5-HT_{1B} Autoreceptors: Molecular Mechanisms and Behavioral Implications

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Abstract

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The serotonergic system is important in modulating a range of emotional behaviors, and disturbances of this system are implicated in psychiatric conditions such as post-traumatic stress disorder, depression, and anxiety. 5-HT_{1B} autoreceptors are located on serotonergic neurons at the presynaptic nerve terminal and serve to regulate synaptic serotonin levels with precise spatial and temporal control. Spanning both behavioral and molecular work, this dissertation delves into the effects of manipulating 5-HT_{1B} autoreceptor levels on contextual fear conditioning and into the signal transduction pathways underlying 5-HT_{1B} receptor activation. We first describe two novel viral vectors for targeting 5-HT_{1B} autoreceptors: one using the serotonin transporter promoter to drive transgene expression to gain neurochemical specificity, and one using an intersectional method that combines a double-floxed and inverted open reading frame virus with a Cre-expressing virus as a neuroanatomical strategy for targeting a single brain circuit. Second, we apply these tools in the study of fear conditioning in both wild type and 5-HT_{1B} receptor knockout mice and find that overexpression of 5-HT_{1B} autoreceptors in nerve terminals throughout the brain decreases fear expression, while overexpression of 5-HT_{1B} receptors specifically in the dorsal raphe nucleus to amygdala circuit increases fear. We then investigate the molecular mechanisms underlying 5-HT_{1B} signaling, looking in particular at the phosphorylation of ERK1/2 following 5-HT_{1B} receptor activation and the pathway proteins that

are involved. Finally, we identify four novel phosphorylation sites in the third intracellular loop of the 5-HT_{1B} receptor and discover that these sites are important for achieving maximal agonist-stimulated activation of phospho-ERK1/2. Taken together, these studies shed light on the intracellular mechanisms by which 5-HT_{1B} receptors function and provide evidence for distinct effects of 5-HT_{1B} receptors on fear behaviors depending on their expression pattern in the brain.

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DEDICATION

To my grandparents, Ye Ye and Nai Nai, who were two of my earliest life teachers, for being my cheerleaders every step along the way – I hope to continue making you proud.

INTRODUCTION

Serotonin in the Brain

Serotonin (5-hydroxytryptamine, 5-HT) was first isolated and characterized in 1948 as the substance in serum responsible for vasoconstriction (Rapport et al 1948a, Rapport et al 1948b). Several years later, it was identified in the mammalian brain and postulated to function as a "neurohumoral agent" (Bogdanski et al 1956, Brodie et al 1955). Since then, serotonergic signaling has been found to modulate many physiological functions in the brain, particularly in various aspects of emotion and cognition. Dysfunction of the serotonergic system is implicated in psychiatric illnesses such as depression, post-traumatic stress disorder, and anxiety. The serotonin transporter (SERT) has been in the research spotlight because treatment of these disorders often includes a selective serotonin reuptake inhibitor (SSRI), which manipulates 5-HT levels in the brain by targeting SERT function.

Serotonergic neurons in the brain have cell bodies that lie in the raphe nuclei of the midbrain but project extensively to nearly every brain region (Jacobs & Azmitia 1992). The dorsal raphe nucleus (DRN) provides significant innervation to the forebrain and has been demonstrated to play a crucial role in stress and emotional behaviors. Previously thought to be comprised of a homogenous cell population, the DRN is actually somatotopically organized, and subpopulations of serotonergic neurons have unique anatomical connections that regulate different types of emotional behaviors (Lowry et al 2008). One reason for this heterogeneity may be that serotonergic neurons arise from distinct neuroprogenitor cells in different rhombomeres, but because molecular markers have not been identified to differentiate these subclasses of serotonergic cells, they are difficult to target and manipulate selectively (Andrade & Haj-Dahmane 2013).

5-HT_{1B} Autoreceptors

Fourteen different 5-HT receptors have been identified to date. With the exception of the ionotropic 5-HT_3 receptor, all other 5-HT receptors are all G protein-coupled receptors (GPCR). The 5-HT_{1B} receptor is a GPCR first discovered and cloned in 1991 (Jin et al 1992, Maroteaux et al 1992, Voigt et al 1991). One human variant of the 5-HT_{1B} receptor has been associated with increased incidence of depression (Ruf & Bhagwagar 2009), and animal studies suggest that drugs targeting the 5-HT_{1B} receptor show promise as adjunctive therapy in combination with selective serotonin reuptake inhibitors (Gardier 2009). Several lines of evidence suggest that the level of 5-HT_{1B} autoreceptor expression is a key determinant of stress reactivity in animal and human models (Hu et al 2010, Kaiyala et al 2003, Murrough et al 2011a, Murrough et al 2011b, Neumaier et al 2002).

5-HT_{1B} receptors are primarily localized to axons and nerve terminals (Boschert et al 1994, Ghavami et al 1999, Riad et al 2000), and exist both as autoreceptors on serotonergic neurons and as heteroreceptors on nonserotonergic cells (Hen 1992). Presynaptic 5-HT_{1B} autoreceptors play a key role in affecting the dynamics of serotonergic neurotransmission throughout the entire brain, providing continuous feedback about synaptic 5-HT levels and real-time adjustments of 5-HT concentrations in a region-specific manner. This acute control can be achieved through 5-HT_{1B}-mediated regulation of 5-HT synthesis (Hjorth et al 1995), 5-HT release (Middlemiss & Hutson 1990), and 5-HT reuptake (Daws et al 2000, Hagan et al 2012, Montanez et al 2013). The localization of 5-HT_{1B} autoreceptors in serotonergic terminals poises them to modulate 5-HT neurotransmission in target regions, particularly those implicated in emotional behaviors (Sari 2004).

Despite multiple important functions at nerve terminals, 5-HT_{1B} autoreceptors are a challenge to study. 5-HT_{1B} autoreceptors and heteroreceptors are identical in sequence and structure but expressed in different neuron types. 5-HT_{1B} autoreceptors are diffusely distributed throughout the brain and intermixed in most brain regions with 5-HT_{1B} heteroreceptors. It is difficult to target 5-HT_{1B} autoreceptors exclusively, especially with conventional methods like pharmacological drug administration, as both 5-HT_{1B} auto- and heteroreceptors would be affected. The only transgenic mice currently available for research have constitutive deletions of the 5-HT_{1B} gene; while these mice have a remarkable phenotype with decreased anxiety and increased aggression (Gingrich & Hen 2001, Groenink et al 2003, Guilloux et al 2011), it is unclear whether these behavioral effects relate to 5-HT_{1B} autoreceptors or heteroreceptors, during early development or adulthood. Indeed, the behavioral and physiological phenotype of these mice does not resemble the effects of acute treatment with a 5-HT_{1B}-selective antagonist (Ase et al 2001, Castanon et al 2000).

Behavioral Effects of Manipulating 5-HT_{1B} Autoreceptors

One approach for manipulating 5-HT_{1B} autoreceptor function is via systemic injections of the brain-penetrant agonist CP-94253, which preferentially activates autoreceptors over heteroreceptors at low doses (McDevitt et al 2011, Sarhan et al 2000). Another method of selectively targeting 5-HT_{1B} autoreceptors is viral-mediated gene transfer. Viruses expressing the 5-HT_{1B} transgene are injected into the DRN, and once expressed, the 5-HT_{1B} receptor is trafficked to the nerve terminals, where they are endogenously located (Clark et al 2002). These techniques have allowed for *in vivo* studies to elucidate the specific contribution of 5-HT_{1B} autoreceptors on behavior.

5-HT_{1B} autoreceptors have been implicated in behavioral measures of anxiety, fear, and depression in a stress-sensitive manner. Rats that overexpress the 5-HT_{1B} transgene in the midrostrocaudal DRN show an increased number of center enterings in the open field test and a decreased fear-potentiated startle response, but no changes in climbing, swimming, treading, or immobility behaviors in the forced swim test (Clark et al 2002, Clark et al 2004). When the caudal DRN is targeted instead, overexpression of 5-HT_{1B} receptors has no effect on anxiety as measured by the open field test; however, in the forced swim test, rats show increased swimming and decreased immobility on the first day, but not on the second day of testing (McDevitt et al 2011). Additionally, overexpression of 5-HT_{1B} receptors decreases fear expression in contextual fear conditioning but has no effect on acquisition; similarly, context-paired administration of a low dose of CP-94253 to preferentially activate 5-HT_{1B} autoreceptors shows the same decrease in fear. Taken together, these data suggest a protective role of 5-HT_{1B} autoreceptors in these emotional behaviors, with differences along the rostrocaudal axis. Interestingly, these anxiolytic and fear-attenuating properties of 5-HT_{1B} autoreceptors are abolished when animals were exposed to stress or received the 5-HT_{1B} antagonist SB224289 prior to behavioral testing (Clark et al 2002, Clark et al 2004, McDevitt & Neumaier 2011).

The relationship between 5-HT_{1B} autoreceptor expression and the behavioral observations are as predicted. In unstressed rats, 5-HT_{1B} mRNA levels in the DRN are inversely correlated with measures of anxiety on both the elevated plus maze (Kaiyala et al 2003) and the open field test (Hiroi & Neumaier 2009). Stress desensitizes 5-HT_{1B} receptor function by reducing their efficacy rather than their surface expression (Bolanos-Jimenez et al 1995) and is correlated with increases in 5-HT_{1B} mRNA levels in the DRN (McDevitt & Neumaier 2011, Neumaier et al 1997); in contrast, SSRI treatments reduce 5-HT_{1B} mRNA levels (Anthony et al

2000, Neumaier et al 1996). However, the mechanisms underlying these behavioral phenomena are not well understood. One hypothesis is that 5-HT_{1B} autoreceptors exert precise spatial and temporal control of synaptic 5-HT levels, for example, by increasing reuptake of 5-HT through SERT when activated (Daws et al 2000, Hagan et al 2012, Montanez et al 2013). Low 5-HT levels then contribute to the decrease in anxiety and fear, which is consistent with reports of increased anxiety and fear resulting from heightened 5-HT levels following acute treatment with SSRIs (Burghardt et al 2007, Griebel et al 1994).

Molecular Mechanisms Underlying 5-HT_{1B} Receptor Signaling

Despite their importance in regulating synaptic 5-HT levels throughout the brain, the biochemical mechanisms by which 5-HT_{1B} receptors function are not well understood. 5-HT_{1B} receptors are $G_{i/o}$-coupled receptors (Bouhelal et al 1988, Schoeffter & Hoyer 1989) and are well known for opening inward rectifying potassium channels (GiRKs) (Andrade et al 1986, Ghavami et al 1997). 5-HT_{1B} receptors can also directly interact with p11, which acts to increase 5-HT_{1B} surface expression and enhances receptor function (Svenningsson et al 2006), and with glycogen synthase kinase-3β (GSK3β), which is important for agonist-induced 5-HT_{1B} receptor activation and internalization (Chen et al 2009, Li & Jope 2010, Zhou et al 2012).

5-HT_{1B} receptors may also signal through various intracellular kinases. 5-HT_{1B} was shown to couple to ERK2 and stimulate p70 S6 kinase (p70S6K); both effects were sensitive to pertussis toxin (PTX) and inhibitors of phosphoinositide 3-kinase (PI3K) (Mendez et al 1999, Pullarkat et al 1998). Akt1 is also indirectly activated by 5-HT_{1B} receptor stimulation, with MAP kinase kinase (MEK1/2) being necessary but not sufficient for this activation (Hsu et al

2001). Both ERK and Akt activation by 5-HT_{1B} receptors can be inhibited by expression of the regulator of G protein signaling 4 (RGS4) protein (Leone et al 2000).

Furthermore, 5-HT_{1B} receptor function can be affected by the presence of other serotonin receptors. 5-HT_{1B} receptors form homodimers when expressed alone and form heterodimers with 5-HT_{1A} and 5-HT_{1D} receptors when coexpressed (Salim et al 2002, Xie et al 1999). Additionally, complex crosstalk between 5-HT_{1B} receptors and the 5-HT_2 receptor subtypes has been demonstrated. Coexpression of 5-HT_{1B} and 5-HT_{2B} receptors changes the mechanism of internalization when compared to expression of either receptor alone (Janoshazi et al 2007). Activation of either 5-HT_{2B} or 5-HT_{2C} receptors inhibits 5-HT_{1B} function through activation of phospholipase C (PLC) and phospholipase A_2 (PLA_2) (Berg & Clarke 2001, Tournois et al 1998), while 5-HT_{2A} receptor activation can attenuate 5-HT_{2B}-mediated inhibition of 5-HT_{1B} receptors (Tournois et al 1998).

One major limitation of many of these mechanistic studies is the use of various cell culture systems. For example, several were performed in non-neuronal cell lines, and subsequent experiments in primary neuronal culture did not implicate the same pathways (Cowen et al 2005). Interaction studies were done by cotransfecting different receptors, which may not be biologically relevant in the brain if they are not endogenously coexpressed in the same cell type or in the same cellular compartment.

Interestingly, the crystal structure of the 5-HT_{1B} receptor has been reported recently, with many extracellular and transmembrane amino acids identified as crucial for agonist binding (Wang et al 2013). Moreover, the crystal structure has permitted studying of functional selectivity of the receptor, particularly its ability to couple to G proteins (Wacker et al 2013).

Comparing this crystal structure with that of more extensively studied GPCRs, such as the β2-adrenergic receptor, may lead to insights about common structure-function relationships.

Specific Aims

The first two chapters of this dissertation focus on the behavioral effects of 5-HT_{1B} autoreceptor manipulation, while the last two chapters delve into the molecular mechanisms underlying 5-HT_{1B} receptor function. In Chapter 1, I describe the characterization of two novel viral vectors that are designed to selectively target 5-HT_{1B} autoreceptors. In Chapter 2, I utilize the viral vectors to investigate the role of 5-HT_{1B} autoreceptors in contextual fear conditioning in both wild type and 5-HT_{1B} knockout mice. In Chapter 3, I use quantitative proteomic methods to seek novel interactors of 5-HT_{1B} receptors and pathway proteins that may be activated downstream of 5-HT_{1B} receptors. Finally, in Chapter 4, I examine the signaling pathway that leads to phosphorylation of ERK1/2 following 5-HT_{1B} receptor activation.

CHAPTER 1

Novel Viral Vector Strategies for Selectively Targeting 5-HT_{1B} Autoreceptors

Introduction

Differentiating between 5-HT_{1B} autoreceptors and 5-HT_{1B} heteroreceptors is crucial in revealing their unique roles in brain circuits and behavior. 5-HT_{1B} autoreceptors are especially challenging to study due to their localization to nerve terminals and the presence of 5-HT_{1B} heteroreceptors intermixed in the same brain regions. Unlike somatodendritic 5-HT_{1A} autoreceptors, which can be differentiated from 5-HT_{1A} heteroreceptors by directly infusing agonists and antagonists into the raphe nuclei where serotonergic cell bodies reside, 5-HT_{1B} autoreceptors must be targeted with more complex strategies. One rudimentary way of activating 5-HT_{1B} autoreceptors is by using the selective agonist CP-94253 at low doses, which preferentially activates autoreceptors over heteroreceptors (McDevitt et al 2011, Sarhan et al 2000). However, it is possible that heteroreceptors may also be affected even at low doses, thus possibly confounding any conclusions based solely on pharmacological experiments. An alternative technique is with the use of viral-mediated gene transfer (Clark et al 2002, Clark et al 2004, Hagan et al 2012, McDevitt et al 2011).

Recombinant viral vectors have been used experimentally for over twenty years, with novel vectors being continuously developed to achieve greater control over transgene expression levels and cell type specificity (Bouard et al 2009, Lowenstein & Castro 2002, Luo et al 2008, Papale et al 2009, Zhang et al 2010). Plasmids encoding the desired transgene are packaged into replication-deficient viral vectors, which may be injected into specific brain structures, and target cells infected with the viral vector then begin expressing the transgene of interest (Papale et al

2009). Herpes simplex virus (HSV), adeno-associated virus (AAV), and lentivirus are three commonly used viral vectors in neuroscience research, each with its own advantages and disadvantages (summarized in Table 1.1), such that the optimal vector to be used is highly dependent on the goals of a particular experiment.

Table 1.1. Comparison of viral vector properties of HSV, AAV, and lentivirus (Bouard et al 2009, Lowenstein & Castro 2002, Luo et al 2008, Papale et al 2009, Zhang et al 2010)

	HSV	**AAV**	**Lentivirus**
Virus type	Double-stranded DNA, enveloped	Single-stranded RNA, enveloped	Single-stranded DNA
Neuron specificity	Yes	Serotype specific	No
Packaging capacity	150 kb	4-5 kb	8-10 kb
Integration into host genome	No	Yes	No
Expression time	Days to weeks	Years	Years
Retrograde expression	Modest	Variable	Variable

In recent years, AAV viruses with a double-floxed inverted open reading frame (DIO) have gained popularity. These constructs have the transgene inserted in the antisense orientation, flanked by two sets of lox sites; when Cre recombinase is present, an inversion occurs at the lox sites to flip the transgene into the sense orientation (Atasoy et al 2008). DIO viruses may be used in combination with transgenic animals expressing Cre in a cell type-specific manner or with retrogradely transported, Cre-expressing canine adenovirus (CAV-Cre) (Saunders et al 2012, Schnutgen et al 2003). This intersectional strategy allows for exquisite control of specificity, as only the cells that both express Cre recombinase and are infected by the DIO virus will express the transgene; this permits the targeting of subpopulations of neurons as well as of monosynaptic neural circuits.

Previous work in our lab has used the neuron-specific HSV to overexpress 5-HT_{1B} receptors in the rat DRN; viral-mediated 5-HT_{1B} receptor expression was shown to localize

correctly to axon terminals and increase autoreceptor activity (Clark et al 2002, Clark et al 2004, Hagan et al 2012). In this chapter, I describe the characterization of two novel viral vectors that improve upon the ability to selectively target 5-HT_{1B} autoreceptors: 1) an HSV viral vector using the SERT promoter to induce transgene expression only in serotonergic cells, and 2) an AAV-DIO viral vector to conditionally express 5-HT_{1B} receptors when used in combination with CAV-Cre viruses or cell type-specific Cre driver mouse lines, such as Pet1-Cre mice that use the serotonergic marker Pet-1 to selectively drive Cre expression.

Methods

Vector construction. To create a serotonin-specific viral gene expression system, we utilized the SERT promoter, as it is expressed exclusively and inclusively in serotonergic neurons. A plasmid containing the 1.7 kb fragment of the human SERT promoter was received as a generous gift from Ovie Wiborg. In rat raphe precursor cells, the 1250 bp proximal to the SERT gene shows the greatest activity (Mortensen et al 1999), so this section, along with GFP and the SV40 polyadenylation sequence, was cloned using PCR. Two SV40 polyA fragments were ligated together, and the ligation reaction was subjected to PCR to form a double polyA site. All PCR fragments were subcloned into the TOPO-Blunt cloning vector. All three components were inserted into the a pHSV-prPUC plasmid, developed by Rachael Neve for use in an HSV gene transfer system (Neve & Geller 1995), to form a cassette. The cassette was cloned the opposite direction of the HSV promoter and tested in cell culture using the serotonergic-like cells CA-77 (Greene & Tischler 1976) and the noradrenergic-like cells PC-12 (Clark et al 1995). Virus was produced from this construct (pSERT-GFP) and injected into rat striatum, ventral tegmental area, and dorsal raphe nucleus had serotonergic specificity when compared against the

non-specific pHSV-GFP construct in which GFP is driven by the endogenous HSV promoter. To create the AAV8-DIO-1B, we constructed a DNA block using annealed synthetic oligonucleotides that was comprised of GSG, 2A skip, and a hemagglutinin (HA) tag with inclusive restriction sites. We then inserted humanized rat 5-HT_{1B} (N357T) without the Kozak sequence, in frame, immediately after the HA tag and mCitrine, in frame, before the GSG sequence. Thus the final construct was: mCitrine-GSG-2A-HA-5-HT_{1B}. The 2A skip was modeled on the porcine teschovirus-1 (Szymczak et al 2004). We utilized the backbone of pAAV-FLEX-hM3D-mCherry (Krashes et al 2011) to gain the AAV origins of replication (LITR/RITR), FLEX double lox sites (Lee & Saito 1998), woodchuck hepatitis virus posttranscriptional response element (WPRE) (Loeb et al 1999, Loeb et al 2002) and antibiotic selectivity. The pAAV-FLEX-hM3D-mCherry was cut with AscI and NheI to remove the hM3D-mCherry portion and insert mCitrine-GSG-2A-HA-5-HT_{1B} in the reverse orientation. The original CAV-Cre was a generous gift from Dr. Richard Palmiter (Hnasko et al 2006), while CAV-GFP is originally from Dr. Eric Kremer (Kremer et al 2000), and then both were propagated and prepared by our lab.

Animal care. All animal procedures were approved by the University of Washington's Institutional Animal Care and Use Committee and carried out in accordance with National Institutes of Health guidelines. Care was taken to minimize animal discomfort. Adult male and female 129S6/SvEvTac, C57BL/6J, and Pet1-Cre mice were housed two to five per cage with access to food and water available ad libitum. Mice were housed on a 12/12 hour light cycle with lights on at 0600 h. All experiments were performed during the light cycle. Mice were 12-24 weeks of age and weighed 15-26 g at the time of surgery.

Stereotaxic surgery. Mice were anesthetized with isoflurane (1-2% in oxygen) or a ketamine/xylazine cocktail (100 mg/kg ketamine and 10 mg/kg xylazine, i.p.), the scalp was shaved and cleaned with betadine and ethanol, and the mice were placed in a Stoelting stereotaxic device. The scalp was incised to reveal the skull, and small holes were drilled at the sites of injection. A 27 gauge needle was slowly inserted, and virus was injected using a microprocessor-controlled pump. The needle was left in position for five minutes following the completion of the injection, and then slowly withdrawn. The scalp was closed with sutures and Vetbond, and mice were placed on a heated pad until they recovered and moved about freely before returning to their home cage. Analgesia was provided using meloxicam (0.4 mg/kg) or ketoprofen (5 mg/kg) administered subcutaneously during surgery.

Injection of viral vectors. The DRN was targeted at an angle of 28° from the vertical with the coordinates AP -4.8, ML +1.5, DV -3.4 from bregma. All viral vectors targeting the DRN were injected at a volume of 1 μL at a rate of 200 nL/min.

Immunohistochemistry. Animals were sedated with Beuthanasia-D (585 mg/kg pentobarbital sodium and 75 mg/kg phenytoin sodium), then intracardially perfused with 1x PBS followed by 4% paraformaldehyde before removing the brains, and the brains were post-fixed in 4% paraformaldehyde for four hours before switching to 1x PBS, then sliced with a vibratome into 40 μm sections. Sections were washed once for ten minutes in 1x PBS/0.5% Triton, then blocked for two hours with 5% normal goat serum (NGS)/1x PBS/0.25% Triton at room temperature with gentle shaking. Primary antibodies were diluted in 2.5% NGS/1x PBS/0.25% Triton and incubated with brain slices overnight at 4°C with gentle shaking. The primary antibody dilutions used were: rabbit anti-GFP 1:400 (Cell Signaling), rabbit anti-HA 1:400 for single-labeling experiments and 1:1000 for double-labeling experiments (Cell Signaling), and

sheep anti-tryptophan hydroxylase (Tph) 1:400 (Millipore). Sections were washed five times with 1x PBS before incubation with secondary antibodies diluted in 2.5% NGS/1x PBS for two hours at room temperature with gentle shaking. The secondary antibody dilutions used were goat anti-rabbit and goat anti-sheep at 1:400 dilutions (Molecular Probes). Brain sections were washed two more times before mounting onto slides with Prolong Gold with DAPI (Molecular Probes).

Results

HSV viral vectors utilize the SERT promoter to drive transgene expression

As shown in Figure 1.1, two plasmids were generated to incorporate the known sequence for the human SERT promoter. The first vector drove expression of eGFP (SERT-GFP), while the second vector drove expression of a HA-tagged 5-HT_{1B} receptor (SERT-1B). The viral vectors included elements necessary for HSV viral expression: the origin of DNA replication (oriS), the IE4/5 promoter, and a packaging signal. To prevent leaky expression in nonserotonergic neurons by the viral vector, the HSV viral elements were placed in a reverse orientation relative to the SERT promoter and eGFP or HA-5-HT_{1B}.

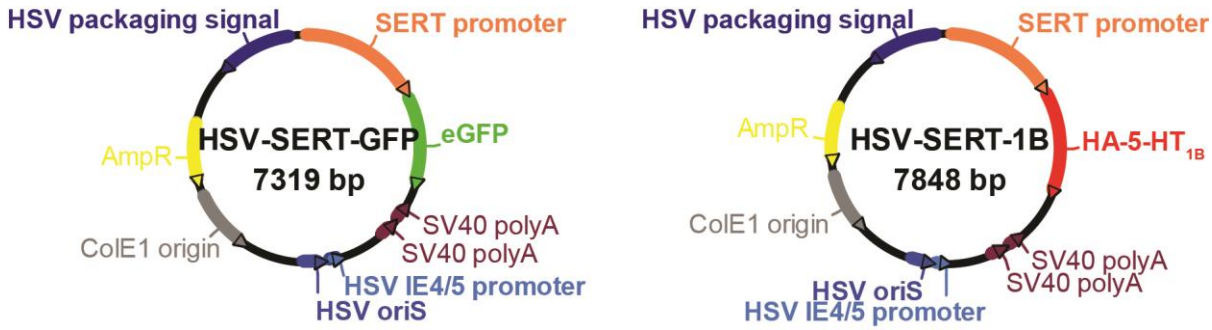


Figure 1.1. Plasmid maps for SERT-GFP (left) and SERT-1B (right) vectors. The human SERT promoter sequence is located directly upstream of the transgene to be expressed. HSV viral elements are the origin of DNA replication (oriS), the constitutive IE4/5 promoter, and the HSV packaging signal.

SERT promoter selectively drives transgene expression in serotonergic cells

To test the expression time course and cell type-specificity of this viral vector, SERT-GFP was injected into the DRN. Immunohistochemistry revealed low GFP expression at day 4, peak expression at day 7, and diminished but readily detectable expression at days 10 and 14 (Figure 1.2a). Immunoreactivity for the serotonergic marker Tph colocalized with GFP, demonstrating that transgene expression was restricted to serotonergic neurons (Figure 1.2b).

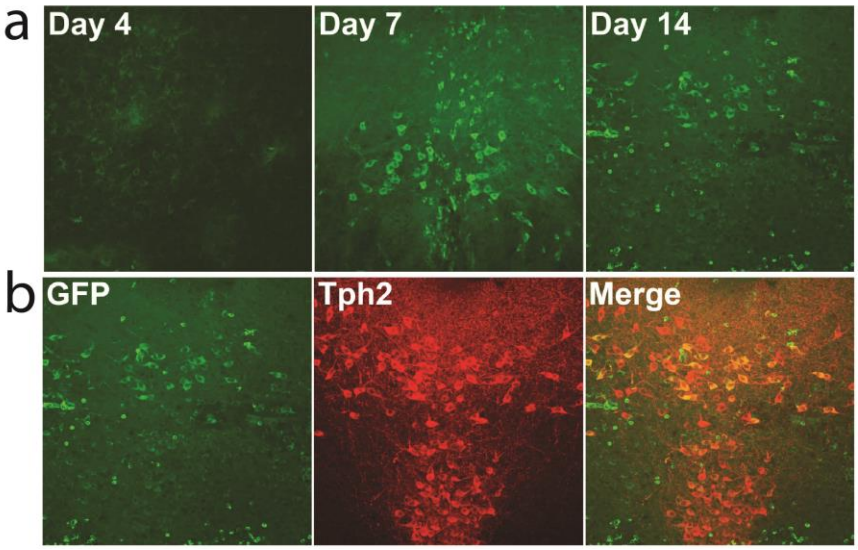


Figure 1.2. HSV viral vectors using the SERT promoter express transgenes selectively in serotonergic neurons, with expression peaking at day 7. SERT-GFP was injected into the DRN of WT mice. (a) Viral expression was assessed at days 4, 7, 10, and 14, with peak expression at day 7. (b) Viral GFP expression colocalized with Tph immunoreactivity, demonstrating the selectivity of using the SERT promoter.

AAV viral vector incorporates DIO construct design for 5-HT_{1B} expression

As shown in Figure 1.3, this novel viral vector (AAV8-DIO-1B) drives the expression of two distinct proteins: mCitrine, a fluorescent protein allowing for easy detection of viral infection, and an HA-tagged 5-HT_{1B} receptor. This bicistronic construct takes advantage of the 2A ribosomal skip peptide, which allows the expression of two proteins encoded by a single messenger RNA (Donnelly et al 2001, Ibrahimi et al 2009, Luke et al 2008, Sharma et al 2012).

This open reading frame (ORF) is inserted in an inverted orientation with respect to the rest of the plasmid, and is flanked by loxP and lox2272 sites as shown; the presence of Cre recombinase will invert the ORF at the lox sites and allow transcription of the protein sequences. The transgene is under the control of the human synapsin (hSyn) promoter, which restricts expression to neurons (Kugler et al 2003, Thiel et al 1991). Of the various AAV serotypes available, AAV8 was chosen because it is known to be a strong driver of transgene expression (Klein et al 2006).

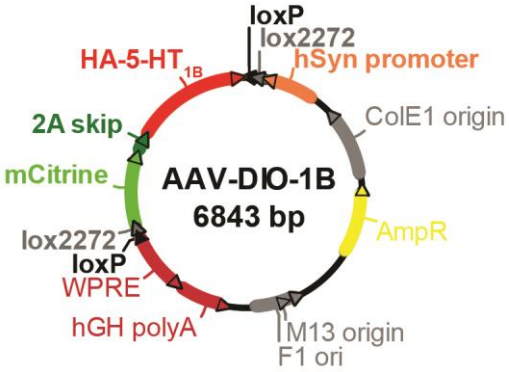


Figure 1.3. Plasmid map for AAV8-DIO-1B vector. The ORF, encoding mCitrine and an HA-tagged 5-HT_{1B} receptor separated by a 2A ribosomal skip peptide, is in an inverted orientation flanked by two sets of lox sites. Cre recombinase flips the ORF into the correct orientation, with transcription driven by the human synapsin promoter, restricting expression of the transgenes to neurons.

AAV8-DIO-1B viral vector expresses mCitrine and HA-tagged 5-HT_{1B} receptors in the presence of Cre recombinase

Hemizygous Pet1-Cre mice received AAV8-DIO-1B viral injections into the DRN. Robust viral expression was observed in the DRN 24 days following surgery. As shown by costaining for HA and Tph, expression of HA-5-HT_{1B} was limited to serotonergic neurons expressing Cre recombinase under the control of the Pet-1 promoter, demonstrating the integrity of the DIO viral construct (Figure 1.4a). Injection of 1 μL of AAV8-DIO-1B was sufficient for expression throughout the DRN (Figure 1.4b). Viral-mediated HA-5-HT_{1B} receptor expression

correctly localized to axon terminals, as evidenced by HA immunoreactivity in neurites (Figure 1.4c). In the DRN, mCitrine was visible without immunohistochemistry, and costaining with HA showed perfect colocalization of the two viral protein products (Figure 1.4d).

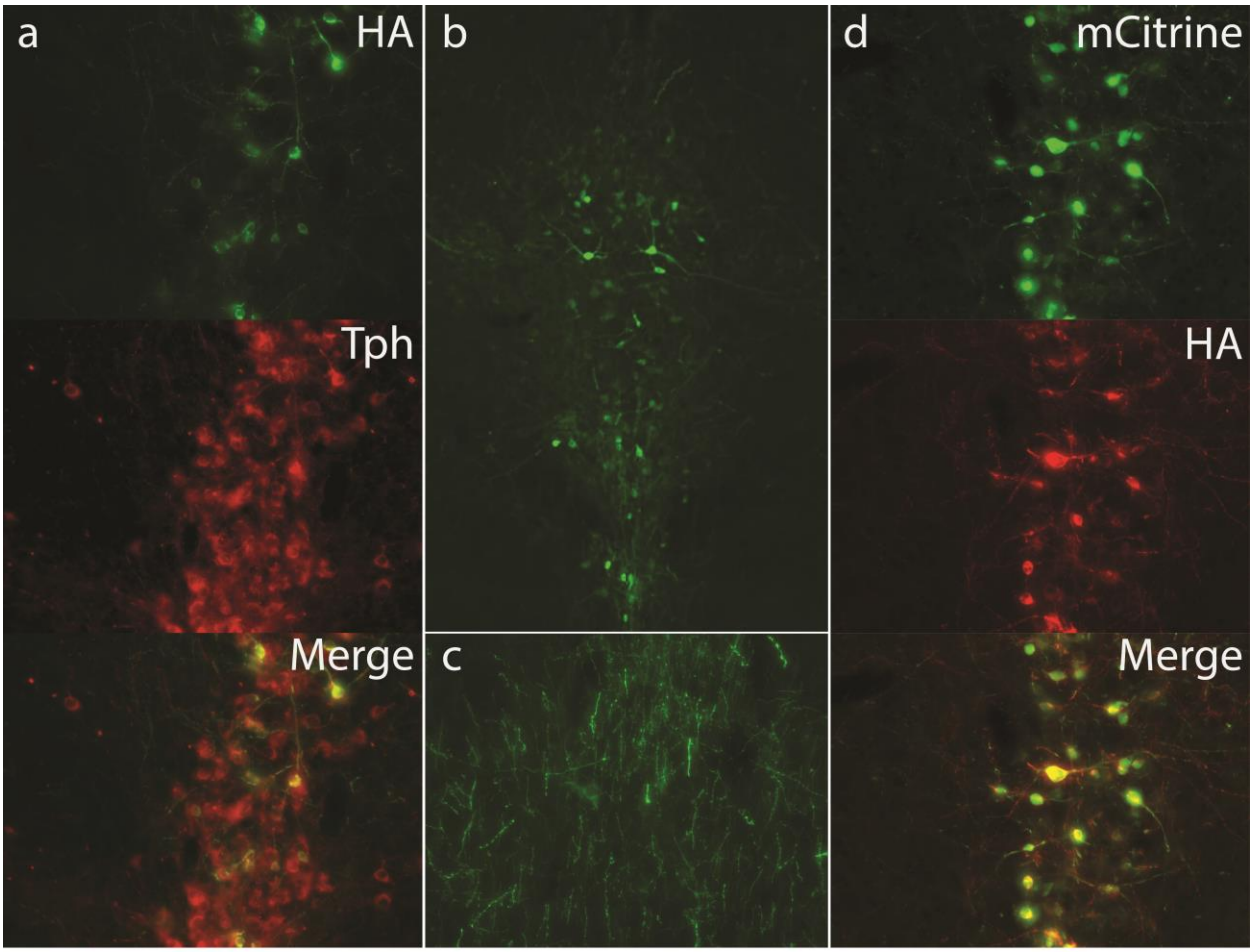


Figure 1.4. Robust mCitrine and HA-5-HT$_{1B}$ expression was seen following injection of AAV8-DIO-1B virus into the DRN of Pet1-Cre mice. Viral expression was observed in the DRN 24 days following surgery. (a) Dual labeling of HA-5-HT$_{1B}$ (green) and Tph (red) showed colocalization only in serotonergic neurons, demonstrating that the DIO viral vector was only expressed in the presence of Cre recombinase. Staining for HA-5-HT$_{1B}$ was prominent throughout the DRN (b), in both cell bodies as well as in neurites (c). (d) Both mCitrine (unstained) and HA (red) were readily detectable, and expression of the two separate proteins were perfectly colocalized.

Discussion

Using the SERT promoter, expression of the HSV viral vectors, SERT-GFP and SERT-1B, is successfully restricted to serotonergic neurons, as shown by dual label immunohistochemistry for GFP, to mark viral expression, and Tph, to mark serotonergic neurons. The peak of viral expression is at day 7, which is in contrast to conventional HSV vectors that peak around day 3 and gradually decline afterward (Carlezon et al 2000, Clark et al 2002, McDevitt et al 2011, Pliakas et al 2001); however, this change in temporal expression is consistent with other HSV vectors that use cell type-specific promoters (Ferguson et al 2011). Development of this serotonin-specific viral vector allows for greater control over viral expression, since a heterogeneous population of cells may be infected by an HSV injection, but only serotonergic neurons will have the necessary elements to induce transgene expression. Additionally, viral expression is easily visualized, suggesting that the SERT promoter is sufficiently strong to drive transgene expression. These qualities are highly advantageous for manipulating 5-HT_{1B} autoreceptor levels, as the viral vector can be injected into serotonergic cell bodies located in the DRN but the receptor itself will be trafficked to the nerve terminals just as endogenous receptors are.

The novel AAV8-DIO-1B viral vector incorporates two important elements. First, because the double-floxed and inverted ORF design requires the presence of Cre recombinase to induce expression, the selection of the source of Cre gives an additional level of control; DIO viral vectors may be used in combination with transgenic mice that express Cre in a cell type-specific way (Krashes et al 2011), or with retrogradely transported CAV-Cre viral vectors, which may be used to study a single circuit in the brain (Boender et al 2014, Nair et al 2013). In Figure 1.4, there is excellent expression of the viral vector using the Pet1-Cre transgenic mouse line.

Second, the 2A ribosomal skip peptide allows the expression of two separate proteins from a single cassette, which may offer an advantage over fluorescently-tagged fusion proteins in that the function of a protein is less likely to be disrupted in its naïve state. Both mCitrine and the HA-tagged 5-HT_{1B} receptor are clearly detectable, suggesting that the 2A ribosomal skip peptide does not significantly impair the translation of either transgene, compared with other polycistronic viral vector approaches such as the use of the internal ribosome entry site, which tends to cause lower expression levels of the second protein encoded in the sequence (Chan et al 2011, de Felipe 2002, Ho et al 2013).

One caveat of viral vector use is the possibility of undesired anterograde or retrograde spread. This potential has been demonstrated for both HSV (McGovern et al 2012, Ugolini et al 1987) and for certain serotypes of AAV (Boulis et al 2003, Burger et al 2004, Rothermel et al 2013). The degree to which this occurs is unknown, especially in comparison to the extent of the primary infection at the injection site; however, robust transgene expression at the target site is readily detected by our lab and others, so any subsequent experimental observations can be attributed at least in part to viral-mediated gene manipulation at the injection site.

These two new viral vectors afford us greater specificity and control over the manipulation of 5-HT_{1B} autoreceptors and circumvent many of the limitations of traditional approaches.

CHAPTER 2

5-HT_{1B} Autoreceptors in Contextual Fear Conditioning

Introduction

5-HT_{1B} autoreceptors modulate a range of emotional behaviors. Previous work in our lab with rodent models revealed that 5-HT_{1B} receptors originating from neurons in the mid-rostrocaudal DRN were important for modulating anxiety behaviors in the open field test and elevated plus maze, while those from the caudal DRN were important in regulating depression-like and fear responses in the forced swim test and fear conditioning paradigms, respectively; however, prior exposure to a strong stressor abolished the ability of 5-HT_{1B} receptors to affect behavior (Clark et al 2002, Clark et al 2004, McDevitt et al 2011, McDevitt & Neumaier 2011).

Importantly, one potential therapeutic application derives from the ability of 5-HT_{1B} autoreceptors to attenuate fear responses (McDevitt et al 2011); contextual fear conditioning in rodents can model some aspects of post-traumatic stress disorder (PTSD) (VanElzakker et al 2014). McDevitt et al. (2011) showed that 5-HT_{1B} receptor overexpression via viral-mediated gene transfer in the caudal DRN significantly decreased freezing behavior when rats were tested 24 hours following the contextual fear conditioning training session; this reduction in fear was also observed when 5-HT_{1B} receptor overexpression occurred after the training session but prior to the test session. Conversely, this effect was absent if training occurred when viral expression was maximal but testing occurred after 5-HT_{1B} receptor overexpression had dissipated. These data indicate that 5-HT_{1B} autoreceptors play a specific role in the expression, rather than the acquisition, of conditioned fear. Most interestingly, if naïve rats were exposed to fear training, then received CP-94253 injections at low doses to preferentially target 5-HT_{1B} autoreceptors 30

minutes prior to the test session, there was a marked decrease in freezing during both that test session as well as a subsequent, drug-free session 24 hours later; this result was only seen when the agonist was administered with the fear context, not when rats were injected and remained in their home cages. These results follow the same principle as the use of D-cycloserine paired with exposure therapy in the treatment of anxiety (Fitzgerald et al 2014, Hofmann et al 2012, Rodebaugh & Lenze 2013), which combines pharmacotherapy with cognitive behavioral therapy disorders, and may have important clinical implications for the treatment of PTSD, as patients usually present with debilitating fear and will only benefit from interventions that target fear expression.

The aforementioned experiments were performed with a non-selective HSV-5-HT_{1B} viral vector injected into the DRN, so it is not possible to say definitively whether 5-HT_{1B} receptor overexpression was limited to only serotonergic neurons in the DRN. In the first portion of this chapter, I will describe experiments where we used the cell type-specific SERT promoter-driven 5-HT_{1B} (SERT-1B) viral vector to investigate whether 5-HT_{1B} autoreceptor overexpression alone is sufficient for the attenuation of fear expression in contextual fear conditioning.

While it has been suggested that conditioned aversive stimuli activate the serotonergic system, and the elicited behaviors are mediated by projections to different brain regions (Deakin & Graeff 1991), the specific circuits involved are still unknown. In the second portion of this chapter, I will discuss our examination of the contribution of 5-HT_{1B} autoreceptors in the DRN-to-amygdala circuit to contextual fear conditioning. The amygdala is a brain area that receives major serotonergic projections and is heavily implicated in various fear and anxiety behaviors (Ciocchi et al 2010, Davis 1992, Ebner et al 2004, Haubensak et al 2010, Herry et al 2010, Johansen et al 2010, Lowry et al 2005). In fact, about 10% of serotonergic neurons in the DRN

project to the amygdala, but nearly all of the neurons that project from the DRN to the amygdala are serotonergic (Ma et al 1991); within the amygdala, serotonergic innervation is densest to the basolateral amygdala and weak in the central amygdala (Vertes 1991). However, it is still unclear how the serotonergic system acts in the amygdala for fear-related responses. One hypothesis has proposed that the DRN-to-amygdala circuit is important for eliciting fear and anticipatory anxiety, while the DRN-to-periaqueductal gray (PAG) circuit is important for preventing inappropriate freezing or fight-or-flight responses (Deakin & Graeff 1991, Graeff et al 1996). Because 5-HT_{1B} autoreceptors modulate serotonergic neurotransmission throughout the brain, it is important to understand their function in specific circuits, which may lead to greater insight about the role of serotonin in various brain regions. The two distinct viral vector strategies utilized in this chapter provide complementary information about the role of 5-HT_{1B} autoreceptors in fear expression, first by using the SERT promoter as a neurochemical identifier of serotonergic neurons, then by using the intersectional AAV8-DIO-1B with CAV-Cre viral vectors to investigate the neuroanatomical connectivity between the DRN and the amygdala.

Methods

Animal care, stereotaxic surgery, injection of viral vectors, and immunohistochemistry. These procedures were carried out essentially as described in Chapter 1. Adult male and female 129S6/SvEvTac mice that either were wild type (WT) or had constitutive deletions of the 5-HT_{1B} receptor (1BKO) were used for these experiments. Mice used in behavioral experiments were 12-16 weeks of age at the time of testing. Bilateral injections of CAV-Cre or CAV-GFP into the amygdala were performed at the coordinates AP -1.25, ML ±2.8, DV -4.6 from bregma, 1 μL per side.

Injection of fluorescent microspheres. Fluorescent microspheres (Molecular Probes) were diluted 1:1 in sterile water and injected bilaterally, 250 nL per side at a rate of 100 nL/sec, to target the amygdala using four test coordinates: 1) AP -1.1, ML ±2.8, DV -4.7; 2) AP -1.25, ML ±2.8, DV -4.6; 3) AP -1.4, ML ±2.8, DV -4.8; or 4) AP -1.55, ML ±2.8, DV -4.6. Animals were sacrificed on day 5 and perfused with 1x PBS followed by 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde for four hours before switching to 1x PBS, sliced, and mounted onto slides with Prolong Gold with DAPI (Molecular Probes).

Contextual fear conditioning. Behavioral experiments began at the time of maximal viral expression: 7 days after surgery for SERT-GFP and SERT-1B mice, and 20-22 days after surgery for AAV8-DIO-1B/CAV-Cre and CAV-GFP mice. Training and testing occurred in a mouse test cage measuring 7 x 7 x 12 in with a shock floor (Coulbourn Instruments) with a 1% acetic acid odor. Scrambled shocks were delivered manually with the Precision Animal Shocker (Coulbourn Instruments). For fear training, mice were placed in the middle of the test cage and allowed to acclimate for two minutes, then received three 2-second, 0.7 mA footshocks every two minutes. Following the third and final footshock, mice remained in the test cage for an additional minute before being returned to their home cage. Twenty-four hours later, mice were placed back in the test cage for a five minute test session. Training and testing sessions were digitally recorded, and freezing was analyzed using ANY-maze software (Stoelting Co.).

Open field test. Mice were placed into a circular open field chamber measuring 45 cm in diameter for five minutes. Behavior was digitally recorded, and ANY-maze software (Stoelting Co.) was used for analysis.

Data analyses. Statistical analyses were performed with GraphPad Prism. Fear expression was measured as percent of time freezing during the five minute test session.

Freezing over the course of the training session was quantified in 60 second bins. Pain sensitivity was measured as the latency from the onset of the first shock to the first episode of freezing. Two-way ANOVA was used to test for statistical significance for behavior, with virus and genotype as the between subjects variables, except for the analysis of freezing during the training session, which used two-way repeated measures ANOVA, with experimental group as the between subjects variable and time as the within subjects variable.

Results

Intra-DRN injection of the SERT-1B viral vector increases levels of 5-HT_{1B} autoreceptors in nerve terminals throughout the brain to reduce the expression of conditioned fear

We tested the effect of viral-mediated expression of 5-HT_{1B} autoreceptors in WT and 1BKO mice on contextual fear conditioning. Analysis of freezing during the five minute test session revealed a significant reduction in freezing due to virus, but no effect of genotype and no interaction between virus and genotype (Figure 2.1; two-way ANOVA, $p = 0.025$ for virus, $p = 0.371$ for genotype, $p = 0.299$ for interaction). WT and 1BKO mice that received the control SERT-GFP viral vector showed similar levels of freezing, which is consistent with previous studies suggesting that typical levels of contextual fear are observed in constitutive 1BKO mice as a result of compensatory adaptations arising from developing without 5-HT_{1B} receptors (Malleret et al 1999). 1BKO mice that received the SERT-1B viral vector into the DRN, which restored expression of 5-HT_{1B} autoreceptors but not 5-HT_{1B} heteroreceptors, showed a dramatic reduction in freezing, while WT mice receiving the SERT-1B viral vector, which led to an overexpression of 5-HT_{1B} autoreceptors in caudal DRN neurons, showed an intermediate

phenotype in between that of the WT controls that received the SERT-GFP vector and of the 1BKO mice receiving the SERT-1B viral vector.

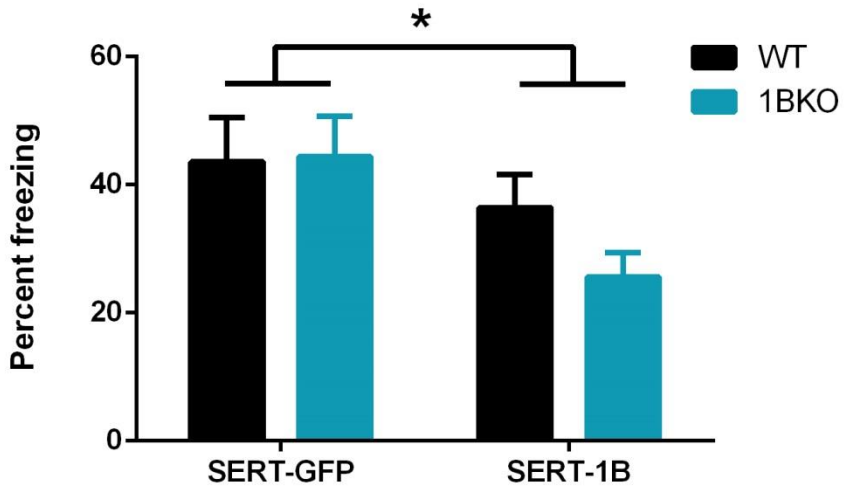


Figure 2.1. Overexpression or selective expression of 5-HT$_{1B}$ autoreceptors reduces the expression of conditioned fear. WT and 1BKO animals receiving the SERT-1B viral vector showed significantly less freezing in the test session following contextual fear conditioning compared to animals receiving the SERT-GFP control vector, and there was no effect of genotype on freezing or interaction between virus and genotype (two-way ANOVA, $p = 0.025$ for virus, $p = 0.371$ for genotype, $p = 0.299$ for interaction).

DRN-to-amygdala projections can be targeted with intersectional viral vector approaches

In order to determine where to target our stereotaxic injections for the intersectional viral vector approach, I injected fluorescent microspheres into the amygdala and examined retrograde transport to the caudal DRN; the most effective coordinates for retrograde transport back to the caudal DRN were AP -1.25, ML ±2.8, DV -4.6 from bregma (Figure 2.2a). These coordinates were used for CAV-Cre injections into the amygdala and were combined with AAV8-DIO-1B injections into the DRN. CAV-Cre was retrogradely transported from the amygdala to the DRN, where it acted to invert the DIO transgene into the correct reading frame, allowing expression of HA-5-HT$_{1B}$ receptors only in DRN neurons that projected to the amygdala.

Immunohistochemistry for HA-tagged 5-HT_{1B} receptors showed immunoreactivity at nerve terminals in the amygdala (Figure 2.2b), as well as in the DRN in both cell bodies and neurites (Figure 2.2c, top), demonstrating not only expression but also correct localization of the receptor to nerve terminals as is seen with endogenous 5-HT_{1B} receptors. Colabeling with Tph (Figure 2.2c, middle) showed some, but not full, colocalization of HA-5-HT_{1B} with Tph (Figure 2.2c, bottom). The number of infected neurons detected was low but consistent with what we would expect with viral expression, especially since only about 10% of neurons from the DRN project to the amygdala (Ma et al 1991).

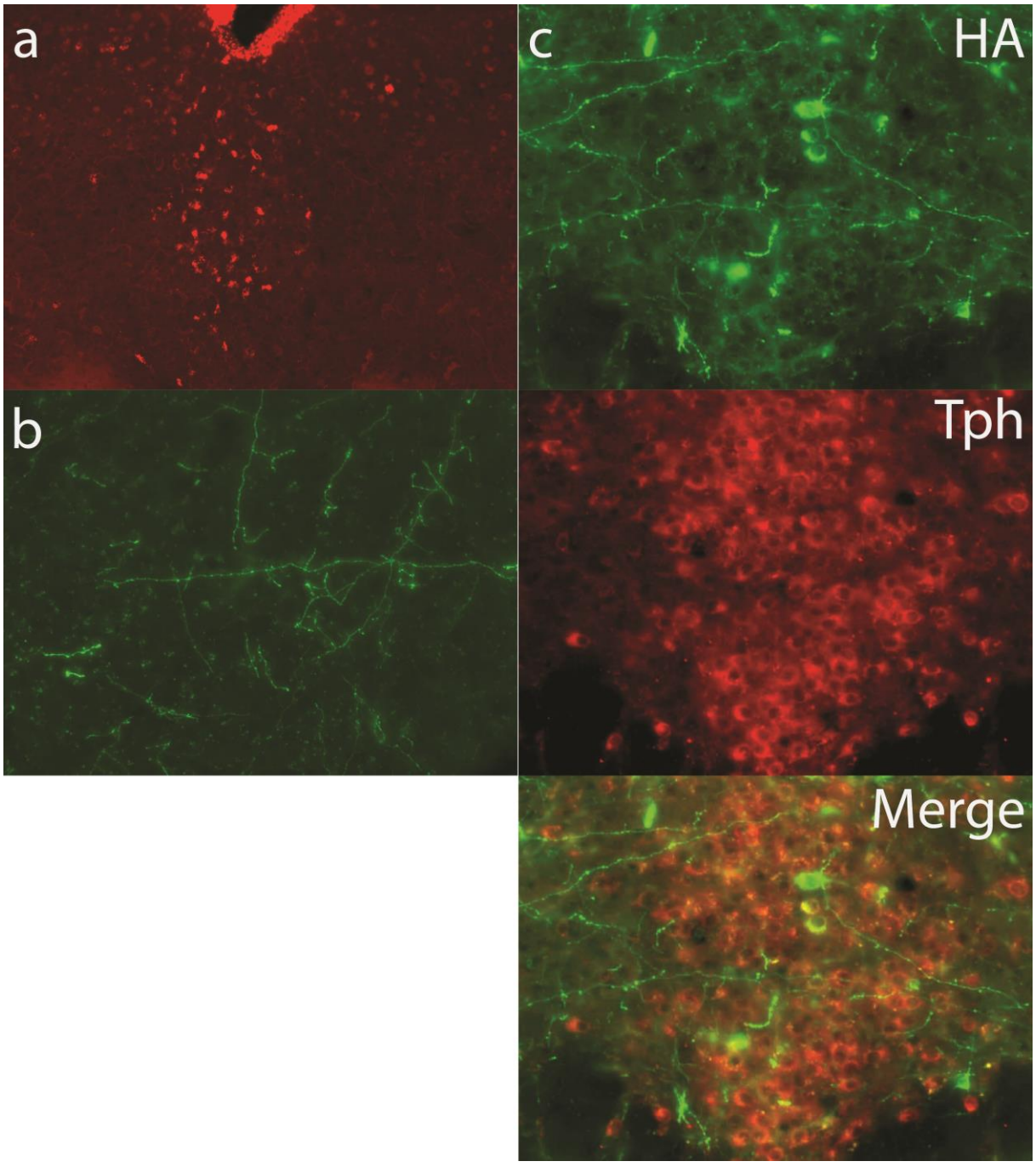


Figure 2.2. DRN-to-amygdala projections are validated with immunohistochemistry. (a) When fluorescent microspheres were injected into several targets within the amygdala, the coordinates AP -1.25, ML ±2.8, DV -4.6 from bregma showed the greatest level of retrograde transport to the caudal DRN. Injection of AAV8-DIO-1B into the DRN combined with injection of CAV-Cre into the amygdala resulted in visible transgene expression at nerve terminals in the amygdala (b; anti-HA staining in green) and in the DRN (c; anti-HA staining in green, anti-Tph staining in red), with HA-5-HT_{1B} receptors in both cell bodies and neurites; HA-5-HT_{1B} colocalizes with Tph immunoreactivity in some, but not all, cells.

5-HT_{1B} receptor overexpression in the DRN-to-amygdala circuit increases fear expression in WT, but not 1BKO, mice

WT and 1BKO mice were injected with AAV8-DIO-1B into the DRN in combination either with CAV-Cre into the amygdala to increase 5-HT_{1B} receptors in the DRN-to-amygdala circuit, or with a CAV-GFP control vector. HA staining of cell bodies in the DRN were only observed when CAV-Cre was injected into the amygdala, indicating that recombination and expression of HA-5-HT_{1B} was Cre-dependent; no HA staining was observed in animals that received CAV-GFP injections into the amygdala. 5-HT_{1B} receptor overexpression in this circuit significantly increased freezing in the test session in WT mice but not in 1BKO mice (Figure 2.3a; two-way ANOVA, two-way ANOVA, $p = 0.04$ for interaction, $p = 0.315$ for virus, $p = 0.127$ for genotype). This was not due to differences between the two groups in anxiety as measured by the open field test (Figure 2.3b; two-way ANOVA, $p = 0.668$ for virus, $p = 0.804$ for genotype, $p = 0.804$ for interaction) or differences in pain sensitivity as measured by the latency to the first freezing episode following the first shock (Figure 2.3c; two-way ANOVA, $p = 0.865$ for virus, $p = 0.246$ for genotype, $p = 0.796$ for interaction). Interestingly, while all groups showed increased freezing over the course of the training session, significant differences were found between 1BKO mice receiving CAV-Cre and both WT mice receiving CAV-Cre at 300, 360, and 420 sec and WT mice receiving CAV-GFP at 420 sec (Figure 2.3d; two-way repeated measures ANOVA, $p < 0.0001$ for time, $p = 0.0079$ for interaction, $p = 0.262$ for experimental group).

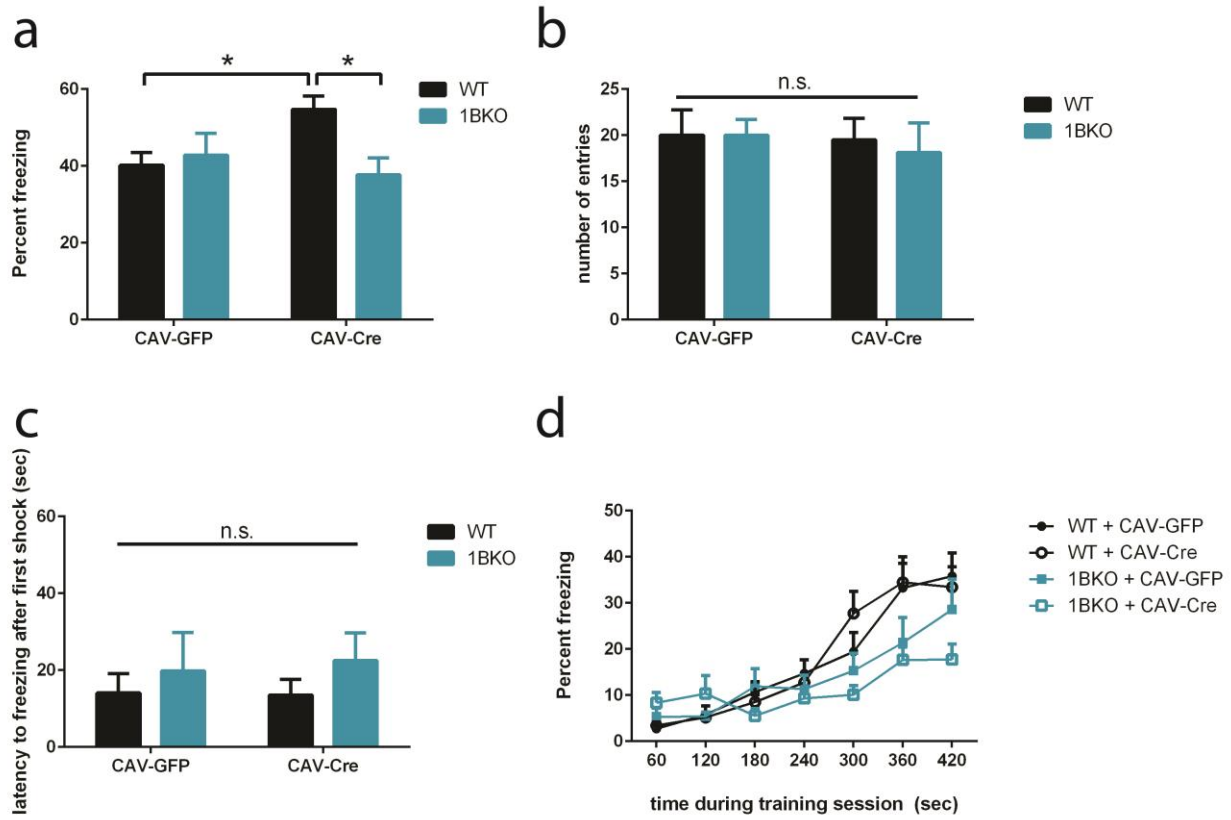


Figure 2.3. Overexpression of 5-HT$_{1B}$ receptors in the DRN-to-amygdala circuit increases fear expression in WT mice. WT and 1BKO mice received injections of AAV8-DIO-1B into the DRN, and either CAV-Cre for 5-HT$_{1B}$ receptor overexpression or CAV-GFP as a control. (a) 5-HT$_{1B}$ receptor overexpression in the DRN-to-amygdala circuit significantly increased freezing following contextual fear conditioning in WT but not 1BKO mice (two-way ANOVA, $p = 0.04$ for interaction, $p = 0.315$ for virus, $p = 0.127$ for genotype). Viral-mediated expression of 5-HT$_{1B}$ receptors in the DRN-to-amygdala circuit had no effect on (b) anxiety in the open field test (two-way ANOVA, $p = 0.668$ for virus, $p = 0.804$ for genotype, $p = 0.804$ for interaction) or on (c) pain sensitivity, as measured by latency to the first freezing episode following the first shock (two-way ANOVA, $p = 0.865$ for virus, $p = 0.246$ for genotype, $p = 0.796$ for interaction). (d) Freezing during the training session increased over time, and freezing in 1BKO mice receiving CAV-Cre was significantly different from WT mice receiving CAV-Cre at 300, 360, and 420 sec and significantly different from WT mice receiving CAV-GFP at 420 sec (two-way repeated measures ANOVA, $p < 0.0001$ for time, $p = 0.0079$ for interaction, $p = 0.262$ for experimental group).

Discussion

Using the serotonin-specific SERT-1B viral vector, 5-HT$_{1B}$ autoreceptor overexpression throughout the brain of WT mice decreases the expression of conditioned fear, which is

consistent with a previous study in our lab that used a nonselective HSV injected in the DRN of rats to drive 5-HT_{1B} receptor expression (McDevitt et al 2011). While conventional HSV viral vectors induce transgene expression in neurons and not glia, expression is not restricted to serotonergic neurons. Since SERT is only expressed in serotonin neurons, we used it to increase 5-HT_{1B} autoreceptors only in serotonin neurons, and this led to a reduction in fear expression; this is consistent with previous studies from our lab (McDevitt et al 2011). Interestingly, injection of SERT-1B into 1BKO mice, which lack both 5-HT_{1B} auto- and heteroreceptors, also results in fear attenuation; with the viral infection, these mice only express 5-HT_{1B} autoreceptors, showing that 5-HT_{1B} autoreceptors alone are sufficient for this effect. Although the virally expressed 5-HT_{1B} receptors are correctly trafficked to the axons and nerve terminals throughout the brain, it is difficult to quantify the amount of receptors that are being expressed and impossible to detect the percent change in 5-HT_{1B} autoreceptor expression as a result of viral infection.

We also developed a method to restrict viral-mediated expression of 5-HT_{1B} receptors to neurons that project from the DRN to the amygdala using intersectional control of transgene expression, which has the advantage of defining a particular brain circuit based on neuroanatomical features. Surprisingly, expressing 5-HT_{1B} receptors only in the DRN neurons that project to the amygdala produced the opposite result of increasing fear expression in WT mice. This observation is not a result of differences in anxiety, fear acquisition, or pain sensitivity between WT mice receiving CAV-Cre and WT mice receiving the control CAV-GFP vector. This interesting finding in the DRN-to-amygdala circuit suggests that 5-HT_{1B} autoreceptors in the amygdala may play a role in promoting fear expression, while other circuits may be responsible for the attenuation of fear. Ethologically, the appropriate expression of fear

responses in the face of danger offers animals a selective advantage (Blanchard et al 1993), so it is possible that 5-HT_{1B} receptors in the DRN-to-amygdala circuit facilitate this process. This may occur through 5-HT_{1B}-mediated reductions in synaptic 5-HT levels, via increased reuptake through SERT (Daws et al 2000, Hagan et al 2012, Montanez et al 2013), suppression of 5-HT synthesis (Hjorth et al 1995), or inhibition of 5-HT release (Middlemiss & Hutson 1990). Indeed, recent reports support this hypothesis, as depletion of 5-HT levels in the amygdala potentiates fear behaviors (Tran et al 2013, Wellman et al 2013), while increased 5-HT levels in the amygdala decreases fear expression (Burghardt & Bauer 2013, Kitaichi et al 2014).

Remarkably, manipulation of 5-HT_{1B} receptors in the DRN-to-amygdala circuit did not have the same effects on 1BKO mice as it did on WT mice. This may be due to developmental differences in 1BKO mice as a result of the constitutive absence of 5-HT_{1B} receptors. Because the amygdala receives strong serotonergic projections from the DRN, the absence of 5-HT_{1B} receptors throughout life may have led to fundamental changes in this circuit, such that reintroduction of 5-HT_{1B} autoreceptors in later life are unable to mediate the same functions as seen in WT mice.

One striking observation resulting from the intersectional use of the AAV8-DIO-1B and CAV-Cre viral vectors is the degree of collateralization observed in the DRN. Injections of AAV8-DIO-1B into the caudal DRN combined with CAV-Cre into the amygdala were expected to increase 5-HT_{1B} autoreceptor expression in the serotonergic nerve terminals in the amygdala; however, immunohistochemical staining for HA shows that the HA-tagged 5-HT_{1B} receptor is abundant in axons and nerve terminals in the DRN as well (Figure 2.2c). It is unknown whether collaterals from these amygdala-projecting neurons synapse onto other amygdala-projecting neurons to form an integrated DRN-to-amygdala circuit, or onto neurons that project to other

brain regions. In the latter case, it is conceivable that serotonergic neurons with projections to the amygdala may also project to and alter the signaling of other serotonergic neurons that send efferents to different brain regions, thereby affecting multiple circuits. Another open question in this scenario is whether these collaterals might have complementary or opposing roles in regulating responses to fear.

One limitation of the intersectional viral vector strategy using AAV8-DIO-1B in combination with CAV-Cre to target the DRN-to-amygdala circuit is the inability to target specific cell types within the DRN. The DRN is a heterogenous structure, containing both serotonergic and nonserotonergic cells (Jacobs & Azmitia 1992). Interestingly, nonserotonergic cells in the DRN also send efferents to the amygdala (Halberstadt & Balaban 2008), so it is possible that 5-HT_{1B} receptor expression in nonserotonergic cells may contribute to the behavioral observations. As shown in Figure 2.2c, viral expression is detected in both Tph-positive and Tph-negative cells; however, due to the low number of cells infected, it is difficult to precisely quantify the percentage of cells that are serotonergic or nonserotonergic. Recent evidence suggests that subdivisions in the DRN amongst serotonergic neurons themselves may have distinct functional roles in behavior, not only in their somatotopic organization (Paul & Lowry 2013), but also in their expression of various genes that had previously been thought to define whether a cell was serotonergic, such as 5-HT_{1A} autoreceptors (Gaspar & Lillesaar 2012, Kiyasova et al 2013). A greater understanding of the organization of the DRN is necessary, as well as an investigation into the anatomical connections between subdivisions of the DRN and the different amygdaloid nuclei.

These new findings shed light on the role of both 5-HT_{1B} autoreceptors and, more broadly, the serotonergic system on fear behaviors. Further work is warranted given the

complexity of the system, particularly the functionally distinct subdivisions of both the amygdala and the DRN (Asan et al 2013, Pare et al 2004, Paul et al 2014, Paul & Lowry 2013, Sierra-Mercado et al 2011) and the collateralization of serotonergic neurons in the DRN (Steinbusch 1981, Waselus et al 2011).

CHAPTER 3

Quantitative Proteomic Approaches for Studying 5-HT_{1B} Activation

Introduction

Although 5-HT_{1B} receptor-mediated inhibition of adenylate cyclase is well established, it is very likely that 5-HT_{1B} receptor activation triggers other signaling pathways and effector proteins that are unknown at the present time. GPCRs are known to have diverse cellular functions and signal through a myriad of distinct mechanisms; conventional methods of probing signal transduction are insufficient for fully exploring all the possible pathways downstream of 5-HT_{1B} receptor activation. Quantitative proteomics is a powerful approach that allows for unbiased identification of novel interactors and signaling proteins.

Mass spectrometry-based proteomics has boomed over the last 15 years as a result of the development of electrospray ionization, which ionizes peptides and allows for their subsequent identification using mass spectrometry (MS) (Fenn et al 1989). Typically, proteins in complex mixtures are digested into peptides with trypsin, then separated based on specific physical properties with high performance liquid chromatography (HPLC) before the mass-to-charge ratios of the peptides are measured by MS (Aebersold & Mann 2003). While liquid chromatography-mass spectrometry (LC-MS) allows the sampling and identification of peptides in complex mixtures, the development of quantitative proteomics approaches like the isotope-coded affinity tag (ICAT) (Gygi et al 1999) and stable isotope labeling by amino acids in cell culture (SILAC) (Ong et al 2002) facilitated quantitative comparisons of thousands of peptides in a single sample. SILAC uses two sets of cells, each receiving a different experimental treatment; the "light" set is cultured in growth medium with normal amino acids, while the "heavy" set is

cultured in growth medium with arginine residues enriched in ^{13}C isotopes (Ong et al 2002, Ong et al 2003). After the cells are treated, the protein lysates from the light and heavy conditions are mixed in a 1:1 ratio for processing, which minimizes variability that might result from processing the two experimental conditions side-by-side. When the samples are analyzed with MS, peptides derived from the heavy condition show a mass shift compared to those from the light condition; the ratio of peak intensities for the light and heavy peptide pairs reflects their relative abundance, thus making it possible to identify proteins that change in abundance as a result of the experimental treatment (Bantscheff et al 2012, Ong & Mann 2005). Replicate experiments often incorporate a 'label-swap' to improve the likelihood of identifying true hits, such that in the "forward" experiment, the light and heavy sets of cells are paired with the control and experimental conditions, respectively, while in the "reverse" experiment, the light set is paired with the experimental condition and the heavy set is paired with the control condition. Triple encoding SILAC uses a slight modification, the addition of a "medium" state with $^{13}C_6$-arginine and $^{2}H_4$-lysine, which allows for the comparison of three different experimental conditions simultaneously (Blagoev et al 2004).

While LC-MS is very sensitive and usually able to identify over 5000 proteins in a complex mixture, there is a limit to its ability to detect proteins that are present but low in abundance (Ebhardt et al 2012, Michalski et al 2011). Studying particular types of proteins may require additional enrichment steps; for example, phosphorylated proteins make up a small percentage of the total protein pool, and without enrichment, typically only phosphopeptides from the most abundant proteins can be identified with LC-MS. With phosphoenrichment techniques, proteins are first separated into fractions based on charge or hydrophobicity, then enriched using metal ions that have a high affinity for the phosphate groups on proteins; methods

such as strong cation exchange (SCX) chromatography with immobilized metal affinity chromatography (IMAC), allows over 13,000 phosphopeptides to be identified from a cell lysate (Villen & Gygi 2008). The combination of SILAC with phosphoenrichment techniques such as SCX/IMAC provides the power to study changes in protein phosphorylation, identifying not only the proteins themselves, but also the specific amino acid residues that are differentially phosphorylated (Olsen et al 2006).

Since little is known about the molecular mechanisms of 5-HT_{1B} receptor signaling, quantitative proteomics is an ideal, unbiased method for exploration. In this chapter, we use SILAC to probe for: 1) proteins that directly interact with the 5-HT_{1B} receptor using immunoprecipitation, and 2) proteins that are regulated in a phosphorylation-dependent manner downstream of 5-HT_{1B} receptor activation using phosphoproteomics.

Methods

Cell culture and drug treatments. Neuro2A (N2A) cells were maintained with growth media consisting of DMEM, 10% fetal bovine serum, 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 G418. N2A-1B cells were grown in SILAC DMEM medium containing either normal isotope abundance amino acids ("light" state), $^{13}C_6$-arginine and $^{2}H_4$-lysine ("medium" state), or $^{13}C_{6,}{}^{15}N_4$-arginine and $^{13}C_{6,}{}^{15}N_2$-lysine ("heavy" state), 10% dialyzed FBS, penicillin and streptomycin at 37°C in 5% CO_2 for at least five doublings. One hour before treatment, cells were switched to serum-free SILAC media, with or without the presence of antagonists as described.

Immunoprecipitation. Cells were lysed in buffer containing 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1% n-dodecyl-β-D-maltoside (DDM), and 1x protease and phosphatase inhibitors, and were further solubilized on an end-over-end rotator for one hour at 4° before pelleting cell debris by centrifugation at 15,000 x g for five minutes. Cell lysates were incubated with directly conjugated mouse monoclonal anti-HA agarose beads (Sigma), with or without competition with 15-fold excess of HA peptide (Sigma), on an end-over-end rotator for four hours at 4°C. Beads were pelleted and washed once with modified lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100). Beads from the three SILAC states were then combined and washed two more times in 1 mL lysis buffer per wash. The proteins were reduced with 1 mM TCEP for ten minutes at 60°C and alkylated with 6 mM chloroacetamide for ten minutes at room temperature. 4X LDS buffer was added to the beads and heated at 70°C for ten minutes prior to separation on a Bolt 4-12% Bis-Tris gel (Novex) with MES buffer.

Basic pH C18-IMAC enrichment of phosphopeptides. N2A-1B cells were grown in triple encoding SILAC medium as described above to obtain five 150 mm dishes (~ 5 x 10^7 cells) in each treatment state. One hour before treatment, cells were switched to serum-free SILAC media. For the first set of cells, light cells received vehicle treatment, and medium- and heavy-labeled cells were treated with the selective 5-HT_{1B} agonist CP-94253 (100 nM) for one or ten minutes, respectively. A separate label-swap replicate set where SILAC labeled cell states and treatments were interchanged was obtained at the same time. Cells were rinsed twice with cold PBS and lysed in 8M urea containing 50 mM Tris pH 8, and 1x protease and phosphatase inhibitors. DNA was sheared by passing lysates 15 times through a 28 gauge needle. The lysates were further sonicated in a cup-horn sonicator (30 sec on, 90 sec off, for ten minutes at

full power). Lysates were centrifuged at 20,000 x g for 15 minutes to remove cell debris. Protein concentrations of clarified lysates were measured using the 660 nm protein assay (Pierce), and equal amounts of protein from each cell state were combined, yielding over 30 mg of protein in each of the two replicate sets. 300 µg Endoproteinase Lys-C (Wako, 1:100 enzyme:substrate ratio) was added to digest proteins at room temperature for three hours. The digest was then diluted to 1.5 M urea with addition of 50 mM Tris pH 8 buffer, and 300 µg of trypsin (Pierce, 1:100 enzyme:substrate ratio) was added to digest proteins overnight at 37°C in a shaking incubator. The digest was centrifuged at 14,000 x g for 15 min to remove precipitated protein; the supernatant was acidified to 1% formic acid to halt protein digestion. Peptides were desalted on a 150 mg 6 cc HLB desalting column (Waters), eluted with 2 x 400 µL 80% acetonitrile 0.1% TFA, dried to remove organic solvent, and resuspended in 20 mM ammonium formate for injection by autosampler onto a Zorbax 300 Å, 5 µm, 9.4 mm × 250 mm Extend-C18 column (Agilent), using a Dionex Ultimate 3000 instrument. The basic reversed phase separation was as described (Mertins et al 2013). Briefly, peptides were separated at 3 mL/min and increasing acetonitrile content with a nonlinear gradient with four different slopes (0% for 2 min; 0% to 10% in 5 min; 10% to 27% in 34 min; 27% to 31% in 4 min; 31% to 39% in 4 min; 39% to 60% in 7 min; 60% for 8 min). Eluted peptides were collected in a 96 x 2 mL deepwell plate with 1.875 mL fractions. Fractions were pooled with a fraction concatenation strategy (Hao et al 2013, Mertins et al 2013) to distribute peptides from the basic pH separation evenly into 24 fractions by their hydrophobicity. Five percent of each of the 24 fractions was saved for whole-proteome analyses; the remaining 95% was further pooled to make 12 fractions and desalted on 10 mg Waters HLB cartridges before IMAC enrichment of phosphopeptides. Peptides were resuspended in 300 µL 80% acetonitrile, 0.1% TFA and incubated for one hour with 10 µL Fe^{3+}

and Ga^{2+} charged Ni-NTA Superflow agarose beads (Qiagen) on an end-over-end rotator at room temperature. The IMAC beads were transferred to equilibrated C18 StageTips, eluted with 500 mM dibasic sodium phosphate pH 7, desalted, evaporated and resuspended in 0.1% TFA, 5% acetonitrile for LC-MS analyses.

Liquid chromatography-mass spectrometry (LC-MS). Peptides are injected on a ~ 10 cm x 75 µm ID column of 3 µm Reprosil C18.aq beads (Dr. Maisch, Germany) with a Dionex Ultimate 3000 RSLCnano for online peptide separation in a gradient of increasing acetonitrile content (3 - 35% B in 90 minutes) and data-dependent acquisition of MS and MS/MS by an Thermo Orbitrap Elite. Profile FTMS spectra were collected (R=30,000 @ 400 m/z) and CID linear ion trap MS/MS data was collected with a Top15 method. The duty cycle of the instrument operated in this way is ~ 2.5 seconds. Data was processed by MaxQuant v. 1.3.0.5 (Cox & Mann 2008) and peptide-spectrum matches obtained with the Andromeda search engine (Cox et al 2011) from a Uniprot Mouse protein database (July 2013). A maximum 1% false discovery rate was applied for protein, peptide, and phosphorylation sites. The minimum Andromeda phosphopeptide score was set at 40. Data was analyzed in the Perseus data analysis package with the 2D Annotation Enrichment function (Cox & Mann 2012).

Results

Immunoprecipitation of 5-HT_{1B} receptors reveals potential direct interactors

Triple encoding SILAC was used to explore direct interactors of 5-HT_{1B} receptors (Figure 3.1). The three conditions explored were: 1) vehicle treatment, 2) 100 nM CP-94253 treatment for one minute, and 3) 100 nM CP-94253 treatment for ten minutes. The results included two

label-swap replicates, where SILAC labeling states and agonist treatment times were exchanged between replicates.

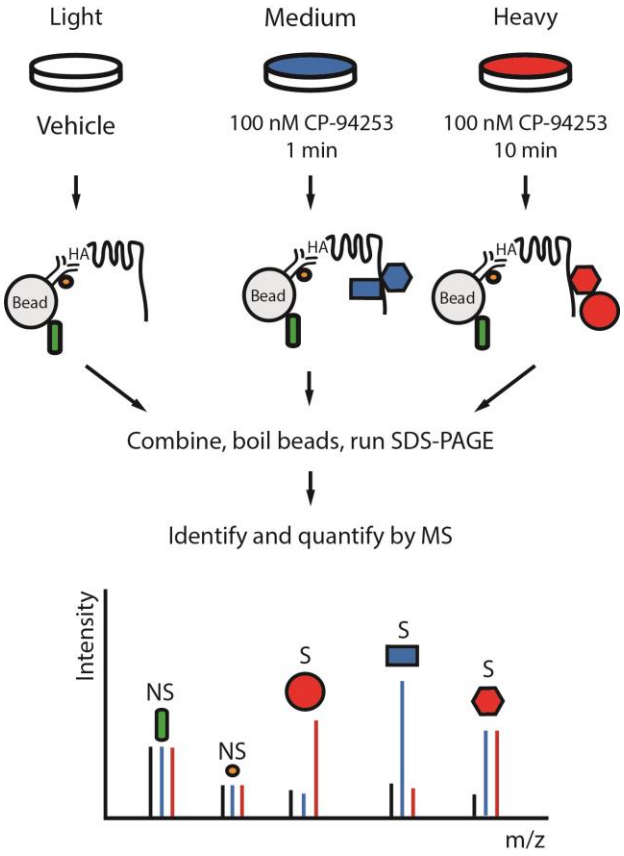
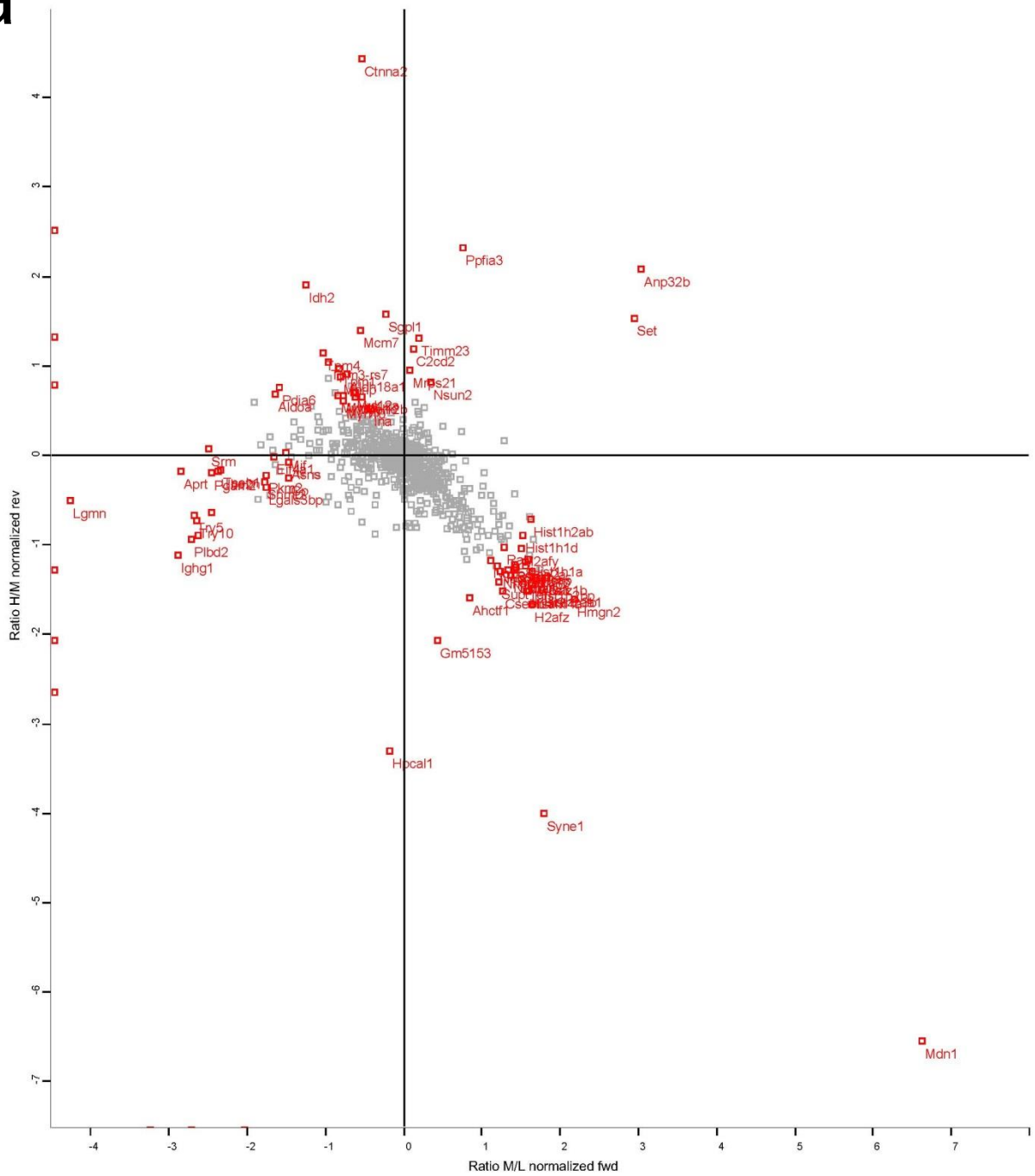


Figure 3.1. Triple encoding SILAC is used to identify potential interactors of 5-HT$_{1B}$ receptors. SILAC labeled cells stably expressing HA-5-HT$_{1B}$ were switched to serum-free SILAC media one hour before treatment with 100 nM CP-94253 for one or ten minutes. Specific interactors were identified using LC-MS.

As shown in Figure 3.2, many significant hits were identified from this experiment, but due to the rate of false positives commonly seen in proteomics experiments, further validation of any potential interacting proteins must be done.

a



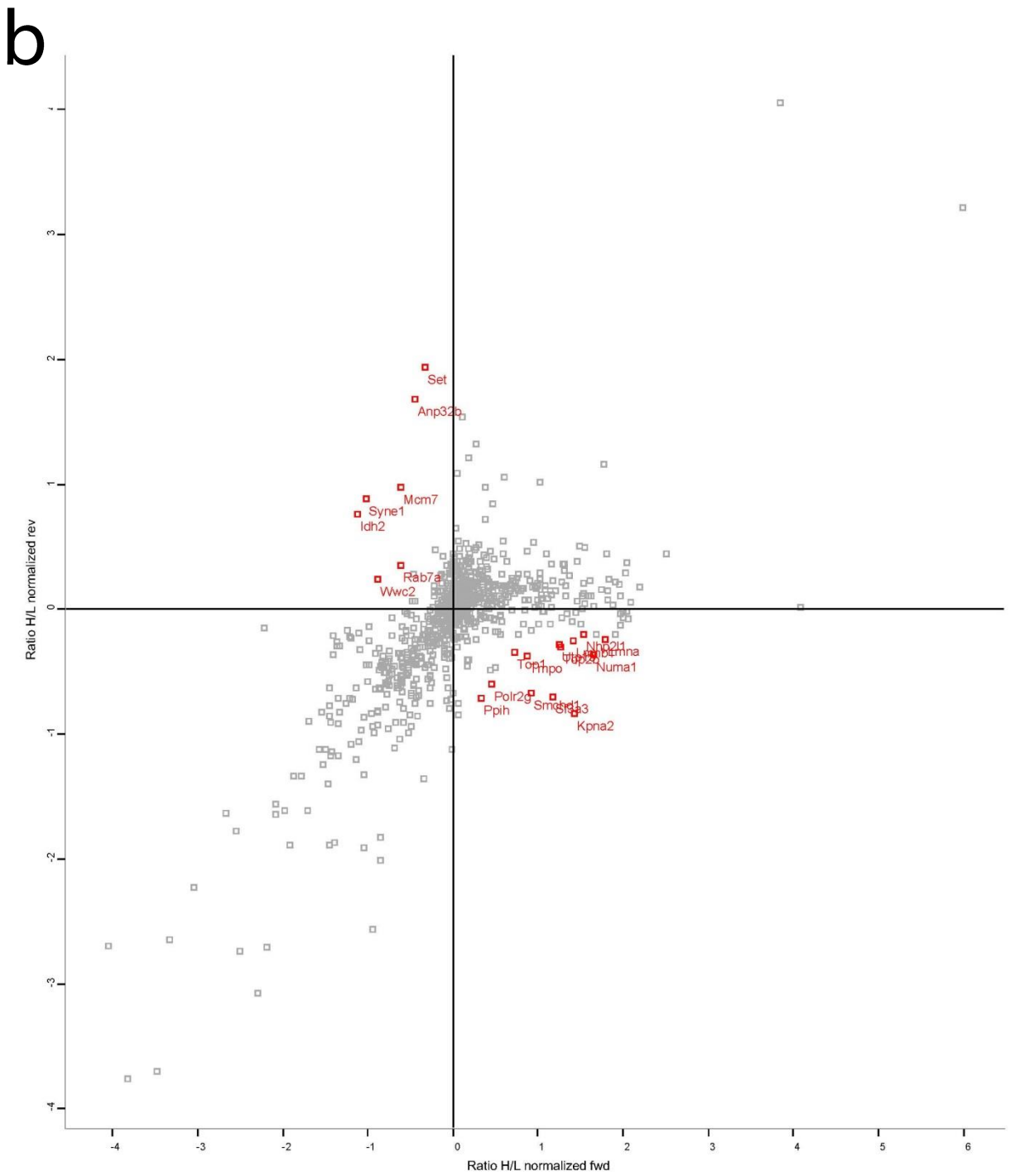


Figure 3.2. Scatterplot of significant hits following immunoprecipitation reveals possible interactors with HA-5-HT$_{1B}$ receptors. Comparison of the medium and light conditions shows proteins that may interact with 5-HT$_{1B}$ receptors at one minute (a), while comparison of the heavy and light conditions shows proteins that may interact with 5-HT$_{1B}$ at ten minutes (b).

Phosphoproteomics identifies possible signaling proteins downstream of 5-HT_{1B} receptor activation as well as putative 5-HT_{1B} receptor phosphorylation sites

Triple encoding SILAC was used to explore proteins that are differentially phosphorylated following 5-HT_{1B} receptor activation (Figure 3.3). The light condition was a vehicle-treated control group, which was compared to treatment with the agonist CP-94253 (100 nM) at one minute and ten minutes in the medium and heavy conditions, respectively. To enhance the identification of phosphopeptides from the samples, basic pH C18-IMAC enrichment was performed prior to running LC-MS.

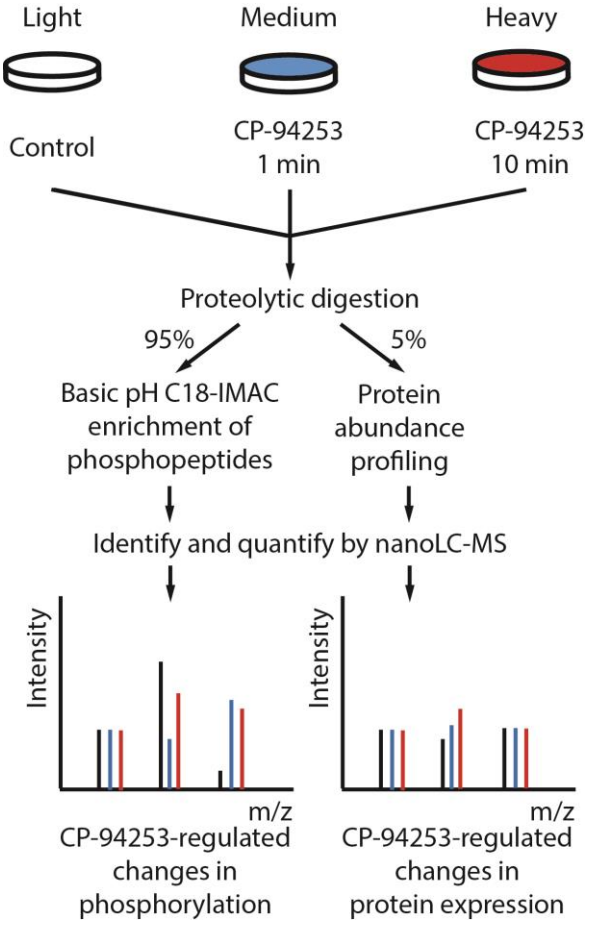


Figure 3.3. Triple encoding SILAC is used to identify possible signaling proteins downstream of 5-HT_{1B} receptor activation. Cells were switched to serum-free SILAC media one hour before treatment with 100 nM CP-94253 for 1 min (medium condition, middle) or 10 min (heavy condition, right), and compared to vehicle-treated controls (light condition, left). Phosphopeptides were enriched from the samples using basic pH C18-IMAC.

The agonist time course phosphoprofiling experiment included two label-swap replicates, where SILAC labeling states and agonist treatment times were exchanged between replicates. One set of samples was reanalyzed as an analytical replicate, generating three distinct groups of data that was then analyzed together as a single dataset. Applying a false discovery rate of 1% at the protein, peptide and PTM site level yielded a combined list of 20,037 phosphorylation sites. The dataset indicated limited regulation at the level of phosphorylated substrates. An analysis of Gene Ontology terms indicated an enrichment of DNA topological modification proteins including TOP2B, TOP2A, TOP1 at the one minute agonist treatment (Figure 3.4, red squares, Benjamini-Hochberg FDR 7.89×10^{-5}). MAPK signaling related kinases and substrates did not exhibit significant regulation, suggesting that there may have been issues with the activation of signaling pathways by agonist treatment in this experiment.

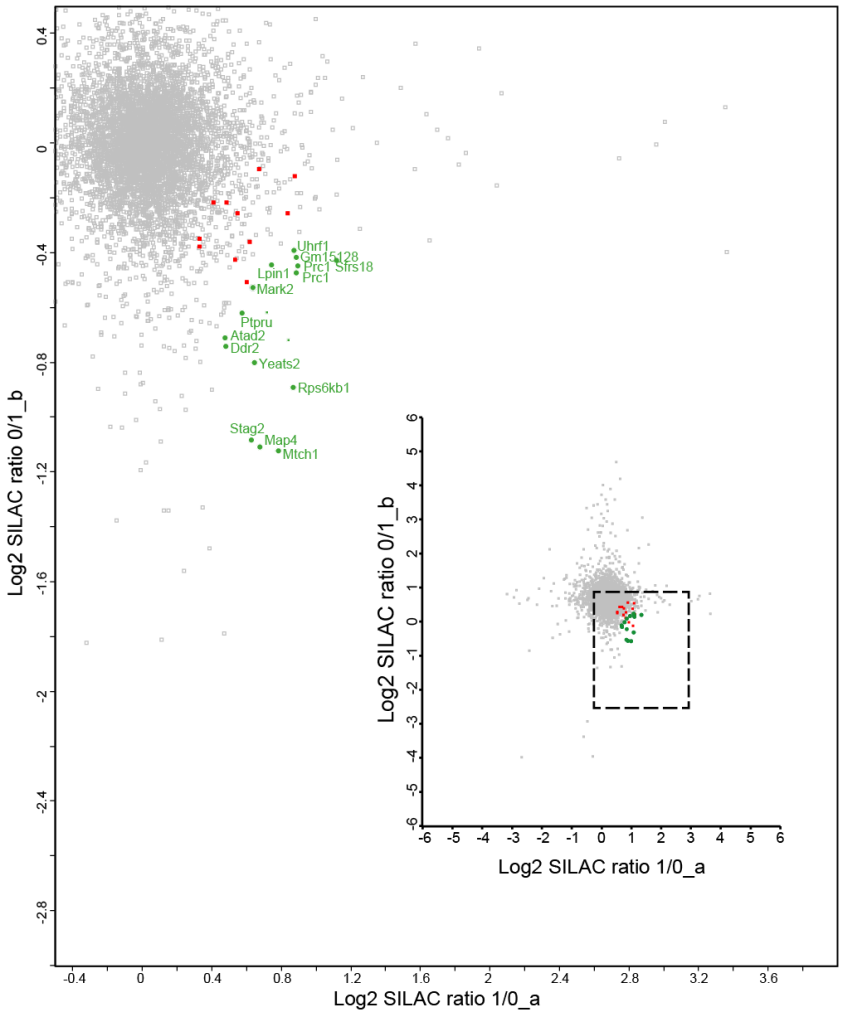


Figure 3.4. Scatterplot for phosphopeptide ratios for one minute agonist treatment versus vehicle-treated N2A-1B cells. The inset shows the full range of the dataset and the dotted line rectangle indicates the magnified region of the scatterplot. 2D annotation enrichment analysis indicates that proteins with functions involved in DNA topological change, chromosome dynamics and histone modification were enriched in phosphorylation after one min of agonist treatment (filled red squares). Phosphopeptides enriched after one minute of agonist treatment compared to vehicle treatment are shown in filled green circles.

Interestingly, global phosphopeptides profiling identified six phosphorylation sites in the 5-HT_{1B} sequence (Table 3.1), and these were used to design the phosphorylation-null 5-HT_{1B} mutant receptors described in Chapter 4.

Table 3.1. Four novel 5-HT$_{1B}$ phosphorylation sites were identified following global phosphopeptide profiling. Phosphorylated serines are highlighted in red in the amino acid sequence, and the residue number and ratio scores for comparing treatment conditions are shown for each phosphopeptide.

5-HT$_{1B}$ phosphopeptides	Res #	Ratio 1/0 a	Ratio 10/0 a	Ratio 10/1 a	Ratio 10/1 b	Ratio 0/1 b	Ratio 0/10 b	Ratio 10/1 c	Ratio 0/1 c	Ratio 0/10 c
AQLITDSPGSTSSVTSINSR	256	1.08	1.57	1.42	0.89	0.73	0.83	0.89	0.89	0.94
VPEVPSESGSPVYVNQVK	277				0.86	0.66	0.73	1.15	0.70	0.60
VPEVPSESGSPVYVNQVK	279	1.02	1.14	1.10	0.92	0.79	0.85	0.87	0.87	1.00
VRVSDALLEK	291	1.02	1.38	1.30	0.78	0.68	0.89	0.95	0.81	0.87

Discussion

Quantitative proteomics is a powerful approach as an unbiased screen for proteins. However, like other large-scale profiling techniques such as DNA and RNA sequencing, the analyses must control for a potentially high rate of false positives resulting from the massive data collected from each experiment. In the immunoprecipitation experiment, although many proteins were identified, it is crucial to validate hits with other biochemical techniques.

The phosphoproteomics pilot experiment was successful in that it identified over 20,000 phosphorylation sites, demonstrating the effectiveness of phosphoenrichment in studying a subpopulation of proteins. Although the initial experiment identified DNA topological modification proteins as highly significant hits, it seems physiologically unlikely that 5-HT$_{1B}$ receptors in neurons would induce changes in DNA, since the receptors are mainly located at the nerve terminals, far from the cell nucleus, and are generally thought to regulate local activity at the synapse. Additionally, MAPK regulation was not observed in this experiment, even though it is a known consequence of 5-HT$_{1B}$ agonist treatment, suggesting that the conditions under which the experiment was performed need to be optimized to generate more robust agonist-

induced responses. Nevertheless, four novel phosphorylation sites of the 5-HT_{1B} receptor were identified, and the importance of these sites are investigated in the subsequent chapter.

CHAPTER 4

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

Introduction

G protein-coupled receptors (GPCRs) represent the largest family of membrane receptor proteins, with their distinctive seven-transmembrane domain structure and canonical signaling through heterotrimeric guanine nucleotide-binding (G) proteins (Gether 2000). Although these receptors were once thought of as "on-off" switches for cell signaling, ongoing research continues to reveal the diversity and complexity of ligand-receptor interactions and structure-function relationships. Receptors may exist in a variety of possible conformations, including multiple "active" conformations, with different ligands stabilizing a particular conformation of the receptor; the stabilized conformation then activates a subset of the total possible downstream effectors (Reiter et al 2012, Urban et al 2007). Even when fully bound by an agonist, a receptor may exist in a dynamic intermediate state between inactive and active (Manglik & Kobilka 2014). In a phenomenon known as "functional selectivity," "ligand-directed signaling," or "biased agonism," different ligands may bind to a receptor to preferentially activate disparate signaling cascades (Wisler et al 2014).

In the classical model of GPCR signaling, the binding of an agonist to a receptor causes a conformational change that stimulates G protein activation via the exchange of GDP for GTP on the α subunit of the heterotrimeric G protein, leading to the dissociation of the Gα from the Gβγ subunits; these subunits then activate or inhibit effector molecules, including adenylate cyclase, phospholipase C (PLC), and ion channels, which ultimately regulate intracellular enzymes, such as protein kinases (Luttrell 2008). There are several major families of Gα subunits, including

$G\alpha_s$ (activates adenylate cyclase), $G\alpha_q$ (activates PLC), and $G\alpha_{i/o}$ (inhibits adenylate cyclase), with each family comprised of a number of subtypes. However, recent discoveries paint an even more complicated picture. For example, activation of some receptors that couple to $G\alpha_i$ has been shown to lead to the rearrangement, rather than the dissociation, of the Gα and Gβγ subunits; this is thought to contribute to the specificity of the response, since certain Gα and Gβγ subunit combinations result in rearrangement while others dissociate (Bunemann et al 2003, Frank et al 2005). Additionally, while all GPCRs may be coupled to G proteins, some signaling pathways are G protein-independent, mediated instead by β-arrestins. In these instances, the agonist-bound receptor is phosphorylated by a G protein-coupled receptor kinase (GRK), which serves as a "barcode" on the GPCR to direct the binding and subsequent actions of arrestins (Liggett 2011, Reiter et al 2012). Arrestins may also trigger desensitization and internalization of the GPCR (Ferguson 2001, Lefkowitz & Shenoy 2005). Ultimately, the signal transduction pathway may regulate anything from kinase and phosphatase activation, to transcription factor binding, to microRNA processing. The multiple layers of complexity allow for both a wide range of possible responses as well as specificity depending on the components that are activated.

Of the multitude of possible signaling pathways, mitogen-activated protein kinases (MAPKs) 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 extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-Terminal kinase (JNK), p38 MAPK, and ERK5 (Keshet & Seger 2010). Remarkably, GPCRs can activate MAPKs via both G protein-dependent (Naor et al 2000) as well as β-arrestin-dependent mechanisms (Luttrell & Miller 2013). In the case of ERK1/2, GPCR activation may lead to the recruitment of some protein intermediates, such as members of

the Raf, Ras, and Src families of protein kinases, which all converge on the MAPK kinase (MAPKK) MEK1/2, which then directly phosphorylates and activates ERK1/2 (Luttrell & Miller 2013, Rozengurt 2007, Sun et al 2007). Phosphorylated ERK1/2 has a diverse set of downstream functions depending on a variety of factors including cell type, cell compartment, and duration of activation (Cargnello & Roux 2011, Keshet & Seger 2010, Luttrell & Luttrell 2003). 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 5-HT_{1B} autoreceptors are also important in fear conditioning, the ability of 5-HT_{1B} receptors to signal via ERK pathways warrants further investigation.

5-HT_{1B} receptors are $G_{i/o}$-coupled receptors that are classically thought to decrease cAMP levels by inhibiting adenylate cyclase (Bouhelal et al 1988, Schoeffter & Hoyer 1989). These receptors preferentially couple to the $G\alpha_{i2}$ subunit (Lin et al 2002), with some evidence showing that they may be constitutively active in inhibiting adenylate cyclase (Ng et al 1993). Several downstream actions of 5-HT_{1B} activation have been previously reported, including activation of ERK1/2, Akt, and p70S6K (Hsu et al 2001, Leone et al 2000, Mendez et al 1999, Pullarkat et al 1998), but little is known about the sequential steps leading to kinase activation. 5-HT_{1B} receptors can also be phosphorylated by GSK3β, which affects 5-HT_{1B}-mediated reduction in cAMP production through the $G\alpha_i$ pathway, as well as receptor internalization (Chen et al 2009, Li & Jope 2010, Zhou et al 2012). The crystal structure of the 5-HT_{1B} receptor was recently solved (Wang et al 2013), which has already led to the study of structure-function relationships; the 5-HT_{1B} receptor has many similar properties of classical GPCRs, such as β_2-adrenergic receptors, and does not seem to exhibit functional selectivity toward either G protein- or β-arrestin-dependent signaling (Wacker et al 2013).

In this chapter, I outline experiments where we investigated the signal transduction pathway leading from 5-HT_{1B} receptor activation to ERK1/2 phosphorylation. I then mutated putative serine/threonine phosphorylation sites of the 5-HT_{1B} receptor in an attempt to identify critical amino acids involved with receptor signaling to ERK1/2.

Methods

Cell culture. These procedures were carried out essentially as described in Chapter 3. Mutant 5-HT_{1B} receptors were transfected as previously described and selection for the stably transfected cell lines was achieved with 500 μg/mL G418.

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 (see Appendix A for primers used) 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.

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 DPBS then 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 DPBS, 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 onto slides with Prolong Gold with DAPI (Molecular Probes).

Drug treatments. Drugs used were: CP-94253, SB224289, gallein, dynasore, PP 1, GW 5074 (Tocris); pertussis toxin (Novex); U0126 (Cell Signaling). 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 treatment, cells were switched to serum-free DMEM, with or without the presence of antagonists as described. Following 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).

Western blot. 4x NuPAGE LDS Sample Buffer (Novex) containing 10 mM DTT was mixed with cell lysates and heated to 70° 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. Membranes were blocked for one hour at room temperature in 1x TBS with 5% nonfat milk. Primary and secondary antibodies were diluted in 1x TBST with either 5% bovine serum albumin (BSA) for detection of phosphorylated proteins, or with 5% nonfat milk for all other proteins. Antibodies were incubated for one hour 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. Antibodies

used were obtained from Cell Signaling and diluted as follows: rabbit anti-phospho-p44/p42 MAPK, 1:2500; rabbit anti-p44/p42 MAPK, 1:10,000; rabbit anti-HA, 1:1000; rabbit anti-phospho-p38, 1:1000; rabbit anti-phospho-JNK, 1:1000; mouse anti-α-tubulin, 1:2500; goat anti-rabbit IgG DyLight 800, 1:15,000; goat anti-mouse IgG DyLight 680, 1:15,000.

Site-directed mutagenesis. Point mutations were introduced into the wild-type rat 5-HT_{1B} receptor sequence using the GeneArt Site-Directed Mutagenesis System (Invitrogen) according to manufacturer's instructions with 20 ng starting DNA per reaction (see Appendix B for primers used). Resulting clones were sequenced through their entire coding sequence to verify the successful introduction of the desired mutation and to confirm that no additional inadvertent mutations had been introduced.

Results

Neuro2A cells do not express 5-HT_{1B} receptors but do express a variety of other serotonin-related genes endogenously

Many previous studies of 5-HT_{1B} receptor signaling used 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), but the results can be hard to interpret in a system that does not mimic conditions in the brain. For example, this was previously seen with 5-HT_{1A} signaling, where findings in non-neuronal cell lines could not be replicated using primary neuronal culture (Cowen et al 2005). To circumvent this issue, 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. As shown in Table 4.1, N2A cells expressed a variety of serotonin-related genes, including 5-HT_{1A},

SERT, Pet-1, and Tph2. 5-HT_{1B} mRNA was essentially absent in untransfected N2A cells but abundant in transfected N2A-1B cells.

Table 4.1. Expression of serotonergic and neuronal genes in untransfected N2A and stably transfected N2A-1B cells. Threshold cycle (Ct) values were normalized to GAPDH. Fold enrichment comparing expression in N2A-1B relative to N2A cells was calculated by the ΔΔCt method.

Gene	Normalized Ct Values		Fold Enrichment
	N2A	N2A-1B	
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

Stably transfected N2A-1B cells express 5-HT$_{1B}$ receptors on the cell surface

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$_{1B}$). As shown in Figure 4.1a, HA is detected in N2A-1B cells but not in N2A cells. Immunocytochemistry for HA verified that a subset of the receptors were present at the cell surface (Figure 4.1b).

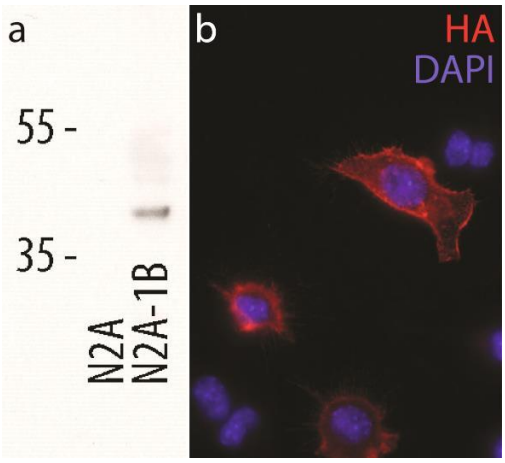


Figure 4.1. HA-tagged 5-HT$_{1B}$ receptors are present in stably transfected N2A-1B cells on the cell surface. (a) Western blot for HA shows the presence of the HA-5-HT$_{1B}$ receptors in N2A-1B cells but not in untransfected N2A cells. (b) Immunocytochemical staining for HA (red) shows HA-5-HT$_{1B}$ receptor staining present at the cell surface.

Selective 5-HT$_{1B}$ receptor agonist treatment 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

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. Phosphorylation of both ERK1 and ERK2 increased in a dose-dependent manner in N2A-1B cells, and this effect was blocked by preincubation with the 5-HT$_{1B}$ antagonist SB224289 (1 μM) for one hour prior to agonist treatment; at 100 nM CP-94253, the highest concentration used, phospho-ERK1 increased by

200%, while phospho-ERK2 increased by 125% (Figures 4.2a, b). Agonist-induced increases in phospho-ERK1/2 were absent in untransfected N2A cells (Figures 4.2c, d). Additionally, total levels of ERK1/2 remained constant in N2A-1B cells, regardless of agonist and antagonist treatments (Figures 4.2e, f).

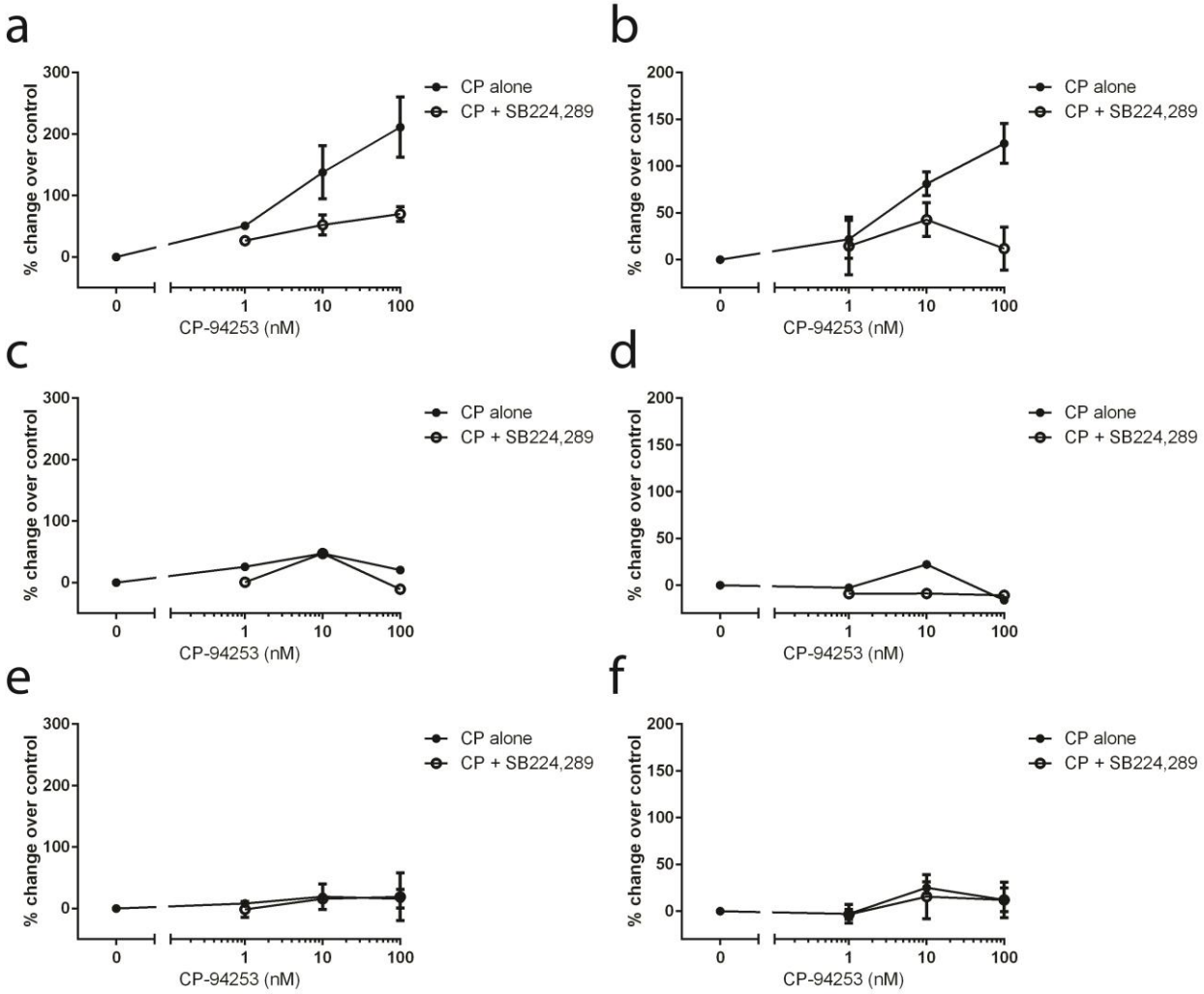


Figure 4.2. CP-94253 treatment increases levels of phospho-ERK1/2 in N2A-1B cells but not in untransfected N2A cells. In stably transfected N2A-1B cells, treatment with 1-100 nM CP-94253 for ten minutes induced phosphorylation of both ERK1 (a) and ERK2 (b) in a dose-dependent manner that was blocked by pretreatment with the antagonist SB224289. In untransfected N2A cells, no change in phosphorylation of ERK1 (c) or ERK2 (d) was observed following agonist treatment. Total levels of ERK1 (e) and ERK2 (f) remained constant following CP-94253 treatment in N2A-1B cells.

CP-94253 treatment has no effect on phosphorylation of other, closely related MAP kinases

Treatment with CP-94253 did not increase phosphorylation of p38 (Figure 4.3a), p54 JNK (Figure 4.3b), or p46 JNK (Figure 4.3c), which are three MAPKs that are closely related to ERK1/2, demonstrating the specificity of the phospho-ERK1/2 response following 5-HT_{1B} receptor activation.

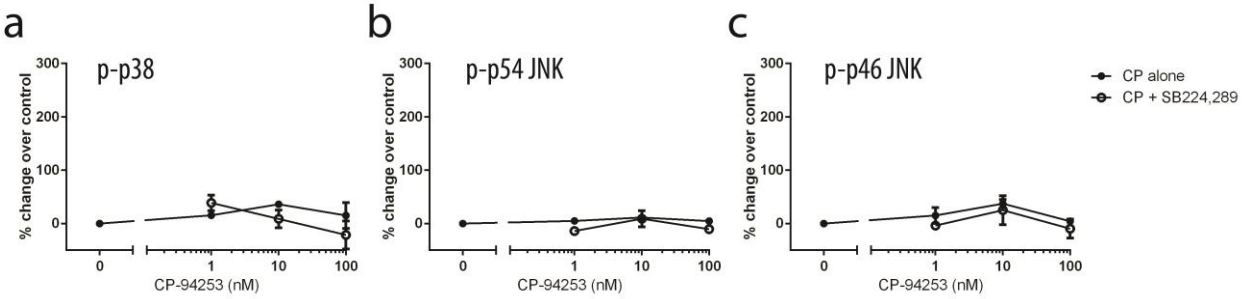


Figure 4.3. CP-94253 treatment has no effect on the phosphorylation state of other, closely related MAPKs. Treatment of N2A-1B cells with CP-94253, with or without the antagonist SB224289, has no effect on levels of phospho-p38 (a), phospho-p54 JNK (b), or phospho-p46 JNK (c).

5-HT_{1B}-mediated phosphorylation of ERK1/2 is reduced when Gα_i signaling, Src kinase, and MEK1/2 are inhibited

To investigate the signaling intermediates between 5-HT_{1B} receptor activation and phosphorylation of ERK1/2, N2A-1B cells were pretreated with pharmacological inhibitors of the targets shown in Figure 4.4a for one hour prior to CP-94253 treatment for ten minutes. Phosphorylation of both ERK1 and ERK2 was reduced in the presence of the Gα_i inhibitor pertussis toxin (Figure 4.4b) and the Src inhibitor PP 1 (Figure 4.4d), and completely blocked by the MEK1/2 inhibitor U0126 (Figure 4.4f). Agonist-induced phosphorylation of ERK1/2 was not sensitive to the G$\beta\gamma$ inhibitor gallein (Figure 4.4c) or the Raf inhibitor GW 5074 (Figure 4.4e). The dynamin inhibitor dynasore increased phosphorylation of ERK1/2 at moderate doses but decreased phospho-ERK1/2 levels at high doses (Figure 4.4g).

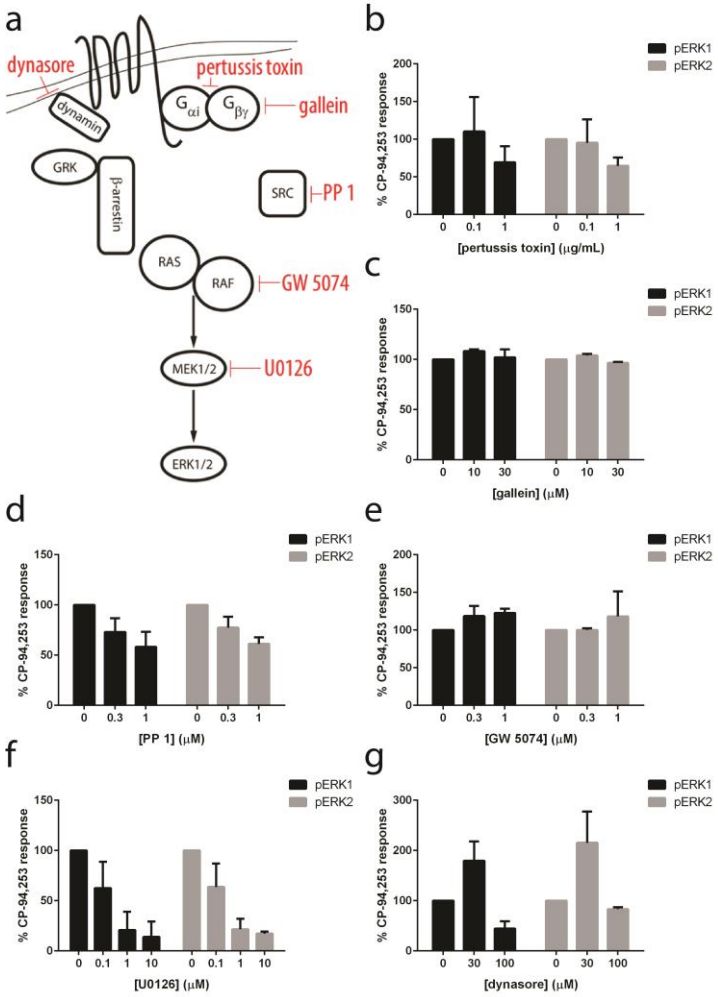


Figure 4.4. The signaling pathway leading to phosphorylation of ERK1/2 following 5-HT_{1B} receptor activation is investigated using various pharmacological inhibitors. (a) Intracellular proteins that may be involved in 5-HT_{1B}-mediated phosphorylation of ERK1/2 are blocked with the specified small molecule inhibitors by pretreatment of N2A-1B cells for one hour prior to agonist treatment with CP-94253 for ten minutes. (b) Pertussis toxin (1 μg/mL) reduces phosphorylation of ERK1/2. (c) Phosphorylation of ERK1/2 is not sensitive to gallein. (d) PP 1 reduces phospho-ERK1/2 levels in a dose-dependent manner. (e) Phosphorylation of ERK1/2 is not sensitive to GW 5074. (f) U0126 reduces phosphorylation of ERK1/2 in a dose-dependent manner, with complete blockade at 10 μM. (g) Dynasore increases phosphorylation of ERK1/2 at 30 μM, but decreases phosphorylation of ERK1 with no effect on ERK2 at 100 μM.

Mutations of 5-HT_{1B} receptor phosphorylation sites alter agonist-induced increases in phospho-ERK1/2

Four novel putative phosphorylation sites on 5-HT_{1B} receptors on three distinct phosphopeptides were discovered by phosphoproteomics (see Chapter 3). Serine residues were mutated to alanine using site-directed mutagenesis to generate three mutant receptors that are unable to be phosphorylated at these sites: 1) S256A, 2) S291A, and 3) S277A+S279A. Three additional mutants were generated to mutate sites on the 5-HT_{1B} receptor known to be crucial for phosphorylation by GSK3β (Chen et al 2009): 1) S154A, 2) T158A, and 3) S154A+T158A. The novel putative phosphorylation sites are all located in the third intracellular loop, while the GSK3β phosphorylation sites are within the second intracellular loop (Figure 4.5).

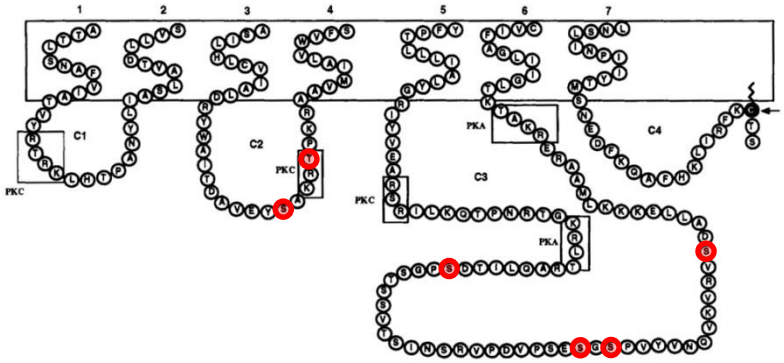


Figure 4.5. Mutant 5-HT_{1B} receptors replace phosphorylation sites with non-phosphorylatable alanine residues. Three mutants target two sites in the second intracellular loop known to be phosphorylated by GSK3β: 1) S154A, 2) T158A, and 3) S154A+T158A. Three other mutants target four novel putative sites in the third intracellular loop: 1) S256A, 2) S291A, and 3) S277A+S279A. (adapted from Ng et al. 1993)

The wild type 5-HT_{1B} receptor and the six mutant receptors were transfected into N2A cells to generate seven stable cell lines: N2A-1B, N2A-$1B_{S256A}$, N2A-$1B_{S291A}$, N2A-$1B_{S277A+S279A}$, N2A-$1B_{S154A}$, N2A-$1B_{T158A}$, and N2A-$1B_{S154A+T158A}$. Immunocytochemical staining for HA confirmed the presence of the receptor at the cell surface (Figure 4.6).

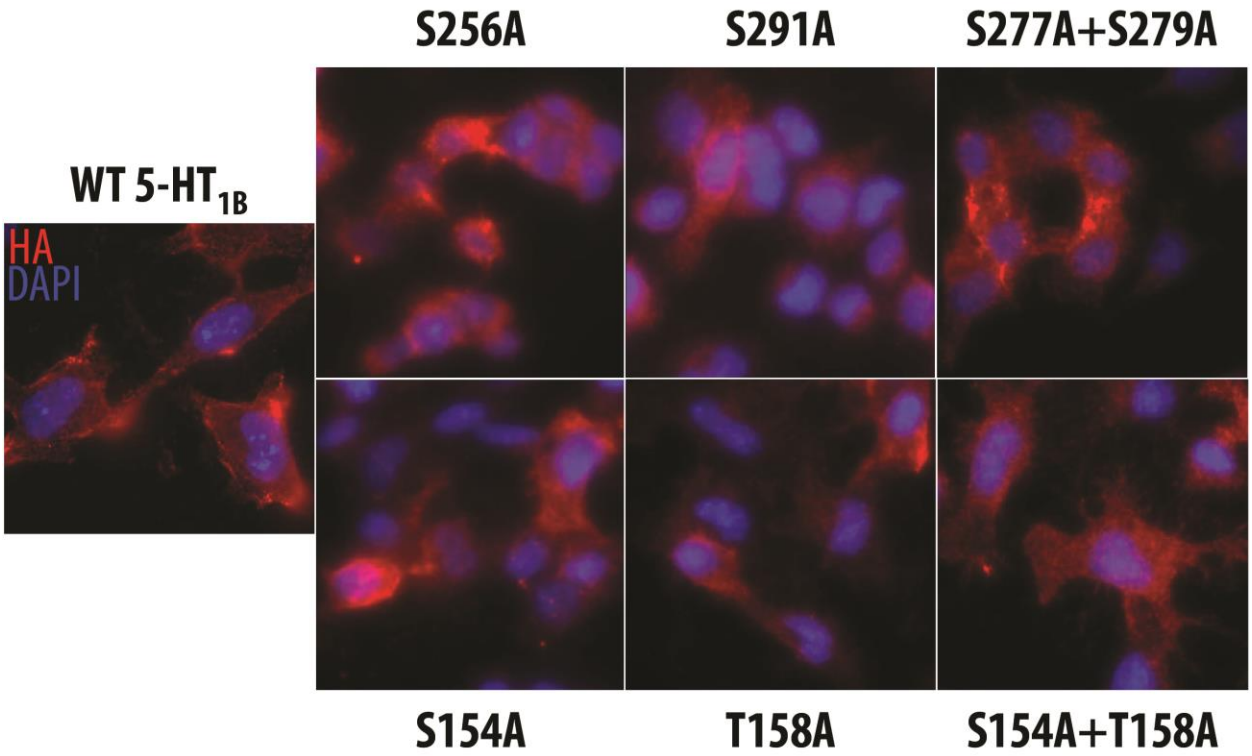


Figure 4.6. Mutant 5-HT$_{1B}$ receptors are expressed at the cell surface. Mutant 5-HT$_{1B}$ receptor plasmids were stably transfected into N2A cells, and immunocytochemical staining for the HA-tagged receptor (red) in unpermeablized cells shows the presence of the receptors at the cell surface.

Cells were treated with 1-100 nM CP-94253 for ten minutes, with or without 1 μM SB224289, then probed for phospho-ERK1/2 expression. All six mutant receptors showed varying levels of phospho-ERK1/2 induction following CP-94253 treatment that was sensitive to blockade by SB2249289. Of the three novel mutants, N2A-1B$_{S256A}$ showed the greatest reduction in phospho-ERK1 levels of 70% compared to wild type N2A-1B cells (Figure 4.7a), while N2A-1B$_{S291A}$ and N2A-1B$_{S277A+S279A}$ showed 55% and 50% reductions in phospho-ERK1, respectively (Figures 4.7c, e). Phospho-ERK2 levels were similar among the wild type and mutant 5-HT$_{1B}$ receptors (Figures 4.7b, d, f).

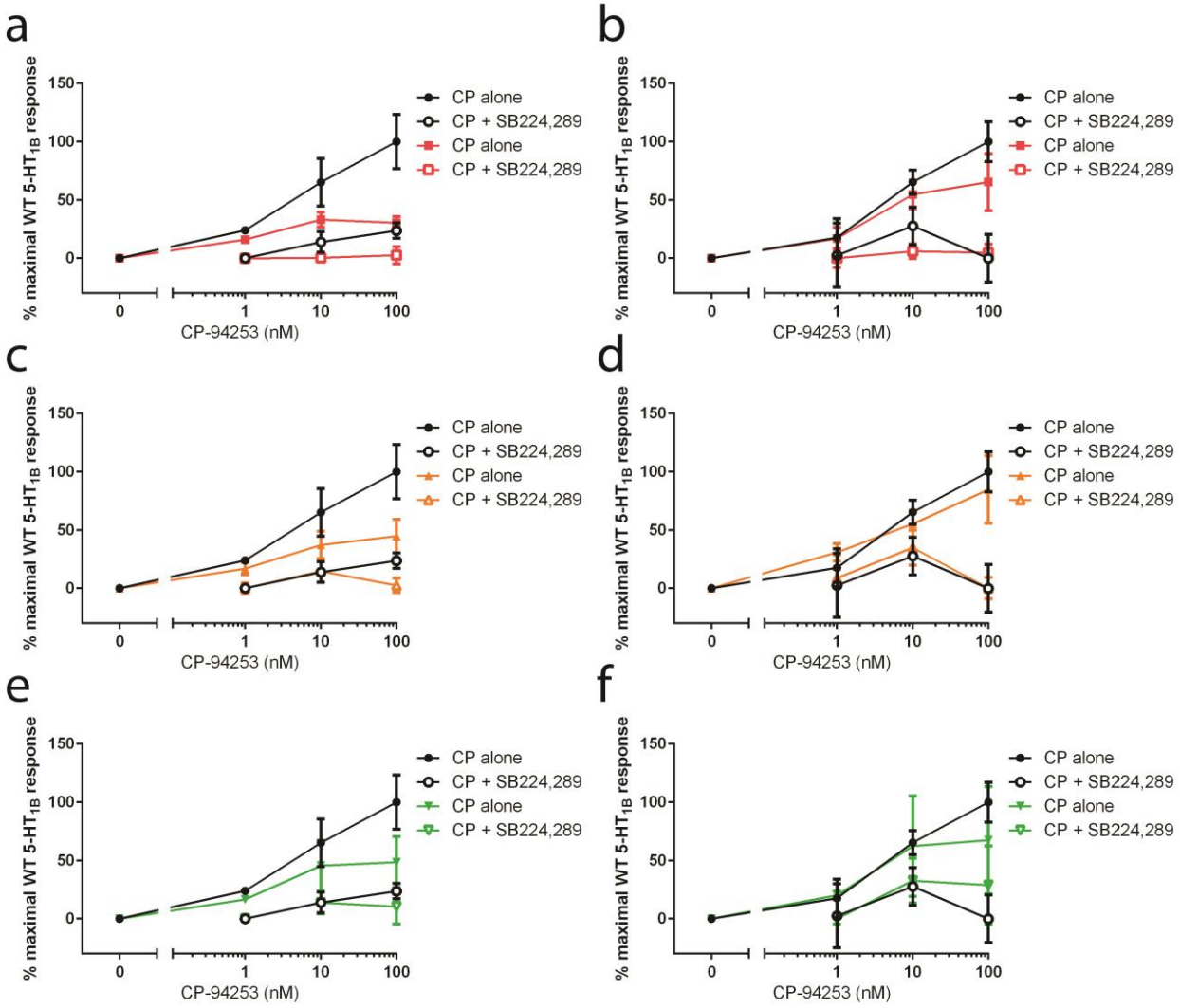


Figure 4.7. Mutations of the 5-HT$_{1B}$ receptor at the newly discovered phosphorylation sites, S256A, S291A, and S277A+S279A, lead to lower levels of phospho-ERK1/2 induction (colored symbols) compared to wild type N2A-1B cells (black symbols). Cells were treated with 1-100 nM CP-94253 for ten minutes, with or without pretreatment with 1 μM SB224289 for one hour, and then examined for phospho-ERK1 (a, c, e) and phospho-ERK2 (b, d, f). N2A-1B$_{S256A}$ (a, b, red symbols) showed a 70% reduction in phospho-ERK1, N2A-1B$_{S291A}$ (c, d, orange symbols) showed a 55% reduction in phospho-ERK1, and N2A-1B$_{S277A+S279A}$ (e, f, green symbols) showed a 50% reduction in phospho-ERK1. Phospho-ERK2 levels did not show the same dramatic reductions in the mutant 5-HT$_{1B}$ receptors compared to the wild type 5-HT$_{1B}$ receptor.

The three mutants targeting sites important for GSK3β phosphorylation, N2A-1B$_{S154A}$, N2A-1B$_{T158A}$, and N2A-1B$_{S154A+T158A}$, showed reductions in phospho-ERK1 of 40%, 50%, and

30%, respectively, compared to wild type N2A-1B cells (Figure 4.8a, c, e). Phospho-ERK2 levels were similar among the wild type and mutant 5-HT_{1B} receptors (Figures 4.8b, d, f).

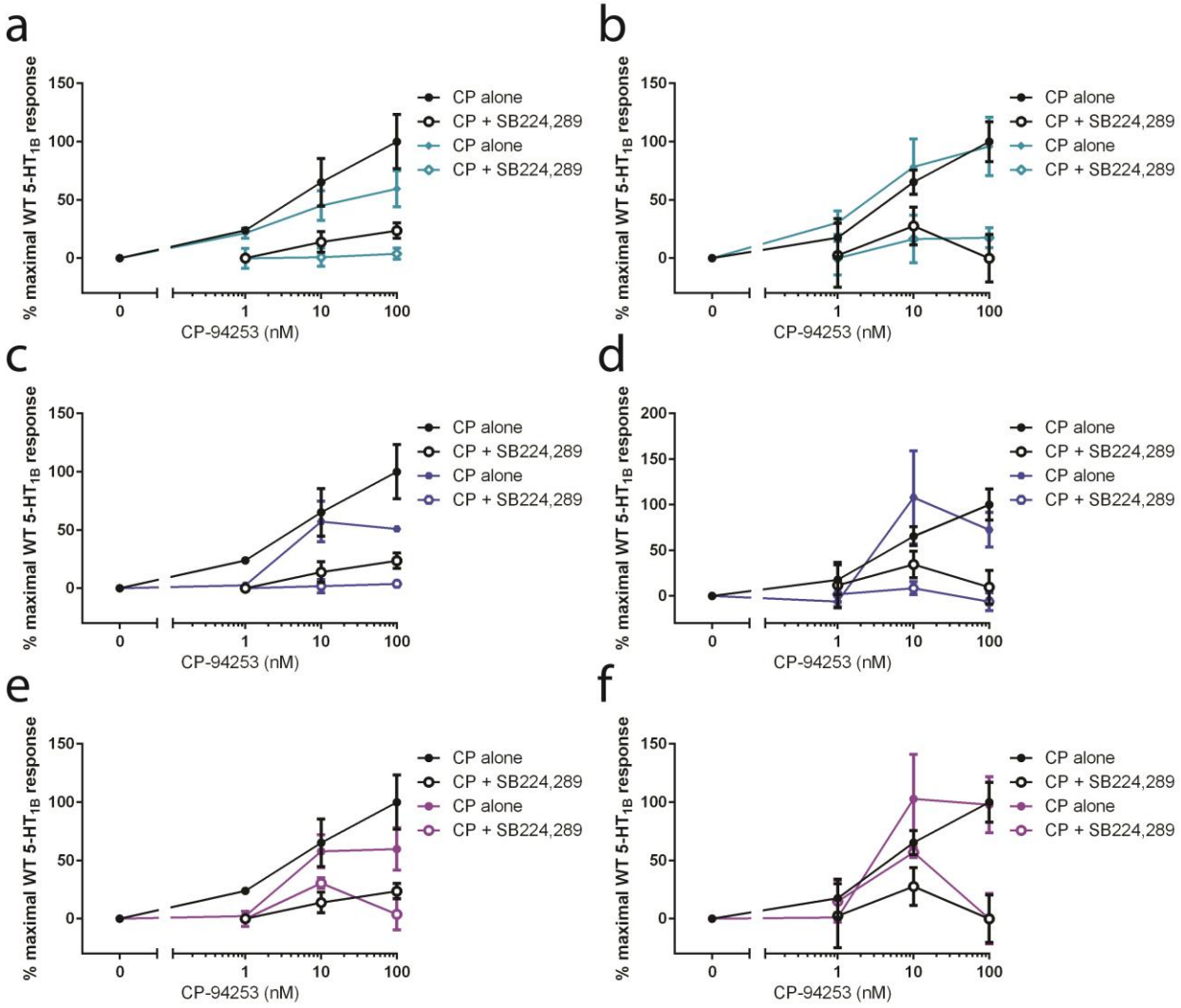


Figure 4.8. Mutations of the 5-HT_{1B} receptor at the sites known to be involved in GSK3β phosphorylation, S154A, T158A, and S154A+T158A, lead to lower levels of phospho-ERK1/2 induction (colored symbols) compared to wild type N2A-1B cells (black symbols). Cells were treated with 1-100 nM CP-94253 for ten minutes, with or without pretreatment with 1 μM SB224289 for one hour, and then examined for phospho-ERK1 (a, c, e) and phospho-ERK2 (b, d, f). N2A-$1B_{S154A}$ (a, b, light blue symbols) showed a 40% reduction in phospho-ERK1, N2A-$1B_{T158A}$ (c, d, dark blue symbols) showed a 50% reduction in phospho-ERK1, and N2A-$1B_{S154A+T158A}$ (e, f, purple symbols) showed a 30% reduction in phospho-ERK1. Phospho-ERK2 levels did not show the same dramatic reductions in the mutant 5-HT_{1B} receptors compared to the wild type 5-HT_{1B} receptor.

In summary, all of the mutant 5-HT_{1B} receptors examined showed reduced levels of phosphorylation of ERK1 following treatment with 100 nM CP-94253 for ten minutes (Figure 4.9a) but no such change in phosphorylation of ERK2 (Figure 4.9b).

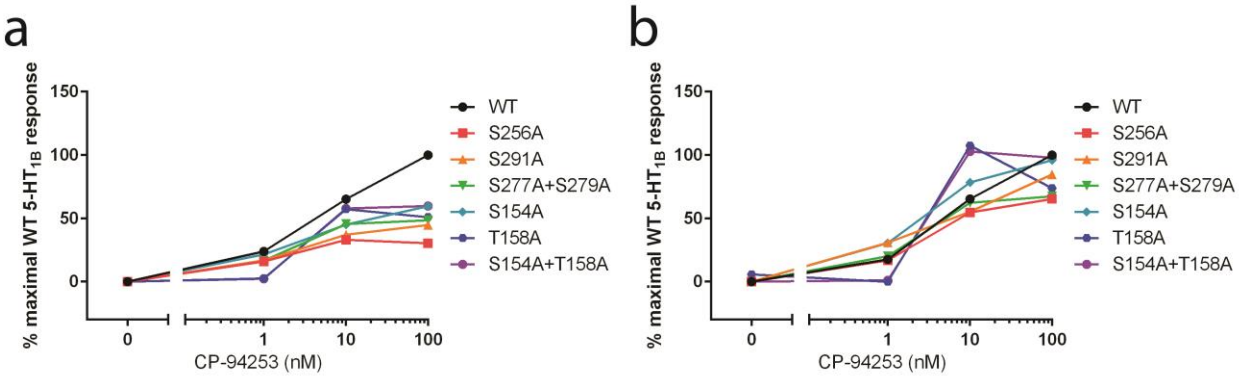


Figure 4.9. Summary of mutant 5-HT_{1B} receptors in activation of phospho-ERK1/2. All mutant 5-HT_{1B} receptors examined showed decreased phospho-ERK1 levels following CP-94253 treatment compared to wild type 5-HT_{1B} receptors (a), but no change in phospho-ERK2 levels (b).

Discussion

Activation of 5-HT_{1B} receptors in N2A cells induces robust phosphorylation of ERK1/2, corroborating studies performed in CHO cells (Mendez et al 1999), but 5-HT_{1B} receptors do not activate p38, p54 JNK, or p46 JNK, which are other MAP kinases in the same family. The use of N2A cells, which endogenously express several key serotonergic and neuronal genes, increases the likelihood that the conclusions drawn from cell culture experiments will be physiologically relevant since many of the same proteins are present and can interact in these cells in the same way as they do in the brain. One major limitation of using N2A cells is the inability to use the endogenous agonist 5-HT to study 5-HT_{1B} receptor signaling selectively due to the demonstrated presence of 5-HT_{1A} receptors and possible presence of others. CP-94253 may potentially be a biased agonist at the 5-HT_{1B} receptor, which would not be able to be

detected in the N2A cell system; however, 5-HT_{1B} receptors tend to be relatively unbiased for a number of other ligands, in contrast to the 5-HT_{2B} receptor, which was shown to have β-arrestin-biased signaling for multiple agonists (Wacker et al 2013).

5-HT_{1B}-mediated phosphorylation of ERK1/2 occurs through the activation of a protein cascade. Inhibiting G protein signaling with pertussis toxin results in approximately 40% blockade of both phospho-ERK1 and phospho-ERK2 at ten minutes, suggesting that there may be G protein-dependent as well as G protein-independent signaling involved. Inhibition of Src family kinases with PP 1 also results in a 40% decrease in phospho-ERK1/2. In contrast, U0126, an inhibitor of MEK1/2, which directly phosphorylates ERK1/2, completely blocks phosphorylation of ERK1/2 in a dose-dependent manner. Gβγ-dependent signaling was examined with the small molecule gallein, which binds to an interaction hotspot on $G\beta_1\gamma_2$ (Lehmann et al 2008); gallein had no effect on agonist-induced phosphorylation of ERK1/2 in N2A-1B cells. This finding suggests that the Gβγ subunit may not be involved in 5-HT_{1B}-mediated phosphorylation of ERK1/2; however, a closely related small molecule inhibitor of Gβγ, M119, was shown to inhibit some aspects of Gβγ-dependent signaling, including PLCβ2 and PI3Kγ, but not ERK1/2 phosphorylation (Bonacci et al 2006). This calls for closer scrutiny with the use of gallein since it may inhibit only some subtypes or combinations of Gβ and Gγ subunits, as well as being unable to inhibit downstream effects if other, distinct interaction domains on the Gβγ are involved instead. Additionally, receptors that signal through rearrangement, rather than dissociation, of the Gα and Gβγ subunits (Bunemann et al 2003, Frank et al 2005) may not be sensitive to small molecules that must directly bind to Gβγ in order to inhibit downstream processes. Treatment with GW 5074 also did not block phosphorylation of ERK1/2; this may be because GW 5074 inhibits c-Raf, whereas another isoform of Raf kinase,

such as B-Raf may be involved in this pathway. Finally, inhibition of internalization with the dynamin inhibitor dynasore at a moderate dose results in an increase in phospho-ERK1/2. This suggests that 5-HT_{1B} receptor internalization serves as a mechanism for desensitization and cessation of downstream signaling to phospho-ERK1/2, which, when blocked, preserves 5-HT_{1B} receptor expression at the cell surface, thereby increasing signaling; this is in contrast to some GPCRs which are internalized and signal through G protein-independent pathways from endosomes (Irannejad & von Zastrow 2014).

5-HT_{1B} receptors are known to be phosphorylated at serine-154 and threonine-158 in the second intracellular loop, and these sites involved in phosphorylation by GSK3β are important for 5-HT_{1B}-mediated reduction in forskolin-stimulated cAMP production (Chen et al 2009). We investigated the importance of these two sites as well as four novel putative phosphorylation sites in the third intracellular loop, serine-256, serine-291, serine-277, and serine-279, on downstream signaling through the ERK1/2 pathway. In all cases, mutations of these phosphorylation sites to alanine resulted in reduced activation of ERK1; in the S256A mutant, phospho-ERK1 levels were reduced by 70% compared to wild type 5-HT_{1B} receptors. The GSK3β phosphorylation sites were also found to reduce phospho-ERK1 levels, implying that GSK3β phosphorylation of 5-HT_{1B} receptors is also important for signaling through ERK. These six amino acids may be sites phosphorylated by GRKs to place "barcodes" on the 5-HT_{1B} receptor to direct downstream signaling; further work investigating the β-arrestin component of the signaling pathway is necessary. Surprisingly, the mutant 5-HT_{1B} receptors showed reduced phospho-ERK1 but not phospho-ERK2 levels. It is possible that 100 nM CP-94253 does not result in maximal activation of ERK2, and higher concentrations of the agonist must be used to detect differences;

indeed, in the case of phospho-ERK1, low doses of CP-94253 resulted in similar levels of induction in both wild type and mutant receptors.

Although 5-HT_{1B} receptors are known to reduce cAMP through $G\alpha_{i/o}$ coupling, little has been known about other downstream effectors of 5-HT_{1B} receptor activation. These results shed light on the ERK1/2 pathway in a neuronal cell line and identify three novel phosphorylation sites that are important for signaling.

CONCLUSIONS

Disturbances of the serotonergic system are implicated in disorders including 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. Understanding the mechanisms by which 5-HT_{1B} autoreceptors regulate this system will shed light on the underlying pathophysiology of various psychiatric disorders and may provide novel therapeutic targets for future treatments of various psychiatric conditions.

In Chapter 1, I described and characterized two novel viral vectors that are uniquely equipped to target 5-HT_{1B} autoreceptors. The SERT-1B virus is ideal for expressing 5-HT_{1B} autoreceptors in the nerve terminals throughout the entire brain, while the AAV8-DIO-1B virus can be used in conjunction with a retrograde virus, such as CAV-Cre, or transgenic mouse lines expressing Cre, to selectively target particular brain circuits. These methods represent a huge leap over traditional pharmacological methods, which were unable to distinguish 5-HT_{1B} autoreceptors from 5-HT_{1B} heteroreceptors.

In Chapter 2, I took advantage of these new tools in both wild type and 5-HT_{1B} knockout mice to investigate the contribution of 5-HT_{1B} autoreceptors to fear expression in contextual fear conditioning, one of many behaviors known to be affected by 5-HT_{1B} receptor function. By injecting SERT-1B into the caudal DRN, the 5-HT_{1B} transgene is expressed selectively in serotonergic neurons and trafficked to nerve terminals throughout the entire brain. This study was the first to establish that 5-HT_{1B} autoreceptors alone are sufficient for mediating a reduction in fear expression, as evidenced by SERT-1B viral injections into constitutive 5-HT_{1B} knockout mice. Examining the role of 5-HT_{1B} receptors from the DRN to amygdala in fear conditioning

by combining AAV8-DIO-1B infusions into the DRN with CAV-Cre infusions into the amygdala was also the first instance of studying 5-HT_{1B} autoreceptors in an isolated circuit. Interestingly, these experiments revealed an increase in fear expression following 5-HT_{1B} overexpression in this circuit in wild type mice. Due to the heterogeneity of both the DRN and the amygdala, further anatomical studies are vital to parsing out the complex connections and relationships between these two regions.

In Chapter 3, I explored quantitative proteomic methods as an unbiased way of seeking novel interactors and pathway proteins following 5-HT_{1B} receptor activation. Although no targets have yet been validated as a result of this new approach, our pilot studies identified some interesting targets and repeating the experiments in the future after optimizing key variables could yield exciting new discoveries. The phosphoproteomic method did, however, reveal several novel putative phosphorylation sites on the 5-HT_{1B} receptor, which were a focus of investigation in the subsequent chapter.

In Chapter 4, I examined the signaling pathway leading to phosphorylation of ERK1/2 following 5-HT_{1B} receptor activation. We found that 5-HT_{1B} receptors signal through G proteins, Src kinase, and MEK1/2 to activate phospho-ERK1/2. Additionally, several amino acid residues in the 5-HT_{1B} receptor were shown to be important for activating this pathway: the novel identification of serine-256, serine-277, serine-279, and serine-291 in the third intracellular loop as phosphorylation sites, and the previously characterized sites important for GSK3β phosphorylation, serine-154 and threonine-158, in the second intracellular loop (Chen et al 2009). Although amino acids in the 5-HT_{1B} receptor sequence important for G protein activity and reduction in cAMP levels have previously been identified (Chen et al 2009, Wacker et al 2013),

this is the first time that specific residues have been investigated for another signal transduction pathway.

These molecular and behavioral results offer a cohesive hypothesis of 5-HT_{1B} autoreceptor function. Upon binding of 5-HT or another agonist, 5-HT_{1B} receptors are phosphorylated at several possible sites in the second and third intracellular loops. 5-HT_{1B} receptor activation leads to phosphorylation of ERK1/2 and increased 5-HT reuptake through SERT, which decreases synaptic 5-HT levels. When 5-HT_{1B} autoreceptors are overexpressed in nerve terminals throughout the entire brain, a reduction in fear expression following contextual fear conditioning is observed; in contrast, when 5-HT_{1B} receptors are overexpressed only in the DRN-to-amygdala circuit, an increase in fear is seen, suggesting that serotonergic signaling in different brain regions may play opposing roles in modulating a single behavior.

Further molecular studies are necessary, particularly to investigate whether β-arrestins are involved in 5-HT_{1B}-mediated phosphorylation of ERK1/2 using short interfering RNAs (siRNAs) to knock down β-arrestin expression. Additionally, because the mutant 5-HT_{1B} receptors show a reduced level rather than a complete absence of phospho-ERK signaling, it is possible that blocking phosphorylation sites on the 5-HT_{1B} receptor interferes with a subset of signaling mechanisms; this can be further examined by using pertussis toxin and β-arrestin siRNAs to distinguish the G protein-dependent and β-arrestin dependent signaling pathways. It would also be of interest to introduce mutations from serine/threonine at the 5-HT_{1B} receptor phosphorylation sites to aspartic acid, which mimics the phosphorylated state, and look into whether the new mutants are more easily activated by 5-HT or constitutively active. If the new mutant 5-HT_{1B} receptors are more likely to activate downstream signaling pathways in the

presence of an agonist, introduction of the mutant receptor into the brain via viral-mediated gene transfer could lead to even more dramatic behavioral results in contextual fear conditioning.

5-HT_{1B} autoreceptors facilitate resilience in a broad range of emotional behaviors, including fear, anxiety, depression, and stress sensitivity. There is a significant knowledge gap regarding the viability of 5-HT_{1B} receptors as a therapeutic target; thus, a greater understanding of its precise functions are absolutely vital, especially for patients who are treatment-resistant or only partial responders to available medications. The results reported here provide clues both for behavioral consequences of 5-HT_{1B} autoreceptor expression, and for molecular mechanisms of 5-HT_{1B} receptor activation.

APPENDICES

Appendix A. 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-HT_{1B}	CACCCTTCTTCTGGCGTCAA	GAGAGCGGGCTTCCACATAG
5-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

Appendix B. Primers used for site-directed mutagenesis of the wild-type 5-HT_{1B} receptor.

Mutant	Forward primer (5' to 3')	Reverse primer (5' to 3')
S256A	CAGTTGATAACAGACGCTCCAG GATCCACG	CGTGGATCCTGGAGCGTCTGTT ATCAACTG
S291A	GTCAAAGTGCGAGTCGCAGAC GCCCTGCTGG	CCAGCAGGGCGTCTGCGACTCG CACTTTGAC
S277A+S279A	AGGTGCCCAGTGAGGCCGGAG CTCCTGTGTACGTGAA	TTCACGTACACAGGAGCTCCGG CCTCACTGGGCACCT
S154A	CACTGATGCGGTGGACTATGCT GCTAAAAGAACTCCCAA	TTGGGAGTTCTTTTAGCAGCAT AGTCCACCGCATCAGTG
T158A	TGGACTATTCTGCTAAAAGAGC TCCCAAAAGGGCGGCCATC	GATGGCCGCCCTTTTGGGAGCT CTTTTAGCAGAATAGTCCA
S154A+T158A	TGATGCGGTGGACTATGCTGCT AAAAGAGCTCCCAAAAGGGCG GC	GCCGCCCTTTTGGGAGCTCTTTT AGCAGCATAGTCCACCGCATCA

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- Investigated role of p38α MAPK pathway in the development of astrocytosis and neuropathic pain behaviors following partial sciatic nerve ligation using conditional knockout mice.

University of Washington, Department of Psychology, Seattle, WA Jun – Aug 2008
Summer Research Rotation, Advisor: Dr. Jeansok J. Kim
- Examined role of thalamo-cortico-amygdala pathway in auditory fear conditioning in rats and the behavioral effects of varying probability of shock in the presence and absence of a conditioned stimulus.

Duke University, Department of Psychological and Brain Sciences, Durham, NC Sep 2007 – May 2008
Independent Study Student, Advisor: Dr. Nestor Schmajuk
- Utilized a neural network approach to model behavioral data on extinction in single versus multiple contexts.

Duke University Medical Center, Department of Hematology, Durham, NC May 2005 – May 2008
Research Assistant, Advisor: Dr. Charles S. Greenberg
- Conducted independent research on pharmacological inhibitors of tissue transglutaminase.
- Developed novel high-throughput fluorescence assay to measure enzymatic activity.
- Characterized two alternatively spliced isoforms of tissue transglutaminase.

HONORS AND AWARDS

University of Washington NIH Institutional Grant for Neurobiology Fellowship (2011-2014)
Serotonin Club Young Investigator Travel Award (2012)
Western Regional Meeting Student Subspecialty Award (2010)
Alexander Grinstein Endowed Fellowship (2009)
Corser Endowed Fund Fellowship (2008)
Psi Chi National Honor Society in Psychology (2007)
Order of Omega Greek Leadership Honor Society (2007)
Donald A. King Student Fellowship, Huntington's Disease Society of America (2005)
National Merit Scholarship Recipient (2004)
Rensselaer Medal (2003)

PUBLICATIONS

Nair SG, Furay AR, Liu Y, and Neumaier JF (2013). Differential effect of viral overexpression of nucleus accumbens shell 5-HT_{1B} receptors on stress- and cocaine priming-induced reinstatement of cocaine seeking. *Pharmacol Biochem Behav* 112C:89-95.

Hagan CE, McDevitt RA, Liu Y, Furay AR, and Neumaier JF (2012). 5-HT_{1B} autoreceptor regulation of serotonin transporter activity in synaptosomes. *Synapse* 66(12):1024-34.

Lai TS, Liu Y, Tucker T, Daniel KR, Sane DC, Toone E, Burke JR, Strittmatter WJ, and Greenberg CS (2008). Identification of chemical inhibitors to human tissue transglutaminase by screening existing drug libraries. *Chem Biol* 15(9):969-78.

Lai TS, Liu Y, Li W, and Greenberg CS (2007). Identification of two GTP-independent alternatively spliced forms of tissue transglutaminase in human leukocytes, vascular smooth muscle and endothelial cells. *FASEB J* 21(14):4131-43.

ORAL PRESENTATIONS

Liu Y (June 2014). Role of 5-HT_{1B} autoreceptors in the expression of conditioned fear. University of Washington Graduate Program in Neurobiology and Behavior Student Symposium, Seattle, WA.

Liu Y (July 2012). Regulation of the serotonin transporter by 5-HT_{1B} autoreceptors. 10th Meeting of the International Society for Serotonin Research, Montpellier, France.

Liu Y (July 2011). 5-HT_{1B} autoreceptors in SERT regulation and fear behaviors. Stress in Seattle Conference, Seattle, WA.

Liu Y (January 2010). Mechanisms underlying astrocytosis following sciatic nerve injury. Western Student Medical Research Forum, Carmel, CA.

ABSTRACTS AND POSTER SESSIONS

Neumaier JF & Liu Y (2014). 5-HT_{1B} receptors activate Erk1/2 in a neuronal cell line. 11th Meeting of the International Society for Serotonin Research, Western Cape, South Africa.

Alvarado CG, Liu Y, Neumaier JF, Montine TJ, Hagan CE (2013). Effects of serotonin transporter deletion on microglial function. Abstracts of the 43rd Annual Meeting of the Society for Neuroscience.

Liu Y & Neumaier JF (2012). 5-HT_{1B} autoreceptors modulate the expression of conditioned fear. Abstracts of the 42nd Annual Meeting of the Society for Neuroscience.

Liu Y, Hagan CE, Neumaier JF (2012). Regulation of the serotonin transporter by 5-HT_{1B} autoreceptors. University of Washington Medical Scientist Training Program Retreat 2012, Leavenworth, WA.

Liu Y & Neumaier JF (2011). Identifying the selective contribution of 5-HT_{1B} autoreceptors to conditioned fear behaviors using cell type-specific viral-mediated gene transfer. Abstracts of the 41st Annual Meeting of the Society for Neuroscience.

Liu Y, Xu M, Chavkin C (2010). Mechanisms underlying astrocytosis following sciatic nerve injury. *J Invest Med* 58(1):108-109. Abstracts of the Western Regional Meeting.

Lai TS, Liu Y, Lee HY, Toone E, Greenberg CS (2006). Human tissue transglutaminase activity is inhibited by protein kinase inhibitors: Potential therapeutic implication for hematological disorders. *Blood* 108(11):461A-462A. Abstracts of the Annual Meeting of the American Society of Hematology.

Lai TS, Liu Y, Yen P, Dorismond V, Greenberg CS (2005). Enhanced expression of two novel GTP-independent alternatively spliced forms of tissue transglutaminase in human platelets and leukocytes. *Blood* 106(11):630A-630A. Abstracts of the Annual Meeting of the American Society of Hematology.