radioligand binding, *in situ* hybridization, and immunohistochemistry. 5-HT_6 receptor expression is particularly enriched in the striatum, far more than in other brain regions. While rats and humans show similar expression profiles, mice express 5-HT_6 receptors at much lower levels and do not show enrichment in the striatum (East et al., 2002; Gerard et al., 1997; Hirst et al., 2003; Ruat et al., 1993). Within the striatum, 5-HT_6 receptors are present on both direct and indirect pathway medium spiny neurons (MSNs) and are occasionally found on cholinergic interneurons (Bonsi et al., 2007; Helboe et al., 2015; Ward et al., 1995). The pharmacology of mouse 5-HT_6 receptors also diverges from that of rat and human receptors. While the receptors for all three species have similar sequence homology, mouse 5-HT_6 receptors demonstrate lower binding affinity for certain ligands due to differences in the binding pocket: specifically

residues 188 in transmembrane domain 5 (TM5) and 290 in TM6 (Hirst et al., 2003; Setola & Roth, 2003). 5-HT$_6$ knockout mice were originally reported to have weight loss (Tecott & Brennan, 2000); however, a subsequent study showed these mice have reduced responses to acute ethanol administration but no changes in weight, health, viability, or lifespan (Bonasera et al., 2006). Additionally, there have been no reports of changes in emotional behavior, cognition, or sensorimotor function. It is likely that the lower expression and different pharmacology of 5-HT$_6$ receptors in mice compared to rats and humans contribute to the subtle phenotype of 5-HT$_6$ knockout mice.

5-HT$_6$ receptors are excitatory GPCRs that couple to G_s proteins to activate adenylyl cyclase (AC) and promote cAMP signaling. These receptors are capable of interacting with multiple different isoforms of AC including AC3, AC5, and AC8; however, AC3 also localizes to primary cilia and has been shown to co-localize with 5-HT$_6$ receptors (Baker et al., 1998; Brodsky et al., 2017). Evidence suggests that 5-HT$_6$ receptors are also capable of activating Fyn kinase and subsequently ERK1/2 signaling, as well as mTOR and Cdk5 signaling pathways (Meffre et al., 2012; Riccioni et al., 2011; Yun et al., 2007). Additional work shows that 5-HT$_6$ receptors can regulate DARPP$_{32}$ phosphorylation, a critical regulatory node that is also involved in dopamine receptor signaling and thus allows for potential crosstalk between serotonergic and dopaminergic pathways (Svenningsson et al., 2002). Both dopamine D_1 and 5-HT$_6$ receptors are excitatory G_s-coupled receptors which allows further synergism between these two signaling mechanisms, particularly in striatal direct pathway MSNs due to their specific expression of D_1 receptors. On the other hand, there is the potential for

opposing interactions between 5-HT_6 and D_2 receptors, which are inhibitory G_i-coupled receptors located on striatal indirect pathway MSNs (Yager et al., 2015).

Manipulations of 5-HT_6 receptor signaling in the brain have a multitude of cognitive and behavioral consequences. Most studies using 5-HT_6 antagonists in rodents have shown pro-cognitive effects in memory tasks (Mitchell & Neumaier, 2005); however, there is less agreement in the literature regarding the effects of 5-HT_6 agonists. Some studies demonstrate a pro-cognitive effect of agonist administration in specific brain regions, while others show impaired cognitive function (Kendall et al., 2011; Loiseau et al., 2008; Meneses et al., 2008; Pereira et al., 2015; Schechter et al., 2008; Woods et al., 2012). It is likely that these discrepancies arise from region- and circuit-specific differences, as well as differences in the behavioral test used. Increased 5-HT_6 expression in dorsomedial striatum impairs the acquisition of an action-outcome learning task but does not affect previously learned behaviors. Conversely, increased expression in the dorsolateral striatum impairs habit formation but does not alter acquisition (Eskenazi & Neumaier, 2011a; Eskenazi & Neumaier, 2011b). When 5-HT_6 receptors are overexpressed specifically in indirect pathway MSNs of the dorsomedial striatum of rats there is an impairment of instrumental learning. 5-HT_6 overexpression in dorsolateral indirect pathway MSNs prevents the acquisition of new behaviors following overtraining (Eskenazi et al., 2015). Ergo, 5-HT_6 receptors appear to exert their influence in both a region- and circuit-specific manner.

Since the dorsal striatum and NAc are critical mediators of drug-taking and reward-seeking behaviors, the abundance of 5-HT_6 receptors in these regions suggests their potential role in addiction mechanisms. Although some studies show no effect of

systemic 5-HT_6 ligands on cocaine self-administration, others indicate that reinforcement and reinstatement of cocaine taking are modulated by 5-HT_6 receptor signaling (Fijal et al., 2010; Frantz et al., 2002; Valentini et al., 2013; van Gaalen et al., 2010). Increased 5-HT_6 expression in the NAc blocks conditioned-place preference to cocaine but does not alter cocaine-induced locomotor sensitization (Ferguson et al., 2008), which demonstrates the ability of these receptors to modulate cocaine reward. More research is needed to achieve a detailed understanding of how 5-HT_6 receptors influence addiction-like behaviors in order to identify potential therapeutic targets.

Specific Aims

The goals of this dissertation are to elucidate the mechanisms by which 5-HT_{1B} receptors influence intracellular signaling and characterize the role of 5-HT_6 receptor signaling in circuit-specific modulation of drug-taking behaviors. This work will enhance our understanding of extra-synaptic serotonin signaling and its role in neuronal function and behavior. In Chapter 2 I describe the generation of neuronal cell lines that can be used to probe the molecular interactions of 5-HT_{1B} receptors and β-arrestin-dependent signaling pathways. In Chapter 3 I utilize these cell lines along with pharmacological methods to further characterize 5-HT_{1B} receptor-mediated activation of ERK1/2. Finally, in Chapter 4 I use viral-mediated gene transfer to probe the circuit-specific effect of increased 5-HT_6 receptor expression on cocaine self-administration.

CHAPTER 2

Generating Cell Lines to Interrogate 5-HT_{1B} Receptor Signaling

Introduction

The serotonergic system is important in modulating a range of emotional behaviors. Disturbances of this system are implicated in psychiatric conditions such as posttraumatic stress disorder, depression, and anxiety. 5-HT_{1B} receptors are primarily localized adjacent to axon terminals and exist both as autoreceptors on serotonergic neurons and as terminal heteroreceptors on nonserotonergic cells (Boschert et al., 1994; Ghavami et al., 1999; Hen, 1992; Riad et al., 2000). They serve to regulate 5-HT synthesis, 5-HT release, and 5-HT reuptake (Daws et al., 2000; Hagan et al., 2012; Hjorth et al., 1995; Middlemiss & Hutson, 1990; Montanez et al., 2014) and modulate a wide variety of behavioral effects (McDevitt et al., 2011; McDevitt & Neumaier, 2011). The diffuse distribution of 5-HT_{1B} receptors throughout the brain and their localization to axon terminals makes studying their signaling properties *in vivo* a challenging endeavor. Much of the mechanistic literature to date has relied on model systems of non-neuronal and inconsistent cell lines. In order to study these receptors more accurately *in vitro*, a neuronal cell line is needed that expresses 5-HT_{1B} receptors. Mouse neuroblastoma cells (Neuro2A) express neuronal genes and provide a more appropriate model system for studying neuronal receptors *in vitro* (Ma'ayan et al., 2009).

Despite research describing numerous interactions of 5-HT_{1B} receptors with other intracellular proteins, the exact nature of downstream signaling cascades remains unclear. GPCRs like the 5-HT_{1B} receptor canonically stimulate G protein activation upon binding with an agonist, during which the Gα subunit exchanges GDP for GTP and

causes the dissociation of the $G\beta\gamma$ subunits. Consequently, these subunits influence the activity of downstream proteins such as AC and ion channels (Luttrell & Luttrell, 2003; Luttrell, 2008). GPCRs are often classified based on the family of Gα subunit to which they bind. $G\alpha_s$ subunits activate AC and increase cAMP signaling, $G\alpha_{i/o}$ subunits inhibit AC and reduce cAMP signaling, and $G\alpha_q$ subunits activate phospholipase C (PLC) and initiate excitatory inositol-3-phosphate (IP_3) and diacylglycerol (DAG) signaling (Luttrell, 2008). 5-HT_{1B} receptors are inhibitory $G\alpha_{i/o}$-coupled receptors (Lin et al., 2002).

After initiating G protein signaling, GPCRs can be phosphorylated by G protein-coupled receptor kinases (GRKs) which recruits β-arrestins to bind to the receptors. β-arrestins can desensitize and inactivate GPCRs by triggering their internalization from the cell membrane; however, it is now known that these proteins also initiate a variety of additional signaling cascades (e.g. ERK1/2 pathways) and can form scaffolds for endosomal signaling complexes (Gesty-Palmer et al., 2006; Luttrell & Lefkowitz, 2002; Luttrell & Miller, 2013; Peterson & Luttrell, 2017). While G protein and β-arrestin signaling were once thought to be independent, recent work indicates that both pathways are needed for certain receptors to engage in noncanonical kinase signaling (Grundmann et al., 2018). Several pharmacological inhibitors of G protein signaling exist for examining this pathway, however no such inhibitors exist for β-arrestins. Previous research has traditionally relied on transient knockdown of β-arrestins using short hairpin RNAs (shRNAs), but these methods do not provide a stable and efficient way to examine the role of β-arrestins *in vitro* (Gesty-Palmer et al., 2006; Peterson & Luttrell, 2017). Appreciating the nature of 5-HT_{1B} receptor interactions with both G protein and β-arrestin pathways is important for understanding the mechanisms of

serotonergic regulation and identifying potential therapeutic targets for psychiatric diseases. In this chapter I describe the creation of Neuro2A cell lines for studying 5-HT_{1B} receptor signal transduction in a neurologically relevant system using stable transfection of 5-HT_{1B} receptor-expressing plasmids, in collaboration with Katie Liu (Liu et al., 2019), and CRISPR-mediated knockout of β-arrestins.

Methods

Cell culture and drug treatments

Neuro2A (N2A) cells were maintained with growth media consisting of Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS), and 1x Antibiotic-Antimycotic (Gibco) at 37°C in 5% CO_2. N2A cells were transfected with a plasmid expressing HA-tagged rat 5-HT_{1B} receptor in a pcDNA3 backbone (N2A-1B) using Lipofectamine LTX (Invitrogen), and selection for the stably transfected cell lines was achieved with 500 µg/mL geneticin (G418). For western blot analysis, cells were lysed with modified RIPA buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate, 1% CHAPS, and 1x protease and phosphatase inhibitors) and briefly vortexed. Cell debris was pelleted by centrifugation at 15,000 x g for five minutes. The protein concentration of the lysate was measured using the 660 nm protein assay (Pierce). For β-arrestin experiments, N2A-1B β-arrestin knockout (KO) cells were grown in media additionally supplemented with 2 µg/ml puromycin. Cell lysates for western blot analysis were prepared as described above.

Quantitative PCR

Cells were detached from growth flasks and pelleted. RNA was purified with the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions, and then treated with TURBO DNase (Ambion). 2 μg of total RNA was reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo) with oligo$(dT)_{18}$ primers. Quantitative PCR was performed using EXPRESS SYBR GreenER (Invitrogen) with 2 μM primers (Appendix 1) and run for 40 cycles on the ViiA 7 Real Time PCR System (Life Technologies). First-strand cDNA synthesis products were diluted 1:20 before adding to the qPCR reaction. Ct values were normalized to that of GAPDH, and fold enrichment was obtained using the ΔΔCt method.

Immunocytochemistry

Cells were plated onto glass coverslips that were pretreated with poly-L-lysine and grown for 24-48 hours before fixation. Cells were then fixed with warm 4% paraformaldehyde-PHEMS buffer for 20 minutes. To achieve cell surface staining, cell membranes were not permeabilized. Cells were washed once with PBS, blocked with 10% BSA for one hour at room temperature, then incubated with rabbit anti-HA antibody (Cell Signaling, 1:4000) overnight at 4°C. After washing cells three times with PBS, cells were incubated with Alexa Fluor 594 goat anti-rabbit IgG secondary antibody (Molecular Probes, 1:4000) for two hours at room temperature. Cells were washed three times with DPBS, then mounted with Prolong Gold with DAPI (Molecular Probes).

Western blot

4x NuPAGE LDS Sample Buffer (Novex) containing 10 mM DTT was mixed with cell lysates and heated to 70°C for ten minutes. Samples were loaded into Bolt 4-12% Bis-Tris gels (Novex) and run for 45 minutes at 140 V. Protein was transferred to a 0.2 µm nitrocellulose membrane with the Mini Trans-Blot Cell (Bio-Rad) for one hour at 80 V or with the Bolt Mini Blot Module (Novex, Life Technologies) for one hour at 30 V. Membranes were blocked for one hour at room temperature in 1x Tris-buffered saline (TBS) with 5% nonfat milk. Primary and secondary antibodies were diluted in 1x TBS with 1% Triton X-100 (TBST), with either 5% bovine serum albumin (BSA) for detection of phosphorylated proteins, or with 5% nonfat milk for all other proteins. For the β-arrestin experiments, Aqua Block buffer (Abcam) was used instead of BSA and nonfat milk. Primary antibodies were incubated for two hours at room temperature with gentle shaking or overnight at -4°C. Secondary antibodies (Dylight 680 and Dylight 800) were incubated for two hours at room temperature with gentle shaking. Protein bands were detected on the Odyssey CLx and analyzed with Image Studio (LI-COR Biosciences). Signal intensity of protein bands were normalized to that of α-tubulin or β-actin. Antibodies from Cell Signaling Technology were diluted as follows: rabbit anti-HA, 1:1000; rabbit anti-β-arrestin 2, 1:1000; mouse anti-α-tubulin, 1:2500; mouse anti-β-actin, 1:2000; goat anti-rabbit IgG DyLight 800, 1:8000; goat anti-mouse IgG DyLight 680, 1:8000. Antibodies from Abcam were diluted as follows: rabbit anti-β-arrestin 1, 1:1000.

Generation and validation of β-arrestin knockout N2A-1B cell lines

Using the CRISPOR program (Haeussler et al., 2016), specific guide sequences were designed, four each for β-arrestin 1 and β-arrestin 2, and ordered as DNA cassettes (Invitrogen GeneArt Gene Synthesis). Each guide sequence was subsequently cloned into a pLentiCRISPRv2 plasmid, a retroviral vector that encodes Cas9, a guide sequence, and puromycin resistance. Retroviral virus-like particles (VLPs) were generated as described elsewhere (Nahabedian et al., 2017). Briefly, HEK293T cells were cotransfected with one of the pLentiCRISPRv2 plasmids, psPAX2 (HIV-1 Gag/Pol/Rev/Tat packaging plasmid), and pMD2.G (vesicular stomatitis virus glycoprotein [VSV-G] envelope plasmid) at a ratio of 1:1:0.5 using the FuGENE 6 transfection reagent (Promega) according to manufacturer's instructions. Forty-eight hours after transfection, HEK293T cell supernatants containing the VLPs were collected and concentrated using Amicon Ultracel 100 K filters (Millipore) to yield approximately 200 μl of concentrated VLPs. N2A-1B cells were plated into six well plates at a density of $2x10^5$ cells/well 24 hours prior to transduction with VLPs. Transduction occurred via spinoculation at 1200 x g for 90 minutes in complete medium supplemented with 500 μg/ml of G418. Twenty-four hours after transduction, cells were passaged into new T75 flasks and cultured in G418-containing media for 48 hours. Subsequently, cells were passaged and cultured in complete media supplemented with both 500 μg/ml of G418 and 2 μg/ml of puromycin to select for transgene expression. Each N2A-1B KO cell line expressed Cas9 and a single guide sequence, with four cell lines specific for β-arrestin 1, four cell lines for β-arrestin 2, and one cell line that expressed a non-targeting control sequence.

Sequences that yielded the most efficient sgRNAs were as follows: non-targeting control, 5'-CCGGGAGATTAACGTTAATT-3'; β-arrestin 1, 5'-GCATTGACCTCGTGGACCCCG-3'; and β-arrestin 2, 5'-GAAGTCGAGCCCTAACTGCA-3'. To validate CRISPR/Cas9 knockout of β-arrestin 1 and β-arrestin 2, each cell line was plated on 60 mm plates and lysed 24 hours later with modified RIPA buffer. Samples were briefly vortexed and cell debris was pelleted by centrifugation at 15,000 x g for 15 minutes. Cell lysates were then collected and analyzed via western blot as described above for the presence or absence of β-arrestin 1 and β-arrestin 2. For β-arrestin 1, signal intensity of protein bands was normalized to that of β-actin, while that of β-arrestin 2 was normalized to α-tubulin.

Results

Neuro2A cells express a variety of neuronal and serotonin-related genes endogenously, and stably transfected N2A-1B cells express 5-HT_{1B} receptors on the cell surface

To model endogenous neuronal signaling pathways, we chose the mouse neuroblastoma Neuro2A cell line and investigated the presence of serotonergic and neuronal markers. RNA was purified from untransfected N2A and stably transfected N2A-1B cells, and ten genes were probed for endogenous expression. N2A cells expressed a variety of serotonin-related genes, including 5-HT_{1A}, SERT, and Tph2 (Table 2.1). 5-HT_{1B} mRNA was essentially absent in untransfected N2A cells but abundant in N2A-1B cells. To verify 5-HT_{1B} protein expression, N2A and N2A-1B cells were lysed and western blotting was performed for the HA-tagged 5-HT_{1B} receptor (HA-5-HT_{1B}). HA is detected in N2A-1B cells but not in N2A cells (Figure 2.1a).

Immunocytochemistry for HA was performed on non-permeabilized N2A-1B cells and verified that a subset of the receptors was present at the cell surface (Figure 2.1b).

Table 2.1: Quantitative RT-PCR measurements of serotonergic and neuronal marker genes in untransfected N2A and stably transfected N2A-1B cells.

Gene	Normalized Ct Values		Fold Enrichment
	N2A cells	N2A-1B cells	
5-HT_{1B}	34.9	24.0	2012.39
5-HT_{1A}	27.1	26.9	1.28
SERT	31.7	32.4	0.79
Pet-1	33.4	34.9	0.47
Tph2	30.8	31.2	0.90
Tph1	27.0	26.9	1.24
AADC	17.8	17.6	1.17
GCH1	23.0	23.1	1.02
VGLUT3	34.0	34.1	1.16
VMAT2	24.5	24.8	0.94

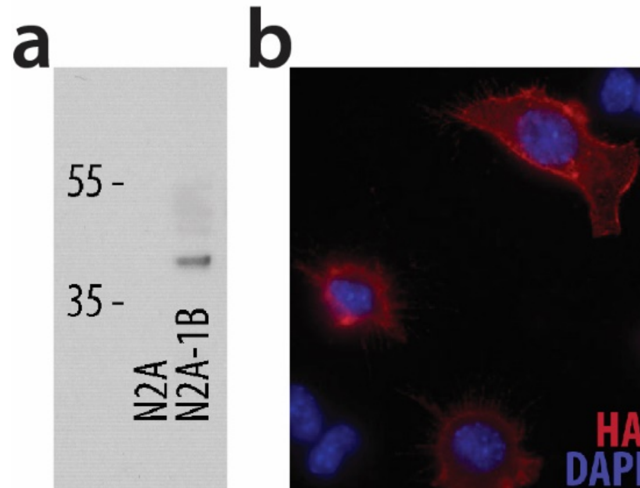

Figure 2.1: HA-tagged 5-HT$_{1B}$ receptors are present in stably transfected N2A-1B cells on the cell surface. (a) Western blot for HA shows the presence of the HA-5-HT$_{1B}$ receptors in N2A-1B cells but not in untransfected N2A cells. (b) Immunocytochemical staining of non-permeabilized N2A-1B cells demonstrates cell surface expression of HA-5-HT$_{1B}$ receptors.

CRISPR/Cas9 enables successful knockout of β-arrestin isoforms in N2A-1B cells

In order to study the roles of β-arrestins in 5-HT$_{1B}$ signaling, we developed stable β-arrestin knockout N2A-1B cell lines using CRISPR/Cas9 gene editing to further investigate the role of β-arrestins in 5-HT$_{1B}$-mediated ERK1/2 activation in neuronal cells (Figure 2.2). β-arrestin knockout lines were generated by transduction of CRISPR/Cas9 lentiviral vectors with sgRNAs specific for β-arrestin 1 (β-Arr1 KO) or β-arrestin 2 (β-Arr2 KO), while N2A-1B control cells were transduced with a non-targeting control sgRNA sequence. Transduction of N2A-1B cells with targeted lentiviral CRISPR/Cas9 vectors induced specific and efficient reduction of β-arrestin 1 and β-arrestin 2 expression, while transduction with a non-targeting control vector ("control"

cells) did not alter β-arrestin expression compared with untransfected N2A-1B cells (Figure 2.3).

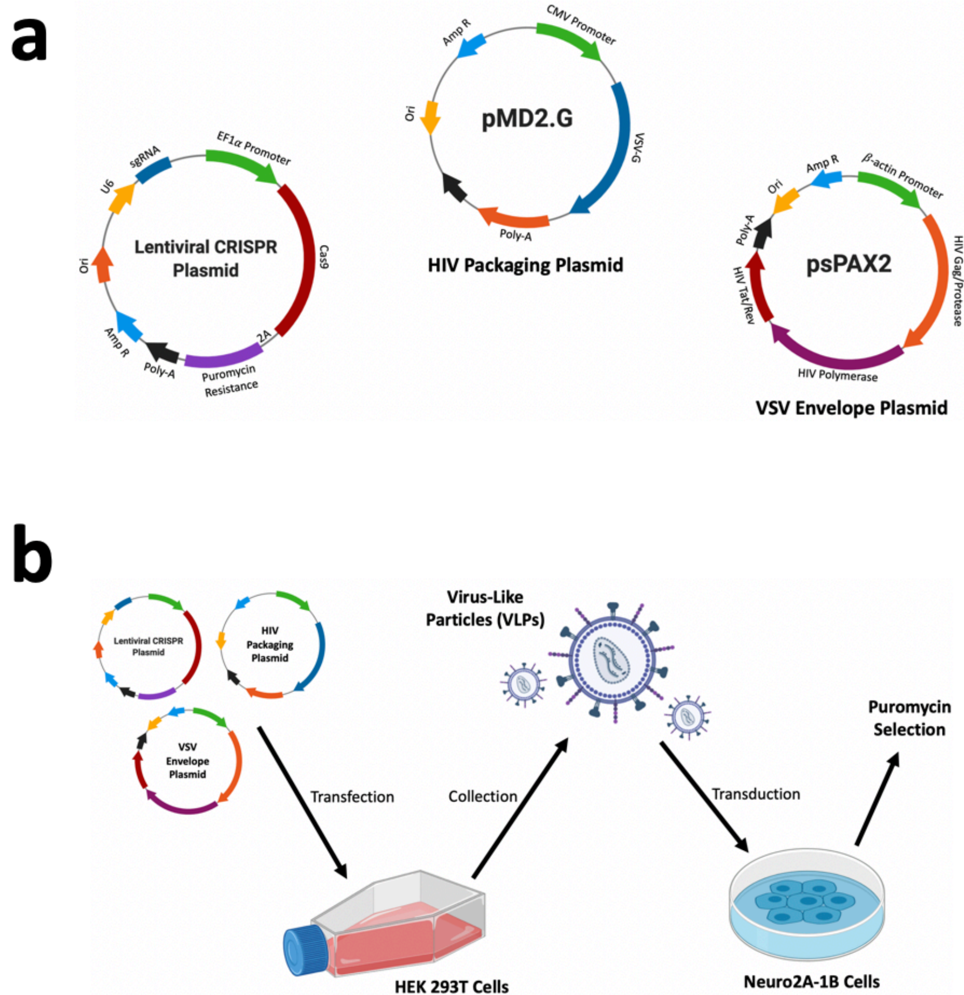

Figure 2.2: Generation of β-arrestin knockout N2A-1B cells with a lentiviral CRISPR/Cas9 system. (a) A pLentiCRISPRv2 plasmid (specific for a β-arrestin isoform or non-targeting sequence), psPAX2 (HIV-1 Gag/Pol/Rev/Tat packaging plasmid), and pMD2.G (vesicular stomatitis virus glycoprotein [VSV-G] envelope plasmid) were used to generate retrovirus-like particles (VLPs). (b) Lentiviral CRISPR/Cas9 vectors were used to generate stable β-arrestin KO N2A-1B cell lines.

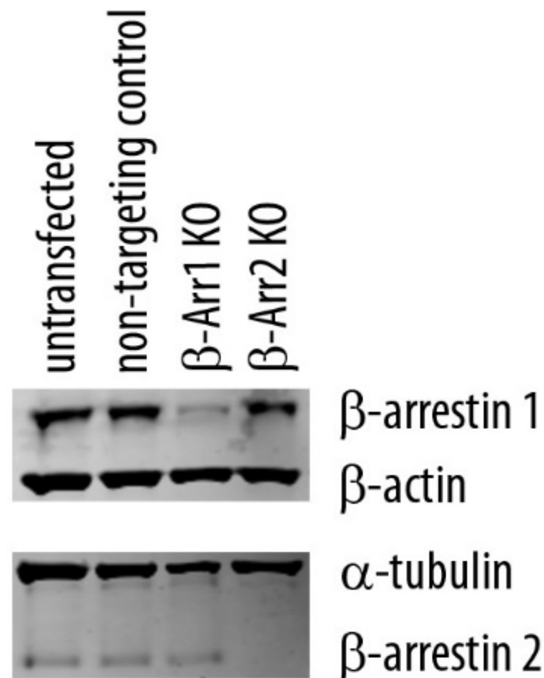

Figure 2.3: Transduction of N2A-1B cells with a lentiviral CRISPR/Cas9 vector induces specific and efficient knockout of β-arrestin 1 and β-arrestin 2. Transduction with a non-targeting control vector does not alter the expression of β-arrestin 1 or β-arrestin 2. Transduction with CRISPR/Cas9 vectors specific for β-arrestin 1 does not alter the expression of β-arrestin 2, and transduction with vectors specific for β-arrestin 2 does not alter the expression of β-arrestin 1.

Discussion

Previous studies of 5-HT_{1B} receptor signaling utilized non-neuronal cell lines (Berg & Clarke, 2001; Janoshazi et al., 2007; Mendez et al., 1999; Pullarkat et al., 1998; Salim et al., 2002; Xie et al., 1999). To mimic endogenous signaling pathways in neurons more faithfully, we chose the mouse neuroblastoma Neuro2A cell line. Compared with non-neuronal lines, the use of N2A cells has advantages because of their endogenous expression of several key serotonergic and neuronal genes, so these may be more physiologically similar to 5-HT_{1B}-expressing neurons *in vivo*. Although

N2A cells do not endogenously express 5-HT_{1B} receptors, transfection and generation of stable cell lines allowed for reliable trafficking to the cell surface. It is important to note that while 5-HT_{1B} receptors are usually localized at axon terminals, N2A cells do not demonstrate this polarized receptor trafficking (Boschert et al., 1994; Ghavami et al., 1999; Hen, 1992; Riad et al., 2000); thus, overall levels of receptor expression in N2A cells do not necessarily correspond to localized levels of receptor expression in neuronal axon terminals.

There are currently no pharmacological inhibitors of β-arrestins, and previous studies have utilized transient knockdown of β-arrestins with shRNAs to investigate their roles in receptor signaling and function (Gesty-Palmer et al., 2006; Peterson & Luttrell, 2017). In order to create stable cell lines lacking β-arrestins in N2A-1B cells, we utilized a retroviral CRISPR/Cas9 system to permanently knockout β-arrestin expression. CRISPR/Cas9 systems involve a Cas9 endonuclease that uses a guide RNA sequence to target a specific gene of interest. Once bound to a gene, Cas9 cleaves both strands of genomic DNA, after which DNA repair mechanisms can introduce a frameshift mutation and premature stop codon that effectively knocks out gene function (Heidenreich & Zhang, 2015; Ran et al., 2013).

While this method can be highly effective, knockout may not be achieved if DNA repair introduces a silent or missense mutation. In the case of the β-arrestin 1 knockout cell line, some cells likely retained β-arrestin 1 expression due to either inefficient editing or lack of a frameshift mutation. However, the overall cell population has dramatically reduced β-arrestin 1 expression, and the β-arrestin 2 knockout cell line showed complete loss of β-arrestin 2 protein (possibly due to more efficient gene

editing). Indeed, certain guide sequences can more efficiently induce gene editing and frameshift mutations (Doench et al., 2014; Haeussler et al., 2016; Hsu et al., 2013).

An additional concern when using CRISPR/Cas9 systems is the potential for off-target gene editing. Guide sequences that share identity with only the target sequence should be selected to avoid editing at unintended sites; however, binding between guide RNAs and genomic sequences with mismatched base pairs can still lead to DNA cleavage (Doench et al., 2014; Hsu et al., 2013). Off-target effects can be minimized by selecting guide sequences that have several characteristics: the fewest potential off-target binding sites, off-target binding sites exclusively within introns or intragenic DNA, off-target binding sites that require a large number of mismatched base pairs, and these mismatched base pairs being proximal to the protospacer adjacent motif (PAM) (Doench et al., 2014; Hsu et al., 2013). Guide sequences for β-arrestin 1 and 2 were selected using the CRISPOR algorithm, which provided a readout for these characteristics and allowed us to select sequences that met these criteria while still binding proximally to the start codon in the first exon of the β-arrestin genes (Haeussler et al., 2016).

The use of VLPs to achieve stable selection and knockout of β-arrestin has advantages over other methods of gene transfer such as transfection or transduction with pure viral vectors like adeno-associated virus (AAV) and lentivirus. This method enables a high efficiency of gene transfer in a rapid timeframe, while allowing for both CRISPR/Cas9 and puromycin resistance genes to be packaged into a single vector and incorporated into the cellular genome. Multiple different constructs can also be

packaged into vectors simultaneously which allows for efficient vector generation and multiplex gene editing (Nahabedian et al., 2017).

Finally, the cell lines generated and described in this chapter can be used for future studies. Investigations into 5-HT_{1B} receptor interactions with other signaling pathways can utilize N2A-1B cells to perform experiments in a neurologically relevant system. Additionally, the β-arrestin knockout cell lines can be used to study the signaling mechanisms of other neuronally expressed GPCRs. The validated guide sequences and lentiviral vectors for β-arrestin gene editing allow for stable β-arrestin knockout in a multitude of cell types, thus enabling investigators to probe GPCR signal transduction in additional systems and contexts. In the next chapter, I will use these cell lines to elucidate the modulation of ERK1/2 signaling by 5-HT_{1B} receptors.

Portions of this chapter were adapted from: Liu Y, Gibson AW*, Levinstein MR, Lesiak AJ, Ong SE, and Neumaier JF. (2019) 5-HT_{1B} receptor-mediated activation of ERK1/2 requires both $G\alpha_{i/o}$ and β-arrestin proteins. ACS Chemical Neuroscience. 10(7): 3143-3153*

**Co-First Authors*

CHAPTER 3

Mechanisms of 5-HT_{1B} Receptor-Mediated Activation of ERK1/2 Signaling

Introduction

Despite its importance in neuromodulation throughout the brain, 5-HT_{1B} receptor function and its underlying biochemical mechanisms are not well understood. 5-HT_{1B} receptors are $G\alpha_{i/o}$-coupled receptors that are well known for inhibiting adenylyl cyclase and opening inward rectifying potassium channels (Andrade et al., 1986; Bouhelal et al., 1988; Ghavami et al., 1997; Schoeffter & Hoyer, 1989). 5-HT_{1B} receptors have been shown to couple to ERK2 and stimulate p70 S6 kinase in a pertussis- and PI3K-sensitive manner in transfected non-neuronal cells (Mendez et al., 1999; Pullarkat et al., 1998). 5-HT_{1B} receptor stimulation also leads to indirect activation of Akt1 in a MEK1/2-dependent manner (Hsu et al., 2001). Furthermore, these receptors directly interact with GSK3β, which is important for agonist-induced 5-HT_{1B} receptor activation and internalization, and with p11, which increases 5-HT_{1B} surface expression and enhances receptor function (Chen et al., 2009; Li & Jope, 2010; Svenningsson et al., 2006; Zhou et al., 2012).

While all G-protein coupled receptors (GPCRs) couple to G proteins to engage canonical signaling pathways such as activation or inhibition of adenylyl cyclase, other signaling pathways are mediated primarily by β-arrestins (Liggett, 2011; Reiter et al., 2012). Recent evidence suggests that this β-arrestin signaling is dependent on G proteins and supports the idea of β-arrestin-G protein coupling (Grundmann et al., 2018). Arrestins may also trigger desensitization and internalization of GPCRs (Ferguson, 2001; Lefkowitz & Shenoy, 2005). Of the multitude of possible signaling

pathways, MAP kinases are ubiquitously expressed, evolutionarily conserved, and activated following extracellular stimulation of a variety of different receptors. MAPKs are serine-threonine kinases, and the most well-studied members of this family are ERK1/2, JNK, p38 MAPK, and ERK5 (Keshet & Seger, 2010). Remarkably, MAPK activation by GPCRs sometimes involve both G protein- and β-arrestin-dependent mechanisms (Luttrell & Miller, 2013; Naor et al., 2000). The relative contribution of different second messenger pathways to the cellular effects of 5-HT_{1B} receptors has not been examined but identifying these pathways may lead to the development of novel ligands with biased signaling effects that might have therapeutic advantages over nonbiased ligands.

Although 5-HT_{1B} receptors have been reported to activate MAPK signaling in non-neuronal cells, our understanding of the molecular mechanisms involved is incomplete. Interestingly, fear behaviors are affected by ERK phosphorylation in the brain (Guedea et al., 2011; Huh et al., 2009; Ishikawa et al., 2012; Vetere et al., 2013; Villarreal & Barea-Rodriguez, 2006). Since we have observed that 5-HT_{1B} autoreceptors are critical in modulating fear responses (Clark et al., 2004; Liu et al., 2015; McDevitt et al., 2011), the relationship between 5-HT_{1B} receptors and ERK signaling warrants further investigation. The purpose of this chapter is to elucidate the signal transduction pathway of 5-HT_{1B} receptors in a neuronal cell line.

Methods

Cell culture and drug treatments

N2A cells were maintained with growth media consisting of Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS), and 1x Antibiotic-Antimycotic (Gibco) at 37°C in 5% CO_2. N2A-1B cells were grown in media additionally supplemented with 500 µg/mL geneticin (G418). Cells were plated in 60 mm plates 48 hours before treatment with growth media consisting of DMEM, 10% dialyzed serum, and 1x Antibiotic-Antimycotic (Gibco), and fed with fresh dialyzed growth media 24 hours before treatment. One hour before agonist treatment, cells were switched to serum-free Opti-MEM to wash out any residual 5-HT, with or without the presence of antagonists as described. Following agonist treatment, cells were lysed with modified RIPA buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate, 1% CHAPS, and 1x protease and phosphatase inhibitors) and briefly vortexed. Cell debris was pelleted by centrifugation at 15,000 x g for five minutes. The protein concentration of the lysate was measured using the 660 nm protein assay (Pierce). Treatment drugs used were: CP-94253 (Koe et al., 1992), SB-224289 (Gaster et al., 1998), gallein (Tocris), pertussis toxin (Novex), and U0126 (Cell Signaling). These drugs were applied one hour prior to the addition of agonists.

For β-arrestin experiments, N2A-1B β-arrestin knockout (KO) cells were grown in media additionally supplemented with 500 µg/mL geneticin (G418) and 2 µg/ml puromycin. Cells were plated in 60 mm plates 24 hours prior to treatment. One hour before agonist treatment, cells were switched to serum-free Opti-MEM, with or without the presence of the selective 5-HT_{1B} antagonist SB-224289 (1 µM), then treated with the selective 5-HT_{1B} agonist CP-94253 (100 nM) for ten minutes. Cell lysates were prepared as described above.

Western blot

4x NuPAGE LDS Sample Buffer (Novex) containing 10 mM DTT was mixed with cell lysates and heated to 70°C for ten minutes. Samples were loaded into Bolt 4-12% Bis-Tris gels (Novex) and run for 45 minutes at 140 V. Protein was transferred to a 0.2 µm nitrocellulose membrane with the Mini Trans-Blot Cell (Bio-Rad) for one hour at 80 V or with the Bolt Mini Blot Module (Novex, Life Technologies) for one hour at 30 V. Membranes were blocked for one hour at room temperature in 1x Tris-buffered saline (TBS) with 5% nonfat milk. Primary and secondary antibodies were diluted in 1x TBS with 1% Triton X-100 (TBST), with either 5% bovine serum albumin (BSA) for detection of phosphorylated proteins, or with 5% nonfat milk for all other proteins. For the β-arrestin experiments, Aqua Block buffer (Abcam) was used instead of BSA and nonfat milk. Primary antibodies were incubated for two hours at room temperature with gentle shaking or overnight at -4°C. Secondary antibodies (Dylight 680 and Dylight 800) were incubated for two hours at room temperature with gentle shaking. Protein bands were detected on the Odyssey CLx and analyzed with Image Studio (LI-COR Biosciences). Signal intensity of protein bands were normalized to that of α-tubulin or GAPDH. Antibodies from Cell Signaling Technology were diluted as follows: rabbit anti-phospho-p44/p42 MAPK (pERK1/2), 1:2500; rabbit anti-p44/p42 MAPK (ERK1/2), 1:2500; rabbit anti-phospho-p38, 1:1000; rabbit anti-phospho-p54 and p46 JNK, 1:1000; mouse anti-α-tubulin, 1:2500; mouse anti-GAPDH, 1:2000; goat anti-rabbit IgG DyLight 800, 1:8000; goat anti-mouse IgG DyLight 680, 1:8000.

Data analysis

In each experiment, independent biological replicates were analyzed in separate assays; the number of independent biological replicates is indicated in the legend for each figure. Signal intensity of protein bands for phospho-ERK1/2 were first normalized to α-tubulin. Signal intensities for samples treated with agonist and antagonist were then compared to signal intensities for vehicle (PBS)-treated controls from the same replicate to yield a percent of unstimulated control. All data were analyzed using a two-way analysis of variance (ANOVA), followed by Dunnett's post-hoc tests when multiple comparisons were made. All statistical analyses were performed using GraphPad Prism 7 or Excel software.

Results

5-HT$_{1B}$ receptor activation increases phosphorylation of ERK1/2 in a dose-dependent manner in N2A-1B cells but not in untransfected N2A cells, with no effect on total levels of ERK1/2 or other closely related MAP kinases

Untransfected N2A and stably transfected N2A-1B cells were treated with the selective 5-HT$_{1B}$ agonist CP-94253 at 1-100 nM for ten minutes. Levels of both ERK1 and ERK2 phosphorylation increased in N2A-1B cells, and this effect was blocked by preincubation with the 5-HT$_{1B}$ antagonist SB-224289 (1 μM) for one hour prior to agonist treatment; at 100 nM CP-94253, the highest concentration used, phospho-ERK1 levels increased by 195%, while phospho-ERK2 levels increased by 146% (Figures 3.1a). Total levels of ERK1/2 remained constant in N2A-1B cells, regardless of agonist and antagonist treatments (Figure 3.1b). Agonist-induced increases in phospho-ERK1/2 were absent in untransfected N2A cells (Figure 3.1c). Treatment with CP-94253 did not

increase phosphorylation of p38, p54 JNK, or p46 JNK (Figures 3.1d, 3.1e, 3.1f). Representative western blot images are shown in Figure 3.2. Additionally, the 5-HT_{1B} agonist CP-93129 produced a similar increase in ERK1/2 phosphorylation that was blocked by preincubation with the antagonist SB-224289 (Figure 3.3).

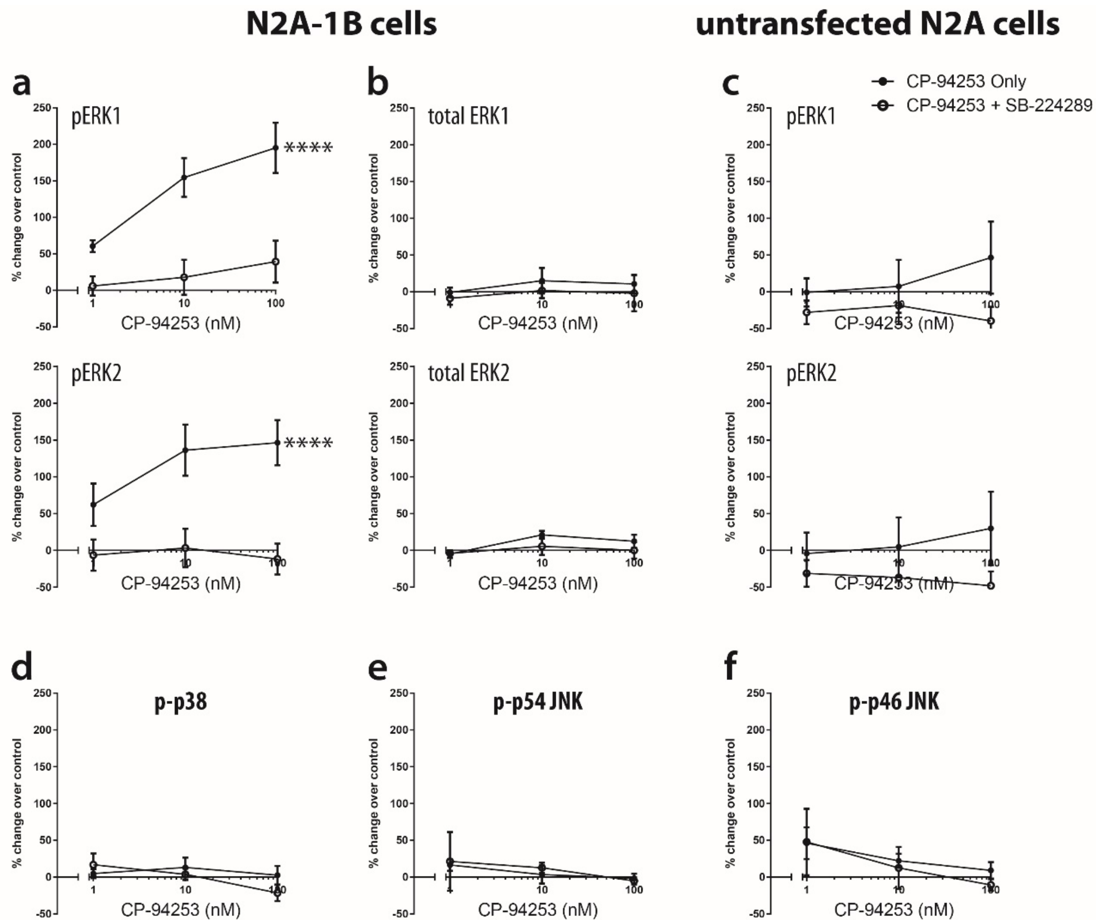

Figure 3.1: CP-94253 increases levels of phospho-ERK1/2, but not phospho-p38 or phospho-JNK, in N2A-1B cells, but not in untransfected N2A cells. (a) Treatment with CP-94253 (1-100 nM) for ten minutes increased phosphorylation of ERK1 and ERK2 in N2A-1B cells compared to unstimulated N2A-1B control cells treated with vehicle (PBS), but this was blocked by pretreatment with the antagonist SB-224289 (pERK1 $F_{1,24} = 34.07$, $p < 0.0001$; pERK2 $F_{1,24} = 28.38$, $p < 0.0001$). (b) No change was observed in total ERK in N2A-1B cells (total

ERK1 $F_{1,18} = 0.89$, $p = 0.36$; total ERK2 $F_{1,18} = 1.79$, $p = 0.20$). (c) No change was observed in phospho-ERK1/2 with agonist treatment in untransfected wild-type N2A cells (pERK1 $F_{1,24} = 3.62$, $p = 0.07$; pERK2 $F_{1,24} = 3.52$, $p = 0.07$). Agonist treatment did not change levels of (d) phospho-p38 ($F_{1,18} = 0.58$, $p = 0.46$), (e) phospho-p54 JNK ($F_{1,18} = 0.06$, $p = 0.81$), and (f) phospho-p46 JNK ($F_{1,18} = 0.21$, $p = 0.65$). Data are expressed as the percent change in pERK signal compared to the no agonist control from each independent biological replicate. Error bars represent SEM and data are averages of 4-5 independent biological replicates (two-way ANOVA). ****$p < 0.00001$

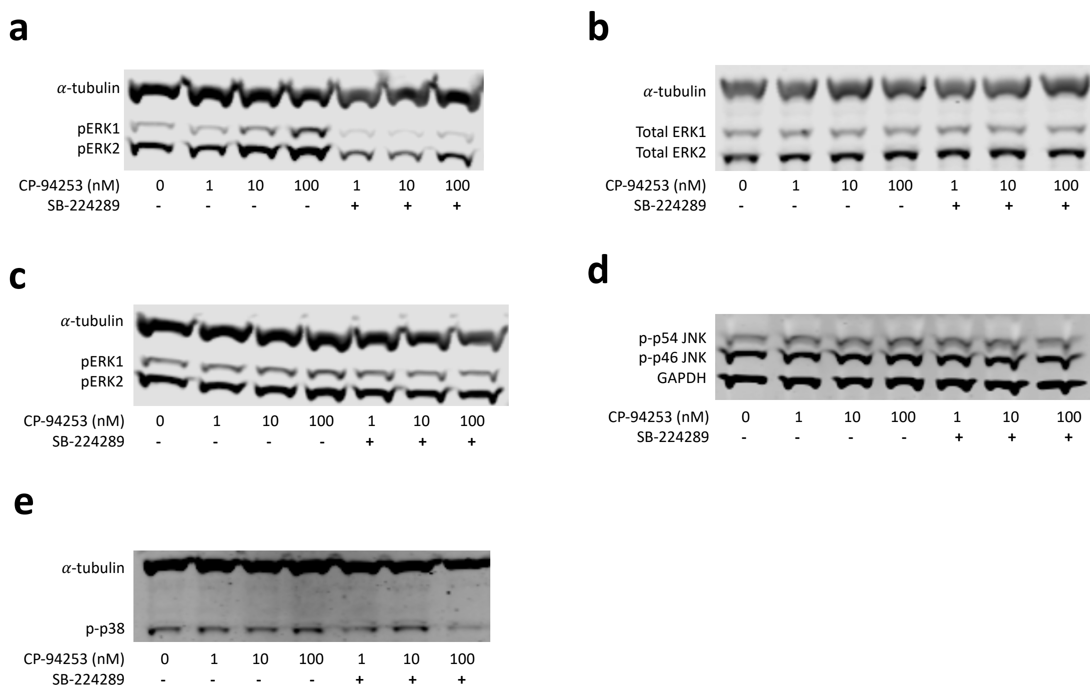

Figure 3.2: CP-94253 increases levels of phospho-ERK1/2, but not phospho-JNK or phospho-p38, in N2A-1B cells, but not in untransfected N2A cells. (a) Treatment with CP-94253 (1-100 nM) for ten minutes increased phosphorylation of ERK1 and ERK2 in N2A-1B cells compared to unstimulated N2A-1B control cells treated with vehicle (PBS), but this was blocked by pretreatment with the antagonist SB-224289. (b) No change was observed in total ERK in N2A-1B cells. (c) No change was observed in phospho-ERK1/2 with agonist treatment in untransfected wild-type N2A cells. Agonist treatment did not change levels of (d) phospho-p54 JNK, phospho-p46 JNK, or (e) phospho-p38.

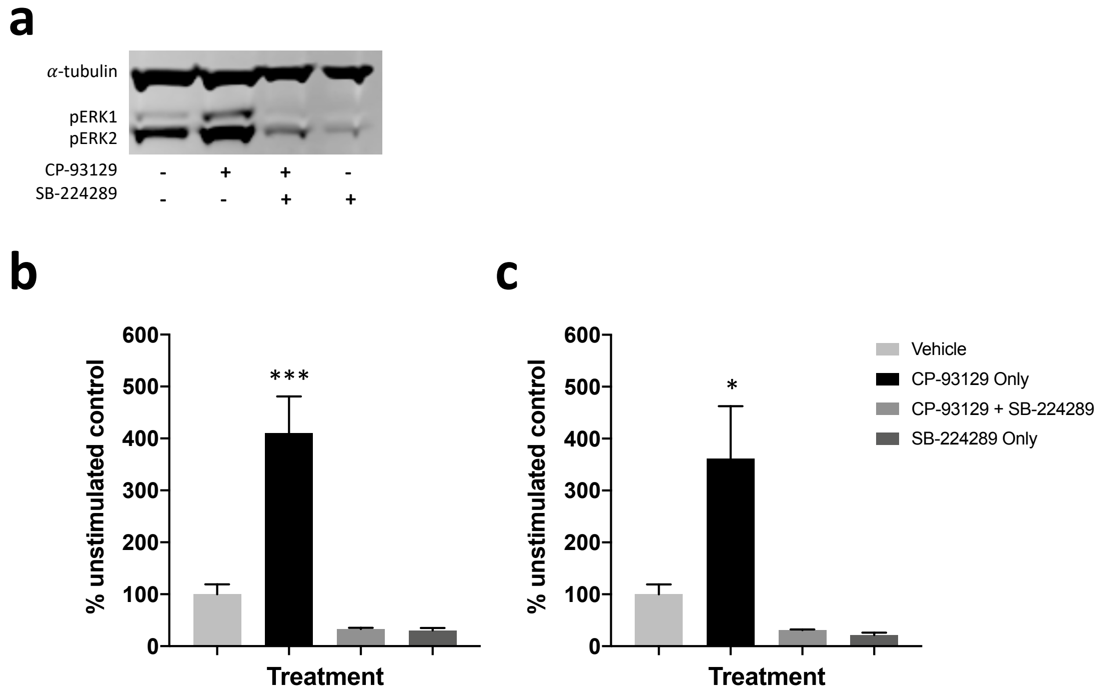

Figure 3.3: CP-93129 increases levels of phospho-ERK1/2 in N2A-1B cells. (a and b) Treatment with CP-94253 (100 nM) for ten minutes increased phosphorylation of ERK1 in N2A-1B cells compared to unstimulated N2A-1B control cells treated with vehicle (PBS) ($p = 0.0008$), but this was blocked by pretreatment with the antagonist SB-224289 ($p = 0.467$ for CP-93129 + SB-224289, $p = 0.437$ for SB-224289 only). (a and c) Treatment with CP-94253 (100 nM) for ten minutes also increased phosphorylation of ERK2 in N2A-1B cells compared to unstimulated N2A-1B control cells treated with vehicle (PBS) ($p = 0.017$), but this was blocked by pretreatment with the antagonist SB-224289 ($p = 0.673$ for CP-93129 + SB-224289, $p = 0.591$ for SB-224289 only). Data are expressed as the percent change in pERK signal compared to the no agonist control from each independent biological replicate. Error bars represent SEM and data are averages of 3 independent biological replicates for all groups (one-way ANOVA with Dunnett's post hoc tests). ***$p < 0.001$, *$p < 0.05$.

5-HT$_{1B}$-mediated phosphorylation of ERK1/2 is a Gα$_i$-dependent process that is reduced with MEK1/2 inhibition

To investigate the signaling intermediates between 5-HT$_{1B}$ receptor activation and phosphorylation of ERK1/2, N2A-1B cells were pretreated with pharmacological inhibitors one hour prior to CP-94253 treatment. Pertussis toxin was used to inhibit Gα$_{i/o}$ signaling, gallein was used to inhibit Gβγ signaling, and U0126 was used to inhibit MEK1/2 signaling. Increased phosphorylation of both ERK1 and ERK2 was blocked when pertussis toxin was present (Figure 3.4a) but was not blocked by gallein (Figure 3.4b). Pretreatment with U0126 also prevented the phosphorylation of ERK1/2 (Figure 3.4c).

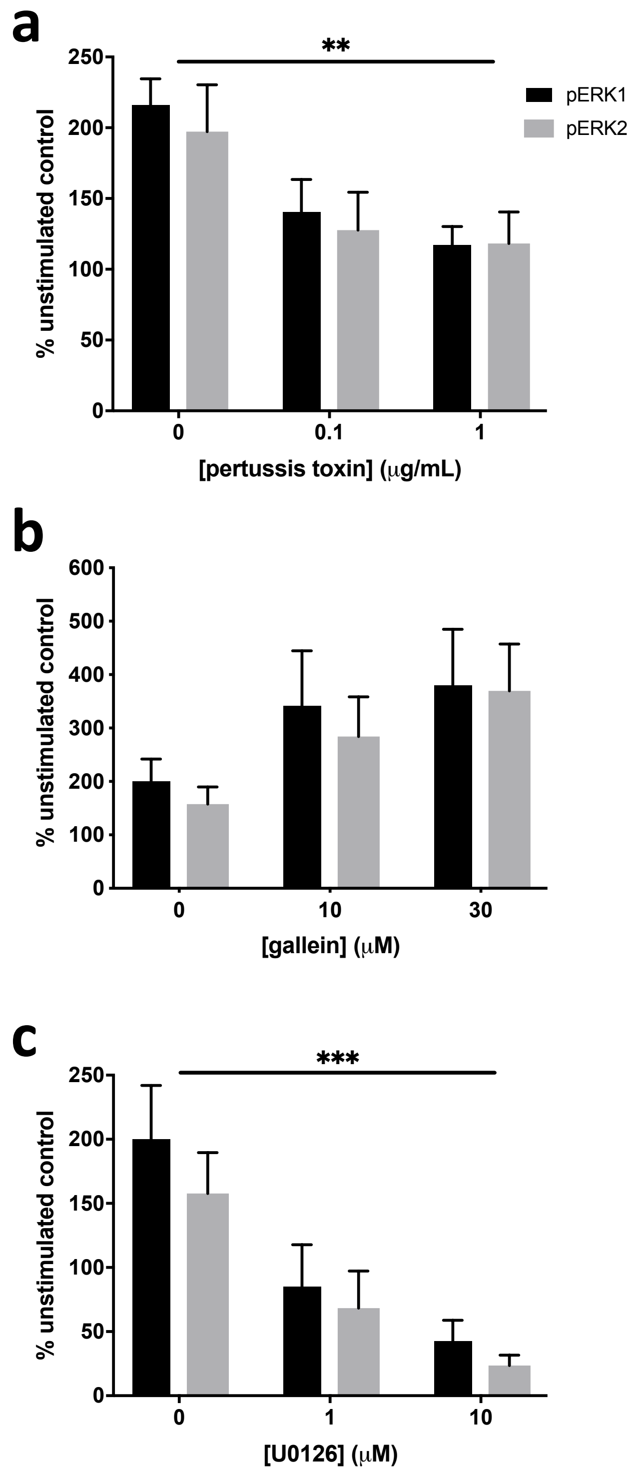

Figure 3.4: Phosphorylation of ERK1/2 by 5-HT$_{1B}$ receptors is sensitive to pertussis toxin and U0126 but not gallein. N2A-1B cells

were pretreated for one hour with inhibitors prior to treatment with 100 nM CP-94253 for ten minutes and compared to unstimulated N2A-1B control cells treated with vehicle (PBS). (a) 5-HT_{1B}-mediated phosphorylation of ERK1/2 was blocked by pertussis toxin (inhibitor effect: $F_{2,18} = 7.98$, $p = 0.0033$). (b) 5-HT_{1B}-mediated phosphorylation of ERK1/2 was not sensitive gallein (inhibitor effect: $F_{2,12} = 3.198$, $p = 0.077$). (c) 5-HT_{1B}-mediated phosphorylation of ERK1/2 was blocked by the MEK1/2 inhibitor U0126 (inhibitor effect: $F_{2,12} = 13.34$, $p = 0.0009$). Data are expressed as the percent change in pERK signal compared to the no agonist control from each independent biological replicate. Error bars represent SEM and data are averages of 3-4 independent biological replicates for each experiment (two-way ANOVA). ***$p < 0.001$, **$p < 0.01$

5-HT_{1B}-mediated phosphorylation of ERK1/2 is dependent on β-arrestins

β-arrestin 1 knockout ("β-Arr1 KO") and β-arrestin 2 knockout ("β-Arr2 KO") N2A-1B cell lines, along with N2A-1B cells transduced with a non-targeting control vector ("control" cells) were used to determine the effect of β-arrestin knockout on ERK1/2 activation following agonist stimulation. Control, β-Arr1 KO, and β-Arr2 KO cells were incubated with or without 100 nM CP-94253 for ten minutes. Agonist treatment significantly increased phospho-ERK1 and phospho-ERK2 levels in control cells, but not in β-Arr1 KO or β-Arr2 KO cells (Figure 3.5). Preincubation with 1 μM SB-224289 blocked both ERK1 and ERK2 activation in control cells, as well as significantly reduced ERK1 phosphorylation in β-Arr2 KO cells and ERK2 phosphorylation in β-Arr1 KO cells (Figure 3.5).

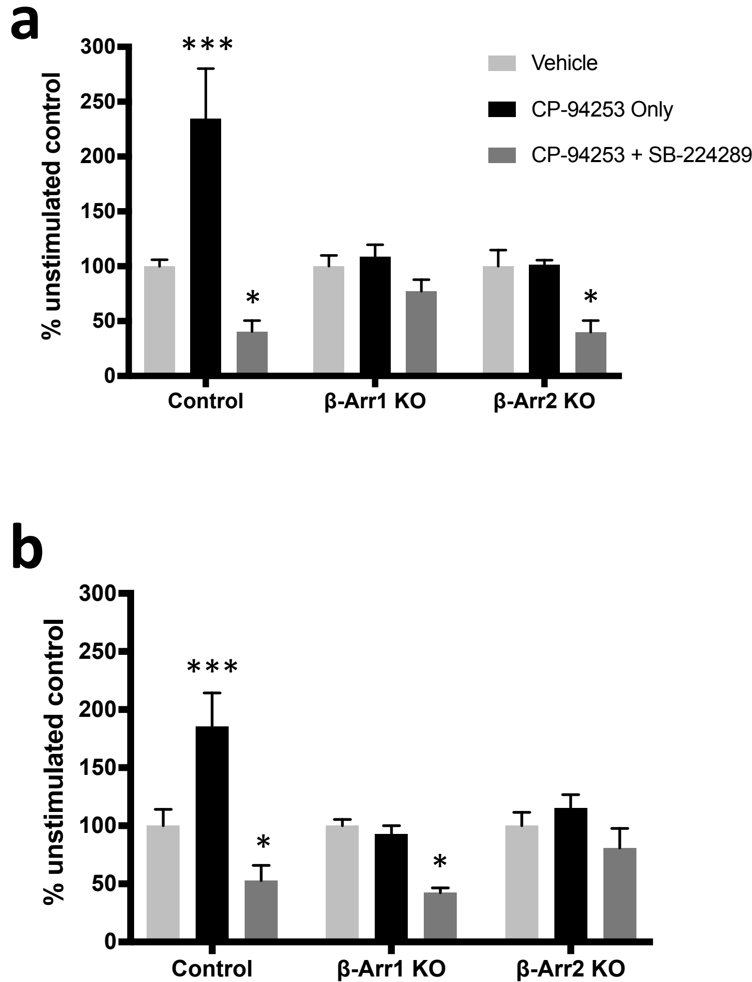

Figure 3.5: 5-HT$_{1B}$-mediated phosphorylation of ERK1/2 is dependent on β-arrestins in N2A-1B cells. N2A-1B control cells, β-arrestin 1 KO cells (β-Arr1 KO), and β-arrestin 2 KO cells (β-Arr2 KO) received treatment with the 5-HT$_{1B}$ agonist CP-94253 (100 nM) alone or pretreatment with the 5-HT$_{1B}$ antagonist SB-224289 (1 μM) 1 hour prior to agonist treatment and compared to unstimulated control cells treated with vehicle (PBS). (a) Levels of phospho-ERK1 significantly differed by treatment ($F_{2,36} = 21.4$, $p < 0.0001$) and cell type ($F_{2,36} = 4.79$, $p = 0.014$), with a significant interaction between treatment and cell type ($F_{4,36} = 7.017$, $p < 0.001$). 5-HT$_{1B}$ receptor stimulation with agonist significantly increased levels of phospho-ERK1 in control cells ($p < 0.0001$) but not in β-Arr1 KO ($p = 0.918$) or β-Arr2 KO cells ($p = 0.997$). (b) Levels of phospho-ERK2 also significantly differed by treatment ($F_{2,36} = 19.3$, $p < 0.0001$) and cell type ($F_{2,36} = 4.362$, $p = 0.020$), with a significant interaction between treatment and cell type ($F_{4,36} = 4.469$, $p = 0.049$). 5-HT$_{1B}$ receptor stimulation significantly increased levels of phospho-ERK2 in control cells (p =

0.0003) but not in β-Arr1 KO ($p = 0.917$) or β-Arr2 KO cells ($p = 0.680$). Furthermore, SB-224289 blocks agonist-induced ERK1/2 activation, reducing phospho-ERK1 levels in control ($p = 0.045$) and β-Arr2 KO cells ($p = 0.043$) (a) and reducing phospho-ERK2 levels in control ($p = 0.048$) and β-Arr1 KO cells ($p = 0.014$) (b). Data are expressed as the percent change in pERK signal compared to the no agonist control from each independent biological replicate. Error bars represent SEM and data are averages of 5 independent biological replicates for all groups (two-way ANOVA with Dunnett's post hoc tests). ***$p < 0.001$, *$p < 0.05$

Discussion

Disturbances of the serotonergic system are implicated in disorders including addiction, fear, anxiety, stress, and depression. Presynaptic 5-HT_{1B} autoreceptors located at serotonergic nerve terminals provide feedback that allows for precise spatial and temporal regulation of synaptic levels of serotonin, thereby affecting serotonergic neurotransmission throughout the entire brain. Postsynaptic 5-HT_{1B} receptors are also localized on axon terminals of diverse but important neuron populations and these may also contribute to potential therapeutic effects of these receptors (McDevitt et al., 2011; McDevitt & Neumaier, 2011). While canonical signaling via inhibition of adenylyl cyclase is well established for this receptor (Barnes & Sharp, 1999; Hamblin et al., 1992), this chapter focuses on noncanonical signaling mechanisms, as they may also be important for mediating the impact of these receptors. Understanding the mechanisms by which 5-HT_{1B} receptors signal may provide new insights into therapeutic opportunities for targeting 5-HT_{1B} receptors in neuropsychiatric disorders.

Activation of these 5-HT_{1B} receptors in N2A-1B cells with CP-94253 induced robust phosphorylation of ERK1/2 but not of other MAP kinases in the same family, demonstrating the specificity of the phospho-ERK1/2 response. We used a combination

of a highly selective 5-HT_{1B} agonist and antagonist in this report to increase our confidence that the 5-HT_{1B} receptor was responsible for inducing ERK1/2 phosphorylation, especially since these cells express low levels of 5-HT_{1A} receptors. Although the degree of biased agonism of CP-94253 for canonical versus noncanonical signaling has not been reported, the structure of the 5-HT_{1B} receptor has been reported to be less likely to lead to biased agonism than other serotonin receptors such as the 5-HT_{2B} receptor, which was shown to display β-arrestin-biased signaling for numerous agonists (Wacker et al., 2013). Additionally, we observed similar inductions in ERK1/2 phosphorylation using a different 5-HT_{1B} receptor agonist, CP-93129. This finding increases our confidence that our results are not due to agonist-specific effects.

We found that pertussis toxin, but not gallein, was able to block ERK1/2 phosphorylation by 5-HT_{1B} receptors completely. This suggests that 5-HT_{1B} receptors must couple to $G\alpha_{i/o}$ in order to activate ERK1/2 signaling, and that this ERK1/2 activation does not depend on $G\beta\gamma$ signaling. Further, CRISPR/Cas9-mediated knockout of either isoform of β-arrestin in N2A-1B cells resulted in blockade of agonist-induced ERK1/2 phosphorylation, suggesting that 5-HT_{1B} receptor-mediated phosphorylation of ERK1/2 is dependent on both β-arrestin 1 and β-arrestin 2 in neuronal cells. β-arrestin 1 and β-arrestin 2 are known to form heterodimers, which influences their localization and signaling properties (Storez et al., 2005). This finding is consistent with several other studies of GPCRs that require both β-arrestin isoforms for signaling, including 5-HT_{2C} and parathyroid hormone 1 (PTH1) receptors (Gesty-Palmer et al., 2006; Labasque et al., 2008), and may explain why both isoforms appear to be necessary for 5-HT_{1B} receptor-mediated ERK1/2 activation. Additionally, GPCRs that

exhibit equivalent affinities for both β-arrestin isoforms can form long-lasting receptor-arrestin complexes that subsequently bind ERK1/2 (Peterson & Luttrell, 2017). Thus, it is possible that 5-HT_{1B} receptors form complexes with β-arrestin to induce phosphorylation of ERK1/2. We were surprised to find that 5-HT_{1B}-mediated ERK1/2 phosphorylation required interactions both with $G\alpha_{i/o}$ and with β-arrestins. However, an intriguing report recently found that when all $G\alpha_{i/o}$ proteins were blocked or deleted by CRISPR/Cas9, β-arrestins still interacted with GPCRs but no longer activated ERK1/2 phosphorylation (Grundmann et al., 2018). β-arrestins may still play important roles in regulating cell surface GPCR density independent of G proteins, but our results lend further support to the complementary involvement of both G proteins and β-arrestins in forming complexes that can mediate noncanonical MAPK signaling by 5-HT_{1B} receptors. We also found that inhibition of MEK1/2 with U0216 prevented phosphorylation of ERK1/2 following 5-HT_{1B} stimulation. While MEK1/2 inhibition reduced levels of phospho-ERK1/2 even in the absence of 5-HT_{1B} agonist (data not shown), this is not surprising given that MEK1/2 is the major upstream activator of ERK1/2 (Luttrell & Luttrell, 2003; Luttrell & Lefkowitz, 2002; Luttrell & Miller, 2013), and our results demonstrate that 5-HT_{1B}-mediated ERK1/2 phosphorylation does not occur in the absence of MEK1/2 signaling.

Interestingly, preincubation with the 5-HT_{1B} antagonist SB-224289 not only prevented agonist-induced phosphorylation of ERK1/2, but also significantly reduced phosphorylation of ERK1 in control and β-Arr2 KO N2A-1B cells, and ERK2 in control and β-Arr1 KO N2A-1B cells. SB-224289 is a potent inverse agonist and reduces the constitutive activity of 5-HT_{1B} receptors (Gaster et al., 1998; Selkirk et al., 1998). Our

results raise the possibility that the constitutive activity of the 5-HT_{1B} receptor influences ERK1 and ERK2 differentially via the two β-arrestin isoforms, with β-arrestin 1 being more important for basal activation of ERK2 and β-arrestin 2 being more important for ERK1 activation. Different GPCR ligands affect not only the affinity of receptors for the β-arrestins but also the affinity of the receptor-arrestin complexes for their downstream effectors (Peterson & Luttrell, 2017). Future experiments should investigate whether there is an interaction between 5-HT_{1B} receptors and ERK1 and ERK2 that is mediated preferentially by β-arrestin 2 and β-arrestin 1, respectively. Furthermore, the possibility that there is constitutive activity of 5-HT_{1B} receptors for β-arrestin-mediated signaling *in vivo* should be examined in future studies.

Taken together, this data show that agonist-induced 5-HT_{1B} receptor activation leads to selective phosphorylation of ERK1/2, with contributions from G protein-dependent signaling through the $G\alpha_{i/o}$ subunit, as well as from interactions with β-arrestins 1 and 2. This work sheds light on the complexity of signal transduction mechanisms that may underlie the diverse functions of the 5-HT_{1B} receptor in neurons.

Portions of this chapter were adapted from: Liu Y, Gibson AW*, Levinstein MR, Lesiak AJ, Ong SE, and Neumaier JF. (2019) 5-HT_{1B} receptor-mediated activation of ERK1/2 requires both $G\alpha_{i/o}$ and β-arrestin proteins. ACS Chemical Neuroscience. 10(7): 3143-3153*

**Co-First Authors*

CHAPTER 4

Circuit-Specific Modulation of Cocaine Taking by 5-HT_6 Receptors

Introduction

Substance use disorders are prevalent neuropsychiatric conditions that are costly on personal and societal levels (Patel et al., 2016; Rehm et al., 2017; Rehm et al., 2009). Addiction is thought to involve maladaptations of the neural processes that normally mediate reward learning in neural circuitry converging in the dorsal and ventral striatum (Hyman et al., 2006), which lead to compulsive substance taking. In particular, the nucleus accumbens (NAc) of ventral striatum is crucial to drug reward and may be an appropriate target for future addiction therapies (Stuber et al., 2011). The NAc is comprised of mostly GABAergic medium spiny neurons (MSNs) which project through two distinct pathways, known as the direct (striatonigral) and indirect (striatopallidal) pathways (Gerfen & Young, 1988; Gerfen et al., 1990; Gerfen et al., 1991; Surmeier et al., 2007). Output via the direct pathway is thought to facilitate actions while the indirect pathway is thought to inhibit actions (Chandra et al., 2015; Macpherson et al., 2014; Yager et al., 2015). The direct pathway MSNs (dMSNs) express mainly D_1 dopamine receptors and the neuropeptide dynorphin (Dyn) whereas the indirect pathway MSNs (iMSNs) express mainly D_2 dopamine receptors and the neuropeptide enkephalin (Enk) (Gerfen & Young, 1988; Gerfen et al., 1990; Gerfen et al., 1991; Hikida et al., 2010). The specific functions and mechanisms underlying drug seeking within these pathways is not well understood, but the relative activity in these pathways likely plays a role in the progression of addiction (Bock et al., 2013; Hikida et al., 2010).

Cocaine's reinforcing properties involve direct interactions with monoamine transporters including SERT (Sora et al., 2001; Uchimura & North, 1990). 5-HT neurons strongly innervate ventral striatum (Dolen et al., 2013; McDevitt et al., 2014; Parsons & Justice, 1993a) and cocaine increases extracellular serotonin in NAc (Andrews & Lucki, 2001; Parsons & Justice, 1993b). By increasing extracellular 5-HT, cocaine enhances signaling at multiple 5-HT receptor subtypes, several of which have been implicated in mediating the reinforcing properties of cocaine in NAc (Muller & Homberg, 2015).

Among the 14 identified serotonin receptors, 5-HT_6 receptors are notable for their abundance in striatum, where they are expressed in both dMSNs and iMSNs (Hirst et al., 2003; Tassone et al., 2011; Ward et al., 1995). They are excitatory $G\alpha_s$-coupled receptors capable of activating AC and initiating cAMP signaling (Nichols & Nichols, 2008). 5-HT_6 receptors also localize to neuronal primary cilia, where they serve to detect extra-synaptic serotonin in the extracellular space (Berbari et al., 2008; Brailov et al., 2000; Hamon et al., 1999; Sterpka & Chen, 2018). These receptors can influence both ciliary and dendritic morphology, which provides additional mechanism for extra-synaptic 5-HT to influence ciliary signaling and neuronal function (Brodsky et al., 2017; Lesiak et al., 2018).

Some reports found no effect of a systemic 5-HT_6 agonist or antagonist on cocaine self-administration (cocaine SA) (Fijal et al., 2010; Frantz et al., 2002; Valentini et al., 2013), while other studies suggested that cocaine reinforcement and reinstatement was regulated by 5-HT_6 receptor activity (Valentini et al., 2013; van Gaalen et al., 2010). We previously showed that viral-mediated increases in 5-HT_6 receptor expression in NAc shell (NAcSh) blocked the acquisition of conditioned place

preference to cocaine without altering psychomotor sensitization (Ferguson et al., 2008). 5-HT_6 receptors also regulate reward motivated learning and the expression of habitual actions (Eskenazi & Neumaier, 2011a; Eskenazi & Neumaier, 2011b). In both these studies, 5-HT_6 receptors were generally increased in MSNs and were not specifically targeted to the direct or indirect pathway. Recently, we observed that increased striatal 5-HT_6 receptor activity had different effects on reward motivated learning using sucrose reinforcers when expression was restricted to either the dMSNs or iMSNs (Eskenazi et al., 2015), suggesting that the distribution of receptors is a key determinant of the behavioral impact of 5-HT_6 receptor signaling.

In general, striatal 5-HT_6 receptors tend to oppose dopamine's effects on drug reward (Eskenazi et al., 2015). Because this might involve opposing the differential activation of both pathways by dopamine, next steps involved the use of phenotype-specific viral vectors to increase expression of 5-HT_6 receptors or GFP selectively in the dMSN's or iMSNs in the NAcSh of rats. We hypothesized that 5-HT_6 receptors in dMSNs and iMSNs would increase and decrease cocaine reinforcement, respectively. In this chapter, I describe experiments that illuminate the role of 5-HT_6 receptors in the circuit-specific modulation of cocaine self-administration.

Methods

Animals

For all experiments, male Long-Evans rats (Charles River, Raleigh, NC) weighing approximately 350 grams were used. Rats were double-housed for one week to acclimate them to the temperature- and humidity-controlled vivarium prior to the

experiment and were kept under a 12-hour light–dark cycle. All experiments were carried out during the light period. Following implantation of intravenous catheters, the rats were housed individually. Food and water were freely available at all times except during the cocaine SA sessions. All experimental procedures were approved by the University of Washington Institutional Animal Care and Use Committee and were conducted in accordance to the guidelines of the "Principles of Laboratory Animal Care" (NIH publication no. 86–23, 1996). A total of 76 rats were used for these experiments, of which 4 were excluded due to failure to learn to self-administer cocaine, 5 were excluded because viral-mediated gene expression was outside the target brain region, and 11 were removed from the study due to health issues (such as lost IV access).

Intravenous catheter placement and intracranial virus-mediated gene transfer

Jugular catheters were implanted as previously described (Nair et al., 2013). Rats were allowed to recover for 10 days before cocaine SA training. During the recovery and training phases, catheters were flushed every 48 hours using sterile gentamicin (0.08 mg/mL). We used replication-deficient herpes-simplex viral (HSV) vectors to increase 5-HT_6 receptor expression in either dMSNs or iMSNs of the NAcSh. The experiment utilized four different viral cassettes for this manipulation: two that express fully functional HA-tagged 5-HT_6 receptors under either the proenkephalin promoter (Enk-5-HT_6) or the prodynorphin promoter (Dyn-5-HT_6), and two control viruses that express GFP alone under either the proenkephalin promoter (Enk-GFP) or the prodynorphin promoter (Dyn-GFP). We have previously confirmed that these viral vectors produce HA-tagged 5-HT_6 receptors in either enkephalin or dynorphin

containing neurons and not glia (Eskenazi et al., 2015; Ferguson et al., 2011; Michaelides et al., 2013). HSV vectors were injected at 400 nl/min using surgical procedures previously described (Eskenazi et al., 2015; Ferguson et al., 2013). The volume of viral vector (2 µl) was chosen based on previous studies in our laboratory to induce discrete infection in the target region (Mitchell & Neumaier, 2008; Mitchell et al., 2007). Using a 10° angle of approach, the NAcSh was targeted using the coordinates relative to bregma: +1.7 mm (anterior-posterior), ± 2.3 mm (medial-lateral), -7.6 mm (dorsal-ventral). To confirm the injection site, rats were perfused as previously described (Eskenazi & Neumaier, 2011a; Eskenazi & Neumaier, 2011b), brains were dissected and post-fixed in 2.5% paraformaldehyde for 6 hours, after which they were placed 30% sucrose in PBS. Tissue sections were made on a Leica Jung CM 3000 cryostat and mounted on slides at 40 µm thickness. Accuracy of injection coordinates was confirmed by visualization of the injection needle tracts and immunological staining. Rats with injection sites outside of the targeted brain region were excluded from the experiments.

Behavioral Apparatus

The rats were trained and tested in standard Med Associates operant chambers (Med Associates, Georgia, VT). Each chamber was equipped with two levers located 9 cm above the grid floor. Lever-presses on the active lever activated the infusion pump whereas lever-presses on the inactive lever had no programmed response. All chambers were kept in sound-attenuating boxes equipped with fans for temperature regulation and to provide white noise. All chambers were connected to a Med

Associates interface, and experimental data were collected using Med-PC software.

Cocaine Self-Administration

The procedure consisted of four phases: fixed-ratio cocaine SA (11 days), progressive ratio (4 days), low-dose responding (3 days) and high-dose responding (3 days). Rats were trained to self-administer cocaine for 2 hours/day (2 one hour sessions with a 5 minute interval between sessions) for 11 days. Cocaine hydrochloride (National Institute on Drug Abuse, Bethesda, MD) was dissolved in sterile injectable 0.9% saline and infused in a volume of 0.1 ml at a dose of 0.75 mg/kg/infusion. Each session started with the turning on of a white house-light and introduction of the levers into the operant chamber. During training, cocaine infusions were earned under a fixed-ratio-1 (FR1), 20 second timeout reinforcement schedule and were accompanied by a compound tone-light cue for 5 seconds. During the 20 second timeout period, lever presses were recorded but did not result in cocaine delivery. A maximum of 20 cocaine infusions/hour was set to prevent cocaine overdose. At the end of each session, the house-light was turned off and the levers retracted.

Progressive Ratio

Following the FR1 sessions, rats were trained to SA cocaine for four days on a progressive ratio (PR) reinforcement schedule, during which the response requirement to earn a cocaine infusion (0.75 mg/kg/infusion) increased after each infusion earned. The response requirement increased incrementally in accordance with the following equation: Response ratio = (5e[injection number x 0.2] - 5) (Richardson & Roberts,

1996). The PR sessions were terminated when the rat failed to receive a cocaine infusion within one hour.

Cocaine Dose-Response Experiments

Following cocaine SA on FR1 and PR schedules, behavioral responding was measured for 3 days each on a low and a high dose of cocaine. The low-dose testing was done during a 2 hour FR1 SA session where each response yielded a cocaine infusion (0.375 mg/kg/infusion). Three days of high-dose (1.5 mg/kg/infusion) FR1 sessions followed immediately after the low dose period.

Cocaine Pharmacokinetic Modeling

Whole-brain levels of cocaine were modeled using a two-compartment mathematical model for rats receiving iv cocaine as previously described (Zimmer et al., 2011), based on an original report that measured extracellular cocaine concentration in the NAc measured by microdialysis (Pan et al., 1991). The average estimated cocaine concentration for each session was calculated between 10-120 minutes after the initiation of the session using the equation:

$$c = \frac{dk}{v(\alpha - \beta)}\left(e^{-\beta t} - e^{-\alpha t}\right)$$

which gives the estimated cocaine brain concentration (*c*) by accounting for the dose of cocaine (*d*), the transport of cocaine between the blood and brain (k = 0.233 min^{-1}), the brain volume (v = 0.151 kg^{-1}), and the removal of cocaine from circulation via redistribution (α = 0.642 min^{-1}) and elimination (β = 0.097min^{-1}). Group means for each

unit dose were calculated from the average values for each animal over the three testing sessions.

Locomotor Activity

On the day following the completion of all cocaine SA behavior, locomotor activity was measured in infrared beam break activity boxes (22 x 45 x 23 cm; San Diego Instruments, San Diego, California) for 30 minutes in a dimly lit room. Locomotor activity was analyzed in 3 minute bins.

Conditioned Place Preference

A separate cohort of rats began place conditioning trials on the eleventh day after viral infusion using a three-chamber conditioned place preference (CPP) apparatus (Medical Associates, St Albans, VT, USA) comprised of two large side chambers (24 x 21 x 21 cm) separated by a small central chamber (12 x 21 x 21 cm). The three chambers differed in lighting (dim, medium or bright), wall color (white, black or gray) and floor texture (grid, rod or solid). Before the onset of the study, light intensities were adjusted so that there was no overall preference by a separate test group of animals for any chamber. We did not use a pre-exposure trial to avoid latent inhibition of associations between the drug effect and the chamber cues (Barot et al., 2007; Tzschentke, 1998). Chamber pairing with drug was randomly assigned in a counterbalanced fashion so any preexisting individual preferences would be randomly distributed.

The first trial of each conditioning day began at 9:00–10:00 am, with each animal receiving an injection of isotonic saline (1 mL/kg, i.p.). After the injection, animals were confined to one of the side chambers of the CPP apparatus for 15 minutes. Three hours after the morning trial, animals received cocaine hydrochloride (NIDA 5 mg/kg in 1 ml/kg saline, i.p.), after which they were placed into the other side chamber for 15 minutes. Conditioning trials were repeated in the same manner for the following 3 days. The day following the three conditioning trials (Day 14), animals were tested for CPP. The animal was placed into the central chamber, and after a 3 minute habituation period the doors were raised and the animal was allowed to explore the entire apparatus for 15 minutes. Time spent in each compartment was recorded automatically.

Immunohistochemistry

Floating sections (40 µm) were washed in 0.5% Triton-X/PBS for 10 minutes, then blocked in 10% normal goat serum (NGS)-Triton-X/PBS for 1 hour. Sections were then incubated in 5% NGS-Triton-X/PBS containing HA (1:400, rabbit, Cell Signaling) with gentle agitation at 4ºC overnight. Next, sections were rinsed 4 times in PBS and incubated with Alexa 488-conjugated, goat anti-rabbit secondary antibody (1:250, Invitrogen, Carlsbad, CA) for 2 hours. Sections were washed 3 times in PBS, mounted on slides and cover-slipped with ProLong Gold Antifade mounting medium (Life Technologies). Images were captured with a Nikon fluorescence microscope and associated ZEN software.

Statistical analyses

Data from the cocaine SA sessions was collected using Med PC IV software. Analysis was performed using GraphPad Prism (Version 5.01). Significance for all cocaine SA data were tested with two-way analyses of variance (ANOVAs; with or without repeated measures, as warranted) followed by Bonferroni post hoc tests. For analysis involving only two samples we used a two-tailed t-test. For all comparisons, we used an alpha value of 0.05.

Results

Pathway-Specific Increase of 5-HT$_6$ Receptors in NAcSh

To increase expression of 5-HT$_6$ receptors in either the direct or indirect pathway neurons selectively, we used HSV viral vectors that have been described (Figure 4.1a) (Eskenazi et al., 2015; Ferguson et al., 2011; Ferguson et al., 2013; Michaelides et al., 2013). These vectors utilize the rat prodynorphin (Dyn) or the rat proenkephalin (Enk) promoter to induce transgene expression of either hemagglutinin (HA) tagged 5-HT$_6$ receptors or GFP in direct and indirect pathway medium spiny neurons (dMSNs and iMSNs), respectively. The dMSN and iMSN manipulations were performed on separate cohorts of animals run at different times and were hence analyzed separately. The medial NAcSh was targeted and accurate injections were confirmed histologically (Figure 4.1b); five animals were excluded due to inaccurate injections. Transgene expression using the Enk and Dyn promoters has been shown by our lab to be present up to two months post-injection (Ferguson et al., 2008) and was confirmed in NAcSh 40 days post-injection using immunohistochemistry (Figure 4.1c). The experimental timeline is illustrated in Figure 4.1d.

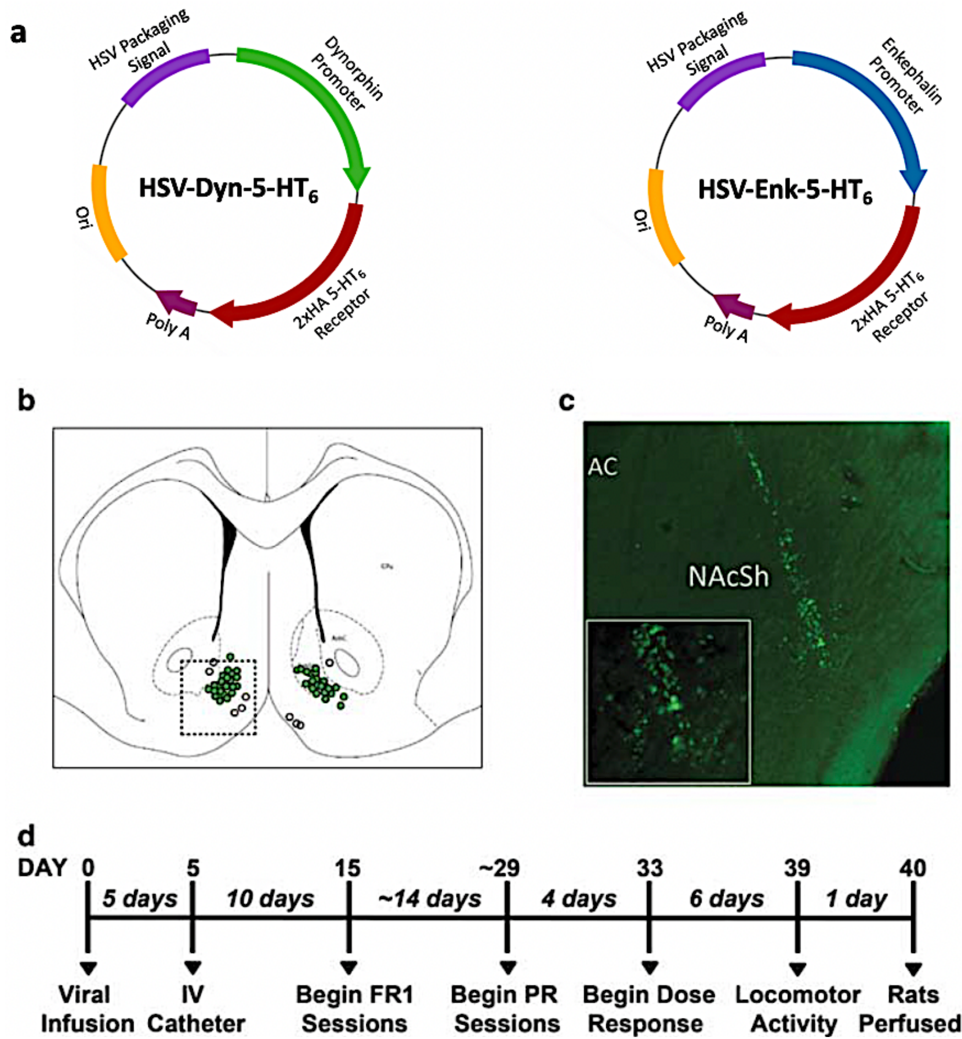

Figure 4.1: Pathway Specific Targeting of 5-HT$_6$ Receptors in NAcSh. (a) Viral vector plasmid maps: plasmid maps for both experimental vectors expressing hemagglutinin (HA)-tagged 5-HT$_6$ receptors via either the pENK promoter (Enk-5-HT$_6$) or the pDYN promoter (Dyn-5-HT$_6$). (b) Diagram depicting locations of viral injections in NAcSh in experimental animals. Hits are shown in green and misses are shown as empty circles. Inset dashed square shows area imaged for Figure 4.1c. (c) Photomicrograph at 5x magnification depicting the immunostaining of cells in the NAcSh infected with experimental virus Enk-5-HT$_6$ more than 40 days past infection. Anti-HA antibody is shown in green. Inset shows same area at 20x magnification (AC: anterior commissure). (d) Timeline of experimental procedure.

Increasing 5-HT$_6$ receptors in NAcSh iMSNs but not the dMSNs decreases operant cocaine SA on a FR1 schedule

In rats treated with Dyn-GFP or Dyn-5-HT$_6$, there was no significant difference between viral vector treatment groups on the number of cocaine infusions (Figure 4.2a), indicating that increased expression of 5-HT$_6$ receptors in dMSNs did not alter cocaine reinforcement at a moderate unit dosage (0.75 mg/kg/infusion). The rats had established stable patterns of cocaine self-administration after the first 8 days; as a result, we examined the final three days of FR1 responding and found no significant difference in average cocaine infusions between these treatment groups (Figure 4.2b). In contrast, in rats expressing either Enk-5-HT$_6$ or Enk-GFP in medial NAcSh there was a significant difference between viral vector treatment groups on the number of cocaine infusions (Figure 4.2c) indicating that increased expression of 5-HT$_6$ receptors in iMSNs decreased cocaine SA on a FR1 reinforcement schedule. We examined the final three days of FR1 responding and the Enk-5-HT$_6$-expressing rats took significantly less cocaine (Figure 4.2d). There was a significant difference between active and inactive lever presses for both the 5-HT$_6$ receptor and GFP groups in the dMSNs (Figure 4.3a), as well as for the 5-HT$_6$ receptor and GFP groups in the iMSNs (Figure 4.3b) across all days of FR1 training.

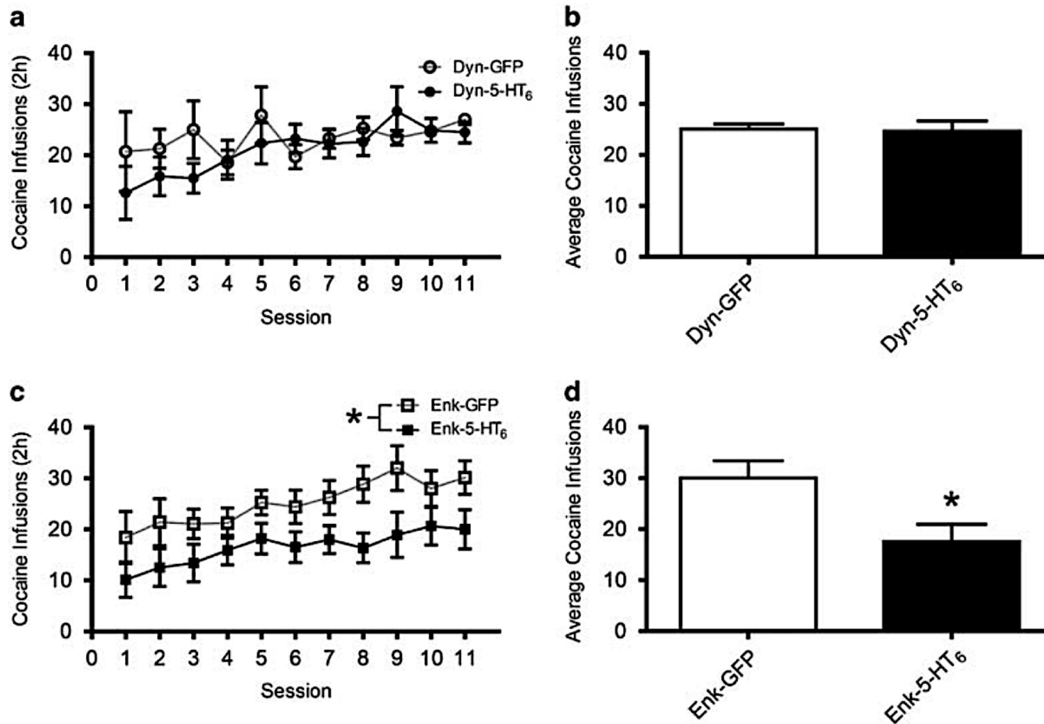

Figure 4.2: Increasing 5-HT$_6$ receptors in NAcSh iMSNs but not the dMSNs decrease operant cocaine SA on a FR1 schedule. (a) Daily FR1 cocaine infusions (Mean ± SEM) do not differ between Dyn-GFP (n = 7) and Dyn-5-HT$_6$ (n = 8) groups (two-way ANOVA, $F_{(1,13)} = 0.87$, p = 0.37). (b) Average FR1 cocaine infusions during the last 3 days of training (Mean ± SEM) do not differ between Dyn-GFP (n = 7) and Dyn-5-HT$_6$ (n = 8) groups (t-test, p = 0.87). (c) Daily FR1 cocaine infusions (Mean ± SEM) are significantly different between Enk-GFP (n = 12) and Enk-5-HT$_6$ (n = 14) groups (two-way ANOVA, $F_{(1,24)} = 5.32$; p = 0.03). (d) Average FR1 cocaine infusions during the last 3 days of training (Mean ± SEM) are significantly different between Enk-GFP (n = 12) and Enk-5-HT$_6$ (n = 14) groups (t-test, p = 0.015). * = $p < 0.05$.

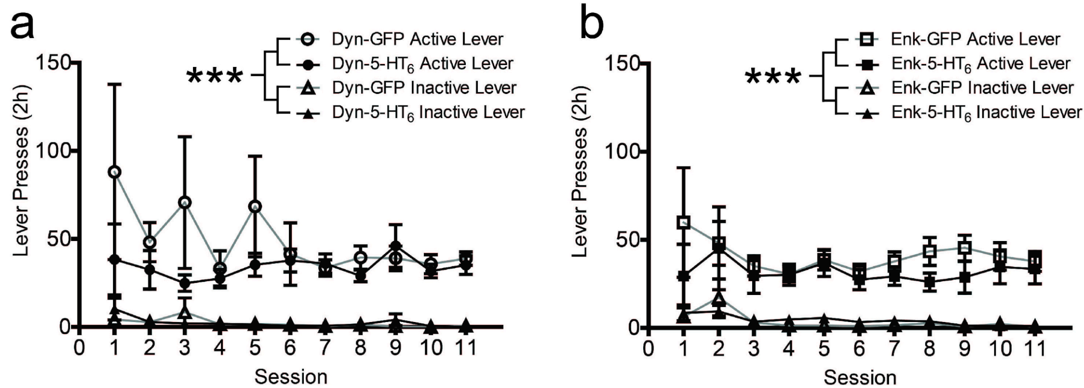

Figure 4.3: Active and inactive lever presses are different for rats with increased 5-HT$_6$ receptors or GFP in both dMSNs and iMSNs. (a) Daily FR1 cocaine active and inactive lever presses (Mean ± SEM) are significantly different between Dyn-GFP (n = 7) and Dyn-5-HT$_6$ (n = 8) groups (two-way ANOVA, $p < 0.001$). (a) Daily FR1 cocaine active and inactive lever presses (Mean ± SEM) are significantly different between Enk-GFP (n =12) and Enk-5-HT$_6$ (n = 14) groups (two-way ANOVA, $p < 0.001$). *** = $p < 0.0001$.

Increased expression of 5-HT$_6$ receptors in iMSNs shifts the dose-preference curve downward

Rats were exposed to two additional doses of cocaine (0.375 mg/kg/infusion, and 1.5 mg/kg/infusion) for three days at each dose, starting with the low dose and followed by the high dose. We also included SA data from the last three training days at the 0.75 mg/kg/infusion dose in our analyses to depict the complete dose-preference curve. All groups had decreasing preference for the highest dose of cocaine as indicated by fewer infusions taken as dose increased. Rats with increased 5-HT$_6$ receptors in the dMSNs did not differ from their GFP counterparts with regard to either the number of cocaine infusions (Figure 4.4a) or the total cocaine taken (Figure 4.4b). However, rats with increased 5-HT$_6$ receptors in iMSNs administered significantly fewer cocaine infusions

at the lower two doses (Figure 4.4c) as well as took less cocaine overall across all doses (Figure 4.4d).

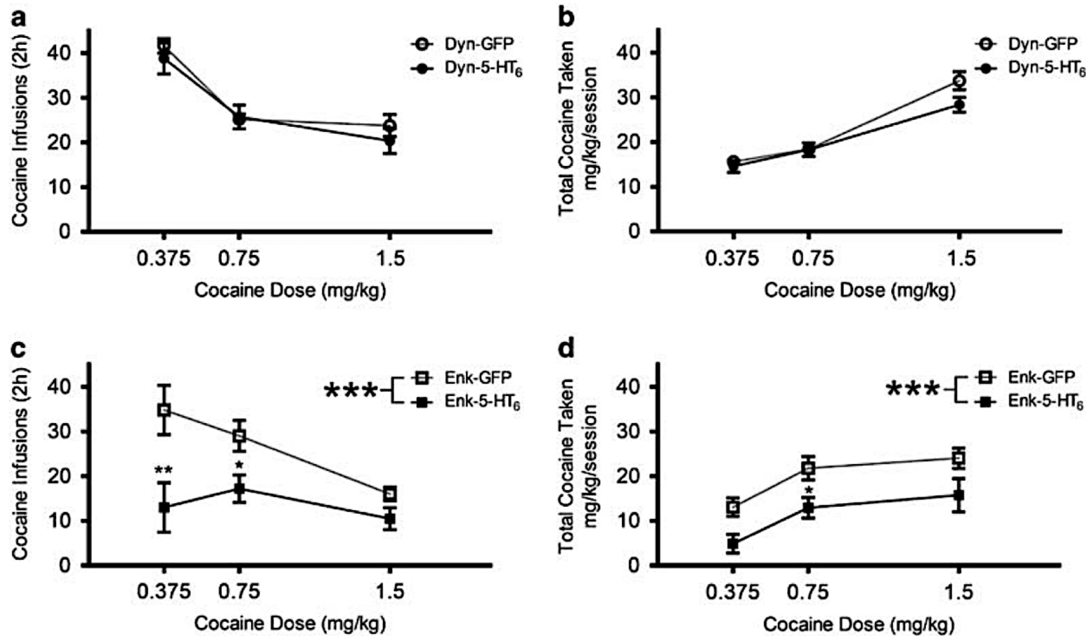

Figure 4.4: Increasing expression of 5-HT$_6$ receptors in iMSNs shifts the dose-preference curve downward. (a) Dose-dependent cocaine infusions (Mean ± SEM) do not differ between rats with Dyn-GFP or Dyn-5-HT$_6$ (Dyn-5-HT$_6$, n = 4-8 depending on dose; Dyn-GFP n= 4-7 depending on dose) at three unit doses of cocaine infusion (0.375 mg/kg/infusion, 0.75 mg/kg/infusion, and 1.5 mg/kg/infusion) (two-way ANOVA, $F_{(1,29)} = 0.77$, $p = 0.39$). (b) Total cocaine received (Mean ± SEM) does not differ between rats with Dyn-GFP or Dyn-5-HT$_6$ at the three cocaine doses (0.375 mg/kg, 0.75 mg/kg, and 1.5 mg/kg) (two-way ANOVA, $F_{(1,29)} = 1.75$, $p = 0.20$). (c) Dose dependent cocaine infusions (Mean ± SEM) are significantly different between rats with Enk-GFP or Enk-5-HT$_6$ (Enk-5-HT$_6$ n = 8-13 depending on dose; Enk-GFP n = 8-13 depending on dose) at the three doses (0.375 mg/kg, 0.75 mg/kg, and 1.5 mg/kg) (two-way ANOVA with Bonferroni post-hoc tests, $F_{(1,52)} = 16.85$; $p = 0.0001$). (d) Total cocaine received (Mean ± SEM) is significantly different between rats with Enk-GFP or Enk-5-HT$_6$ at the three doses (0.375 mg/kg, 0.75 mg/kg, and 1.5 mg/kg) (two-way ANOVA with Bonferroni post-hoc tests, $F_{(1,52)} = 14.9$, $p = 0.0003$). * = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$.

Increasing expression of 5-HT$_6$ receptors in the NAcSh iMSNs changes patterns of cocaine taking

To assess whether increased 5-HT$_6$ receptors in either dMSNs or iMSNs affects the pattern of cocaine taking, we examined the time-course (15 minute intervals) of FR1 operant responding for a unit dose of 0.75 mg/kg/infusion. The number of infusions per 15 minute bin over the course of the session did not change for rats with increased 5-HT$_6$ receptors in dMSNs (Figure 4.5a). However, the average number of infusions per 15 minute bin over the course of the session was significantly decreased in rats with increased 5-HT$_6$ receptors in iMSNs (Figure 4.5b). We also examined the interval from the initiation of the session until the first response within each session. We found no change in the average time to first response for the group with increased 5-HT$_6$ receptors in dMSNs (Figure 4.5c), but increased 5-HT$_6$ receptors in iMSNs significantly increased the interval prior to the initial lever press of each session (Figure 4.5d).

Increasing expression of 5-HT$_6$ receptors in iMSNs reduces the preferred brain cocaine concentration

To further probe the pattern of cocaine taking among rats, we used a previously described pharmacokinetic model to estimate local brain concentration of cocaine in NAc (Pan et al., 1991; Zimmer et al., 2011). For reference, examples of how the model predicted brain cocaine concentration during the SA session are shown for Enk-GFP and Enk-5-HT$_6$ (Figure 4.5e and f, respectively). Estimated tissue cocaine concentrations were not different between Dyn-5-HT$_6$ and Dyn-GFP rats at any unit dose tested (Figure 4.5g). However, rats with increased 5-HT$_6$ receptors in iMSNs

titrated around significantly lower cocaine concentrations as compared to their GFP counterparts (Figure 4.5h).

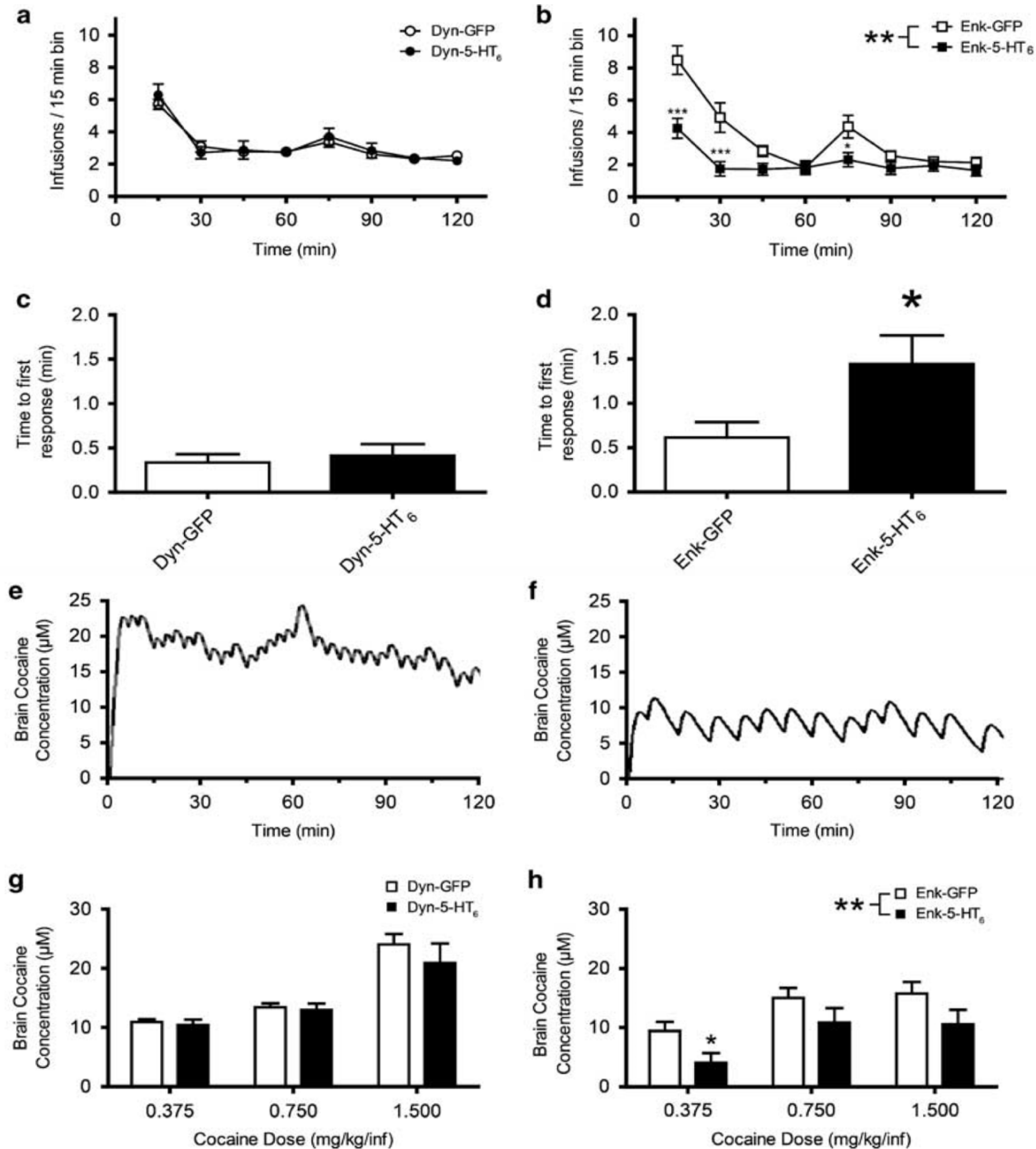

Figure 4.5: Increasing expression of 5-HT$_6$ receptor in the NAcSh iMSNs changes patterns of cocaine taking and reduced the preferred brain cocaine concentration. (a) Average cocaine infusions (Mean ±

SEM) per 15 minute bin on mid-dose testing days does not differ between rats expressing either Dyn-GFP (n = 7) or Dyn-5-HT_6 (n = 8) (two-way ANOVA, $F_{(1,11)} = 2.56$; $p = 0.14$). (b) Average cocaine infusions (Mean ± SEM) per 15 minute bin on mid-dose testing days is significantly different between rats expressing either Enk-GFP (n = 13) or Enk-5-HT_6 (n = 13) (two-way ANOVA with Bonferroni post-hoc tests, $F_{(1,24)} = 9.07$, $p = 0.006$). (c) Average time to initial response in minutes does not differ betweeen Dyn-GFP (n = 7) and Dyn-5-HT_6 (n = 8) rats on all testing days (t-test, $p = 0.63$). (d) Average time to initial response in minutes is significantly different between Enk-GFP (n = 13) and Enk-5-HT_6 (n = 14) rats on all testing days (t-test, $p = 0.035$). Example brain cocaine concentration modeling at 0.75 mg/kg/infusion are shown for reference Enk-GFP (e) and Enk-5-HT_6 (f). (g) Average estimated brain cocaine concentrations across doses (0.375 mg/kg/infusion, 0.75 mg/kg/infusion, and 1.5 mg/kg/infusion) does not differ between Dyn-5-HT_6 rats (n = 4-8 depending on dose) and Dyn-GFP rats (n= 4-7 depending on dose) (two-way ANOVA, $F_{(1,29)} = 1.94$, $p = 0.18$). (h) Average estimated brain cocaine concentrations across doses (0.375 mg/kg/infusion, 0.75 mg/kg/infusion, and 1.5 mg/kg/infusion) is significantly different between Enk-5-HT_6 rats (n = 8-14 depending on dose) and Enk-GFP rats (n = 8-14 depending on dose) (two-way ANOVA with Bonferroni post-hoc tests, $F_{(1, 54)} = 7.427$, $p = 0.009$). * = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$.

Increasing expression of 5-HT_6 receptors in either the NAcSh dMSNs or iMSNs MSNs does not affect cocaine SA on PR reinforcement schedule

On a progressive ratio (PR) reinforcement schedule, increased 5-HT_6 receptor expression in dMSNs had no significant effect on the total number of cocaine infusions when averaged over 4 PR testing days (Figure 4.6a) or average active-lever presses (Figure 4.6b). Similarly, increased 5-HT_6 receptor expression in iMSNs had no significant effect on the average number of cocaine infusions over four PR testing days (Figure 4.6c) or active-lever presses (Figure 4.6d). Thus, we conclude that the motivation to self-administer cocaine at the dose of 0.75 mg/kg/infusion was not directly affected by 5-HT_6 receptors in either pathway.

Increasing expression of 5-HT$_6$ receptors in NAcSh iMSNs increases conditioned place preference for cocaine at a low dose

To examine whether increased 5-HT$_6$ receptors in the indirect pathway altered the sensitivity to the rewarding properties of cocaine, we tested the effects of expressing 5-HT$_6$ receptors or GFP in iMSNs on the ability of cocaine to support CPP to a low dose of cocaine (5 mg/kg) that we previously found was too low to produce a consistent CPP in control rats (Barot et al., 2007; Neumaier et al., 2002). The rats with increased 5-HT$_6$ receptor expression in iMSNs spent a significantly longer period exploring the cocaine paired chamber during testing (Figure 4.6e).

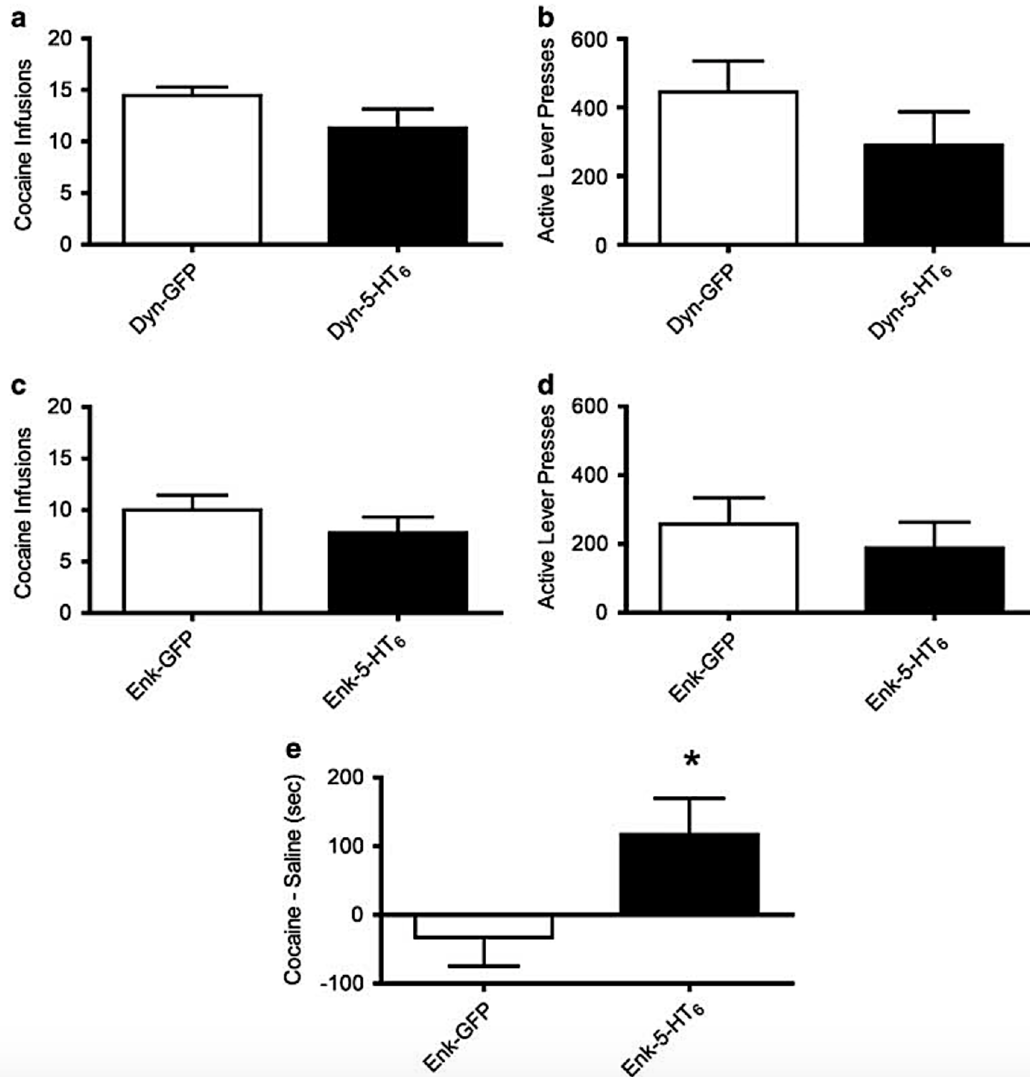

Figure 4.6: Increasing expression of 5-HT$_6$ receptors in either the NAcSh dMSNs or iMSNs does not affect operant responding on a PR schedule. (a) Cocaine infusions (Mean ± SEM) for the average of four PR sessions does not differ between Dyn-GFP (n = 7) and Dyn-5-HT$_6$ (n = 7) (t-test, p = 0.14). (b) Active lever presses (Mean ± SEM) for the average of four PR sessions do not differ between Dyn-GFP (n = 7) and Dyn-5-HT$_6$ (n = 7) rats (t-test, p = 0.26). (c) Cocaine infusions (Mean ± SEM) for the average of four PR sessions does not differ between Enk-GFP (n = 12) and Enk-5-HT$_6$ (n = 14) rats (t-test, p = 0.31). (d) Active lever presses (Mean ± SEM) for the average of four PR sessions does not differ between Enk-GFP (n = 12) and Enk-5-HT$_6$ (n = 14) rats (t-test, p = 0.53). (e) Increasing expression of 5-HT$_6$ receptors the iMSNs increases preference for cocaine at a low dose. CPP score (cocaine time – saline

time [seconds]) shown for rats with either Enk-GFP (n = 8) or Enk-5-HT_6 (n = 8) (t-test, $p = 0.04$). * = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$.

Increasing expression of 5-HT_6 receptors in dMSNs or iMSNs does not influence locomotor activity

We tested whether increasing 5-HT_6 receptors in dMSNs or iMSNs altered spontaneous locomotor activity in a subset of rats from all groups. All of the groups demonstrated similar locomotor activity patterns with no significant between-group differences (direct pathway: Figure 4.7a; indirect pathway: Figure 4.7b).

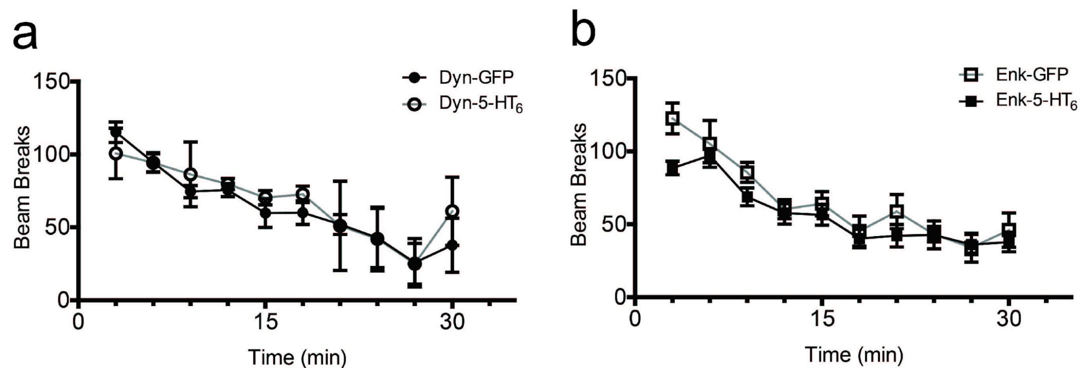

Figure 4.7: Increasing expression of 5-HT_6 receptor in either the dMSNs or iMSNs does not change locomotor activity. (a) Average beam breaks per 3 minute bin does not differ between rats with either Dyn-GFP (n = 4) or Dyn-5-HT_6 (n = 3) (two-way ANOVA, $F_{(1,5)} = 0.30$; $p = 0.61$). (b) Average beam breaks per 3 minute bin does not differ between rats with either Enk-GFP (n = 3) or Enk-5-HT_6 (n = 6) (two-way ANOVA, $F_{(1,10)} = 2.42$; $p = 0.15$).

Discussion

The segregation of direct and indirect pathway MSNs in dorsal and ventral striatum is a fundamental feature of brain reward circuitry and the functional implications of the divergent roles of these pathways is currently a topic of great interest. It has been

known for many years that both iMSNs and dMSNs express 5-HT_6 receptors (Ward et al., 1995), but past studies involving 5-HT_6 receptors and psychostimulants did not address these distinct pathways (de Bruin et al., 2013; Eskenazi & Neumaier, 2011a; Eskenazi & Neumaier, 2011b; Valentini et al., 2013; van Gaalen et al., 2010), and hence the circuitry mechanisms involved were not evaluated. Therefore, we investigated the relative contribution of 5-HT_6 receptors in each pathway on operant behaviors reinforced by cocaine.

Previously, the Neumaier lab has shown that increasing 5-HT_6 receptors in both pathways of NAcSh interfered with learning of a conditioned place preference for cocaine, and that systemic treatment with a selective 5-HT_6 antagonist had the opposite effect (Ferguson et al., 2008). However, that study did not observe any direct effects on drug reward. Furthermore, increasing 5-HT_6 receptors in both pathways of dorsomedial striatum interfered with acquisition of action-outcome learning when sucrose was used as the reinforcer (Eskenazi & Neumaier, 2011b; Mitchell et al., 2007). In dorsomedial striatum, selective expression of 5-HT_6 receptors in indirect pathway MSNs alone was sufficient to interfere with action-outcome operant learning (Eskenazi et al., 2015). In contrast, 5-HT_6 receptor signaling in dorsolateral striatum facilitates omission training in a contingency-specific manner, but does not affect the learning acquisition of action-outcome learning (Eskenazi & Neumaier, 2011a). Thus, the impact of 5-HT_6 receptors on striatum-dependent behavior depends both on the pathway being manipulated and the subregion that is targeted.

The central finding of this chapter is that increased expression of 5-HT_6 receptors in the iMSNs reduced cocaine SA under an FR1 reinforcement schedule by about 50%

when using a 0.75 mg/kg unit dose. The most obvious explanation for this result is that the animals had lower motivation to take cocaine, but increasing 5-HT_6 receptors in iMSNs had no effect on the “break point” under a PR reinforcement schedule at a dose of 0.75 mg/kg, suggesting that their motivation to take cocaine was not likely altered at this dose. In contrast, increasing expression of 5-HT_6 receptors in the dMSNs of NAcSh had no effect on cocaine SA under either FR1 or PR reinforcement schedules. Although the control rats in the dMSN experiments self-administered slightly more cocaine than the rats in the control iMSN experiments, these cohorts were run at entirely separate times and the difference can be attributed to biological variation. While it is possible that increasing 5-HT_6 receptor expression may modulate break point at higher or lower doses of cocaine, the present evidence suggests that there were no changes in motivation using the PR “break point” method.

Another possible explanation for our central observation is that the rats were more sensitive to the reinforcing properties of cocaine and therefore required less to attain a preferred subjective response to the drug. We applied a previously developed and later refined method for modeling the pharmacokinetics and tissue concentration of cocaine to estimate the brain cocaine concentration that each animal titrated to and presumably preferred for each unit dose of cocaine tested (Pan et al., 1991; Zimmer et al., 2011). This analysis suggested that the Enk-5-HT_6 rats preferred a lower tissue concentration of cocaine, further supporting the notion that they are more sensitive to the reinforcing properties of cocaine.

We further tested this idea using a different behavioral model by performing a cocaine CPP experiment using conditions that enhance the sensitivity to detecting

differences in reward as compared to aversion — i.e. low dose and brief pairing of cocaine with the context (Barot et al., 2007; Ettenberg, 2004; Pliakas et al., 2001). Rats with increased 5-HT_6 receptors in iMSNs developed a stronger preference for a typically sub-threshold dose of cocaine (5mg/kg i.p.) (Barot et al., 2007; Neumaier et al., 2002), suggesting that these rats were more sensitive to the rewarding effects of cocaine at this dose.

It is also conceivable that increasing 5-HT_6 receptors in the iMSNs altered the processing of aversive information. However, we found no differences in operant self-administration at the highest cocaine dose (1.5 mg/kg unit dose), which is most likely to generate aversive effects. We did not test place conditioning with high dose cocaine and delayed pairing, which is a sensitive method for detecting aversion (Barot et al., 2007; Ettenberg, 2004; Pliakas et al., 2001), because there were no differences in cocaine SA at the highest unit dose tested. Further, we found no differences in operant responding on a PR reinforcement schedule. Taken together, this suggests that these results are not likely due to changes in the processing of aversive information. Another explanation is that increased 5-HT_6 receptors in NAcSh altered motor activity and interfered with cocaine taking, but there were no changes in locomotion following these manipulations nor were there differences at 1.5 mg/kg cocaine, a unit dose that would be most likely to induce motor deficits. In a previous study there was no evidence that increased 5-HT_6 receptors altered the acute locomotor response to cocaine or sensitization over several days, although in that study we expressed 5-HT_6 receptors using a pathway-nonselective viral vector (Ferguson et al., 2008).

While we observed no change in PR responding at 0.75 mg/kg, which is usually interpreted to mean that there was no change in motivation to take cocaine at this dose, Enk-5-HT_6 animals displayed a consistent pattern of delaying the initial cocaine infusions during each test session across multiple days. It is unlikely that increased 5-HT_6 receptors impaired the animal's association between cocaine availability and the active lever because there was no declination in the delay to initial lever press over multiple days. However, most of the differences between Enk-GFP and Enk-5-HT_6 treated rats occurred during the beginning of each session, as the experimental animals lacked the initial burst of cocaine taking that is typical of cocaine SA (Belin et al., 2009; Zimmer et al., 2011). The higher initial rate of cocaine infusions may be taken to attain a preferred cocaine level in the brain, followed by a second phase of slower cocaine taking to maintain a preferred cocaine concentration in the brain. If this interpretation is correct, 5-HT_6 receptors in the NAcSh indirect pathway have an impact on the initial loading phase of cocaine SA and it is not surprising that there was no change in break point under a progressive ratio schedule, which depends on an extended cocaine SA session. In a recent study, we found that increased 5-HT_6 receptors in iMSNs in dorsomedial striatum delayed the time to initial lever press only on the first day of training for sucrose responding (Eskenazi et al., 2015), which is a similar result but this effect was not sustained over multiple days as it was in the present study using cocaine SA. If 5-HT_6 receptors in iMSNs of the NAcSh produce increased sensitivity to lower doses of cocaine, this might in turn reduce the likelihood of developing compulsive addiction-like behaviors which are associated with higher infusion rates of cocaine SA during early experience with drug taking (Belin et al., 2009). This interpretation is

supported by previous work showing that activating the indirect pathway fosters resilience to compulsive cocaine use (Bock et al., 2013) and reducing indirect pathway activity can facilitate psychomotor sensitization to cocaine (Ferguson et al., 2011).

One parsimonious way to interpret all of this data together is to consider the distribution of 5-HT_6 receptors in both of these output pathways. Whereas dopamine differentially activates dMSNs via D_1 receptors and inhibits iMSNs via D_2 receptors, serotonin will excite both pathways as both populations of MSNs express endogenous 5-HT_6 receptors (Ward et al., 1995).Thus, dMSNs are activated by both the endogenous $G\alpha_s$-coupled D_1 and 5-HT_6 receptors, leading to an accumulation of cAMP (Dobi et al., 2011; Surmeier et al., 2007). However, D_2 and 5-HT_6 receptors in iMSNs have opposite effects on adenylate cyclase activity and will tend to oppose one another. Therefore, 5-HT_6 receptors in the iMSNs are positioned to interfere with behaviors that are supported by dopamine action in NAcSh. Presumably balanced activation of endogenous 5-HT_6 receptors in both pathways simultaneously interferes with dopamine actions by reducing the differential activation of these two pathways. It is not known whether exposure to abused drugs alters the expression levels of 5-HT_6 in these pathways differentially, but this is a topic under active exploration.

The finding that increased 5-HT_6 receptor expression in the iMSNs of NAcSh leads to an increase in the sensitivity to the reinforcing properties of cocaine while maintaining normal reward motivation properties has implications toward the field of drug addiction research. While similar studies have focused on the role of 5-HT_6 receptors modulating dopamine in the NAcSh during cocaine reinforcement (Valentini et al., 2013), or the role of the indirect pathway in reducing cocaine reinforcement (Hikida

et al., 2010), no studies have focused on the pathway specific roles for 5-HT_6 receptors. Our findings suggest that 5-HT_6 receptors are a potential target for treatment of drug addiction, in that if receptors are selectively up-regulated or stimulated in the iMSNs, the amount of drug taken by individuals might reduce, thereby impeding the progression toward compulsive, unregulated drug use.

*Portions of this chapter were adapted from: Brodsky M**, *Gibson AW**, *Smirnov D, Nair SG, and Neumaier JF. (2016) Striatal 5-HT_6 receptors regulate cocaine reinforcement in a pathway-selective manner. Neuropsychopharmacology. 41(9): 2377-2387*

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CHAPTER 5

Conclusions and Future Directions

5-HT neurotransmission regulates emotional behaviors through the modulation of neuronal circuits. Both synaptic and volume transmission of 5-HT can influence neuronal signaling, and diffusion of 5-HT in the extracellular space can activate extra-synaptic 5-HT receptors that serve important roles in neuromodulation. Two extra-synaptic 5-HT receptors, the 5-HT_{1B} and 5-HT_6 receptors, display unique cellular localization that enables them to detect extracellular 5-HT and subsequently regulate neurotransmitter release and circuit function. 5-HT_{1B} receptors are located on axon terminals as autoreceptors on serotonergic neurons or heteroreceptors on non-serotonergic neurons, where they act to inhibit the release of neurotransmitters. 5-HT_6 receptors localize to neuronal primary cilia and are particularly abundant in the striatum of the brain, where they serve as excitatory receptors that detect serotonin around neuronal soma. Investigating both the signaling properties of these receptors and the ways in which they influence circuit-specific behaviors will expand our understanding of serotonin biology and illuminate potential therapeutic targets for psychiatric treatments.

In Chapter 2 I validated the creation of neuronal cell lines that have stable expression of 5-HT_{1B} receptors and lack β-arrestin proteins. These cell lines enable the *in vitro* characterization of 5-HT_{1B} receptor signaling using a neuronal system, and the stable knockout of either β-arrestin isoform establishes a method for probing the dynamics of GPCR signal transduction. The use of CRISPR/Cas9 technology improves upon previous methods for β-arrestin knockdown, specifically through stable knockout with a lower probability of off-target effects. These cell lines can be used for future

experiments focused on aspects of 5-HT_{1B} receptor signaling, and to further examine the interactions between this receptor, G proteins, and β-arrestins.

In Chapter 3 I used these new cell lines to elucidate the mechanisms of 5-HT_{1B} receptor-mediated phosphorylation of ERK1/2. This work sheds light on the downstream effects of 5-HT_{1B} receptor activation and is the first study to demonstrate ERK1/2 activation by this receptor in a neuronal cell line. Inhibition of $G\alpha$ and MEK1/2, as well as knockout of β-arrestin 1 and 2 prevented ERK1/2 phosphorylation following 5-HT_{1B} receptor activation. Since β-arrestins are known to induce receptor internalization and form endosomal signaling scaffolds, the findings laid out in this chapter offer a hypothesis for 5-HT_{1B} receptor dynamics. 5-HT_{1B} receptors bind 5-HT and can initiate canonical G protein signaling, after which they bind to β-arrestin, become internalized, and engage in ERK1/2 signaling through endosomal signaling scaffolds requiring both β-arrestin and $G\alpha$ proteins. Activated ERK1/2 proteins can then interact with intracellular proteins to influence neurotransmission or translocate to the nucleus and regulate gene expression. This process could influence the delayed efficacy of SSRIs, in addition to the desensitization yet continued expression of 5-HT_{1B} receptors following stress. Novel therapies that affect receptor internalization or ERK1/2 signaling could be developed to treat a variety of psychiatric conditions including depression, anxiety, and panic disorders.

In Chapter 4 I described the pathway-specific effect of increased 5-HT_6 receptor expression on cocaine self-administration. 5-HT_6 receptors in the indirect pathway of the NAc regulate the sensitivity to the reinforcing properties of cocaine, a finding that has implications for the treatment of addiction. It is possible that by influencing 5-HT_6

signaling in accumbal iMSNs one can reduce drug taking and thus prevent overdose or the transition from drug taking to addiction. Regulation of NAc output pathways by 5-HT_6 receptors could also play an important role in other aspects of reward-seeking behavior and may be potential target for the treatment of other psychiatric disorders.

Additional research is needed in order to fully understand how 5-HT_{1B} receptors influence neurotransmission and circuit activity. It remains unclear whether these receptors engage in ERK1/2 signaling from endosomes or from the cell surface, and whether specific β-arrestin isoforms are required for internalization versus ERK1/2 activation. Further studies that explore these aspects will provide a more complete picture of 5-HT_{1B} autoreceptor and heteroreceptor regulation of axon terminals. It is also possible that 5-HT_{1B} receptors affect other signaling cascades (e.g. Akt pathways) and future studies should be done to explore this as well. If it is revealed that 5-HT_{1B} receptors cycle between cell surface- and endosome-specific signaling it would have a profound impact on our understanding of the pharmacology of extra-synaptic 5-HT at axon terminals.

We now know that 5-HT_6 receptors can influence the indirect striatal pathway to alter drug-taking behavior; however, an effect of direct pathway 5-HT_6 receptors has yet to be revealed. The study outlined in Chapter 4 leaves open the possibility of a ceiling effect of increased 5-HT_6 receptor signaling in the direct pathway. 5-HT_6 receptors could have opposing effects on drug-taking via dMSNs and iMSNs, and a loss of 5-HT_6 signaling in dMSNs rather than an increase could demonstrate this experimentally. CRISPR/Cas9 vectors offer the potential for pathway-specific knockout of 5-HT_6 receptors, and future experiments could use this approach to investigate these

receptors in the direct striatal pathway. Additionally, the roles of 5-HT_6 receptors in other facets of the addiction cycle should be explored. Pathway-specific overexpression or knockout of 5-HT_6 receptors could be used to evaluate relapse to drug-taking using cocaine reinstatement models. Finally, the ability of these ciliary receptors to modulate behaviors in a pathway-specific manner raises the possibility that primary cilia in general regulate neuronal circuits and complex behaviors. Experimental modifications to downstream mediators of ciliary cAMP signaling like AC3 could provide insight into how these organelles regulate brain function.

Although *in vivo* receptor overexpression and selective drug treatments can provide valuable insight into 5-HT signaling, these approaches have several limitations. Drug treatments lack cell type or pathway specificity, and for receptors localized to cellular compartments such as axon terminals and primary cilia, overexpression can sometimes result in leakage outside of these compartments. Cell type-specific knockout of 5-HT receptors using CRISPR/Cas9 methods could enable more accurate examination of endogenous receptor function. To this end, the Neumaier lab is currently validating viral vectors for Cre recombinase-dependent CRISPR/Cas9-mediated knockout of both 5-HT_{1B} and 5-HT_6 receptors. This approach can be used in future experiments to build upon previous studies and the findings presented here.

Overall, this dissertation reveals important mechanisms of extra-synaptic serotonin signaling and the ways in which it modulates neuronal circuits and behaviors. Although different in their pharmacological properties and localizations, both 5-HT_{1B} and 5-HT_6 receptors work to influence behaviors by translating extra-synaptic 5-HT into intracellular signals that alter the activity of neurons and circuits. The findings described

here expand our knowledge of serotonin biology, provide additional insight into potential therapeutic targets for psychiatric conditions, and generate new hypotheses for future research into the nature of serotonergic systems.

APPENDICES

Appendix 1: Primers used for qPCR in untransfected N2A and stably transfected N2A-1B cells.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
$5\text{-}HT_{1B}$	CACCCTTCTTCTGGCGTCAA	GAGAGCGGGCTTCCACATAG
$5\text{-}HT_{1A}$	GCCAACTATCTCATCGGCTCC	TGGTACAGAGCAGCCATGGG
SERT	TCACGGTGCTTGGCTACATG	GGCAAAGAATGTGGATGCTGG
Pet-1	GTCGGAGATGGTCTTTTTAAGG	TGCCACAACTGGATCTGC
Tph2	CTACCCGACTCATGCTTGCC	CAGGAAGTCTCTTGGGCTCAG
Tph1	AAGAAATTGGCCTGGCTTC	GTTTGCACAGCCCAAACTC
AADC	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA
GCH1	GCCTCACCAAACAGATTGC	CACGCCTCGCATTACCAT
VGLUT3	TTTGTCCCCTCATTGTTGGT	GCGCTGCTATGAGGAACAC
VMAT2	TGCTGAAGGACCCATACATTC	CACATGGTCTCCATCATCCA

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