Investigating the neuroprotective potential of short-term 5-HT$_7$ receptor activation against neuronal excitotoxicity

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

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Abstract

The pathophysiology of cerebral ischemia or acute ischemic stroke involves what is called neuronal "excitotoxicity", where an initial failure in intracellular energy metabolism causes excessive pre-synaptic glutamate release that over-stimulates ionotropic receptors, particularly the N-Methyl-D-aspartate (NMDA) receptor. Pharmaceutical research endeavours in the past decade that developed and tested therapeutics against glutamate neurotoxicity have exhibited dose-limiting adverse CNS effects in several large scale stroke clinical trials. Given their involvement in glutamatergic neurotransmission, serotoninergic compounds offer a method to modulate glutamate neurotoxicity without evoking unacceptable side effects.

A novel neuroprotective mechanism against NMDA induced excitotoxicity involves cross-talk between 5-hydroxytryptamine (serotonin) type 7 ($5\text{-}HT_7$) receptor and a growth factor receptor called the platelet-derived growth factor β receptor (PDGFRβ). In primary hippocampal neurons, long-term (24 hour) activation of the $5\text{-}HT_7$ receptor by small molecule ligands increase the expression of PDGFRβ that in turn phosphorylates a downstream effector which differentially regulates NMDA receptor subunit expression to prevent NMDA-induced neurotoxicity. Direct activation of platelet-derived growth factor (PDGF)β receptors in primary hippocampal and cortical neurons inhibits NMDA receptor activity and attenuates NMDA receptor-induced toxicity. This study aimed to determine if such a neuroprotective pathway could be active with short-term application of a 5-HT7 receptor agonist. Western blot analysis revealed that 2 to 4 h activation of 5-HT7 receptors in primary hippocampal neurons increased tyrosine 1021 phosphorylation (PLCγ binding site responsible for down regulating NR2B containing

NMDA receptors), as well as TrkB-FL receptor expression and phosphorylation. In addition, sustained increases in PLCγ1 expression and Akt activation were observed. Cell viability assays performed in primary hippocampal neurons showed that 2 and 4 h pre-treatment with LP 12 was neuroprotective against NMDA insults.

Similar results were observed in HT22 cells. After extensive characterization of the cell line, we determined that differentiated HT22 cells were susceptible to NMDA-induced excitotoxicity. Similar to primary neurons, HT22 cells treated with 4 h LP 12 resulted in an increase in tyrosine 1021, ERK1/2, PLCγ$_1$, and Akt threonine 308 phosphorylation. MTT viability assays in differentiated HT-22 cells suggested that neuroprotection against NMDA can be observed with 4 h LP 12 pre-treatment and that a sustained rise in 5-HT$_7$ receptor expression occurs during neuronal excitotoxicity.

These findings expand the potential role of 5-HT$_7$ receptors as a drug target beyond neuropsychiatric illnesses and provide further evidence for the possibility of producing growth factor receptor-dependent neuroprotective effects using small-molecule ligands of G protein-coupled receptors for neuronal excitotoxicity.

Acknowledgements

I would like to thank my supervisor, Dr. Michael Beazely, for his unconditional support and guidance throughout this great program. The accomplishments and milestones I have been able to achieve are a testament to his exemplary mentorship. It has been an unforgettable journey and I am glad I can rely on you for guidance, advice and friendship. My warm wishes extend to my wonderful advisory committee members, Dr. John Mielke and Dr. Marianna Foldvari who have given invaluable feedback and support throughout the course of my masters. John, your contributions and mentorship throughout my young adulthood have been invaluable--thank you for always being there, and thank you for giving me a chance. I would like to acknowledge the wonderful support system at the School of Pharmacy without whom this milestone would not have been realized: Nyasha Gondora for her diligent and persevering professional attitude, Dr. Maryam Vasefi for her mentorship and training, Dr. Jeff Kruk, and Golam Saffi and the continuous administrative support from Sarah Rae. I would like to highlight valuable contributions from Roger Chen and Spencer Berg who had a direct impact on some key experiments I performed in my masters. My sincere gratitude extends to Dr. Jamie Joseph and Dr. Murray Cutler for sharing their wisdom with me. Thank you Leonard Angka and Eric Lee for making #LoveIsOnYourSide--you guys are forever in my hearts and I am thankful, there is finally some tangible love on your side. Lastly, I thank my wonderful mum and dad for supporting my life choices and always being by my side, I love you dearly.

Dedication

To my late grandfather, Ahmed Nazir Mirza (1934-2008) aka "Superman Pa," who left us too soon after a debilitating episode of acute ischemic stroke.

Table of Contents

ix

List of Figures

Chapter 5

List of Tables

Abbreviations

5-CT, 5-carboxamidotryptamine
5-HT; 5-hydroxytryptamine or serotonin
AC, adenylyl cyclases
sAHP, afterhyperpolarization
AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BDNF, brain-derived neurotrophic factor
Cdc42, cell division control protein 42
CNS, central nervous system
CREB, cAMP response element binding protein
CaMKII, Ca2+-calmoduline-dependent protein kinase II
CDK5, cycline-dependent kinase 5
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
IP3-kinase, phosphatidylinositol 3-kinase
LTP, long-term potentiation
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
RhoA, Ras homolog gene family, member A
RTKs, receptor tyrosine kinases

Chapter 1: Introduction

This chapter begins with a brief review of all the different topics pertinent to the research question in this thesis. The scope of this chapter is to firstly introduce three key components of the research: GPCRs, RTKs, and neuronal excitotoxicity. Second, the chapter will aim to explain the cross-talk between the three components and create rationale for my hypothesis.

1.1 G-protein coupled receptors and serotonin signalling

1.1.1 G-protein coupled receptors

G-protein coupled receptors represent a large and diverse family of transmembrane receptors which all function by transducing signals from external stimuli to internal responses that may be either physiological or pathophysiological in nature [1]. All GPCRs have an extracellular amino terminus, seven membrane-spanning α-helices, and a cytosolic carboxyl terminus [1]. GPCRs are bound to a select variety of heterotrimeric G proteins (as their name suggests) in the cytosol that, upon activation, are responsible for signal transduction [1]. Heterotrimeric G proteins are composed of α, β, and γ subunits and upon activation the α subunit separates from the βγ complex and both may then interact with effector proteins [1]. Ligand binding to a GPCR incurs a conformational change of the receptor to an active state where the cytosolic guanine exchange factor domain in the GPCR results in the release of bound GDP and the binding of GTP to the Gα subunit, thus activating and separating the Gα subunit from the βγ complex [1, 2]. The intrinsic GTPase activity of the Gα subunit can terminate a transduced signal by hydrolyzing the bound GTP to GDP leading to the reattachment of the Gα subunit with the βγ

complex and subsequent reassociation with the GPCR [1, 2]. The variation in cellular and physiological consequences of G protein activation is largely a result of the effectors the $G\alpha$ subunit interacts with [2], though $G\beta\gamma$ dimers have also been shown to modulate downstream effectors [3]. To date, there are 16 known $G\alpha$ subunits, where the four most prominently found and rigorously studied are classified into four subgroups: $G\alpha_s$ (stimulate adenylate cyclase, AC), $G\alpha_{i/o}$ (inhibit AC), $G\alpha_q$ (activate phospholipase C, PLC) and $G\alpha_{12}$ (activate Rho guanine-nucleotide exchange factors, Rho GEFs) [1,2].

For the majority of GPCRs, inactivation occurs when certain threonine and serine residues on their C-terminal tails are phosphorylated, obstructing their physical association with the G protein binding domains [2]. This phosphorylation can be achieved by active protein kinase A (PKA) and protein kinase C (PKC) [4] and/or GPCR kinases (GRKs) [4]. Upon phosphorylation of GPCRs, β-arrestin molecules interact with the phosphorylation site and link the complex to clathrin-coated pits, leading to the internalization of GPCRs into endosomes [4,5]. Once internalized, the association of β-arrestins to GPCRs in endosomes determines if the receptor is recycled back to the membrane or undergoes lysosomal degradation [4,5].

1.1.2 Serotonin and serotonin receptors

5-hydroxytryptamine (5-HT) or serotonin is a monoamine isolated in the late 1940s from blood serum and was first characterized mainly as a vasoconstrictor [6]. In mammals, 5-HT is widely distributed in blood, platelets, brain and nervous tissue, gastrointestinal enterochromaffic cells and smooth muscle [6]. Interestingly, 5-HT exerts its strongest actions in the brain even though only only 1% of the body's total 5-HT is produced in the central nervous system [7].

2

Neurons that produce 5-HT (serotonergic neurons) are concentrated in the raphe nuclei of the midbrain and their fibers diffusely project to the cerebral cortex, limbic system, hypothalamus, hippocampal formation, and the spinal cord [8]. 5-HT has been recognized as a major CNS neurotransmitter that is involved in the regulation of anxiety and aggression, circadian rhythms and sleep, mood and perception, pain, and feeding behaviour [9]. Fourteen structurally and pharmacologically distinct mammalian 5-HT receptor subtypes (divided into seven main classes 5-HT_1 to 5-HT_7) have been identified to date [10]. All the 5-HT receptors are of the rhodopsin family of GPCRs with the exception of the 5-HT_3 receptor which is a ligand-gated ion channel. 5-HT_1 and 5-HT_5 are $G\alpha_i$-coupled receptors, 5-HT_2 receptors are $G\alpha_q$-coupled and finally $5\text{-HT}_{4,6\&7}$ are $G\alpha s$-coupled (stimulate AC) where the 5-HT_7 receptor has also been reported to couple with $G\alpha_{12}$ [10, 11]. Table 1 presents a summary of all 5-HT receptor G protein coupling, their major signal transduction mechanism(s), and what neurochemical event occurs (excitation or inhibition) at the level of a single neuron.

Table 1: Overview of 5-HT Receptor Subtypes As adapted from [10, 11]

5-HT Receptor Family	Gα Protein Unit Coupling	Signal Transduction Mechanism	Response at the single neuron level
5-HT$_1$	Gαi/o	Inhibits AC (decreasing cellular cAMP levels)	Inhibitory
5-HT$_2$	Gαq	Activates PLCβ to produce IP$_3$ and DAG, increasing cellular cAMP levels	Excitatory
5-HT$_3$	Ligand-gated Na$^+$ and K$^+$ cation channel	Depolarization of plasma membrane	Excitatory
5-HT$_4$	Gαs	Stimulate AC,	Excitatory
5-HT$_5$	Gαi/o	Inhibits AC	Inhibitory
5-HT$_6$	Gαs	Stimulate AC	Excitatory
5-HT$_7$	Gαs or Gα12	Stimulate AC, Stimulate Cdc42 and RhoA (involved with cytoskeleton remodelling)	Excitatory

1.2 5-HT$_7$ receptors

1.2.1 5-HT$_7$ receptor properties

The 5-HT$_7$ receptor is the most recently identified member of the 5-HT receptor family. To date 5-HT$_7$ receptors have at least five splice variants (5-HT$_{7a-e}$) in human (contains a,b, d), mouse (contains a,b,c) and rat (contains a,b,c,e) which differ in their C-terminal tail however differences in their pharmacology and/or functionality have not yet been elucidated [12-14].

One established 5-HT$_7$ receptor signalling pathway is the activation of Gα_s protein which *in vitro* can activate all AC isoforms [15]; though it has been reported that the 5-HT$_{7a}$ isoform can specifically stimulate AC1 and AC8 [16]. Upon activation of AC, there is a surge in the levels of the intracellular second messenger molecule, cyclic adenosine monophosphate (cAMP); this activates the enzyme PKA which phosphorylates downstream effector proteins [16]. PKA can catalyze the activation of several intracellular signalling cascades which include the neuroprotective extracellular signal-regulated kinases (ERK) and Akt (protein kinase B) pathways [16].

5-HT$_7$ receptors are also coupled to the Gα_{12} proteins that mainly activate Rho GEFs (among many other effectors) to ultimately initiate downstream signalling from Rho family of small GTPases (mainly Rho and Cdc42) [16]. Activation of Gα_{12} by 5-HT$_7$ receptors appears to selectively activate RhoA and Cdc42 and is involved in cytoskeletal remodelling [16]. 5-HT$_7$ receptor-dependent activation of Cdc42 has now been shown to stimulate neurite outgrowth in primary cultures of cortical, hippocampal and striatal neurons in pathways that also involve the activation of ERK and the mammalian target of rapamycin (mTOR) [17]. A recent study by

5

Kobe *et al*. in 2012 showed that $5\text{-}HT_7/G\alpha_{12}$ signalling in the hippocampus is more pronounced in cultured organotypic hippocampal slices from juvenile mice compared to older mice as measured by assessing neuronal morphology, synaptogenesis, and synaptic plasticity [18]. This same study further confirmed the strong developmental regulation of $5\text{-}HT_7/G\alpha_{12}$ signalling in the hippocampus by reporting a progressive decrease in $5\text{-}HT_7$ and $G\alpha_{12}$ protein levels in older neuronal cultures [20], further supporting their role in activity dependent structural and functional plasticity

$5\text{-}HT_7$ receptors can exist as homodimers but can also form heterodimers with the $G\alpha_i$ coupled $5\text{-}HT_{1A}$ receptors in neuroblastoma N1E-115 cells and mouse hippocampal neurons [19]. $5\text{-}HT_7/5\text{-}HT_{1A}$ heterodimerization increases internalization kinetics of $5\text{-}HT_{1A}$ receptors (thus decreased activation of $G\alpha_i$ signalling) but spares the $G\alpha_s$ signalling of $5\text{-}HT_7$ receptors [21]. The physiological consequences of $5\text{-}HT_7$ receptor heterodimer formation has not been fully investigated.

1.2.2 5-HT₇ receptor functions

Various techniques and pharmacological compounds have been used to study $5\text{-}HT_7$ receptor activation and deactivation *in vitro* and *in vivo*. The recent development of selective $5\text{-}HT_7$ agonists (AS-19, LP-44, LP-12, LP-211, and E-55888), antagonists (SB-258719, SB-269970, and SB-656104) and $5\text{-}HT_7$ receptor knockout mice have been used to study the signalling of the receptor as well as its involvement in CNS pathologies like anxiety, depression, schizophrenia, sleep disorder, epilepsy, and obsessive compulsive disorder (OCD) (see reviews [20-22]). The $5\text{-}HT_7$ receptor is expressed in both the CNS and the PNS. In the PNS, the $5\text{-}HT_7$

receptor is predominately found in smooth muscle cells and is involved with smooth muscle relaxation [23]. In the CNS, the 5-HT$_7$ receptor is extremely abundant in the cortex, hypothalamus, thalamus and the hippocampus [24]. Much information has become available regarding the physiological role of the 5-HT$_7$ receptor activation in the CNS being pertinent to regulating circadian rhythms (facilitates circadian phase shift and REM sleep) [25, 26], thermoregulation (induces hypothermia) [27, 28], and cognitive processes like memory (facilitates long and short-term memory consolidation) [20, 29-31].

Given the established presence of 5-HT$_7$ receptors in key cerebral areas coupled with the finding that several antipsychotics and antidepressants have high affinity for the 5-HT$_7$ receptor, much attention has been dedicated to elucidating the role of 5-HT$_7$ receptors in neuro-psychiatric disorders like depression, anxiety, schizophrenia and sleep disorders [32, 33]. Despite requiring further validation, pre-clinical studies completed to date support using 5-HT$_7$ receptor antagonists to ameliorate effects of schizophrenia, anxiety and especially depression. For depression, the pharmacological inhibition of the 5-HT$_7$ receptor synergistically enhances the anti-depressive effects of clinically used antidepressants [34, 35]. New individual therapies for neuropsychiatric disorders targeting the blockade of 5-HT$_7$ receptors in combination with using current antidepressants are currently under investigation [22]. 5-HT$_7$ receptor blockade is one of the key pharmacological features of vortioxetine, an atypical antidepressant that was approved by the U.S. FDA for the treatment of major depressive disorder in 2013 [36].

1.3 Neurotrophic factors and receptor tyrosine kinases (RTKs)

In general, neurotrophic factors or growth factors bind to cell surface receptors or receptor tyrosine kinases (RTKs) and initiate signaling cascades like phosphatidylinositol 3-kinase (PI3K) /Akt (cell survival), MAP Kinase (neurite outgrowth and neuronal differentiation), and PLC-γ (activity dependent plasticity) that result in the proliferation and growth of different cell types [37, 38]. Strictly speaking, the two major families of neurotrophic factors are the neurotrophin family and the glial cell line-derived neurotrophic factor (GDNF) family [38]. The neurotrophins comprise of nerve growth factor (NGF), neurotrophin-3, neurotrophin 4 (NT-3, NT-4) that bind to the tropomyosin related kinase (Trk) family of receptor tyrosine kinases A, B and C that can all activate the previously mentioned signalling cascades [38]. These particular neurotrophins can also bind to the p75 neurotrophin receptor that can initiate cell death pathways [44]. In contrast, the glial cell line-derived neurotrophic factors (GDNF) comprise of GDNF, neurturin, and persephin which all exhibit distant homology with the transforming growth factor beta (TGFβ) family of ligands [38]. They signal through a receptor complex composed of the Ret tyrosine kinase and glycosyl-phosphatidyl inositol-anchored coreceptor (GFRα family: GFRα1, α2, α3, and α4) making them potent axon-promoting growth factors *in vivo* for developing sympathetic and parasympathetic neurons [38].

Any growth factor that promotes neuronal survival and growth could be described as a neurotrophic factor. Binding of the neurotrophic factor causes receptors to dimerize, resulting in the intracellular kinase catalytic domains coming into close proximity and becoming phosphorylated [39]. The phosphorylation of the kinase domain activation site leads to the trans-

autophosphorylation of several other tyrosine kinase residues in the intracellular tail, allowing the phosphorylated tyrosine residues to become docking sites for signaling enzymes and adaptor proteins, which predominately initiate pro-survival proliferative signaling cascades mentioned earlier [39]. Based on this classic definition, factors known for their hormone functions and peripheral effects like insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) should also be considered in the scheme of neurotophic factors which act on RTKs. Two important growth factors are relevant to this proposed research: platelet-derived growth factor (PDGF) and brain-derived neurotrophic factor (BDNF) which bind to RTKs called PDGF receptors (PDGFRs) and tropomyosin-receptor kinase (Trk) neurotrophic receptors, respectively.

1.3.1 Platelet derived growth factor receptors

PDGFs are powerful mitogens that act on a range of cell types which include CNS neurons, smooth muscle, fibroblasts and vascular endothelial cells [39]. PDGFs have been divided into four subtypes (A to D) with PDGF-A and PDGF-B being the most extensively researched [37]. PDGFs are typically found as homodimers . PDGF-BB can bind to PDGFβ receptors (PDGFRβ) whereas the α receptor binds all but PDGF-DD [37]. The work presented in this thesis is pertinent to the PDGFβ receptors given their exclusive expression in pyramidal neurons of the hippocampus [40] (it should be noted that PDGFα receptors do express in the hippocampus albeit showing higher expression in non-neuronal cells [37]). Upon PDGFβ receptor activation, the trans-autophosphorylation of several tyrosine residues on the cytoplasmic

9

tails serve as docking sites for some key signalling enzymes and adaptors that generally initiate proliferative signalling (see Table 2). PDGFβ receptors are found in caveolae and ligand activation can also quickly internalize the receptor in a clathrin-dependent manner [39, 41].

The physiological role of the PDGFβ receptor is critical in mammalian development and normal function. PDGFβ receptor knockout mice display severe perinatal defects that include kidney failure, cardiovascular defects, limited ability to repair wounds, and pulmonary abnormalities [42]. In the CNS, PDGF signalling is required for proliferation of oligodendrocytes and the production of myelin [37]. Conversely, excessive activity of the PDGFβ receptor activity is implicated in atherosclerosis and fibrosis as well as tumor formation and progression [43].

1.3.2 Neurotrophins and TrkB receptors

Neurotrophin binding to Trk receptors predominantly promotes survival and maintenance of neurons especially during pathophysiological CNS events that cause neuron damage [43]. Each Trk receptor subtype has different affinities for different neurotrophins: TrkB receptors can bind BDNF, NT-3, or NT-4 [44], while TrkA receptors bind NGF, and TrkC receptors bind NT-3 [43]. Similar to PDGFRβ, the TrkB receptor dimerizes upon ligand binding leading to kinase domain activation and transautophosphorylation of its intracellular tyrosine residues that act as docking sites for signalling enzymes and adapter proteins (see Table 3).

Three alternatively spliced isoforms of the TrkB receptor in humans are responsible for much of the neurotrophin signalling. The first isoform is the full length (TrkB-FL) receptor that dimerizes upon ligand binding and is responsible for intracellular signalling. The other two isoforms are the truncated TrkB-T1 and TrkB-Shc which have a ligand binding and

transmembrane domain but lack the intracellular kinase domain. Despite being truncated, TrkB-T1 and TrkB-Shc are able to modulate the activity of the full-length TrkB receptor by forming heterodimers with the full length receptor and also by competing for BDNF [38, 45]. Synaptic activity that causes a strong post-synaptic calcium ion influx (such as in long-term potentiation) can cause clathrin-dependent internalization of BDNF bound TrkB receptors [46]. Additionally, and much like the PDGFβ receptor, ligand binding also causes clathrin-dependent internalization of TrkB receptors but internalization does not mean deactivation given internalized TrkB receptors still participate in downstream signalling [47].

Table 2. PDGFβ receptor signalling as adapted from [39]

Signaling Molecule	RTK phosphorylation site	Pathways activated and signaling consequences
PDGFβ receptor kinase domain activation site	Y857	Activation of receptor kinase activity and trans-autophosphorylation of other tyrosine residues on the cytoplasmic tail.
PLCγ1	Y1021 and Y1009	Ca^{2+} signaling, mitagenicity and in some cell migration, inhibition of NMDA and GABAA receptors
PI3K	Y740 and Y751	Akt/PKB pathway
Grb2	Y716 and Y775	Sos/Ras/MAPK pathway
Grb7	Y716 and Y775	Migration of neuronal cells in embryo
Stat5	Y579, Y581 and Y775	Activation and translocation to nucleus leads to gene expression related to proliferation, differentiation and apoptosis
Shc	Y579, Y740, Y751, Y771	Grb2/Ras/MAPK pathway
Nck	Y751	Proliferation
SHP-2	Y1009 and Y763	Negatively: dephosphorylates autophosphorylation site Tyr740/Tyr751/Tyr771. Positively: Grb2/Ras/MAPK pathway, Src/MAPK pathway

Table 3. TrkB receptor signaling as adapted from [48]

Signaling Molecule	RTK phosphorylation site	Pathways activated and signaling consequences
Shc which then binds Grb2/SOS	Y515	Activation of the *MAPK* signalling cascade promoting neuronal differentiation and growth through extracellular signal-regulated kinase (ERK).
		Activation of the phosphatidylinositol 3-kinase (PI3K) cascade promoting survival and growth of neurons through Ras or GRB-associated binder 1 (GAB1).
PLCγ1	Y816	Generation of inositol-1,4,5-trisphosphate (Ins(1,4,5)P3) (IP3) and diacylglycerol(DAG).
		DAG stimulates protein kinase C (PKC) isoforms and IP3 promotes the release of Ca2+ from internal stores and subsequent activation of Ca2+/calmodulin (Ca2+/CaM)-dependent protein kinases (CaMKII, CaMKK and CaMKIV).

1.4 GPCR regulation of RTKs

Apart from the ligand-dependent activation of RTKs described previously, several studies to date have shown that dimerization and transautophosphorylation of RTKs can occur in a ligand-independent manner. The most common type of ligand-independent RTK activation is GPCR-induced transactivation. Examples of GPCR mediated activation of RTKs outside the nervous system include angiotensin II (agonist of angiotensin receptor II) activating PDGFβ receptors in vascular smooth muscle cells [49], thrombin (agonist of protease-activated receptor) activating insulin growth factor-I receptor (IGFR-1) in aortic smooth muscle cells [50] and endothelin-1 or lysophosphatidic acid (LPA) or thrombin induced activation of epidermal growth factor receptor (EGFR) in rat-1 fibroblasts [51] (see review [52] among many others).

In the nervous system, agonists of GPCRs can also activate RTKs in a transient fashion i.e., short exposure of GPCR to agonist bringing about a short-lived activation of a RTK. Some prominent examples include dopamine 4 (D4) and the dopamine 2 (D2) receptor stimulation activating PDGFRs in Chinese hamster ovary (CHO-K1) cells [53]. Activation of 5-HT$_{1A}$ receptor with 5-HT transactivates PDGFRβ and TrkB in human SH-SY5Y neuroblastoma cell line as well as mouse primary cortical neurons [54, 55]. Similarly, D2-class dopamine receptor transactivation of PDGF receptors in *ex vivo* hippocampal neurons [56].

1.4.1 5-HT$_7$ regulation of PDGFβ receptor

Research continues to clearly elucidate the physiological relevance and exact mechanisms of ligand-independent transactivation of RTKs [55]. A related series of studies described in Beazely lab (University of Waterloo School of Pharmacy) suggest that long-term (2-24 h) exposure of 5-HT$_7$ receptors to 5-HT, 5-carboxamidotryptamine (5-CT) or 5-HT$_7$ receptor selective agonist (LP 12) increase PDGFRβ expression and basal phosphorylation at the PLCγ binding site in primary mouse hippocampal, and cortical neurons as well as the SH-SY5Y neuroblastoma cell line [57]. Figure 1 (adopted from [62]) shows that 24 hour treatment of primary hippocampal neurons with 300 nM LP 12 increased PDGFRβ receptor expression; an increase that was blocked by SB 269970 (an inverse 5-HT$_7$ receptor agonist) and SB 258719 (neutral 5-HT$_7$ receptor antagonist) (see Figure 1A and B). Similar increases in PDGFRβ expression were seen after 24 h treatment of primary cortical neurons and SH-SY5Y neuroblastoma cells by 300 nM LP 12 (Figure 1C and D). Accordingly, Vasefi *et al.,* (2012) investigated if LP 12 was able to increase PDGFRβ expression as well as phosphorylation at the

PLCγ binding site at shorter time frames (4 h to 18 h). In primary mouse hippocampal neurons, a significant bimodal effect on PDGFRβ expression at 8 h and 24 h LP 12 treatment was shown, though the non-significant increase at 4 h should not be ignored (Figure 2A). In primary mouse cortical neurons, an LP 12 time-course only shows a significant increase on PDGFRβ expression at 24 hours (Figure 2B). Interestingly, 4 h and 24 h LP 12 administration showed significant increases in PDGFRβ expression in SH-SY5Y cells (Figures 2B and 2C are unpublished data sets that belong to first author of [62]).

In summary, Vasefi *et al* (2012) has shown that 24 hour treatment with a selective 5-HT$_7$ receptor agonist like LP 12 is sufficient to significantly increase the expression of PDGFRβ expression and phosphorylation at the PLCγ binding site where the natural ligand PDGF-BB was not involved. The LP 12 administration time course for 4 h to 8 h 5-HT$_7$ exposure to agonist was inconclusive where additional experiments that test the same mechanism between 1-8 h agonist exposure form an important part of the current investigation.
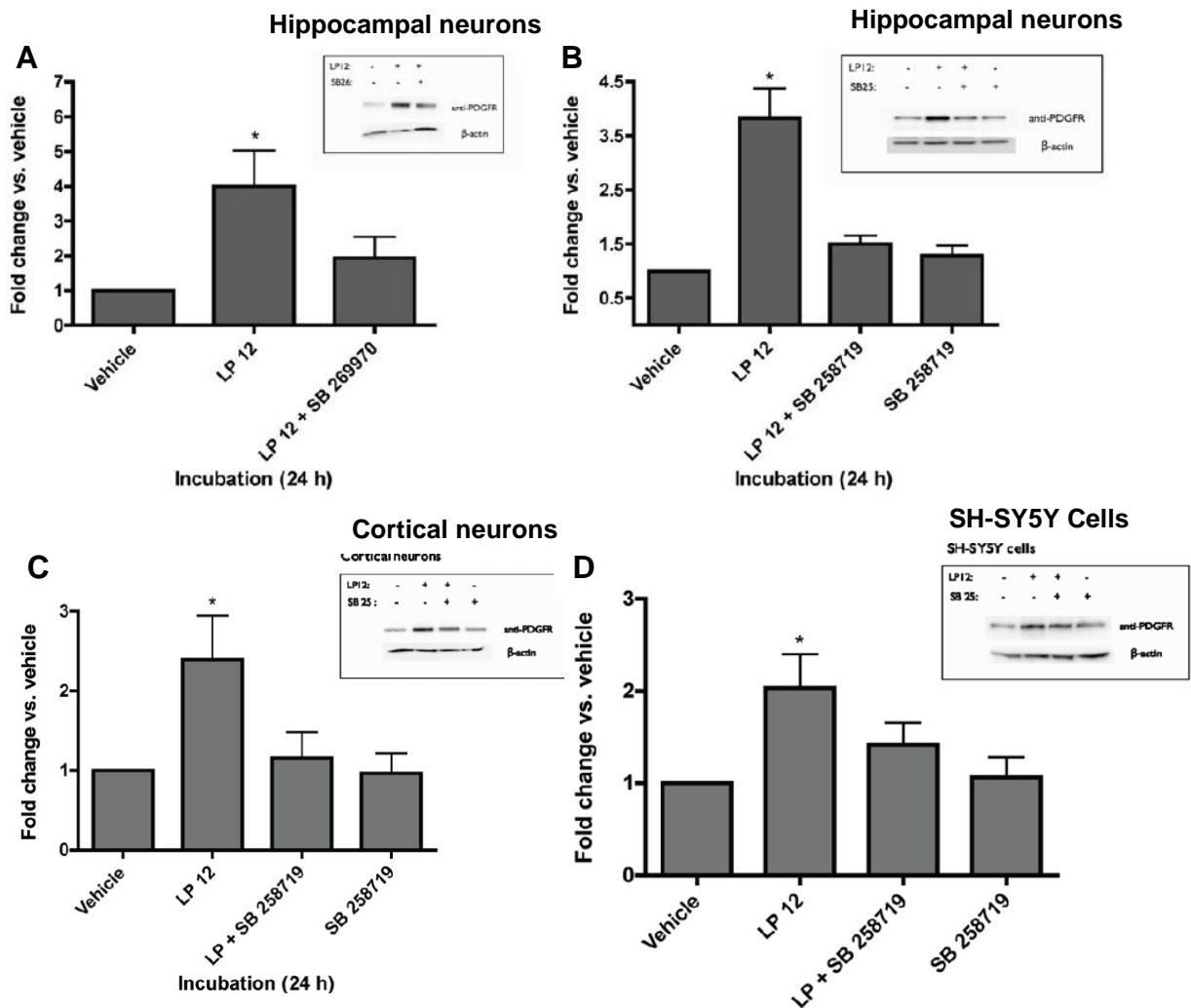
Figure 1. 24 h LP 12 treatment increases PDGFβ receptor expression in primary hippocampal and cortical neurons as well as in SH-SY5Y neuroblastoma cell line.

A) Hippocampal neurons were incubated for 24 h with LP 12 (300 nM) with or without or 1 μM SB 269970, a 5-HT7 receptor inverse agonist. LP 12 increased PDGFβ receptor expression and this was reduced by co-incubation with SB 269970 (n = 7). 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 μM, n = 5). C) 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 μM, n = 4). D) LP 12 (300 nM) was added to SHSY5Y cells for 24 h in the presence or absence of the neutral 5-HT7 receptor antagonist SB 258719 (1 μM, n= 5). For A* p <0.01, ANOVA analysis with Dunnett's post-test. For B-D * p < 0.05, ANOVA analysis with Dunnett's post-test. The fold-change in PDGFRβ expression was determined by quantification of Western blots. Figure As adapted from [57].

15
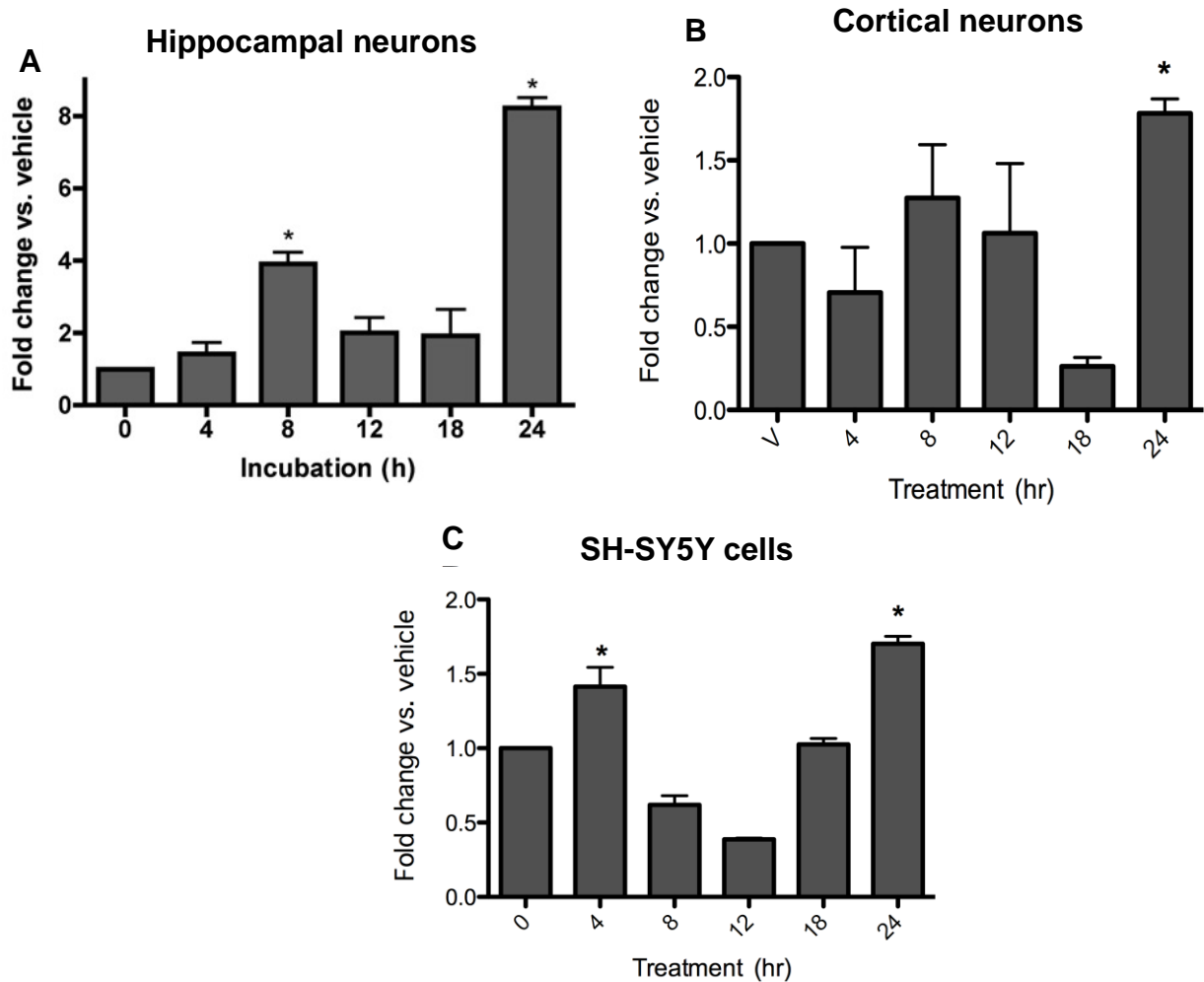
Figure 2. LP 12 time course for PDGFβ receptor expression in hippocampal and cortical neurons and SH-SY5Y neuroblastoma cell line.

A) Hippocampal cultures were incubated with 300 nM LP 12 for 4-24 h, n=3. (* $p < 0.05$, ANOVA analysis with Dunnett's post-test) The fold-change in PDGFβ receptor expression was determined by quantification of Western blots, n = 3 (*$p < 0.05$, ANOVA with Dunnett's post-test) [57] The cortical cultures (B) and SH-SY5Y cells (C) were incubated with 300 nM LP12 for 0–24h. The fold-change in PDGFβ receptor expression was determined by quantification of Western blots, n = 3 (*$p < 0.05$, ANOVA with Dunnett's post-test) (Data not published but belongs to first author of [62].

16

1.4.2 5-HT$_7$ regulation of TrkB receptors

Multiple GPCRs have the ability to transactivate TrkB receptors and exert trophic effects in neurons. Adenosine A2A receptors [58, 59], 5-HT$_{1a}$ receptors [55], dopamine 1 (D1) receptors, human secretin receptor (SCTR) [60], and CB1 cannabinoid receptor [61] have all been shown to modulate TrkB receptor expression and signalling in neuronal systems. Evidence of 5-HT$_7$ receptors modulating TrkB activity was scarce until a physiological role for 5-HT$_7$ receptors in a form of respiratory motor plasticity, termed phrenic motor facilitation (pMF), was discovered [62, 63]. Phrenic motor facilitation is a general term used to describe a long lasting firing of phrenic motor neurons elicited by acute exposure to intermittent hypoxia [68]. 5-HT$_7$ receptor agonists induce pMF via Epac (exchange protein directly activated by cAMP)-Akt-mTORC1 signalling, a mechanism where new synthesis of an immature TrkB isoform is required, but not new BDNF synthesis [64]. Work done by this group suggests that for 5-HT$_7$ receptor agonist dependent pMF to occur, i) ERK and Akt signalling must be active but no TrkB expression is neccessary in the early phase of pMF, and ii) in the late phase of pMF TrkB transactivation along with PI3K activation is necessary [69].

Interestingly, a recent study from Beazely lab has reported that 5-HT$_7$ receptor selective agonist, LP 12, increases the expression of TrkB and phosphorylation of its tyrosine residue Y816 (that binds PLCγ) in the SH-SY5Y and RGC-5 cell lines and to some extent in primary mouse cortical cultures [65]. Using a similar experimental set up (same agonist and antagonist concentration as well as treatment time schemes) as Vasefi, *et al.* (2012) where the focus was 5-HT$_7$-PDGFβ receptor signalling, this paper reported 5-HT$_7$ dependent significant increases in

TrkB-FL expression and phosphorylation in SH-SY5Y and RGC-5 cells with 24 h agonist exposure [71]. Conversely, with 2 h agonist exposure similar increases in TrkB-FL expression were observed in SH-SY5Y and RGC-5 cells and phosphorylation at Y816 was only significantly increased in RGC-5 cells [71]. The transactivation effects of LP 12 treatment in the short-term (15 min to 4 h) was also assessed in this study where there was a transient rise in Y816 phosphorylation at 15 min in SH-SY5Y cells but a more sustained rise between one and four hours in RGC-5 cells [71]. Although preliminary, these results suggested that, in addition to PDGFβ receptors, 5-HT$_7$ receptors may also positively regulate TrkB receptors.

1.5 N-Methyl-D-aspartate (NMDA) glutamate receptors, RTKs, and GPCRs

Growth factors and neurotrophin receptors like PDGFRβ and TrkB, respectively, as well as certain GPCRs have long been known to modulate the activity of an excitatory ion channel in the CNS called the N-Methyl-D-aspartate (NMDA) receptor that has implications in almost all the prominent CNS neurodegenerative and neurological disorders. This section will elaborate on the cross-talk between the three systems in the context of NMDA receptor mediated neuronal excitotoxicity.

1.5.1 Glutamatergic neurotransmission and NMDA receptors

The ionotropic glutamate channels mediate most of the fast synaptic transmission in the CNS [66]. There are three main subtypes of the glutamate receptor (each named after a chemical agonist): i) NMDA (N-methyl-D-aspartic acid), ii) AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionate) and iii) KA (Kainic Acid) [72]. The neurotransmitter glutamate

18

activates all three receptor subtypes, however, AMPA acts only at AMPA receptor, NMDA acts only at NMDA receptor and so on. NMDA and AMPA receptors each have distinct physiological properties and often coexist at the same synapse, as a result most glutamate mediated excitatory post-synaptic potentials (EPSPs) have components contributed by both receptors [72]. The AMPA-gated channels are permeable to both Na^+ and K^+ and most are not permeable to Ca^{2+} [72]. The net effect of AMPA-gated channel activation at normal negative membrane potential is the influx of Na^+ into cell causing a rapid and large depolarization of the post synaptic membrane which renders the NMDA channel active by removing the constitutive channel blocker, Mg^{2+}, from the NMDA channel ion pore [72]. Consequently, the NMDA-gated channels cause excitation of a cell by admitting Na^+ but differ from AMPA receptors in two ways: i) NMDA receptors are permeable to Ca^{2+}, and ii) the inward ionic current through them is voltage dependent i.e., they will only fire upon membrane depolarization and removal of the Mg^{2+} block [72]. Glutamate activation of AMPA receptors and consequently of NMDA receptors, NMDA receptor dependent calcium influx, and the trafficking of AMPA receptors in the CA1 region of the hippocampus form the basis of synaptic plasticity [67, 68].

The NMDA receptors are a heterogeneous group of ion channels that exist as cation-selective tetramers formed by homo- and hetero-oligomeric assembly of subunits with common structural features that include [66]:

- an extracellular amino (N')-terminal,
- three transmembrane domains (M1, M3 and M4) ,
- a channel lining re-entrant 'p-loop' (M2) and
- an intracellular carboxy (C')-terminal.

The two extracellular segments S1 and S2 (a binding pocket created by two regions present in the N-terminus and the extracellular loop between M3 and M4) have been shown to comprise the ligand binding domain on the NR2 subunits which is responsible for binding both the neurotransmitter and competitive agonists/antagonists of the receptors [72]. The re-entrant M2 loop comprises of the channel pore and determines calcium permeability of the channel and magnesium blockade of the ion pore [72].

NMDA receptors are comprised of three major subfamilies of subunits: the ubiquitously expressed NR1 subunit, four distinct NR2 sub-units (A–D), and two NR3 members (A and B) [72]. Glutamate/glycine responsive NMDA receptors require both, dimers of NR1 and dimers of NR2 subunits in order to be functional [72]. Generally, NR2A and NR2B containing NMDA receptors (paired with NR1) are the predominant receptors expressed in the forebrain and hippocampus, NR2C is expressed largely in cerebellar granule cells, and NR2D expression is limited to diencephalon and midbrain for early development [72]. NMDA receptors that complex with NR3 subunits generally are not functional [72]. The current consensus in NMDA receptor neurobiology is that much of the NR2A containing NMDA receptors are predominantly expressed at synaptic sites whereas NR2B containing NMDA receptors are located at extrasynaptic sites in the adult CNS [72, 73, 74].

1.5.2 NMDA receptors and neuronal excitotoxicity

Large concentrations of glutamate present for excessive amounts of time can "excite neurons to death". The neurodestructive potential of glutamate (coined excitotoxicity) was first

established by the demonstration that an overdose of systemic glutamate destroys hypothalamic

nuclei in immature monkeys and rodents [69]. Follow-up research demonstrated that high

concentrations of glutamate [100–500 mM] induced cell death *in vitro* and that similar

extracellular concentrations are present in the rodent brain and spinal cord during ischemia [70,

71]. Subsequently, AMPA and NMDA receptor-mediated, glutamate-induced cell death *in vitro*

and *in vivo* models of ischemia was described [72]. The excessive release of the excitatory amino

acid glutamate in excitotoxicity is dependent on the type of insult (acute or chronic) to the CNS

which varies between diseases. For instance, in acute ischemic stroke and brain injury severe

ATP depletion and energy impairment occurs earlier on in the ischemic cascade which

excessively activates NMDARs causing excessive influx of Ca^{2+} and Na^+ with the efflux of K^+

(intracellular ionic imbalance), overloading mitochondria resulting in free radical production and

activation of other downstream proteases, lipases and nucleases that lead to predominantly a

necrotic form of neuronal injury and gradual apoptotic cell death [73].

Unlike acute forms of excitotoxicity seen in ischemic stroke or traumatic brain injury, a

slower, more subtle form of excitotoxicity is implicated in progressive neurodegenerative

disorders like Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis,

HIV associated dementia, glaucoma, and amyotrophic lateral sclerosis [74]. In these disease

modalities a more chronic exposure to elevated glutamate concentrations slowly and

cumulatively triggers apoptotic-like cell death in neurons that is mediated by extra-synaptic

NMDARs (NR2B-containing) [75-77] where the synaptic (NR2A containing) NMDARs are

thought to be pro-survival [78, 79] (See Figure 3). Conversely, in acute ischemic stroke the

excessive release of glutamate in synaptic and extrasynaptic sites causes global populations of

NMDA receptors to be activated causing pro-apoptotic neuronal calcium ion concentrations to

rise [85]. The mechanisms by which NR2B subunits initiate cell death and NR2A subunits

preserve synaptic activity are largely due to differences in the signalling proteins directly bound

or indirectly coupled to the long C-terminal domains of these subunits [85]. While NR2A

subunits bind to or associate with signalling proteins that activate Akt, ERK, and CREB, NR2B

subunits in extrasynaptic sites serve as a hub for a plethora of death signalling proteins [85] that

have been summarized in Table 4.
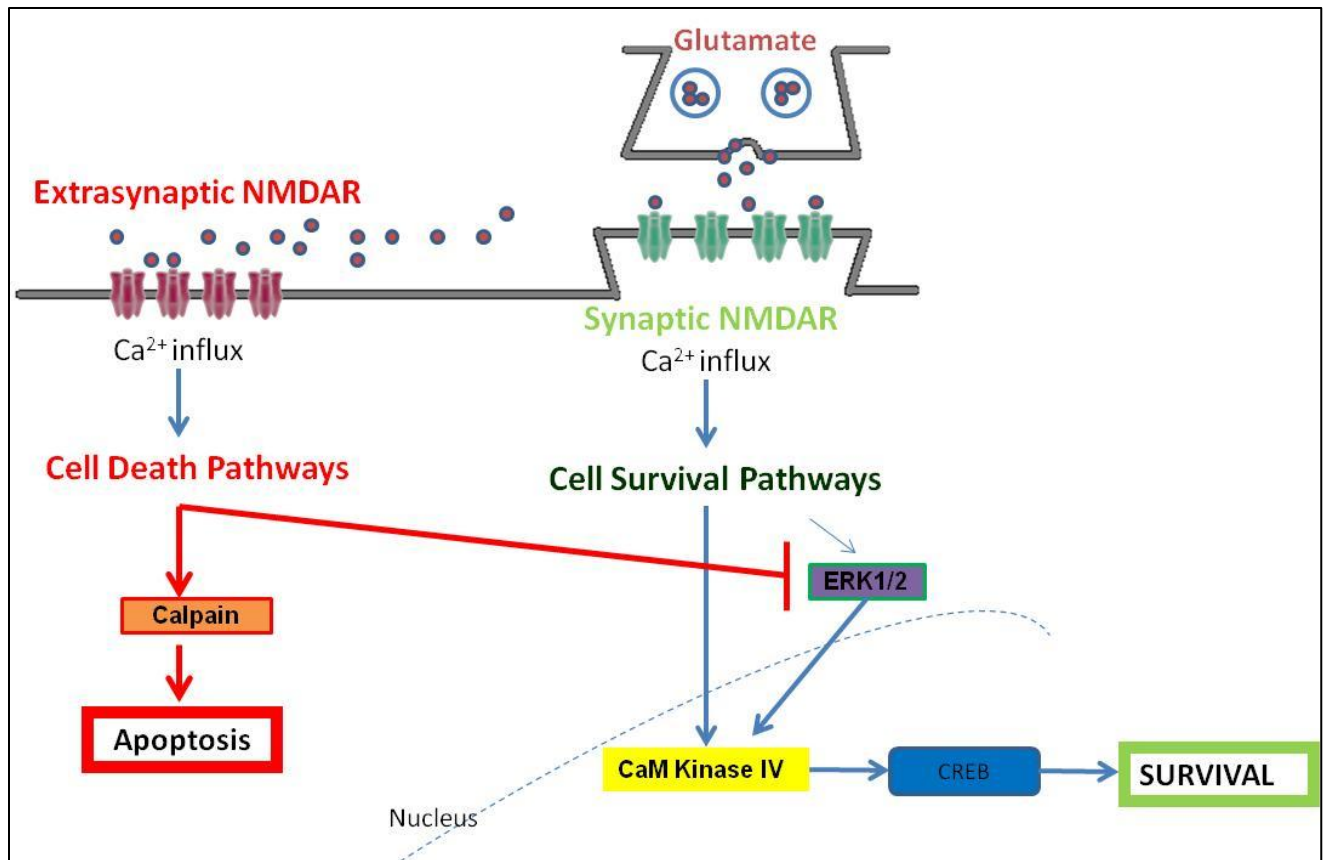
Figure 3. NMDAR subunit dependent mechanisms of neuronal excitotoxicity

As adapted from [84, 85].

Table 4. Extrasynaptic NMDA receptors and associations with death signalling proteins. Information as adapted from reviews [80, 81].

Signaling molecules associated with NR2B containing NMDA receptors	General mechanisms of action in excitotoxicity
DAPK1 (death-associated protein kinase 1)	Excessive Ca^{2+} influx in excitotoxicity causes calcineurin to de-phosphorylate DAPK1(inactive when phosphorylated) which augments NR2B subunits to futher mediate excitotoxicity and also binds to ERK to suppress any survival signalling
nNOS (neuronal nitric oxide synthase)	Indirectly bound toNR2B subunits by PSD-95 protein where a NR2B-PSD95-nNOS death complex is formed which produces severe oxidative stress via production oxygen and nitrogen oxide free radicals.
Calpain	A death promoting protease that mediates protein cleavage specifically of substrates that bind to cdk5 (cyclin dependent kinase): p35 and p25. The truncation of neuroprotective p35 to p25 redirects cdk5 towards neuronal death.
STEP (striatal-enriched protein tyrosine phosphatase)	Calpain mediated truncation of p38 bound STEP (survival signalling through MAPK) to inactive STEP33 contributes to neuronal death
PTEN (phosphatase and tensin homolog deleted on chromosome ten)	A death promoting protein phosphatase bound to NR1 subunit of extrasynaptic NR2B containing NMDA receptors. De-phosphorylates plasma membrane protein PIP3 into PIP2 which prevents Akt translocation into nucleus and survival signallling.

1.5.3 Current Treatments for NMDA receptor mediated neuronal excitotoxicity

The clinical failure and side effects of competitive antagonists and several NMDA receptor ion-channel blockers against neuronal excitotoxicity, in conditions like ischemic stroke, are well documented in current literature that has synthesized two decades of failed clinical attempts [82]. The primary reasoning behind the failure of these compounds pertains to their mode of action: competitive agonists compete with glutamate in all areas of the brain which also blocks normal brain function and ion-channel blockers to date have possessed an undesirably high affinity for the Mg^{2+} site that also blocks normal neuronal function [74, 82].

Given the mechanisms of excitotoxic damage, a clinically acceptable anti-excitotoxic drug needs to have a two-fold mechanism of action in which i) excessive activation of NMDARs must be blocked and ii) normal functioning of NMDARs must be kept intact to avoid adverse side effects. To date, only one compound, memantine, is being tested for several neurodegenerative disorders given its approval for mild to moderate Alzheimer's disease (a putative treatment for chronic neuronal excitotoxicity) [83]. Memantine is currently undergoing clinical trials for glaucoma, neuropathic pain, substance abuse, multiple sclerosis an epilepsy and acute ischemic stroke [clinicaltrials.gov]. Memantine exerts its effects by binding preferentially at or near the Mg^{2+} binding site within the ion pore [84]; this finding coupled with strong electrophysiological kinetic data suggests that memantine preferentially blocks NMDAR activation if the probability of the ion channel to be in open configuration is much higher, i.e., under excitotoxic conditions. Memantine likely only acts under pathological conditions in low doses without substantially affecting normal neuronal synaptic function though high doses of memantine are contraindicated for patients at risk of acute ischemic stroke as it is able to block global populations of NMDA receptors [85].

1.5.4 RTKs and neuronal excitotoxicity

In the last decade several pre-clinical studies have put forth consistent findings of growth factor receptors and their agonists (like PDGFβ receptor/PDGF-BB) and neurotrophin receptors and their agonists (like TrkB/BDNF) exerting neuroprotective effects against neuronal excitotoxicity in different model systems. With regards to PDGF and PDGF receptors, a few studies initially reported that there was an increased expression of PDGF receptor ligands,

especially PDGF-B chain isoform mRNA transcripts, as well as PDGF receptors in neurons and support cells surrounding the lesioned area of the CNS in animal models of stroke [86, 87]. Subsequent to these findings it was shown that administration of PDGF-BB inhibits NMDA-evoked currents and excitatory postsynaptic potentials that are mediated by the NR2B-containing NMDA receptors in primary CA1 hippocampal neurons [88, 89], one of the proposed mechanisms in studies that showed neuroprotection of hippocampal neurons from glutamate or NMDA-induced excitotoxicity [90, 91]. Apart from the direct inhibition of the NR2B subunit expression in NMDA-excitotoxicity, Beazely *et al.*, (2009) also showed that PDGF-BB administration leads to phosphorylation of downstream pro-survival transcription factor ERK and also prevented the NR2B receptor inhibition of ERK and CREB phosphorylation.

There is strong evidence for cross-talk between PDGFRβ and the NMDA receptor that is able to explain the pre-clinical neuroprotection achieved thus far against glutamate or NMDA induced excitotoxicity *in vitro*. On the other hand, the neuroprotective mechanisms by which BDNF brings about neuroprotection against acute ischemia *in vivo* (see studies [92, 93]) did not suggest TrkB modulation of NMDA receptors as a mechanism of action. In other studies, 7,8-dihydroxyflavone (7,8-DHF), a small-molecule flavone derivative that binds with high affinity and specificity to TrkB, has been shown to exert neuroprotection against glutamate-induced toxicity in the hippocampal HT-22 cell line [94] and in an *in vivo* model of cerebral ischemia and reperfusion (I/R) injury in rats [95]. The proposed mechanism of neuroprotection in studies outlined thus far are heavily in favour of the antioxidant properties of BDNF (or 7,8-DHF) that is able to increase cellular glutathione levels and reduces ROS production as well as the activation

of the signaling pathways MAPK, PI3K and PLCγ. In normal physiology, post synaptic

activation of TrkB receptors via BDNF causes them to interact with NMDA receptors via a

protein tyrosine kinase called Fyn which increases the open probability of the NMDA ion

channel (see review [48]). BDNF levels are significantly increased above baseline values during

the first 4 h and up to 24 h after ischemic insult *in vivo* [96, 97] thus, it remains to be seen if the

neuroprotective effects of BDNF or TrkB activation could also be a result of direct inhibition of

specific NMDA receptor subunits as is the case with PDGF-BB.

1.5.5 GPCRs and neuronal excitotoxicity

There is ample evidence of GPCRs regulating NMDA receptor activity in normal brain

physiology. GPCRs involved in dopaminergic, GABA-ergic, cannabinergic, cholinergic

neurotransmission are responsible for NMDA receptor regulation in a sub-type specific manner

[98]. In fact, all serotonin receptors take some part in regulating NMDA receptor activity.

Examples include 5-HT$_{2B}$ receptors being involved in enhanced pre-synaptic firing to depolarize

NMDA receptors [99] and 5-HT$_{1A}$ receptors being involved in hyperpolarization of post-synaptic

terminals which inhibits NMDA currents [100]. No attempt to date has resulted in a

comprehensive list of GPCRs that can act as strategic targets for neuronal excitotoxicity. For

instance, the inhibitory property of 5-HT$_{1A}$ receptors against NMDA receptors was grounds for

clinical assessment, albeit unsuccessful, in an acute ischemic stroke trial [101]. The role of 5-

HT$_7$ receptors in neuronal excitotoxicity is merely speculative at this stage given their ability to

enhance hippocampal NMDA currents in acute treatments (5-10 min) [102] but have indirect

(RTK dependent) inhibitory effects on NMDA receptors with longer activation [103].

1.6 Targeting 5-HT7 receptors and RTKs for neuronal excitotoxicity

Based on the findings that 5-HT$_7$ receptor-selective and non-selective agonists can increase expression and phosphorylation of PDGFRβ, and that PDGFRβ are able to selectively modulate extrasynaptic NR2B subunits during excitotoxicity, Vasefi *et al.,* (2013) showed that 24 hour pre-administration of 5-HT$_7$ receptor agonist, LP 12, is neuroprotective against NMDA-induced excitotoxicity in primary hippocampal neurons. Vasefi *et al.,* utilized the kinase inhibitors STI-571 and AG1296 to confirm that the neuroprotection was indeed dependent on kinase activity of the PDGFβ receptor as both the inhibitors prevented LP-12 induced neuroprotection and blocked the reduced expression of the NR1 and NR2B subunit. The involvement of the 5-HT$_7$ receptor in this pathway was confirmed by the observations that two 5-HT$_7$ receptor antagonists (SB 258719 and inverse agonist SB 269970) blocked all of LP 12's effects [103]. See Figure 4 for a schematic of the proposed mechanism by which long-term 5-HT$_7$ agonists can modulate NMDA receptor activity.
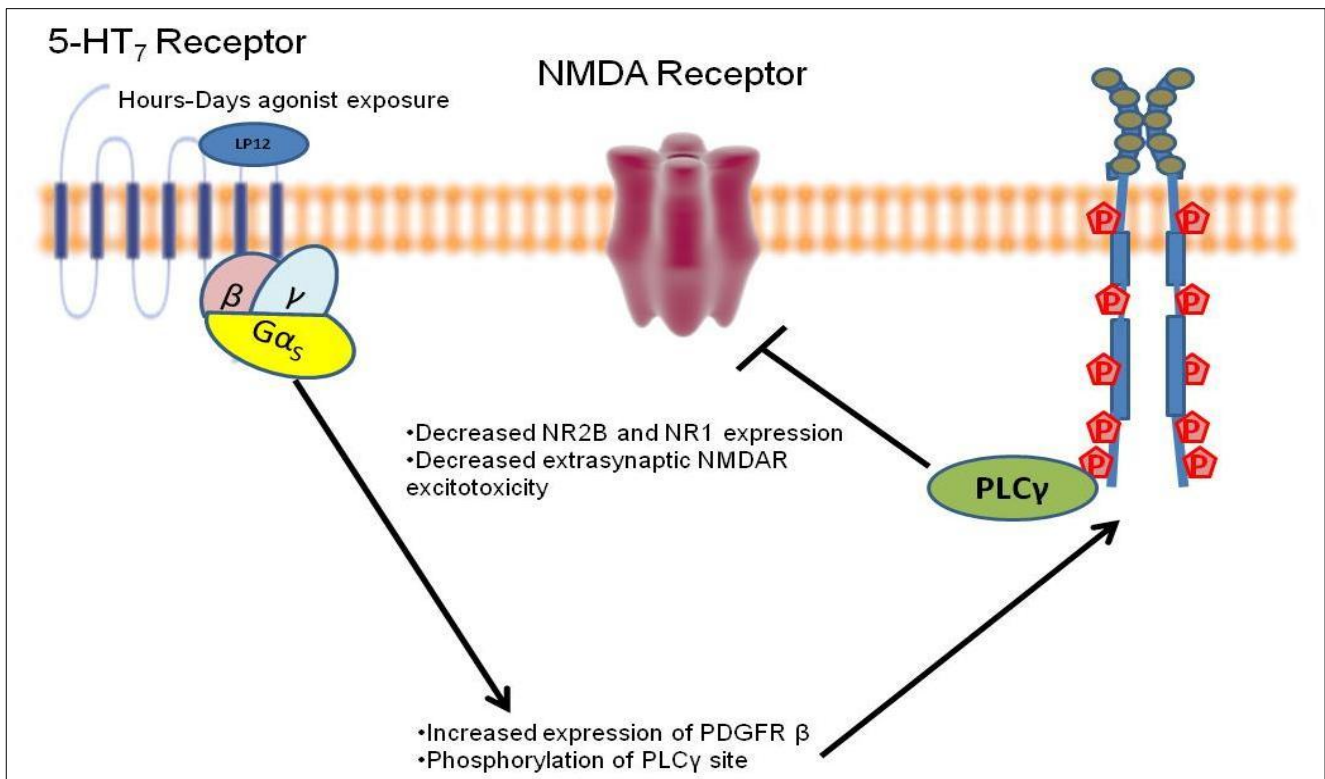
Figure 4. Long-term activation of 5-HT7 receptors is neuroprotective against NMDA excitotoxicity

Figure as adapted from [103].

Given that 5-HT$_7$ neuroprotection is dependent on RTKs such as PDGFRβ and potentially even TrkB [65], this mechanism opens up an opportunity to use selective small molecule agonists for 5-HT$_7$ receptors that can cross the blood brain barrier (BBB) to enhance endogenous growth factor signals before, during or shortly excitotoxic insults. The clinical use of growth factors for neurological diseases is limited by major adverse effects, short *in vivo* half-lives, poor delivery and the inability of these large proteinaceous ligands to penetrate the BBB [104, 105]. Vasefi *et al.,* (2013) have put forth a mechanism that is neuroprotective against excitotoxicity after 24 h pre-incubation with LP 12, however, this time frame is far too long to significantly rescue damaged neurons in the acute phase of neuronal excitotoxicity. The objectives presented in this thesis are ultimately to study if short-term (15 min to 4 h) activation of 5-HT$_7$ receptors is able to similarly protect neurons against neuronal excitotoxicity.

28

Chapter 2: Objectives and Hypothesis

The objectives of this project are:

1. Characterize the short-term (15 min to 4 h) effects of LP 12 on PDGFβ and TrkB receptor expression and signalling activity in neuronal cell lines and primary neuronal cells

2. Determine if short-term pre-treatment with LP 12 is neuroprotective against excitotoxicity and whether if the neuroprotection is PDGFβ and/or TrkB receptor dependent

These objectives have been made to fulfill the hypothesis that:

Short-term (1-4 h) 5-HT$_7$ agonist treatment of primary neurons and differentiated HT-22

cells will be neuroprotective against NMDA-induced excitotoxicity via increases in

PDGFRβ and TrkB expression and basal signaling activity (phosphorylation)

Chapter 3: Materials and Methods

3.1 Reagents and Antibodies

LP 12 (4-(2-Diphenyl)-N-(1,2,3,4-tetrahydronaphthalen-1-yl)-1-
piperazinehexanamide hydrochloride), NMDA (N-methyl-D-aspartate), L-glutamic acid, PDGF-
BB, glycine, and other chemical reagents were purchased from Sigma (St. Louis, MO, USA).
AG1296 (6,7-Dimethoxy-2-phenylquinoxaline). 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-HT7
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) and the NMDA receptor antagonist MK-
801 (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate
were obtained from Tocris (Ellisville, MO, USA). The antibodies used, their molecular weight,
isoform and and manufacturer are listed in Table 5 below:

Table 5. Antibodies

Antibody	Isotype	Molecular weight (kDa)	Manufacturer
PDGFβ receptor	Rabbit	190	Santa Cruz
PDGFβ receptor phospho-tyrosine 1021	Rabbit	190	Santa Cruz
TrkB-FL, TrkB-Shc, TrkB-T1	Rabbit	145, 105, 95	Santa Cruz
phospho-TrkB Y816	Rabbit	145	Abcam
NR1	Rabbit	120	Cell Signaling
NR2A	Rabbit	180	Cell Signaling
NR2B	Rabbit	190	Cell Signaling

Table 5 continued			
β-Actin	Mouse	45	Santa Cruz
Cytochrome C	Rabbit	14	Cell Signaling
PLCγ1	Rabbit	150	Cell Signaling
p-ERK1/2	Rabbit	42, 44	Cell Signaling
ERK1/2	Rabbit	42, 44	Cell Signaling
5-HT$_7$	Rabbit	50	Rockland and Fisher
p-Akt Threonine 308	Rabbit	60	Cell Signaling
Akt	Rabbit	60	Cell Signaling
Anti-mouse horseradish peroxidase (HRP) enzyme-conjugated IgG	Goat	Secondary	Fisher
Anti-rabbit horseradish peroxidase (HRP) enzyme-conjugated IgG	Goat	Secondary	Fisher
Anti-rabbit Dylight 488	Goat	Fluorescently labeled secondary	Fisher

3.2 Cell Culture

3.2.1 SH-SY5Y neuroblastoma cell line

Neuroblastoma cells were cultured in DMEM and HAM's F12 (1:1) (Fisher #SH20361), 10% fetal bovine serum (Fisher), 100 U/ml penicillin and 100 μg/ml streptomycin (together referred to as Pen Strep). Cells were maintained at a temperature of 37°C in a humidified atmosphere of 95% air and 5% CO_2, and media were changed every 3-5 days. Cells were trypsinized with 0.25% trypsin/0.1% EDTA, and passages 1-12 were used. For drug treatments and experimentation, cells were plated without antibiotics and serum starved for 24 h once they had reached 80% confluency.

3.2.2 HT-22 murine hippocampal cell line

HT-22 cells were cultured and maintained using the same reagents and in the same conditions as SH-SY5Y cells. For drug treatments and experimentation, HT-22 cells were seeded (at experiment dependent seeding densities) in full growth media for 24 h, after which the cells were differentiated in neurobasal media (Gibco, Life Technologies) containing N2 supplement and 2 mM L-glutamine for an additional 24 h. Drug treatments were performed in neurobasal media.

3.2.3 Primary hippocampal neuron cell culture

Hippocampal and cortical neurons were isolated from embryonic day 17-19 fetuses of CD1 mice and placed in cold dissection media (33 mM glucose, 58 mM sucrose, 30 mM HEPES, 5.4 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 0.34 mM Na_2HPO_4, 4.2 mM $NaHCO_3$, 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 35mm culture dishes and grown at 37°C in a humidified atmosphere containing 5% CO2. For cell attachment right after dissection, cells were plated in plating media (DMEM, 10% fetal bovine serum (FBS) and 10% horse serum) for 4 h which followed a media change to feeding media (Neurobasal media supplemented with B27) and feeding media was changed twice per week. In order to inhibit growth of non-neuronal cells, FUDR (0.081 mM 5-fluoro-2 deoxyuridine) and 0.2 mM uridine were added 3-4 days after plating for 24 h once the cells reached confluency. Drug treatments were performed 10-12 days after plating.

3.3 Western Blot

3.3.1 Whole cell lysate preparation

For all cell culture experiments, after drug treatment cells were washed with chilled phosphate-buffered saline (PBS), and lysed in chilled lysis buffer containing 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, and 1% Triton X-100; supplemented with Halt Protease and Phosphatase Inhibitor prior to use. In order to solubilize NMDA receptors, the lysis buffer for HT-22 cells contained 0.05% SDS in supplement to the Triton X-100 and for hippocampal neurons the lysis buffer contained an additional 0.5% SDS in supplement to the Triton X-100. Cells were scraped, mechanically homogenized using a syringe and centrifuged at 14,000 x g for 20 min at 4°C and the supernatants were collected.

3.3.2 BCA protein assay and sample preparation

BCA protein assay (Thermo) was used to quantify concentration of protein in samples. Samples were prepared so 25-35 µg total protein could be mixed and heated with 3x loading buffer (240 mM Tris-HCl at pH 6.8, 6% w/v SDS, 30% v/v glycerol, 0.02% w/v bromophenol blue, 50 mM DTT, and 5% v/v β-mercaptoethanol) for 15 min at 75°C.

3.3.3 SDS-PAGE and immunodetection

Protein samples were loaded into 1.5 mm thick polyacrylamide gel wells. Proteins were separated by SDS-PAGE in electrophoresis buffer (25 mM Tris base, 190 mM glycine, 3.5 mM sodium dodecyl sulfate), followed by electroblotting of proteins onto nitrocellulose membranes

with chilled transfer buffer (25 mM Tris base, 190 mM glycine, 20% v/v methanol) for either 90 min or 16 h. Membranes were then blocked with 5% non-fat milk in Tris-buffered saline (20 mM Tris base, 150 mM NaCl, pH 7.6) plus 0.1% Tween (TBS-T) for 1 h at room temperature or overnight at 4 °C, followed by incubation with primary antibody (usually made in blocking buffer or just TBS-T) for 1 h at room temperature or overnight at 4 °C. Membranes were washed three times with TBS-T, and then incubated with secondary antibody conjugated to horse radish peroxidase (HRP) in blocking buffer for 1 h at room temperature (1:5000 for anti-mouse primary antibody, 1:10000 for anti-rabbit primary antibody). Membranes were washed three additional times with TBS-T. Western chemiluminescent substrate (Luminata Crescendo - Millipore) was used to visualize proteins on a Kodak 4000MM Pro Imaging Station. Densitometric analyses on blots were performed using Kodak Molecular Imaging software (Carestream Health, USA). After imaging, membranes were stripped and re-probed with other appropriate antibodies.

3.4 MTT Toxicity Assay

For neuroprotection assays that required primary hippocampal neurons, isolated hippocampal neurons were counted and plated in 96-well plates at 20×10^3 cells/well and grown for 10-12 days. After treatment, hippocampal neurons were washed and incubated in feeding media for 24 h to allow for cell death after which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in an amount equal to 10% of the culture medium volume were added to each well and the cells were incubated for an additional 3 h at 37 °C in 5% CO_2. 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 and 690 nm. All results are expressed as a percent reduction of MTT relative to untreated controls.

For neuroprotection assays that required HT-22 cells, a standard seeding density of 2.5 x 10^3 cells/well was used in 96-well plates for 24 h and then differentiated for an additional 24 h. For experiments that did not require differentiation of HT-22 cells, 2.5 x 10^3 cells/well were seeded in 96-well plates and serum starved for 4 h prior to treatment. After treatment of differentiated HT-22 cells (incubating in neurobasal media) MTT was added for 3 h and a read out was taken as described above. After treatment of undifferentiated HT-22 cells, a washout was performed with serum and phenol red free DMEM (Fisher# SH3027201) after which MTT reagent was added for 3 h and a read out was taken.

3.5 Immunofluorescence

Immunofluorescence was performed to look for qualitative and preliminary evidence of TrkB receptors in differentiated and undifferentiated HT-22 cells. Briefly, HT-22 cells were seeded at 35,000 cells/well in 6 well plates for 24 h and differentiated for an additional 24 h (Corning). After differentiation, cells were washed twice in PBS (with calcium and magnesium) and pre-fixed with 4% (w/v) paraformaldehyde (Sigma) for 2 min, aspirated and fixed again with 2% (w/v) paraformaldehyde for 20 min at room temperature pressure. Cells were rinsed twice in PBS and then washed in wash buffer (0.1% Bovine Serum Albumin (BSA, Rockland, Gilbertsville,PA) in PBS). Cells were then permeabilized and blocked using 0.3 % Triton X-100 (Sigma) and 10 % FBS all dissolved in PBS for 45 min at room temperature then washed with PBS. After blocking, cells were coated with TrkB primary antibody at 1:200 dilution in dilution

35

buffer (PBS, 1% bovine serum albumin (BSA), 0.3% Triton X-100, and 0.01% sodium azide) overnight with agitation at 4 °C. Next day, cells were washed twice with PBS and stained with secondary antibody (Dylight 488) in dilution buffer at 1:1000 for 1 h. Cells were washed twice in PBS and mounted with a cytoplasmic counterstain, CellMask Plasma Membrane Stain Deep Red (Invitrogen), for 10 min and visualized on a Zeiss laser scanning confocal microscope LSM Z710 (Zeiss, Germany) with 63x oil-based objective.

3.6 Phase Contrast Microscopy

In order to visually assess treatment effects on morphology of differentiated HT-22 cells, cells were seeded at a density of 30 x 10^3 cells/per well in 6-well plates and differentiated for 24 h. Treatments were performed and images of cells were captured using a 10x objective over an inverted phase contrast microscope IX53 (Olympus) attached to a DP25 digital camera (Olympus).

3.7 Statistical analysis

Data were analyzed using GraphPad Prism 5.0 (GraphPad Software, USA). Student's t-test were used when comparing two data sets, or one-way analysis of variance (ANOVA) with Bonferroni's post hoc test for comparing three or more data sets. Unless otherwise stated, the results are presented as mean ± SEM and each n value denotes independent experiments. For graphs, $*=p < 0.05$, $**=p<0.01$ and $***=p<0.001$.

Chapter 4: Results

4.1 Short-term LP 12 treatment induces changes in PDGFRβ and TrkB-FL expression and activity in primary hippocampal neurons

Short-term time course experiments of 0.25 to 4 h of 300 nM LP 12 treatments were performed in primary hippocampal neurons. The concentration of 300 nM LP 12 was previously shown to maximally increase PDGFβ receptor expression at 24 h [103]. This concentration also resulted in a significant downregulation of the 5-HT$_7$ receptor [103]. The aggregate time course data indicated that LP 12 caused a sustained phosphorylation of PDGFβ receptors at tyrosine 1021 (Y1021) between 2 and 4 h (Figure 4A). Total PDGFβ receptor expression was only significantly elevated above baseline at 2 h (Figure 4B). In addition to PDGFβ receptor signalling, assessment of TrkB receptor phosphorylation at tyrosine 816 (Y816) revealed significant increases at 2 h and 4 h (Figure 4C) where TrkB expression was also increased at 2 h and 3 h but not 4 h (Figure 4D). Using the same membranes from the time course, changes in expression of NR2B subunits, PLCγ1, 5-HT$_7$ receptor, as well as Akt phosphorylation at threonine 308 (Thr308) were also measured. Given the increased phosphorylation of Y1021 between 2 h and 4 h, along with the elevated Y816 phosphorylation at 2 h, it was expected that i) NR2B total expression be reduced (Figure 5A) ii) PLCγ1 expression be elevated at 2 h and 4 h (Figure 5B), and iii) at least one downstream signalling protein be active; this was the case with pAkt Thr308 (Figure 5D). Changes in 5-HT$_7$ receptor expression revealed a significant increase in expression at 1 h followed by a sustained return to baseline until 4 h (Figure 5C).
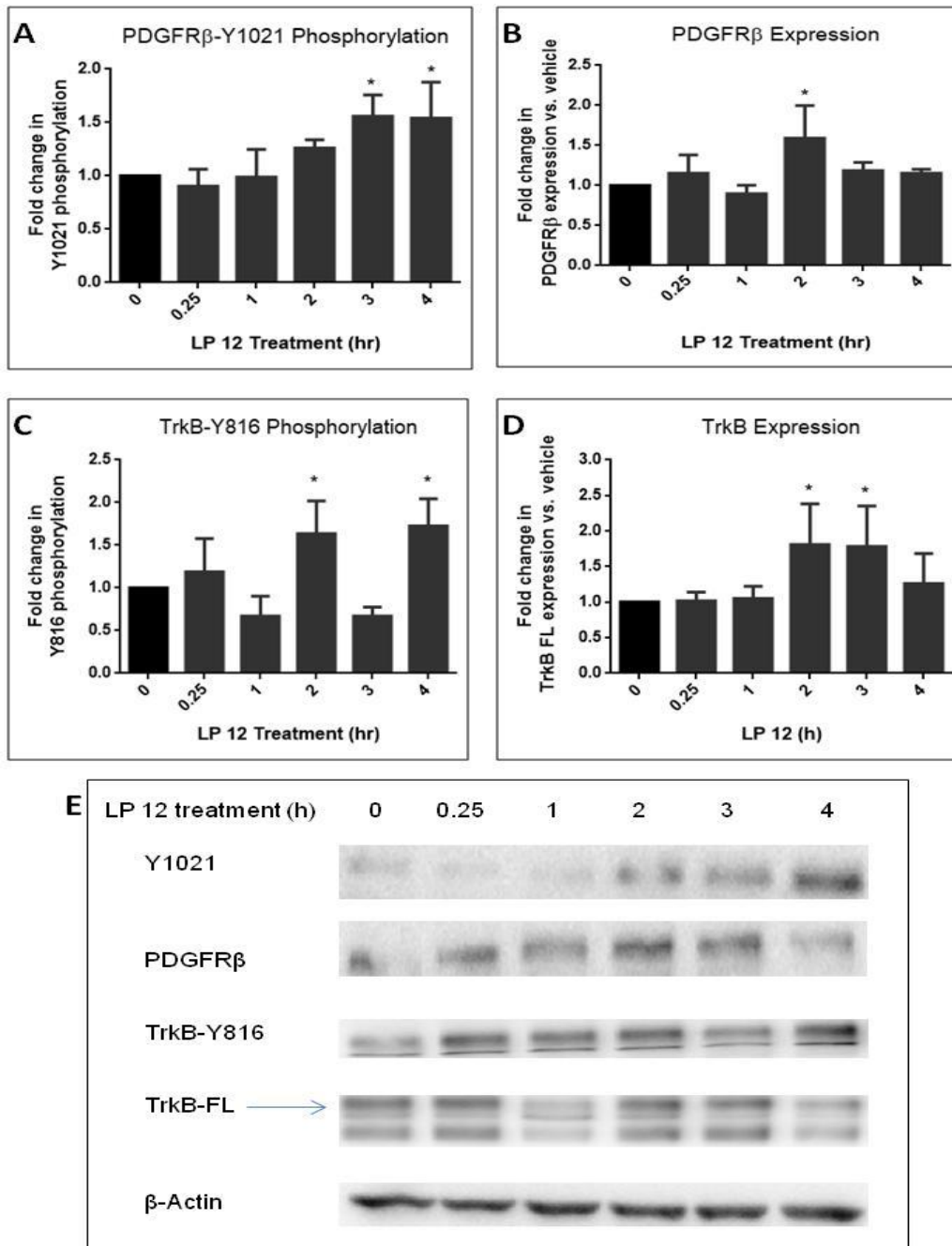
Figure 5. Short term 5-HT7 receptor activation increases PDGFRβ and TrkB-FL expression and phosphorylation in hippocampal neurons.

Cultured hippocampal neurons were treated with selective 5-HT7 receptor agonist LP 12 (300 nM) for 0.25 h to 4 h. (**A, B**) The phosphorylation of the PDGFβ receptors was determined using an anti-phospho-Y1021 PDGFβ receptor antibody and was normalized to total PDFGβ receptor expression (n = 4), The level of PDGFβ receptor expression is displayed as a fold change vs. vehicle-treated neurons. and was normalized to β-actin (n = 4). (**C, D**) The phosphorylation of the TrkB-FL receptor was determined using an anti-phospho-Y816 TrkB receptor antibody and was normalized to total TrkB-FL receptor expression (n = 4), the level of TrkB-FL receptor expression is displayed as a fold change vs. vehicle-treated neurons. and was normalized to β-actin (n = 4). *p<0.05, ANOVA analysis with Bonferroni's post-hoc test. **E**) Representative Western blots for Y1021, PDGFβ receptor, Y816, TrkB-FL, and β-actin.

38
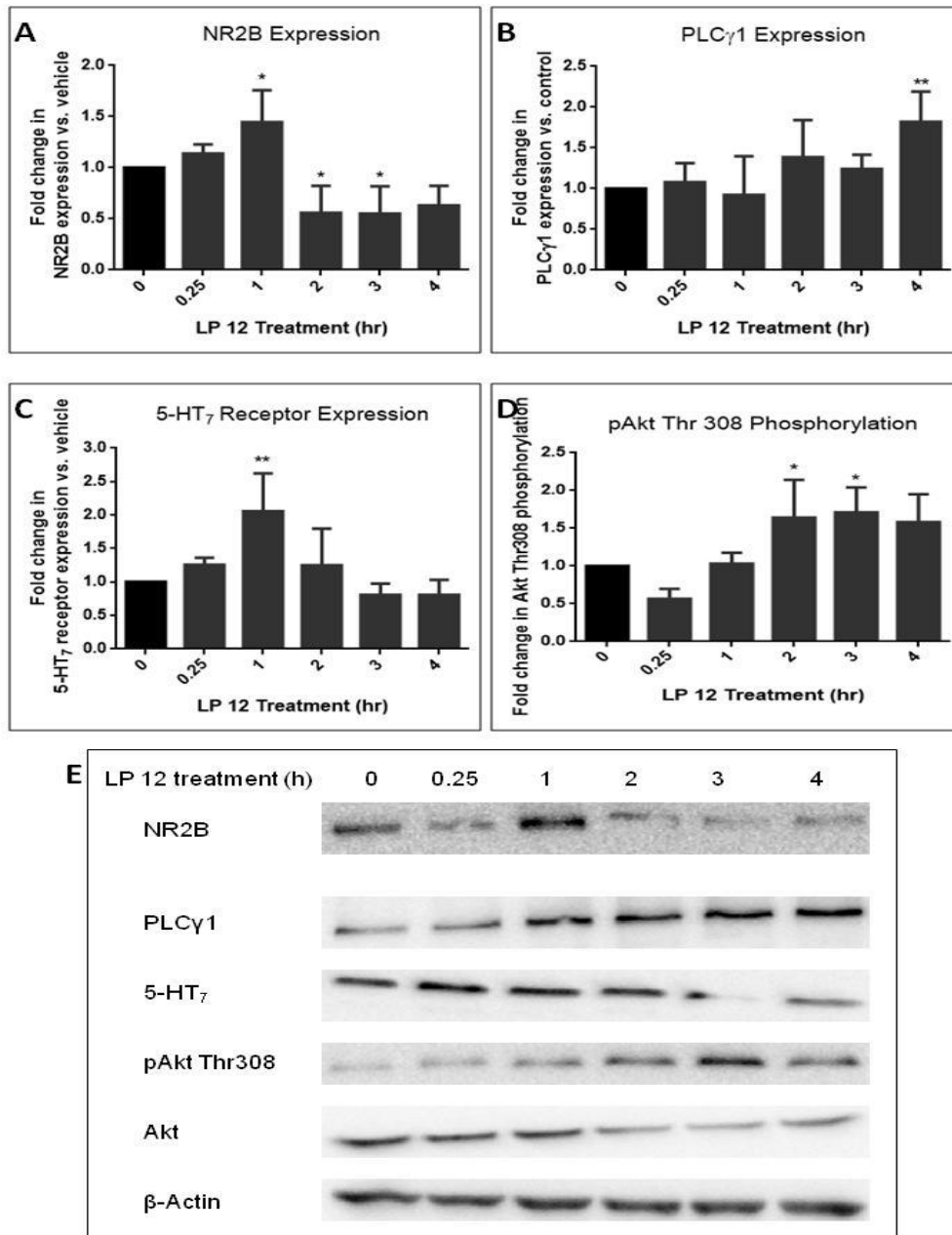
Figure 6. Short-term activation of 5-HT7 receptors increases PLCγ1 expression and Akt phosphorylation which reduces NR2B expression in hippocampal neurons.

Cultured hippocampal neurons were treated with selective 5-HT7 receptor agonist LP 12 (300nM) for 0.25 h to 4 h. (**A**) The level of NR2B subunit receptor expression is displayed as a fold change vs. vehicle-treated neurons. and was normalized to β-actin (n = 4). (**B**) the level of PLCγ1 expression is displayed as a fold change vs. vehicle-treated neurons and was normalized to β-actin (n = 4). (**C**) the level of 5-HT$_7$ receptor expression is displayed as a fold change vs. vehicle-treated neurons and was normalized to β-actin (n = 5). (**D**) The phosphorylation of Akt at threonine 308 (pAkt Thr308) was determined using an anti-phospho-Akt Thr308 antibody and was normalized to total Akt expression (n = 4), *p<0.05, **p<0.01, ANOVA analysis with Bonferroni's post-hoc test. **E**) Representative Western blots for NR2B, PLC γ1, 5-HT$_7$ receptor, pAkt Thr308, Akt and β-actin.

39

To determine if the changes in expression and phosphorylation of TrkB-FL and PDGFβ receptor were indeed 5-HT$_7$ receptor dependent in primary hippocampal neurons, the selective 5-HT$_7$ receptor antagonist, SB 258719 (5 μM), was applied 30 min prior to LP 12 treatment for 2 h and 4 h treatments only (due to primary neuronal culture constraints not all time points could be assessed). With 2 h agonist exposure, SB 258719 blocked increases in TrkB-FL expression and Y816 phosphorylation (Figure 6A & B), as well as blocking increases in PLCγ1 expression (Figure 6C) and pAkt Thr308 (Figure 6E). The LP 12 induced reduction in NR2B subunit was not blocked by SB 258719 at 2 h (Figure 6D).

At 4 h of LP 12 treatment, SB 258719 blocked the increases in TrkB Y816 and PDGFRβ Y1021 phosphorylation, respectively (Figure 7A & B). Increases in PLCγ1 expression and pAkt Thr308 phosphorylation were also blocked by SB 258719 (Figure 7C & E). At 4 h of LP 12 treatment NR2B levels were decreased below baseline and SB 258719 blocked this from occurring in the presence of LP 12 (Figure 7D). Taken together, these results suggest that the observed changes in protein and phosphorylation levels induced by LP 12 are indeed 5-HT$_7$ receptor dependent.

Figure 7. Two hour changes in TrkB-FL and PDGFR expression and phosphorylation are 5-HT7 receptor dependent in hippocampal neurons

Cultured hippocampal neurons were treated with selective 5-HT7 receptor agonist LP 12 (300 nM), 5 μM SB 258719, or both where SB was treated 30min prior to LP 12 treatment for 2 h. (**A, B**) The phosphorylation of the TrkB-FL receptor was determined using an anti-phospho-Y816 TrkB receptor antibody and was normalized to total TrkB-FL receptor expression (n = 4), the level of TrkB-FL receptor expression is displayed as a fold change vs. vehicle-treated neurons and was normalized to β-actin (n = 4). (**C, D, E**) The level of PDGFβ receptor expression (n=5), PLCγ1 expression (n=5) and NR2B expression (n=6) are displayed as a fold change vs. vehicle-treated neurons. and were normalized to β-actin. (**F**) The phosphorylation of Akt at threonine 308 (pAkt Thr308) was determined using an anti-phospho-Akt Thr308 antibody and was normalized to total Akt expression (n = 6), *p<0.05, **p<0.01, ANOVA analysis with Bonferroni's post-hoc test. **G**) Representative Western blots for Y816, TrkB-FL, PDGFβR, PLC γ1, NR2B, pAkt Thr308, Akt and β-actin.
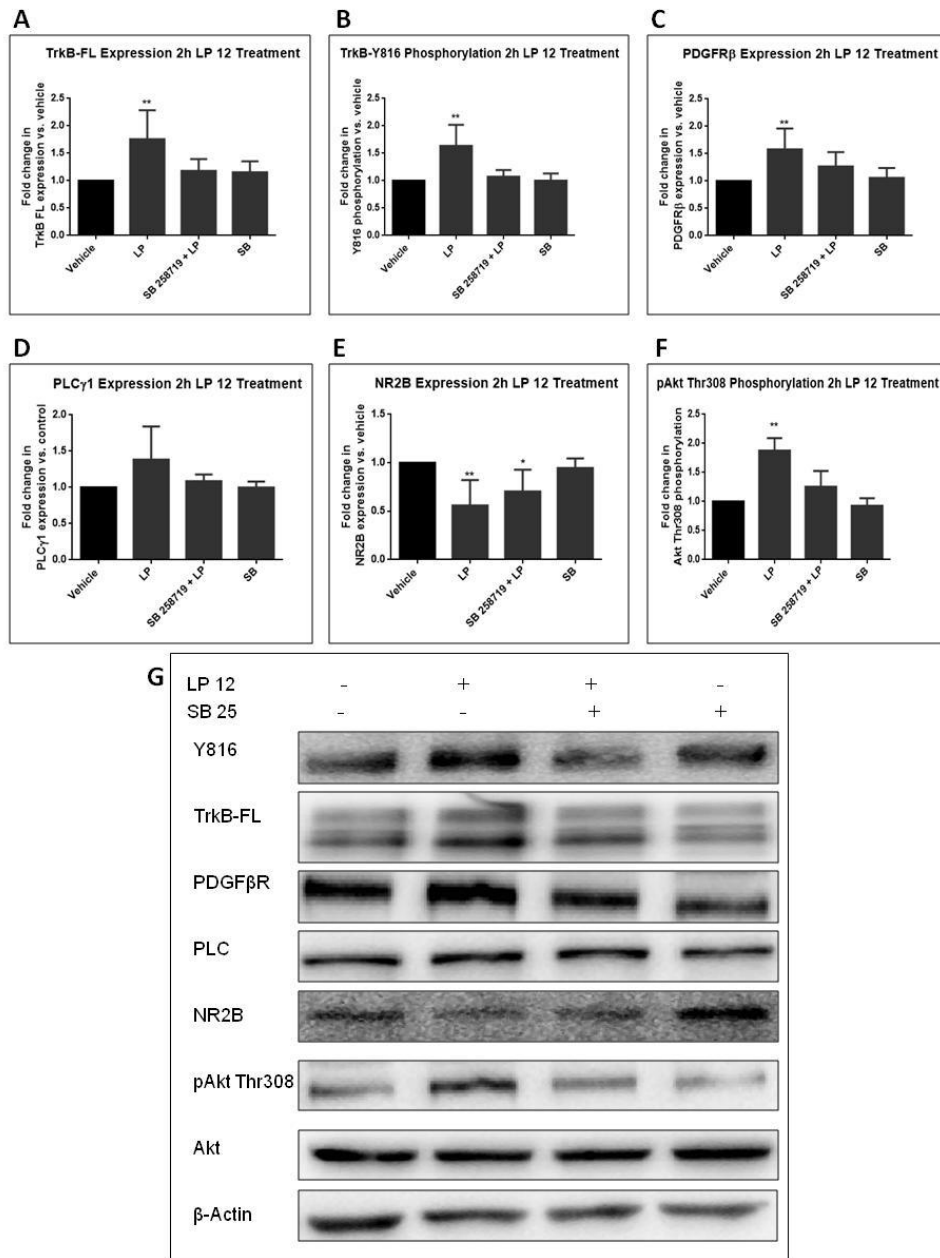
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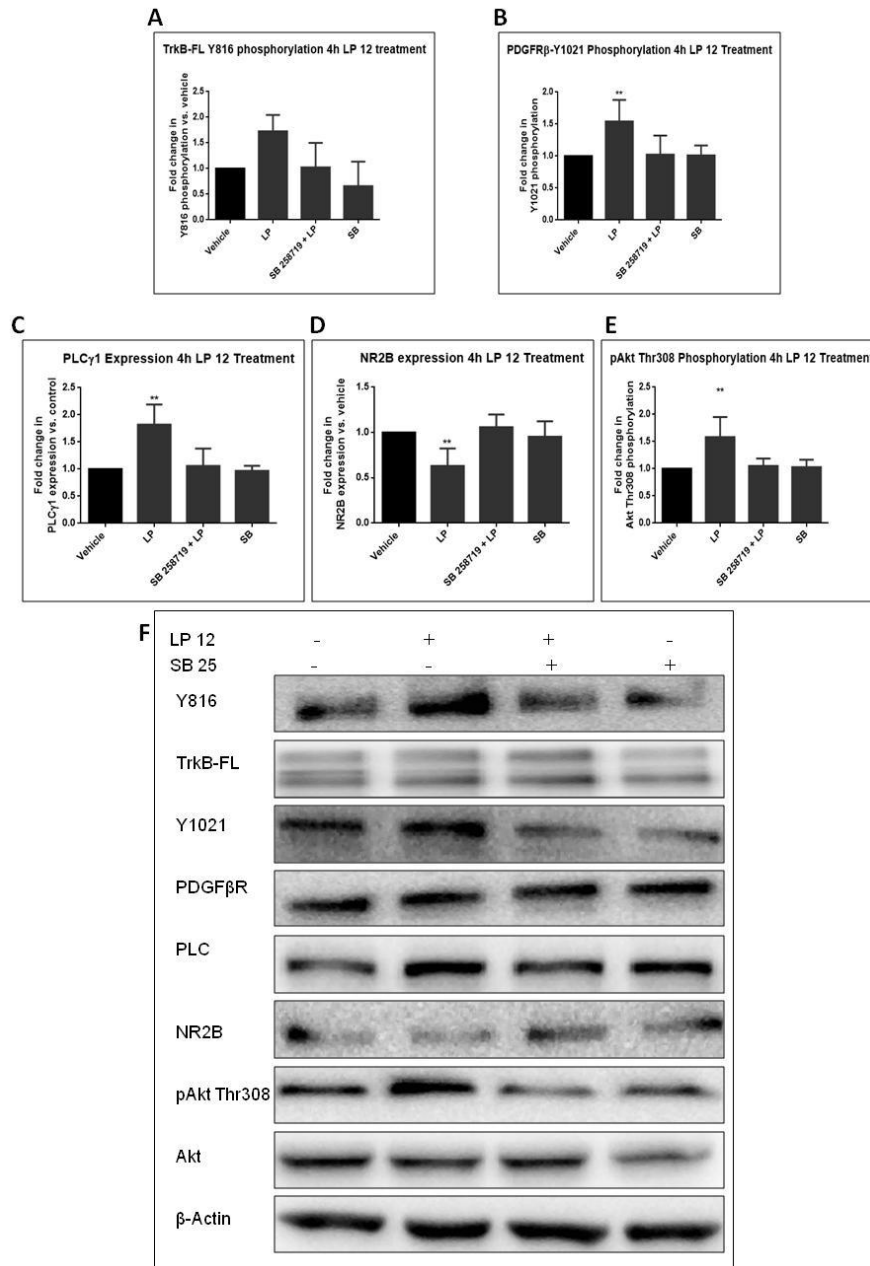
Figure 8. Four hour changes in TrkB-FL and PDGFR expression and phosphorylation are 5-HT7 receptor dependent in hippocampal neurons
Cultured hippocampal neurons were treated with selective 5-HT7 receptor agonist LP 12 (300 nM), 5 μM SB 258719, or both where SB was treated 30 min prior to LP 12 treatment for 4 h. (**A, B**) The phosphorylation of the TrkB-FL receptor was determined using an anti-phospho-Y816 TrkB receptor antibody and was normalized to total TrkB-FL receptor expression (n = 6). The phosphorylation of the PDGFβ receptors was determined using an anti-phospho-Y1021 PDGFβ receptor antibody and was normalized to total PDFGβ receptor expression (n = 6). (**C, D**) The level of PLCγ1 expression (n=6) and NR2B expression (n=6) are displayed as a fold change vs. vehicle-treated neurons. and were normalized to β-actin. (**E**) The phosphorylation of Akt at threonine 308 (pAkt Thr308) was determined using an anti-phospho-Akt Thr308 antibody and was normalized to total Akt expression (n = 6), *p<0.05, **p<0.01, ANOVA analysis with Bonferroni's post-hoc test. **F**) Representative western blots for Y816, TrkB-FL, Y1021, PDGFβR, PLC γ1, NR2B, pAkt Thr308, Akt and β-actin.

42

4.2 Short-term activation of 5-HT7 receptors is neuroprotective against NMDA excitotoxicity in hippocampal neurons

Given the rapid changes in TrkB and PDGFβ receptor expression and phosphorylation at 2 h and 4 h and the decreased NR2B subunit expression, we hypothesized that LP 12 would protect primary hippocampal neurons from NMDA/glycine induced excitotoxicity, similarly to what was previously shown at 24 h [103]. MTT toxicity assays were performed in hippocampal neurons which were pre-treated with 300 nM LP12 for either 2 or 4 h followed by NMDA (100 µM) and co-agonist, glycine (1 µM), to induce NMDA excitotoxicity. As the hypothesis is that LP 12's effects are RTK-dependent, specifically, PDGFβ receptor-dependent, 5 µM STI-571 (imatinib mesylate) was applied 30 min prior to LP 12 treatment. As a positive control, 10 ng/mL PDGF-BB was applied 10 min prior to NMDA/glycine insult as it would demonstrate direct neuroprotective effects of PDGFβ receptor activation against excitotoxicity. Hippocampal neurons pre-treated with LP 12 for 2 or 4 h remained more viable than neurons in the NMDA alone group (Figure 8 A & D). Additionally, the extent of neuroprotection was reduced in neurons pre-treated with STI-571 suggesting that the neuroprotection against excitotoxicity was likely due to LP 12-induced increases in signalling activity of RTKs like the PDGFβ receptor. Finally, neurons that were treated with PDGF-BB directly were protected as previously described at Beazely lab [89, 103].
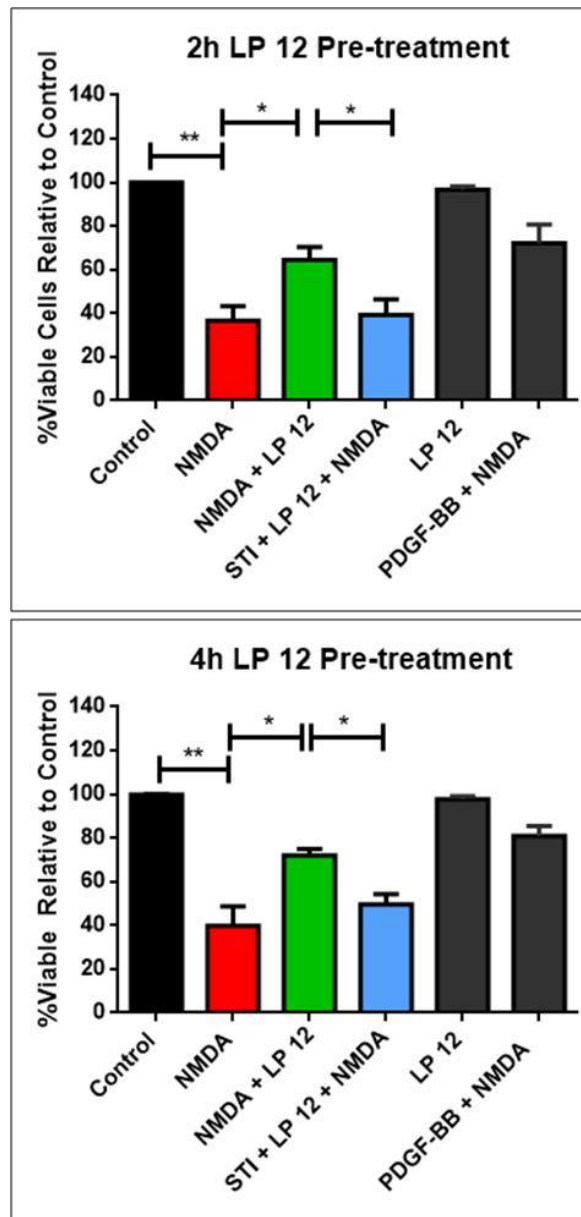
Figure 9. Two and four hours LP 12 pre-treatment protects hippocampal neurons from NMDA excitotoxicity

Hippocampal cultures were pre-treated with vehicle or 300 nM LP 12 for 2 h or 4 h followed by 100 μM NMDA/1 μM glycine for 10 min. To determine if the neuroprotective effects of the 5-HT7-receptor agonist required PDGFβ receptor kinase activity, cultures were co-pre-treated with 5 μM STI-571 (imatinib mesylate) followed by the same LP 12 pre-treatment and respective NMDA/glycine insult. For the PDGF-BB + NMDA bar, 10 ng/mL PDGF-BB was added for 10 min prior to respective NMDA/glycine treatment. The number of viable cells were determined 24 h later by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, n=3 *p < 0.05, **p < 0.01 ANOVA analysis with Bonferroni's post-test (for all LP 12 bars, second last bar is a single, representative experimental result).

4.3 Investigating the susceptibility of differentiated HT-22 cells to NMDA mediated excitotoxicity

Amongst researchers studying neuronal excitotoxicity and ionotropic glutamate receptors the consensus is that cell lines of neuronal origin typically do not possess functional ionotropic glutamate receptors [106]. Many studies to date have attempted to differentiate cell lines with different growth medias, growth factors, and agents like retinoic acid that collectively act at the transcriptional level to induce distinct phenotypic changes in cell lines (however these changes do not always have functional outcomes) [107]. As far as ionotropic glutamate receptors in cell lines are concerned, mRNA transcripts for the NR1 subunit in cell lines like PC12 (adrenal medulla) [113], SH-SY5Y (human neuroblastoma) [108], and oligodendroglial lineage cells [113] have been reported but no evidence of NR2 subunit mRNA or protein has been found in these cell lines.

Two recent publications have made a direct claim that differentiating immortalized mouse hippocampal neuronal HT-22 cells renders them sensitive to glutamate excitotoxicity by inducing expression of NMDA receptors [109, 110]. These papers show evidence of NR1 subunits expressing after differentiation (other subunits were not assessed) and the NMDA receptor channel blockers MK-801 and memantine exerted neuroprotection against 50 µM glutamate or 1 mM homocysteine over 24 h (concentrations typically used in neuronal excitotoxicity experiments in primary neuronal cells) [116]. It is widely accepted across literature that HT-22 cells do not express functional ionotropic glutamate receptors and are susceptible to death only after high doses of glutamate (1 mM-10 mM) over several hours through a mechanism independent of ionotropic glutamate receptors (see recent review [113]).

45

We decided to investigate the possibility of HT-22 cells expressing NMDA receptors and being susceptible to NMDA excitotoxicity upon differentiation as reported in a single isolated publication [116].

4.3.1 HT-22 Cells are susceptible to NMDA and glutamate toxicity in a dose and time dependent manner

The susceptibility of undifferentiated and differentiated HT-22 cells to NMDA and glutamate was tested using the MTT assay. First, in accordance with literature, we found that 24 h incubation with high millimolar concentrations of glutamate (L-glutamic acid) in undifferentiated HT-22 cells significantly reduced cell viability (EC50 5.8 ±0.37mM) (Figure 9A). In contrast, NMDA was not able to exert any significant cytotoxic effects in undifferentiated HT-22 cells (Figure 9A). HT-22 cells that were differentiated for 24 h in neurobasal media and N2 supplement displayed a different sensitivity to 24 h treatments with both glutamate and NMDA. Both glutamate and NMDA significantly reduced cell viability in a concentration dependent manner in differentiated cells; EC50 of ~1.36 ± 0.14mM and ~0.98 ± 0.09 mM, respectively (Figure 9B). Interestingly, the EC50 for glutamate induced reduction in cell viability of differentiated cells is more than ten folds higher than reported by He *et al.* [116].

Using the EC50 values from Figure 9B, further MTT assays were used to assess if short-term (10 min to 4 h) glutamate or NMDA treatment could reduce cell viability in differentiated HT-22 cells. Differentiated HT-22 cells were exposed to 2 mM glutamate or 1 mM NMDA (with 10 µM glycine co-treatment) over a time course of 10 min to 4 h followed by MTT reagent

46

application. Between 2 and 4 h, both NMDA and glutamate significantly reduced cell viability, though not below 50% as was the case with 24 h treatments (Figure 9C).

An important question left to be answered was whether if the sensitivity of differentiated HT-22 cells to glutamate and NMDA was dependent on the amount of time the cells were allowed to differentiate. Time course experiments were performed using the MTT assay to assess if HT-22 cells were still sensitive to 4 h NMDA and glutamate treatment when differentiated for either 6 h, 12 h, or 48 h. The time course revealed that 1 mM NMDA and 2 mM glutamate treatment for 4 h resulted in the greatest reduction in cell viability in HT-22 cells that were differentiated for 24 h (Figure 9D) but further differentiation (48 h) did not result in additional toxicity.
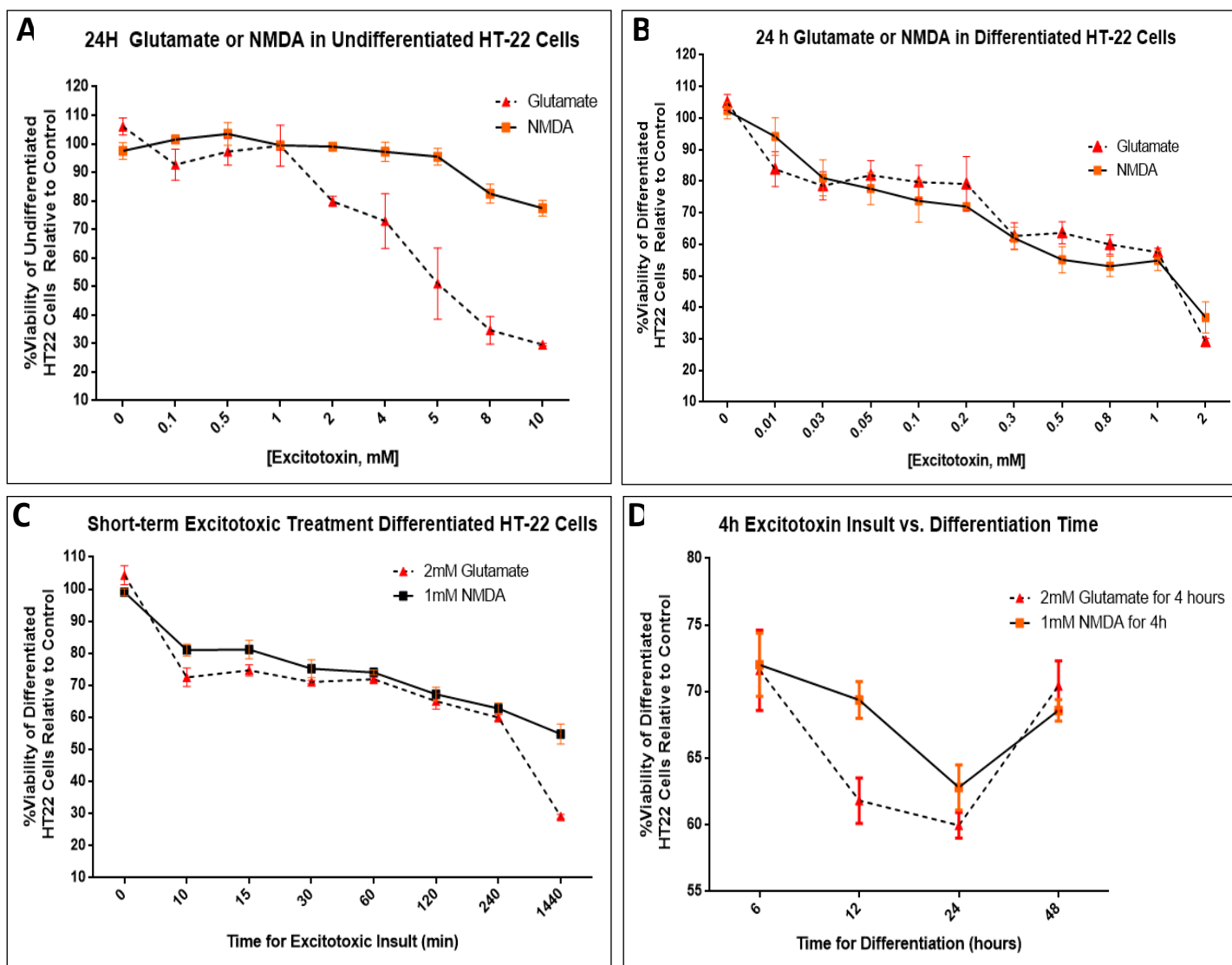
Figure 10. Differentiation renders HT-22 cells susceptible to NMDA and glutamate toxicity

(**A**) Undifferentiated HT-22 cells were treated with 0.1 mM to 10 mM glutamate or NMDA (with 10 μM glycine) for 24 h. (**B**) Differentiated HT-22 cell were treated with 0.01 mM to 2 mM glutamate or NMDA (with 10 μM glycine) for 24 h. (**C**) To assess if a shorter exposure with NMDA and glutamate would exert any toxic effects on differentiated HT-22 cells, 1 mM NMDA (with 10 μM glycine) or 2 mM glutamate was applied for 10 min to 240 min (1440 h time point obtained from **B**). (**D**) HT-22 cells were differentiated for 6h , 12 h, 24 h or 48 h and treated with 1 mM NMDA (with 10 μM glycine) or 2 mM glutamate for 4 h.
The number of viable cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. All experiments were performed twice in quadruplets.

48

4.3.2 Investigating the presence of functional NMDARs and RTKs in differentiated HT-22 cells

MTT toxicity assays showed a clear trend towards differentiation induced phenotypic changes in the HT-22 cell line. Literature to date on undifferentiated HT-22 cells largely shows that high concentrations of glutamate causes the failure of the cystine/glutamate antiporter which brings about ROS mediated oxidative stress; a non-ionotropic glutamate receptor dependent form of glutamate cytotoxicity [106]. The next line of experiments in HT-22 cells investigated if i) differentiation for 24 h causes expression of functional NR1 and NR2 subunits, and ii) the differentiated cells possess functional PDGFRβ and TrkB-FL receptors.

Initial Western blot screening comparing undifferentiated and differentiated HT22 cells revealed conclusive presence of NR1 subunit, TrkB-FL and its truncated isoforms (TrkB-Shc, TrkB-T1), PDGFβ receptor, and 5-HT$_7$ receptors in both types of HT-22 cells (Figure 10). Interestingly, no conclusive evidence of NR2B or NR2A subunits expressing in either type of HT-22 cells was obtained, although densitometry analysis for these blots did result in a net positive intensity where extremely faint intensity bands were noticed particularly for the NR2B subunit in differentiated HT-22 cells (Figure 10).
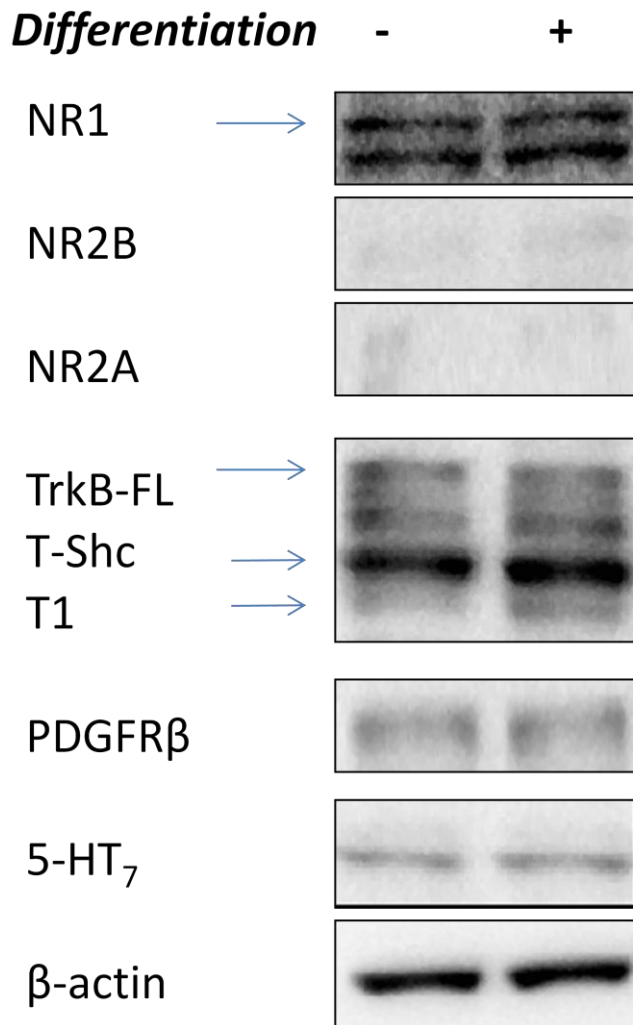
Figure 11 NMDARs and RTKs in differentiated and undifferentiated HT-22 cells

Differentiated or undifferentiated HT-22 cells were lysed and evaluated by Western blot analysis (n=3).

Given no conclusive evidence of NR2 subunits was found through Western blot characterization, a number of reasons pertinent to methodical issues have been suggested in the discussion section of this document. The NR1 subunits were robustly expressed in both differentiated and undifferentiated cells, as has previously been reported [106].

Evidence of functional TrkB-FL or any of its isoforms has not been reported to date in HT-22 cells [94] unless artificially transfected [111]. The distinct finding of TrkB-FL receptors

and its isoforms in both differentiated and undifferentiated cells warranted an investigation into their functionality. First, preliminary immunohistochemistry experiments were performed to visually detect the presence of TrkB receptors in undifferentiated and differentiated HT-22 cells. The presence of TrkB receptors was easily detected in both differentiated and undifferentiated cells under a confocal microscope (Figure 11). Differentiated HT-22 cells were treated with a range of concentrations of BDNF for 10 min, lysed and subject to western blotting. Differentiated cells exerted no response to BDNF treatment as measured by TrkB-Y816 phosphorylation, TrkB-FL expression, ERK phosphorylation and Akt Thr308 phosphorylation (Figure 11). This key experiment suggested the possibility of HT-22 cells expressing non-functional TrkB receptors, although given there was no positive control it is also possible the treatment time of 10 min may not have been enough to phosphorylate TrkB-FL receptor.

The functionality of PDGFβ receptors was also assessed by applying increasing concentrations of the natural ligand PDGF-BB for 10 min in differentiated HT-22 cells. Western blot analysis clearly revealed that PDGFβ receptors were active and functional in this cell line given the sharp (and large) increases in tyrosine 1021, ERK, and Akt Thr 308 phosphorylation, respectively (Figure 12A, C, and D). Upon ligand mediated activation of the tyrosine 1021 site, the total expression of PDGFβ receptors plummeted well below baseline (Figure 12B) and interestingly, the expression of NR1 subunit was significantly reduced with 10 ng/mL PDGF-BB application (Figure 12E).
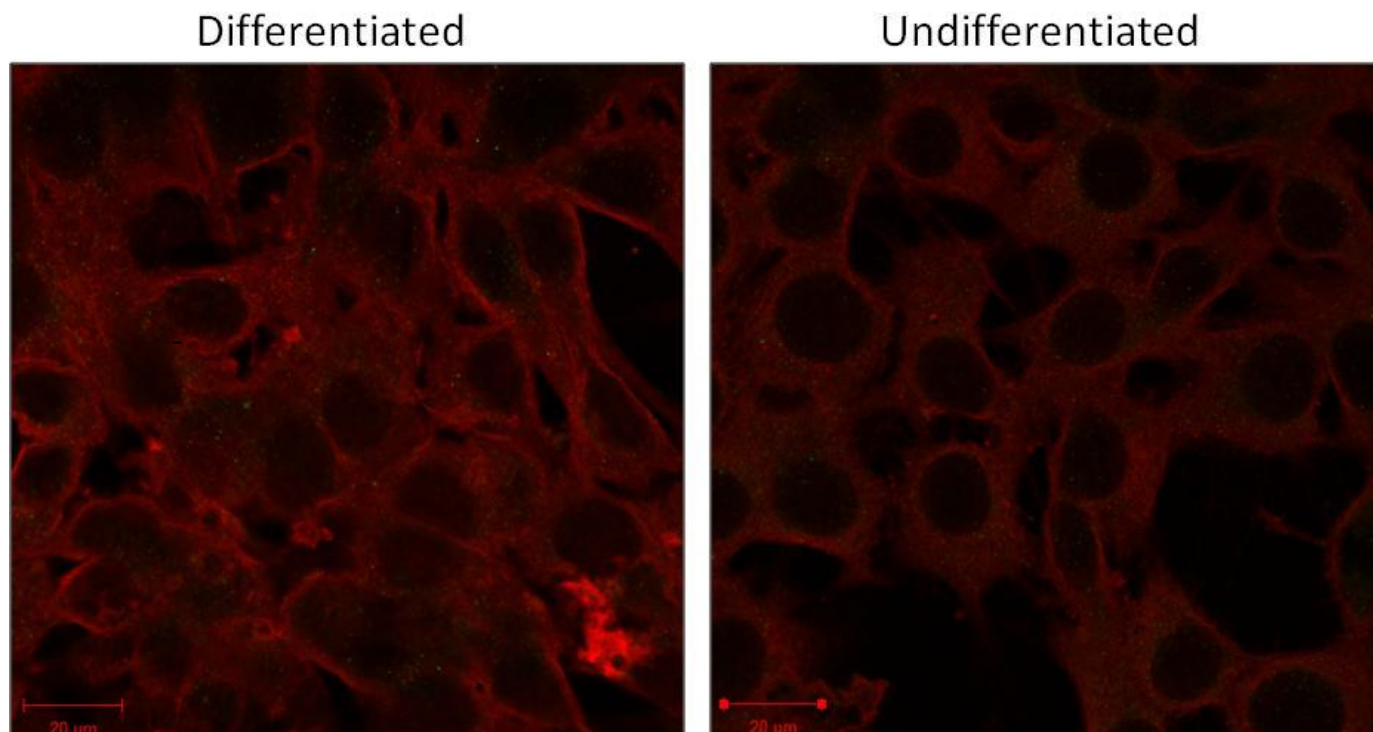
Figure 12. Immunohistochemistry reveals presence of TrkB receptors in differentiated and undifferentiated HT-22 cells

HT-22 cells were either left undifferentiated (R panel) or differentiated for 24 h (L panel). Cell cytoplasm were directly stained with deep red CellMask plasma membrane stain, and the TrkB receptor was detected using an anti-TrkB receptor antibody and a secondary antibody conjugated to Dylight 488 (green). The images displayed are representative of three independents wells per condition. *Imaging was performed by Ding Wen (Roger) Chen, Foldvari Lab, University of Waterloo School of Pharmacy.
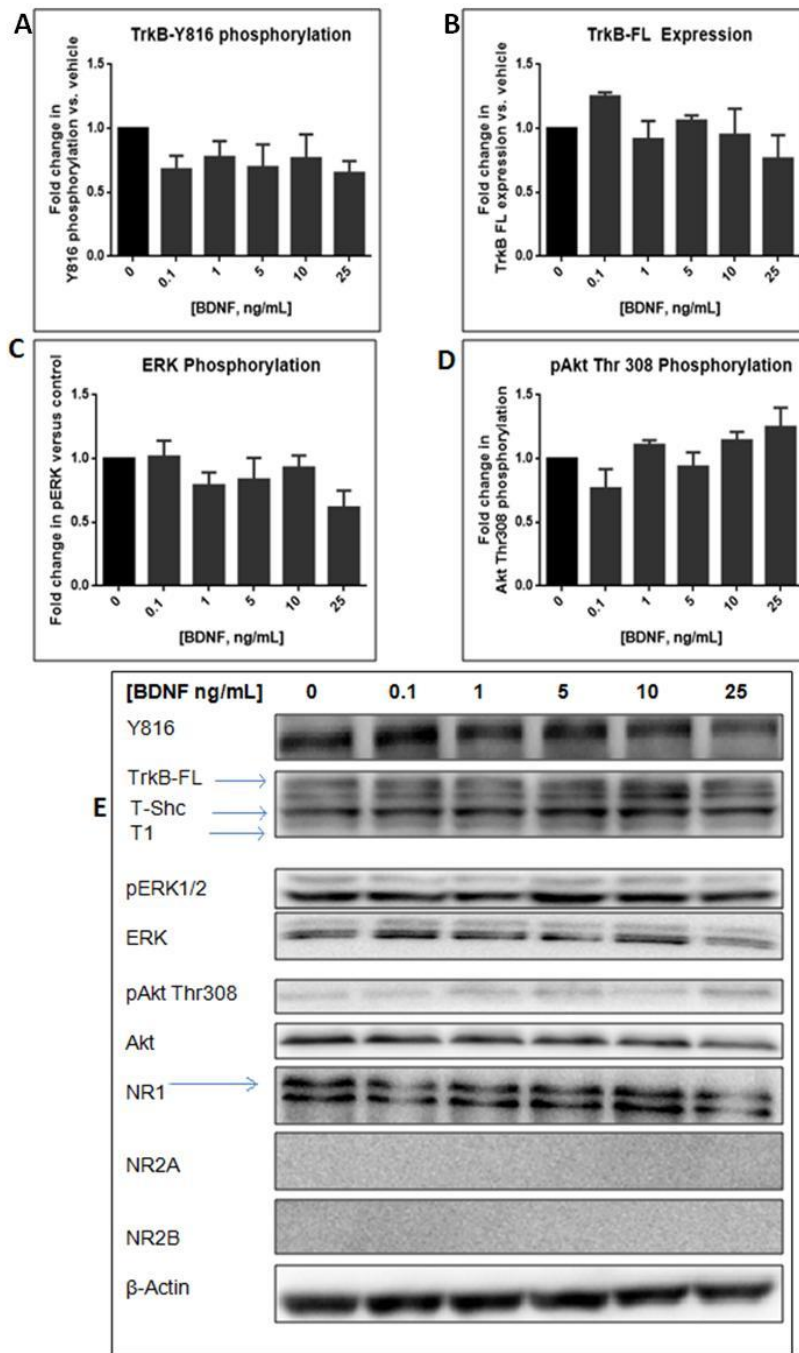
Figure 13. TrkB receptors in differentiated HT-22 cells are not responsive to BDNF

Differentiated HT-22 cells were treated with 0.1 ng/mL to 25 ng/mL BDNF for 10 minutes and subject to Western blotting. (**A, B**) The phosphorylation of the TrkB-FL receptor was determined using an anti-phospho-Y816 TrkB receptor antibody and was normalized to total TrkB-FL receptor expression (n = 3). TrkB-FL receptor expression is displayed as fold change vs. vehicle treated HT-22 cells and normalized to β-actin. (**C**) The phosphorylation of ERK was determined using the anti-pERK1/2 antibody and was normalized to total ERK expression (n=3). (**D**) The phosphorylation of Akt at threonine 308 (pAkt Thr308) was determined using an anti-phospho-Akt Thr308 antibody and was normalized to total Akt expression (n = 3). ANOVA analysis with Bonferroni's post-hoc test. (**E**) Representative western blots for Y816, TrkB-FL, pERK, ERK, pAkt Thr308, Akt, NMDA subunits and β-actin are shown.

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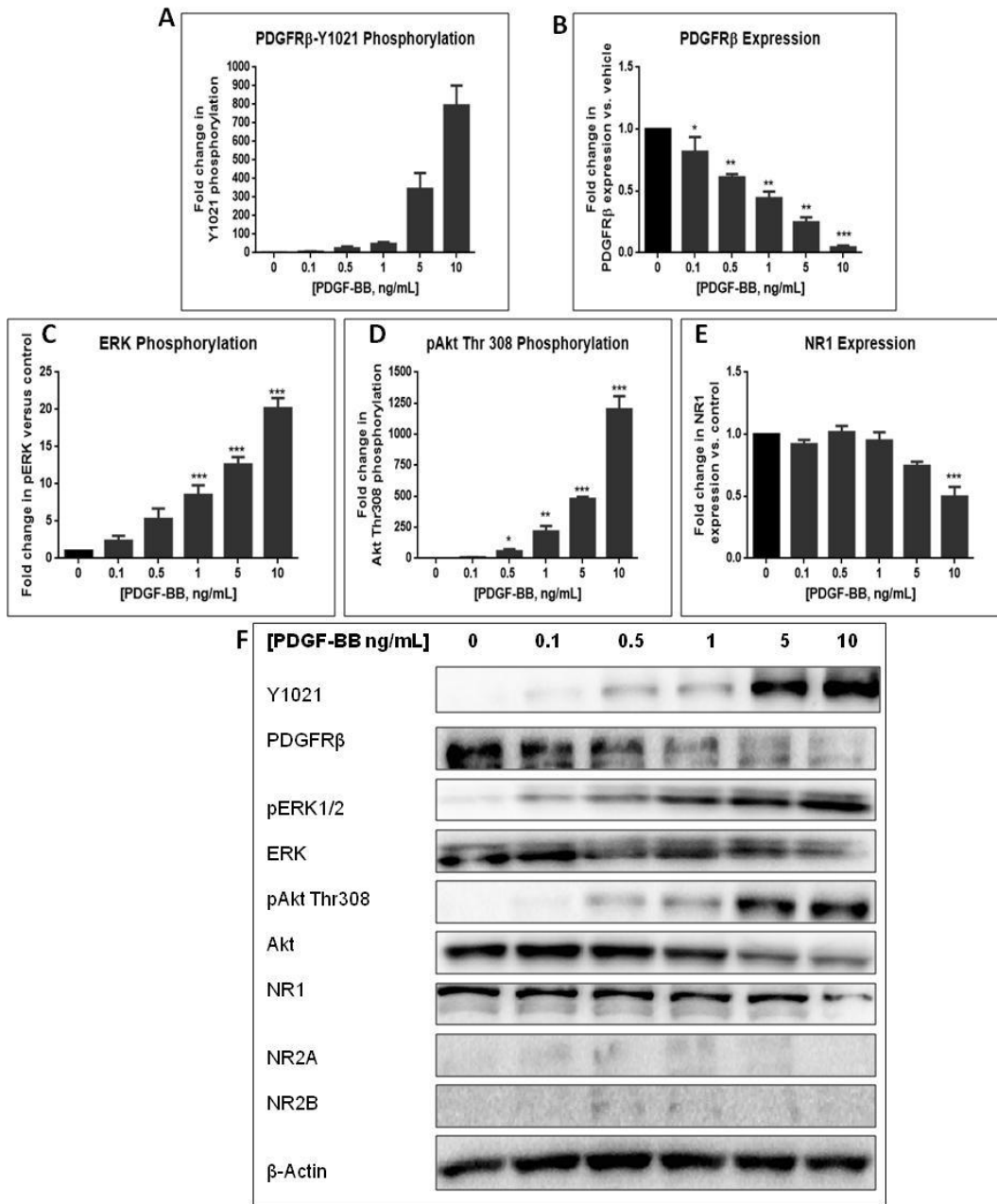
Figure 14. Differentiated HT-22 cells express functional PDGFβ receptors
Differentiated HT-22 cells were treated with 0.1 ng/mL to 10 ng/mL BDNF for 10 minutes and subject to western blotting. (**A, B**) The phosphorylation of the PDGFβ receptors was determined using an anti-phospho-Y1021 PDGFβ receptor antibody and was normalized to total PDFGβ receptor expression (n = 3), where total PDGFβ receptor expression is displayed as fold change vs. vehicle and normalized to β-actin (n=3). (**C**) The phosphorylation of ERK was determined using the anti-pERK1/2 antibody and was normalized to total ERK expression (n=3). (**D**) The phosphorylation of Akt at threonine 308 (pAkt Thr308) was determined using an anti-phospho-Akt Thr308 antibody and was normalized to total Akt expression (n = 3). (**D**) The expression of NR1 subunit is displayed as fold change vs. vehicle and normalized to β-actin (n=3). *p<0.05, **p<0.01, ANOVA analysis with Benferroni's post-hoc test. (**E**) Representative western blots for Y1021, PDGFRβ, pERK, pAkt Thr308, NMDAR subunits, and β-actin are shown.

54

4.4 Short-term LP 12 treatment increases PDGFβ receptor signalling activity in differentiated HT-22 cells

Even though no evidence of functional ionotropic NMDA receptors was found in differentiated HT-22 cells, a rapid decrease of the NR1 subunit expression was seen with 10 ng/mL PDGF-BB application (Figure 12E). Vasefi *et al.* reported a decrease in NR1 subunit expression with 24 h LP 12 application in primary hippocampal neurons [103], but Beazely *et al.*, reported no significant changes in NR1 subunit expression with 10 min PDGF-BB application to CA1 hippocampal neurons [89]. Given the presence of functional 5-HT7 receptors, PDGFβ receptor and potentially a small subset of active NMDA receptors in the differentiated cell line, the next step was to investigate if LP 12 could induce short-term (1-4 h) changes in Y1021 phosphorylation in differentiated HT-22 cells.

A comprehensive time course with LP 12 was not performed in differentiated HT-22 cells based on strong evidence that 300 nM LP 12 treatment for 4 h significantly activates the PDGFRβ -Y1021 site in primary hippocampal neurons (Figure 4A), primary cortical cultures (supplementary Figure 18C & D) and SH-SY5Y neuroblastoma cells (supplementary Figure 18A & B). Differentiated HT-22 cells were treated with a range of concentrations of LP 12 for 4 h to see if 300 nM would be the ideal concentration for activating 5-HT$_7$ receptors as measured by phosphorylation of Y1021, ERK, Akt Thr308 and PLCγ1, respectively. Compared to other concentrations, 300 nM LP 12 considerably increased Y1021, PLCγ1, ERK. and Akt Thr308 and phosphorylation, respectively (Figure 13 A, B, D, E). Interestingly, 5-HT$_7$ receptor expression was well below baseline with 300 nM LP 12 (Figure 13C).
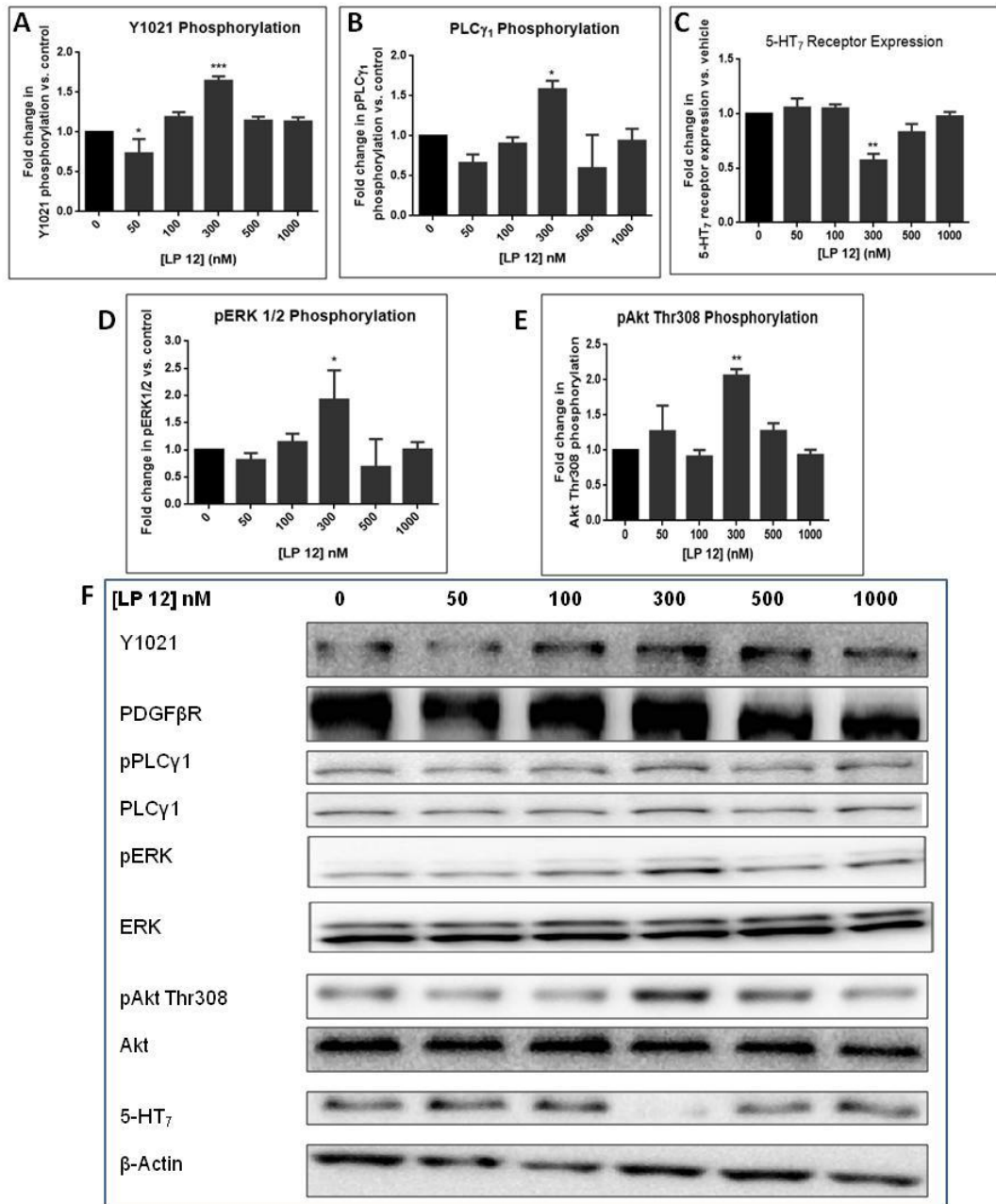
Figure 15. Four hour LP 12 treatment in differentiated HT-22 cells increasing PDGF receptor signalling

Differentiated HT-22 cells were treated with 50 nM to 1000 nM LP 12 for 4 h and subject to Western blotting. (**A**) The phosphorylation of the PDGFβ receptors was determined using an anti-phospho-Y1021 PDGFβ receptor antibody and was normalized to total PDFGβ receptor expression (n= 4) (**B**) The phosphorylation of the pPLCγ1 was determined using an anti-phospho-PLCγ1 antibody was normalized to total PLCγ1 expression (n=4). (**C**) The expression of 5-HT$_7$ receptor is displayed as fold change vs. vehicle and normalized to β-actin (n=4). (**D**) The phosphorylation of ERK was determined using the anti-pERK1/2 antibody and was normalized to total ERK expression (n=4). (**E**) The phosphorylation of Akt at threonine 308 (pAkt Thr308) was determined using an anti-phospho-Akt Thr308 antibody and was normalized to total Akt expression (n=4). *p<0.05, **p<0.01, ANOVA analysis with Bonferroni's post-hoc test. (**F**) Representative western blots for Y1021, PDGFRβ, pPLCγ1, PLCγ1, pERK, ERK, pAkt Thr308, Akt, 5-HT$_7$ receptor, and β-actin.

56

4.5 Short-term 5-HT$_7$ receptor activation is neuroprotective against NMDA excitotoxicity in differentiated HT-22 cells

Some preliminary MTT assays performed in undifferentiated HT-22 cells showed PDGF-BB mediated neuroprotection against 3 h hydrogen peroxide insult, as well as 8 h glutamate insult (see supplementary Figure 19A & B), suggesting PDGFβ receptor activation in HT-22 cells is indeed effective against oxidative stress; a finding that has recently been validated [112]. The next aim was to determine if changes in PDGFβ receptor signalling that was seen with 5-HT$_7$ receptor activation in differentiated HT-22 cells (Figure 13) would be neuroprotective against a 4 h NMDA insult. MTT assays were performed on differentiated HT-22 cells that were pre-treated with 300 nM LP 12 for 4 h after which an excitotoxic insult of 1 mM NMDA/10 μM glycine was given to cells for an additional 4 h. In order to determine if any neuroprotective activity would be due to PDGFβ receptor tyrosine kinase activity, 5 μM of a selective tyrosine kinase inhibitor, AG 1296, was applied 30 min prior to LP 12. Additionally, any effects of the NMDA receptor ion channel blocker, MK-801, against NMDA excitotoxicity was also assessed by applying 20 μM, 30 min prior to NMDA/glycine insult.

LP 12 pre-treatment in differentiated HT-22 cells significantly protected cells against NMDA where the neuroprotection was dependent on the kinase activity of the PDGFβ receptor as AG 1296 co-treatment with LP 12 blocked any survival effects (Figure 14). Interestingly, these assays also revealed that cells treated with MK-801 prior to NMDA insult were significantly more viable than cells treated with NMDA alone (Figure 14), further complicating explanations to the negative findings of NR2 subunits in differentiated cells.
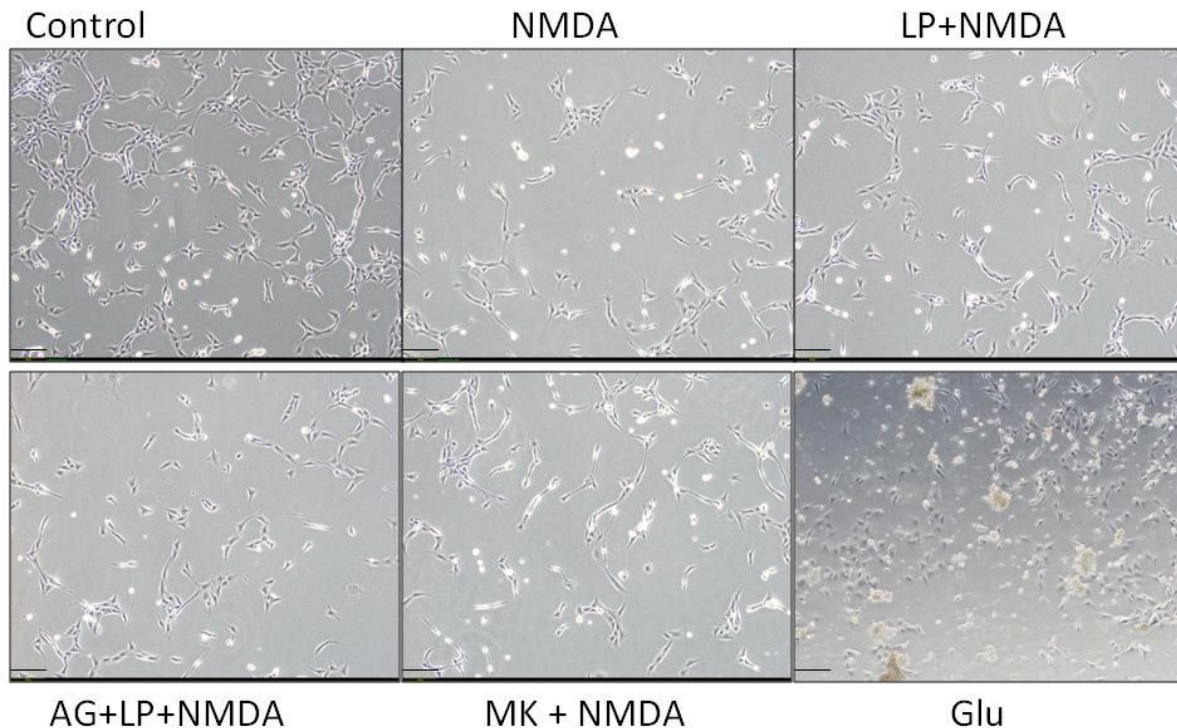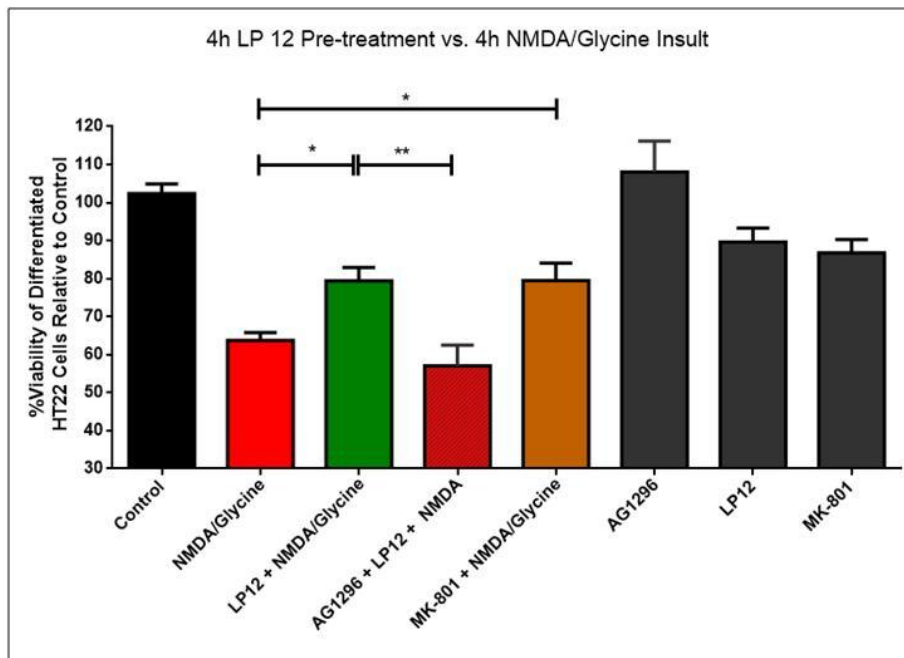
Figure 16. LP 12 is neuroprotective against NMDA excitotoxicity in differentiated HT-22 cells
Differentiated HT-22 cells were pre-treated with vehicle or 300 nM LP 12 for 4 h followed by 1 mM NMDA/10 μM glycine for an additional 4 h. To determine if the neuroprotective effects of the 5-HT7-receptor agonist required PDGFβ receptor kinase activity, cultures were co-pre-treated with 5 μM AG 1296 followed by the same LP 12 pre-treatment and respective NMDA/glycine insult. NMDA receptor ion channel blocker, MK 801 (20 μM) was treated 30 min prior to NMDA/glycine insult. The number of viable cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, n=3 *p < 0.05, **p < 0.01 ANOVA analysis with Bonferroni's post-test. Phase-contrast photomicrographs of representative conditions are shown, bottom right image is an example of 2mM glutamate cytotoxicity after 4 h; images obtained with 10x objective; scale bars represent 50 μM.

58

The mechanism by which LP 12 can keep its pro-survival activity sustained over a course of 8 h and prove to be neuroprotective against NMDA had to be investigated via Western blot analysis. Differentiated cells were pretreated with 4 h of LP 12 or co-pretreated with SB 258719 (30 min prior to LP 12), both in the presence or absence of a 4 h application of NMDA. Given the longer time frame of this experiment the data is hard to interpret from the perspective of 5-HT_7 receptor mediated activation of PDGFβ receptor signalling, however it does show conclusively that in the NMDA insult alone group there is elevated expression of PDGFβ receptors, 5-HT_7 receptors, and the NR1 subunit (Figure 15 A, B and C). To date, it is well understood that the PDGFβ receptor system is up-regulated *in vivo* as a response to ischemia but no study has evaluated 5-HT_7 receptor mRNA or expression levels during an excitotoxic insult. The activity of tyrosine 1021 was found to be below baseline in all conditions that contained NMDA (data not shown), but PDGFβ receptor expression was elevated in all these conditions except for SB 25/LP 12/NMDA condition (Figure 15A), perhaps suggesting a compensatory mechanism in PDGFβ receptor signalling during excitotoxicity. Last, the elevated levels of phosphorylated Akt in the LP+NMDA group (Figure 15D) provides some indication of pro-survival signalling being sustained until the end of the 8 h treatment.
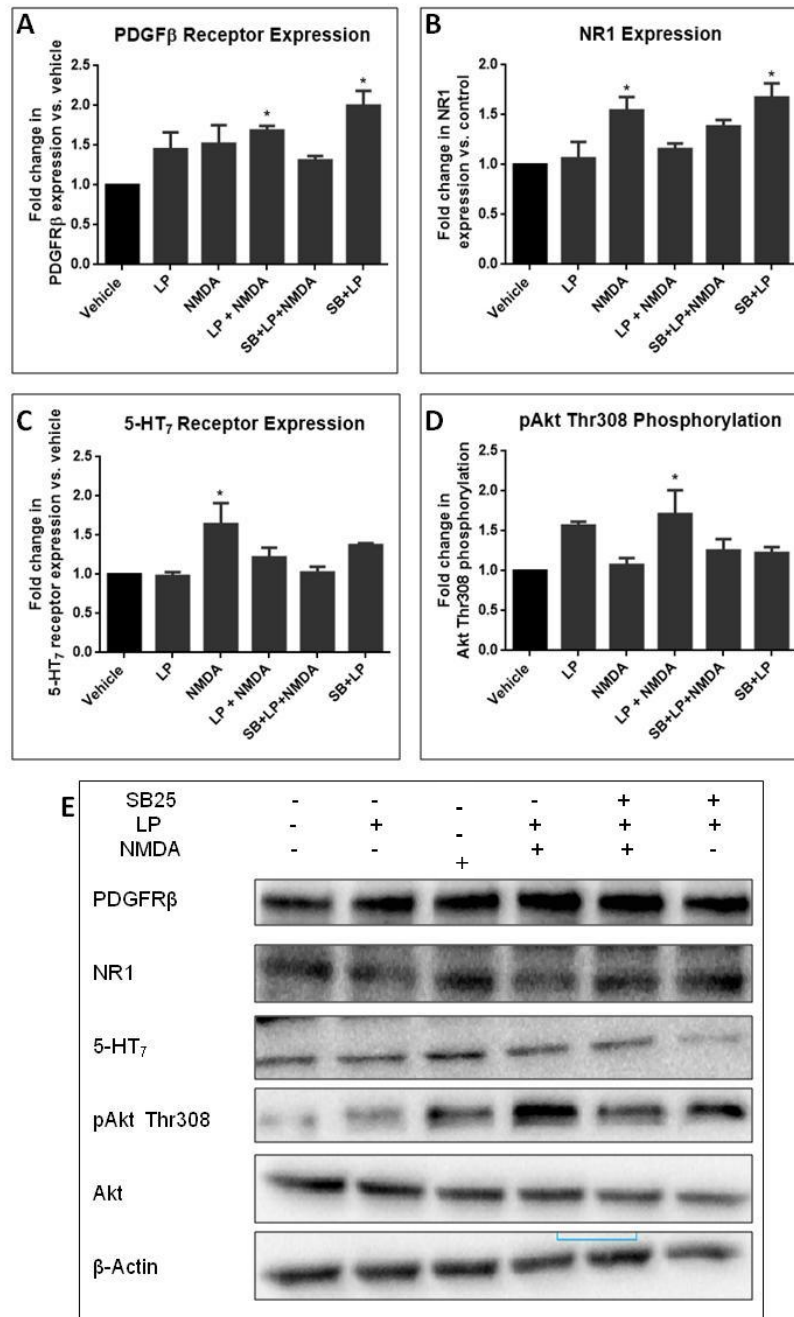
Figure 17. 5-HT$_7$ receptor, PDGFβ receptor and NR1 subunit expression is elevated during NMDA excitotoxicity

Differentiated HT-22 cells were treated with 300 nM LP 12 for 4 h or co-pretreated with 5 μM SB 258719, 30 min prior to LP 12 treatment. The cells were then subject to 4 h insult of 1 mM NMDA/ 10 μM glycine and subject to Western blotting. (**A**) The expression of PDGFβ receptor is displayed as fold change vs. vehicle and normalized to β-actin (n=3). (**B**) The expression of NR1 subunit is displayed as fold change vs. vehicle and normalized to β-actin (n=3). (**C**) The expression of 5-HT$_7$ receptor is displayed as fold change vs. vehicle and normalized to β-actin (n=3). (**D**) The phosphorylation of Akt at threonine 308 (pAkt Thr308) was determined using an anti-phospho-Akt Thr308 antibody and was normalized to total Akt expression (n=3). *p<0.05, **p<0.01 relative to control, ANOVA analysis with Bonferroni's post-hoc test. (**F**) Representative Western blots for PDGFRβ, NR1, 5-HT$_7$, pAkt Thr308, Akt, and β-actin.

60

4.6 Supplementary Figures
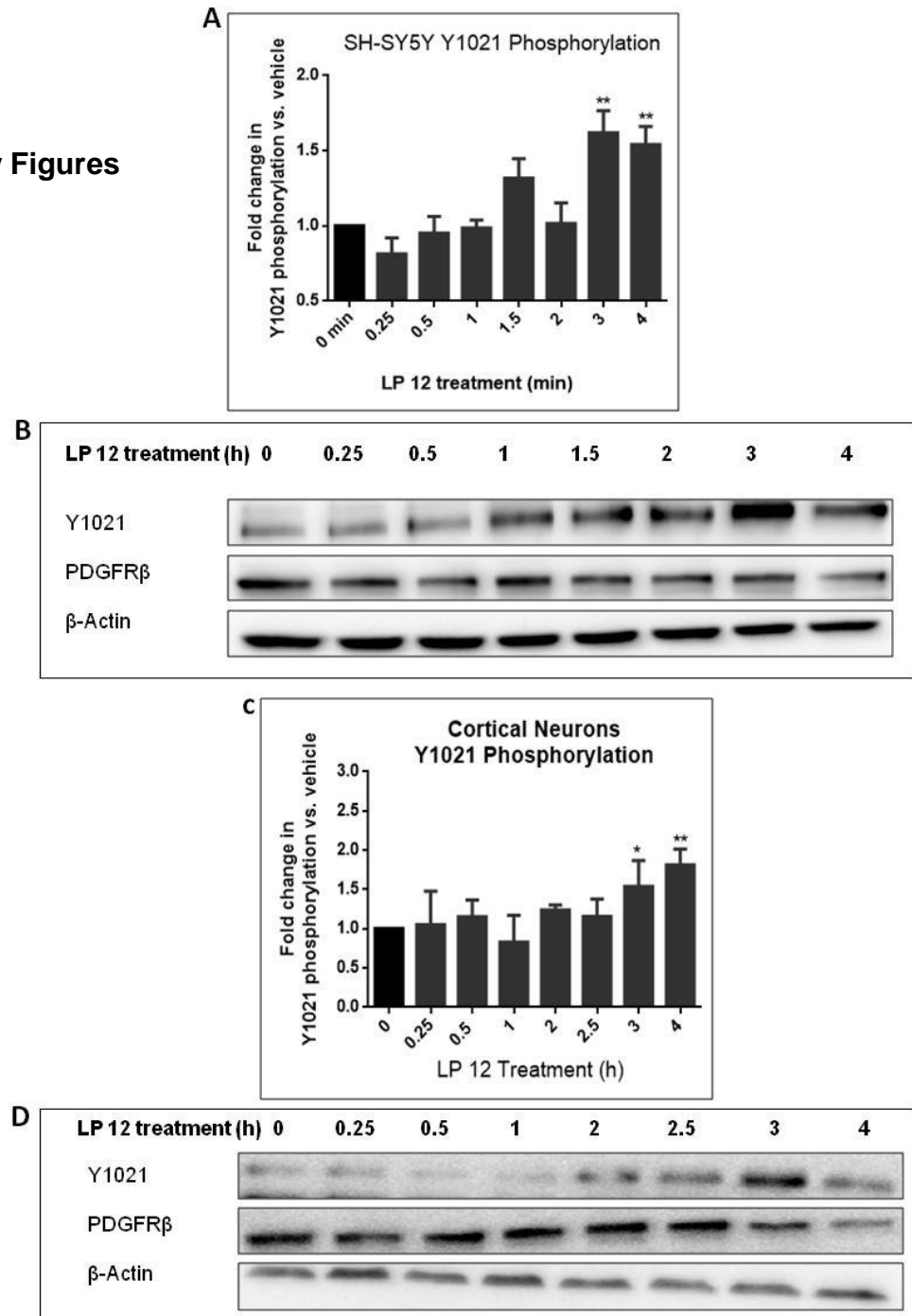
Figure 18. Short-term LP 12 time courses in SH-SY5Y cells and primary cortical cultures
(**A**) SH-SY5Y cells were treated with 300 nM LP 12 for 0.25 h to 4 h and subject to Western blotting. The phosphorylation of the PDGFβ receptors was determined using an anti-phospho-Y1021 PDGFβ receptor antibody and was normalized to total PDFGβ receptor expression (n= 4), (**B**) Representative blots for **A. (C)** Primary cortical cultures were treated on the 10th day *in vitro* with 300 nM LP 12 for 0.25 h to 4 h and subject to Western blotting. The phosphorylation of the PDGFβ receptors was determined using an anti-phospho-Y1021 PDGFβ receptor antibody and was normalized to total PDFGβ receptor expression (n= 5). (**D**) Representative western blots for **C.** *p<0.05, **p<0.01 relative to control, ANOVA analysis with Benferroni's post-hoc test.

61
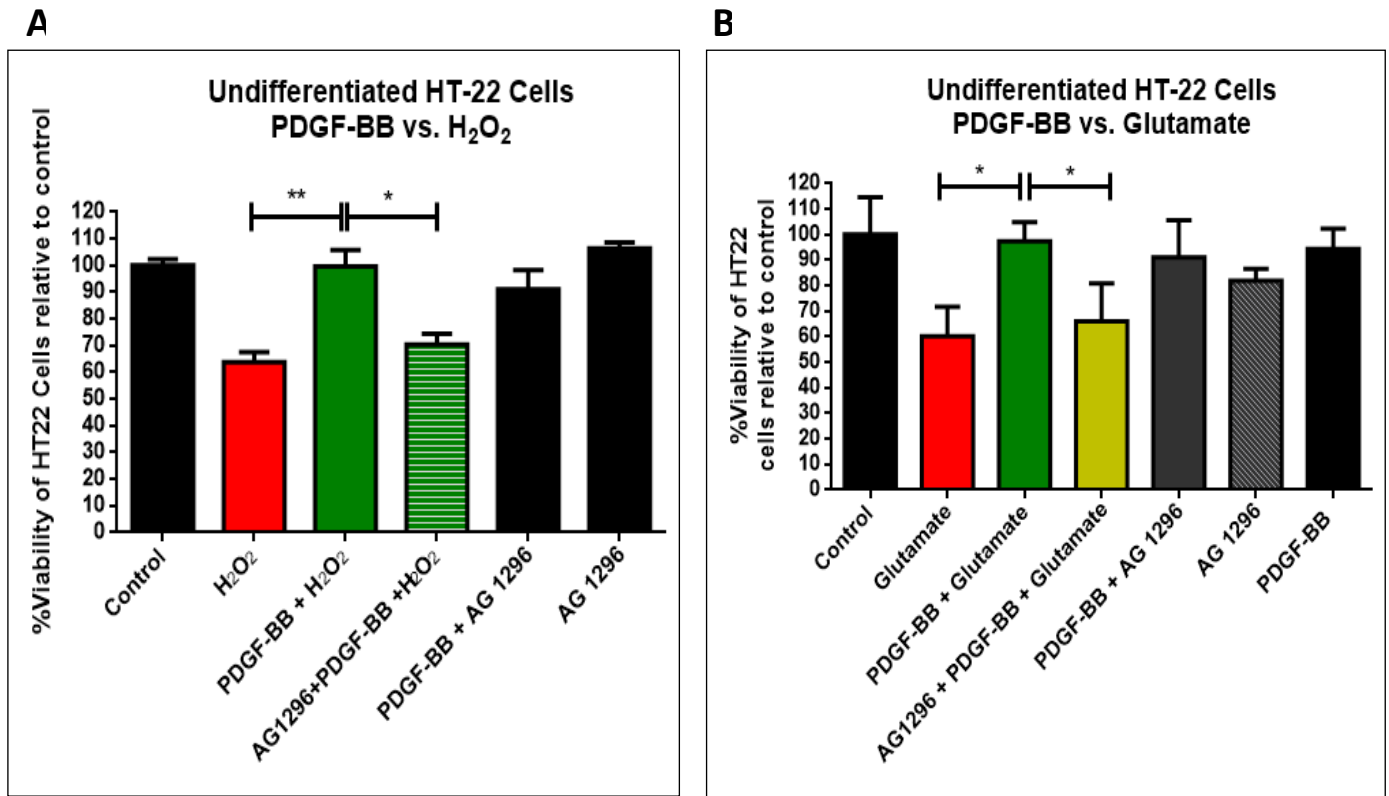
Figure 19. PDGF-BB protects against glutamate and H_2O_2 oxidative stress in undifferentiated HT-22 cells

(A) Undifferentiated HT-22 cells were pre-treated with a 10 min application of 10 ng/mL PDGF-BB or co-pretreated with 5 μM of a PDGFRβ kinase inhibitor, AG 1296, thirty min prior to LP 12 treatment. Cells were then subject to 3 h treatment with 200 μM H_2O_2. **(B)** Undifferentiated HT-22 cells were pre-treated with a 10 min application of 10 ng/mL PDGF-BB or co-pretreated with 5 μM of a PDGFRβ kinase inhibitor, AG 1296, thirty min prior to LP 12 treatment. Cells were then subject to 5 mM glutamate insult for 8 h.

The number of viable cells were determined in both **(A)** and **(B)** by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, n=3 *p < 0.05, **p < 0.01 ANOVA analysis with Bonferroni's post-test.

Chapter 5: Discussion

The results presented in this thesis have added a different dimension to the findings of Vasefi *et al.,* who discovered a novel pathway by which PDGFβ receptors can be activated in a ligand-independent manner using 5-HT$_7$ receptor agonists. The ultimate aim of this project was to ameliorate the effects of neuronal excitotoxicity by achieving the same GPCR-RTK-NMDAR cross-talk with short-term exposure to 5-HT$_7$ receptor agonists. The following sections will summarize and provide commentary on the results of this project.

5.1 5-HT7 receptor activation increases neurotrophic signalling in neurons

The first objective of this project was to collect evidence of significant 5-HT$_7$-PDGFRβ/TrkB-NMDAR cross-talk pathway occurring with short-term agonist (LP 12) exposure, across different cell models. The most compelling evidence of this pathway came from 1 h to 4 h treatment of primary hippocampal neurons where the PDGF system was activated between 2 and 4 h as levels of Y1021 phosphorylation were maximal at 4 h (Figure 5A). Additionally, in primary hippocampal neurons, the selective 5-HT$_7$ receptor inhibitor, SB 258719, blocked increases in Y1021 phosphorylation at 4 h, suggesting the mechanism was 5-HT$_7$ receptor-dependent. In differentiated HT-22 cells, SH-SY5Y cells, and primary cortical neurons, 5-HT$_7$ receptor activation also increased PDGFβ receptor phosphorylation and its subsequent downstream activity at 4 h (Figure 15, supplementary Figure 18 A-D). Interpreting the results of the short-term time course (15 min to 4 h) in this project and that of the long-term time course performed by Vasefi *et al.* [57] suggests that the PDGF neurotrophic system begins

to elevate in activity around 2 h of LP 12 incubation and peaks at 4 h, 8 h and again at 24 h (Figure 20A). In contrast, LP 12 treatment in primary hippocampal neurons showed increased TrkB receptor activity at 2 h and then again at 4 h where TrkB receptor expression was also elevated at 2 and 3 h but not 4 h (Figure 5 C & D). Samarajeewa *et al.,* previously showed that TrkB phosphorylation and expression is elevated significantly above baseline with 24 h LP 12 treatment in primary cortical neurons, retinal ganglion cell line (RGC-5), and SH-SY5Y cells [65]. The short-term time courses of Samarajeewa *et al.,* in SH-SY5Y cells only revealed elevated TrkB expression but not Y816 phosphorylation between 1 h and 2 h whereas in RGC-5 cells both TrkB expression and phosphorylation were elevated in the same time frame [65]. Collectively, it is possible that short-term (2 to 4 h) as well as long-term (24 h) $5\text{-}HT_7$ activation also elevates TrkB receptor activity above baseline however time points between 4 h and 24 h are yet to be investigated (Figure 20B).
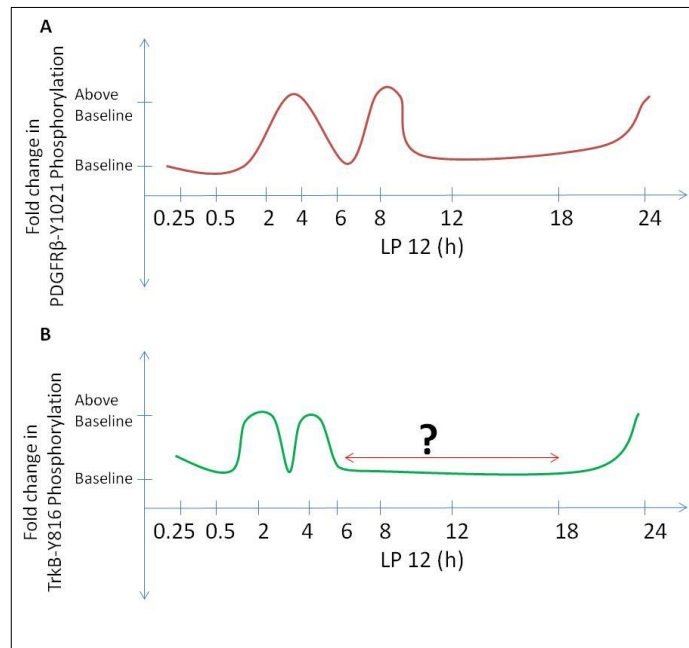
Figure 20. Time course of 5-HT7 receptor activation and PDGFRβ and TrkB neurotrophic activity
Schematic representing time course of 5-HT$_7$ receptor activation as a function of increased PDGFRβ and TrkB activity

Ligand-independent intracellular activation of RTKs by GPCRs is usually coined the term "transactivation", an event that is frequently reported in literature to be acute, and transient in nature. One of the first, and best, studies to have demonstrated transactivation involve D2-class dopamine receptor transactivation of PDGFβ receptors [53]. In this study, the activation of either D2L (long isoform) or D4 dopamine receptors in Chinese hamster ovary (CHO) cells results in the phosphorylation of ERK1/2 downstream of PDGFβ receptors that were transactivated in a Gα$_{i/o}$ and Src-dependent manner [53]. Another prominent example includes 5-HT (serotonin) induced phosphorylation of ERK1/2 as well as PDGFβ and TrkB receptor phosphorylation in SH-SY5Y cells where all phosphorylation events depend on NADPH oxidase but only the RTK phosphorylation is dependent on ROS [55]. Current literature points to a myriad of factors that are involved in transactivation which occurs in many *in vitro* models, these

65

include: timing and choice of ligand, several common effector proteins involved between the GPCR and the RTK, role of β-arrestins in enhancing or downregulating the transactivation, dimerization of RTKs and localization between the GPCR and the RTK [113].

Unlike transactivation that occurs rapidly and only lasts a few minutes, results of this project adds to the growing number of studies that report a late onset yet longer lasting form of ligand independent activation of RTKs via GPCRs. This particular process is an exception to the "acute RTK transactivation rule" where for instance, activation of adenosine receptors or D1dopamine receptors in striatal neurons (using 1 µM of a D1/D5 agonist SKF38393) increases TrkB receptor phosphorylation after 3 h where phosphorylation remains elevated up to 6 h [114]. Additionally, in CHO cells D2L and D3 dopamine receptors increased Akt and GSK-3β phosphorylation for more than 2 h after a 10 min treatment with dopamine, a process that was dependent on the RTK insulin like growth factor-1 (IGF-1) receptor [115]. As shown in Figure 20 it is clear that short-term and long-term activation of 5-HT$_7$ receptor activation by selective agonists leads to ligand independent increases in PDGFβ and TrkB receptor activity that has physiologically relevant consequences as measured by increased PLCγ1 levels (Figure 7D and 8C), decreased NR2B levels (Figure 7E and 8D) and increased Akt phosphorylation (Figure 7F and 7E). The exact intracellular signalling components that lead to short-term and long-term activation of PDGFβ and TrkB receptors via 5-HT$_7$ receptors are currently unknown and should be subject to immediate investigation. As far as this particular cross-talk pathway is concerned, some key questions remain unanswered thus far: i) why is it that 5-HT$_7$ receptor dependent ligand-independent elevation in neurotrophic systems occur in two different time periods? ii)

66

Unlike at 24 h, why do increases in PDGFβ and TrkB receptor activity not overlap over the short-term time course i.e., why don't they occur at the same time points?

The mechanistic questions regarding the role of 5-HT7 receptor activation on neurotrophic factor signalling can be answered by assessing similar intracellular aspects that recent transactivation studies have investigated. First, given Vasefi *et al.* and Samarajeewa *et al.* concluded that long term activation of PDGFβ and TrkB receptors by 5-HT$_7$ receptors were predominantly Gα$_s$-dependent respectively [57, 65], intracellular intermediates that are downstream of PKA activation should be investigated. Additionally, an experiment is required to answer whether if LP 12 induced short-term increases in PDGFβ and TrkB receptor activity are mutually exclusive of each other or if one is dependent on the other. For TrkB, since the phosphorylation and expression were elevated at 2 h in primary hippocampal neurons, a Trk tyrosine kinase inhibitor, K-252a, should be used to block Y816 activity to see if the PDGFβ receptor continues to increase in phosphorylation between 2 h and 4 h after LP 12 treatment. Conversely, PDGFβ receptor kinase activity should be blocked, via AG-1296, and it should be assessed if TrkB receptor phosphorylation still rises at 2 h and 4 h. These experimental conditions will clearly deduce if the short-term changes in PDGFβ and TrkB receptor phosphorylation are mutually exclusive or whether if blocking the activity of one could change the profile of another in the short-term. Given only the PLCγ1 sites on the RTKs were examined in this project (and the phosphorylation states were found to be elevated), it is quite possible that the intracellular mechanism for both short-term and long-term 5-HT$_7$ receptor activation involves an increase in Ca^{2+} influx [114]. The exact role of calcium influx should be unraveled by pre-

67

incubating the same set of experimental conditions with a calcium chelator , such as BAPTA, and an ionophore, such as ionomycin, that can increase intracellular Ca^{2+} influx. Last, the role of β-arrestins and phosphodiesterases that regulate the de-phosphorylation of GPCRs and RTKs should be tested, respectively.

In the context of transactivation and neuronal survival, it is easier to postulate the physiological relevance of transactivation being pertinent to a safety system that delays neuronal death in the absence of growth factors, but this explanation is hard to interpret over a longer time course (hours to days). The ability of 5-HT$_7$ receptor agonists to concurrently increase the activity of PDGFβ and TrkB receptors in a relatively short time frame such as 4 h gives rise to the intriguing possibility that 5-HT$_7$ receptors may play a key role in response to acute neuronal stress like excitotoxicity.

5.2 Short-term activation of 5-HT7 receptors is neuroprotective against neuronal excitotoxicity

The second objective of this project was to decipher if any short-term changes in PDGFβ or TrkB receptor systems caused by 5-HT$_7$ receptor agonists would prove to be neuroprotective against excitotoxicity. Indeed, the short-term increases in RTK signalling in primary hippocampal neurons and differentiated HT-22 cells increased the expression of PLCγ1 binding site as well as the activation of Akt, two events that would be pre-requisites for inhibiting the expression of NR2B subunits and its downstream pro-death signalling, respectively. Subsequent to all the pre-treatment effects of LP 12, 4 h application of LP 12 protected primary hippocampal neurons as well as differentiated HT-22 cells against NMDA insults (Figures 9 & 16, respectively). Indeed, in primary hippocampal neurons the expression of the extrasynaptic NR2B

subunit appears to be reduced over a sustained period of 2 h to 4 h after agonist application (Figure 6A) which further strengthens the proposed mode of neuroprotection by Vasefi *et al.* [103]. Beazely *et al.*, demonstrated that PDGFβ receptor expression is mainly extrasynaptic and that they co-localize with NR2B subunits, providing a possible explanation as to why PDGF-BB application in hippocampal neurons selectively decreases the cell surface expression of NR2B subunits and phosphorylates certain tyrosine sites that increase their internalization kinetics [89]. The exact mechanism by which PDGFβ receptors inhibit NR2B subunits should be further investigated.

Vasefi *et al.,* did not investigate the role of TrkB receptors, but Samarajeewa *et al.* showed both rapid (transactivation) and short-term increases (2 h) in TrkB receptor expression and activity with LP 12 in various models [65]. This project unravelled an intriguing finding that 5-HT$_7$ agonists can cause short-term and concurrent increases in both PDGFβ receptor and TrkB receptor signalling which may incur a synergistic effect against neuronal excitotoxicity. However, the neuroprotection data using MTT assays only utilized RTK inhibitors that were specific for PDGFβ receptors (Figure 8) but the lack of neuroprotection with 2 and 4 h application of LP 12 in the presence of imatinib indicates the neuroprotection is PDGFβ receptor specific and counters the theory that both TrkB and PDGFβ receptor signalling are required to happen concurrently in order to prevent NMDA excitotoxicity.

The mechanisms by which PDGFβ receptors and TrkB receptors prevent neuronal excitotoxicity may be mechanistically and temporally distinct. The PDGFβ receptors physically interact with extrasynaptic NR2B containing pools of NMDA receptors given they do not

69

colocalize with PSD-95 (synaptic marker) or with synaptic NR2A subunits [89], and that PDGFβ

receptor activation reduces expression of NR2B subunits as found in this project along with

several other reports [37, 89, 103]. This key feature of the PDGF system inhibiting extrasynaptic

NMDA receptor activity mimics the action of the pharmacological agent memantine which only

blocks the NMDA receptor ion channel during excitotoxicity, while sparing synaptic NMDA

receptor activity. Indeed, the neuronal and vascular PDGF system is up-regulated rapidly *in vivo*

in response to acute ischemia for neuroprotection and injury repair, respectively [86, 116]. In

contrast, the activation of the neurotrophin system in acute excitotoxicity is believed to be

dependent on the synaptic activity of NR2A containing NMDA receptors which leads to CREB

phosphorylation, leading to the delayed transcription and synthesis of BDNF [75] which can then

act on Trk receptors and also causes transcription of pro-survival genes against both

extrasynaptic NMDA receptor signalling and downstream cell death [117]. Given the enhanced

activity of extrasynaptic NMDA receptors during neuronal excitotoxicity, and the short-term

downregulation of NR2B subunits by PDGFβ receptors, it is very likely that the PDGF system is

first in line to sustain synaptic activity by preventing NR2B subunit dependent decreases in

CREB and ERK phosphorlation [89] which subsequently allows for the synthesis of BDNF and

the long-term trophic effects of TrkB activation and neuronal survival. Given 5-HT$_7$ receptor

expression is likely elevated during excitotoxicity (Figure 17), and that its activation between 2

and 24 h upregulates PDGFβ receptor activity as well as TrkB receptor activity, it is plausible to

suggest that 5-HT$_7$ receptors can play a critical role in response to ischemia and other forms of

neuronal excitotoxicity (see schematic in Figure 21).
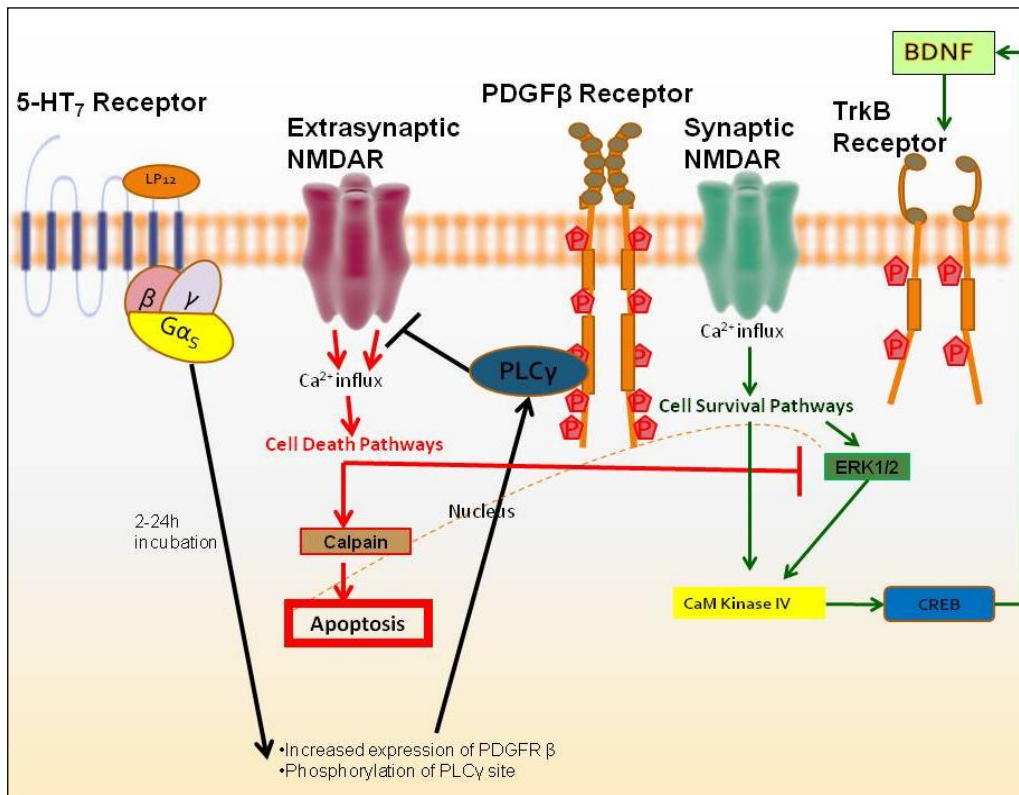
Figure 21. 5-HT$_7$ receptors: short-term and long-term activation against neuronal excitotoxicity

Schematic proposing that 5-HT$_7$ receptor activation under excitotoxic conditions activates PLCγ1 on PDGFβ receptors that inhibits the activity of extrasynaptic NMDA receptors which is no longer able to inhibit synaptic NMDA receptor activity that is required for BDNF-TrkB mediated neuroprotection.

5.3 5-HT$_7$ receptors agonists for neuronal excitotoxicity

The research presented in this project gives direct evidence for targeting the 5-HT$_7$ receptor system to (ligand-independently) upregulate the activity of PDGFβ and TrkB receptors with only short-term agonist application. In cell biology it is no longer a mystery that GPCRs can activate (transiently and long-term) RTKs intrinsically without the need of growth factors and in neurobiology specifically, these systems are beginning to be well characterized. Several clinical attempts to target neurological disorders with growth factors had great premise but limited success due to i) the prolonged deleterious effects of direct activation of RTKs and ii) problems with effectively delivering large proteinaceous growth factors to the brain. Short-term use of 5-

71

HT$_7$ receptor agonists for the treatment of acute brain injuries, which all inherently involve excitotoxicity, may be beneficial given its robust involvement in concurrently upregulating two neurotropic systems that govern the brain's natural responses to injury. However, the use of agonists like LP 12 or its recently more bioavailable and brain penetrable analogues, LP 211, for neuronal excitotoxicity is still an avenue that needs strengthening through more *in vitro* and *in vivo* investigation (see section 5.4). In order for the 5-HT$_7$ receptor to become a new therapeutic target for neuronal excitotoxicity the following research questions need to be addressed well before the pre-clinical stage:

- How long do the effects of LP 12 last in neuronal cultures and is extrasynaptic NMDA receptor expression and activity restored past 24 h?
- Can the short-term effects of LP 12 on RTKs be replicated with post-NMDA treatment instead of pre-treatment?
- What are the intermediate signalling components of short-term and long-term 5-HT$_7$ receptor activation of PDGFβ and TrkB receptors?

5.4 Limitations and future directions

This project had numerous methodical limitations which affect the inferences that can be made out of the results presented. First, in primary hippocampal neurons the excitotoxicity incurred was merely a 10 min NMDA/glycine exposure after which a washout was performed and neurons were allowed to undergo demise for 24 h. Neuroprotection against this form of *in vitro* excitotoxicity is not sufficient evidence for pre-clinical investigation of the agonist given physiologically the insult is typically more robust. In order to warrant the pre-clinical development of LP 12 or analogs, the neuroprotective effects of the agonists need to be assessed using higher NMDA concentrations for longer periods of time or even in the presence of glutamate instead of NMDA. In addition to increasing the intensity of the excitotoxic insult, it is

imperative to assess if the neuroprotection studies done in this project can be replicated using the MAP-2 assay as it will indicate neuronal survival specifically. Second, evidence presented that 5-HT_7 receptors upregulate during excitotoxicity was only drawn from experiments done in differentiated HT-22 cells that were shown to be susceptible to NMDA excitotoxicity. Given no expression of functional NMDA receptors was detected in this cell line, this piece of evidence should still be considered unconfirmed until mRNA analysis or further Western blot analysis of NR2 subunit transcripts is performed in differentiated HT-22 cells. Last, experiments were not performed (in neuronal cultures or cell lines) to conclusively deduce if short-term effects of LP 12 on RTK activation are indeed $G\alpha_s$ dependent or whether if $G\alpha_{12}$ activation also plays a role.

In addition to experiments that could not be performed for various reasons, a key limitation that needs to be elaborated upon is the differentiated HT-22 cell line used in this project. Even though experiments suggest the differentiated cell line possesses functional PDGFβ receptors as well as 5-HT_7 receptors, the lack of NR2 subunit detection via western blot impedes the conclusions that can be made out of this project (thus far). More sensitive tools like real time-polymerase chain reaction (rt-PCR) need to be employed to sensitively detect any levels of NR2 subunit mRNAs in the differentiated cell line. Additionally, cell lysis and Western blot protocols need to be optimized to detect NR2 subunits where a higher concentration of SDS (up to 0.5%) can be used to lyse differentiated cells, and a higher amount of protein can be loaded (40-45 ug) to detect any expression. The data presented in this thesis on the cell line being susceptible to excitotoxicity is indeed plausible given differentiation of any cell line is never wholesome, i.e., not every immortalized cell is able to escape the cell cycle and sustain a post-mitotic fate. The

reduction in viability with 1 mM NMDA for 4 h was on average around 35% suggesting only a subset of cells may have been undergoing the differentiation necessary to possess functional NR2 subunits, which meant not enough protein was extracted from differentiated cells to be detected via western blot. Similar observations supporting only a subset of cells undergoing differentiation were reported by Zhao *et al.* who assessed homocysteine toxicity in differentiated HT-22 cells [110]. Experiments in the very near future at Beazely lab will undoubtedly reveal whether if this cell line can be used as a model to study NMDA excitotoxicity. In addition to the lack of NR2 subunits detected in HT-22 cells, data presented here shows no response of these cells to a 10 min application of BDNF (Figure 13). This experiment suffered heavily from not having a positive control (perhaps the use of SH-SY5Y cells alongside HT-22 cells) and the short application time of 10 min with BDNF. In literature, while variable treatment times with BDNF are used in cell lines and primary neurons, it is accepted that any changes in ERK or Akt activation can be delayed till 30 minutes post treatment [117]. An additional experiment needs to be performed with a longer exposure to BDNF to conclusively rule out the cell line possessing non-functional TrkB receptors.

The ultimate future direction of this research will be to utilize electrophysiology tools to measure miniature excitatory postsynaptic currents (mEPSCs) in hippocampal neurons (or differentiated HT-22 cells if found to be successfully differentiated) subject to both short and long-term treatment with LP 12. This series of experiments will conclusively unravel the time course by which 5-HT$_7$ receptors may promote, inhibit or have no effect on NMDA receptor activity.

5.5 Conclusion

The prolonged activation of the 5-HT$_7$ receptor inhibits NMDA receptor function by upregulating PDGFβ receptor expression and activity. PDGFβ receptors activation is a biological event that occurs in response to neuronal excitotoxicity, but the most toxic form of neuronal excitotoxicity occurs in an acute and short-term time frame where prolonged activation with a GPCR to upregulate its activity may not be a favourable clinical strategy. This research shows evidence of short-term activation of 5-HT$_7$ receptors upregulating both PDGFβ and TrkB receptor signalling activity which also proves to be neuroprotective against NMDA excitotoxicity, further suggesting that 5-HT$_7$ receptor modulation of NMDA receptors is a novel therapeutic target for a multitude of neurodegenerative and neurological pathologies.

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