

# 5-HT<sub>7</sub> Receptor Neuroprotection against Excitotoxicity in the Hippocampus

by

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## **AUTHOR'S DECLARATION**

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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## Abstract

**Introduction and Objectives:** The PDGF $\beta$  receptor and its ligand, PDGF-BB, are expressed throughout the central nervous system (CNS), including the hippocampus. Several reports confirm that PDGF $\beta$  receptors are neuroprotective against N-methyl-D-aspartate (NMDA)-induced cell death in hippocampal neurons. NMDA receptor dysfunction is important for the expression of many symptoms of mental health disorders such as schizophrenia.

The serotonin (5-HT) type 7 receptor was the most recent of the 5-HT receptor family to be identified and cloned. 5-HT receptors interact with several signaling systems in the CNS including receptors activated by the excitatory neurotransmitter glutamate such as the NMDA receptor. Although there is extensive interest in targeting the 5-HT7 receptor with novel therapeutic compounds, the function and signaling properties of 5-HT7 receptors in neurons remains poorly characterized.

**Methods:** The SH-SY5Y neuroblastoma cell line, primary hippocampal cultures, and hippocampal slices were treated with 5-HT7 receptor agonists and antagonists. Western blotting was used to measure PDGF $\beta$  receptor expression and phosphorylation as well as NMDA receptor subunit expression and phosphorylation levels. Real-time RT-PCR was used to measure mRNA level of PDGF $\beta$  receptor in neuronal cultures. Cell death assays (MAP2, MTT) were used to measure the neuroprotective effects of 5-HT7 and PDGF $\beta$  receptor activation.

**Results:** My research involved elucidating the molecular mechanisms of neuroprotection after 5-HT7-induced PDGF $\beta$  receptor upregulation. I demonstrated that 24 h treatment with the selective 5-HT7 receptor agonist, LP 12, increased not only the expression but also the activation of PDGF $\beta$  receptors as measured by the phosphorylation of tyrosine 1021, the phospholipase C $\gamma$  binding site. Activation of the 5-HT7 receptor also selectively changed the expression and phosphorylation state of the NR2B

subunit of the NMDA receptor. Activation of 5-HT<sub>7</sub> receptors was neuroprotective against NMDA-induced toxicity in primary hippocampal neurons and this effect required PDGF $\beta$  receptor kinase activity. Thus, long-term (24 h) activation of 5-HT<sub>7</sub> receptors was neuroprotective via increasing the expression of a negative regulator of NMDA activity, the PDGF $\beta$  receptor. In contrast, acute activation (5-30 min) of 5-HT<sub>7</sub> receptor increased NMDA evoked current and altered NMDA receptor subunit phosphorylation in hippocampal neurons in a manner that was different from what we observed in our 24 h experiments.

Conclusions: I identified two 5-HT<sub>7</sub> receptor to NMDA receptor pathways: acute activation of the receptor increased NMDA-evoked currents whereas long-term 5-HT<sub>7</sub> agonist treatment prevented NMDA-induced excitotoxicity in a PDGF $\beta$  receptor-dependent manner. This research is significant in the ongoing advances for the treatment of mental health disorders, such as schizophrenia and depression, that involve the 5-HT, glutamate, and neuronal growth factor systems.



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## **Dedication**

This thesis is dedicated to my husband, Mahdi, my son, Parsa, and my daughter, Sara, who have always stood by me!

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## List of Abbreviations

5-CT, 5-carboxamidotryptamine  
5-HT; 5-hydroxytryptamine or serotonin  
AC, adenylyl cyclases  
sAHP, afterhyperpolarization  
AMPA,  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid  
CaMKII, Ca<sup>2+</sup>-calmoduline-dependent protein kinase II  
CDK5, cycline-dependent kinase 5  
CNS, central nervous system  
CREB, cAMP response element binding protein  
DA, dopamine  
DAG, diacyl-glycerol  
DRN; dorsal Raphe nucleus  
ERK, extracellular signal-regulated kinases  
EPSPs, excitatory postsynaptic potentials  
GAP, GTPase-activating protein  
GPCR, G protein-coupled receptor  
iGluRs, ionotropic glutamate receptors  
LTP, long-term potentiation  
NE, norepinephrine  
NMDA, N-methyl-D-aspartate  
mGluRs, metabotropic glutamate receptors  
PDGF, platelet-derived growth factor  
PI3-kinase, phosphatidylinositol 3-kinase  
PIP2, phosphatidylinositol 4,5-bisphosphate  
PKA, protein kinase A  
PLC, phospholipase C  
RTKs, receptor tyrosine kinases  
SCN, suprachiasmatic nucleus  
SSRIs, selective serotonin reuptake inhibitors  
TCAs, Tricyclic antidepressants

# Chapter 1

## General Introduction

The most complex organ in the body is the brain, whose complexity and processing depends on the interactions between billions of neurons. The most common method of communication between brain cells occurs through the actions of transmembrane receptors and neurotransmitters (organic chemicals released from neurons). Transmembrane protein receptors are activated upon binding with a chemical signal or ligand. The activation of a receptor by a ligand changes intracellular chemical levels (second messengers) that initiate several signaling pathways, which collectively result in changes in cellular responses. There are three main classes of transmembrane receptors: ion channels, G protein-coupled receptors (GPCRs), and receptor tyrosine kinases (Hazell *et al.* 2012).

GPCRs are one of the largest classes of cell surface receptors (Hino *et al.* 2012) and contain seven putative hydrophobic transmembrane domains connected by three intracellular loops and three extracellular loops (Raymond *et al.* 2001). The extracellular GPCR domains consist of glycosylated amino termini that contain cysteine residues while the intracellular carboxyl termini contain sites for G-protein and regulatory proteins interaction as well as phosphorylation sites recognized by serine-threonine kinases (Raymond *et al.* 2001). G proteins, or guanosine nucleotide-binding proteins, are able to bind both guanosine triphosphate (GTP) and guanosine diphosphate (GDP) and possess intrinsic GTPase activity that slowly hydrolyzes bound GTP to GDP (Brown and Sihra 2008). In the inactive state, GDP is bound to the  $\alpha$  subunit, and the  $\alpha$  subunit is associated with  $\beta$  and  $\gamma$  subunits (forming a “heterotrimeric” G protein) as well as the GPCR. The binding of an agonist to the GPCR results in a conformational change that stimulates the  $\alpha$  subunit to exchange GDP for GTP.  $G\alpha$ -GTP then dissociates from both the receptor and the  $G\beta\gamma$  subunits, and both  $G\alpha$ -GTP and  $G\beta\gamma$  are then able to interact with effector proteins

(Peng *et al.* 2003). G protein effectors include adenylyl cyclases (AC), phospholipase (PLC),  $\text{Ca}^{2+}$  channels, and several others. The intrinsic GTPase activity of the  $\alpha$  subunit hydrolyzes GTP to GDP, and the  $\alpha$  subunit reassembles with the  $\beta\gamma$  subunits to reform the heterotrimer and to reassociate with the GPCR (Rasmussen and Haberman 2011). There are three major types of  $G\alpha$  subunits that differentially affect second messengers and signaling pathways:  $G\alpha_s$  (stimulates AC),  $G\alpha_{i/o}$  (inhibits AC), and  $G\alpha_q$  (activates  $\text{PLC}\beta$ ).

### 1.1 Serotonin receptors

Rapport *et al.* discovered serotonin (5-HT, 5-hydroxytryptamine) in 1948 (Rapport *et al.* 1948; Raymond *et al.* 2001). 5-HT plays important roles in the central nervous system (CNS) with respect to the regulation of a variety of functions including sleep (Markov and Goldman 2006) and pain (Butkevich *et al.* 2005). The number of different physiological roles for 5-HT can be explained based on its wide localization in the CNS and the variety of its receptor subtypes and isoforms (5-HT1 to 5-HT7 receptors, many having multiple isoforms) (Hoyer *et al.* 2002).

Because 5-HT is one of the most important neurotransmitters in the mammalian CNS, its dysregulation is associated with a number of mental health diseases such as schizophrenia and depression. Serotonin is produced by neurons in the raphe nuclei and secreted from serotonergic nerve terminals. Serotonin receptors play an important role in brain development, and 5-HT signaling affects synaptic plasticity and neurogenesis in both the developing and adult brain. All seven subtypes of 5-HT receptors are expressed in the CNS (Meltzer *et al.* 2003; Millan 2006; Raymond *et al.* 2001; Bockaert *et al.* 2006). The 5-HT receptor subfamilies overlap with respect to their pharmacological properties, amino acid sequences, and the second messenger signaling pathways they initiate (Hoyer *et al.* 1994). Most 5-HT receptors are GPCRs, with the exception of 5-HT3, which is an ionotropic receptor (Martinowich and Lu 2008). 5HT1 and 5HT5 receptors are  $G\alpha_{i/o}$ -coupled receptors that typically inhibit adenylyl cyclase (AC); 5HT4, 6, and 7 receptors

are  $G\alpha_s$ -coupled receptors that typically stimulate AC. Activation of AC leads to the elevation of cAMP production, resulting in protein kinase A (PKA) activation. Phospholipase C (PLC)  $\beta$  is activated by a  $G\alpha_q$ -coupled 5-HT<sub>2</sub> receptor whereas the 5-HT<sub>3</sub> receptor is a ligand-gated ion channel (Millan *et al.* 2008). PLC activation results in the accumulation of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to produce IP<sub>3</sub> and diacyl-glycerol (DAG). Generation of IP<sub>3</sub> increases intracellular Ca<sup>2+</sup> levels and DAG activates Ca<sup>2+</sup> and phospholipid-dependent protein kinase (PKC) (Raymond *et al.* 2001).

### **1.1.1 5-HT<sub>7</sub> receptor function**

The most recently discovered 5-HT receptor is the 5-HT type 7 receptor. At the time of its discovery in 1993, selective ligands had not yet been developed (Hedlund 2009). After the discovery of selective agonists such as AS-19 (Meltzer *et al.* 2011), it became possible to investigate the function and localization of 5-HT<sub>7</sub> receptors (Ikeda *et al.* 2006; Thomas and Hagan 2004).

5-HT affects a variety of membrane-bound receptors, and its receptors are expressed in both the central and peripheral nervous system as well as in non-neuronal tissue. 5-HT<sub>7</sub> receptors are expressed in smooth muscle and promote relaxation within the vasculature in the gastrointestinal tract. The highest 5-HT<sub>7</sub> receptor densities are found in both neurons and support cells in the thalamus, the hypothalamus, the suprachiasmatic nucleus, the prefrontal cortex, the hippocampus, and the amygdala (Mahe *et al.* 2004b; Thomas and Hagan 2004). Based on its initial identification in the suprachiasmatic nucleus, one of the first roles proposed for 5-HT<sub>7</sub> was the regulation of sleep/wake cycles (Lovenberg *et al.* 1993).

5-HT<sub>7</sub> receptors are  $G\alpha_s$ -coupled receptors; their activation leads to an increase both in adenylyl cyclase activity and in the intracellular level of cAMP, a second messenger. Activation of the 5-HT<sub>7</sub> receptor also increased intracellular Ca<sup>2+</sup>-stimulated AC in the rat cerebral cortex and

hippocampus (Mons *et al.* 1998; Mork and Geisler 1990), and this effect was independent of PKC (Baker *et al.* 1998). The 5-HT<sub>7</sub> receptors endogenously expressed by cultured hippocampal neurons in rat also stimulated MAP kinase (ERK1/2), and this effect was not sensitive to pertussis toxin, indicating that G $\alpha_{i/o}$ -proteins are not involved in this pathway (Errico *et al.* 2001).

**Table 1.1 5-HT receptor subtypes and the CNS**

Family	Distribution	Type	Mechanism	Response
5-HT <sub>1</sub>	Blood Vessels  CNS	Gi/Go -protein coupled	Decreasing cellular levels of cAMP	Inhibitory
5-HT <sub>2</sub>	Blood Vessels  CNS GI Tract Platelets PNS Smooth Muscle	Gq/G11 protein coupled	Increasing cellular levels of cAMP	Excitatory
5-HT <sub>3</sub>	CNS  GI Tract PNS	Ligand-gated Na <sup>+</sup> and K <sup>+</sup> cation channel	Depolarizing plasma membrane	Excitatory
5-HT <sub>4</sub>	CNS  GI Tract PNS	Gs -protein coupled	Increasing cellular levels of cAMP	Excitatory
5-HT <sub>5</sub>	CNS	Gi/Go -protein coupled	Decreasing cellular levels of cAMP	Inhibitory
5-HT <sub>6</sub>	CNS	Gs -protein coupled	Increasing cellular levels of cAMP	Excitatory
5-HT <sub>7</sub>	Blood Vessels  CNS GI Tract	Gs -protein coupled	Increasing cellular levels of cAMP	Excitatory

Seven 5-HT receptor subtypes are expressed in the CNS, each with its own unique signaling and functional properties. Activation of GPCRs stimulates intracellular second messenger cascades.

### 1.1.2 5-HT7 receptor variants

In humans, the serotonin receptor encoded by the HTR7 gene is located on chromosome 10 (10q21-q24) (Gelernter *et al.* 1995) and possesses three exons separated by two introns (found in the second internal loop and in the intracellular carboxyl terminal) (Heidmann *et al.* 1997; Shen *et al.* 1993; Ruat *et al.* 1993), which are transcribed into three different splice variants (Raymond *et al.* 2001; Heidmann *et al.* 1997). Four major 5-HT7 receptor splice variants have been identified in mammals (5-HT7a-d) (Heidmann *et al.* 1997; Jasper *et al.* 1997; Stam *et al.* 1997), of which humans express three: 5-HT7a/b/d (Heidmann *et al.* 1997). In humans, the 5-HT7a and 5-HT7b receptors are in the major isoforms (Heidmann *et al.* 1997). The 5-HT7a is a full-length receptor (445 aa), while 5-HT7b contains 432 aa, which is truncated by 13 aa because of the introduction of an in-frame stop codon. The 5-HT7c and 5-HT7d receptors contain 479 aa due to the retention of an extra exon (exon C in rat and exon D in humans, which are located in the second intron) that results in a longer carboxyl terminal end (35 aa in rat and 47 aa in human) (Krobert and Levy 2002; Heidmann *et al.* 1997; De Martelaere *et al.* 2007). Although the level of expression differs between isoforms, no significant functional differences, such as signal transduction and distribution, have yet been observed (Krobert *et al.* 2001).

PDZ proteins interact with the C terminal end of GPCRs and contribute to the signal transduction properties and trafficking of GPCRs (Chetty *et al.* 2009). Notably, the 5-HT7a and 5-HT7d receptors do not contain PDZ motifs, whereas 5-HT7b and 5-HT7c receptors do contain PDZ motifs (Heidmann *et al.* 1998). The PDZ domain contains approximately 90 aa and helps to anchor transmembrane proteins to the cytoskeleton and hold together signaling complexes (Ranganathan and Ross 1997).

5-HT7 receptors displayed inverse agonist responses when treated with a number of antagonists (Romero *et al.* 2006), and they were rapidly desensitized in recombinant systems by both agonists

and antagonists (Krobert *et al.* 2006). As measured by 5-HT-stimulated cAMP accumulation in 5-HT7 receptor-expressing cells, several antipsychotic agents with affinities for the 5-HT7 receptor, including risperidone, appeared to “inactivate” 5-HT7 receptors after brief exposure to these agents (Toohey *et al.* 2009; Vasefi *et al.* 2012). Toohey *et al.* (2009) hypothesized that these neuroleptics induced a stable 5-HT7 conformation that prevents the receptor activation of  $G\alpha_s$ .

Although 5-HT7 receptors have been identified as  $G\alpha_s$ -coupled in HEK293 and NIH3T3 cells, there is some evidence that 5-HT7 receptors may also couple to  $G\alpha_{12}$  (Kvachnina *et al.* 2005).

The pharmacology of 5-HT7 receptors involves several unique aspects, including very high basal activity and rapid desensitization and downregulation by agonists. Additionally, 5-HT7 receptors can be inactivated by antagonists, including therapeutically useful antipsychotic medications (Krobert and Levy 2002; Mahe *et al.* 2004b). Serotonin receptors interact with several other signaling systems in the CNS, including the excitatory neurotransmitter glutamate. Clinical data, animal studies, electrophysiological data, and biochemical data have revealed several points of crosstalk between 5-HT receptors and glutamate pathways.

### **1.1.3 Schizophrenia**

#### **1.1.3.1 Definition**

Schizophrenia is a chronic and severe brain disorder that affects about 1 % of the population worldwide (Huang *et al.* 2012). Its symptoms can be divided into three categories: (A) positive symptoms that are not normally observed in patients, such as hallucinations; (B) symptoms involving loss of normal functioning, such as emotional flattening; (C) cognitive symptoms, such as disordered thinking (Kay *et al.* 1987; Huang *et al.* 2012).

Several neuroimaging techniques have revealed that schizophrenia is characterized by abnormal brain structure and atypical responses to cognitive tasks (Vita *et al.* 2006; Glahn *et al.* 2008;

Ellison-Wright and Bullmore 2009). Schizophrenia is described as a neurodegenerative disease that derives from neurodevelopmental deficits. Evidence suggests a disturbance of glutamatergic neurotransmission and especially NMDA receptor-mediated signaling in schizophrenia patients (Fatemi and Folsom 2009; Belforte *et al.* 2010). The psychotomimetic agents ketamine and phencyclidine (PCP) induce symptoms of schizophrenia by blocking NMDA receptor activity (Javitt and Zukin 1991; Goff and Coyle 2001; Arguello and Gogos 2006), and NMDA receptor antagonists induce schizophrenic behaviour in rodents (Goff and Coyle 2001; Arguello and Gogos 2006; Gainetdinov *et al.* 2001). Genetic and pharmacological studies show that glutamatergic dysfunction, especially with the involvement of NMDA receptors, is a significant factor in the abnormalities seen in schizophrenia (Aida *et al.* 2012). Ketamine and PCP do not cause neurotoxic effects in rats until the rats reach puberty (Stone *et al.* 2007), possibly because neuronal connections involved in the neurotoxicity of NMDA receptor antagonists are not fully developed until late adolescence (Olney *et al.* 1999; Stone *et al.* 2007). This evidence supports the current view of schizophrenia as a disorder that likely begins in early development but does not manifest until late adolescence or early adulthood (Murray and Lewis 1987; Stone *et al.* 2007). Glutamate levels are notably higher in the cortex of schizophrenic patients (Olbrich *et al.* 2008). Goto *et al.* (2012) reported that treatment with atypical antipsychotic drugs reduces glutamate levels in early-stage schizophrenia (Goto *et al.* 2012). The levels of cortical glutamate released and the expression of NMDA receptors would be expected to change depending on the stage of the illness (Stone *et al.* 2007).

### **1.1.3.2 Risk factors**

One of the strongest factors contributing to the development of schizophrenia is genetics (Owen *et al.* 2005). Molecular genetic studies of schizophrenic patients indicated that specific genes may increase the risk of developing the disease (Harrison and Owen 2003). Other researchers suggested that deletions or duplication of DNA sequences within genes is linked to schizophrenia



(Walsh *et al.* 2008; Xu *et al.* 2008). However, fetal environmental factors, such as maternal infection and stress (Brown 2006), as well as childhood environmental factors may also affect the incidence of schizophrenia (McGrath *et al.* 2008; March *et al.* 2008).

### **1.1.3.3 Treatment**

Antipsychotic drugs that block D2-class dopamine receptors are the primary treatment for schizophrenia (Kapur and Mamo 2003). They are categorized as two classes: typical and atypical (van Os and Kapur 2009). Typical antipsychotics such as chlorpromazine, also called first-generation antipsychotics, block D2-receptors with very high affinity (Seeman 2006). Typical antipsychotics reduce positive symptoms of schizophrenia such as delusions but also cause several adverse effects such as pseudo-Parkinsonism. Atypical antipsychotics, known as second-generation antipsychotics, such as risperidone, are effective for treating both positive and negative symptoms and result in fewer side effects than do typical antipsychotics (Widschwendter and Fleischhacker 2005; van Os and Kapur 2009). Despite the benefits of atypical over typical antipsychotics, the successful treatment of schizophrenia remains poor. Improving and increasing the efficacy of this type of treatment with fewer side effects requires the discovery of additional methods of intervening in the pathology of the disease.

### **1.1.4 Depression**

#### **1.1.4.1 Definition**

Depression affects one in six individuals during their lifetime and has an impact on a person's thoughts, emotions, and behaviour (Kessler *et al.* 2005). Diagnostic criteria include a depressed mood for longer than 6 months, abnormal appetite, abnormal sleep patterns, and suicidal thoughts.

#### **1.1.4.2 Risk factors**

For depression related to life events, risk factors include job problems and relationship issues. Non-psychiatric illnesses such as chronic pain and medical therapies such as high blood pressure treatment can cause a depressed mood in a significant number of patients (Saravane *et al.* 2009 ; Shaffery *et al.* 2003). Investigating the pathophysiology of depression is a challenge because symptoms of depression such as guilt and suicidal thoughts are impossible to reproduce in animal models (Krishnan and Nestler 2008). However, there are animal behavioural tests and pharmacological studies that can help with the study of depression. Anatomically, the limbic system is involved in depression, and therapy may exert an influence on that system (Berton and Nestler, 2006). Clinical studies have shown that activity in the prefrontal cortex and amygdala is increased in depressed patients and also in healthy volunteers who are experiencing sadness (Drevets 2001; Ressler and Mayberg 2007). The volume of post-mortem gray matter and the number of cells in the prefrontal cortex are also decreased (Sheline 2003).

#### **1.1.4.3 Treatment**

The primary targets of currently available antidepressant drugs are the monoamine system (Berton and Nestler 2006). Tricyclic antidepressants (TCAs) such as desipramine are used to treat depression. TCAs inhibit 5-HT and/or norepinephrine (NE) and/or dopamine (DA) reuptake by blocking the transport of the neurotransmitter in order to increase their synaptic concentration (Elhwuegi 2004; Holtzheimer and Nemeroff 2006). Monoamine oxidase inhibitors such as phenelzine inhibit monoamine oxidase and keep 5-HT and NE levels elevated by preventing their metabolism (Youdim *et al.* 2006). Selective serotonin reuptake inhibitors (SSRIs) such as citalopram target 5-HT transporters in order to prevent serotonin uptake from the synaptic area (Holtzheimer and Nemeroff 2006).

### 1.1.5 5-HT7 receptors in CNS disease

5-HT7 receptors are implicated in numerous CNS functions, such as cognition (Cifariello *et al.* 2008), learning and memory (Perez-Garcia and Meneses 2005; Liy-Salmeron and Meneses 2007), and sleep (Thomas *et al.* 2003), as well as in specific disease states, including migraine headaches (Agosti 2007), epilepsy (Witkin *et al.* 2007), and anxiety (Wesolowska *et al.* 2006b). The extensive distribution of 5-HT7 receptors in selected brain areas suggests that they are important in learning and memory.

Because of the high affinity of 5-HT7 receptors for several atypical antipsychotics such as clozapine and resperidone as well as for antidepressants such as citalopram, it has been suggested that the 5-HT7 receptor might play a role in mental health diseases and their treatment (Hedlund 2009). Genetic associations between 5-HT7 genes and schizophrenia have also been reported (Ikeda *et al.* 2006). The relationship between 5-HT7 receptors and mental health has provided an incentive for the investigation of 5-HT7 receptor antagonists as pharmaceutical therapies (Mullins *et al.* 1999; Sleight *et al.* 1995).

Traditional neuroleptic drugs have been predominantly D2-class dopamine receptor antagonists (Seeman *et al.* 1987). However, many neuroleptics antagonize several other classes of GPCRs, such as the 5-HT7 receptor (Hedlund 2009). In schizophrenics, 5-HT7 receptor mRNA levels are downregulated in the dorsolateral prefrontal cortex but not in the hippocampus (Hedlund 2009). The 5-HT7 receptor antagonist SB 656104 (Uzun *et al.* 2005; Galici *et al.* 2008) inhibits a number of drug-induced psychosis models (Galici *et al.* 2008) while clozapine, an atypical neuroleptic, regulates 5-HT7 receptor expression in HeLa cells (Zhukovskaya and Neumaier 2000). The pharmacological blockade of 5-HT7 receptors or the deletion of the 5-HT7 receptor gene also leads to antidepressant-like behavioural effects, such as reduced immobility in both a forced swimming test and a tail suspension test in mice (Hedlund 2009; Bonaventure *et al.* 2007;

Wesolowska *et al.* 2006b; Wesolowska *et al.* 2006a). The levels of 5-HT7 receptor mRNA were increased in rat hippocampus after acute stress (Yau *et al.* 2001). Several antidepressants also have an affinity for 5-HT7 receptors (Lucchelli *et al.* 2000; Hedlund 2009), including amisulpiride (Abbas *et al.* 2009).

In addition to possible antipsychotic and antidepressant actions, the selective 5-HT7 receptor antagonist SB 269970 induced an anxiolytic effect in rats and mice (Hagan *et al.* 2000). 5-HT7 receptor antagonists may also reduce pain transmission as SB 269970 increased the number of shocks tolerated by the animal in the Vogel test as well as the number of entries onto open arms and the amount of time spent in those arms but did not produce these effects in knockout mice (Hedlund 2009). Although both pharmacological and genetic studies of animal models support a role for 5-HT7 receptor signaling as a possible avenue for the treatment of mental health diseases, the exact role of 5-HT7 receptor signaling in CNS dysfunction is unknown (Hedlund 2009). Further research will aid in an understanding of the impact of the 5-HT7 receptor signaling on cognitive function and help to elucidate possible uses for treating specific cognitive disorders.

## **1.2 PDGF receptors**

Platelet-derived growth factor (PDGF) initially purified from human platelets (Heldin and Westermark 1999) binds to PDGF receptors (PDGFRs) that are receptor tyrosine kinases (RTKs) and plays a key role in the regulation of cell proliferation, differentiation, and survival (Blume-Jensen and Hunter 2001). RTK activation occurs through growth factor binding and the dimerization of the receptor (Ullrich and Schlessinger 1990; Lemmon and Schlessinger 2010). The binding of growth factors in the extracellular domain of the RTK brings the two receptors into close proximity, stimulates tyrosine kinase activity, and results in multiple phosphorylated tyrosine residues that facilitate several downstream signaling pathways (Clayton *et al.* 2005).

### 1.2.1 PDGF receptor expression and function

PDGF exists as dimers of four polypeptides: PDGF-A, -B, -C, and -D. These either homo-dimerize or hetero-dimerize into PDGF-AB, -AA, -BB, -CC, and -DD. The PDGF isoforms act through PDGF  $\alpha$  and  $\beta$  receptors (Fredriksson *et al.* 2004). The molecular sizes of  $\alpha$  and  $\beta$  receptors are 170 kDa and 180 kDa, respectively (Heldin and Westermark 1999). The  $\alpha$ -receptor binds to either A- or B-chains of PDGF whereas the  $\beta$ -receptor binds to B-chains with high affinity. PDGF receptors are widely expressed throughout the CNS (Valenzuela *et al.* 1997). PDGF $\beta$  receptors are found almost exclusively in pyramidal neurons in the hippocampus (Smits *et al.* 1991; Beazely *et al.* 2009) whereas the PDGF $\alpha$  receptor and its primary ligand, PDGF-AA, are found in non-neuronal cells (Yeh *et al.* 1993; Fruttiger *et al.* 1996; Woodruff *et al.* 2004). Both PDGF $\alpha$  and PDGF $\beta$  are expressed in the hippocampus (Heldin *et al.* 1998).

After PDGF binding to their receptors, the tyrosine kinase domains transphosphorylate one another on multiple tyrosine residues. Effectors such as phospholipase C (PLC)  $\gamma$ , phosphatidylinositol 3-kinase (PI3-kinase), Src family tyrosine kinases, tyrosine phosphatases such as SHP-2, and a GTPase-activating protein (GAP) for Ras are activated by the phosphorylation of the PDGF receptor (Heldin 1992; Heldin and Westermark 1999). Grb2/Sos adaptor proteins become activated after binding directly to the PDGF receptor or indirectly through Shc protein. Grb2/Sos converts inactive Ras-GDP to active Ras-GTP. Upon RTK phosphorylation, Shc binds to the Grb2/Sos complex and activates the Ras-MAPK signaling pathway. Activation of Ras is an important factor in several cellular responses (Heldin and Westermark 1999). Distinct signal transduction pathways may thus be initiated through each SH2 domain-containing proteins activated by PDGF receptor binding (Heldin and Westermark 1999). Stat family molecules also bind to the activated PDGF receptor and become phosphorylated (Heldin and Westermark 1999). The Stat family of transcription factors can affect the transcription of specific genes after tyrosine phosphorylation (Heldin *et al.* 1998). Thus, by direct

activation of effector proteins or via cytoplasmic adaptor proteins, RTKs initiate a wide variety of downstream signaling pathways.

### **1.2.2 PDGF receptors and neuroprotection**

The PDGF $\beta$  receptor is involved in neuroprotection against ischemic events (Egawa-Tsuzuki *et al.* 2004). Rapid increases in PDGF-B chain mRNA transcripts have been reported after rat brain focal ischemia (Ohno *et al.* 1999). The expression of PDGF-A and -B mRNA and PDGF-BB and -AB dimers has been reported to increase in neurons and support cells surrounding areas damaged during ischemia (Krupinski *et al.* 1997). In addition to the upregulation of PDGF $\beta$  receptors and PDGF-B chain expression after ischemic events, exogenously applied PDGF-BB is also neuroprotective with respect to focal ischemia (Sakata *et al.* 1998) and glutamate- and NMDA-induced cell death in hippocampal neurons (Egawa-Tsuzuki *et al.* 2004; Tseng and Dichter 2005).

The neuroprotective effect of PDGF involves the inhibition of N-methyl-D-aspartate (NMDA) receptors, specifically the NMDA receptors containing NR2B subunits (Beazely *et al.* 2009). Interestingly, dopamine receptor activation in either the hippocampus or prefrontal cortex increases the PDGF $\beta$  receptor activity and results in an inhibition of NMDA currents, suggesting a relationship between dopaminergic hyperactivity and NMDA receptor hypoactivity (Valenzuela *et al.* 1996; Lei *et al.* 1999; Beazely *et al.* 2009; Kotecha *et al.* 2002). An understanding of the role of neuronal PDGF signaling can therefore help explain the neurodevelopment impairment associated with schizophrenia (Carter 2006).

With respect to depression, NMDA receptors may be an essential component of the pathogenesis and treatment (Petrie *et al.* 2000; Hashimoto 2010). A number of studies have reported increased spinal fluid and plasma glutamate levels in patients with major depressive disorders (Kim *et al.* 1982; Altamura *et al.* 1993). Many therapeutically useful antidepressant medications decrease

NMDA receptor activity, and NMDA receptor antagonists exhibit antidepressant activity (Petrie *et al.* 2000; Hashimoto 2009). Focusing on specific NMDA receptor subunit antagonists as potential antidepressants is an interesting possibility. The specific NR2B-subunit antagonist Ro 25-6981 acts as an antidepressant in rodents (Maeng *et al.* 2008), and the antagonist CP-101,606 shows promising results in treatment-resistant depressed patients (Preskorn *et al.* 2008). It remains unclear whether and how PDGF signaling may be involved in the regulation of NMDA receptor activity in the context of mental illness.

### **1.2.2 GPCR regulation of PDGF receptor expression and activity**

Serotonin is involved in the regulation of multiple cellular functions (Wilkinson *et al.* 1991), including the promotion of cell growth (Martinowich and Lu 2008). Growth factor receptors can be activated when a specific ligand interacts with the binding site on the receptor (Saito *et al.* 2001). As described above, the binding of the PDGF to the receptor leads to dimerization and phosphorylation in the receptor and triggers numerous signaling pathways and cell responses. However, it has been reported that a ligand-binding domain is not a requirement for receptor dimerization (Kwatra *et al.* 1992). This finding suggests that dimerization and autophosphorylation of the growth factor receptor may occur in a ligand-independent manner. Several studies have also demonstrated that GPCR agonists, such as angiotensin II, endothelin-1, and thrombin, stimulate RTK activity even in the absence of an exogenous growth factor (Wetzker and Bohmer 2003; Lowes *et al.* 2002; Saito *et al.* 2001). Nebigil *et al.* (2000) demonstrated that, in the absence of the ligand, activation of the 5-HT<sub>2B</sub> G $\alpha_q$ -coupled receptor triggers the phosphorylation of the PDGF $\beta$  receptor (Nebigil *et al.* 2000). This effect involved the activation of c-Src kinase by 5-HT to stimulate the phosphorylation and activation of the PDGF receptor (Nebigil *et al.* 2000). Liu *et al.* (2007) reported that 5-HT leads to the autophosphorylation of the PDGF $\beta$  receptor by activating 5-HT transporters in smooth muscle cells of the pulmonary artery (Liu *et al.* 2007a).

5-carboxamidotryptamine (5-CT) activates  $G\alpha_{i/o}$ -coupled receptors, such as 5-HT1 and 5-HT5 receptors, and  $G\alpha_s$ -coupled receptors, such as 5-HT7 receptors (Eglen *et al.* 1997; Hoyer and Martin 1997). Kruk *et al.* (2013) recently showed that 5-CT transactivates PDGF $\beta$  receptors in neuronal cell cultures (Kruk *et al.* 2013b). Numerous studies have shown that the transactivation of RTKs for a number of GPCRs occurs through exclusively intracellular signaling pathways (Daub *et al.* 1996). An example of such a pathway is the D2-class dopamine receptor transactivation of PDGF receptors in hippocampal neurons (Kotecha *et al.* 2002). The transactivation of RTK receptors by GPCR initiation may regulate a population of RTKs that differ from those activated by direct ligand binding (Heeneman *et al.* 2000).

### **1.3 Glutamate receptors**

Metabotropic glutamate receptors (mGluRs) and ionotropic glutamate receptors (iGluRs) are the two major types of glutamate receptors. mGluRs are coupled to G-proteins, and their action is mediated by second-messenger systems. iGluRs are ligand-gated ion channels of three types: AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), kainate, and NMDA receptors. All are involved in excitatory neurotransmission (Dingledine *et al.* 1999).

#### **1.3.1 NMDA receptor subunits**

NMDA receptors are composed of two compulsory NR1 subunits and at least two additional subunits of the following types: NR2A, NR2B, NR2C, NR2D, and NR3 (Paoletti and Neyton 2007). The properties of an NMDA receptor are dependent on its subunit composition (Dingledine *et al.* 1999). For example, NR1 plays a central role in NMDA receptor trafficking at the synapse and is essential for surface expression (Nong *et al.* 2003; Perez-Otano *et al.* 2001). Extracellular ligand-binding sites for glutamate are located in the NR2 subunits (Laube *et al.* 1997). The dominant NR2 forms in the hippocampus are NR2A and NR2B (Groc *et al.* 2006; Al-Hallaq *et al.* 2007). NMDA receptors are constitutively blocked by extracellular  $Mg^{2+}$  ions and



open only upon depolarization and agonist binding (Mayer *et al.* 1984; Nowak *et al.* 1984). The binding of glutamate as the agonist and glycine as the co-agonist is necessary for channel opening (Laube *et al.* 1997). However, the binding of glycine alone induces internalization of the NMDA receptor (Nong *et al.* 2003). AMPA, kainate, and NMDA receptors are permeable to Na<sup>+</sup> (influx) and K<sup>+</sup> (outflux) ions, but only the NMDA receptor is permeable to Ca<sup>2+</sup> (Arundine and Tymianski 2003). NR1/NR2A and NR1/NR2B channels have a stronger voltage dependence on Mg<sup>2+</sup> ions than do NR1/NR2C and NR1/NR2D (Monyer *et al.* 1994). NMDA receptor activity and trafficking are regulated by several protein kinases, including protein kinase A (PKA), protein kinase C (PKC), Ca<sup>2+</sup>-calmodulin-dependent protein kinase II (CaMKII), cyclin-dependent kinase 5 (CDK5), and members of the Src families of tyrosine kinases (Wang *et al.* 2006). NMDA receptor responses are also regulated by Zn<sup>2+</sup> ions, nitric oxide, polyamines, steroids, and protons (Waxman and Lynch 2005).

### **1.3.2 NMDA receptors and excitotoxicity**

Calcium influx through NMDA receptors plays a critical role in synaptic plasticity, a cellular mechanism involved in learning and memory. However, excessive calcium influx is detrimental to neurons. Excitotoxicity is caused by the excessive release of glutamate from presynaptic terminals and/or by the inhibition of reuptake systems (Chen and Wyllie 2006). The overactivation of NMDA receptors results in an excessive influx of Ca<sup>2+</sup> ions (Choi *et al.* 1987). Most extrasynaptic NMDA receptors contain the NR2B subunit and synaptic NMDA receptors are primarily NR2A-containing receptors (Groc *et al.* 2006; Tovar and Westbrook 2002; Groc *et al.* 2007). Extrasynaptic NR2B-containing NMDA receptors directly cause excitotoxic cell death through an influx of Ca<sup>2+</sup> (Hardingham and Bading 2002; Zhang *et al.* 2007), and synaptic NR2A-containing receptors, despite also increasing Ca<sup>2+</sup> levels, provide neuroprotection during ischemia (Liu *et al.* 2007b; von Engelhardt *et al.* 2007). The involvement of glutamate

excitotoxicity has been suggested with respect to numerous brain disorders, including stroke and Alzheimer's disease, but the exact mechanism(s) remain unclear.

### **1.3.3 Mental health diseases related to NMDA receptors**

The NMDA receptor has been implicated in multiple neuronal functions, ranging from synapse plasticity to learning and memory (Dingledine *et al.* 1999). Glutamate is very important for the physiological balance of the CNS and dysfunctional NMDA receptors are strongly linked to the pathophysiology of mental disorders (Tsai *et al.* 2002).

In schizophrenia, it is the hypofunction of glutamatergic systems that is associated with schizophrenia symptoms (Carlsson and Carlsson 1990; Toru *et al.* 1994). In the brains of schizophrenic patients, the K<sup>+</sup>-dependent release of glutamate is decreased (Sherman *et al.* 1991), and the number of glycine binding sites is increased (Ishimaru *et al.* 1994). Interestingly, D-cycloserine, a partial agonist at the glycine binding site, increases symptoms, and this effect is reduced by the antagonistic properties of this agent (Cascella *et al.* 1994).

Researchers have recently determined that ketamine (a non-competitive NMDA receptor antagonist) produced an antidepressant effect in animal models (Maeng *et al.* 2008). Such findings suggest that reducing glutamatergic activity in the brain can improve depressive symptoms (Krishnan and Nestler 2008). An examination of postmortem depressed individuals revealed a decreased allosteric coupling of the glycine site to NMDA receptors (Nowak and Wender 1994). Taken together, these data suggest that NMDA receptor antagonists have an antidepressive-like effects in animal models and in humans (Trullas and Skolnick 1990; Moryl *et al.* 1993).

### **1.3.4 NMDA receptor and serotonin**

Serotonin (5-HT) is a major monoamine neurotransmitter in the mammalian central nervous system (CNS). Multiple physiological, therapeutic, and pathological pathways are dependent on serotonergic signaling. The serotonergic system in the prefrontal cortex (PFC) is involved in the control of emotion and cognition under both normal and pathological conditions (Buhot 1997; Davidson *et al.* 2000). At least 15 different 5-HT receptor isoforms are expressed in humans (O'Neil and Emeson 2012). 5-HT regulates neuronal growth, survival, and maturation (Lotto *et al.* 1999; Whitaker-Azmitia *et al.* 1996) and also influences the neuronal activity of the glutamatergic system in adults (Hoyer 2002; Verge and Calas 2000).

### **1.3.5 NMDA and 5-HT7 receptors**

5-HT7 receptors are highly expressed in the suprachiasmatic nucleus (Thomas and Hagan 2004) but have also been identified in both neurons and support cells in the prefrontal cortex, the hippocampus, the thalamus, the hypothalamus, and the amygdala (Mahe *et al.* 2004b; Thomas and Hagan 2004). The regulation of sleep/wake cycles was one of first roles proposed for 5-HT7 (Lovenberg *et al.* 1993). 5-HT7 receptors have four isoforms (A, B, C, and D), which exhibit no differences in their respective pharmacology, signal transduction, or tissue distribution (Heidmann *et al.* 1997; Jasper *et al.* 1997). 5-HT7 receptors mediate 5-HT-induced hypothermia in guinea pigs (Marcos *et al.* 2009), with clear evidence of the receptor crosstalk that is common between 5-HT receptors and other receptors (Marcos *et al.* 2009).

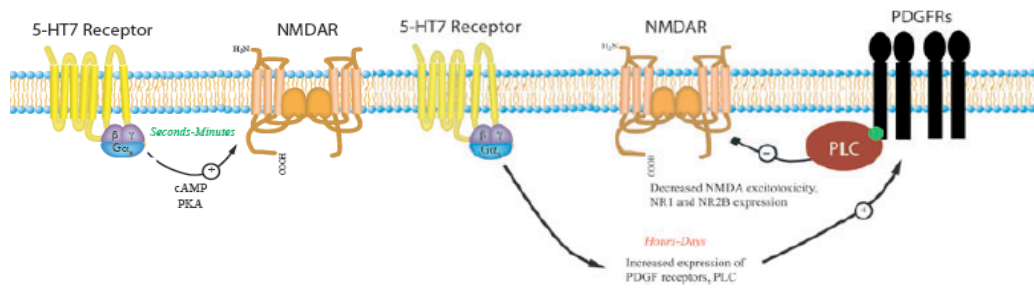
As mentioned above PDGF $\beta$  receptors inhibit NMDA receptors through the selective internalization of NR2B-containing NMDA receptors (Beazely *et al.* 2009), which are internalized after phosphorylation at the tyrosine 1452 site in the hippocampus. The inhibition of NMDA receptor currents by PDGF $\beta$  receptors is due to the activation of phospholipase C $\gamma$  (PLC $\gamma$ ), the release of intracellular calcium, and the stability of the actin cytoskeleton (Henke *et*

*al.* 1999) and also involves decrease in the surface expression of NR2B subunits of the NMDA receptor (Beazely *et al.* 2009).

Conflicting evidence has been found with respect to the effects of 5-HT7 receptors on neuronal and synaptic activity. The decrease in the slow afterhyperpolarization (sAHP) caused by 5-HT7 receptors leads to increased synaptic excitability in the hippocampus (Bacon and Beck 2000; Tokarski *et al.* 2005), possibly through the inhibition of calcium-dependent potassium channels (Gill *et al.* 2002). However, other reports have suggested that 5-HT7 receptors decreased glutamatergic signaling. The circadian phase advances in the dorsal raphe nucleus (DRN) involve an inhibition of glutamatergic neurotransmission (Duncan and Congleton 2010), and the 5-HT7 receptors in the DRN antagonize the NMDA-receptor-dependent synaptic release of both serotonin and glutamate (Harsing *et al.* 2004). The glutamate-mediated increase in intracellular calcium was inhibited by the activation of 5-HT7 receptors in rat suprachiasmatic nuclei (SCN) (Smith *et al.* 2001), and the amplitude of glutamate excitatory postsynaptic potentials (EPSPs) decreased after the activation of 5-HT7 receptors in mouse SCN (Quintero and McMahon 1999). Recently, Kobe and coworkers demonstrated that the activation of 5-HT7 receptors increases the number of dendrites and promotes the formation of synapses in primary hippocampal cultures but does not affect the magnitude of the LTP (Kobe *et al.* 2012). However, in 5-HT7 receptor knockout mice, a reduced induction of LTP has been demonstrated (Roberts *et al.* 2004).

The ability of 5-HT7 receptors to regulate the expression of PDGF receptors, as discussed in the results section, may help explain the apparent lack of clarity associated with the effects of 5-HT7 receptors on glutamatergic signaling. It should be noted that in some of the studies cited above, 5-HT7 receptor agonists were applied for 5 min to 10 min (Harsing *et al.* 2004; Quintero and McMahon 1999) whereas in others, animals/tissues were treated for hours to days (Kobe *et al.* 2012). Furthermore, in none of the studies cited above were selective 5-HT7 agonists used, and only Kobe *et al.* (2012) used a selective 5-HT7 receptor antagonist in their research.

NMDA receptors are tetrameric channels in the hippocampus with NR2A and NR2B being the dominant NR2 forms (Al-Hallaq *et al.* 2007). Studies have shown that antagonists of NMDA and 5-HT7 receptors produce an effective treatment for both schizophrenia and depression. It is unclear how the antagonists of NMDA and 5-HT7 receptors affect signaling mechanisms. With respect to behavioural models, there are interactions between 5-HT, 5-HT receptors, and NMDA receptors. Little is known about 5-HT receptor subtypes, which regulate NMDA receptor activity. 5-HT1A receptors have been found to inhibit NMDA receptor currents (Yuen *et al.* 2005) while 5-HT3 receptor activation in cortical slices has been shown to reduce NMDA currents (Liang *et al.* 1998). Low concentrations of 5-HT2A/2C agonists also increased NMDA currents in a PKC-dependent manner in prefrontal cortical slices (Arvanov *et al.* 1999). A number of questions remain unanswered, including how 5-HT7 receptors regulate NMDA receptor activity; whether 5-HT7 and other 5-HT receptors target a specific subgroup of NMDA receptors (i.e., NR2A- or NR2B-containing NMDA receptors) and if so, what mechanism(s) are involved.



**Figure 1.1 Schematic displaying the proposed model by 5-HT7 receptor agonists indicating 5-HT7 receptor has potential for both positive and negative regulation of NMDA signaling.**

## Chapter 2

# **Acute 5-HT7 receptor activation increases NMDA-evoked current and differentially alters NMDA receptor subunit phosphorylation in hippocampal neurons**

### **2.1 Summary**

N-methyl-D-aspartate (NMDA) receptors are regulated by several G-protein coupled receptors (GPCRs) as well as receptor tyrosine kinases. Serotonin (5-HT) type 7 receptors are expressed throughout the brain including the thalamus and hippocampus. Long-term (2-24 h) activation of 5-HT7 receptors promotes the expression of neuroprotective growth factor receptors, including the platelet-derived growth factor (PDGF)  $\beta$  receptors, which can protect neurons against NMDA-induced neurotoxicity.

Acute (5 min) treatment of isolated hippocampal neurons with the 5-HT7 receptor agonist 5-carboxamidotryptamine (5-CT) enhances NMDA-evoked peak currents and this increase in peak currents is blocked by the 5-HT7 receptor antagonist, SB 269970. In hippocampal slices, acute 5-HT7 receptor activation increases NR1 NMDA receptor subunit phosphorylation and differentially alters the phosphorylation state of the NR2B and NR2A subunits. NMDA receptor subunit cell surface expression is also differentially altered by 5-HT7 receptor agonists: NR2B cell surface expression is decreased whereas NR1 and NR2A surface expression are not significantly altered.

## 2.2 Introduction

Several studies have examined the ability of serotonin (5-HT) receptors to modulate NMDA receptor activity. For example, in isolated cortical neurons, activation of 5-HT<sub>1A</sub> receptors inhibits NMDA receptor currents (Yuen *et al.* 2005) and 5-HT<sub>3</sub> receptor activation reduces NMDA currents in cortical slices (Liang *et al.* 1998). In contrast, in *Xenopus* oocytes, 5-HT<sub>2</sub> receptor activation increases NMDA receptor currents (Blank *et al.* 1996) and in prefrontal cortical slices, 5-HT<sub>2A/2C</sub> agonists enhance NMDA-evoked responses (Arvanov *et al.* 1999).

The effect of 5-HT<sub>7</sub> receptor ligands on NMDA-evoked currents remains unknown, however recent studies provide clear evidence for the regulation of glutamatergic signaling by 5-HT<sub>7</sub> receptors. 5-HT<sub>7</sub> receptors inhibit NMDA-induced neurotransmitter release in the dorsal Raphe nucleus (DRN) and the physiological role of 5-HT<sub>7</sub> receptors in circadian rhythms is linked to an inhibition of glutamate-dependent events (Harsing *et al.* 2004). In the suprachiasmatic nucleus, glutamate excitatory postsynaptic potentials (EPSPs) and glutamate-induced intracellular calcium levels are both inhibited by 5-HT<sub>7</sub> receptor activation (Quintero and McMahon 1999; Smith *et al.* 2001). Taken together, these studies suggest that 5-HT<sub>7</sub> receptor activation decreases NMDA and/or glutamate receptor signaling. In contrast, 5-HT<sub>7</sub> receptor knock-out mice display a reduced induction of long-term potentiation (LTP), the magnitude of LTP, and hippocampus-associated learning compared to wild-type (Roberts *et al.* 2004). Therefore although there is evidence that 5-HT<sub>7</sub> receptors negatively regulate NMDA/glutamate signaling, deletion of 5-HT<sub>7</sub> receptors decreased the magnitude of NMDA receptor-dependent events such as LTP.

We recently identified the 5-HT<sub>7</sub> receptor as a regulator of platelet-derived growth factor (PDGF)  $\beta$  receptor expression and activity (see Chapter 3). Activation of PDGF $\beta$  receptors by PDGF-BB selectively inhibits NR2B-containing NMDA receptor currents and this may be a mechanism of PDGF $\beta$  receptor-mediated neuroprotection (Beazely *et al.* 2009). Intriguingly, 5-HT<sub>7</sub> receptor-



induced upregulation of PDGF $\beta$  receptor is also sufficient to protect neurons against NMDA-induced excitotoxicity (Figure 4.9, see Chapter 4). Thus, we proposed that long-term activation of 5-HT7 receptors initiates pathways that ultimately negatively regulate NMDA receptor signaling.

To clarify the direct effects of 5-HT7 receptor activation on NMDA receptor signaling we examined the effects of 5-HT7 receptor agonists and antagonists on NMDA-evoked currents, NMDA receptor subunit phosphorylation, and NR subunit cell surface expression. In isolated hippocampal neurons, application of the 5-HT7 receptor agonist, 5-CT, resulted in a rapid, robust, and sustained increase in peak NMDA-evoked currents. 5-HT7 receptor agonist treatment also differentially altered NMDA receptor subunit phosphorylation and cell surface expression. These data, along with our other work, suggest a model for differential NMDA receptor regulation by 5-HT7 receptors over the short- and long-term.

### **2.3 Materials and Methods**

Reagents and antibodies – 5-CT and LP 12 (4-(2-Diphenyl)-N-(1,2,3,4-tetrahydronaphthalen-1-yl)-1-piperazinehexanamide hydrochloride) were purchased from Sigma (St. Louis, MO, USA). The 5-HT7 receptor antagonists SB 258719 ((R)-3,N-Dimethyl-N-[1-methyl-3-(4-methylpiperidin-1-yl)propyl]benzene sulfonamide) and SB 269970 (R-3-(2-(2-(4-methylpiperidin-1-yl)ethyl)-pyrrolidine-1-sulfonyl)-phenol) were purchased from Tocris (Ellisville, MO, USA). Antibodies used include anti-NR1, anti-phospho-896-NR1, anti-phospho-897-NR1, anti-NR2A, anti-phospho-pan-NR2A, anti-NR2B, anti-phospho-1252-NR2B, anti-phospho-1336-NR2B, and anti-phospho-1472-NR2B were purchased from Millipore (Billerica, MA).

Cell isolation and whole-cell recording – CA1 neurons were isolated from hippocampal slices of postnatal day 14-21 Wistar rats as previously described (Wang and MacDonald 1995). The

extracellular solution (ECF) was composed of 140 mM NaCl, 1.3 mM CaCl<sub>2</sub>, 25 mM N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES), 33 mM glucose, 5.4 mM KCl, and 0.5 μM tetrodotoxin, and 0.5 μM glycine, with pH of 7.3-7.4 and osmolarity ranging from 320-330 mOsm. Recordings were done at room temperature. The intracellular solution consisted of 11 mM ethyleneglycol-bis-(α-amino-ethyl ether) N,N'-tetra-acetic acid (EGTA) as intracellular calcium chelating buffer, 10 mM HEPES, 2 mM MgCl<sub>2</sub>, 2 mM tetraethyl ammonium chloride (TEA-Cl) to block K<sup>+</sup> channel, 1 mM CaCl<sub>2</sub>, 140 mM CsF, and 4 mM K<sub>2</sub>ATP. NMDA currents were evoked by rapid application of NMDA solution delivered from a multi-barreled fast perfusion system for 2 sec in every minute.

Western blot – Hippocampal slices were prepared, treated with drugs for 5 min, and homogenized chilled lysis buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 30 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.5% SDS, and 1% triton-X-100; supplemented with Halt Protease and Phosphatase Inhibitor (Thermo, Fisher, Markham, Ontario) prior to use. Lysates were centrifuged at 14,000 x g for 20 min at 4°C and the supernatant was collected. The supernatant was subjected to SDS-PAGE and proteins were transferred to nitrocellulose membranes, blocked with 5% non-fat dry milk in Tris-buffered saline and 0.1% Tween for 1 h at room temperature or overnight at 4°C, and incubated in primary antibodies for 1 h at room temperature or overnight at 4°C. Membranes were washed three times in Tris-buffered saline with 0.1% Tween-20, incubated with HRP-conjugated secondary antibodies for 1 h at room temperature, washed again, and bound antibodies were visualized by the enhanced chemiluminescence using the chemiluminescent substrate (Millipore, Etobicoke, Ontario). Images of Western blots were taken on the Kodak 4000MM Pro Imaging Station, and densitometric analyses were performed using the Kodak Molecular Imaging software. Membranes were then stripped and reprobed with other antibodies.

Surface Biotinylation Assay – Hippocampal slices were incubated for 5 min with 5-HT7 receptor agonists and antagonists. Slices were washed in ice-cold ECF and incubated with 0.5 mg/ml Sulfo-NHS-LC-biotin for 30 min. The biotin reaction was quenched by washing with 10 mM glycine. Slices were washed twice more and homogenized in lysis buffer. Lysate protein concentrations were normalized and lysates were incubated with streptavidin beads overnight at 4 °C (Sigma). Beads were washed three times in lysis buffer and boiled in loading buffer for 5 min before separation by SDS-PAGE.

Statistical Analysis – Statistical analysis of the data was performed using the Prism® GraphPad program. For electrophysiology, graphs and sample tracings were made using the Origin® program. Significance level was set at  $\alpha = 0.05$ . Data was analyzed by one-way ANOVA or Student's t-test where appropriate.

Animals – All animal experiments were performed in agreement with the guidelines of the policies on the Use of Animal at the University of Waterloo and the University of Western Ontario.

## **2.4 Results**

Application of the 5-HT7 receptor agonist, 5-CT, to isolated hippocampal neurons for 5 min robustly increased NMDA-evoked peak currents (Vasefi *et al.* 2013b). This effect occurred within minutes after 5-CT application and the increase in peak NMDA-evoked currents was sustained even after 5-CT was washed out (minutes 10-30). In addition to 5-HT7, 5-CT also activates 5-HT1 and 5-HT5 receptors (Hoyer *et al.* 1994; Eglen *et al.* 1997). However, the increase in NMDA-evoked currents by 5-CT was completely blocked by the 5-HT7 receptor-selective antagonist, SB 269970 (Vasefi *et al.* 2013b).

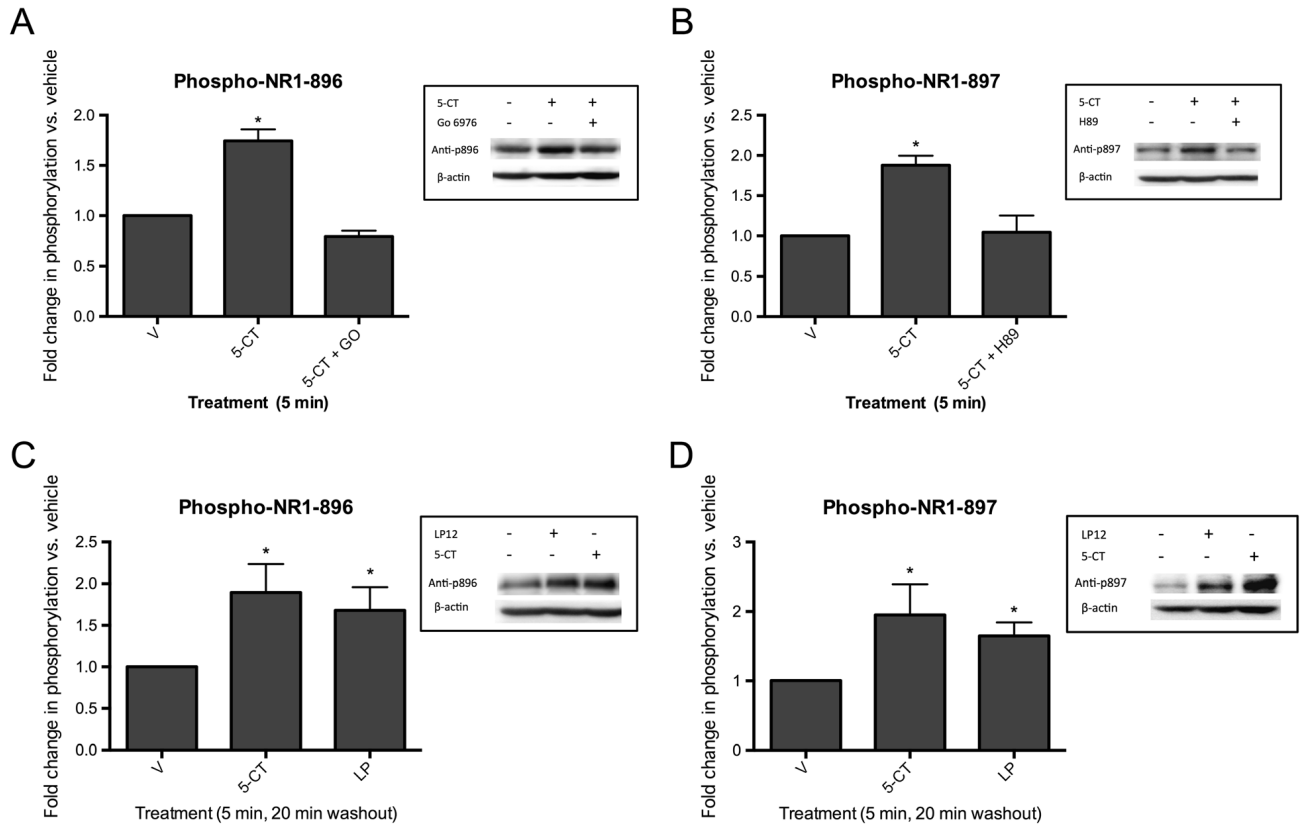
To determine if 5-HT7 receptor activation alters NMDA receptor subunit phosphorylation we incubated hippocampal slices with 50 nM 5-CT in the absence or presence of the 5-HT7 receptor antagonist, SB 258719 (Mahe *et al.* 2004b). 5-HT7 receptors are coupled to  $G\alpha_s$  in several cell lines (Thomas *et al.* 1999a, Crider *et al.* 2003, Mahe *et al.* 2004a, Andressen *et al.* 2006, Romero *et al.* 2006) and the application of 5-CT to hippocampal slices robustly increased NR1 receptor phosphorylation at the PKA phosphorylation site, serine 896 (Table 2.1). 5-CT application also increased the phosphorylation of the NR1 subunit at PKC-site serine 897 (Table 2.1). The increase in the PKC-site serine 896 phosphorylation was blocked by the PKC inhibitor Go 6983 (Figure 2. 1A) and the increase in the PKA-site serine 897 phosphorylation was blocked by the PKA inhibitor, H89 (Figure 2.1B). A 5 min application of 5-CT resulted in a sustained elevation of NMDA-evoked current for at least 20 min after washout (Vasefi *et al.* 2013b). To determine if the observed changes in NR1 subunit phosphorylation were similarly sustained we treated hippocampal slices with 5-CT for 5 min, washed the slices, and waited an additional 20 min before homogenizing the tissue in lysis buffer. The phosphorylation state of both serine 896 and 897 remained significantly elevated above control for 20 min after treatment with 5-CT (Figure 2.1C, D). As mentioned above, 5-CT will also activate 5-HT1 and 5-HT5 receptors (Eglen *et al.* 1997; Hoyer *et al.* 1994). To confirm that the observed changes in NMDA receptor subunit phosphorylation were indeed due to the activation of the 5-HT7 receptor we incubated acutely-dissected hippocampal slices with the 5-HT7-selective agonist, LP 12 (Leopoldo *et al.* 2007) in the absence or presence of the 5-HT7 receptor antagonists, SB 269970 and SB 258719 (Thomas *et al.* 1999b). Similar to the PKA phosphorylation site, serine 897 was phosphorylated (Figure 2.2A, C) and this increased phosphorylation was sustained for at least 20 min (Figure 2.1D). Interestingly, we observed an even more robust increase in the phosphorylation of the adjacent, PKC-phosphorylated serine 896 (Figure 2.2B, C). However, the magnitude of the increase in serine 896 compared to serine 897 may be due to the lower basal phosphorylation state at serine 896. The increase in serine 896 phosphorylation was also observed for at least 20 min after LP 12

treatment (Figure 2.1C). The increased phosphorylation by LP 12 at both sites was blocked by SB 258719 (Figure 2.2) (Mahe *et al.* 2004b).

**Table 2.1 5-CT treatment differently alters NMDA receptor subunit phosphorylation.**

NMDA subunit/ phosphorylation site	Treatment (5 min)		
	5-CT	5-CT + SB	SB 269970
NR1/896, n = 8	2.07 ± 0.39**	0.91 ± 0.31	1.06 ± 0.28
NR1/897, n = 8	1.51 ± 0.13**	1.00 ± 0.39	0.95 ± 0.11
NR2A/total, n = 10	0.71 ± 0.07**	0.97 ± 0.37	0.86 ± 0.17
NR2B/1472, n = 5	2.79 ± 0.37**	1.49 ± 0.61	2.86 ± 1.33
NR2B/1252, N = 10	1.39 ± 0.49	1.50 ± 0.23	1.53 ± 0.31
NR2B/1336, n = 7	1.25 ± 0.19	1.98 ± 0.31	2.36 ± 0.36**

Hippocampal slices were treated with 50 nM 5-CT in the absence or presence of 1  $\mu$ M SB 269970 for 5 min. Changes in the phosphorylation state for each antibody were normalized to control (vehicle-treated slices) and to total NR subunit expression. For each experiment a  $\beta$ -actin antibody was used to confirm equal loading. Data presented are the average and standard error. \*  $p < 0.5$ , \*\*  $p < 0.01$ , compared to vehicle.

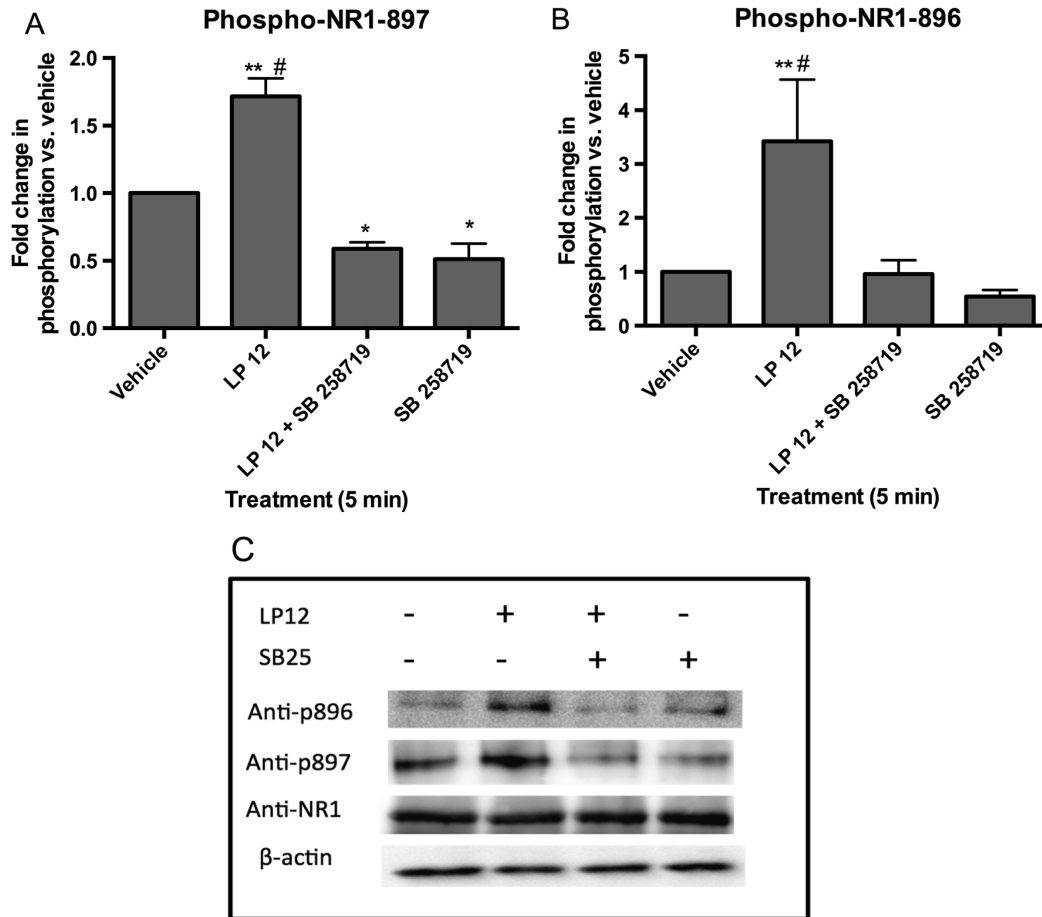


**Figure 2.1 5-CT treatment of hippocampal slices increases NR1 phosphorylation at serines 896 and 897.**

A) Hippocampal slices were treated with 50 nM 5-CT in the absence or presence of 1  $\mu$ M Go 6983 (GO) for 5 min. Increases in the phosphorylation state at serine 896 were normalized to control (vehicle-treated slices) and to total NR1. Data represent the average and standard error of 10 independent experiments. \*  $p < 0.5$  compared to vehicle, ANOVA analysis with Dunnett's post-test. INSET: representative Western blots showing serine 896 phosphorylation and the  $\beta$ -actin loading control. B) Hippocampal slices were treated with 50 nM 5-CT in the absence or presence of 10  $\mu$ M H89 for 5 min. Data represent the average and standard error of 10 independent experiments. \*  $p < 0.5$  compared to vehicle, ANOVA analysis with Dunnett's post-test. INSET: representative Western blots showing serine 897 phosphorylation and the  $\beta$ -actin loading control. C) Hippocampal slices were treated with 50 nM 5-CT or 300 nM LP 12 for 5 min, the slices were washed and incubated in drug-free buffer for an additional 20 min before lysis. Data represent the average and standard error of 8 independent experiments. \*  $p < 0.5$  compared to vehicle, ANOVA analysis with Dunnett's post-test. INSET: representative Western blots showing serine 896 phosphorylation and the  $\beta$ -actin loading control. D) Slices were treated as in C. Data represent the average and standard error of 4 independent experiments. \*  $p < 0.5$  compared to vehicle, ANOVA analysis with Dunnett's post-test. INSET: representative Western blots showing serine 897 phosphorylation and the  $\beta$ -actin loading control.

Application of LP 12 also differentially affects NR2 subunit phosphorylation. While LP 12 significantly reduced the phosphorylation state of the NR2A subunit (Figure 2.3A, B), LP 12 increased NR2B phosphorylation at tyrosine 1472 (Figure 2.3C, D). However the phosphorylation of NR2B at other tyrosine residues, 1252 and 1336, remained unchanged, with LP 12 inducing a  $0.97 \pm 0.12$  ( $n = 12$ ) and  $0.93 \pm 0.11$  ( $n = 10$ ) fold change, respectively, compared to control. The effects of LP 12 on the phosphorylation of NR2A and NR2B-Y1472 were both blocked by SB 258719 (Figure 2.3). 5CT treatment similarly enhanced NR2B-Y1472 phosphorylation and decreased NR2A phosphorylation (Table 2.1).

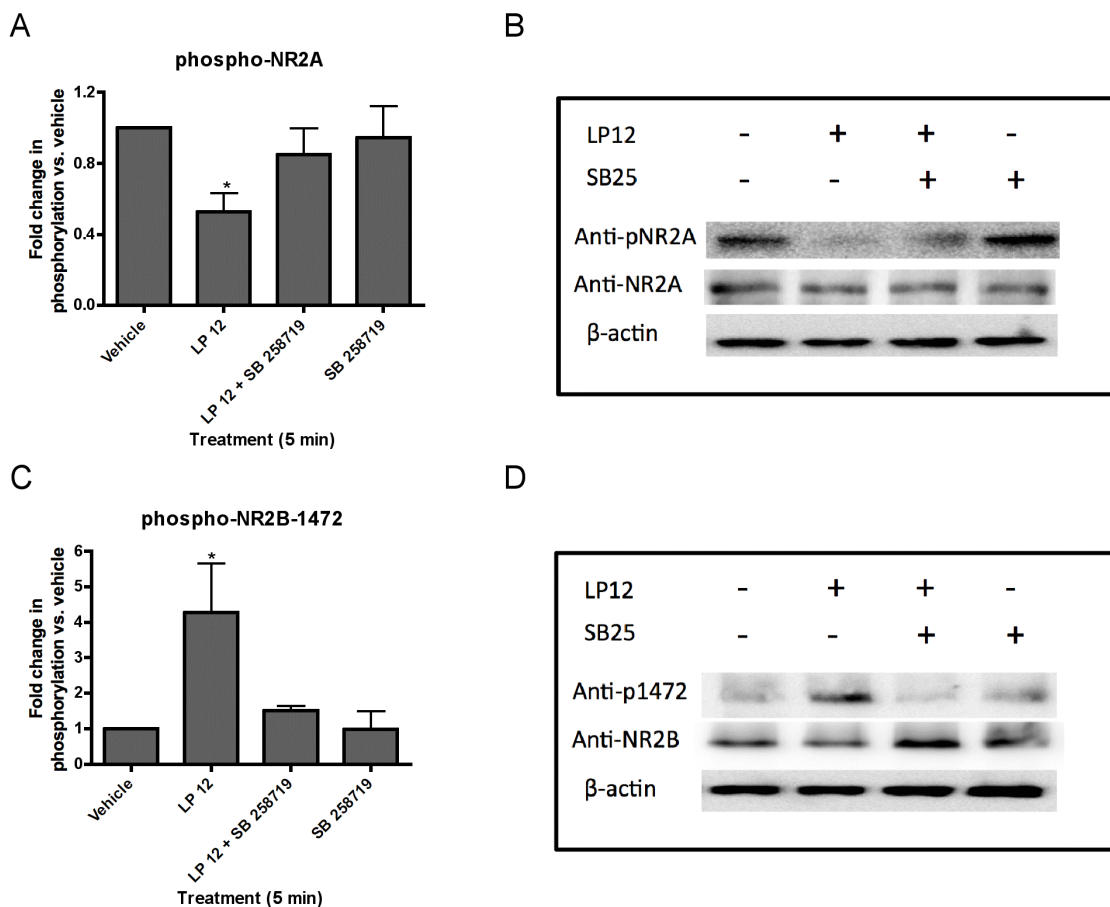
Several pathways regulate NMDA receptor subunit cell surface expression. To investigate the effects of 5-HT7 receptor agonist treatment on the surface expression of the NMDA receptors subunits, we treated hippocampal slices with LP 12 in the absence or presence of SB 258719 for 5 min. In hippocampal slices LP 12 treatment did not significantly change the cell surface expression of NR1 ( $0.92 \pm 0.13$  vs. control) and NR2A ( $1.05 \pm 0.13$  vs. control), but NR2B cell surface expression was reduced by approximately 25% (Figure 2.4).



**Figure 2.2 5-HT7 receptor activation increases NR1 subunit phosphorylation.**

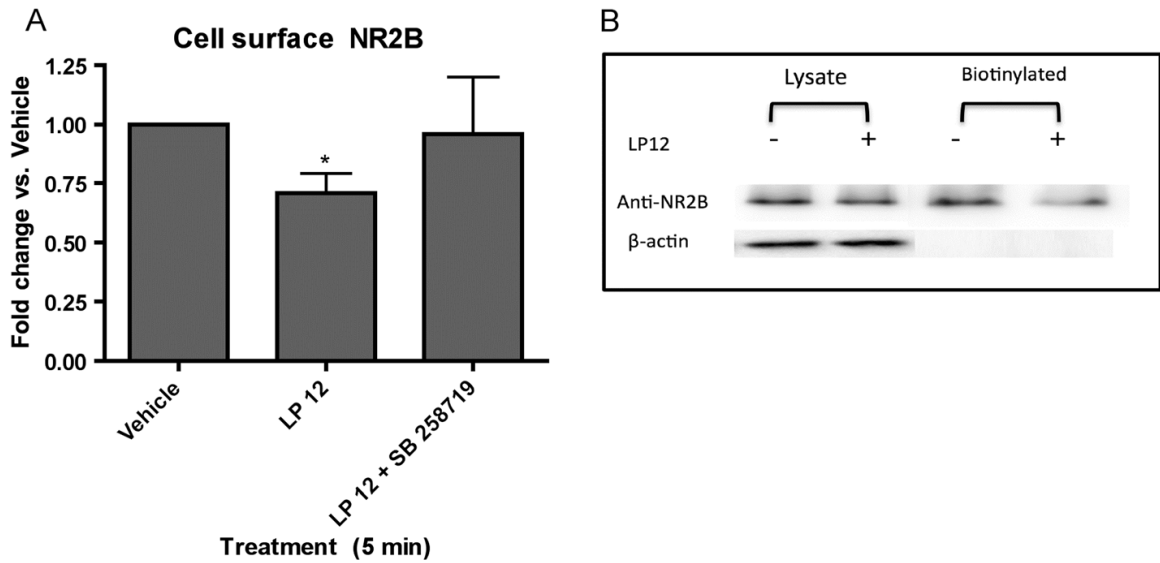
A) Hippocampal slices were treated with 300 nM LP 12 in the absence or presence of 1  $\mu$ M SB 258719 for 5 min. Increases in the phosphorylation state at serine 897 were normalized to control (vehicle-treated slices) and to total NR1. Data represent the average and standard error of 9 independent experiments. \*\*  $p < 0.01$  LP 12 vs. vehicle, #  $p < 0.01$  vs. LP 12 vs. LP 12 + SB 258719, \*  $p < 0.5$  compared to vehicle, ANOVA analysis with Bonferroni's post-test. B) Hippocampal slices were treated as in A and Western blots were performed using an anti-phospho-serine 896 antibody. Data represent the average and standard error of 10 independent experiments. \*\*  $p < 0.01$  LP 12 vs. vehicle, #  $p < 0.01$  vs. LP 12 vs. LP 12 + SB 258719, ANOVA analysis with Bonferroni's post-test. C) Representative Western blots showing serine 896 and serine 897 phosphorylation, total NR1, and  $\beta$ -actin.





**Figure 2.3 5-HT7 receptor activation differentially alters NR2 subunit phosphorylation.**

A) Hippocampal slices were treated with 300 nM LP 12 in the absence or presence of 1  $\mu$ M SB 258719 for 5 min. Decreases in the phosphorylation state of NR2A were normalized to control (vehicle-treated slices) and to total NR2A. Data represent the average and standard error of 7 independent experiments. \*  $p < 0.05$  LP 12 vs. vehicle ANOVA analysis with Dunnett's post-test. B) Representative Western blots using anti-phospho-pan-NR2A, NR2A, and  $\beta$ -actin. C) Hippocampal slices were treated as in A. Increases in the phosphorylation state of NR2B at tyrosine 1472 were normalized to control (vehicle-treated slices) and to total NR2B. Data represent the average and standard error of 6 independent experiments. \*  $p < 0.05$  LP 12 vs. vehicle ANOVA analysis with Dunnett's post-test. D) Representative Western blots using anti-phospho-Y1472-NR2B, NR2B, and  $\beta$ -actin.



**Figure 2.4 LP 12 application results in the selective reduction of the surface expression of NR2B.**

A) CA1 hippocampal slices were treated with vehicle, 300 nM LP 12, or LP 12 + 1  $\mu$ M SB 258719 for 5 min. The slices were biotinylated and lysates were incubated overnight with streptavidin beads. Data represent the average and standard error of 6 independent experiments.

\*  $p < 0.05$  LP 12 vs. vehicle ANOVA analysis with Dunnett's post-test. B) Total lysates or biotinylated samples were blotted using antibodies against NR2B or  $\beta$ -actin.

## 2.5 Discussion

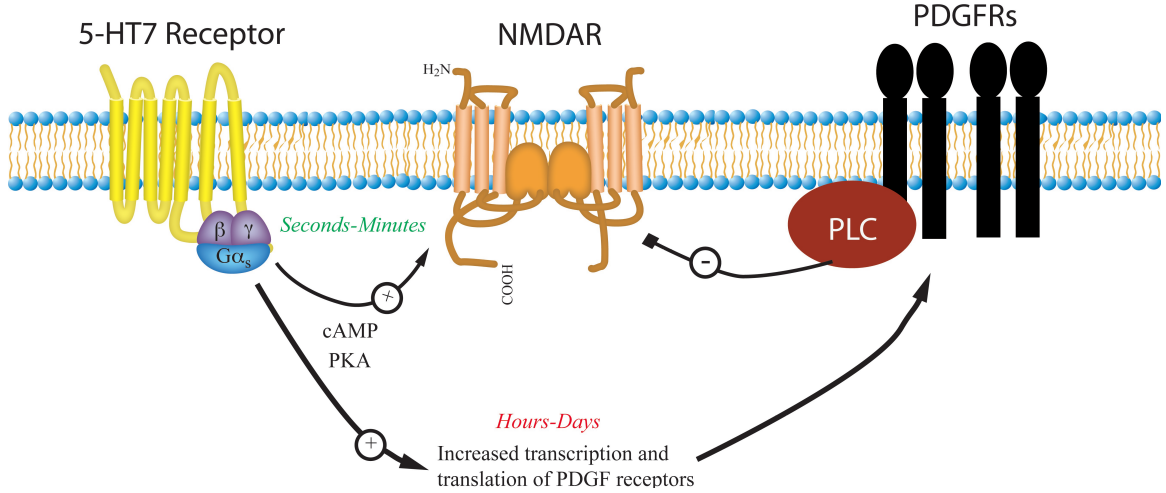
5-HT7 receptor knock-out mice have a reduced induction of LTP (Roberts *et al.* 2004). Our finding shows that increased NMDA-evoked currents by the acute 5-HT7 receptor activity may contribute to our understanding of how 5-HT7 receptors modulate synaptic plasticity. 5-HT7 receptor-mediated increases in NMDA-evoked currents may also explain how 5-HT7 receptor activation potentiates bursting frequency in the CA3 hippocampal neurons (Bacon and Beck 2000) and increases CA1 extracellular population spike amplitude in CA1 hippocampal neurons (Tokarski *et al.* 2003). Conversely, there is evidence that 5-HT7 receptor agonists decrease glutamate-induced increases in intracellular calcium (Smith *et al.* 2001) and the amplitude of glutamate EPSPs (Quintero and McMahon 1999). Based on the findings reported here and our other work describing the ability of long-term 5-HT7 receptor agonist treatment to upregulate PDGF $\beta$  receptors (Figure 4.9) (Vasefi *et al.* 2012), we propose that the activation of 5-HT7 receptors may regulate NMDA receptor activity via two temporally distinct pathways (Figure 2.5). Acute (5-10 min) activation of 5-HT7 receptors increases NMDA-evoked currents whereas long-term (2-24 h) activation of 5-HT7 receptors upregulates PDGF $\beta$  receptors, a receptor tyrosine kinase that inhibits NMDA receptor activity (Valenzuela *et al.* 1996, Vasefi *et al.* 2013a).

5-HT7 receptors are involved in aspects of learning and memory that are hippocampus dependent (Meneses 2004). For example, 5-HT7 receptor knock-out mice display impaired contextual fear-conditioning (Roberts *et al.* 2004) and display a reduced ability to recognize new environments (Sarkisyan and Hedlund 2009). These and other studies have promoted interest in 5-HT7 receptors as a potential drug target in Alzheimer's disease and are further supported by a study demonstrating an increase in memory formation by the 5-HT7 receptor agonist, AS 19 (Perez-Garcia and Meneses 2005). NMDA receptors are crucial components of learning and memory

pathways in the hippocampus. Thus, the positive linkage between 5-HT7 receptors and NMDA receptor activity may explain how 5-HT7 receptors promote learning and memory as well as their involvement in LTP.

All three 5-HT7 receptor splice variants identified in rats are positively coupled adenylate cyclase and display some level of constitutive activity (Leopoldo *et al.* 2011). Therefore it is not surprising that the activation of 5-HT7 receptors in hippocampal slices increases the phosphorylation of the NR1 subunit at serine 897. On the NR2B subunit, tyrosine 1472 is required/involved in CaMKII binding and activation (Nakazawa *et al.* 2006) and this phosphorylation site is linked to spinal pain transmission (Katano *et al.* 2011). Interestingly, 5-HT7 receptor agonists promote pain after formalin injection in animal models (Rocha-Gonzalez *et al.* 2005). However others have found anti-nociceptive effects of 5-HT7 receptors (Dogrul and Seyrek 2006). These differences may be due to distinct central and peripheral pain pathways that contain 5-HT7 receptors.

In summary, we have now identified two pathways downstream of 5-HT7 receptors that ultimately regulate NMDA receptor activity/signaling (Figure 2.5). We have demonstrated that the upregulation of PDGF $\beta$  receptors by long-term treatment with 5-HT7 receptor agonists is sufficient to protect hippocampal neurons against NMDA excitotoxicity (Figure 4.9) (Vasefi *et al.* 2012; Vasefi *et al.* 2013a) whereas acute activation of 5-HT7 receptors increases NMDA-evoked currents. These findings may help to explain why previous reports identified 5-HT7 receptors as both positive and negative regulators of NMDA receptor signaling.



**Figure 2.5 Proposed model for NMDA receptor regulation by short- and long-term activation of 5-HT7 receptors.**

Prolonged (2 to 24 h) activation of 5-HT7 receptors up regulates PDGF receptor activation and expression in hippocampal neurons, cortical neurons, and SH-SY5Y cells. The upregulation of PDGF $\beta$  receptors is sufficient to inhibit NMDA-induced neuronal cell death in primary cultures. In addition, NMDA receptor subunit expression is reduced. In contrast, acute (5 min) activation of 5-HT7 receptors increases NMDA-evoked currents and differentially regulates NMDA receptor subunit phosphorylation and cell surface expression. Figure modified from (Vasefi *et al.* 2013a).

## Chapter 3

### Activation of 5-HT7 receptors increases neuronal platelet-derived growth factor beta receptor expression

#### 3.1 Summary

Several antipsychotics have a high affinity for 5-HT7 receptors yet despite intense interest in the 5-HT7 receptor as a potential drug target to treat psychosis, the function and signaling properties of 5-HT7 receptors in neurons remain largely uncharacterized. In primary mouse hippocampal and cortical neurons, as well as in the SH-SY5Y cell line, incubation with 5-HT, 5-carboxamidotryptamine (5-CT), or 5-HT7 receptor-selective agonists increases the expression of platelet-derived growth factor (PDGF) receptors. The increased PDGF receptor expression is cyclic AMP-dependent protein kinase (PKA)-dependent, suggesting that 5-HT7 receptors couple to  $G\alpha_s$  in primary neurons. Interestingly, up-regulated PDGF receptors display an increased basal phosphorylation state at the phospholipase C $\gamma$ -activating tyrosine 1021. This novel linkage between the 5-HT7 receptor and the PDGF system may be an important GPCR-neurotrophic factor signaling pathway in neurons.

#### 3.2 Introduction

5-HT receptors can activate many intracellular signaling pathways and there are numerous examples of crosstalk between 5-HT receptors, growth factor receptors, and ion channels (Raymond *et al.* 2001; Shah and Catt 2004). For example, several signaling pathways between the 5-HT system and the platelet-derived growth factor (PDGF) receptor have been identified. In the mouse fibroblast cell line LMTK- transfected with the human 5-HT receptor subtype 2B, Nebigil *et al.* showed that this receptor can acutely induce PDGFR $\beta$  phosphorylation within 5

min (Nebigil *et al.* 2000), a process called transactivation. In addition, the serotonin transporter (5-HTT) may be involved in 5-HT-induced PDGFR activation in smooth muscle cells (Liu *et al.* 2007a; Ren *et al.* 2010).

Despite preliminary evidence and hypotheses for the involvement of 5-HT7 receptors in CNS physiology and mental health, much remains to be elucidated about 5-HT7 receptor signaling as it impacts brain function. Here, we demonstrate that prolonged activation of 5-HT7 in cultured hippocampal neurons increases the activity and expression of PDGF receptors, thereby establishing an additional linkage between serotonergic and neurotrophic factor signaling in neurons.

### **3.3 Materials and Methods**

#### **3.3.1 Reagents and antibodies**

5-HT, 5-CT, H89, LP 12 (4-(2-Diphenyl)-N-(1,2,3,4-tetrahydronaphthalen-1-yl)-1-piperazinehexanamide hydrochloride), SB 269970 (R-3-(2-(2-(4-Methylpiperidin-1-yl)ethyl)-pyrrolidine-1-sulfonyl)-phenol) and WAY 100635 (N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl) cyclohexanene carboxamide trihydrochloride) were purchased from Sigma (St. Louis, MO, USA). The 5-HT7 receptor antagonists SB 258719 ((R)-3,N-Dimethyl-N-[1-methyl-3-(4-methylpiperidin-1-yl)propyl]benzene sulfonamide) was obtained from Tocris (Ellisville, MO, USA). Antibodies used include those raised against PDGF $\beta$  receptor (Epitomics, California, USA), PDGF $\beta$  receptor phospho-tyrosine 1021 and  $\beta$ -actin (Santa Cruz, CA, USA), and  $\alpha$ -actinin (Sigma).

#### **3.3.2 Cell culture**

Hippocampal and cortical neurons were isolated from E17-19 mouse fetuses (CD-1 mice, Harlan Lab, USA) and placed in cold dissection media (33 mM glucose, 58 mM sucrose, 30 mM HEPES, 5.4 mM KCl, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 0.34 mM Na<sub>2</sub>HPO<sub>4</sub>, 4.2 mM NaHCO<sub>3</sub>,

0.03 mM phenol red, pH 7.4, 320-335 mOsm/kg). The hippocampi or cortex were digested in 0.25% trypsin/0.1% EDTA for 30 min at 37°C. Cell mixtures were plated on collagen-coated culture dishes and grown at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were fed twice per week with plating media (MEM, supplemented with 28 mM glucose, 5.4 mM NaHCO<sub>3</sub>, 100 units/ml insulin, 10% fetal bovine serum, 10% horse serum, pH 7.6, 320-335 mOsm/kg) for the first week and with feeding media (plating media without serum) in subsequent weeks. FUDR (0.081 mM 5-fluoro-2-deoxyuridine (Sigma) and 0.2 mM uridine to MEM), a mitotic inhibitor, was added 3-5 days after and drug treatments were performed 14 to 21 days after plating. For SH-SY5Y cells, cells were plated in 6-well culture plates with complete growth media consisting of DMEM and Ham's F12 (Thermo, Fisher, Markham, Ontario) in a 1:1 ratio, supplemented with 2.5 mM glutamate, and 10% fetal bovine serum (Sigma). Upon reaching confluency, cells were serum-deprived for 24 h prior to drug treatments.

### **3.3.3 Real-time PCR analysis**

RNA isolation (BioRad Aurum RNA Mini kit) and reverse transcription (iScript cDNA synthesis kit; BioRad, Hercules CA) were used on extracts of primary hippocampal neurons. Primers for the PDGF $\beta$  receptor (forward primer 5'- GCG GGT GGT GTT CGA GGC TTA T -3', 5'- CCT GTG GCT CAA GGA CAA CCG T -3' and reverse primer 5'- AGG TCT CTG CAG GTA GAC CAG GTG -3', 5'- GAG GTC TCT GCA GGT AGA CCA GGT -3', IDT, San Diego, California, USA) were used to determine PDGF $\beta$  receptor mRNA expression and levels were normalized to  $\beta$ -actin expression. SSO Fast EvaGreen real-time PCR supermix was purchased from BioRad.

### **3.3.4 Western blot and analysis**

After drug treatment, cells were washed with chilled PBS, and lysed in chilled lysis buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 30 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerophosphate, 1 mM sodium orthovanadate, and 1% triton-X-100; supplemented with Halt Protease and Phosphatase Inhibitor (Thermo, Fisher, Markham, Ontario)



prior to use. Cell lysates were subjected to SDS-PAGE and proteins were transferred to nitrocellulose membranes. Membranes were blocked, washed, incubated with primary and secondary antibodies and visualized by the enhanced chemiluminescence using the chemiluminescent substrate (Millipore, Etobicoke, Ontario). Statistical analysis of the data was completed using the Prism® GraphPad program.

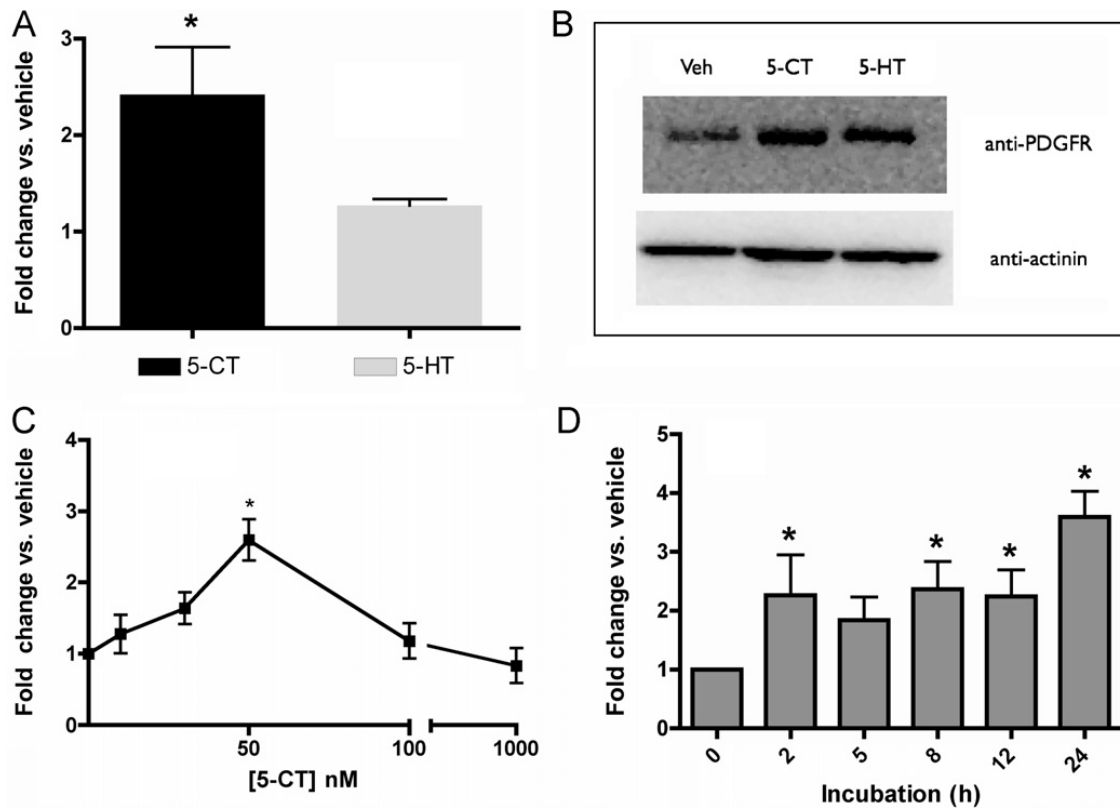
### **3.3.5 Animals**

All animal experiments were performed in agreement with the guidelines of the policies on the Use of Animal at the University of Waterloo.

## **3.4 Results and Discussion**

In cultured hippocampal neurons, treatment with 5-carboxamidotryptamine (5-CT), a 5-HT<sub>1</sub>, 5, and 7-selective agonist (Voronezhskaya *et al.* 2008) increased PDGF $\beta$  receptor expression to a greater extent than 5-HT itself (Figure 3.1A). The increase in PDGF $\beta$  receptor expression by 5-CT was dose dependent with a maximal effect observed at a concentration of 50 nM (Figure 3.1C). 24 h incubation robustly increased PDGF $\beta$  receptor expression and an increase in PDGF $\beta$  receptor levels was detected as early as 2 h after application of 5-CT to the hippocampal cultures (Figure 3.1D). Although 5-HT<sub>7</sub> receptors are expressed in both neurons and non-neuronal cells, the PDGF $\beta$  receptor is expressed primarily in pyramidal neurons in the hippocampus. (Beazely *et al.* 2009; Smits *et al.* 1991). GPCRs such as D<sub>2</sub>-class dopamine receptors can transactivate PDGF $\beta$  receptors, a rapid (3-10 min) process that leads to a phosphorylation of the PDGF receptor and activates downstream signaling pathways, including a phosphorylation of mitogen-activated protein kinase (MAPK) and inhibition of NMDA receptor currents (Beazely *et al.* 2006; Kotecha *et al.* 2002; Oak *et al.* 2001).

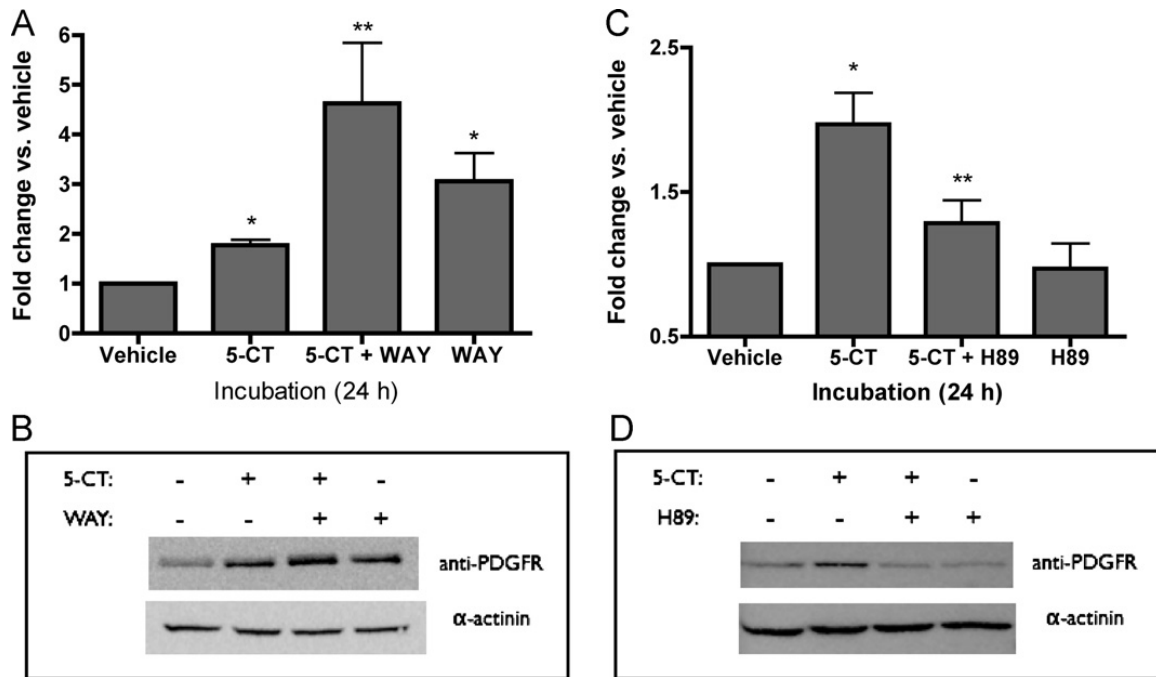
Although more subtype-selective than 5-HT, 5-CT also activates 5-HT1 and 5-HT5 receptors (Voronezhskaya *et al.* 2008). To further identify the specific 5-HT receptor subtype mediating the increase in PDGF $\beta$  receptor expression, we first examined the ability of 5-CT to increase PDGF $\beta$  receptor levels in the presence of the 5-HT1A antagonist, WAY 100635. WAY 100635, applied 30 min prior to the application of 5-CT, did not block the increase in PDGF $\beta$  receptor expression (Figure 3.2A, B). Interestingly, WAY 100635 alone increased PDGF $\beta$  receptor expression, and WAY 100635 + 5-CT together additively enhanced PDGF $\beta$  receptor expression (Figure 3.2A, B). We next incubated hippocampal cultures with the cyclic AMP-dependent protein kinase (PKA) inhibitor, H89. H89 blocked the upregulation of PDGF $\beta$  receptor by 5-CT (Figure 3.2C, 3D), suggesting that the serotonin receptor responsible for PDGF $\beta$  receptor upregulation is coupled to G $\alpha_s$  and an increase in cyclic AMP levels. Furthermore, application of the direct adenylate cyclase activator, forskolin (10  $\mu$ M) also increased PDGF $\beta$  receptor expression and activation in primary hippocampal cells (Figure 3.3A, B).



**Figure 3.1 Activation of 5-HT receptors in primary hippocampal neurons increases the expression of PDGF $\beta$  receptors.**

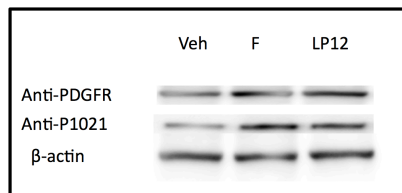
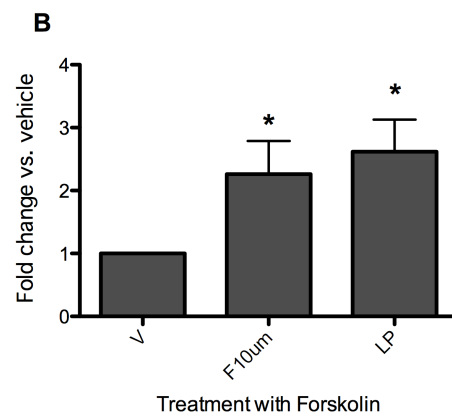
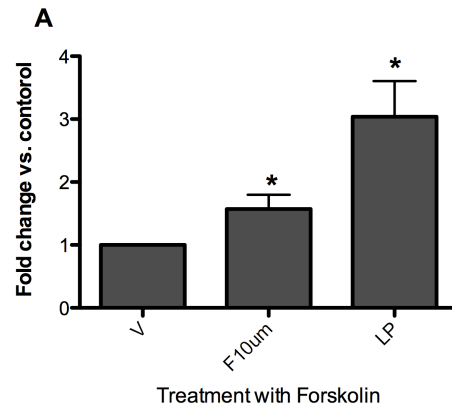
A) Primary hippocampal neurons were treated with 50 nM 5-CT or 50 nM 5-HT for 24 h. The increase in the expression level of PDGF $\beta$  receptors is expressed as a fold change vs. vehicle-treated neurons (control). Data represented the average and standard error of 6 independent experiments. \*  $p < 0.05$ , Student's unpaired t-test. B) Western blots showing PDGF $\beta$  receptor expression and  $\alpha$ -actinin expression as a loading control. C) Concentrations of 10-1000 nM 5-CT were added to hippocampal cultures for 24 h. The fold-change in PDGF $\beta$  receptor expression was determined by quantification of Western blots,  $n = 5-7$ . \*  $p < 0.05$ , ANOVA analysis with Dunnett's post-test. D) Hippocampal cultures were incubated with 50 nM 5-CT for 2-24 h. The fold-change in PDGF $\beta$  receptor expression was determined by quantification of Western blots,  $n = 5$ . (\*  $p < 0.05$ , ANOVA analysis with Dunnett's post-test).

The ability of H89, but not WAY 100635, to block 5-CT-mediated increases in PDGF $\beta$  receptor expression suggests that the G $\alpha_s$ -coupled 5-HT7 receptor may be responsible for mediating the increase in PDGF $\beta$  receptor expression. To investigate this we employed the 5-HT7 receptor-selective agonist LP 12 (Leopoldo *et al.* 2008), the 5-HT7 receptor inverse agonist, SB 269970 (Mahe *et al.* 2004b), and the neutral antagonist SB 258719 (Mahe *et al.* 2004b). Similar to 5-CT, 24 h treatment of hippocampal neurons with 300 nM LP 12 increased PDGF $\beta$  receptor expression and this increase was blocked by SB 269970 (Figure 3.4A) and by SB 258719 (Figure 3.4B). Both SB 269970 and SB 258719 also blocked 5-CT-induced increases in PDGF $\beta$  receptor expression. In addition to increasing PDGF $\beta$  receptor expression, PDGF $\beta$  receptor mRNA levels were also increased by nearly 45% in primary hippocampal neurons (Figure 3.4C). Interestingly, the time-course of LP 12 administration displayed a bimodal effect on PDGF $\beta$  receptor expression, with significant increases at 8 h and then again at 24 h (Figure 3.4D). However, The LP 12 administration time-course showed a significant increase on PDGF $\beta$  receptor expression at 24 h in the cortical culture (Figure 3.5A) and at 4h and 24 h in the SH-SY5Y cells (Figure 3.5B). LP 12 has a very high affinity for 5-HT7 receptors ( $K_i = 0.13$  nM) (Leopoldo *et al.* 2007). However we observed the greatest increases in PDGF $\beta$  receptor expression at 300 nM in both hippocampal and cortical neurons, and at 200 nM in the SH-SY5Y cells. At a concentration of 300 nM, we would expect significant binding to both 5-HT1A receptors ( $K_i = 61$  nM) (Leopoldo *et al.* 2007) as well as D2 dopamine receptors ( $K_i = 224$  nM) (Leopoldo *et al.* 2007). However the ability of both 5-CT and LP 12 to increase PDGF $\beta$  receptor expression, the inability of the 5-HT1A receptor antagonist, WAY 100635 to block these effects, and the complete inhibition of both LP-12- and 5-CT-induced increases in PDGF $\beta$  receptor expression by the 5-HT7 antagonists SB 269970 and SB 258719 suggests that the observations reported herein are indeed mediated by the 5-HT7 receptor.



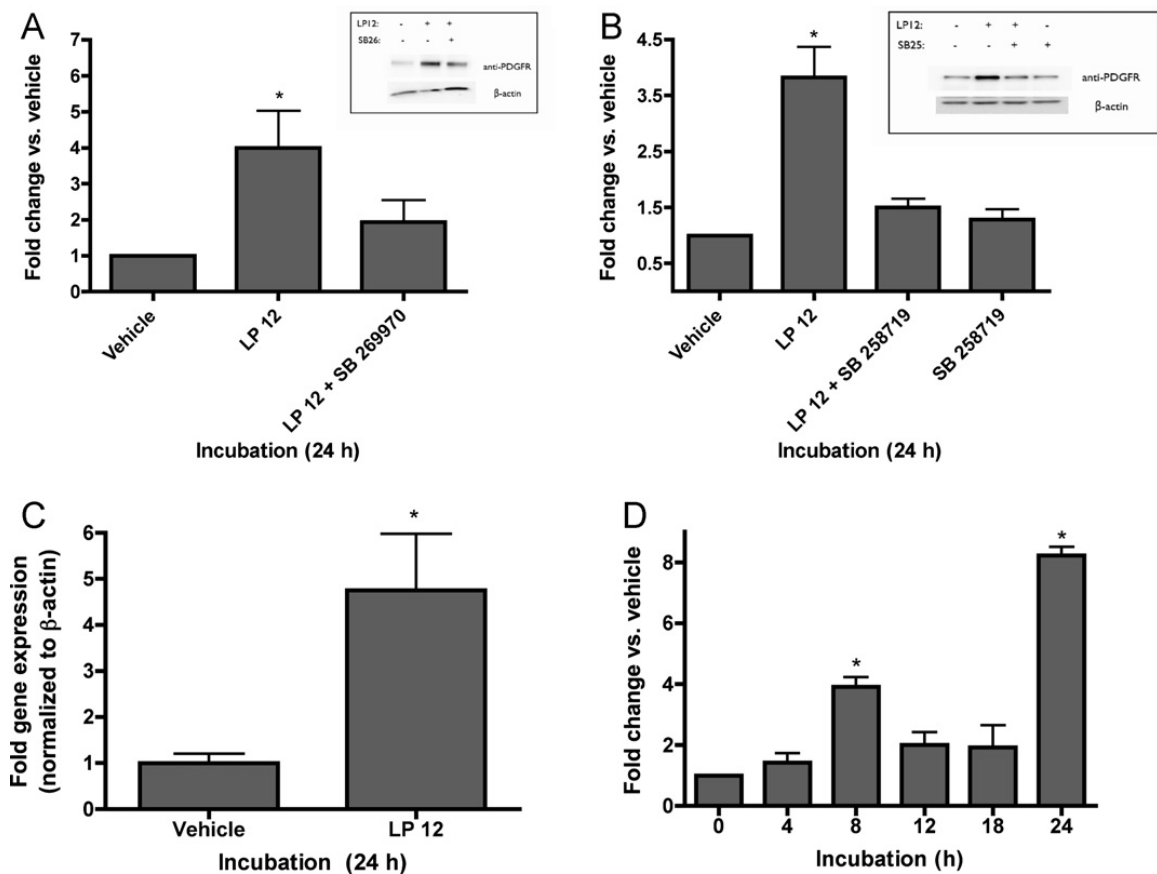
**Figure 3.2 5-CT-induced up-regulation of PDGF $\beta$  receptor is PKA-dependent and 5-HT $_{1A}$  receptor-independent.**

A-D) Cultured hippocampal neurons were incubated overnight with 5-CT (50 nM) with or without or 1  $\mu$ M WAY100635 (A, B) or 10  $\mu$ M H89 (C, D) to antagonize 5-HT $_{1A}$  receptors or to inhibit PKA, respectively. 5-CT increased PDGF $\beta$  receptor expression to control (\*  $p < 0.05$  vs. control, \*\*  $p < 0.05$  vs. 5-CT-treated cells, ANOVA analysis with Bonferroni's post-test,  $n = 8$  for experiments A, B and  $n = 5$  for experiments C, D). B and D show representative blots for the data presented in A and C.  $n = 8$  for experiments A, B and  $n = 5$  for experiments C, D.



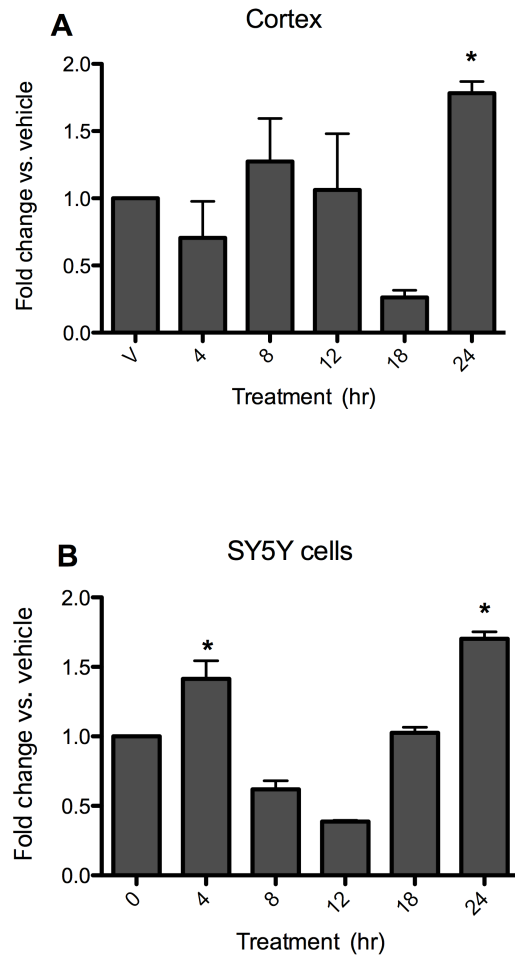
**Figure 3.3 Forskolin up regulates PDGF $\beta$  receptor expression in hippocampal culture.**

A-C) Cultured hippocampal neurons were incubated overnight with LP 12 (300 nM) and forskolin (10  $\mu$ M). Forskolin increased PDGF $\beta$  receptor expression (A) and activation at Y1021 (B) in the hippocampal culture  $n = 3$ . (\*  $p < 0.05$ , ANOVA analysis with Dunnett's post-test). C shows representative blots for the data presented in A and B.



**Figure 3.4 5-HT7 receptor activation increases PDGF $\beta$  receptor expression in primary hippocampal neurons.**

A) Hippocampal neurons were incubated for 24 h with LP 12 (300 nM) with or without or 1  $\mu$ M SB 269970, a 5-HT7 receptor inverse agonist. LP 12 increased PDGF $\beta$  receptor expression and this was reduced by co-incubation with SB 269970 ( $n = 7$ ). \*  $p < 0.01$ , ANOVA analysis with Dunnett's post-test. B) LP 12 (300 nM) was added to hippocampal cultures for 24 h in the presence or absence of the neutral 5-HT7 receptor antagonist SB 258719 (1  $\mu$ M,  $n = 5$ ). \*  $p < 0.05$ , ANOVA analysis with Dunnett's post-test. INSETS: Representative western blots showing PDGF $\beta$  receptor expression and  $\beta$ -actin as a loading control. C) Hippocampal cultures were incubated with vehicle or 300 nM LP 12 for 24 h. The mRNA was isolated using Aurum RNA Mini kit. PDGF $\beta$  receptor mRNA levels were normalized to  $\beta$ -actin. \*  $p < 0.05$ , Student's unpaired t-test. D) Hippocampal cultures were incubated with 300 nM LP 12 for 4-24 h. The fold-change in PDGF $\beta$  receptor expression was determined by quantification of Western blots,  $n = 3$ . (\*  $p < 0.05$ , ANOVA analysis with Dunnett's post-test).



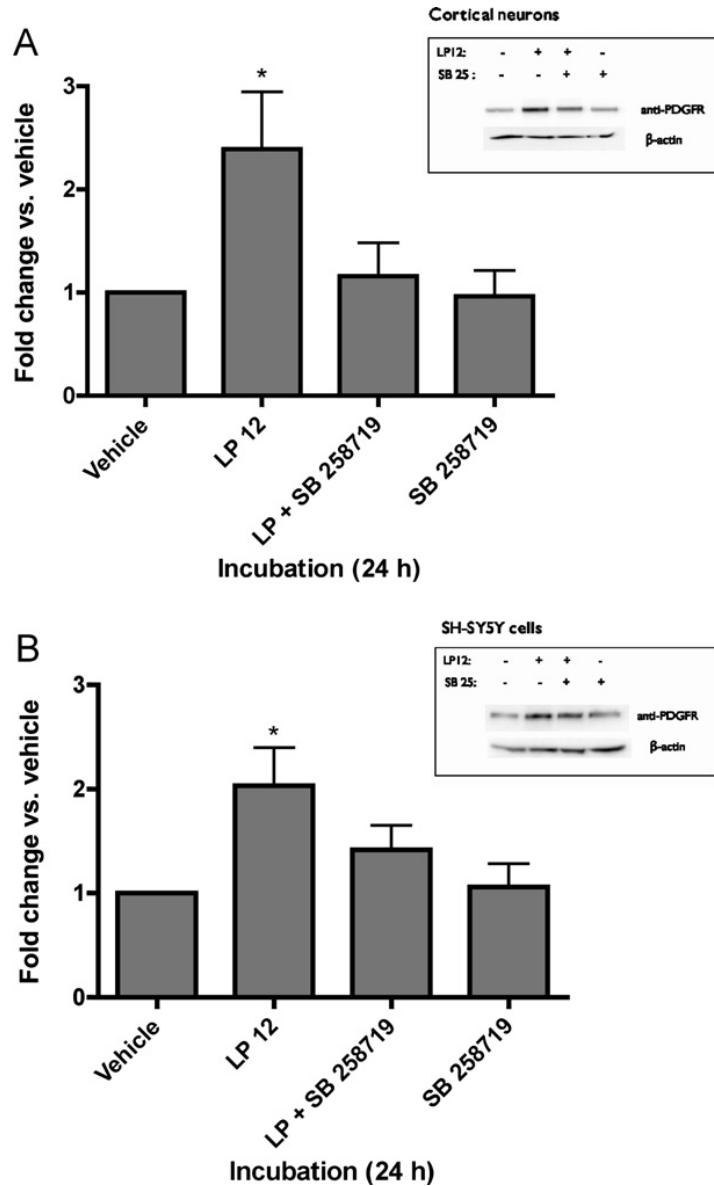
**Figure 3.5 5-HT7 receptor activation increases the expression of PDGF $\beta$  receptor in cortical culture and SH-SY5Y cells for 24 h.**

The cortical cultures (A) and SH-SY5Y cells (B) were incubated with 300 nM LP12 for 0–24 h. The fold-change in PDGF $\beta$  receptor expression was determined by quantification of Western blots,  $n = 3$  (\* $p < 0.05$ , ANOVA with Dunnett’s post-test).



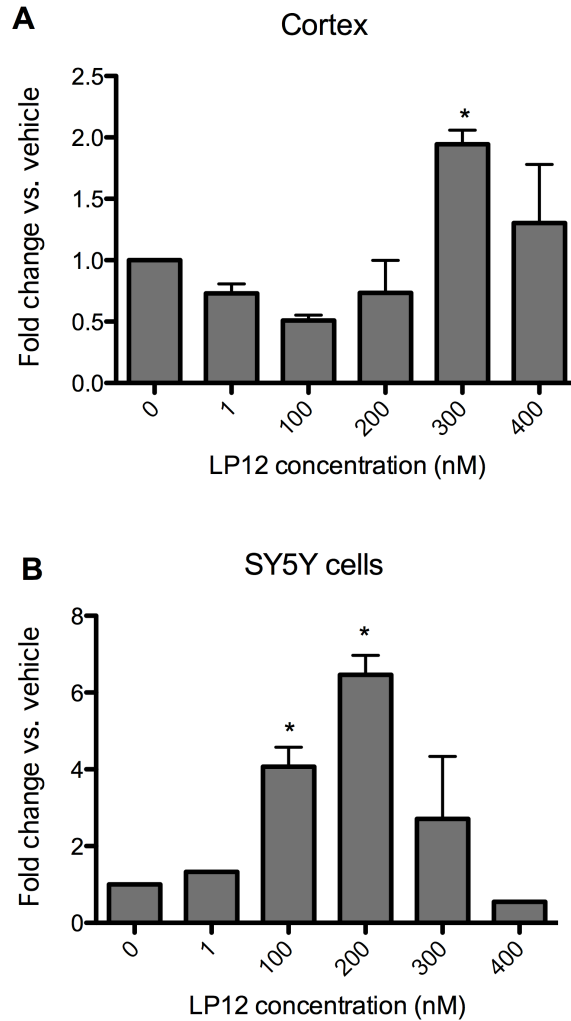
PDGF $\beta$  receptors are expressed throughout the CNS, including the hippocampus (Beazely *et al.* 2009) and the cortex (Beazely *et al.* 2009). In primary cortical neurons, 24 h incubation with LP 12 increases PDGF $\beta$  receptor expression and is blocked by SB 258719 (Figure 3.6A). Similar results were observed in the SH-SY5Y cell line (Figure 3.6B). Similar to primary hippocampal neurons, maximal effects on PDGF $\beta$  receptor expression were observed at 300 nM LP 12 in primary cortical neurons and 200 nM LP 12 in SH-SY5Y cells (Figure 3.7A, B).

PDGF $\beta$  receptors are receptor tyrosine kinases that activate numerous intracellular signaling pathways when the receptor is phosphorylated at specific tyrosine residues (Heldin *et al.* 1998). Phosphorylation of PDGF $\beta$  receptors at the PLC $\gamma$ -activating tyrosine 1021 is associated with the inhibition of NMDA receptor currents (Beazely *et al.* 2009; Lei *et al.* 1999; Valenzuela *et al.* 1996) and is one of the most robust phosphorylation sites for PDGF $\beta$  receptors transactivated by dopamine receptors in the hippocampus and prefrontal cortex (Beazely *et al.* 2006). To determine if the PDGF $\beta$  receptors, after upregulation by 5-HT $_7$  receptors, have a higher basal phosphorylation we monitored the phosphorylation state at Y1021. In addition to an increase in receptor expression, the basal Y1021 phosphorylation of PDGF $\beta$  receptors is much higher after LP 12 incubation of hippocampal cultures (Figure 3.8). Similar results were observed with 5-CT in hippocampal cultures and with LP 12 in cortical cultures and SH-SY5Y cells (Figure 3.9A, B).



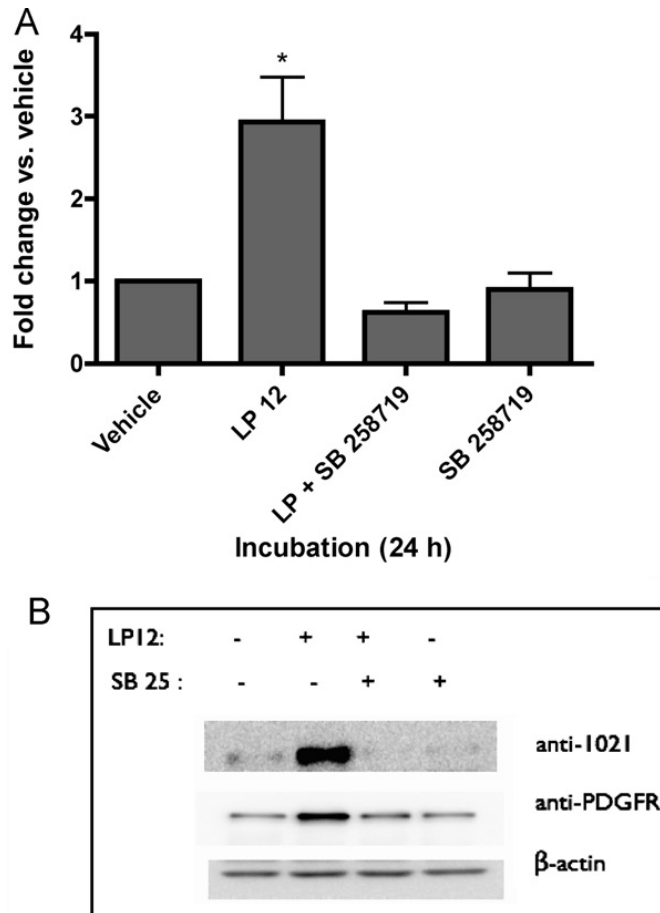
**Figure 3.6 5-HT7 receptor activation increases PDGF $\beta$  receptor expression in primary cortical neurons and SH-SY5Y cells.**

A) LP 12 (300 nM) was added to cortical cultures for 24 h in the presence or absence of the neutral 5-HT7 receptor antagonist SB 258719 (1  $\mu$ M, n = 4). \* p < 0.05, ANOVA analysis with Dunnett's post-test INSET: Representative western blots. B) LP 12 (300 nM) was added to SH-SY5Y cells for 24 h in the presence or absence of the neutral 5-HT7 receptor antagonist SB 258719 (1  $\mu$ M, n = 5). \* p < 0.05, ANOVA analysis with Dunnett's post-test INSET: Representative western blots.



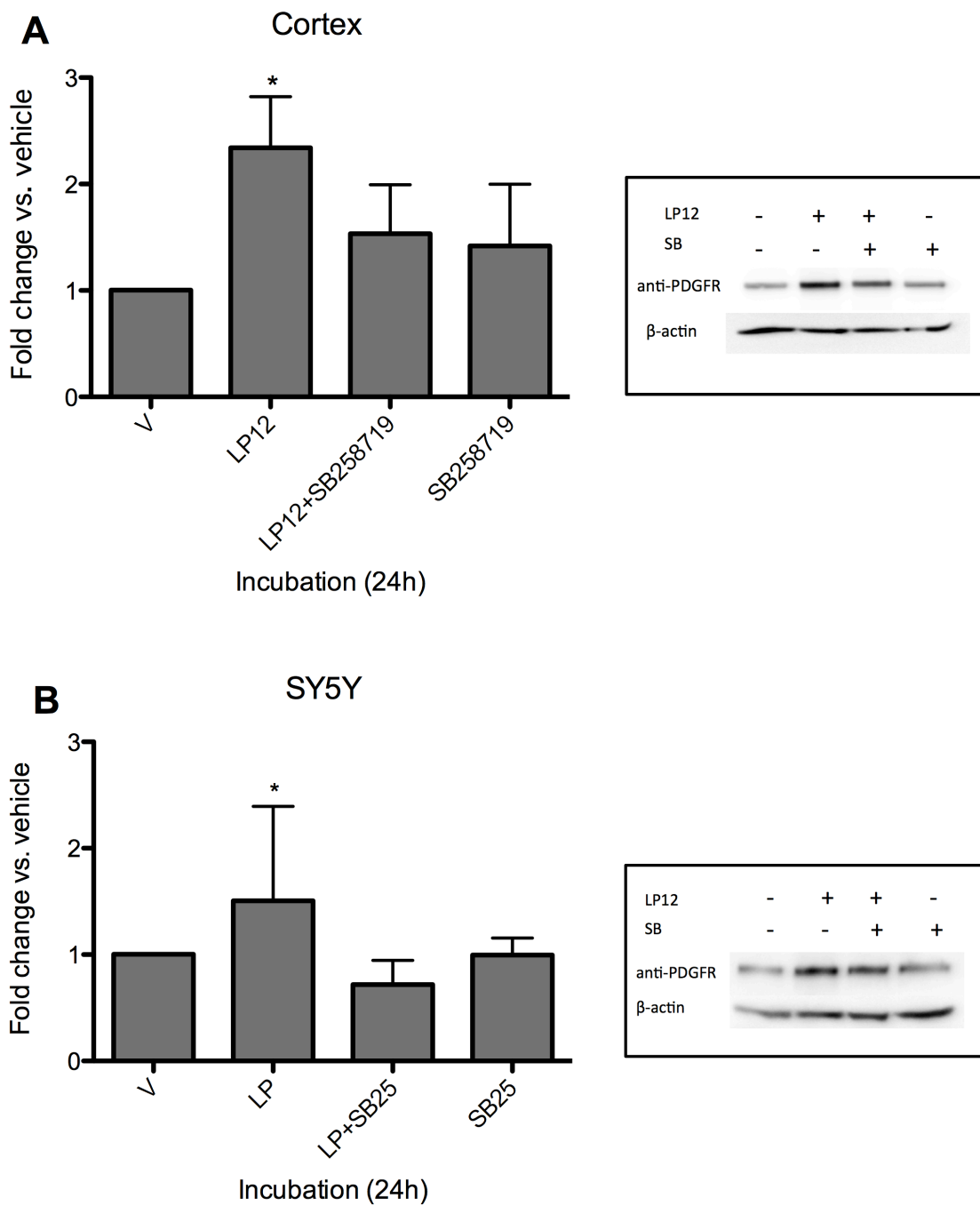
**Figure 3.7 Activation of 5-HT7 receptor in cortical culture and SH-SY5Y cell line is dose dependent.**

Concentrations of 1-400 nM LP12 were added to the cultures for 24 h. The fold-change in PDGF $\beta$  receptor expression was determined by quantification of Western blots, n = 5-7. \* p < 0.05, ANOVA analysis with Dunnett's post-test.



**Figure 3.8 The basal activity level of up-regulated PDGF $\beta$  receptors is increased.**

A, B) Cultured hippocampal neurons were incubated overnight with LP 12 (300 nM) with or without or 1  $\mu$ M SB 258719, a selective 5-HT<sub>7</sub> receptor antagonist. Western blot membranes were probed with anti-PDGFB receptor antibodies, stripped, and reprobred with anti-phospho-1021 PDGF $\beta$  receptor antibodies. Blots are representative of 7 independent experiments. \*  $p < 0.05$ , ANOVA analysis with Dunnett's post-test.



**Figure 3.9 5-HT7 receptor activation increases the expression of PDGFβ receptor in cortical culture and SH-SY5Y cells.**

The cells were incubated overnight with LP 12 (300 nM) with or without or 1 μM SB 258719, a selective 5-HT7 receptor antagonist. Western blot membranes were probed with anti-PDGFRβ receptor antibodies. Blots are representative of 8 independent experiments. \*  $p < 0.05$ , ANOVA analysis with Dunnett's post-test.

There are several lines of evidence linking the 5-HT system to growth factor receptor signaling. As noted by Andrae *et al.* in their review, “little is known about PDGF receptors transcriptional regulation” (Andrae *et al.* 2008). This report suggests that PDGF $\beta$  receptor expression, at least in hippocampal and cortical neurons and SH-SY5Y, may be cyclic AMP-dependent and regulated by 5-HT<sub>7</sub> receptors. We have identified the 5-HT<sub>7</sub> receptor as a regulator of PDGF $\beta$  receptor expression. Activation of 5-HT<sub>7</sub> receptors increases PDGF $\beta$  receptor expression and increases the basal activity of the receptor in hippocampal and cortical neurons and in the SH-SY5Y cell line.

## Chapter 4

### 5-HT7 receptor neuroprotection against NMDA-induced excitotoxicity is PDGF $\beta$ receptor-dependent

#### 4.1 Summary

The serotonin (5-HT) type 7 receptor is expressed throughout the CNS including the hippocampus. Long-term (2-24 h) activation of 5-HT7 receptors regulates growth factor receptor expression, including the expression of PDGF $\beta$  receptors. Direct activation of PDGF $\beta$  receptors in hippocampal and cortical neurons as well as primary neurons inhibits N-methyl-D-aspartate (NMDA) receptor activity and attenuates NMDA receptor-induced neurotoxicity. Our objective was to investigate whether the 5-HT7 receptor-induced increase in PDGF $\beta$  receptor expression would be similarly neuroprotective. We demonstrate that 5-HT7 receptor agonist treatment in primary hippocampal neurons also increases the expression of phospholipase C (PLC)  $\gamma$ , a downstream effector of PDGF $\beta$  receptors associated with the inhibition of NMDA receptor activity. To determine if the upregulation of PDGF $\beta$  receptors is neuroprotective, primary hippocampal neurons were incubated with the 5-HT7 receptor agonist LP 12 for 24 h. Indeed, LP 12 treatment prevented NMDA-induced neurotoxicity and this effect was dependent on PDGF $\beta$  receptor kinase activity. Treatment of primary neurons with LP 12 also differentially altered NMDA receptor subunit expression, reducing the expression of NR1 and NR2B, but not NR2A. These findings demonstrate the potential for providing growth factor-dependent neuroprotective effects using small molecule G protein-coupled receptors ligands.

#### 4.2 Introduction

Clinically used antipsychotic drugs antagonize 5-HT7 receptors and compounds with 5-HT7 receptor antagonist activity have been investigated as potential treatments for schizophrenia and

other psychoses (Uzun *et al.* 2005; Galici *et al.* 2008) and clinical trials are ongoing with novel 5-HT7 receptor antagonists, JNJ-18038683 and Lu AA21004, to treat depression (Bonaventure *et al.* 2012; Mork *et al.* 2012). Despite intense clinical research into drugs that activate or antagonize 5-HT7 receptors, very little is known about their molecular pharmacology, particularly their signaling roles in neurons. With respect to neuronal activity 5-HT7 receptors are reported to increase excitability in the hippocampus, possibly by decreasing slow afterhyperpolarizations (sAHPs) (Bacon and Beck 2000; Tokarski *et al.* 2005) or by inhibiting calcium-dependent potassium channels (Gill *et al.* 2002). 5-HT7 receptors have also been linked to the stimulation of afterdepolarizations in the thalamus (Chapin and Andrade 2001a; Chapin and Andrade 2001b). Thus, there is evidence that 5-HT7 receptors can significantly affect neuronal activity but the molecular mechanisms remain unknown.

We have demonstrated that 2-24 h 5-HT7 receptor agonist treatment increases the expression of PDGF $\beta$  receptors in primary hippocampal and cortical neurons, as well as in the SH-SY5Y cell line. The upregulated PDGF $\beta$  receptors displayed an increase in basal phosphorylation at tyrosine 1021, the PLC $\gamma$  docking site and the phosphorylation site associated with PDGF-induced inhibition of NMDA-evoked currents (Valenzuela *et al.* 1996; Beazely *et al.* 2009; Vasefi *et al.* 2012). Both the expression of PDGF $\beta$  receptors and the increase in their phosphorylation level were maximally increased after 24 h incubation, but increases were observed as early as 2 h.

The expression of the PDGF ligands, PDGF-A and -B mRNA and the PDGF-BB and -AB protein levels are increased in neurons and support cells surrounding areas damaged during ischemic events (Krupinski *et al.* 1997). In addition to PDGF $\beta$  receptor and PDGF-B chain expression being upregulated after ischemic events, exogenously-applied PDGF-BB is also neuroprotective in focal ischemia (Sakata *et al.* 1998) and against glutamate- and N-methyl-D-aspartate (NMDA)-induced cell death in hippocampal neurons (Tseng and Dichter 2005; Egawa-Tsuzuki *et al.* 2004). We have demonstrated previously that PDGF $\beta$  receptors selectively inhibit NR2B-containing



NMDA receptor currents and that PDGF $\beta$  receptor-mediated neuroprotection against NMDA-induced toxicity is associated with the inhibition of NR2B-containing NMDA receptors (Beazely *et al.* 2009). Acute treatment of hippocampal slices with PDGF-BB also selectively reduces the cell surface localization of the NR1 and NR2B NMDA receptor subunits, but not NR2A (Beazely *et al.* 2009).

There are several challenges involved in the clinical use of growth factors to treat CNS disorders. Growth factors are proteins that are relatively large (compared to small molecule drugs) which prevents both oral administration and their ability to passively cross the blood-brain barrier. Given the ability of 5-HT7 receptor agonists to upregulate the expression and basal phosphorylation of PDGF $\beta$  receptors, our objective was to determine if simply upregulating PDGF $\beta$  receptors would result in neuroprotective effects against NMDA-induced toxicity. We demonstrate that the 5-HT7 agonist LP 12 increased PDGF $\beta$  receptor expression on pyramidal neurons in primary hippocampal cultures and LP 12 treatment for 24 h prevented NMDA-induced cell death in a PDGF $\beta$  receptor-dependent manner. Interestingly, 5-HT7 receptor activation decreased the expression of NR1 and NR2B NMDA receptor subunits but not the expression of NR2A. Thus, 5-HT7 receptor activation reduces NR2B expression and increases the expression of the PDGF $\beta$  receptor, a selective inhibitor of NR2B-containing NMDA receptors.

### **4.3 Materials and Methods**

Reagents and antibodies – LP 12 (4-(2-Diphenyl)-N-(1,2,3,4-tetrahydronaphthalen-1-yl)-1-piperazinehexanamide hydrochloride), NMDA (N-methyl-D-aspartate), PDGF-BB, glycine, and other chemical reagents were purchased from Sigma (St. Louis, MO, USA). AG1296 (6,7-Dimethoxy-2-phenylquinoxaline) and Ro 25-6981 (( $\alpha$ R, $\beta$ S)- $\alpha$ -(4-Hydroxyphenyl)- $\beta$ -methyl-4-(phenylmethyl)-1-piperidinepropanol maleate) were purchased from Cedarlane (Burlington, ON). Imatinib mesylate (STI-571) (4-[(4-methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-

pyridinyl)-2-pyrimidinyl]amino]phenyl]-methanesulfonate-benzamide) was purchased from Novartis (Basel, Switzerland). The 5-HT<sub>7</sub> receptor antagonists SB 258719 ((R)-3,N-Dimethyl-N-[1-methyl-3-(4-methylpiperidin-1-yl)propyl]benzene sulfonamide) and SB 269970 ((2R)-1-[(3-Hydroxyphenyl)sulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl] pyrrolidine hydrochloride) were obtained from Tocris (Ellisville, MO, USA). Antibodies used include those raised against PDGF $\beta$  receptor (Epitomics, California, USA), PDGF $\beta$  receptor phospho-tyrosine 1021, PLC $\gamma$ ,  $\beta$ -actin (Santa Cruz, CA, USA), anti-NR1, anti-NR2A, and anti-NR2B (EMD Millipore, Billerica, MA). All secondary antibodies including Dylight 488 were obtained from Fisher (Ottawa, ON).

Primary cell culture - Hippocampal neurons were isolated from E17-19 fetuses and placed in cold dissection media (33 mM glucose, 58 mM sucrose, 30 mM HEPES, 5.4 mM KCl, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 0.34 mM Na<sub>2</sub>HPO<sub>4</sub>, 4.2 mM NaHCO<sub>3</sub>, 0.03 mM phenol red, pH 7.4, 320-335 mOsm/kg). The hippocampi were separated from the brain and digested in 0.25% trypsin/0.1% EDTA for 20 min at 37°C. The resulting cell mixtures were plated on poly-D-lysine (PDL) (Sigma) coated culture dishes and grown at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were plated in plating media (DMEM (Fisher), 10% fetal bovine serum (FBS) and 10% horse serum (HS) (Sigma)) and fed twice per week with feeding media (Neurobasal media supplemented with B27 (Life Technologies, Burlington, ON)). FUDR (0.081 mM 5-fluoro-2-deoxyuridine) and 0.2 mM uridine (Sigma) were added 3-5 days after plating for 24 h once the cells reached confluency to inhibit the growth of non-neuronal cells. Drug treatments were performed 10 to 14 days after plating.

Western blot - After drug treatment, cells were washed with chilled phosphate-buffered saline (PBS), and lysed in chilled lysis buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 30 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerophosphate, 1 mM sodium orthovanadate, and 1% Triton X-100; supplemented with Halt Protease and Phosphatase Inhibitor (Thermo, Fisher, Markham, Ontario) prior to use). For NMDA receptor subunit blotting, lysis

buffer was supplemented with 1% SDS and 1% Triton X100 to solubilize the receptors. Cells were scraped, homogenized and centrifuged at 14,000 x g for 20 min at 4°C and the supernatant was collected. Homogenates were subjected to SDS-PAGE and proteins were transferred to nitrocellulose membranes, blocked with 5% non-fat dry milk in Tris-buffered saline and 0.1% Tween-20 for 1 h at room temperature or overnight at 4°C, and incubated in primary antibodies for 1 h at room temperature or overnight at 4°C. Membranes were washed three times in Tris-buffered saline with 0.1% Tween-20, incubated with HRP-conjugated secondary antibodies for 1 h at room temperature, washed again, and bound antibodies were visualized by the enhanced chemiluminescence using Luminata Crescendo substrate (Millipore, Etobicoke, Ontario). Images of Western blots were taken on a Kodak 4000MM Pro Imaging Station, and densitometric analyses were performed using Kodak Molecular Imaging software. Membranes were then stripped and reprobed with additional antibodies.

**Immunofluorescence** - Primary hippocampal neurons were grown on sterile coverslips in Petri dishes and treated with LP 12 for 24 h. Hippocampal neurons were fixed by 4% (w/v) paraformaldehyde (Sigma) for 15 min and rinsed three times with PBS. The cultures were permeabilized using 0.3% Triton X-100 (Sigma) for 15 min and then washed with PBS. Hippocampal neurons were blocked in 4% (w/v) bovine serum albumin (Rockland, Gilbertsville, PA) for 1 h and incubated with primary antibody overnight with agitation at 4°C. The coverslips were washed three times with PBS, labeled with secondary antibody (Dylight 488) at 1:2000 (Thermo) for 1 h, mounted with Prolong Gold antifade solution with DAPI (Invitrogen, Carlsbad, CA), and visualized on Zeiss Axiovert 200 microscope and LSM 510 META software (Carl Zeiss Canada Ltd., Mississauga, ON) with 63x oil-based objective.

**MAP2 cell death assay** - 10-14-day-old hippocampal neurons on 12-well plates were pretreated for 24 h with drugs, washed, and incubated with vehicle or NMDA/glycine for 5 min. The cells were washed and returned to culture medium overnight. After 24 h, cells were fixed in 4%

paraformaldehyde in PBS, permeabilized with 0.4% Triton in PBS, blocked for 30 min with 10% bovine serum albumin in PBS, and incubated with anti-MAP2 antibodies (1:5000, Sigma) overnight at 4 °C. The following day, the cells were washed in 0.4% Triton/PBS three times and incubated with anti-mouse IgG horseradish peroxidase-conjugated antibody (Amplex Elisa Development Kit, Molecular Probes). Cells were washed three times and incubated with Amplex UltraRed substrate for 30 min. The reaction was then terminated with Amplex UltraRed Stop Solution. Absorption at 565 nm was measured and data were normalized to control samples.

MTT toxicity assay - Hippocampal neurons were plated at density of  $4 \times 10^5$  per mL in 96-well plates and grown for 10-14 days. The cells were pretreated with drugs for 24 h and exposed to NMDA/glycine for 5 min. Cells were washed and incubated in feeding media for 24 h. MTT in an amount equal to 10% of the culture medium volume was added to each well and the cells were incubated for additional 3 h at 37 °C in 5% CO<sub>2</sub>. After incubation, the resulting formazan crystals were solubilized with MTT reagent solution (10% Triton X-100 and 0.1 N HCl in anhydrous isopropanol) in each well and the absorbance was recorded at 570 nm. All results were expressed as a percent reduction of MTT relative to untreated controls.

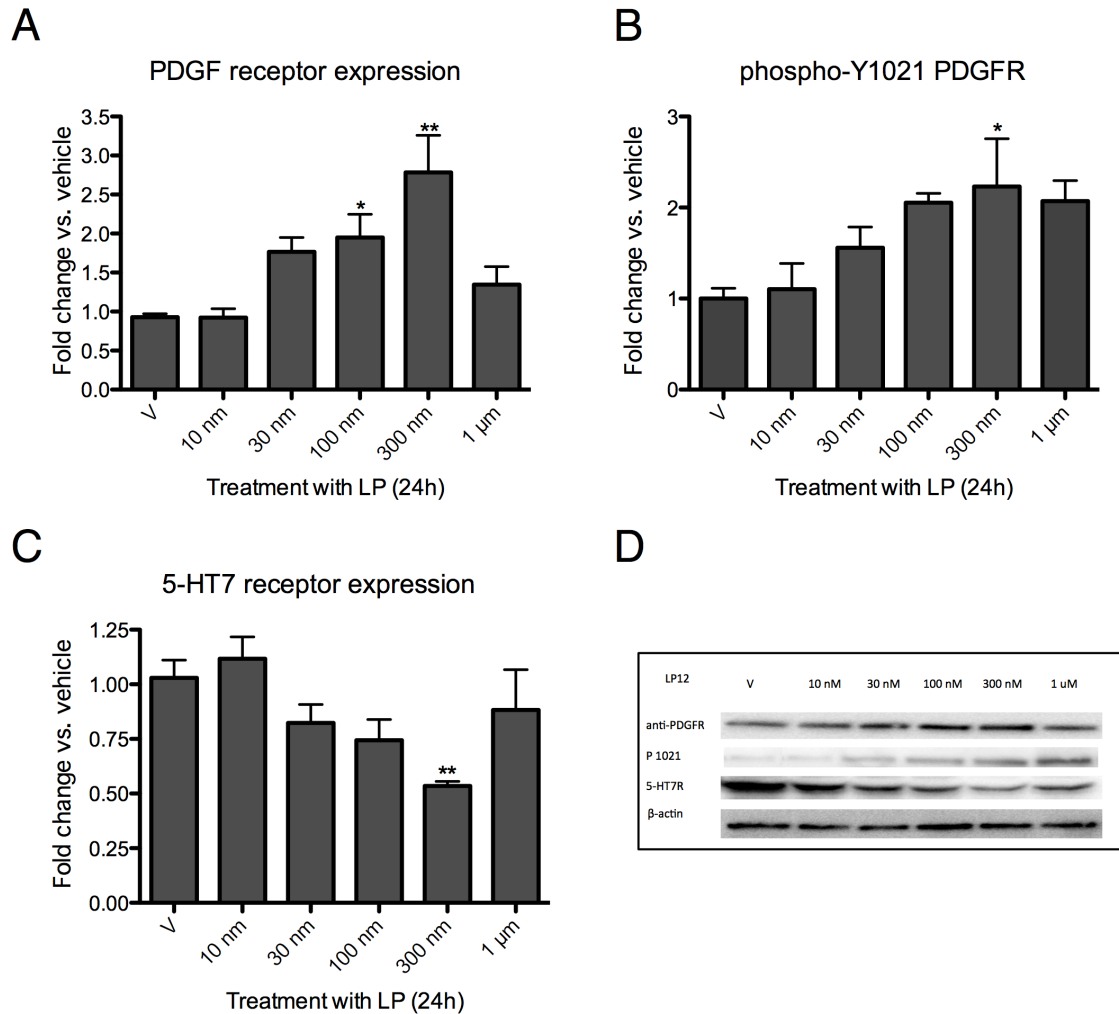
Statistical Analysis - Statistical analysis of the data was completed using Prism® GraphPad program. All data is reported as mean  $\pm$  SEM. Significance level is set at  $\alpha = 0.05$ . Data was analyzed by one-way ANOVA.

Animals - All animal experiments were performed in agreement with the guidelines of the policies on the Use of Animal at the University of Waterloo. All animal protocols were approved by the University of Waterloo animal care committee.

#### 4.4 Results

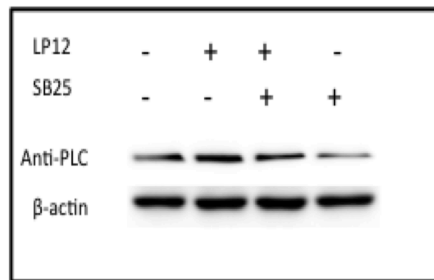
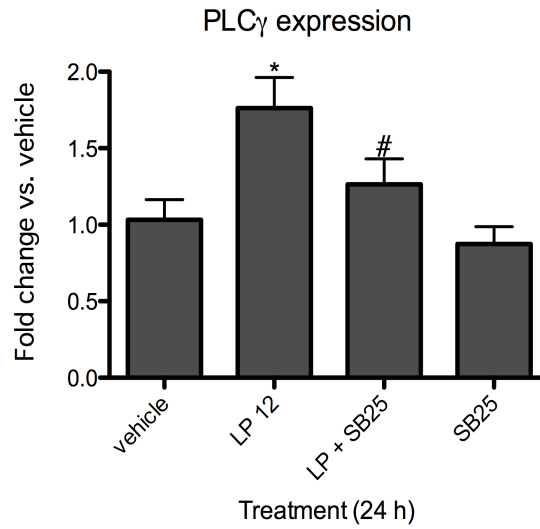
We recently reported that long-term (2-24 h) activation of 5-HT<sub>7</sub> receptors with 5-HT, 5-carboxamidotryptamine (5-CT), or the 5-HT<sub>7</sub>-selective agonist, LP 12 (Leopoldo *et al.* 2008), increased PDGF $\beta$  receptor expression in primary mouse hippocampal and cortical neurons and in SH-SY5Y cells (Figure 3.4A, B and 3.6) (Vasefi *et al.* 2012). The increase in PDGF $\beta$  receptor expression after LP 12 incubation was dose-dependent with significant increases observed at concentrations of 100 and 300 nM (Figure 4.1A, D).

The increased expression of PDGF $\beta$  receptors was accompanied by an increase in the basal PDGF $\beta$  receptor phosphorylation state at tyrosine 1021 (Figure 3.8, Vasefi *et al.* 2012) as well as other tyrosine residues (tyrosine 1009 and tyrosine 771). Similar to PDGF $\beta$  receptor expression, tyrosine 1021 phosphorylation was maximally increased at 300 nM LP 12 (Figure 3A, B). Similar to other G $\alpha_s$ -coupled receptors, 5-HT<sub>7</sub> receptors are reported to be internalized and down-regulate after exposure to agonist (Krobert *et al.* 2001). Interestingly, despite the fact that LP 12 has a reported affinity of 0.13 nM for the 5-HT<sub>7</sub> receptor, we only observed a significant reduction in 5-HT<sub>7</sub> receptor expression after 24 h treatment with 300 nM LP 12 (Figure 4.1C, D), the same concentration that maximally increased the expression of PDGF $\beta$  receptors. LP 12 also increased the expression of phospholipase C (PLC)  $\gamma$ , ERK, and CREB in primary hippocampal cultures and this increase was blocked by the 5-HT<sub>7</sub> receptor antagonist SB 258719 (1  $\mu$ M) (Figure 4.2A, B and 4.3A, C).



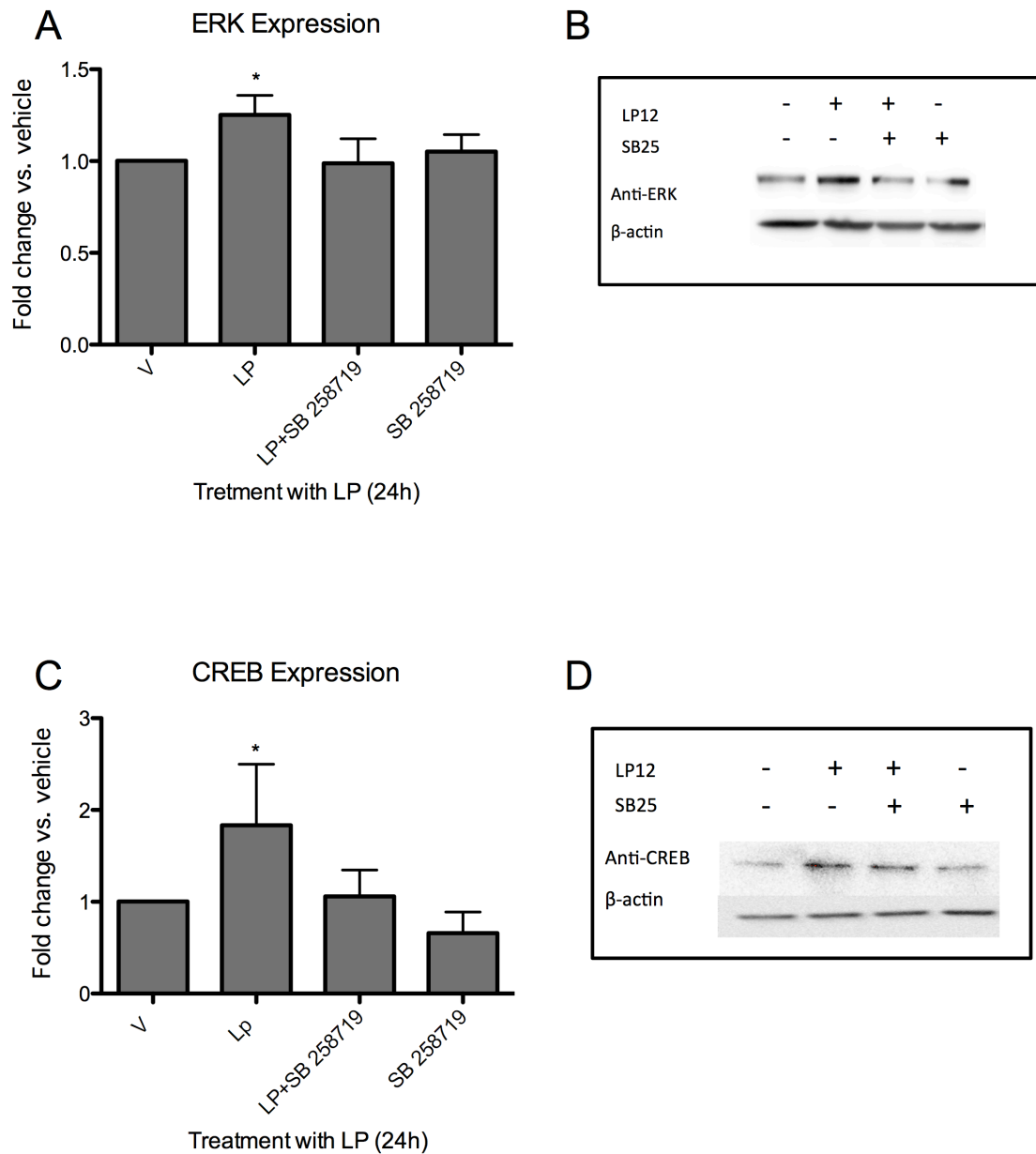
**Figure 4.1 Effect of LP 12 on PDGFβ and 5-HT7 receptor in primary hippocampal neurons.**

A) Neurons were treated with 10 nM to 1 μM LP 12 for 24 h. The level of PDGFβ receptor expression is displayed as a fold change vs. vehicle-treated neurons. Immunoreactivity for the PDGFβ receptor was normalized to β-actin. n = 3 \* p < 0.05, \*\* p < 0.01, ANOVA analysis with Dunnett's post-test. B) Neurons were treated as in A. The phosphorylation of the PDGFβ receptors was determined using an anti-phospho-Y1021 PDGFβ receptor antibody and was normalized to total PDGFβ receptor expression. n = 3 \* p < 0.05, ANOVA analysis with Dunnett's post-test. C) Neurons were treated as in A and B. Immunoreactivity for the 5-HT7 receptor was normalized to β-actin. n = 4 \*\* < 0.01, ANOVA analysis with Dunnett's post-test. D) Representative Western blots for the PDGFβ receptor, phospho-Y1021, the 5-HT7 receptor, and β-actin.



**Figure 4.2 Effect of LP 12 on PLC $\gamma$  expression in primary hippocampal culture.**

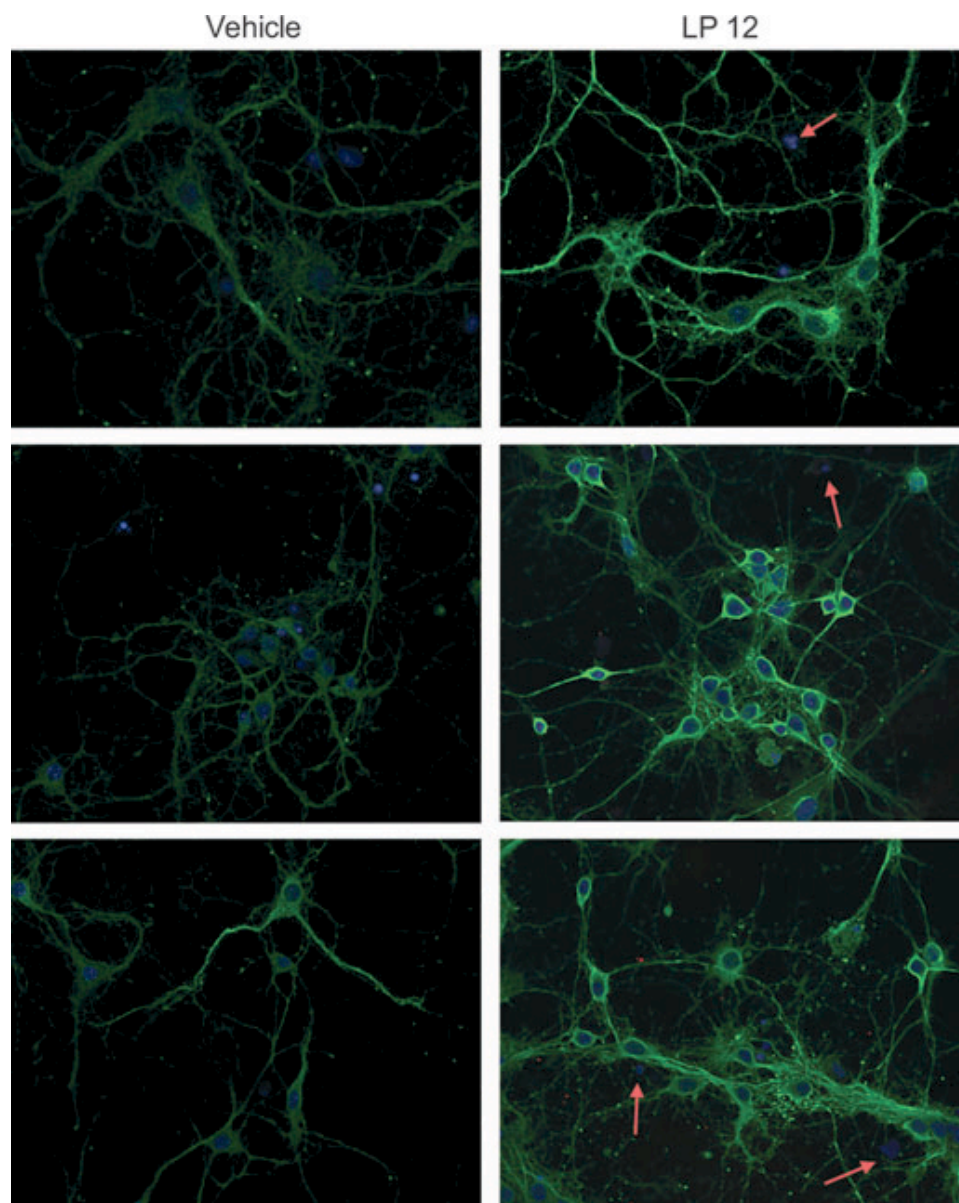
Hippocampal cultures were treated with vehicle or 300 nM LP 12, 1  $\mu$ M SB 258719, or both for 24 h. The level of PLC $\gamma$  expression is expressed as the fold change vs. vehicle-treated cells. Immunoreactivity for PLC $\gamma$  was normalized to  $\beta$ -actin,  $n = 3$  \*  $p < 0.05$  vehicle vs. LP 12, #  $p < 0.05$  LP 12 vs. LP 12 + SB 258719, ANOVA analysis with Bonferroni's post-test. Representative Western blots are shown.



**Figure 4.3 The 5-HT<sub>7</sub> receptor activation increases expression of ERK and CREB.**

The hippocampal cultures were treated with LP 12 (300nM) for 24 h with or without or 1  $\mu$ M SB 258719, a selective 5-HT<sub>7</sub> receptor antagonist. Western blot membranes were probed with anti-ERK antibodies (A, B) and anti-CREB antibodies (C, D). Blots are representative of 8 independent experiments. \*  $p < 0.05$ , ANOVA analysis with Dunnett's post-test.





**Figure 4.4 LP 12 increases PDGF $\beta$  receptor expression in pyramidal neurons in primary hippocampal cultures.**

Cells were cultured on glass coverslips and maintained at 37°C for 14-21 days *in vitro* at which time cells were treated with the control (vehicle) or with 300 nM LP 12 (LP 12) for 24 h. Nuclei were directly stained with DAPI (blue) and the PDGF receptors were detected with an anti-PDGF receptor antibody and secondary antibody conjugated to Dylight 488 (green). For the LP 12-treated images, the red arrow indicates a non-neuronal cell, which shows a DAPI-stained nucleus without PDGF $\beta$  receptor immunoreactivity. The images displayed are representative of six independent experiments.

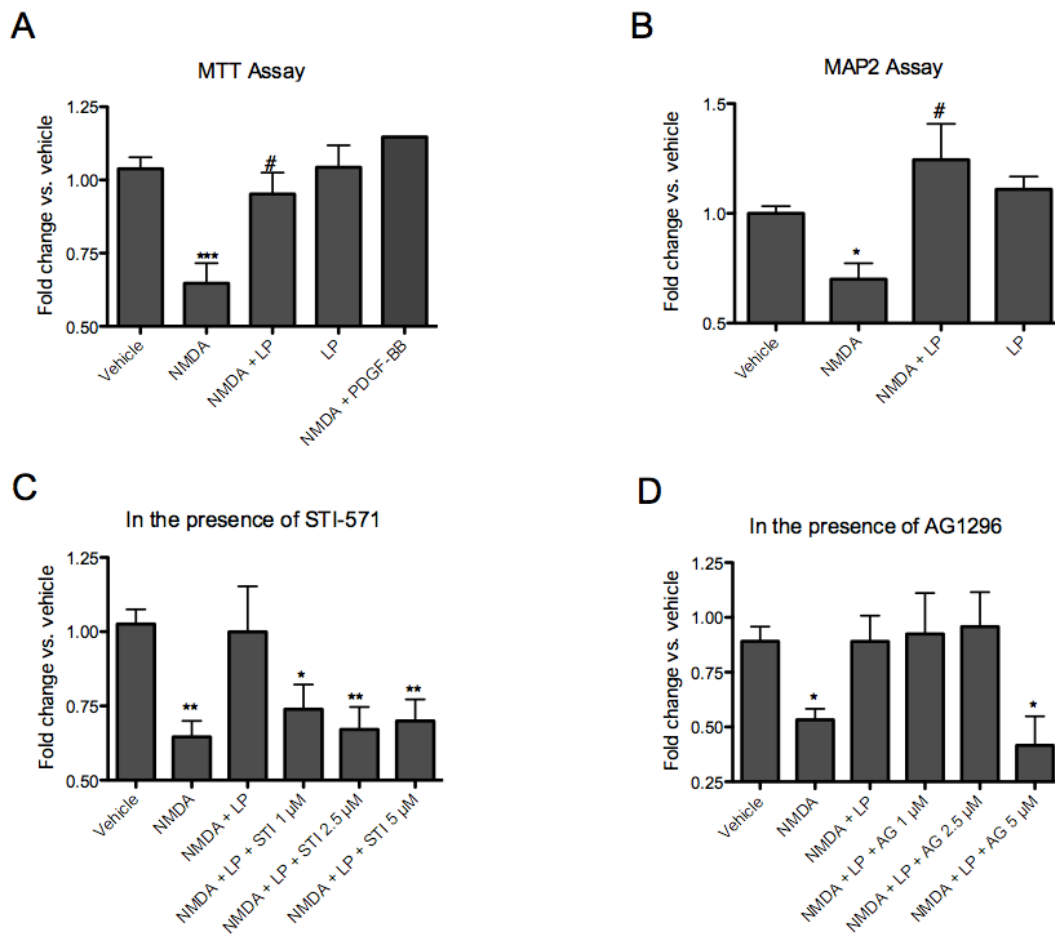
There are two PDGF receptor isoforms,  $\alpha$  and  $\beta$ , and in the hippocampus, the  $\beta$  isoform appears to be primarily expressed in pyramidal neurons (Smits *et al.* 2001; Beazely *et al.* 2009). PDGF $\beta$  receptors do not appear to significantly colocalize with PSD-95 or the NR2A subunit of the NMDA receptor, but have a relatively higher colocalization with the NR2B subunit (Beazely *et al.* 2009.). To qualitatively assess if the expression pattern of PDGF $\beta$  receptors after 24 h LP 12 treatment displayed a similar expression pattern compared to untreated cells, primary hippocampal neurons were treated for 24 h with vehicle or 300 nM LP 12, fixed, and incubated with anti-PDGF $\beta$  receptor antibodies. After LP 12 treatment, PDGF $\beta$  receptor expression was still observed primarily on pyramidal neurons compared to non-neuronal cells (Figure 4.4). Similar to our previous results, PDGF $\beta$  receptors were expressed on both neuronal cell bodies and processes.

Direct activation of PDGF $\beta$  receptors by PDGF-BB produces neuroprotective effects in cultured hippocampal neurons against glutamate- and NMDA-induced neurotoxicity (Tseng and Dichter 2005; Beazely *et al.* 2009). To determine if 24 h treatment with LP 12 could similarly promote neuroprotective effects by upregulating PDGF $\beta$  receptor expression we incubated primary hippocampal neurons for 24 h with 300 nM LP 12 followed by 100  $\mu$ M NMDA/1  $\mu$ M glycine for 10 min. 24 h post-NMDA treatment we measured the remaining number of live cells using both MTT (Figure 4.5A) and MAP2 (Figure 4.5B) assays (Carrier *et al.* 2006; Beazely *et al.* 2009). As a positive control we also treated neurons with PDGF-BB (10 ng/mL) for 10 min to directly activate PDGF $\beta$  receptors prior to NMDA treatment (Figure 4.5A). LP 12 treatment for 24 h was indeed able to prevent NMDA-induced cell death (Figures 4.5A, B). To determine if the neuroprotective effects of LP 12 required PDGF $\beta$  receptor activity we repeated the cell death assay in the absence or presence of PDGF $\beta$  receptor kinase inhibitors. STI-571 inhibits PDGF $\beta$  receptor kinase activity *in vitro* with an IC<sub>50</sub> value of  $\sim$ 100 nM (Roussidis *et al.* 2007) and

inhibits PDGF $\beta$  receptor signaling with an IC<sub>50</sub> value in the mid- $\mu$ M range (Roussidis *et al.* 2007; Weigel *et al.* 2009). STI-571 attenuated the neuroprotective effects of LP 12 at concentrations of 1 and 5  $\mu$ M (Figure 4.5C). Similarly, AG1296 prevented LP 12 induced cell death at 5  $\mu$ M, a concentrations similar to its IC<sub>50</sub> for inhibiting PDGF-induced cell proliferation (Kovalenko *et al.* 1994) (Figure 4.4D). Both STI-571 and AG1296 blocked the neuroprotective effects elicited by LP 12, suggesting that these neuroprotective effects are PDGF $\beta$  receptor-dependent.

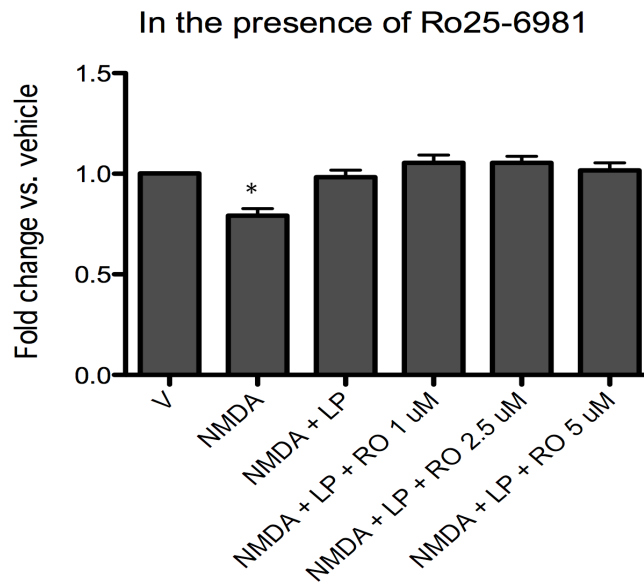
The neuroprotective effects elicited by LP 12 were similar to those observed in neurons pretreated with the NR2B subunit-selective antagonist, Ro 25-6981 (Beazely *et al.* 2009) (Figure 4.6).

5-HT<sub>7</sub> receptor activation increases PDGF $\beta$  receptor and PLC $\gamma$  expression and reduces the expression of 5-HT<sub>7</sub> receptors as shown in Figure 4.1 and 4.2. Other 5-HT receptor subtypes have been shown to differentially affect the expression of NMDA receptor subunits. For example, *in vivo*, treatment with the 5-HT<sub>6</sub> receptor ligand, SB-271047, differentially alters NMDA receptor subunit expression in the striatum (Marcos *et al.* 2010). To determine if the application of 5-HT<sub>7</sub> receptor agonists altered NMDA receptor subunit expression in primary hippocampal neurons we applied 300 nM LP 12 for 24 h. 5-HT<sub>7</sub> receptor activation reduced the expression of the NR1 and NR2B subunits but not the expression of NR2A (Figure 4.7). The changes in NR1 and NR2B expression were blocked by the 5-HT<sub>7</sub> receptor antagonist, SB 258719 (1  $\mu$ M, Figure 4.7A, B, E, F). To determine if the observed change in NR2B subunit expression was due to the activation of the PDGF $\beta$  receptor we incubated the neurons with LP 12 in the absence or presence of STI-571. STI-571 prevented LP 12-induced decreases in both NR1 and NR2B subunit expression (Figure 4.8).



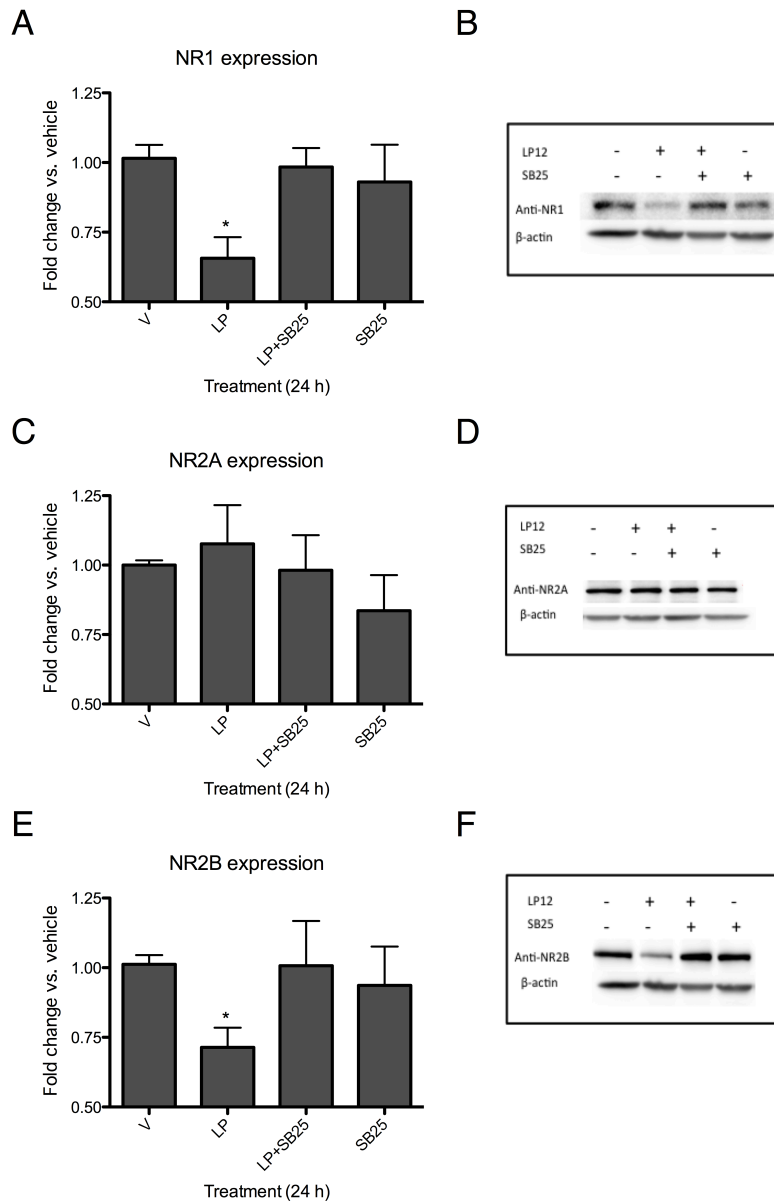
**Figure 4.5 Activation of 5-HT7 receptors is neuroprotective via PDGF $\beta$  receptor activity.**

Hippocampal cultures were pretreated with vehicle or 300 nM LP 12 for 24 h followed by 100  $\mu$ M NMDA/1  $\mu$ M glycine for 10 min. For the PDGF-BB bar, 10 ng/ml PDGF-BB was added for 10 min prior to NMDA treatment. The number of cells was determined 24 h later by both MTT (A, n = 8) and MAP2 (B, n = 7) assays. Panel A, \*\*\* p < 0.001, vehicle vs. NMDA, #, p < 0.05, NMDA vs. NMDA + LP, ANOVA analysis with Bonferroni's post-test (For PDGF-BB, the last bar in panel A is a single, representative experimental result). Panel B, \* p < 0.05, vehicle vs. NMDA, #, p < 0.01, NMDA vs. NMDA + LP, ANOVA analysis with Bonferroni's post-test. To determine if the neuroprotective effects of the 5-HT7 receptor agonist required PDGF $\beta$  receptor kinase activity, cultures were co-pretreated with 1 or 5  $\mu$ M STI-571 (C, n = 13) or 1, 2.5, or 5  $\mu$ M AG1296 (D, n = 4) and 300 nM LP 12 for 24 h followed by 100  $\mu$ M NMDA/1  $\mu$ M glycine for 10 min. Cell viability was determined 24 h later by MTT assay. Panel C and D, \* p < 0.05, \*\* p < 0.01 compared to vehicle, ANOVA analysis with Dunnett's post-test.



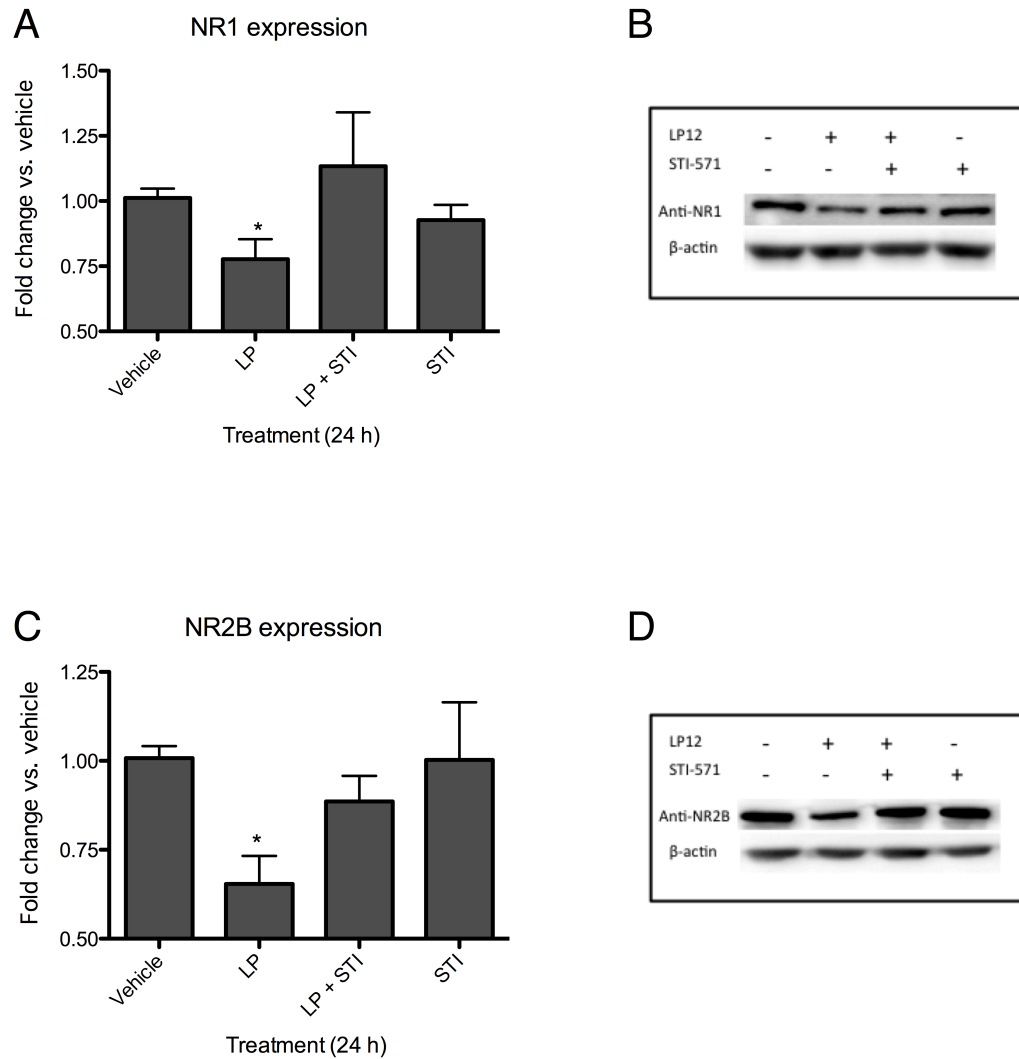
**Figure 4.6 Activation of 5-HT7 receptors is neuroprotective similar to NR2B subunit-selective antagonist, Ro 25-6981.**

To determine if the neuroprotective effects of the 5-HT7 receptor agonist is similar to NR2B subunit-selective antagonist, cultures were co-pretreated with 1, 2.5 or 5  $\mu$ M Ro25-6981, n = 7) and 300 nM LP 12 for 24 h followed by 100  $\mu$ M NMDA/1  $\mu$ M glycine for 10 min. The number of cells was determined 24 h later by MTT assay. \*  $p < 0.05$ , compared to vehicle, ANOVA analysis with Dunnett's post-test.



**Figure 4.7 The 5-HT<sub>7</sub> receptor activation alters NMDA receptor subunits expression in primary hippocampal neurons.**

A, C) Primary hippocampal neurons were incubated with vehicle or 300 nM LP 12 (LP), 1  $\mu$ M SB 258719 (SB 25), or both for 24 h. Western blots were quantified and bands were normalized to the loading control  $\beta$ -actin. Data are expressed as the fold-change in the NR subunit expression vs. vehicle. NR1 (n = 9), NR2A (n = 12), NR2B (n = 14). Representative western blots are shown for each subunit in (B, D, F). \*  $p < 0.05$ , ANOVA analysis with Dunnett's post-test.



**Figure 4.8 Inhibition of PDGF $\beta$  receptor kinase activity blocks the LP 12-induced decrease in NR2B expression.**

(A, C) Primary hippocampal neurons were incubated with vehicle or 300 nM LP 12 (LP), 5  $\mu$ M STI-571, or both for 24 h. Western blots were quantified and bands were normalized to the loading control  $\beta$ -actin. Data are expressed as the fold change in the NR1 (A, n = 4) or NR2B (C, n = 7) subunit expression versus vehicle, \* $p < 0.05$ , ANOVA analysis with Dunnett's post-test. Representative western blots are shown in (B and D).

## 4.5 Discussion

There is evidence that the brain uses the PDGF system as an endogenous neuroprotective mechanism to counter neuronal insults. Focal ischemia in rat brain causes an increase in PDGF-B mRNA that peaks at 24 h post-ischemia (Iihara *et al.* 1996). PDGF $\beta$  receptor expression also rises rapidly after ischemia in rat brain (Iihara *et al.* 1996). In addition to PDGF $\beta$  receptor and PDGF ligand being upregulated after ischemic events, exogenous PDGF-BB is also neuroprotective in focal ischemia (Sakata *et al.* 1998; Arimura *et al.* 2012) and against glutamate- and NMDA-induced cell death in cultured neurons (Tseng and Dichter 2005; Egawa-Tsuzuki *et al.* 2004; Beazely *et al.* 2009). Several studies have demonstrated that activation of synaptic pools of NMDA receptors promotes the phosphorylation of both cAMP-response element binding protein and extracellular signal-regulated kinase, and promotes cell survival, whereas the activation of extrasynaptic NMDA receptor pools promotes cell death (Soriano and Hardingham 2007). Others have suggested that it is the NMDA receptor composition, specifically the NR2 subunit, that dictates whether NMDA receptor activation will promote cell death (NR2B) or cell survival (NR2A) (Lai *et al.* 2011). We have previously demonstrated that PDGF $\beta$  receptors selectively inhibit NR2B-containing NMDA receptor currents and that PDGF $\beta$  receptor neuroprotection against NMDA-induced toxicity is similar to that observed with the NR2B antagonist Ro 25-6981 (Beazely *et al.* 2009). In addition to the effects of PDGF $\beta$  receptors on NR2B-containing NMDA receptors, additional NMDA receptor-independent neuroprotective mechanisms downstream of PDGF $\beta$  receptors have been reported. These include the activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway (Peng *et al.* 2008), increased expression of glutamate transporters on neurons and support cells (Figiel *et al.* 2003), and the involvement of transient receptor potential (TRP) C1 and TRPC6 channels (Yao *et al.* 2009a; Yao *et al.* 2009b).

In the current study, the kinase inhibitors STI-571 and AG1296 are able to prevent LP 12-induced neuroprotection and changes in NMDA receptor subunit expression. The IC<sub>50</sub> values for the



inhibition of PDGF $\beta$  receptor kinase activity *in vitro* are in the 100-nM range; however, their IC<sub>50</sub> values for inhibiting PDGF $\beta$  receptor-induced effects on, for example, cell growth, are in the low- to mid- $\mu$ M range (Kovalenko *et al.* 1994; Roussidis *et al.* 2007; Weigel *et al.* 2009). We observed a significant effect on LP 12-induced neuroprotection at 1  $\mu$ M for STI-571 and 5  $\mu$ M for AG1296 and believe that this suggests that PDGF $\beta$  receptor kinase activity is required for the effects of LP 12. However the interpretation of these results is made more difficult given the long-term (24 h) experimental design. In the current work, LP 12 and the kinase inhibitors are co-incubated over the entire 24 h period. To further complicate the interpretation, 5-HT<sub>7</sub> receptors are being desensitized/downregulated by 300 nM LP 12 (Figure 4.1C) (Krobert *et al.* 2006) and PDGF $\beta$  receptor expression and phosphorylation state are increasing (Figures 4.1A, B). Additional studies will be required to determine the temporal relationship between these events.

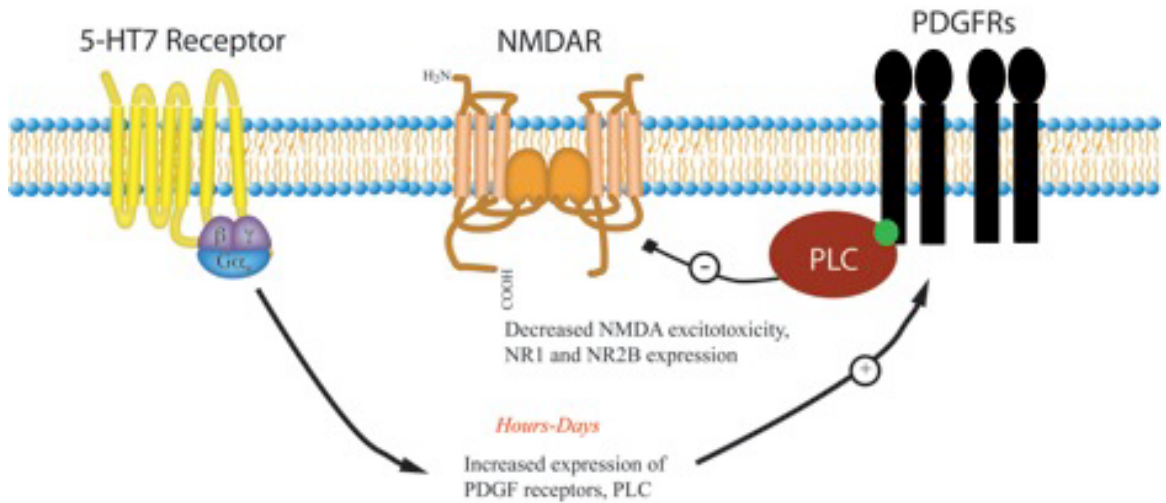
There is conflicting evidence for the effect(s) of 5-HT<sub>7</sub> receptor on neuronal and synaptic activity. There is evidence that 5-HT<sub>7</sub> receptors increase synaptic excitability in the hippocampus (Bacon and Beck 2000; Gill *et al.* 2002; Tokarski *et al.* 2005). However other reports suggest that 5-HT<sub>7</sub> receptors decrease glutamatergic signaling. Circadian phase advances in the dorsal raphe nucleus (DRN) involve an inhibition of glutamatergic neurotransmission (Duncan and Congleton 2010) and 5-HT<sub>7</sub> receptors in the DRN antagonize NMDA receptor-dependent synaptic release of both serotonin and glutamate (Harsing *et al.* 2004). The glutamate-mediated increase in intracellular calcium is inhibited by 5-HT<sub>7</sub> receptor activation in rat SCN (Smith *et al.* 2001) and the amplitude of glutamate excitatory postsynaptic potentials (EPSPs) is decreased after 5-HT<sub>7</sub> receptor activation in mouse suprachiasmatic nucleus (Quintero and McMahon 1999). Recently, Kobe *et al.* demonstrated that the activation of 5-HT<sub>7</sub> receptors increased the number of dendrites and promoted synapse formation in primary hippocampal cultures, but did not affect the magnitude of long-term potentiation (LTP). However, 5-HT<sub>7</sub> receptor knock-out mice display a reduced induction of LTP (Roberts *et al.* 2004). 5-HT<sub>7</sub> regulation of PDGF $\beta$  receptor expression

may explain the lack of clarity with respect to the effects of 5-HT7 receptors on glutamatergic signaling. In some of the studies cited above, 5-HT7 receptor agonists were applied for 5-10 min (Harsing *et al.* 2004; Quintero and McMahon 1999) whereas in other animals or tissues studies the treatment time ranged from hours to days, a time period sufficient for the upregulation of PDGF $\beta$  receptors (Duncan and Congleton 2010; Kobe *et al.* 2012). Furthermore, none of the studies cited above used selective 5-HT7 agonists and only Kobe *et al.* used a selective 5-HT7 receptor antagonist in their study.

5-HT7 receptors display inverse agonist responses when treated with several antagonists (Romero *et al.* 2006). Furthermore, 5-HT7 receptors are rapidly desensitized in recombinant systems by both agonists and antagonists (Krobert *et al.* 2006) and indeed we saw a robust reduction in 5-HT7 receptor expression after prolonged exposure to the 5-HT7 receptor agonist, LP 12. Several antipsychotic agents with affinities for the 5-HT7 receptor, including risperidone, appear to “inactivate” 5-HT7 receptors (as measured by 5-HT-stimulated cyclic AMP accumulation in 5-HT7 receptor-expressing cells) after a brief exposure to these agents (Toohey *et al.* 2009). Toohey *et al.* (2009) hypothesize that these neuroleptics induce a stable 5-HT7 conformation that prevents receptor activation of G $\alpha_s$ . However, in this study the neutral antagonist, SB 258719 did not change PDGF $\beta$  receptor or NMDA receptor expression after when used alone. Similarly, in our previous work examining the effects of LP 12 on promoting PDGF $\beta$  receptor expression, neither of the 5-HT7 receptor antagonists used altered PDGF $\beta$  receptor expression when used alone (Vasefi *et al.* 2012).

Notwithstanding the effectiveness of growth factors to protect neurons against a variety of insults *in vitro* (Beazely *et al.* 2009) and in animal models (Wu *et al.* 2004), there are several obstacles that prevent their clinical use. For example, growth factors such as PDGF are several tens of kilodaltons in size and do not readily cross the blood-brain barrier. Certain growth factors including PDGF may increase the risk of glioma induction or proliferation (Shih and Holland

2006; Ellis *et al.* 2011; Ellis *et al.* 2012b; Ellis *et al.* 2012a). Furthermore, PDGF causes several direct effects on the vasculature, including cerebral vasospasm in rats (Shiba *et al.* 2013). Could these obstacles be avoided by promoting the signaling of endogenous growth factors and their receptors in situ, rather than attempting to introduce exogenous proteins into the brain? Our results demonstrate that 5-HT7 receptor activation not only increased the expression of PDGF $\beta$  receptors, but also increased the expression of PLC $\gamma$  and caused a down-regulation of 5-HT7 receptors in primary hippocampal neurons. We demonstrated that long-term (24 h) activation of 5-HT7 receptors resulted in neuroprotective effects against NMDA-induced toxicity and that this effect was PDGF $\beta$  receptor-dependent (Figure 4.9). Furthermore, 5-HT7 agonists selectively decreased the expression of the NR2B and NR1 subunits of the NMDA receptor. The findings suggest that targeting GPCRs may be a valid strategy to exploit the neuroprotective effects of growth factor receptors in neurons.



**Figure 4.9 Schematic displaying the proposed pathway whereby 5-HT7 receptor agonists provide neuroprotection against NMDA toxicity via their effects on PDGF $\beta$  receptor-expression.**

Long-term (24 h) activation of 5-HT7 receptors increases the expression of PDGF $\beta$  receptors and PLC $\gamma$  as well as an increase in the PLC $\gamma$ -activating site on the PDGF $\beta$  receptor, Y1021 (green circle). 5-HT7 receptor activation prevents NMDA-induced toxicity and differentially regulates NMDA receptor subunit expression: NR1 and NR2B subunit expression is reduced but NR2A subunit expression remains unchanged. Both the neuroprotective effects and the effects on NMDA receptor subunit expression induced by 5-HT7 agonists are blocked by PDGF $\beta$  receptor kinase inhibitors.

## Chapter 5

### General Discussion

Twenty percent of Canadians will experience a mental illness during their lifetime (Public Health Agency of Canada, 2012). The 5-HT<sub>7</sub> receptor may represent a useful pharmacological target for the treatment of psychiatric disorders such as schizophrenia and depression (Nikiforuk 2012). Schizophrenia affects 1% of the Canadian population (Perala *et al.* 2007). Current medication includes antagonists of the dopamine D<sub>2</sub> receptor: antipsychotic drugs that prevent the positive symptoms of this disease and are much less effective at treating the negative or cognitive symptoms (Ritsner and Kurs 2002). A medical need exists for additional progress in the treatment of schizophrenia. A number of antipsychotic drugs have a high affinity for 5-HT<sub>7</sub> receptors, which indicates the possibility that some of the effects of these drugs could be mediated by 5-HT<sub>7</sub> receptors (Leopoldo *et al.* 2011). Pharmacological studies indicate that 5-HT<sub>7</sub> receptor antagonists demonstrate both antipsychotic and pro-cognitive activity in preclinical animal models and thus may have therapeutic potential for use in the treatment of schizophrenia (Horisawa *et al.* 2013; Waters *et al.* 2012); however, the potential role of 5-HT<sub>7</sub> receptors is unclear.

#### 5.1 5-HT<sub>7</sub> receptor neuroprotection

##### 5.1.1 Long-term (24 h) activation of 5-HT<sub>7</sub> receptors

PDGF $\beta$  receptors play an important role in mitogenic signaling pathways; however, direct and prolonged activation of PDGF $\beta$  receptors leads to hyperplasia and cancer (Heldin and Westermark 1999). Nevertheless, activation of PDGF $\beta$  receptors after 24 h 5-HT<sub>7</sub> agonist treatment is neuroprotective via PDGF $\beta$  receptor. The question is whether it would be possible to

achieve the beneficial, neuroprotective effects of PDGF $\beta$  receptor signaling while avoiding potential unregulated cell growth.

In addition to long-term (24 h) 5-HT<sub>7</sub> receptor-induced upregulation of PDGF $\beta$  receptors and an increase in their basal phosphorylation state, PDGF $\beta$  receptors can also be acutely transactivated by GPCRs such as the dopamine receptor D<sub>4</sub> (Nair and Sealson 2003; Oak *et al.* 2001). ERK1/2 activity promotes cell survival and proliferation and PDGF $\beta$  receptors activate ERK1/2 (Heldin and Westermark 1999). Dopamine D<sub>4</sub> and D<sub>2</sub> receptors increase ERK1/2 activity in Chinese hamster ovary CHO-K1 cells (Oak *et al.* 2001). However, Oak *et al.* (2001) determined that dopamine-induced ERK1/2 activity was blocked by selective tyrosine kinase inhibitors AG1295 and tyrphostin A9 (Oak *et al.* 2001). This finding demonstrates that dopamine transactivates PDGF $\beta$  receptors and that dopamine receptor-induced ERK1/2 activity is dependent on PDGF $\beta$  receptor activity in the CHO cells (Oak *et al.* 2001).

A recent publication from our lab demonstrated that 5-CT is able to acutely transactivate the PDGF $\beta$  receptor; however, this effect was mediated by 5-HT<sub>1A</sub>, not 5-HT<sub>7</sub>, receptors (Kruk *et al.* 2013b). In hippocampal cultures, the expression of PDGF $\beta$  receptors was increased by 5-CT after 24 h treatment, and the maximal effect was observed at a concentration of 50 nM (Figure 2.1). 5-CT activates 5-HT<sub>1</sub>, 5-HT<sub>5</sub>, and 5-HT<sub>7</sub> receptors; however, the expression of PDGF $\beta$  receptors was not blocked by WAY 100635, a 5-HT<sub>1A</sub> receptor antagonist (Figure 3.2). Rather, the WAY 100635 compound actually potentiated the effects of 5-CT. The ability of WAY 100635 to act as a potent dopamine receptor agonist (in HEK 293 cells) may explain the increased level of PDGF $\beta$  receptor expression created by WAY 100635 (Wenk 2003).

The 5-HT<sub>7</sub> receptor increases cAMP formation via G $\alpha_s$  protein (Bard *et al.* 1993; Lovenberg *et al.* 1993; Adham *et al.* 1998). Activation of the 5-HT<sub>7</sub> receptor increases cAMP levels in rabbit and human corneal epithelium (Neufeld *et al.* 1982; Crider *et al.* 2003). This effect is blocked by

SB269970, a selective 5-HT7 receptor antagonist (Grueb *et al.* 2012). 5-HT4 and 5-HT6 receptors increase cAMP levels as well; however, it is likely that the cAMP formation observed is via the 5-HT7 receptor because the 5-HT4 and 5-HT6 receptors are not expressed in the corneal epithelium and the effects are blocked by SB269970 (Pauwels 2000; Crider *et al.* 2003). The stimulation of 5-HT7 receptors with 5-CT is also positively coupled to the cAMP and PKA formation in corneal epithelial and endothelial cells (Grueb *et al.* 2012). In our studies, the PKA inhibitor H89 blocked 5-CT-mediated increases in the expression of the PDGF $\beta$  receptor, an effect that suggests that the  $G\alpha_s$  pathway is responsible for the increased expression of PDGF $\beta$  receptors in hippocampal neurons (Figure 3.2).

5-CT activates 5-HT1 ( $G\alpha_{i/o}$ -coupled), 5-HT5 ( $G\alpha_{i/o}$ -coupled), and 5-HT7 ( $G\alpha_s$ -coupled) receptors; however, our pathway suggests that 5-CT increases PDGF $\beta$  receptor expression through a  $G\alpha_s$ -coupled receptor. The selective 5-HT7 receptor agonist LP 12 increases the expression of PDGF $\beta$  receptors in hippocampal and cortical neuron cultures and in an SH-SY5Y cell line after 24 h, and this effect is blocked by both 5-HT7 receptor antagonists: SB 269970 and SB 258719 (Figure 3.4).

Figure 3.4C (rt-PCR experiment) suggests that the expression level of the PDGF $\beta$  gene and the mRNA level of the PDGF $\beta$  receptor were both increased significantly in a primary hippocampal culture after treatment with LP 12 for 24 h. In addition to the LP 12-mediated increase in expression, LP 12 treatment also increases PDGF $\beta$  receptor phosphorylation at tyrosine (Y) 1021, the PLC $\gamma$  binding site (Figure 3.8). The phosphorylation of Y1021 is associated with the inhibition of the NMDA receptor (Beazely *et al.* 2009; Lei *et al.* 1999; Valenzuela *et al.* 1996), and phosphorylation of this site is increased after dopamine receptor transactivation of the PDGF $\beta$  receptor and subsequent inhibition of NMDA-evoked currents in hippocampal and cortical slices (Kotecha *et al.* 2002; Beazely *et al.* 2006).

Reduced levels of 5-HT7 receptors in spinal nerve injury suggest that selective 5-HT7 receptor antagonists may act as analgesics with respect to nerve injury pain (Amaya-Castellanos *et al.* 2011). However, none of the studies used selective 5-HT7 receptor ligands or investigated the effects of long-term (> 24 h) treatment. Krobert *et al.* (2006) reported that 5-HT7 receptor signaling was desensitized by both agonists and antagonists (Krobert *et al.* 2006). The data presented in this work indicate that long-term (24 h) treatment with LP 12 upregulates PDGF $\beta$  receptor expression and activation but downregulates 5-HT7 receptor expression in a mouse hippocampal culture (Figure 4.1). This finding suggests that long-term treatment with a 5-HT7 receptor agonist might alleviate pain by downregulating the 5-HT7 receptor.

PDGF-BB protects neurons from NMDA-induced cell death (Beazely *et al.* 2006), and the PDGF system may decrease the risk of ischemic brain injury (Egawa-Tsuzuki *et al.* 2004). To identify whether 5-HT7 upregulated from PDGF $\beta$  receptors was neuroprotective, primary cultures were treated with LP 12 followed by NMDA/glycine treatment. The results show that LP 12 treatment did indeed prevent NMDA-induced cell death and that the neuroprotective effect of LP 12 was blocked by STI-571 and AG1296, two PDGF $\beta$  receptor kinase inhibitors (Figure 4.5).

PDGF $\beta$  receptors are expressed in primary hippocampal neurons (Smits *et al.* 2001; Beazely *et al.* 2009). Growth factors and neurotrophic factors, as therapeutic agents, cannot permeate the blood-brain barrier (BBB) because of their size, and they cannot be administered orally because of degradation in the gastrointestinal tract. In addition, hyperplasia and cancer might be promoted by high doses of exogenous growth factors such as PDGF (Heldin and Westermark 1999). Small molecules that can activate GPCRs and transactive PDGF $\beta$  receptors can permeate the BBB (Hardebo and Owman 1980). Treatment with LP 12 for 24 h increased the PDGF $\beta$  receptors on both neuronal cell bodies and cell processes but not on non-neuronal cells (Figure 4.4). 5-HT7 receptor ligands also increased the phosphorylation of PDGF $\beta$  receptors at Y1021 in neurons. However, the magnitude of the increase in PDGF $\beta$  receptor expression and phosphorylation may



not be great enough to cause hyperplasia and cancer. Thus, GPCR promotion of PDGF $\beta$  receptor signaling may allow for *in situ* promotion of PDGF $\beta$  receptor activity without many of the adverse consequences. These findings suggest that targeting GPCRs may be a strategy for exploiting the neuroprotective effects of growth factor receptors in neurons.

The findings of this research represent key contributions to the field of 5-HT receptor regulation of NMDA receptor activity. All 5-HT receptor subtypes have been reported to regulate NMDA receptors (Marcos *et al.* 2010). NMDA receptor activation is inhibited by the function of 5-HT1A receptors in several regions of the brain (Licata *et al.* 1998; Murase *et al.* 1990; Schmitz *et al.* 1995; Strosznajder *et al.* 1996; Yuen *et al.* 2008). The activation of 5-HT1 receptors leads to the inhibition of presynaptic glutamatergic transmission in the spinal cords of rats (Singer *et al.* 1996). 5-HT also increases the basal neuronal firing rate through the activation of 5-HT2 receptors and decreases the firing rate through the activation of 5-HT1 receptors (Licata *et al.* 1995). The 5-HT2B receptor is involved in presynaptic inhibition and causes several behavioural effects in the CNS. The activation of 5-HT2 receptors appears to facilitate NMDA-induced membrane depolarization; the evidence suggests that it enhances phosphoinositide hydrolysis, which leads to the production of IP3, DAG, and Ca<sup>2+</sup> (Berridge 1987; Chuang 1989; Nahorski 1988). The depolarization of NMDA using the grease-gap recording technique is enhanced by 5-HT through the action of 5-HT2 receptors (Rahman and Neuman 1993), and the effect of 5-HT2 receptors on NMDA depolarization has been examined using the agonist of 2,5-dimethoxy-4-iodoamphetamine (DOI) that is selective for the 5-HT1C and 5-HT2 agonist, m-trifluoromethylpiperazine (TEMPP), a 5-HT1B and 5-HT1C agonist, and 8-OH-DPAT, a 5-HT1A agonist.

The 5-HT4 receptor increases cAMP levels and is expressed in the hippocampal, cortical, and striatal regions. This evidence suggests that 5-HT4 has an indirect effect on NMDA receptors. 5-HT4 receptors play an important role in the regulation of the release of hippocampal

acetylcholine (King *et al.* 2008), and their activation induces neuronal excitability (Ansanay *et al.* 1996; Bockaert *et al.* 1998).

Several studies have shown that 5-HT<sub>6</sub> receptors are involved in learning and memory (Woods *et al.* 2012; Fone 2008). The activation of the 5-HT<sub>6</sub> receptor also alters NMDA receptor subunit expression in the striatum in a learning model (Marcos *et al.* 2010). The psychotomimetic effects of the NMDA receptor antagonist are attenuated by the 5-HT<sub>6</sub> receptor antagonist (Pitsikas *et al.* 2008).

NR2A subunits are expressed primarily in the synaptic area and are associated with glutamate-induced neuroprotection. NR2B subunits are expressed in the extrasynaptic area and are related to glutamate-mediated hippocampal toxicity (Beazely *et al.* 2009). PDGF $\beta$  receptors do not significantly colocalize with PSD-95, a synaptic marker, or with the NR2A subunit of NMDA receptors (Beazely *et al.* 2009), suggesting that their expression is primarily extrasynaptic.

Prolonged treatment with a 5-HT<sub>7</sub> receptor agonist changes the expression and the phosphorylation profile of NMDA receptor subunits. 5-HT<sub>7</sub> receptor activation reduced the expression of NR1 and NR2B subunits, and this effect was blocked by SB 258719 and STI-571 (Figures 4.7, 4.8). These results suggest that the neuroprotective effect of LP 12 requires the kinase activity of the PDGF $\beta$  receptor and that the 5-HT<sub>7</sub> receptor may be colocalized with the PDGF $\beta$  receptor and the NR2B-containing NMDA receptor in the extrasynaptic area. Ro 25-6981, an NR2B subunit-selective antagonist, elicits a neuroprotective effect similar to that produced by LP 12 (Figure 4.6).

In addition to increasing PDGF $\beta$  receptor expression, LP 12 treatment also increased the expression of PLC $\gamma$  in hippocampal cultures (Figure 4.2). Interestingly, the phosphorylation of the PDGF $\beta$  receptor at Y1021, the primary PLC  $\gamma$  binding and activation site, is required for the neuroprotective effect of PDGF $\beta$  receptors against NMDA-induced cell death (Figure 4.9)

(Beazely *et al.* 2009). Activation of PDGF receptors leads to a decrease in the NMDA-evoked current in hippocampal neurons (Valenzuela *et al.* 1996; Lei *et al.* 1999), and recombinant PDGF $\beta$  receptors also inhibit NMDA-evoked currents in oocytes (Valenzuela *et al.* 1996). A PLC inhibitor abolished the depression of the NMDA current in hippocampal neurons after treatment with PDGF-BB or quinpirole (Lei *et al.* 1999; Kotecha *et al.* 2002). Neuregulin receptor tyrosine kinase activation has also been reported to inhibit NMDA currents by activating a PLC-dependent pathway (Gu *et al.* 2005). These data suggest that the activation of PDGF $\beta$  receptors at Y1021 by 5-HT7 receptors may decrease NMDA currents in hippocampal neurons. Further experiments should involve an analysis of NMDA-evoked currents in neurons after LP 12 treatment.

NMDA receptors can either kill neurons or support neuronal health (Hardingham and Bading 2010). Activation of all NMDA receptors results in ERK activation followed by ERK inactivation (Chandler *et al.* 2001). Activation of synaptic NMDA receptors promotes ERK signaling pathway and increases the phosphorylation of the transcription factor CREB to promote neuroprotection (Hetman and Kharebava 2006). Extrasynaptic NMDA receptor activation induces the deactivation of ERK and CREB in hippocampal and cortical neurons and leads to neurodegeneration (Zhang *et al.* 2007). 5-HT7 receptor agonist treatment (24 h) increases both ERK and CREB expression (Figure 3.3), and these may also contribute to the neuroprotective effect of PDGF receptors in hippocampal cultures. PDGF $\beta$  receptors directly increase the phosphorylation of ERK and indirectly decrease the ability of NR2B subunits to promote ERK and CREB dephosphorylation (Beazely *et al.* 2009). The increased level of ERK and CREB expression through the activation of 5-HT7 receptors may be a result of the downregulation of NR2B-containing NMDA receptors in a hippocampal neuron culture.

### **5.1.2 Acute (5 min to 10 min) activation of 5-HT7 receptors**

In contrast to the long-term activation of 5-HT7 receptors, acute 5-CT treatment (5 min) increases NMDA-evoked currents in hippocampal neurons, and this effect has been shown to be blocked by 5-HT7 receptor antagonists (Vasefi *et al.* 2013b). The application of 5-CT and LP 12 increases NR1 receptor phosphorylation at serine 897 and serine 896 (Figure 2.1). Increases in the serine 897 PKA phosphorylation site and the serine 896 PKC phosphorylation were blocked by H89 or Go6083, respectively (Figure 2.1). Acute activation of 5-HT7 receptors promotes NMDA receptor phosphorylation and evoked currents in the hippocampal slices. However, long-term treatment with LP 12 decreases NMDA receptor expression through tyrosine kinase activity.

While 5-HT7 receptor agonists have been found to promote pain after formalin injections in animal models (Rocha-Gonzalez *et al.* 2005), others have reported that 5-HT7 receptors produce anti-nociceptive effects (Dogrul and Seyrek 2006). On the other hand, the phosphorylation of NR2B at Y1472 is linked to spinal pain transmission (Katano *et al.* 2011). In our experiments, treatment with LP 12 acutely induced NR2B phosphorylation at Y1472 (Figure 2.3) and reduced NR2A subunit phosphorylation (Figure 2.3). The 5-HT7 receptor antagonist SB 258719 inhibited these changes in NR2 subunit phosphorylation. LP 12 also reduced surface expressions of NR2B in hippocampal slices (Figure 2.4). This finding suggests that the acute activation of 5-HT7 receptors could be linked to pain transmission through the promotion of the phosphorylation of Y1472; however, the level of NR2B surface expression is decreased in the hippocampus. On the other hand, the long-term activation of 5-HT7 receptors could help reduce pain by decreasing the level of 5-HT7 receptors in neurons.

## **5.2 Conclusions**

In contrast to the long-term activation of 5-HT7 receptors, acute activation (minutes) of 5-HT7 receptors robustly enhances NMDA-evoked currents in isolated hippocampal neurons. Thus, 5-

HT7 receptors regulate NMDA receptors differently depending on short-term activation vs. long-term agonist treatment (Figure 2.5). The prolonged activation of 5-HT7 receptors may inhibit NMDA receptor function by decreasing NMDA receptor expression and upregulating PDGF $\beta$  receptors, a potent negative regulator of NMDA signaling. With respect to ischemic neuronal damage, PDGF $\beta$  receptor activation reduces neuronal death and enhances cell proliferation. This research revealed that LP 12, a selective 5-HT7 receptor agonist, provides a neuroprotective effect through the inhibition of NMDA-induced cell death. These findings highlight the potential for temporally differential regulation of NMDA receptors by 5-HT7 receptors.

This research represents a significant advance in the ongoing quest for effective treatments for mental health disorders such as schizophrenia and will improve the efficient use of drug therapies to strengthen the overall health of patients. 5-HT7 receptor ligands could possibly be used to treat a number of mental health diseases, including schizophrenia. The present study involved the identification of molecular mechanisms and pathways for neuroprotection after 5-HT7-induced PDGF $\beta$  receptor upregulation and the regulation of NMDA receptor activity. Prolonged (24 h) activation of 5-HT7 receptors increases the expression and activation of PDGF $\beta$  receptors at tyrosine 1021, the PLC $\gamma$  binding site. Such long-term activation of 5-HT7 receptors offers neuroprotective benefits by increasing the expression of a negative regulator of NMDA activity: the PDGF $\beta$  receptor. In addition, activation of the 5-HT7 receptor also selectively changes the expression and phosphorylation state of the NR2B subunit of the NMDA receptor. Interestingly, acute activation (5 min) of 5-HT7 receptors altered NMDA receptor subunit phosphorylation and increased NMDA-evoked currents in the hippocampal neurons. Thus, two distinct 5-HT7 receptor-NMDA receptor regulatory mechanisms have been identified: acute activation of the receptor increased NMDA-evoked currents whereas long-term 5-HT7 agonist treatment prevented NMDA-induced excitotoxicity in a PDGF $\beta$  receptor-dependent manner. These findings

may help to explain why previous reports have identified 5-HT7 receptors as both positive and negative regulators of NMDA receptor signaling.

### **5.3 Future direction and considerations**

#### **5.3.1 NMDA receptor currents**

The electrophysiology data demonstrate that 5-CT increased NMDA-evoked currents (5 min to 30 min) (Vasefi et al. 2013b). However, long-term LP 12 activation decreased NR2B-containing NMDA receptor expression in the hippocampal culture. It would be interesting to measure NMDA receptor miniature excitatory postsynaptic currents (mEPSCs) in hippocampal cultures subjected to 24 h LP 12 treatment. Measuring these currents during 24 h LP 12 incubation would also help to identify whether 5-HT7 receptors switch from promoting to inhibiting NMDA receptor activity over time.

With the use of the MTT assay, this research has demonstrated that, after 24 h treatment with LP 12, 5-HT7 receptors have a neuroprotective effect against NMDA. The results indicate that 5-CT and 5-HT7 receptors increase the expression and activation of PDGF receptors as early as 2 h. The 5-HT7 receptor activation could be neuroprotective from 2 h to 12 h of a 24 h incubation. For future investigation, shorter-term cell survival experiments would help to determine how long an incubation with 5-HT7 ligands would be required in order to provide neuroprotection.

In all of the above experiments, cells were treated with LP 12 for 24 h and NMDA was applied for 5 min. It would be interesting to treat the cells with NMDA prior to LP 12 treatment and the measure the neuroprotective effect of LP 12 in the neuron culture. The results of this experiment would help predict whether 5-HT7 receptor activation could improve post-injury neuronal health.

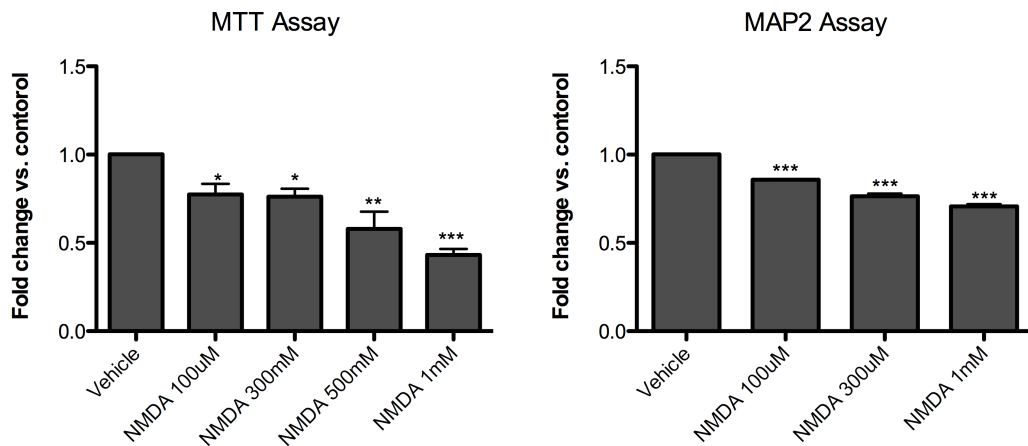
## Appendix A

### Supplementary Data

This appendix contains additional data of interest. Some these data are negative results or data that were the result of collaboration with other research groups.

#### NMDA

NMDA receptors are important in the pathogenesis of schizophrenia (Tolis *et al.* 2002). The NMDA glutamate receptor, a main subtype of the excitatory ligand-gated ion channel, has been implicated in multiple neuronal functions such as learning and memory (Dingledine *et al.* 1999).



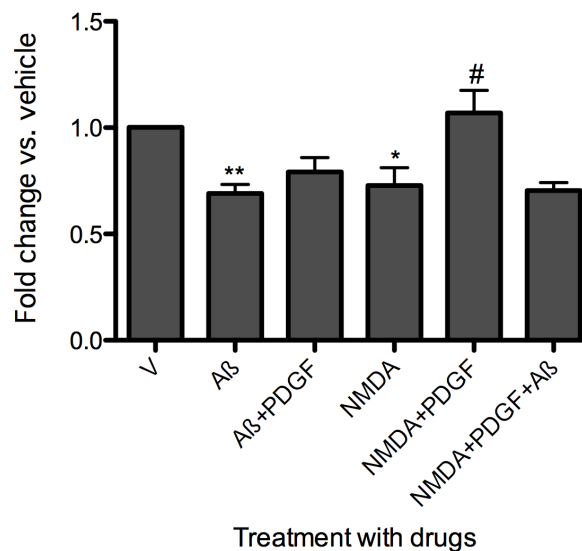
**Figure Apx A. 1 NMDA-induced cell death in hippocampal cultures.**

NMDA was used to induce cell death in a neuron culture. NMDA could kill SH-SY5Y cells, hippocampal and cortical cultures from 100 µM to 1 mM for 5 min. The hippocampal cultures were treated with 100 µM, 300 µM, and 500 µM of NMDA for 5 min. The number of live cells was counted using the MTT and MAP2 test. \*\*\* $p < 0.001$ , ANOVA analysis with Duunett's post-test.

## $\beta$ -amyloid

Alzheimer disease (AD) is a neurodegenerative disease in which  $\beta$ -amyloid plaque formation causes a decline in cognitive function and memory.  $\beta$ -amyloid plaque is toxic and responsible for pathological effects of AD (Wenk 2003). The following experiments were conducted in collaboration with Hui (Lucy) Liu and Dr. Zoya Leonenko. The results determined that  $\beta$ -amyloid attenuates the neuroprotective effects of PDGF and LP 12 against NMDA-induced excitotoxicity. I also measured the neuroprotective effects of melatonin (M) and cyclodextrin (CD) against  $\beta$ -amyloid toxicity.

### Interaction between $\beta$ -amyloid and PDGF-BB

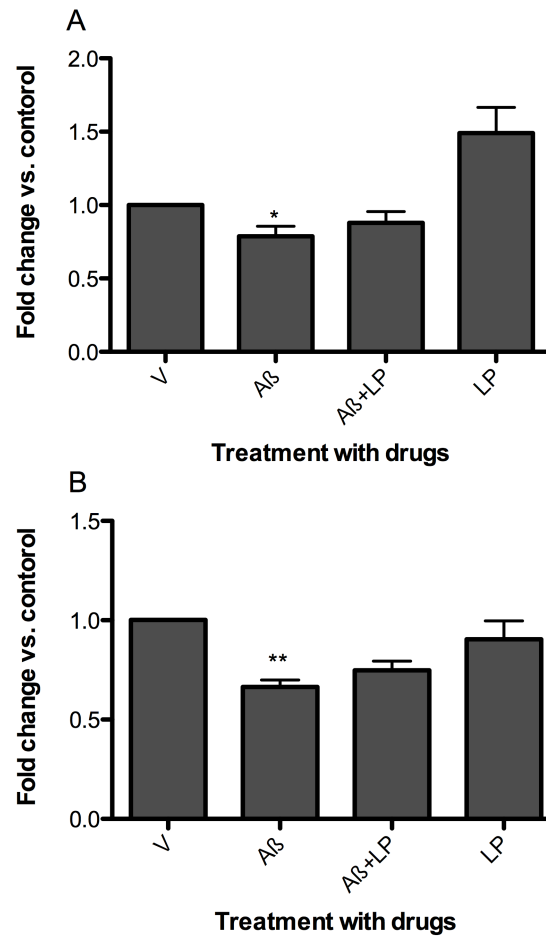


**Figure Apx A. 2  $\beta$ -amyloid inhibits PDGF-BB neuroprotection against NMDA-induced excitotoxicity.**

To determine whether PDGF-BB was neuroprotective against  $\beta$ -amyloid, the SH-SY5Y cells were treated with the vehicle or 5  $\mu$ M of  $\beta$ -amyloid (A $\beta$ ) for 10 min followed by 50 ng of PDGF-BB for 10 min. For the test represented by the NMDA bar in the figure, 100  $\mu$ M NMDA/1  $\mu$ M glycine was added for 10 min after PDGF-BB treatment. Cells were incubated with fresh serum-free media. The number of cells was determined 24 h later by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. PDGF-BB for 10 min reduced NMDA-induced cell death, and this effect was blocked by  $\beta$ -amyloid in the cell culture. \*\* $p < 0.01$ , vehicle versus NMDA, #  $p < 0.05$ , NMDA versus NMDA + PDGF-BB, ANOVA analysis with Bonferroni's post-test.



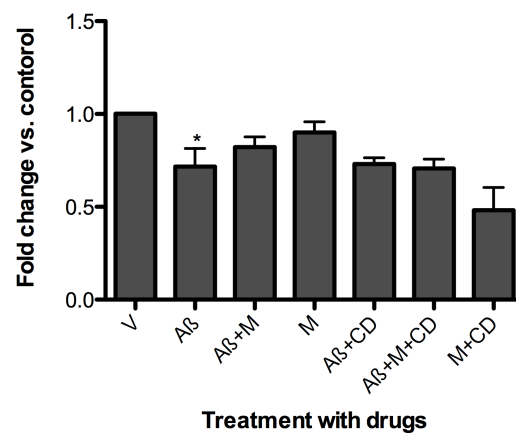
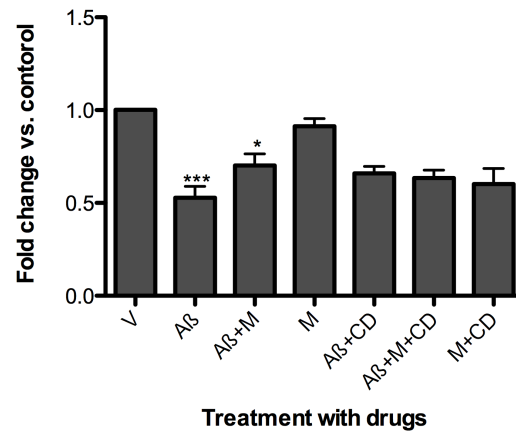
## $\beta$ -amyloid and LP 12



**Figure Apx A. 3  $\beta$ -amyloid attenuates LP 12 neuroprotection in the cell cultures.**

These data indicate that LP 12 (24 h treatment) was neuroprotective against NMDA-induced toxicity in a hippocampal culture. To determine whether LP 12 was neuroprotective against  $\beta$ -amyloid, the SH-SY5Y cells (A) and RGC cells (B) were treated for 10 min with  $\beta$ -amyloid (5  $\mu$ M), followed by 24 h of LP 12 (300 nM). Cells were incubated with fresh serum-free media. The number of cells was determined 24 h later by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. LP 12 could not protect the cells against  $\beta$ -amyloid. \*\* $p < 0.01$ , ANOVA analysis with Bonferroni's post-test.

## $\beta$ -amyloid and melatonin/cyclodextrin



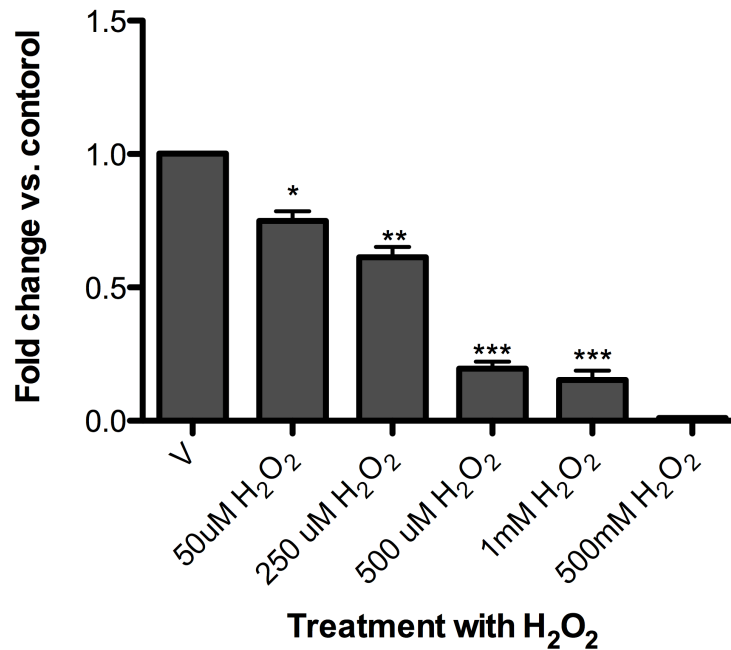
**Figure Apx A. 4 Melatonin is neuroprotective against  $\beta$ -amyloid.**

To evaluate whether melatonin (M) and cyclodextrin (CD) are neuroprotective against  $\beta$ -amyloid (A $\beta$ ), the RGC cells were treated with 5  $\mu$ M CD for 1 h. RGC cells were washed and added 5  $\mu$ M  $\beta$ -amyloid for 2 h (A) and 24 h (B). Melatonin 50  $\mu$ M were added 45 min prior to  $\beta$ -amyloid treatment. Melatonin was able to protect the RGC cells against  $\beta$ -amyloid.  $n = 8$  \* $p < 0.05$ , ANOVA analysis with Bonferroni's post-test.

## Hydrogen peroxide

In previous studies, oxidative stress was shown to be involved in the death of RGC cells and to be associated with ocular neurodegenerative disorders (Li *et al.* 2012). It has also been reported that a high level of hydrogen peroxide ( $H_2O_2$ ) caused cellular damage and cell death (Burton and Jauniaux 2011). However, D1 dopamine receptor agonists reduced  $H_2O_2$ -induced injury in RGC cells (Li *et al.* 2012). This section describes our examination of whether the 5-HT7 receptor agonist LP 12 can attenuate  $H_2O_2$  toxicity in RGC cells. These experiments were conducted in collaboration with Anshula Samarajeewa in order to determine whether LP 12 is neuroprotective against  $H_2O_2$ -induced cell death.

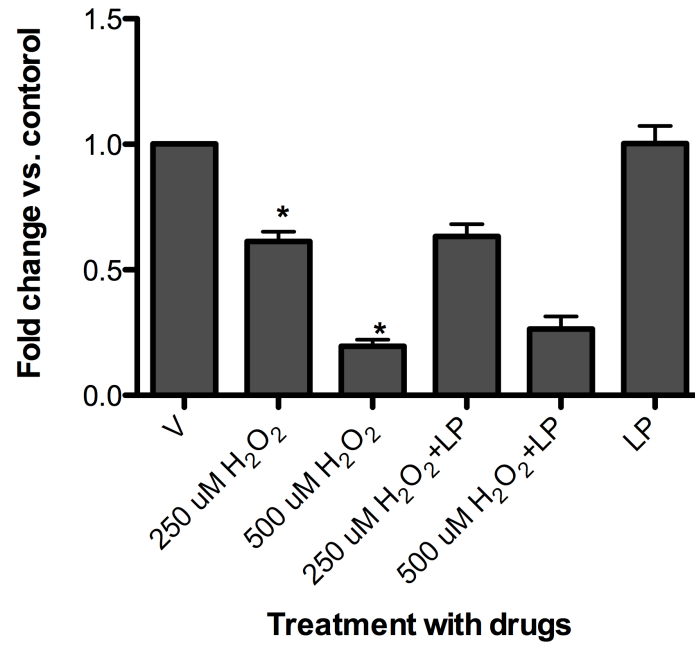
### $H_2O_2$ and RGC cells



**Figure Apx A. 5  $H_2O_2$ -induced toxicity in RGC cells.**

RGC cells were incubated with  $H_2O_2$  (50  $\mu$ M to 500 mM) for 30 min, followed by an MTT assay to measure the cell viability in the culture.  $H_2O_2$  toxicity occurred in a dose-dependent manner. N = 8 \*  $p < 0.05$ , ANOVA analysis with Bonferroni's post-test.

## H<sub>2</sub>O<sub>2</sub> and LP 12



**Figure Apx A. 6 LP 12 did not affect H<sub>2</sub>O<sub>2</sub>-induced cell death.**

RGC cells were incubated with vehicle or H<sub>2</sub>O<sub>2</sub> (30 min) and 300 nM LP 12 (24 h), or both. The number of live cells was measured using an MTT assay. These results indicate that LP 12 did not protect RGC cells against H<sub>2</sub>O<sub>2</sub>. n = 8, ANOVA analysis with Bonferroni's post-test.

## Appendix B

### Research Activity Resulting from this program

- **Selected Refereed Reports**

Jeff Kruk, **Maryam S. Vasefi**, J. J. Heikkila, and M. A. Beazely. “Reactive oxygen species are required for 5-HT-induced transactivation of neuronal platelet-derived growth factor and TrkB receptors but not for ERK1/2 activation,” *PLOS ONE*, 2013.

**Maryam S. Vasefi**, Kai Yang, Jerry Li, J. Kruk, J. J. Heikkila, M. F. Jackson, J. F. MacDonald, and M. A. Beazely. “5-HT<sub>7</sub> receptor activation increases NMDA-evoked currents in isolated hippocampal neurons,” *Molecular Brain*, 2013.

**M. S. Vasefi**, J. Kruk, J. J. Heikkila, and M. A. Beazely. “5-HT<sub>7</sub> receptor-mediated neuroprotection against NMDA-induced excitotoxicity is PDGF $\beta$  receptor-dependent,” *Journal of Neurochemistry*, 2013.

J. Kruk, **M. S. Vasefi**, H. Liu, J. Heikkila, and M. A. Beazely. “5-HT<sub>1A</sub> receptors transactivate the platelet-derived growth factor receptor type beta in neuronal cells,” *Journal of Cellular Signaling*, 2013.

Tarek Mohamed, Jacky Yeung, **Maryam S. Vasefi**, Michael A. Beazely, and Praveen P. Rao. “Development and evaluation of multifunctional agents for potential treatment of Alzheimer’s disease: Application to a pyrimidine-2,4-diamine template,” *Bioorganic & Medicinal Chemistry Letters*, 2012.

**M. S. Vasefi**, J. Kruk, H. Liu, M. A. Beazely, and J. J. Heikkila. “Activation of 5-HT7 receptors increases neuronal platelet-derived growth factor  $\beta$  receptor expression,” *Neuroscience Letters*, 2012.

- **Articles in Progress or Submitted for Publication**

Jeff Kruk, **Maryam S. Vasefi**, J. J. Heikkila, and M. A. Beazely. “Fluoxetine-induced transactivation of the platelet-derived growth factor type  $\beta$  via serotonin receptors reveals a heterologous desensitization mechanism,” *Molecular and Cellular Neuroscience* (submitted).

**Maryam S. Vasefi**, and Micheal Beazely. “Serotonin receptor subtype regulation of N-methyl-D-aspartate receptors,” *Brain Research* (in progress).

Hui Liu, Golam T. Saffi, **Maryam S. Vasefi**, Zoya Leonenko, Vince Choi, Jeff S. Kruk, and Michael A. Beazely. “The inability of PDGF-BB to protect neuronal cells from  $\beta$ -amyloid toxicity reveals a novel mechanism of  $\beta$ -amyloid toxicity” (in progress).

Lollita Goldman, Ansula Samarajeewa, **Maryam S. Vasefi**, Azita Koshmashki, Chandi..... “5-HT7 receptors upregulates TrkB in neuron models” (in progress).

- **Selected Poster Presentations**

**Maryam S. Vasefi**, and Michael A. Beazely. “Regulation of NMDA receptors by 5-HT7 receptor ligands,” SONA conference, University of Waterloo, Waterloo, May 13, 2013.

Anshula Samarajeewa, Azita Kouchmeshky, **Maryam S. Vasefi**, and Michael A. Beazely.

“Prospective intervention for Glaucoma: 5-HT7 receptor activation increases TRKB expression and phosphorylation,” SONA conference, University of Waterloo, Waterloo, May 13, 2013.

Jeff S. Kruk, **Maryam S. Vasefi**, John J. Heikkila, and Michael A. Beazely. “Transactivation of platelet-derived growth factor type beta receptors is subject to heterologous desensitization in neuronal culture,” SONA conference, University of Waterloo, May 13, 2013.

**Maryam S. Vasefi** and Michael A. Beazely. “5-HT7 receptor-induced neuroprotection is PDGF receptor-dependent,” Great Lakes GPCR Retreat conference, London, October 17-19, 2012.

Jeff J. Kruk, **Maryam S. Vasefi**, and Michael A. Beazely. “Monoamine transporter inhibitors can transactivate the platelet-derive growth factor receptor type beta in neuronal cells,” Great Lakes GPCR Retreat conference, London, October 17-19, 2012.

**Maryam S. Vasefi**, Kai Yang, and Michael A. Beazely. “5-HT7 receptor neuroprotection against excitotoxicity in the hippocampus,” AFPC, Quebec City, June 2012.

**S. M. Vasefi** and M. Beazely. “5-HT7 receptor neuroprotection against excitotoxicity in the hippocampus.” CREST, McMaster University, Hamilton, March 11-12, 2011.

Jerry Li, **Maryam S. Vasefi**, and Michael A. Beazely. “Serotonin receptor type 7 regulation of NMDA receptor subunit phosphorylation in the hippocampus,” School of Pharmacy, University of Waterloo, Kitchener, 2010.

M. A. Beazely, **S. M. Vasefi**, J. Kruk, and K. Yang. “Bidirectional regulation of NMDA receptor signaling by type 7 serotonin receptors,” First Presenter, Great Lakes GPCR Retreat conference, University of Toronto, Leslie Dan Faculty of Pharmacy, Toronto, 2010.

- **Non-Refereed Contributions**

**S. M. Vasefi**. “Bidirectional regulation of NMDA receptor signaling by type 7 serotonin receptors,” University of Waterloo Graduate Student Research Conference, Waterloo, April 27, 2011.

**S. M Vasefi**. “5-HT7 receptor neuroprotection against excitotoxicity in the hippocampus,” Ph.D. research proposal presentation, University of Waterloo, Waterloo, 2010.

**S. M Vasefi**. “Regulation of PDGF receptor expression by 5-HT type 7 receptors: A mechanism for an indirect inhibition of NMDA receptor signaling?” School of Pharmacy Journal Club presentation, University of Waterloo, Kitchener, 2010.



## Appendix C

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