Transactivation of platelet-derived growth factor receptor type β: Mechanisms and potential relevance in neurobiology

by

Jeffrey Stephen Kruk

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AUTHOR'S DECLARATION

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

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Abstract

In the absence of ligand, certain growth factor receptors can be activated via G protein-coupled receptor (GPCR) activation in a process termed transactivation. Serotonin (5-HT) receptors can transactivate the receptor tyrosine kinase (RTK) platelet-derived growth factor (PDGF) β receptors in smooth muscle cells, but it is not known if similar pathways occur in neuronal cells. Here, it is shown that 5-HT can transiently increase the phosphorylation of PDGFβ receptors in a time- and concentration-dependent manner in SH-SY5Y neuroblastoma cells. This transactivation pathway was pertussis-toxin sensitive, and was dependent on phospholipase C activity, intracellular calcium signaling and subsequent protein kinase C activation. Exogenous application of non-lethal concentrations of H_2O_2 induced the phosphorylation of PDGFβ receptors in a concentration-dependent fashion, similar to that observed with 5-HT. Further investigation revealed reactive oxygen species (ROS) production as a necessary component in the transactivation pathway, as scavenging ROS eliminated PDGFβ receptor phosphorylation. NADPH oxidase was determined to be the likely source of ROS given that the NADPH oxidase inhibitors diphenyleneiodonium chloride and apocynin abrogated PDGFβ receptor transactivation. The role of Src tyrosine kinase was also investigated, and its location in this signaling cascade was found to be downstream of calcium signaling, but upstream of NADPH oxidase activity. In addition, the activation of ERK1/2 in this system was elucidated to be independent of PDGFβ receptor transactivation. Interestingly, 5-HT also transactivated TrkB receptors, another RTK whose function is implicated in clinical depression. Expectedly, the enzymes in this mechanism were consistent with those revealed in 5-HT-to-PDGFβ receptor signaling. This cross-talk between 5-HT and RTKs such as TrkB and PDGFβ receptors identifies a potentially important signaling link between the serotonergic system and neurotrophic factor signaling in neurons that could have implications in mental health disorders including depression.

Furthermore, although transactivation pathways are commonly initiated by a GPCR, recent reports have demonstrated that selective serotonin reuptake inhibitors (SSRIs) were able to block 5-HT-induced transactivation of PDGFβ receptors, suggesting that in addition to GPCRs, monoamine transporters may also be involved in RTK transactivation. SH-SY5Y cells pretreated with the SSRI fluoxetine blocked 5-HT-induced transactivation of the PDGFβ receptors, but not PDGF-induced PDGFβ receptor activation. Upon further examination, it was discovered that during the pretreatment period, fluoxetine itself was transiently transactivating the PDGFβ receptor via $5\text{-}HT_2$ receptors. By the end of the pretreatment period, the effects of fluoxetine on PDGFβ receptor phosphorylation had

returned to baseline, and a subsequent transactivating stimulus (5-HT) failed to "re-transactivate" the PDGFβ receptor. Additional investigations demonstrated that 5-HT pretreatment can block dopamine-induced PDGFβ receptor transactivation, but not PDGF-induced PDGFβ receptor activation. This is the first demonstration of the heterologous desensitization of an RTK via a transactivation pathway, and this phenomenon is specific for transactivation pathways because in all cases the PDGFβ receptor ligand PDGF-BB was able to directly stimulate receptor activity in spite of GPCR agonist pretreatment. Heterologous desensitization in transactivation signaling reveals a previously unknown short-term "blackout" period wherein no further transactivation signaling can occur to potentially exploit the mitogenic effects of RTK activation.

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Lastly, I would also like to thank my family for their unwavering support over the last four years. It is truly appreciated.

Dedication

This thesis is dedicated to graduate students everywhere. I know what you are going through.

- Basic research is 99% exasperation and 1% dumb luck. We really don't know a millionth of one percent about anything… but we're trying. *(Apologies to T.A. Edison)*

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Chapter 1
Introduction

Of all the systems in the human body, the nervous system is by far the most complex. The notion that roughly one billion neurons forming trillions of synaptic connections define the personality of an individual is remarkable. And yet, although we can macroscopically map out various regions of the nervous system and assign to them specific roles, the functional aspects of this system inevitably come down to the signaling capabilities of each individual cell. Similar to other organ systems, signaling molecules and their receptors drive the cellular function of both neurons and glial cells in the nervous system. In this chapter, G protein-coupled receptors, receptor tyrosine kinases, their respective ligands, and various intracellular effectors are discussed in general and in the context of a particular phenomenon termed transactivation. These elements also partake in the biochemical pathophysiology of mental disorders such as depression, and this is contemplated here and throughout this thesis.

1.1 G protein-coupled receptors

1.1.1 G protein-coupled receptor activation

G protein-coupled receptors (GPCRs) are metabotropic, signal-transducing proteins with characteristic seven transmembrane domains [1]. They make up a large, diverse family of receptors that respond to a vast array of stimuli including hormones, neurotransmitters, lipids, and sensory stimuli [2, 3]. In the absence of agonist, the energy required to break intramolecular bonds between transmembrane domains in the inactive state in part determines the level of basal activity [4]: those receptors with strong intramolecular bonds in the inactive state have low basal activity [5]. Agonist binding to the GPCR that is able to achieve the required activation energy induces a conformational change from an inactive to active state [4]. This exposes the G protein-binding domain in the cytosol to heterotrimeric G proteins, the proteins responsible for initiating signal transduction [1]. Heterotrimeric G proteins are composed of an α, β and γ subunit that are assembled when inactive. There are 16 known α subunits, with the three most studied being $G\alpha_i$ which negatively regulates adenylate cyclase activity, while $G\alpha_s$ stimulates adenylate cyclase activity, and $G\alpha_q$ activates phospholipase C [6]. The cytosolic portions of GPCRs contain guanine exchange factor domains,

which catalyze a replacement of GDP with GTP on the α subunit, thus activating it [6]. The α subunit separates from the βγ complex and interacts with the appropriate effector [6]. To terminate G protein signaling, the α subunit is deactivated by its intrinsic GTPase activity, or more rapidly by other GTPase-activating proteins (GAPs), which hydrolyze GTP to GDP. This results in the α subunit rejoining the βγ complex [6]. Interestingly, the βγ complex can also participate in its own signaling pathways, separate from α subunit activity [7].

1.1.2 G protein-coupled receptor deactivation

Signaling from GPCRs can be terminated by receptor phosphorylation at specific serine and threonine residues, thus impeding G protein association with the receptor and the initiation of further signal transduction [6]. GPCR amino acid segments that match a consensus sequence are subject to phosphorylation by activated PKA and PKC, which are non-selective in phosphorylating active or inactive GPCRs [8]. G protein-coupled receptor kinases (GRKs), on the other hand, are also serine/threonine kinases that specifically target activated GPCRs [9]. The resulting phosphorylated residues on GPCRs serve as docking points for β-arrestin molecules, which are necessary for full receptor deactivation [8]. Moreover, β-arrestin C-termini have affinity for clathrin triskelia which associate with the receptor complex [5]. The association of clathrin with β-arrestin-GPCR complexes leads to formation of a coated pit and internalization of the membrane containing the GPCR [6], and prevents further agonist binding to the GPCR. Internalized vesicles containing the GPCR can then be recycled back to the membrane, or targeted for destruction in lysosomes [5]. The affinity of β-arrestins to the receptor in endosomes seems to dictate the recycle versus degradation fate of internalized GPCRs [5].

1.2 Serotonin signaling

1.2.1 Serotonin in the CNS

Derived from the amino acid tryptophan [10], serotonin (5-HT) is a crucial neurotransmitter that has been associated with sleep/wake cycles [11], cognition [12], and memory [13]. The major source of 5-HT in the CNS is derived from serotonergic neurons in the raphe nuclei located in the brainstem, and project to several important areas including neocortex, cerebellum, striatum, amygdala,

hippocampus, motor systems, and spinal cord [14-16]. 5-HT synthesis begins with the amino acid tryptophan being hydroxylated to 5-hydroxytryptophan by the enzyme tryptophan hydroxylase [17]. 5-hydroxytryptophan is then decarboxylated to 5-hydroxytryptamine (i.e. 5-HT) by L-aromatic amino acid decarboxylase [17]. Synthesized 5-HT is packaged into vesicles by the vesicular monoamine transporter and awaits a signal for release into the synaptic cleft [18].

When an action potential reaches a presynaptic terminal, voltage-gated calcium channels in this structure respond by opening to allow an influx of calcium, which initiates neurotransmitter vesicle fusion with the membrane [19]. These synaptic vesicles dock at the membrane and are primed by formation of a SNARE complex consisting of SNAP-25 and syntaxin proteins on the plasma membrane, and synaptobrevin proteins on the vesicle [19]. Once this complex is stabilized by complexin, calcium influx from the depolarization binds synaptotagmin proteins on the vesicle, which catalyze a pore opening and neurotransmitter release [19].

Upon release, 5-HT diffuses across the synaptic cleft where it binds to receptors on the post-synaptic cell [19]. The type of 5-HT receptors found on the post-synaptic cell will dictate the particular cellular effect that will be elicited. This could be a change in membrane potential via ionotropic receptor activity or initiation of a signal transduction pathway [18]. Released 5-HT may also bind to receptors on the pre-synaptic cell, and this will be discussed in subsequent sections.

1.2.2 Serotonin reuptake

5-HT does not remain in the synaptic cleft indefinitely; the majority is removed from the synapse by the serotonin transporter (5-HTT). The 5-HTT pumps 5-HT back into the presynaptic cell where it is either repackaged into vesicles or degraded by monoamine oxidase A via oxidation to 5-hydroxyindoleacetic acid [12].

The serotonin transporter is a roughly 630-amino acid monoamine transporter responsible for clearing 5-HT from the synaptic space [20, 21]. It contains twelve transmembrane domains with both termini within the cytosol [21, 22]. Newly synthesized 5-HTT protein is processed in the endoplasmic reticulum and Golgi with a series of glycosylation steps necessary for full protein function [22]. 5-HT reuptake is dependent on Na^+ and Cl^- ions [22]. One model for 5-HTT mechanics suggests that Na^+ binds along with 5-HT to the transporter, which subsequently undergoes a conformational change that releases Na^+ and 5-HT into the cytoplasm [23]. Upon binding K^+, the transporter is returned to its

outward-facing conformation. Cl^- is also present and seems to be a necessary co-factor for these actions [23].

Intriguingly, there was interest in an allelic variation in a promoter region of the 5-HTT gene. Investigations were conducted into correlations in affective disorders with the "short" allele, which was posited to be associated with an increase in depression episodes and a decrease in 5-HTT mRNA expression as compared to the "long" allele. Some studies support this correlation while others do not [24-26]. Nevertheless, the role of 5-HT in CNS pathology is of particular interest given its potential role in depression, schizophrenia, anxiety and drug addiction, and the fact that there are several examples of very successful drugs that target the 5-HT system to alleviate these pathological effects [12, 27]. As such, the study of 5-HT and its function is an ongoing area of intense active research.

1.2.3 Serotonin receptors

5-HT receptors include both ionotropic and metabotropic subtypes. 5-HT_3 receptors are non-selective ion channels, permeable to sodium, potassium, and calcium [12], and the remainder are G protein-coupled receptors (GPCRs). The $G\alpha_s$-coupled 5-HT receptors include 5-HT_4, 5-HT_6 and 5-HT_7, while 5-HT_1 and 5-HT_5 subtypes are linked to $G\alpha_{i/o}$ [12, 28]. Finally, the $G\alpha_q$-coupled 5-HT_2 receptors complete the repertoire [12, 28]. In the following sections, the 5-HT_{1A} and 5-HT_2 receptors are discussed, as they are implicated in the transactivation pathway elucidated here.

1.2.3.1 5-HT_{1A} receptors

5-HT_{1A} receptors are abundantly found throughout the CNS including in the hippocampus, cortex, raphe nuclei, thalamus, and hypothalamus, although there are few to none located in the cerebellum and basal nuclei [12, 28, 29]. One pool of these receptors is situated postsynaptically in various limbic areas where they evoke hyperpolarizations via links with G protein-coupled potassium channels [12, 28, 30]. 5-HT_{1A} receptors also mediate norepinephrine and acetylcholine release from respective neurons [12]. Another pool of 5-HT_{1A} receptors, located especially in the raphe nuclei, is found on dendrites and cell bodies of serotonergic neurons and these receptors act as autoreceptors [12, 28]. This pool also inhibits neuron firing through activation of potassium channels and inhibition of voltage-dependent calcium channels [12, 28].

5-HT_{1A} knockout mice exhibit a variety of phenotypes such as increased responses to stress [30, 31], anti-depressant-like behavior [32], increased anxiety [32, 33], including a fear of new or stressful environments [33]. These mice also show some cognitive abnormalities in learning and memory, although some of these reports have since come under scrutiny [34].

1.2.3.2 5-HT_2 receptors

5-HT_2 receptors are $G\alpha_q$-coupled receptors and are subdivided into three isoforms. 5-HT_{2A} receptors exist as somatodendritic heteroreceptors located in cortex, pons, limbic areas and basal nuclei [12, 28, 35, 36]. When active, they cause a depolarization of the plasma membrane and contract vascular smooth muscle [28, 37]. These receptors also affect memory and sleep patterns [12, 38], and antagonists aid in treating negative symptoms of schizophrenia [12]. In 5-HT_{2A} receptor knock-out experiments, mice demonstrate decreased nociception, and abnormal sleep patterns [39, 40]. 5-HT_{2A} receptors may also modulate neural and behavioral responses of addictive substances [41, 42] and psychoses from hallucinogens such as LSD and psilocybin [28]. Also found in the CNS, 5-HT_{2B} receptors are expressed in the cerebellum, hypothalamus, and amygdala [12, 28]. They also serve as heteroreceptors [12] and regulate motor behavior, anxiolysis, hyperphagia, and pain perception [28, 43]. 5-HT_{2B} receptors are often overexpressed in pulmonary hypertension [28, 44]. Finally, like 5-HT_{2A} receptors, 5-HT_{2C} receptors are similarly found in cortex, hippocampus, amygdala, striatum, and substantia nigra, but also prominently in choroid plexus [12, 28]. Neuron depolarizations result from activation of these receptors, as well as inhibition of dopamine and norepinephrine release [12, 45]. 5-HT_{2C} receptor knock-out mice are obese, have convulsions as well as cognitive impairment [28, 46].

1.3 Platelet-derived growth factor receptor signaling

1.3.1 Platelet-derived growth factors

Platelet-derived growth factors (PDGFs) are potent mitogens that act on a variety of cell types including smooth muscle, vascular endothelial cells, fibroblasts and neurons [47]. They are characterized into four subtypes (A, B, C, and D) [48], with PDGF-A and PDGF-B being the most extensively studied. Of the four PDGF ligands, only PDGF-B and possibly PDGF-D can bind to the β

receptor, while the α receptor binds all but PDGF-D [48, 49]. PDGFs are typically found as homodimers, bridged via disulfide bonds in an antiparallel arrangement; however, heterodimers such as PDGF-AB also exist [47, 49]. PDGFs usually act in a paracrine fashion by binding PDGF receptors in local populations of cells, although they have been shown to act as autocrine factors in cancerous tissue [48].

PDGF ligands are divided into two subfamilies based on protein structure and post-translational processing of the propeptide: PDGF-A and PDGF-B compose the first subfamily whose propeptide N-terminal sequences are cleaved prior to excretion [48, 50]. These ligands also contain a C-terminal basic retention sequence that readily binds extracellular matrix, restricting the activity of these ligands to the immediate surroundings unless the retention sequences are subsequently cleaved off thus making the ligands are much more soluble [48]. The second subfamily is composed of PDGF-C and PDGF-D, which lack a basic retention sequence and are processed extracellularly [48]. They contain an N-terminal CUB domain that may act similar to retention sequences by binding extracellular matrix, but these sequences must be cleaved off for the protein to be active [48, 50].

1.3.2 Platelet-derived growth factor receptors

Platelet-derived growth factor (PDGF) receptors belong to the class III receptor tyrosine kinases (RTKs). They have been shown to be important in embryonic development of neurons and glial cells, as well as having roles in mature central nervous and vascular system function [49, 50]. These transmembrane receptors are divided into two main receptor types: PDGFα and PDGFβ at approximately 170 and 180 kDa respectively [50]. Post-translational glycosylation accounts for 40-50 kDa of this weight; however, receptors have been found to be functional without this modification [51, 52]. Both PDGF receptor types (α and β) are found in the central nervous system [48]. Five extracellular immunoglobulin (Ig)-like domains that are associated with ligand binding as well as an intracellular tyrosine kinase domain are required for activation of the receptor (Figure 1.1) [48, 50]. Binding of the ligand dimer to a receptor causes the recruitment of another receptor to form a receptor dimer, and all three receptor combinations are possible (αα, αβ, and ββ) [49, 50]. Ligand binding appears to be occurring at receptor Ig-like domains 1-3, and domain 4 is important at stabilizing receptor-receptor interaction [47]. With the formation of the receptor dimer, the kinase catalytic domains come within close proximity and become phosphorylated at Y849 and Y857 of the α and β receptors, respectively [49, 50]. This phosphorylation increases the activity of the kinase domains and

leads to trans-autophosphorylation of several other tyrosine residues on the cytoplasmic tail [49, 50]. These residues become docking sites for signaling enzymes and adaptor proteins, which predominately initiate proliferative signaling cascades (see Table 1 and the reviews by Heldin *et al.* [47, 49]). The proteins that bind phosphorylated PDGF receptors contain a SH2-binding domain – a domain that has affinity for phosphorylated tyrosine residues [53].

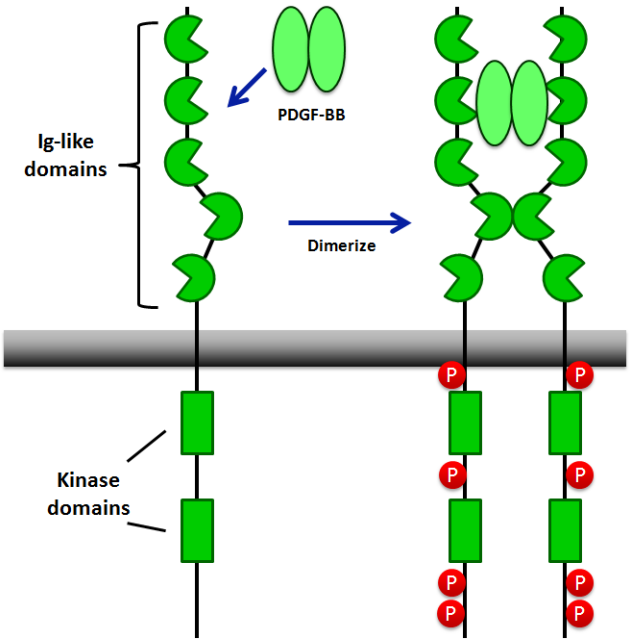


Figure 1.1. PDGFβ receptor structure. PDGFβ receptors possess five extracellular immunoglobulin (Ig)-like domains that mediate ligand binding and receptor dimerization. Intracellular kinase domains are flanked by several tyrosine residues that, when phosphorylated, participate in signal transduction. Adapted from Heldin *et al.*, 1998 [47].

1.3.3 Downstream PDGF receptor signaling

Phosphatidylinositide 3-kinases (PI3K) belong to three classes of enzymes, of which class I is relevant here. Members of this class are heterodimers composed of a p85 regulatory subunit and a p110 catalytic subunit [54]. The regulatory subunit is unique to this class, and notably has SH2 and p110-binding domains [54]. The catalytic subunit has a p85 and Ras-binding domains, as well as the catalytic domain [55]. In its basal state, PI3K is inactivated by is regulatory subunit [54]. However, when bound to phosphotyrosine (such as on phosphorylated RTKs) via SH2 domains, the p85 suppression is relieved and the enzyme is activated [55]. PI3K can also be activated by Ras and GPCRs [55]. When active, PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which initiates signaling pathways (notably Akt) that

lead to cell cycle progression, growth, survival (inhibiting apoptosis), and motility [48, 54]. PI3K binds Y731/742 and Y740/751 of PDGFα and PDGFβ receptor, respectively [49].

Phospholipase C (PLC) is a calcium-dependent cytoplasmic protein (Figure 1.2) whose isoforms can be activated by both GPCRs (including via $G\alpha_q$ and $G\beta\gamma$ subunits) and receptor tyrosine kinases [56, 57]. Translocation to the plasma membrane is a necessary step in activation, where it carries out the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) [56, 57]. Y988/1018 and Y1021/1009 of the α and β receptors bind and activate PLCγ, and via increased cytosolic calcium and DAG, lead to activation of protein kinase C [49].

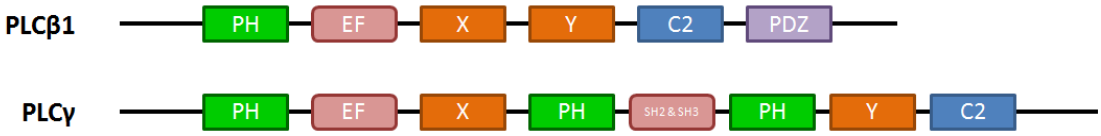


Figure 1.2. Phospholipase C structure.
At the N-terminal end, pleckstrin homology (PH) domains allow binding to phosphoinositides within the plasma membrane. EF motifs are helix-loop-helix structures that bind calcium, and are part of the catalytic core of the enzyme along with X, Y, and C2. Two SH2 and one SH3 domains are found in between the X and Y domains of PLCγ. These are unique to this isoform and SH2 domains allow for binding to phosphotyrosine and activation of PLCγ, while SH3 domains can bind dynamin and actin. PDZ domains assist in forming protein complexes and anchoring proteins to the cytoskeleton. Adapted from Vines, 2012 [56].

Other proteins that bind PDGF receptor and become activated include the non-receptor tyrosine kinase Src kinase, which binds Y572 and Y579, and SHP-2 binds Y720/754 and Y1009/763 [49]. SHP-2 is a phosphatase responsible for regulating PDGF receptor activity by dephosphorylating (thus inactivating) the receptor [49]. Other factors that bind PDGF receptors include: Grb2/Sos and GAP (GTPase-activating protein), which activate and deactivate (respectively) the G protein Ras; the transcription factor Stat5; and many other adaptor proteins (see Table 1) [49].

Table 1: PDGF receptor-associated signaling molecules

Signaling molecule	PDGFα receptor phosphorylation site	PDGFβ receptor phosphorylation site
PI3K	Y731 Y742	Y740 Y751
PLCγ	Y988 Y1018	Y1021 Y1009
c-Src	Y572	Y579
SHP-2	Y720 Y754	Y1009 Y763
Grb2	Unknown	Y716 Y775
Grb7	Unknown	Y716 Y775
GAP	None	Y771
Shc	Unknown	Y579 Y740 Y751 Y771
Stat5	Unknown	Y579 Y581 Y775
Nck	Unknown	Y751
PDGF receptor kinase domain activation site	Y849	Y857

Table of signaling molecules that bind phosphorylated PDGF receptors with associated phosphorylation site. Adapted from Heldin and Westermark, 1999 [49].

1.3.3.1 Protein kinase C

Although protein kinase C (PKC) is not directly activated by PDGF receptors, it does play a role in downstream signaling and is one of the important cellular enzymes in signal transduction. It is a single polypeptide and exists as several different isoforms: PKCα, PKCβ and PKCγ comprise the conventional (cPKC) isoforms (Figure 1.3), and require calcium ions, which target the enzyme to the plasma membrane where they bind phosphatidylserine and DAG, thus activating the enzyme [58, 59]. Novel isoforms (nPKCs) consist of PKCδ, PKCϵ, PKCη, and PKCθ and do not require calcium, while

atypical isoforms (aPKCs) PKCζ and PKCλ do not require DAG or calcium [58]. A pseudosubstrate domain keeps the enzyme inactive when calcium and/or DAG are absent by blocking the active site [58, 59].

PKC has three main domains: an N-terminal regulatory region, a hinge region and a C-terminal catalytic region [58, 59]. The regulatory region contains binding domains for calcium (for cPKCs), diacylglycerol (for cPKCs and nPKCs), phorbol esters and phosphatidylserine [58]. The catalytic region houses the ATP and substrate binding sites, where it phosphorylates substrates at serine/threonine residues [59].

PKC function is also regulated by phosphorylation at three sites. Upon completion of protein synthesis, PKC is phosphorylated at a site on its activation loop to prevent degradation [58]. A second phosphorylation occurs in a C-terminal turn motif which creates a stable conformation necessary for enzyme function [58]. A third phosphorylation occurs at a site in the C-terminal hydrophobic region in cPKC and nPKC isoforms but its function remains to be fully explored [58]. The enzyme is then subject to regulation by the isoform-specific cofactors and the pseudosubstrate domain [58, 59]. Active PKCs mediate a wide variety of signal transduction pathways, binding several enzymes including Raf1, STAT, HSP25, and PLD [58].

Figure 1.3. Conventional protein kinase C
The pseudosubstrate (PS) domain contains the consensus sequence for the kinase domain, but lacks the target serine/threonine, thus regulating its activity. C1 domains bind DAG or phorbol esters, while C2 domains bind calcium in cPKCs. The hinge region allows bending of the protein to permit the PS to bind the kinase domain. Adapted from Zeng *et al.*, 2012 [58].

1.3.3.2 Receptor deactivation

PDGF ligand stimulation of PDGF receptors also has the outcome of internalizing the receptor. PDGF receptors are found concentrated in caveolae, and ligand-activated receptors are rapidly removed from the plasma membrane via clathrin-dependent internalization [47]. The fate of PDGF receptors will be either to be separated from the ligand and recycled back to the cell surface, or to be degraded upon fusion of its endosome with a lysosome [48, 49]. As is the case for many other proteins, ubiquitination of activated PDGF receptors leads to increased rate of degradation in

proteasomes, and consequently, mutated ubiquitination sites result in longer receptor half-lives [47, 49]. In addition, the level of kinase activity of the receptor and the phosphorylation status of Y579 have also been implicated in the rate of internalization [47].

1.3.3.3 Inhibition of PDGF receptor function

Preventing downstream signaling from PDGF receptors can be attained by several means. Pharmaceutical inhibition of PDGF receptors is achieved by blocking the ATP-binding site, thus preventing kinase activity. Many tyrphostins can perform this function, but these drugs often have other targets besides PDGF receptors [60]. Specific inhibition of receptor function can be achieved by using antibodies targeting the extracellular domain, thus preventing ligand binding. In spite of this, it is probable that this would not prevent PDGF receptor transactivation (discussed below), as extracellular domains are not involved in this pathway [61]. Targeting the ligands themselves with antibodies would also eliminate receptor activation through sequestration and preventing their binding to the receptor [47].

1.3.3.4 PDGF receptors in development and normal function

Activation of PDGF receptors can lead to cell growth by activating the MAP kinase pathway through Ras activation [47]. On a tissue level, effects of PDGF signaling in development include proliferation and development of lung smooth muscle, proliferation and differentiation of mesenchymal cells in intestinal villus formation, hair follicle formation, and testis formation [48]. In the CNS, oligodendrocyte precursors respond to PDGF by proliferating and producing myelin [48]. Absence of PDGF signaling can result in insufficient or no myelination of some nerve fibers [48].

PDGF receptors have been shown to be associated with focal adhesions via Na^+/H^+ exchanger regulatory factors (NHERFs), which bind directly with PDGF receptors [48]. These factors link PDGF receptors to focal adhesion kinases, the actin cytoskeleton, and integrins [48]. PDGF signaling also affects actin organization and disassembly of stress fibers [50], which are necessary for cell migration [48].

Moreover, PDGF signaling can increase wound repair. Although expression of PDGF receptors is low in smooth muscle and fibroblasts, inflammation induces their upregulation [50]. Subsequently,

PDGF signaling increases the rate of new epithelium and vascular regeneration, leading to faster wound healing [50].

PDGF receptors and ligands are crucial in maintaining cell proliferation in embryonic development: mice with either receptor type or PDGF-A or PDGF-B knocked out results in embryonic or perinatal death [49]. Kidney function is critically undermined as mesangium fails to develop, leading to essentially non-functional glomeroli in both PDGF-B and PDGFβ receptor knock-out mice [62, 63]. Deficiencies in the cardiovascular system in general are also seen, with heart and vasculature defects leading to bleeding due to lack of pericytes around the endothelium of small vessels [49, 64]. In addition, lung function is compromised in PDGF-A knock-out mice, with defective alveolar development, leading to symptoms of emphysema, and death occurring within a month after birth [65].

1.3.3.5 PDGF receptors in disease

PDGF activity has been implicated in the formation of atherosclerosis. An injury in a blood vessel increases inflammation and aggregation of platelets at the site [48]. PDGFs released from platelets or activated immune cells cause smooth muscle migration and proliferation from the tunica media to the intima, the endothelial, lumen-facing layer of blood vessels. This contributes to plaque growth into the lumen [48, 50], and restricts blood flow through the site. In addition, overactivity of PDGF signaling has been linked to various cases of fibroses including liver, heart, kidney, and dermis [48]. For instance, in lung fibrosis, inflammation results from irritants in alveoli [48]. PDGF receptor expression increases from the effects of inflammation and activated immune cells release PDGF [48]. Proliferation of myofibroblasts and accumulation of extracellular matrix result [50], leading to scar formation and eventual decrease in oxygen diffusion efficiency [66]. In addition, overactivity of PDGF signaling leads to tumor formation and progression. In malignant gliomas, an autocrine loop develops as cells express both PDGF ligands and receptors [50, 67]. How these genetic changes arise remains to be elucidated, but in many cases, gene mutations or amplification of receptors or ligands are found [48].

1.3.4 Transactivation

In addition to ligand-induced activation of PDGF receptors, another mechanism of activation has recently emerged. GPCR signaling has been shown to mediate PDGF ligand-independent PDGF receptor activation in a process known as transactivation (Figure 1.4) [61, 68]. In fact, several GPCR ligands can transactivate PDGF receptors in numerous systems including angiotensin II [69, 70], endothelin [71], dopamine [68], sphingosine-1-phosphate [72], lysophosphatidic acid [73, 74], and leukotrienes [75]. PDGF receptor transactivation pathways most likely involve exclusively intracellular signaling cascades, but other RTK transactivation pathways involve extracellular release of factors that act on the RTK [76, 77]. 5-HT itself transactivates the PDGFβ receptor in pulmonary artery smooth muscle cells and in LMTK$^-$ mouse fibroblast cells [78, 79]. However, the mechanistic pathway(s) that lead to PDGFβ receptor transactivation by 5-HT remain largely uncharacterized and it is not known if 5-HT induces transactivation of PDGFβ receptors in neurons.

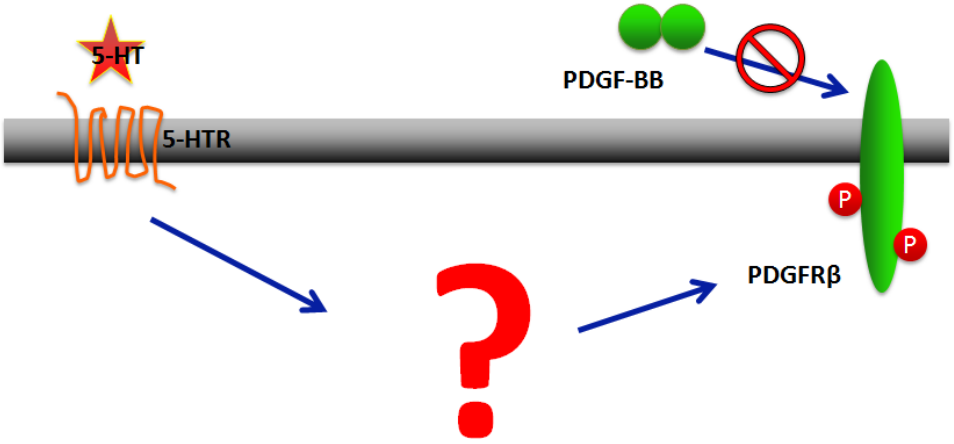


Figure 1.4. 5-HT-induced transactivation of PDGF receptors.
Certain GPCRs such as 5-HT receptors mediate an intracellular signaling cascade that results in PDGF receptor phosphorylation. This ligand-independent transactivation pathway has not been characterized in neurons, but is believed to involve many enzymes described in other pathways. One of the objectives of this study is to identify some of these enzymes involved.

1.3.4.1 EGFR transactivation

The model of epidermal growth factor (EGF) receptor transactivation follows a slightly different mechanism compared to transactivation seen with other RTKs: GPCR activation leads to an intracellular signaling pathway that activates membrane-bound metalloproteases [80]. These cleave

membrane-bound heparin-binding EGF-like and release it extracellularly, which binds the extracellular ligand-binding domains of the EGF receptor thus activating it [80]. Although not a wholly intracellular mechanism, this so-called "triple-membrane-passing signaling" [80] (Figure 1.5) is considered transactivation by virtue of the fact that the process is initiated by a GPCR.

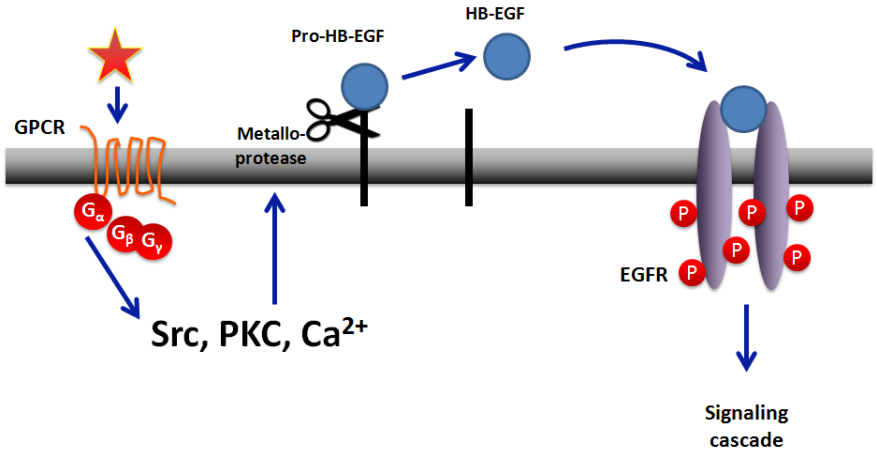


Figure 1.5. Triple-membrane-passing signaling.
Activation of a GPCR from an extracellular ligand results in signaling via intracellular mediators that in turn, activate metalloproteases. These cleave extracellular pro-heparin-binding epidermal growth factor (HB-EGF) which then diffuses away to interact with epidermal growth factor receptor (EGFR). Phosphorylation of EGFR results in further intracellular growth factor signaling. Adapted from Wetzker and Bohmer, 2003 [80].

1.4 TrkB receptor signaling

1.4.1 Neurotrophins

Neurotrophic factors are proteins that promote survival and maintenance of neurons [81]. These members are composed of neurotrophins, ciliary neurotrophic factor, and glial cell line-derived neurotrophic factors [82-84]. Within the family of neurotrophins, there are four main proteins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4), although other neurotrophins do exist in non-mammalian species [82, 85]. It should be mentioned that neurotrophin-5 was discovered in human but it was later determined that it was the species homolog of *Xenopus* neurotrophin-4 [82, 86].

Neurotrophins are translated as propeptides that must be cleaved by proteases for full activity at neurotrophic receptors [82]. However, there is evidence that proneurotrophins may bind the p75 neurotrophin receptor and induce apoptosis [87]. Active neurotrophins exist as non-covalently-bound homodimers, except for BDNF, which dimerizes with a monomer of NT-3 or NT-4 [82, 88]. Neuron development is enhanced by neurotrophins, and the age and location of the neuron can determine to which neurotrophin it responds. For example, NT-3 is responsible for survival effects in sympathetic neuroblasts and newly differentiated neurons, whereas mature neurons depend on NGF [82].

1.4.2 TrkB receptors

Most neurotrophin signaling is initiated by binding and activating one of three Trk neurotrophic receptors (A to C), which belong to the family of receptor tyrosine kinases [81, 82]. Due to the differences in amino acid sequence in the extracellular ligand-binding Ig-like domains, each receptor subtype has different affinities for different neurotrophins [81]. TrkB receptors can be activated by the ligands BDNF, NT-3, or NT-4 [89], while TrkA receptors have higher affinity for NGF, and TrkC receptors bind NT-3 [81]. Like the PDGFβ receptor, TrkB receptors dimerize upon ligand binding, followed by kinase domain activation and trans-autophosphorylation of tyrosine residues outside of the kinase domain (Figure 1.6) [81, 90]. TrkB receptor phospho-Y515 binds Shc, which then binds Grb2/Sos and leads to activation of the Ras-MAPK pathway [90]. Ras activity promotes neuronal survival and differentiation [82]. Grb2 also mediates the formation of a complex involving Grb-associated binder-1 protein, and insulin receptor substrate 1 and 2 [90]. This complex mediates the activation of PI3K and subsequently Akt, leading to cell survival effects [90]; unlike with PDGF receptor, PI3K does not directly bind to phosphorylated TrkB receptors [90]. On the other hand, TrkB receptor phospho-Y816 binds and activates PLCγ, which increases cytosolic calcium concentrations and DAG, and subsequently activates PKC isoforms [90].

TrkB receptors can be trafficked to and from the membrane based on which signaling molecules are present. Synaptic activity that increases intracellular calcium concentrations via NMDA or calcium channels lead to CaMKII activation and induces fusion of intracellular TrkB receptor-containing vesicles with the plasma membrane, even in absence of BDNF [91]. Binding of ligand to TrkB receptors leads to endocytosis, and this can be enhanced by increases in calcium concentrations from synaptic activity as well [91]. Internalized TrkB is still active and can participate in downstream signaling since the phosphorylated C-terminal tails are still in the cytoplasm [91].

Human TrkB receptor signaling is dependent upon three alternatively spliced isoforms: the full-length TK+, and 2 truncated (TK-) forms, T1 and T-Shc. While T1 and T-Shc lack the intracellular kinase domain and therefore do not participate in the downstream proliferative signaling pathways typical of RTKs, they can modulate the activity of the full length TrkB receptor [90, 91].

Previously, Trk receptors were reported to be transactivated by adenosine A_{2A} receptors and many of the proteins involved in that pathway are similar to those required for 5-HT-induced transactivation of the PDGFβ receptor [92-94]. However, it is unknown if any other GPCRs can trigger transactivation of TrkB receptors.

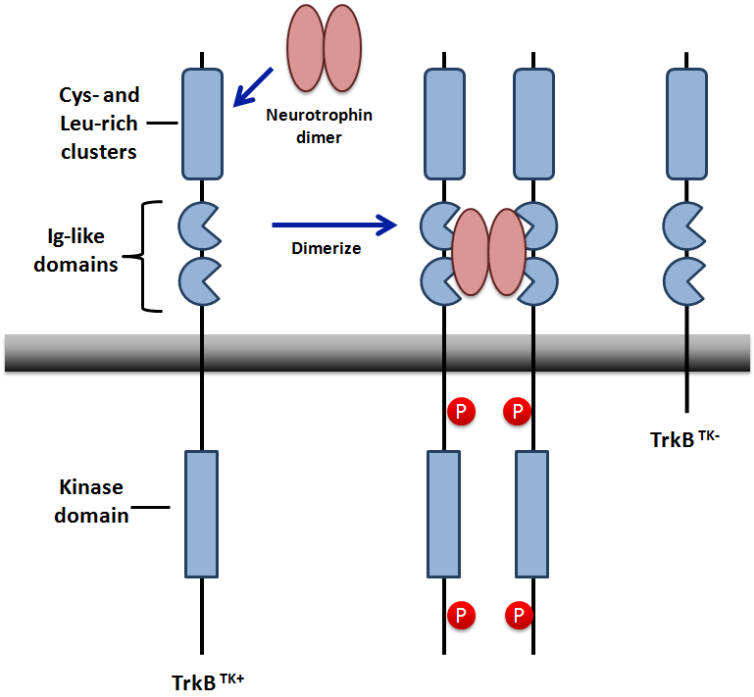


Figure 1.6. TrkB receptor structure.
TrkB receptors bind several neurotrophins at the two extracellular Ig-like domains. For TK+ receptors, an intracellular kinase domain phosphorylates other intracellular tyrosine residues that initiate cell signaling. TK- receptors lack the intracellular kinase domain and phosphotyrosines; thus, they do not themselves participate in cell signaling, but they can modulate the activity of TK+ receptors by dimerizing with them. See text for details. Adapted from Skaper, 2008 and Minichiello, 2009 [82, 90].

1.5 Depression

1.5.1 Manifestations and anatomy

Clinical depression is a historied mental disease that will affect one in six individuals in their lifetime according to some statistics [95]. Diagnoses date back as far as to Hippocrates in 400 BC, who described the disease as *melancholia* [96]. The main points of diagnosis are listed below in Table 2.

Note that diagnoses are subjective; there is some overlap of symptoms with related disorders such as bipolar disorder and anxiety disorders [97].

Table 2. Depression manifestations

Depressed mood
Anhedonia
Irritability
Difficulty concentrating
Abnormal appetite
Abnormal sleep patterns
Low energy
Low self-esteem
Suicidal thoughts

Criteria for clinical depression diagnosis. Based on symptoms listed in the *Diagnostic and Statistical Manual of Mental Disorders IV*. Several symptoms should persist for at least 2 weeks [96-98].

Risk factors for depression include prior episodes, family history, medical co-morbidity, stress, and substance abuse [98]. Despite these criteria, there is no solid genetic link to depression [97]: there is no single "smoking gun" gene, and if a genetic link is definitively found, there will most likely be several genes responsible. The limbic system is one brain region implicated in depression, which may be where antidepressant drugs are acting [99]. Brain scans also point to increased activity in prefrontal cortex and amygdala in depressed individuals and in healthy volunteers in a state of induced sadness [100, 101]. Post-mortem analyses have discovered a decrease in gray matter volume and cell number in the prefrontal cortex, limbic, and basal nuclei (including nucleus accumbens, caudate, and hippocampus) in many but not all cases of depression [100, 102].

The study of depression, and indeed mental diseases in general, has the unfortunate inconvenience of a lack of an ideal animal model. We can study the effects of drugs on animal brains, and animals can be genetically or psychologically modified to manifest neurological disorders [96], but these modifications themselves may be confounding. Cognitive tests are difficult to administer to a model that has no vocal or linguistic capacity with which to describe a feeling. Thus, a combination of animal and human studies is necessary for the clearest understanding of mental disorders.

1.5.2 Treatments for depression

Being a highly prevalent disorder, much effort has been put into the development of pharmaceuticals that alleviate the symptoms of depression. In spite of this, 20-40% of patients do not respond to a given antidepressant [103] . Currently used drugs mainly target the monoamine system, and are summarized in Table 3.

Table 3. Commonly prescribed antidepressants

Drug class Generic (Trade) names	Mechanism of action
TCA Amitriptyline (Elavil) Clomipramine (Anafranil) Desipramine (Norpramin) Doxepin (Sinequan) Imipramine (Tofranil) Nortriptyline (Aventyl) Trimipramine (Surmontil, Rhotrimine)	Inhibitors of serotonin and/or norepinephrine and/or dopamine transporters May also affect neurotransmitter receptor function
MAOI Phenelzine (Nardil) Selegiline (Eldepryl) Tranylcypromine (Parnate)	Inhibitors of monoamine oxidase
SSRI Citalopram (Celexa) Fluoxetine (Prozac) Fluvoxamine (Luvox) Paroxetine (Paxil) Sertraline (Zoloft)	Inhibitors of serotonin transporters
SNRI Duloxetine (Cymbalta) Venlafaxine (Effexor)	Inhibitors of serotonin and norepinephrine transporters

Selected pharmaceuticals with antidepressant action available in Ontario, Canada, per the Ontario Drug Benefit Formulary, Edition 41. Mechanisms of action are listed for each drug class [99, 104].

Tricyclic antidepressants (TCAs) were among the first drug classes available to treat depression [99]. In general, these drugs prevent the reuptake of 5-HT and/or norepinephrine (NE) into the cell by blocking the 5-HTT and NE transporter (NET) [18, 104], thus allowing these monoamines to remain in the synaptic space for longer periods of time. Unfortunately, these drugs often target other receptors such as alpha adrenergic, muscarinic cholinergic, and histamine receptors [18, 104], leading to undesirable or intolerable side effects. Although drugs in this class do alleviate symptoms of depression and continue to be prescribed, they have been replaced as first-line treatments in favor of those with fewer side effects [104].

Monoamine oxidase inhibitors prevent the mitochondria-bound monoamine oxidase from metabolizing 5-HT and NE, keeping the cellular pools of these monoamines from being depleted [105]. However, there is a risk of developing a tyramine-induced hypertensive emergency through concurrent consumption of high tyrosine-containing foods. Monoamine oxidase inhibitors prevent metabolism of tyramine, a catecholamine-releasing agent. This can result in an increase of catecholamines into systemic circulation, overactivity of adrenergic receptors, and critical increases in systemic blood pressure [106]. Therefore, these drugs are usually not first-line options for depression, and with decreased use, hypertensive emergencies are now less common [106].

The selective serotonin reuptake inhibitors (SSRIs) have become the first-line choice for the treatment of depression, based on safety and efficacy [104, 107]. These drugs selectively target the 5-HTT, with significantly less affinity for NET, acetylcholine or histamine receptors [104]. As such, they have fewer side effects and are generally tolerated by a greater number of people [18, 104, 108]. Along the same lines are the dual serotonin-norephinephrine reuptake inhibitors (SNRIs). These provide the therapeutic benefits of TCAs without the adverse side effects, and are alternatives to SSRI use [104]. One meta-analysis even suggests that some SNRIs are more efficacious than certain SSRIs [108].

1.5.3 Hypotheses of depression etiology

1.5.3.1 Monoamine hypothesis

The monoamine hypothesis of depression posits that a deficiency in monoamine signaling in the CNS can result in depression, and that resolving this deficiency ameliorates symptoms of the disease [97, 109, 110]. Drugs that work to increase monoamine signaling should therefore alleviate depression

symptoms, and this hypothesis was devised from the observed antidepressant effects of iproniazid, a MAOI, and imipramine, a TCA, in the 1950s [18, 97, 111]. Further, reserpine depletes a neuron's available monoamine stores by blocking the vesicular monoamine transporter, thus preventing monoamine packaging and release into the synapse, and the effects of this drug include an induction of depressive symptoms [112]. However, the main criticism of this hypothesis is that the effects of the antidepressant do not appear for several weeks after starting the drug regimen, when one would expect the effects to occur within hours or days at the most [18, 113]. Clearly, the mechanism is more complex than once thought, and a modification to the initial hypothesis was required: the antidepressant-induced acute increase in monoamine signaling is simply the first step in a lengthy process at the synapse [114]. A current theory suggests that the increased monoamines (particularly 5-HT) in the synapse caused by reuptake inhibition bind to pre-synaptic autoreceptors, whose normal function is to decrease further 5-HT release into the synapse through negative feedback (Figure 1.7) [18, 114]. Somatodentritic 5-HT_{1A} autoreceptors and terminal 5-$HT_{1B/1D}$ autoreceptor activity in the raphe nuclei have been implicated in depression by causing hyperpolarization of the neuron to inhibit its firing [18, 114]. 5-HTT blockade by long-term SSRI therapy leads to a slow desensitization of these 5-HT autoreceptors [115, 116] by increasing synaptic 5-HT concentrations, which progressively bind more of these receptors over time (thus accounting for the lag time between starting treatment and observable effects), a result that is blocked by 5-HT_1 antagonists [114, 116]. Less inhibition of 5-HT neuron firing, along with decreased 5-HT uptake through 5-HTT blockade, increases 5-HT signaling capacity in the synapse, thus improving symptoms of depression.

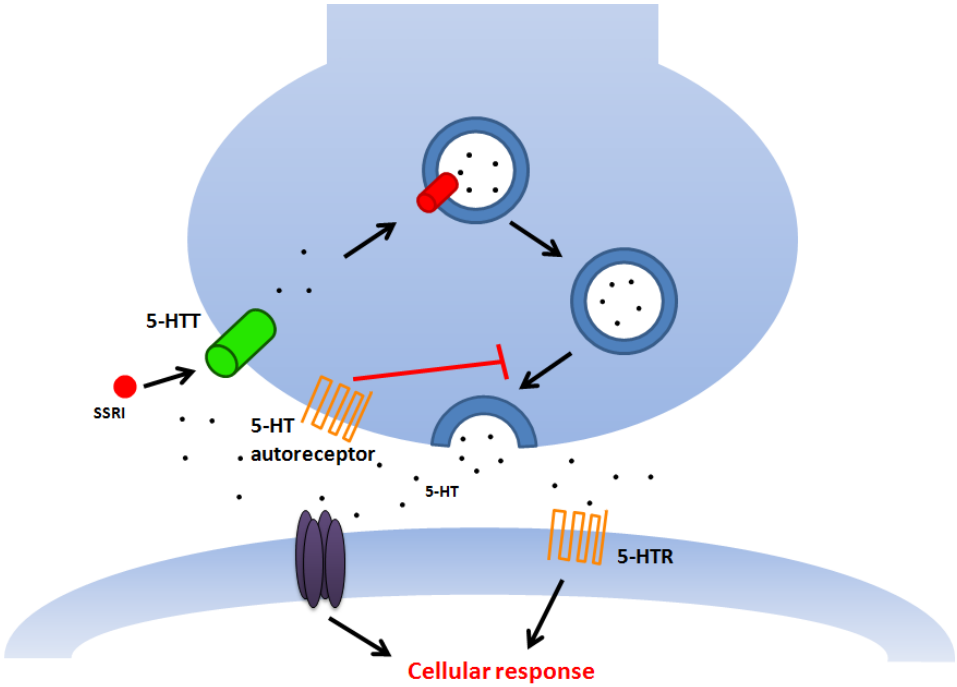


Figure 1.7. Monoamine hypothesis signaling.
A depolarized presynaptic neuron will release its neurotransmitter into the synaptic space. 5-HT binding to autoreceptors inhibits further neurotransmitter release. Selective serotonin reuptake inhibitors (SSRIs) act on 5-HT transporters (5-HTT) to decrease 5-HT reuptake. This has the effect of increasing synaptic 5-HT concentrations, which leads to desensitization of the 5-HT autoreceptors through increased 5-HT binding. With less inhibitory 5-HT autoreceptors and in combination with 5-HTT inhibition, synaptic 5-HT levels are maximized. Adapted from Elhwuegi, 2004 [18].

1.5.3.2 Neuroendocrine factors

The hypothalamus-pituitary-adrenal (HPA) axis (Figure 1.8) is a well-known mechanism of stress response, and may be compromised in depressed individuals [96]. The amygdala and hippocampus positively and negatively (respectively) regulate paraventricular nucleus (PVN) activity in the hypothalamus [96]. The PVN is an integration center for stress-related information, and secretes corticotropin-releasing hormone (CRH) when stimulated [96]. CRH targets the anterior pituitary, which releases adrenocorticotropic hormone (ACTH) [96]. ACTH stimulates the adrenal cortex to release cortisol, a glucocorticoid whose increase in blood concentration correlates with increased stress [117]. Acute increases in cortisol levels negatively regulate the HPA axis, and may increase activity of the hippocampus [96], thus providing a negative feedback. On the other hand, chronic

stress increases blood cortisol levels over a longer time period, and this constant hypercortisolemia may be neurodegenerative in the hippocampus with consequences such as loss of dendritic branches and spines (thus less synapses), and reduced neurogenesis [96, 99]. This leads to decreased hippocampal inhibition of the PVN, and continued activity of this axis, which maintains high cortisol levels in blood [96, 99]. Justifiably, there is active research in understanding the role of the HPA axis in depression. Current evidence suggests an increase in CRH-releasing neuroendocrine cells in the PVN occurs in depression, while antidepressants and electroconvulsive therapy work to reduce CRH concentration [104, 118]. However, not all depressed patients show abnormal HPA axis physiology [96], suggesting this may not be the only causative factor of depression.

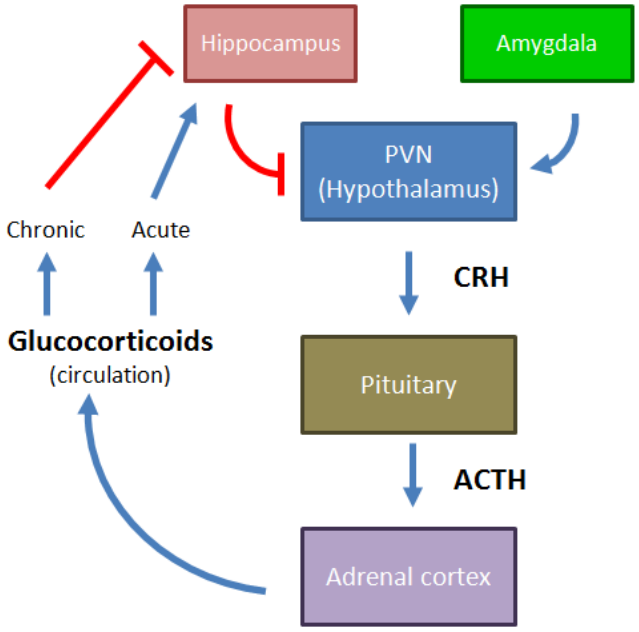


Figure 1.8. HPA axis.
Stress initiates signaling in the HPA axis. The main structures include the amygdala, hippocampus, the paraventricular nucleus (PVN) of the hypothalamus, and the anterior pituitary. Hormones include corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and glucocorticoids. See text for details. Adapted from Nestler *et al.*, 2002 [96].

1.5.3.3 Neurotrophic factor hypothesis

Another more recent hypothesis considers a role for neurotrophic factors in depression. This hypothesis states that a decrease in neurotrophic factor signaling, particularly in the hippocampus, underlies depression pathophysiology [96, 97, 119]. BDNF is the most suspected factor involved, as it is expressed in limbic regions and regulates brain plasticity [97]. Evidence that supports this idea suggests that acute or chronic stress induces a reduction in hippocampal BDNF signaling, and long-term antidepressant use increases BDNF expression [96, 120-123]. Decreased BDNF expression has

been implicated in changes to neuronal dendrites including decreased dendritic branches and complexity [124]. Moreover, direct injection of BDNF into the hippocampus produces antidepressive results in rodents [122]. One issue with the neurotrophic hypothesis is the depressive effects of BDNF seen in the VTA and nucleus accumbens [125], so there are likely other factors involved. In addition, not all antidepressants positively modulate BDNF levels: some antidepressants lead to increased BDNF expression, while others show no effect [120].

1.5.3.4 Neurogenesis

Despite erroneous lay claims to the contrary, neurogenesis is seen continually in the adult brain in at least two main locations: the subgranular zone of the hippocampus and the subventricular zone lining the lateral ventricles [113, 126]. Neurogenesis is thought to slow or stop in depression, and is postulated as an effect of overactive endocrine function as described in section 1.5.3.2. This could lead to atrophy and decreased gray matter volume as seen in some depressed patients [100, 127]. Conversely, certain antidepressant therapies seem to result in induction or restart of neurogenesis in the hippocampus [127-130]. This induction of new neuron production is posited to be caused by antidepressant-induced increases in BDNF signaling and has been linked to the neurotrophic factor hypothesis [97]; however, a mechanism has yet to be established.

1.5.3.5 Other depression research

There is also research investigating the role of DNA itself in depression. Methylated cytosine typically indicates inactive/repressed DNA, and methylated DNA on glucocorticoid receptor promoters decreases receptor expression and correlates with increased anxiety [97]. In addition, histone acetylation is an indicator of transcriptional activity, and may also be an antidepressant target [97]. This is corroborated by evidence suggesting that histone deacetylase inhibitors produce antidepressant effects [131, 132] and antidepressant activity increases histone acetylation at BDNF promoters by downregulating deacetylases [113, 131]. Alternatively, electroconvulsive therapy in rodents also increases histone acetylation at BDNF promoters, and correlates with an increase in BDNF expression [133]. Electroconvulsive therapy involves placing electrodes on the scalp and running current to induce a general seizure [104]. Although historically connoted to be barbaric, safety and efficacy have since improved with more strategic electrode placement, and response rates

vary between 50-90% [104, 134, 135]. But there is still a risk of confusion, memory, and cardiovascular complications [136-138]. In contrast, deep brain stimulation involving electrodes implanted in the brain have shown to provide instant decrease in depressive symptoms, especially when stimulating the nucleus accumbens – a region important for reward, and the anhedonic (i.e. lack of capacity to feel pleasure) effects of depression [139]. This method does not involve inducing seizures, and provides potentially encouraging evidence of therapeutic value to depressed patients who have had prior failures with pharmacological, psychological, and electroconvulsive therapies [139].

1.6 Objectives

This project was divided into two objectives that sought to investigate the role, if any, of 5-HT in PDGFβ receptor transactivation in neurons. Transactivation is a relatively recent discovery in signal transduction as a means of cross-talk between GPCRs and RTKs, but the mechanistic details have yet to be fully elucidated.

The first objective was to determine whether 5-HT elicits PDGF receptor phosphorylation in neurons in the short term (i.e. within minutes), and if so, to determine the molecular mechanism (i.e. enzymes involved) of this phenomenon. There are many examples of transactivation in other cell types using other GPCR agonists, but the mechanism involved is somewhat vague and fragmented. Here, an attempt to discover insights into this mechanism in a systematic and thorough manner from GPCR to PDGF receptor in neurons is executed. Many GPCR agonists such as 5-HT can lead to cell proliferation; therefore, these 5-HT-mediated cellular growth effects may be attributable to growth factor receptor transactivation.

For the second objective, a piece of evidence was investigated that showed fluoxetine disrupting 5-HT-mediated PDGF receptor phosphorylation in smooth muscle. This might have greater implications if it could be shown to be true in neurons, where clearly 5-HT and fluoxetine play an important role in normal and abnormal brain function. If fluoxetine, a widely prescribed antidepressant, does negatively affect 5-HT signaling in neurons, this could have an important impact in our understanding of the mechanism of action of fluoxetine and of disease states such as depression or other affective disorders.

Chapter 2
Materials and methods

2.1 Reagents and Antibodies

PDGF-BB, fluoxetine (*N*-methyl-3-[(4-trifluoromethyl)phenoxy]-3-phenylpropylamine hydrochloride), dopamine (3,4-dihydroxyphenethylamine hydrochloride), DCFH-DA (2',7'-dichlorodihydrofluorescin diacetate), and EGTA (ethylene glycol-*bis*(2-aminoethylether)-*N*,*N*,*N*',*N*'-tetraacetic acid) were purchased from Sigma (St. Louis, MO). Serotonin (5-HT; 5-hydroxytryptamine hydrochloride), *N*-acetyl-L-cysteine, diphenyleneiodonium chloride, Go 6983 (3-[1-[3-(dimethylamino)propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione), pertussis toxin, WAY 100135 ((*S*)-*N*-*tert*-butyl-3-(4-(2-methoxyphenyl)-piperazin-1-yl)-2-phenylpropanamide dihydrochloride), citalopram (1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-5-isobenzofurancarbonitrile hydrobromide), and LY 272015 1-[(3,4-dimethoxyphenyl)methy]-2,3,4,9-tetrahydro-6-methyl-1*H*-pyrido[3,4-*b*]indole hydrochloride, U73122 (1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione), PP2 (3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine), PP3 (1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine), AG 1296 (6,7-dimethoxy-2-phenylquinoxaline), and 8-hydroxy-DPAT (8-hydroxy-2-dipropylaminotetralin hydrobromide) were purchased from Cedarlane (Burlington, ON). 5-CT (5-carboxamidotryptamine maleate), LP 12 (4-(2-diphenyl)-*N*-(1,2,3,4-tetrahydronaphthalen-1-yl)-1-piperazinehexanamide hydrochloride), apocynin (4'-hydroxy-3'-methoxyacetophenone), clorgyline (*N*-[3-(2,4-dichlorophenoxy)propyl]-*N*-methyl-prop-2-yn-1-amine), and BAPTA-AM (1,2-*bis*(2-aminophenoxy)ethane-*N*,*N*,*N*',*N*'-tetraacetic acid tetrakis(acetoxymethyl ester)) were purchased from Santa Cruz (Santa Cruz, CA). Imatinib (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). Antibodies against PDGF-BB, β-actin, TrkB, 5-HT_{1A} receptor, 5-HTT, PDGFβ receptor and PDGFβ receptor phosphorylation sites Y1021, Y1009, Y751 were also purchased from Santa Cruz. Antibodies against Src, phospho-Src Y418, phospho-TrkB Y816, ERK1/2 and phospho-ERK1/2 were purchased from Cedarlane.

2.2 SH-SY5Y neuroblastoma cell line

Complete growth media consisted of DMEM and Ham's F12 in a 1:1 ratio (Fisher #SH20361), 10% fetal bovine serum (Sigma), 100 U/ml penicillin, and 100 µg/ml streptomycin. 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. Prior to drug treatments and experimentation, cells were plated without antibiotics and serum starved for 24 h.

2.3 RGC-5 retinal ganglion cell line

Cultures were grown in complete growth media consisting of low-glucose DMEM (Fisher #SH30021), 10% horse serum (Sigma), and penicillin/streptomycin. Cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at a temperature of 37°C, and media was changed every 3-4 days. For experimentation, cells were plated without antibiotics, and prior to drug treatments, serum concentration was lowered to 2% for 24 h.

2.4 Primary mouse cortical neuron cultures

CD-1 (Harlan, Indianapolis, IN) time-pregnant mice were sacrificed using cervical dislocation. E17 to E19 mouse embryos were removed from pregnant CD-1 mice and transferred to chilled 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). Embryos were removed from uterine sacs and placed in fresh dissection media. Embryos were then decapitated and brains were removed from the skulls and placed in fresh dissection media. The cortex was removed, separated, washed, and trypsinized with 0.25% trypsin/0.1% EDTA for 20 min at 37°C. After trypsinization, cells were washed with warm dissection media and suspended in warm plating media [DMEM/F12 (Fisher #SH30023), supplemented with 10% fetal bovine serum, 10% horse serum, penicillin/streptomycin]. Cells were triturated and strained with a 100 µm-mesh nylon strainer. Cells were then plated on poly-D-lysine-coated culture dished and grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were plated with plating media for the first 2-4 h until attached and then with feeding media consisting of Neurobasal medium and B-27 supplement (Life

Technologies, Burlington, ON) without serum. Media were changed twice per week. Drug treatments were performed 7-10 days after plating the cells to allow cells to mature. The overgrowth of non-neuronal cells was inhibited by means of a mitotic inhibitor (81 μM 5-fluoro-2'-deoxyuridine and 200 μM uridine), which was added for 24 h once cells reached confluency. All animal experiments were performed in agreement with the guidelines of the policies on the Use of Animals at the University of Waterloo.

2.5 Conditioned media evaluation

Following drug treatments but prior to cell lysis, conditioned cell culture media was collected, Complete Mini Protease Inhibitor Cocktail (Roche, Laval, QC) was added, and samples were kept on ice. Samples were centrifuged at 1000 x g for 30 s at 4°C to remove any large debris. Samples were then concentrated by centrifugation in Amicon Ultra-4, 10 kDa filter devices (Millipore, Billerica, MA) at 3000 x g for 30 min at 4°C. The concentrate was then added to loading buffer for western blotting, and membranes were probed for PDGF-BB.

2.6 Western blotting

Following drug treatments, cells were washed with ice-cold PBS, and then lysed with chilled lysis buffer [20 mM Tris-HCl at pH 7.5; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 30 mM sodium pyrophosphate; 1 mM β-glycerophosphate; 1 mM sodium orthovanadate; 1% NP-40/IGEPAL CA-630 (Sigma); supplemented with Halt Protease and Phosphatase Inhibitor (Thermo) prior to use]. Cells were scraped, homogenized, and centrifuged at 13,000 x g for 20 min at 4°C to remove insoluble debris and the supernatant was collected. Total protein was measured using a BCA protein assay (Thermo). Samples were heated in 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 and 20-40 μg total protein was loaded into polyacrylamide gel wells. Proteins were separated by SDS-PAGE using electrophoresis buffer (25 mM Tris base, 190 mM glycine, 3.5 mM sodium dodecyl sulfate), followed by transfer of proteins to a membrane by electroblotting with transfer buffer (25 mM Tris base, 190 mM glycine, 20% v/v methanol). 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 added to blocking buffer 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. 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 of images were performed using Kodak Molecular Imaging software. After imaging, membranes were stripped and re-probed with other appropriate antibodies. Molecular weights of analyzed proteins are as follows: PDGFβ receptor, 180 kDa; ERK1/2, 42/44 kDa; Src kinase, 60 kDa; PDGF-B (monomer), 14 kDa; TrkB, 145 kDa; β-actin, 43 kDa.

2.7 Immunoprecipitation

Cell lysates were collected in non-denaturing lysis buffer containing NP-40/IGEPAL CA-630 and centrifuged at 13,000 x g for 20 min at 4°C to remove insoluble debris and the supernatant was collected. Total protein was measured using a BCA protein assay (Thermo). Samples were normalized to equal protein concentrations and volumes, and were pre-cleared by adding irrelevant (IgG control) antibody and agarose beads for 1-2 h at 4°C with agitation to minimize non-specific binding to beads or IgG. Samples were centrifuged at 1000 x g and 4°C for 1 min. The supernatant was collected for the actual immunoprecipitation experiment. The pre-cleared pellet was washed and centrifuged three times with lysis buffer and run as a bead/IgG control by western blotting. Supernatants were then incubated overnight with anti-PDGFβ receptor antibodies at 4°C with agitation. Agarose beads coated with protein A/G were then added for 2 h at 4°C with agitation to bind protein-antibody complexes. Samples were centrifuged at 1000 x g and 4°C for 1 min to collect the immunoprecipitate and washed 3 times with lysis buffer followed by centrifugation. Supernatants were discarded while loading buffer was added to the pellets, which were then subjected to western blotting as described above. Membranes were probed for PDGFβ receptors and 5-HT_{1A} receptors (50 kDa) or 5-HTT (90 kDa).

2.8 DCFH assay

Reactive oxygen species (ROS) production was quantified by measuring the conversion of non-fluorescent 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA) to fluorescent

dichlorodihydrofluorescein (DCF) when exposed to ROS. RGC-5 cells were treated with 0.2 mM DCFH-DA for 60 min to allow accumulation and deacetylation in cells. Cells were then washed with warm PBS and warm, fresh media was added. Drugs were added for stated time intervals, and resulting fluorescence was quantified using a SpectraMax M5 multimode microplate reader (Molecular Devices) with fluorescent excitation and emission wavelengths set at 480 nm and 530 nm, respectively. Fluorescent images were taken with a Kodak 4000MM Pro Imaging Station (Carestream Health, Inc.) with excitation and emission wavelengths set at 470 nm and 530 nm, respectively.

2.9 PDGF-BB ELISA

The ELISA was performed using the Amplex ELISA Development Kit (Life Technologies), and the protocol was adapted slightly from the manufacturer's instructions. A 10 μg/ml rabbit anti-PDGF-BB antibody solution in 0.1 M sodium bicarbonate was added to wells of a 96-well plate and incubated overnight at 4°C. Wells were washed three times with phosphate-buffered saline (PBS) plus Tween 20, and a 1% w/v bovine serum albumin (BSA) solution in PBS was added to the wells overnight at 4°C to block unbound plastic. Wells were washed another three times with PBS plus Tween. Conditioned media or purified PDGF-BB protein diluted in PBS with 0.1% BSA (positive control) or 0.1% w/v BSA in PBS (negative control) was added to the wells for 1 h at room temperature. Wells were washed three times with PBS plus Tween and 50 ng/ml mouse anti-PDGF-BB antibody diluted in 0.1% w/v BSA in PBS was added to wells for 30 min at room temperature. Wells were washed three times with PBS plus Tween and 50 ng/ml goat anti-mouse IgG antibody conjugated to horse radish peroxidase was added to wells for 30 min at room temperature. Wells were washed three times with PBS plus Tween and Amplex UltraRed reagent was added for 30-60 min at room temperature and protected from light. Reactions were stopped by adding Amplex UltraRed Stop Solution, and the plate was read with using a SpectraMax M5 multimode microplate reader (Molecular Devices) with fluorescent excitation and emission wavelengths set at 530 nm and 590 nm, respectively.

2.10 MTT cell viability assay

SH-SY5Y cells were seeded at equal concentrations and grown to 90% confluency. Cells were then serum starved overnight prior to drug treatments. After drug treatments, media was changed to serum-

free, phenol red-free DMEM/F12 and cultures were returned to the cell culture incubator for 24-48 h to allow mitochondrial enzyme deactivation in dead cells. PBS supplemented with 12 mM MTT reagent (thiazolyl blue tetrazolium bromide: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was added to cell culture media in 96-well plates in an amount equal to 10% of the initial cell culture media volume. Plates were returned to the cell culture incubator for 2-4 h for the reaction to occur. Live cells produce insoluble, purple formazan crystals via mitochondrial enzyme action, which can be observed by light microscopy inside cells, while dead cells do not. Cells were then lysed and crystals dissolved in solubilization buffer (0.1 M HCl, 10% Triton X-100 in propan-2-ol) on a gyratory plate shaker. Plates were read at 570 nm absorbance and background absorbance at 690 nm was subtracted from these values. For experiments involving hydrogen peroxide, H_2O_2 concentration was verified by titration with potassium permanganate/sulfuric acid.

2.11 Data analysis and statistics

Data statistics were evaluated with GraphPad Prism software using Student's *t*-test for comparing two data sets, or analysis of variance (ANOVA) with Tukey's post hoc test for comparing three or more data sets, all with statistical significance set at $p<0.05$. For graphs, * = $p<0.05$ compared to vehicle-treated cells, and # = $p<0.05$ compared to agonist alone-treated cells. Data consisted of 3-12 independent experiments.

Chapter 3
Mechanism of 5-HT-mediated neuronal PDGFβ receptor transactivation

3.1 Results

In this section, the focus is on elucidating the potential enzymes responsible for PDGFβ receptor transactivation in neuronal cultures. Firstly, transactivated PDGFβ receptor responses were compared with that of direct PDGF ligand-mediated signaling. This is followed by an attempt at a logical discovery process of the mechanism by which PDGFβ receptor transactivation arises. Finally, an exploration of the transactivation of another receptor tyrosine kinase, TrkB receptor, and its possible role in physiology are examined. A proposed mechanism is presented on page 86 to which the reader can refer.

3.1.1 5-HT transiently increases PDGFβ receptor phosphorylation in SH-SY5Y cells and primary neuron cultures

Direct activation of PDGFβ receptors by PDGF-BB ligand results in a robust phosphorylation of the receptor at multiple tyrosine residues [49]. In SH-SY5Y cells, PDGF-BB application caused a rapid and sustained phosphorylation of PDGFβ receptors at tyrosine 1021 (Y1021) over a 20-min time course (Figure 3.1). Application of 5-HT (0.1 μM) to SH-SY5Y cells also caused an initial rapid increase in PDGFβ receptor phosphorylation at Y1021 that peaked at 5 min but returned to near baseline by 15 min (Figure 3.2). 5-HT application also resulted in phosphorylation of Y1009 and Y751, which followed a similar trend to Y1021 (Figure 3.3 and Figure 3.4). Primary mouse cortical neuron cultures also responded to 5-HT with a transient increase in phosphorylation at Y1021 (Figure 3.5). Interestingly, increasing concentrations of 5-HT increased PDGFβ receptor phosphorylation at Y1021 in a concentration-dependent manner until 0.1 μM, after which higher concentrations failed to increase phosphorylation (Figure 3.6). In contrast, PDGF-BB robustly increased receptor phosphorylation at Y1021 in a concentration-dependent manner (Figure 3.7).

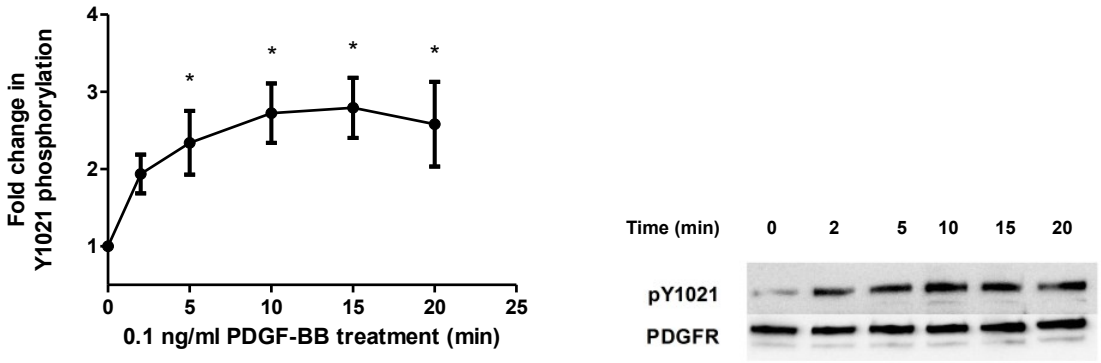


Figure 3.1. PDGF-BB induces sustained PDGFβ receptor phosphorylation in the short term.
SH-SY5Y cells were treated with 0.1 ng/ml PDGF-BB for 0, 2, 5, 10, 15, or 20 min. Proteins were separated and resolved by SDS-PAGE, transferred to a membrane and probed for PDGFRβ and phospho-PDGFRβ Y1021. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in pY1021 immunoreactivity compared to vehicle-treated cells (* = $p<0.05$ compared to vehicle-treated cells; $n = 7$).

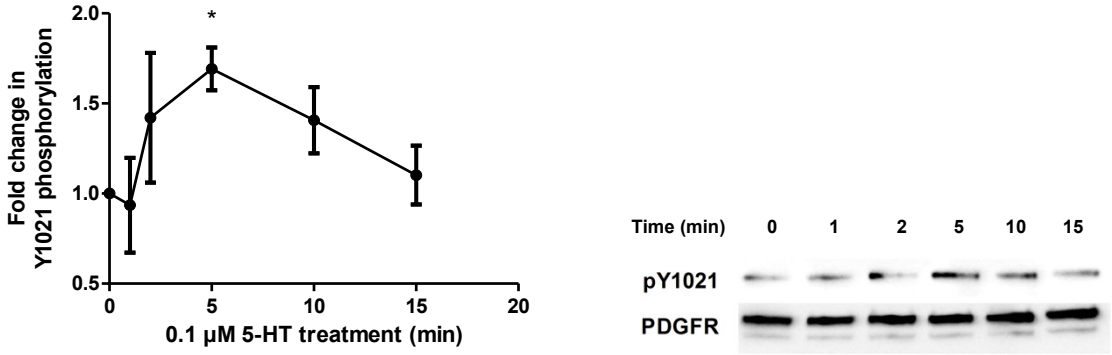


Figure 3.2. 5-HT induces transient PDGFβ receptor phosphorylation at Y1021.
SH-SY5Y cells were treated with 0.1 µM 5-HT for 0, 1, 2, 5, 10, or 15 min. Proteins were separated and resolved by SDS-PAGE, transferred to a membrane and probed for PDGFRβ and phospho-PDGFRβ Y1021. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in pY1021 immunoreactivity compared to vehicle-treated cells (* = $p<0.05$ compared to vehicle-treated cells; $n = 4$).

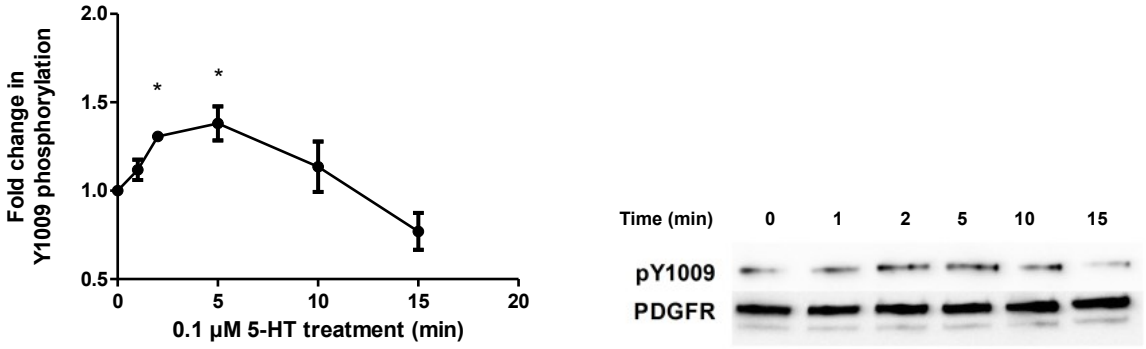


Figure 3.3. 5-HT induces transient PDGFβ receptor phosphorylation at Y1009.
SH-SY5Y cells were treated with 0.1 µM 5-HT for 0, 1, 2, 5, 10, or 15 min. Proteins were separated and resolved by SDS-PAGE, transferred to a membrane and probed for PDGFRβ and phospho-PDGFRβ Y1009. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in pY1009 immunoreactivity compared to vehicle-treated cells (* = $p<0.05$ compared to vehicle-treated cells; $n = 4$).

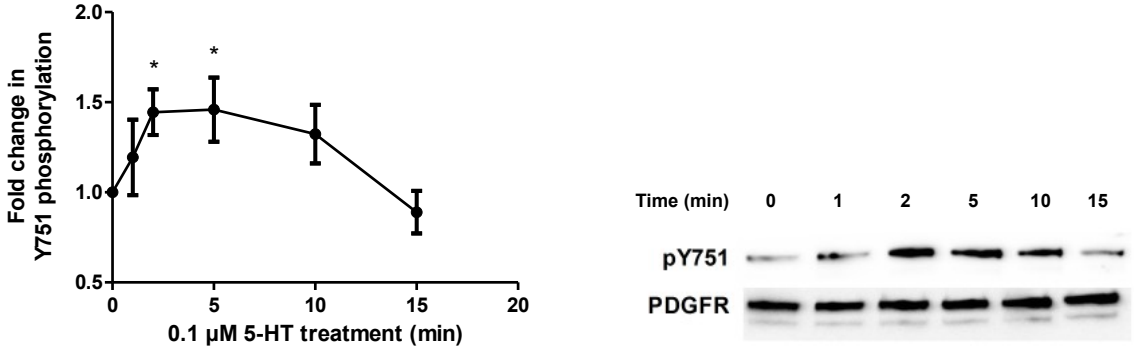


Figure 3.4. 5-HT induces transient PDGFβ receptor phosphorylation at Y751.
SH-SY5Y cells were treated with 0.1 µM 5-HT for 0, 1, 2, 5, 10, or 15 min. Proteins were separated and resolved by SDS-PAGE, transferred to a membrane and probed for PDGFRβ and phospho-PDGFRβ Y751. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in pY751 immunoreactivity compared to vehicle-treated cells (* = $p<0.05$ compared to vehicle-treated cells; $n = 4$).

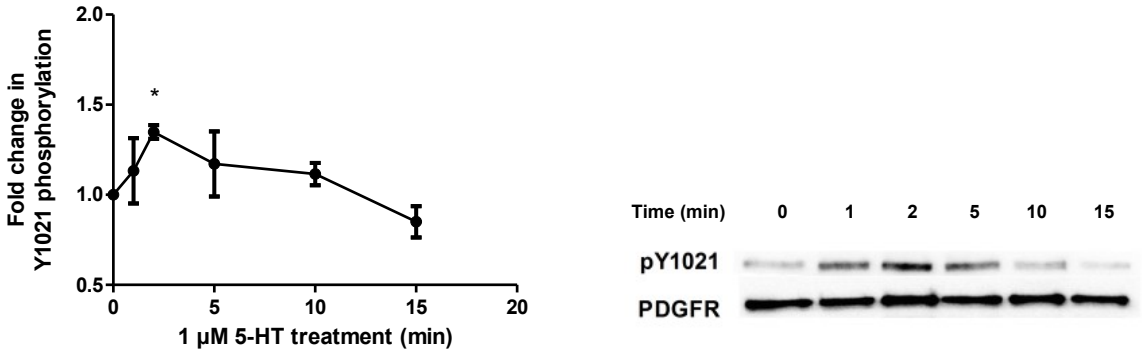


Figure 3.5. 5-HT induces transient PDGFβ receptor phosphorylation at Y1021 in primary neuron cultures.

7 DIV mouse cortical cultures were treated with 1 µM 5-HT for 0, 1, 2, 5, 10, or 15 min. Proteins were separated and resolved by SDS-PAGE, transferred to a membrane and probed for PDGFRβ and phospho-PDGFRβ Y1021. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in pY1021 immunoreactivity compared to vehicle-treated cells (* = $p<0.05$ compared to vehicle-treated cells; $n = 3$).

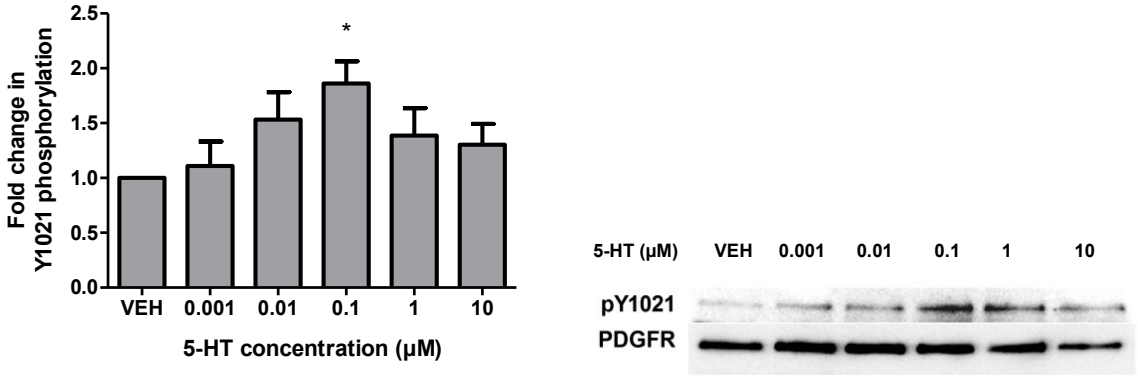


Figure 3.6. 5-HT induces a concentration-dependent response in PDGFβ receptor phosphorylation.
SH-SY5Y cells were treated with vehicle (VEH) or 0.001 μM to 10 μM 5-HT for 5 min. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Membranes were probed for PDGFRβ and phospho-PDGFRβ Y1021. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in pY1021 immunoreactivity compared to vehicle-treated cells (* = $p<0.05$ compared to vehicle-treated cells; $n = 5$).

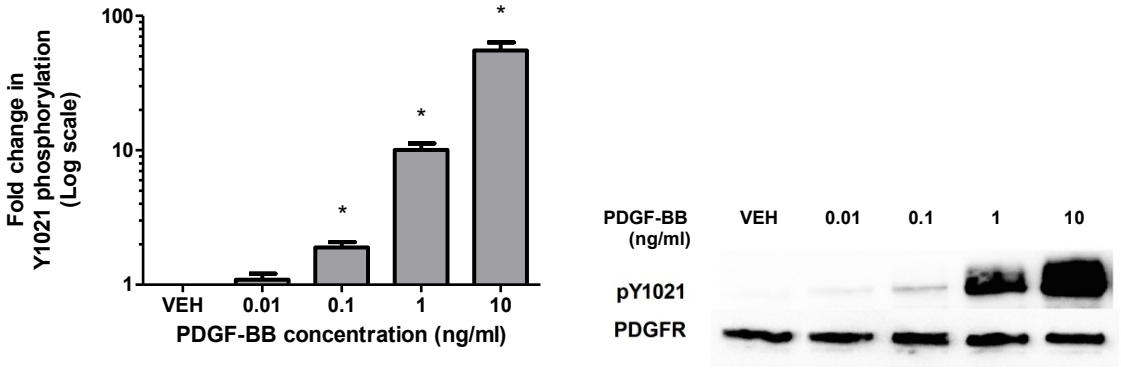


Figure 3.7. PDGF-BB induces concentration-dependent increase in PDGFβ receptor phosphorylation.
SH-SY5Y cells were treated with vehicle (VEH) or 0.01 to 10 ng/mL PDGF-BB for 5 min. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Membranes were probed for PDGFRβ and phospho-PDGFRβ Y1021. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in pY1021 immunoreactivity compared to vehicle-treated cells (* = $p<0.05$ compared to vehicle-treated cells; $n = 5$).

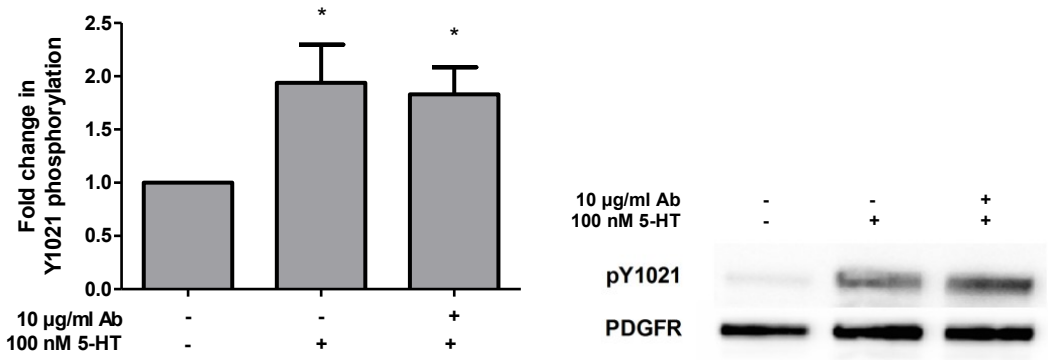


Figure 3.8. 5-HT-induced PDGFβ receptor phosphorylation is not affected by anti-PDGF-BB antibodies.
SH-SY5Y cells were incubated with 10 µg/ml anti-PDGF-BB antibody prior to 5 min treatment with 0.1 µM 5-HT. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Membranes were probed for PDGFRβ and phospho-PDGFRβ Y1021. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in pY1021 immunoreactivity compared to vehicle-treated cells (* = $p<0.05$ compared to vehicle-treated cells; $n = 5$).

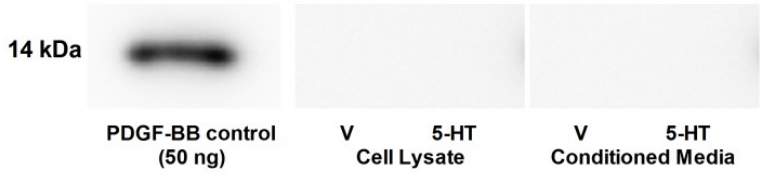


Figure 3.9. 5-HT-induced PDGFβ receptor phosphorylation is not due to PDGF-BB release.
Conditioned cell culture media from SH-SY5Y cells was collected after 5 min vehicle (V) or 0.1 µM 5-HT treatments and concentrated to 60x with Amicon protein concentrators (Millipore). Anti-PDGF-BB antibodies failed to detect any PDGF-B (monomer) protein at 14 kDa in either cell lysate or conditioned media but did detect 50 ng of PDGF-BB as a positive control ($n = 3$).

3.1.2 5-HT-induced PDGFβ receptor transactivation is not dependent on PDGF-BB ligand

Although transactivation of the PDGFβ receptor is thought to involve only intracellular signaling, a possible explanation for the effect of 5-HT on PDGFβ receptor phosphorylation may involve the release of PDGF ligands into the extracellular environment, which would then bind and activate PDGFβ receptors. To determine if PDGF-BB ligand was released as a result of 5-HT application, anti-PDGF-BB antibodies were added during the 5-HT application to sequester any released ligand and prevent it from activating its receptor. As a control, it was confirmed that PDGF-BB antibodies blocked PDGF-BB-induced phosphorylation at Y1021 (data not shown). However, PDGF-BB antibodies failed to block PDGFβ receptor phosphorylation after 5-HT application (Figure 3.8). In addition, conditioned media was collected and analyzed for PDGF-BB content. No PDGF-BB was detected in either vehicle or 5-HT-treated cells, suggesting that no PDGF-BB was released from cells upon 5-HT application (Figure 3.9). Conditioned media was also analyzed for PDGF-BB content by ELISA; however, no PDGF-BB protein was detected (data not shown). Moreover, antibodies failed to detect PDGF-BB protein from cell lysates (Figure 3.9). These data suggest that PDGF-BB ligand is not being produced by SH-SY5Y cells, nor is any released when cells are treated with short-term application of 5-HT, nor is there any in the culture media itself. Thus, transactivation of the PDGFβ receptor is through a mechanism that is independent of PDGF-BB.

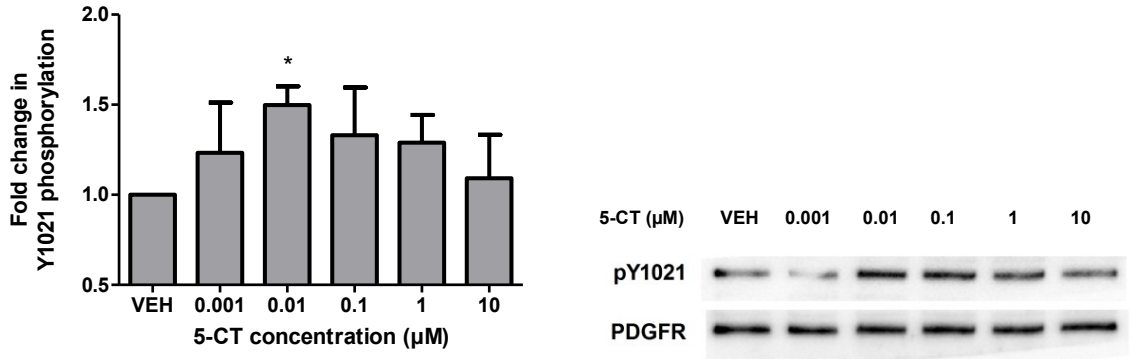


Figure 3.10. 5-CT induces a concentration-dependent response in PDGFβ receptor phosphorylation.
SH-SY5Y cells were treated with vehicle (VEH) or 0.001 μM to 10 μM 5-CT for 5 min. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Membranes were probed for PDGFRβ and phospho-PDGFRβ Y1021. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in pY1021 immunoreactivity compared to vehicle-treated cells (* = $p<0.05$ compared to vehicle-treated cells; $n = 5$).

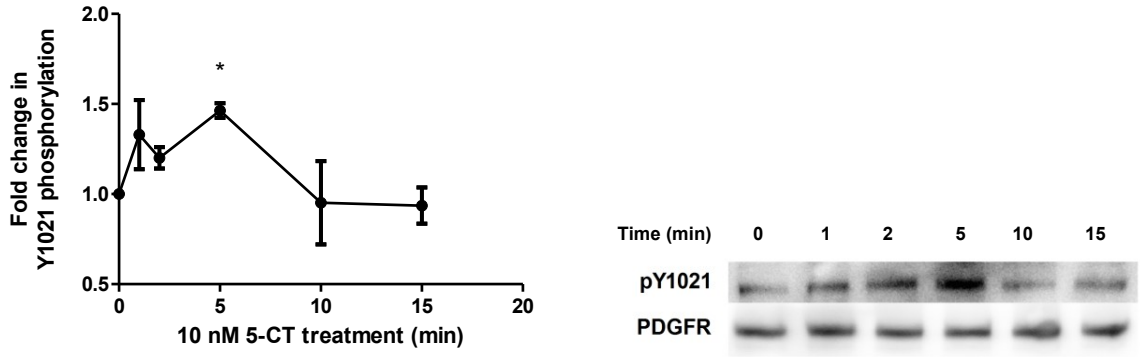


Figure 3.11. 5-CT induces transient PDGFβ receptor phosphorylation at Y1021.
SH-SY5Y cells were treated with 10 nM 5-CT for 0, 1, 2, 5, 10, or 15 min. Fold-phosphorylation data for immunoreactivity with antibodies directed against tyrosine 1021. Proteins were separated and resolved by SDS-PAGE, transferred to a membrane and probed for PDGFRβ and phospho-PDGFRβ Y1021. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in pY1021 immunoreactivity compared to vehicle-treated cells (* = $p<0.05$ compared to vehicle-treated cells; $n = 4$).

3.1.3 PDGFβ receptor transactivation is dependent on pertussis toxin-sensitive 5-HT receptors

To elucidate which 5-HT receptors were involved in the transactivation of PDGFβ receptors, cells were treated with the 5-HT analog, 5-carboxamidotryptamine (5-CT), which only activates the $G\alpha_i$-coupled 5-HT_1 and 5-HT_5, and the $G\alpha_s$-coupled 5-HT_7 receptor subtypes [140]. Acute 5-CT treatment maximally increased PDGFβ receptor phosphorylation at Y1021 at a concentration of 10 nM (Figure 3.10), a 10-fold lower concentration than was required to induce phosphorylation with 5-HT. Similar to 5-HT, maximal phosphorylation was observed with a 5-min incubation at this concentration (Figure 3.11).

It was recently reported that long-term (2 to 24 h) 5-HT_7 receptor activation increased PDGFβ receptor expression in SH-SY5Y cells as well as in primary mouse hippocampal and cortical neurons [140]. Since SH-SY5Y cells express 5-HT_7 receptors [140], it was questioned whether the effects of 5-CT could be mediated by $G\alpha_s$-coupled 5-HT_7 receptors; therefore, cells were treated with the 5-HT_7 receptor-specific agonist LP 12. Acute LP 12 application was unable to increase PDGFβ receptor phosphorylation at Y1021 at any concentration tested (Figure 3.12). To confirm whether 5-CT was acting via $G\alpha_i$-coupled receptors to induce PDGFβ receptor phosphorylation in SH-SY5Y cells, cultures were pretreated with increasing concentrations of pertussis toxin (Ptx). Ptx acts by adding ADP-ribosyl groups to susceptible G proteins ($G\alpha_i$ and $G\alpha_o$), thus inactivating them [141]. According to previous studies, Ptx blocked ERK1/2 activation induced by dopamine receptor-mediated transactivation of PDGFβ receptors in Chinese hamster ovary cells [68], and it was confirmed that overnight Ptx pretreatment was able to block dopamine-induced ERK1/2 phosphorylation (Figure 3.13) as well as PDGFβ receptor phosphorylation (Figure 3.14) in SH-SY5Y cells. Ptx pretreatment also prevented 5-HT- and 5-CT-induced PDGFβ receptor transactivation in a concentration-dependent manner (Figure 3.15 and Figure 3.16, respectively). The inability of the 5-HT_7 receptor agonists to transactivate the PDGFβ receptor, coupled with the ability of Ptx to block transactivation, suggests that 5-CT (and 5-HT) transactivated the PDGFβ receptor via the $G\alpha_i$-coupled 5-HT_1 or 5-HT_5 receptors.

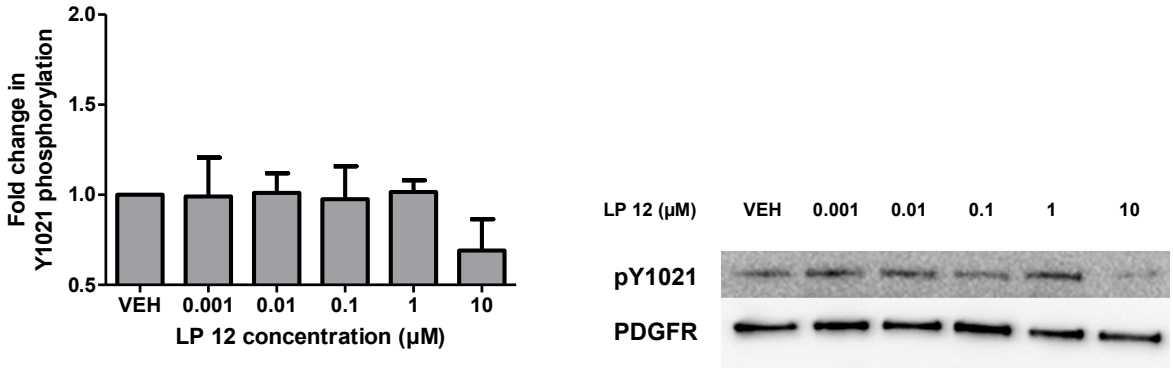


Figure 3.12. LP 12 does not induce a response in PDGFβ receptor phosphorylation.
SH-SY5Y cells were treated with vehicle (VEH) or 1 nM to 10 μM of the 5-HT_7 agonist LP 12 for 5 min. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Membranes were probed for PDGFRβ and phospho-PDGFRβ Y1021. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in pY1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (n = 6).

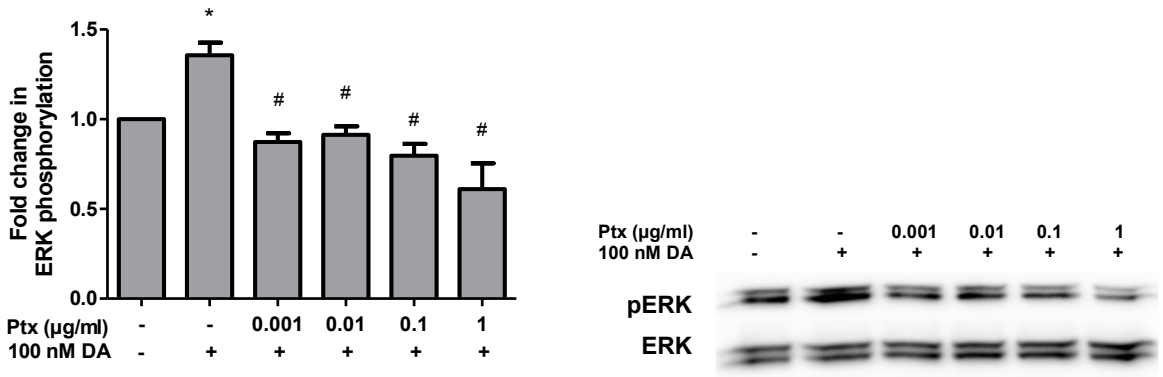


Figure 3.13. Pertussis toxin completely inhibits dopamine-induced ERK1/2 phosphorylation.
SH-SY5Y cells were incubated overnight with 0.001 to 1 µg/mL pertussis toxin (Ptx) followed by 2 min treatment with 100 nM dopamine (DA). Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in combined phospho-ERK1/2 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and ERK1/2 are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with DA-treated cells; $n = 5$).

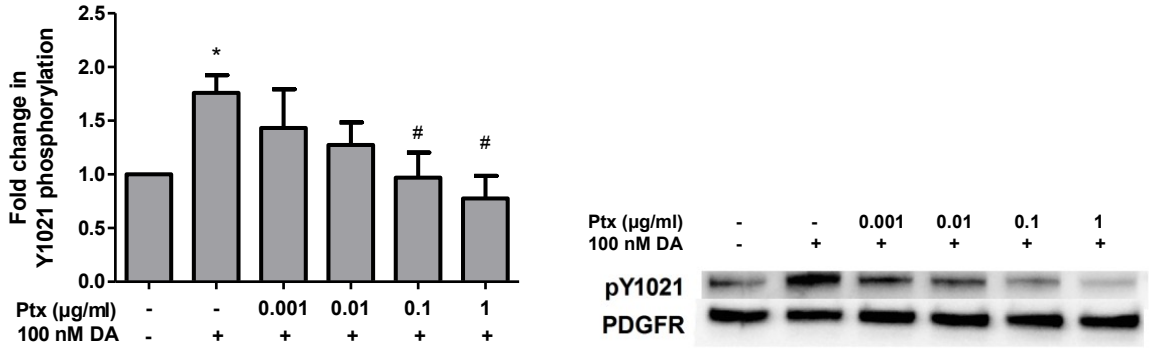


Figure 3.14. Pertussis toxin inhibits dopamine-induced PDGFβ receptor phosphorylation in a concentration-dependent manner.
SH-SY5Y cells were incubated overnight with 0.001 to 1 µg/mL pertussis toxin (Ptx) followed by 2 min treatment with 100 nM dopamine (DA). Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with DA-treated cells; $n = 4$).

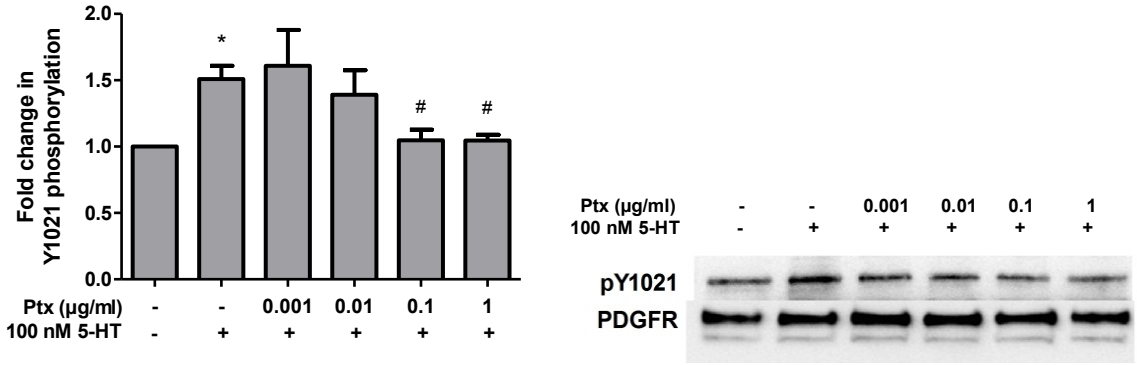


Figure 3.15. Pertussis toxin inhibits 5-HT-induced PDGFβ receptor phosphorylation in a concentration-dependent manner.
SH-SY5Y cells were incubated overnight with 0.001 to 1 µg/mL pertussis toxin (Ptx) followed by 5 min treatment with 100 nM 5-HT. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 5$).

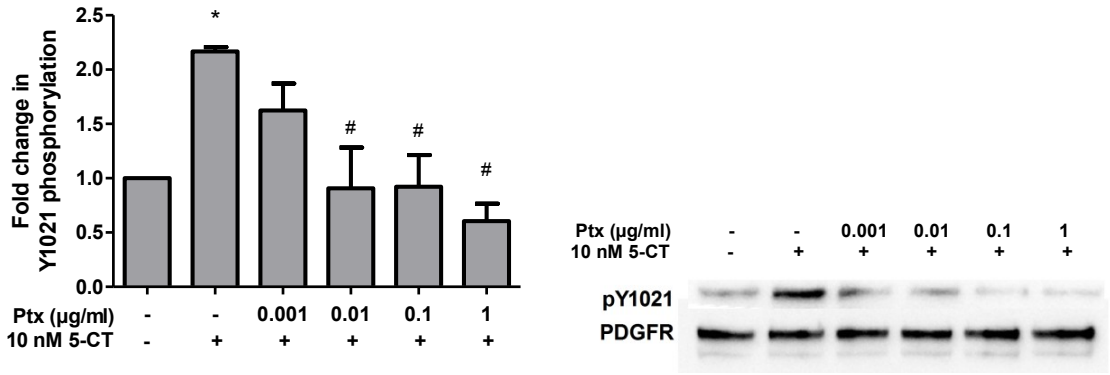


Figure 3.16. Pertussis toxin inhibits 5-CT-induced PDGFβ receptor phosphorylation in a concentration-dependent manner.
SH-SY5Y cells were incubated overnight with 0.001 to 1 µg/mL pertussis toxin (Ptx) followed by 5 min treatment with 10 nM 5-CT. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-CT-treated cells; $n = 3$).

3.1.4 Involvement of 5-HT_{1A} receptors in PDGFβ receptor transactivation

Since SH-SY5Y cells express 5-HT_{1A} receptors [142], the 5-HT_{1A} receptor antagonist, WAY 100135, was applied prior to 5-HT application to determine whether 5-HT was transactivating PDGFβ receptors via 5-HT_{1A} receptors. WAY 100135 was indeed able to attenuate 5-HT-induced PDGFβ receptor transactivation at Y1021 at a concentration of 100 nM (Figure 3.17). However, at concentrations below (10 nM) and above (1 μM) the 100 nM concentration, a significant block was not observed (Figure 3.17). WAY 100135 also completely blocked 5-CT-induced PDGFβ receptor transactivation (Figure 3.18). To confirm the involvement of 5-HT_{1A} receptors in this transactivation pathway, the 5-HT_{1A}-selective agonist 8-hydroxy-DPAT (DPAT) was applied to SH-SY5Y cells. Similar to 5-HT, DPAT increased PDGFβ receptor phosphorylation in a concentration-dependent manner with a peak effect observed at 10 nM and a significant increase observed at 1 nM (Figure 3.19), just above the reported K_d value of 0.5 nM for 5-HT_{1A} receptors [143, 144]. At concentrations above 10 nM DPAT did not significantly increase receptor phosphorylation, comparable to the trends observed with 5-HT (see Figure 3.6).

Previous studies have identified physical protein-protein interactions between receptors involved in other cross-talk pathways, and these interactions may mediate signal transduction. In smooth muscle cells, 5-HT induces a complex formation between PDGFβ receptor and the 5-HT transporter [78], and sphingosine-1-phosphate type 1 receptors are constitutively complexed with PDGFβ receptors in both smooth muscle cells and mouse embryonic fibroblasts [145, 146]. This led to a query into whether PDGFβ receptors physically interact with 5-HT_{1A} receptors, the target of DPAT; thus, PDGFβ receptors were immunoprecipitated from SH-SY5Y cell lysates under non-denaturing conditions. Although 5-HT_{1A} receptor expression was observed in the lysate control, no 5-HT_{1A} immunoreactivity was observed in the immunoprecipitate in either vehicle-treated or 5-HT-treated conditions (Figure 3.20) suggesting these two receptors do not physically interact as part of this transactivation pathway.

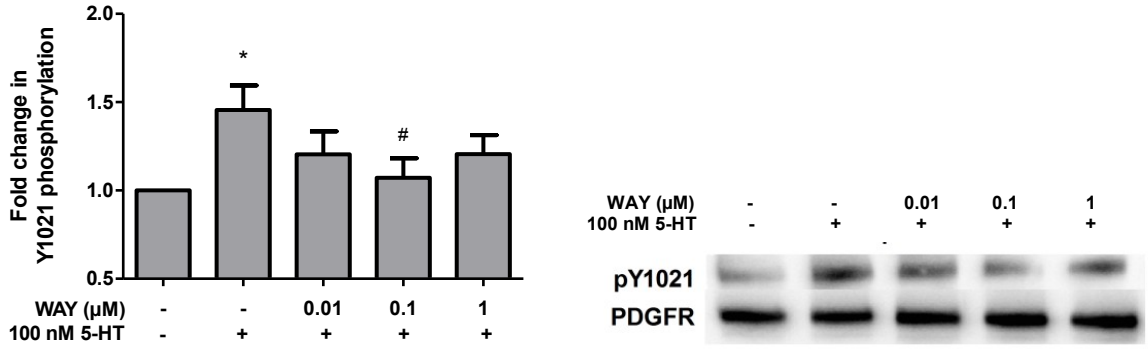


Figure 3.17. 5-HT$_{1A}$ inhibition prevents PDGFβ receptor phosphorylation by 5-HT.
SH-SY5Y cells were treated for 5 min with vehicle, 100 nM 5-HT, with or without 0.01, 0.1, or 1 μM WAY 100135 (WAY). Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 9$).

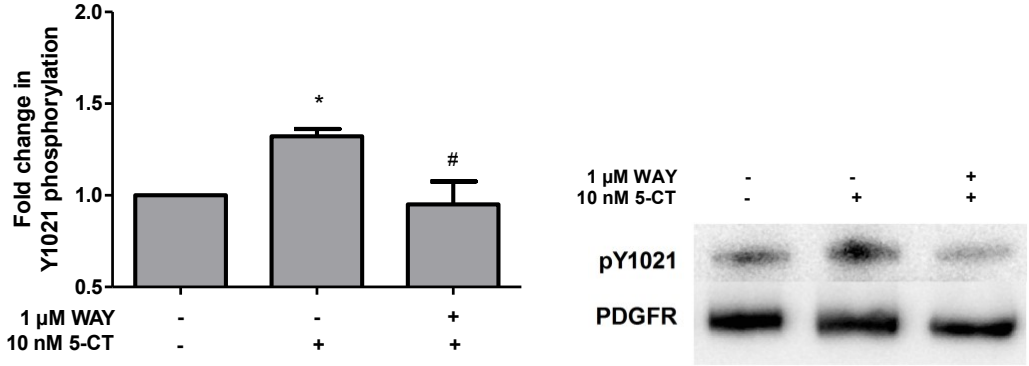


Figure 3.18. 5-HT$_{1A}$ inhibition prevents PDGFβ receptor phosphorylation by 5-CT.
SH-SY5Y cells were treated for 5 min with vehicle, 10 nM 5-CT, with or without 1 μM WAY 100135. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-CT-treated cells; $n = 5$).

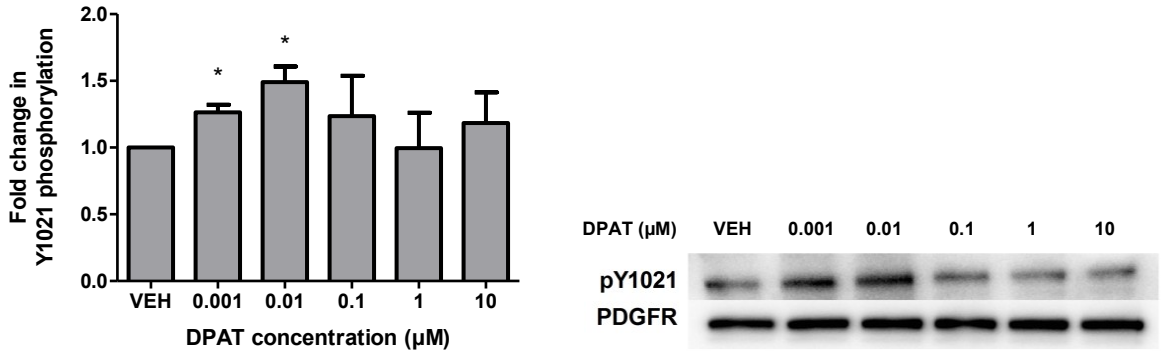


Figure 3.19. DPAT induces a concentration-dependent response in PDGFβ receptor phosphorylation.
SH-SY5Y cells were treated with vehicle (VEH) or 0.001 μM to 10 μM of the 5-HT_{1A} agonist DPAT for 5 min. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Membranes were probed for PDGFRβ and phospho-PDGFRβ Y1021. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in pY1021 immunoreactivity compared to vehicle-treated cells (* = $p<0.05$ compared to vehicle-treated cells; $n = 7$).

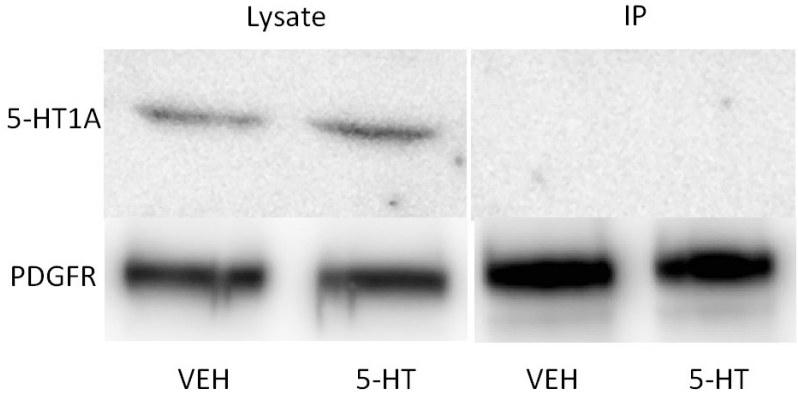


Figure 3.20. PDGFβ and 5-HT_{1A} receptors do not associate.
SH-SY5Y cells were treated with either vehicle (VEH) or 100 nM 5-HT for 5 min. Following drug treatments, cell lysates were collected and subjected to immunoprecipitation under non-denaturing conditions. PDGFβ receptor was pulled down, and both immunoprecipitates and lysates were subjected to Western blot and probed for PDGFβ and 5-HT_{1A} receptors. Representative blots are shown ($n = 3$).

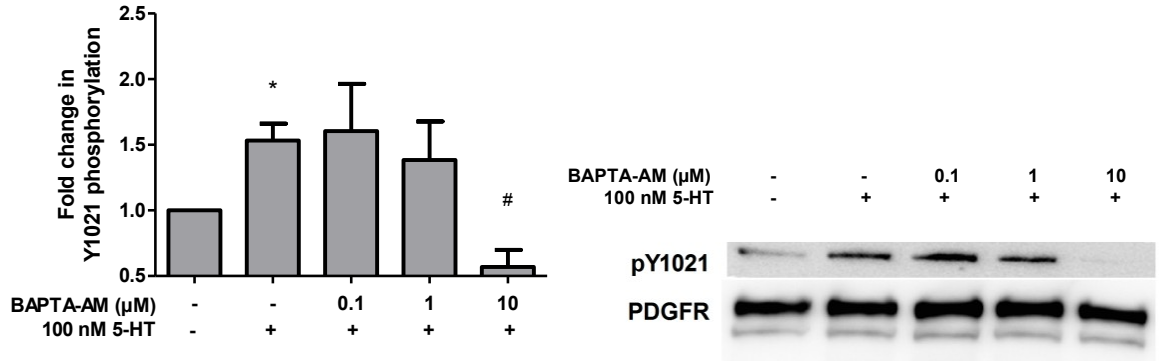


Figure 3.21. PDGFβ receptor transactivation is blocked by membrane-permeable calcium chelation.
SH-SY5Y cells were treated with vehicle, 100 nM 5-HT for 5 min, with or without a 10 min pretreatment of 0.1, 1, or 10 μM BAPTA-AM. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 4$).

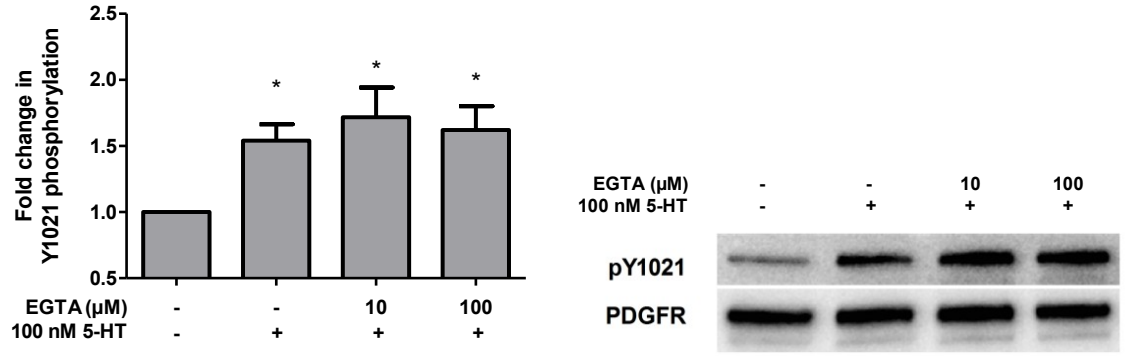


Figure 3.22. PDGFβ receptor transactivation is not blocked by extracellular calcium chelation.
SH-SY5Y cells were treated with vehicle, 100 nM 5-HT for 5 min, with or without a 10 min pretreatment of 10 or 100 μM EGTA. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 5$).

3.1.5 5-HT signaling to PDGFβ receptors is calcium-dependent

Calcium signaling was shown to play an important role in PDGFβ receptor transactivation induced by sphingosine-1-phosphate [72]. To determine if intracellular calcium signaling was required for PDGFβ receptor transactivation by 5-HT in neurons, the cell-permeable calcium chelator BAPTA-AM was used to sequester intracellular calcium. Upon 5-HT application, cells pretreated with 10 μM BAPTA-AM showed a significant decrease in PDGFβ receptor phosphorylation at Y1021 (Figure 3.21). BAPTA-AM, which preferentially chelates calcium over magnesium [147], passes into the cytoplasm where it is cleaved to non-permeable BAPTA by cytoplasmic esterases and becomes locked inside the cell [148]. Thus, BAPTA-AM is effectively an intracellular calcium chelator. However, 5-HT-induced PDGFβ receptor phosphorylation was unaffected by 10 or even 100 μM EGTA, a non-permeable (therefore extracellular) calcium chelator (Figure 3.22) that also has higher affinity for calcium over magnesium [147]. Given the role of calcium signaling in 5-HT-induced PDGFβ receptor transactivation, a potential mechanism for calcium release was sought. Activation of phospholipase C (PLC) leads to intracellular calcium release via phosphatidylinositol 4,5-bisphosphate (PIP_2) cleavage and subsequent inositol 1,4,5-trisphosphate (IP_3)-induced opening of calcium channels on the endoplasmic reticulum [149, 150]. PDGFβ receptor phosphorylation at Y1021 by 5-HT was blocked by the PLC inhibitor U73122 at a concentration of 1 μM (Figure 3.23). Taken together, these results indicate that 5-HT-induced PDGFβ receptor transactivation involves PLC-dependent intracellular calcium release.

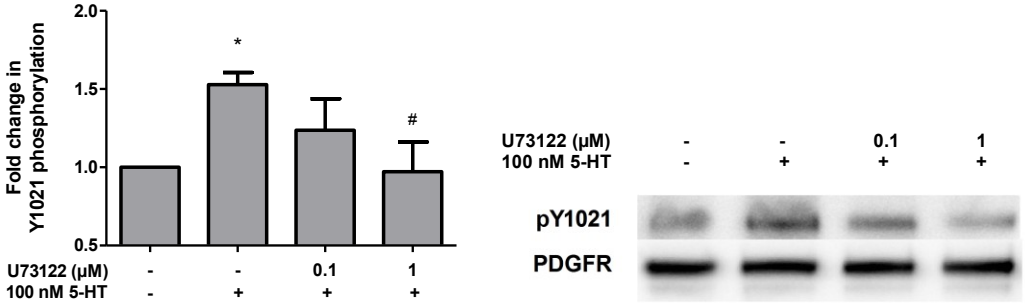


Figure 3.23. PDGFβ receptor transactivation is inhibited by PLC inhibition.
SH-SY5Y cells were treated with vehicle, or 100 nM 5-HT for 5 min, with or without pretreatment with 0.1 or 1 μM U73122. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 6$).

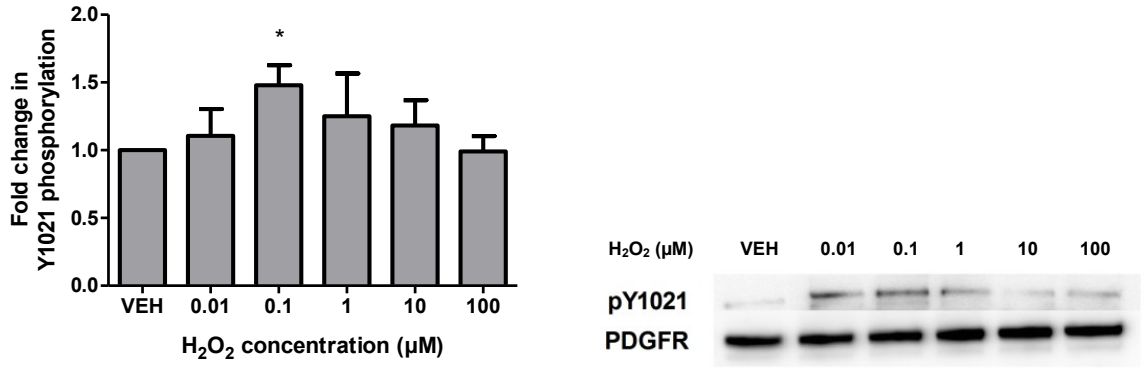


Figure 3.24. H_2O_2 increases PDGFβ receptor phosphorylation in SH-SY5Y cells.
SH-SY5Y cells were treated with vehicle (VEH) or 0.01 to 100 μM H_2O_2 for 5 min. Following drug treatments, cell lysates were evaluated by Western blot analysis as described in Materials and methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 5$).

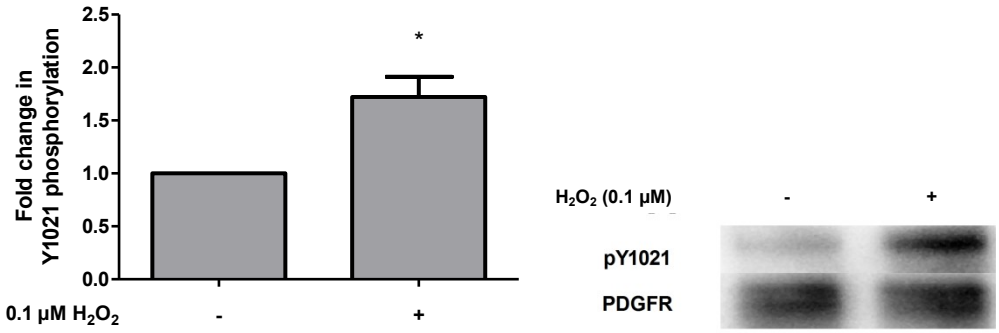


Figure 3.25. H_2O_2 increases PDGFβ receptor phosphorylation in primary cultured neurons.
Primary cultured cortical neurons were treated with vehicle (VEH) or 0.1 μM H_2O_2 for 5 min. Following drug treatments, cell lysates were evaluated by Western blot analysis as described in Materials and methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 4$).

3.1.6 H_2O_2 increases PDGFβ receptor phosphorylation in SH-SY5Y cells and primary neuron cultures

Based on transactivation pathways described in other systems [78, 151], it was postulated that reactive oxygen species (ROS) are also involved in the 5-HT-induced transactivation of RTKs described here. A dose-response analysis of H_2O_2 application to SH-SY5Y cells for 5 min had peak Y1021 phosphorylation of PDGFβ receptor at a concentration of 0.1 μM (Figure 3.24). This concentration was also sufficient to cause transactivation in primary mouse cortical neuron cultures (Figure 3.25). To verify the involvement of ROS in 5-HT-induced transactivation of PDGFβ receptor, cells were pretreated with the ROS scavenger, *N*-acetyl-L-cysteine, followed by 100 nM 5-HT for 5 min (Figure 3.26). *N*-acetyl-L-cysteine (1000 μM) abrogated PDGFβ receptor phosphorylation, suggesting that ROS are involved in this 5-HT-mediated transactivation pathway. But because H_2O_2 can cause cell damage and death at high concentrations, it was necessary to verify that the low concentrations of H_2O_2 used here were not adversely affecting cell viability. By using the MTT cell viability assay, cultures were determined to be unaffected by H_2O_2 at 0.1, 1, and 10 μM, even after overnight treatment (Figure 3.27). Only at 100 μM or higher was cell viability significantly decreased.

As an aside, direct evidence of ROS production in 5-HT-mediated transactivation of PDGFβ receptor was also sought. A DCFH assay was employed to directly measure intracellular ROS production in RGC-5 cells, which also show 5-HT-induced PDGFβ receptor transactivation (data not shown). The assay was attempted with SH-SY5Y cells; however, this cell line was not tolerant to the assay reagents. Using a dose-response analysis of 5-min H_2O_2-mediated fluorescence, the assay was able to detect ROS-induced fluorescence at a concentration of 0.1 mM H_2O_2 or higher (Figure 3.28A). However, with 5-min 5-HT application, no change in fluorescence was seen (Figure 3.28B). This was not entirely unexpected, since 0.1 μM H_2O_2 (i.e. 1000-fold less than minimum sensitivity) showed optimal PDGFβ receptor transactivation (see Figure 3.24) with the same fold-increase in phosphorylation over vehicle as transactivation mediated by 5-HT (see Figure 3.6). Thus, this assay was not sensitive enough to measure intracellular ROS production in the high nanomolar range that is predicted with transactivation events.

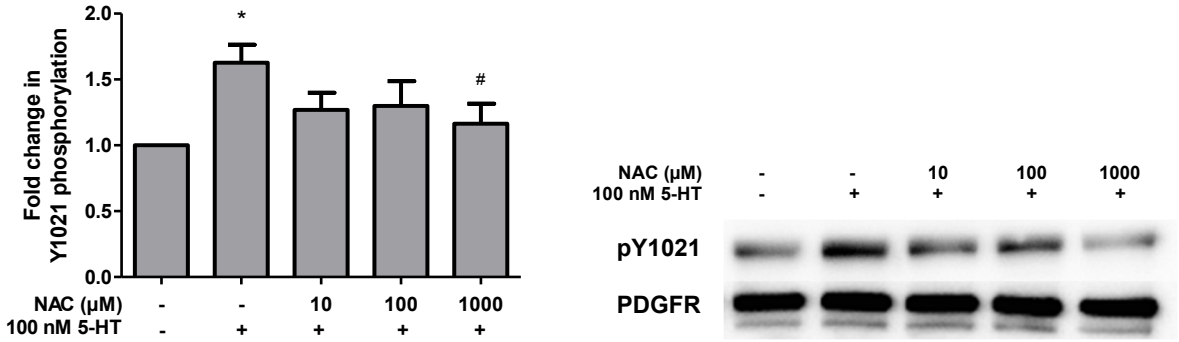


Figure 3.26. PDGFβ receptor transactivation is sensitive to ROS scavenging.
SH-SY5Y cell cultures were pretreated with vehicle or 10, 100, or 1000 μM of the ROS scavenger *N*-acetyl-L-cysteine (NAC) for 45 min followed by treatment with vehicle or 100 nM 5-HT for 5 min. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 6$).

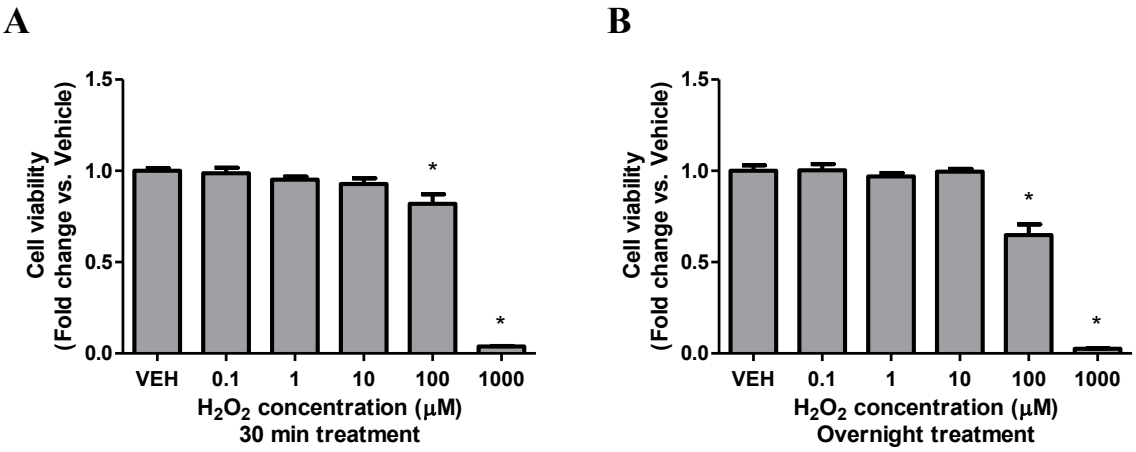


Figure 3.27. H_2O_2 concentrations that induce transactivation do not affect cell viability.
Cells were treated with H_2O_2 at various concentrations either for 30 min (A) or overnight (B). Media was changed and cultures were returned to the incubator for 24 h prior to adding MTT reagents. Cell viability was assessed by absorbance of the resulting solution at 570 nm (* = $p<0.05$ compared to vehicle-treated cells; $n = 4$).

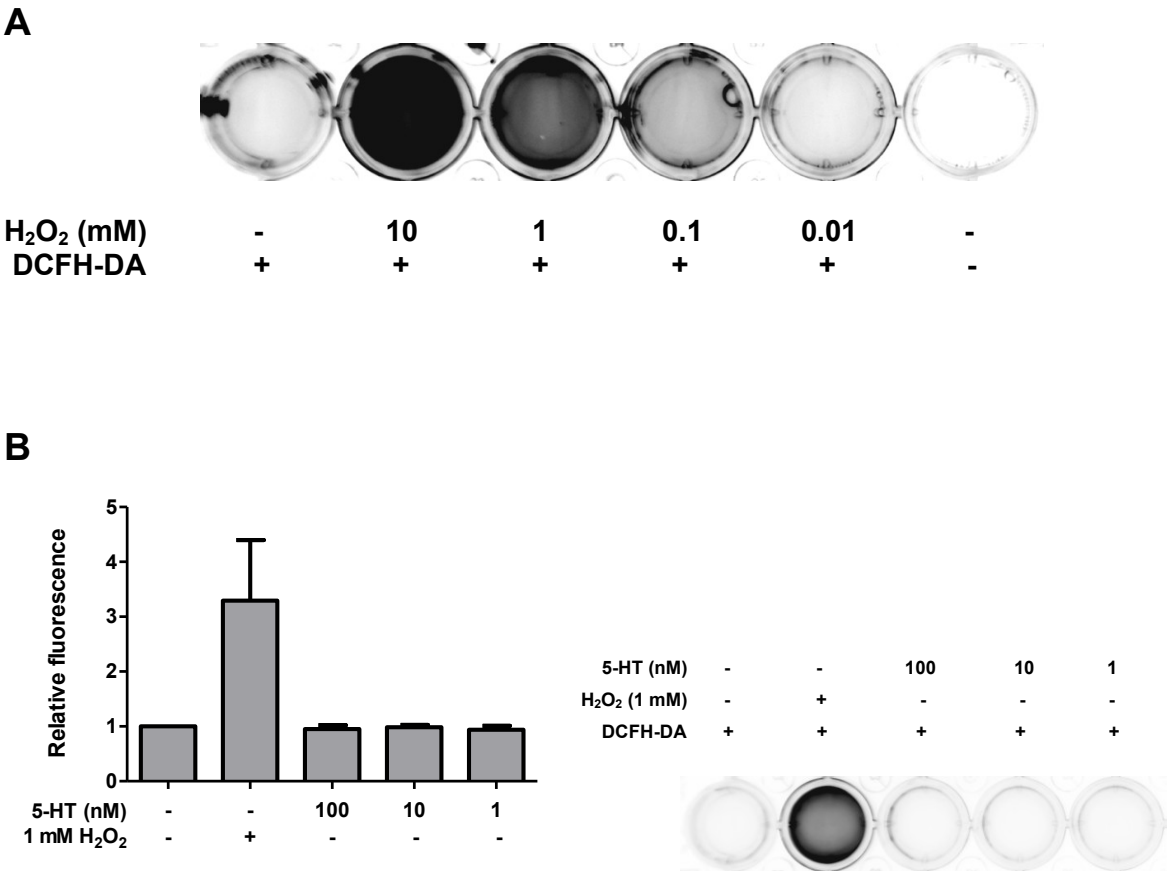


Figure 3.28. DCFH assay measures ROS production.
(A) RGC-5 cells were treated with 0.2 mM DCFH-DA for 60 min. Cells were washed with PBS and treated with varying concentrations of H_2O_2 for 5 min. An increase in fluorescence was detected in 0.1 to 10 mM wells. (B) RGC-5 cells were treated with 0.2 mM DCFH-DA for 60 min. Cells were washed with PBS and treated with vehicle, H_2O_2 or 5-HT for 5 min. Resulting DCF production was measured by fluorescent plate reader and represents a fold-change in fluorescence vs. vehicle-treated cells. Representative images were taken by fluorescent imaging (n = 5).

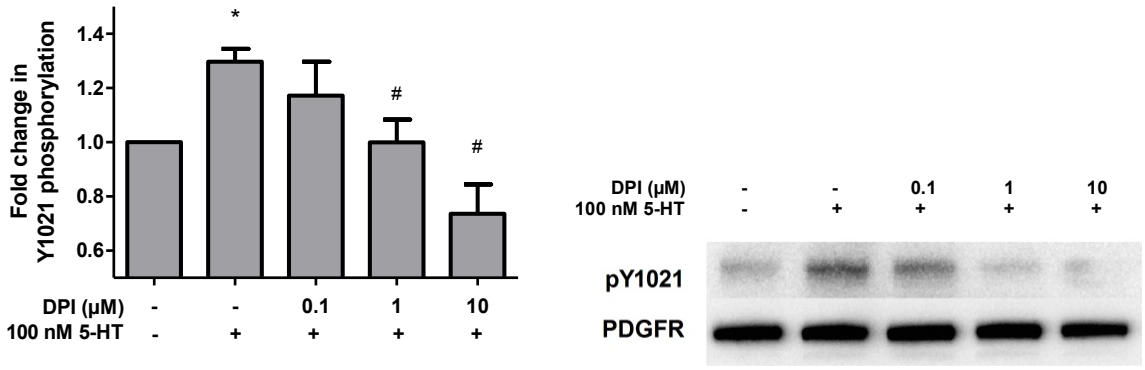


Figure 3.29. PDGFβ receptor transactivation is sensitive to diphenyleneiodonium chloride.
SH-SY5Y cell cultures were pretreated with vehicle or 0.1, 1 or 10 μM of the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI) for 45 min followed by treatment with vehicle or 100 nM 5-HT for 5 min. Following drug treatments, cell lysates were evaluated by immunoblot analysis as described in Materials and methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 5$).

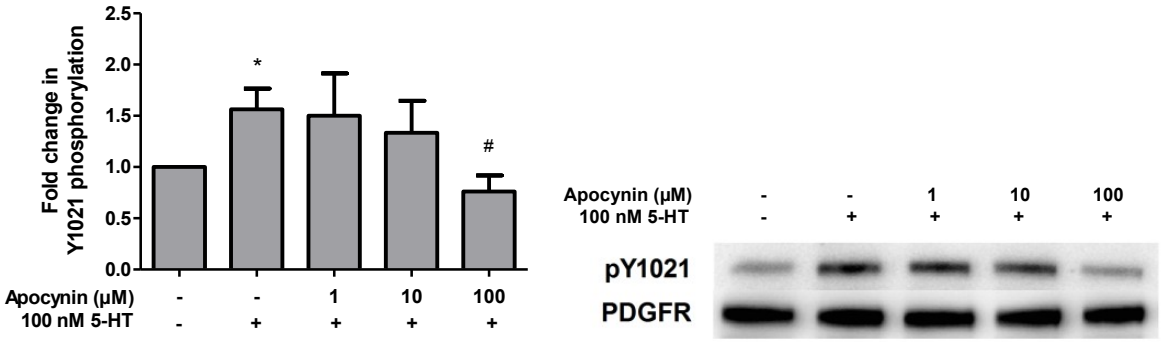


Figure 3.30. PDGFβ receptor transactivation is sensitive to apocynin.
SH-SY5Y cell cultures were pretreated with vehicle or 1, 10 or 100 μM of the NADPH oxidase inhibitor apocynin for 45 min followed by treatment with vehicle or 100 nM 5-HT for 5 min. Following drug treatments, cell lysates were evaluated by immunoblot analysis as described in Materials and methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 4$).

3.1.7 5-HT-induced PDGFβ receptor transactivation involves NADPH oxidase and protein kinase C

To investigate a potential source of ROS, NADPH oxidase was considered since it was implicated in other transactivation systems [71, 152, 153]. Treatment with the NADPH oxidase inhibitors diphenyleneiodonium chloride (1 μM) and apocynin (100 μM) in SH-SY5Y cells blocked PDGFβ receptor transactivation by 5-HT (Figure 3.29 and Figure 3.30). Diphenyleneiodonium chloride may be chemically modifying certain subunits [154] while apocynin prevents assembly of the oxidase [155], suggesting that the complete, functional oxidase is necessary for PDGFβ receptor transactivation. NADPH oxidase components were previously shown to be activated by protein kinase C (PKC) [156], either directly or via Rap1A and Rac1/2 [157, 158]. Thus, PKC involvement in this transactivation pathway was examined not only for its ability to activate NADPH oxidase components, but also for its potential as a downstream effector of PLC activity (see Figure 3.23). It is well known that the PLC products diacylglycerol and calcium are necessary for activation of calcium-dependent PKC isoforms [159]. When cells were pretreated with the PKC inhibitor Go 6983 (0.1 μM), 5-HT failed to transactivate the PDGFβ receptor (Figure 3.31). Overall, these findings suggest that 5-HT treatment results in the activation of PKC via PLC and calcium release, which leads to activation and assembly of NADPH oxidase components, resulting in the production of ROS and ultimately phosphorylation of PDGFβ receptor.

In addition to NADPH oxidase, monoamine oxidase A (MAO-A) was also considered as a potential source of ROS. Upon being transported back into the presynaptic neuron, 5-HT can be degraded by one of two MAO enzymes. The MAO-A isoform preferentially targets 5-HT, which is metabolized to 5-hydroxyindoleacetic acid with ROS as a by-product [160]. Pretreatment with the MAO-A inhibitor clorgyline also inhibited 5-HT-induced PDGFβ receptor phosphorylation (Figure 3.32). However, since many systems involving GPCR agonists transactivating RTKs do not involve MAO as does 5-HT, this may be a unique factor in 5-HT-mediated transactivation pathways. Thus, this experimental direction was abandoned in favor of NADPH oxidase, which is predicted to be an effector of a wider range of transactivating agonists compared to MAO.

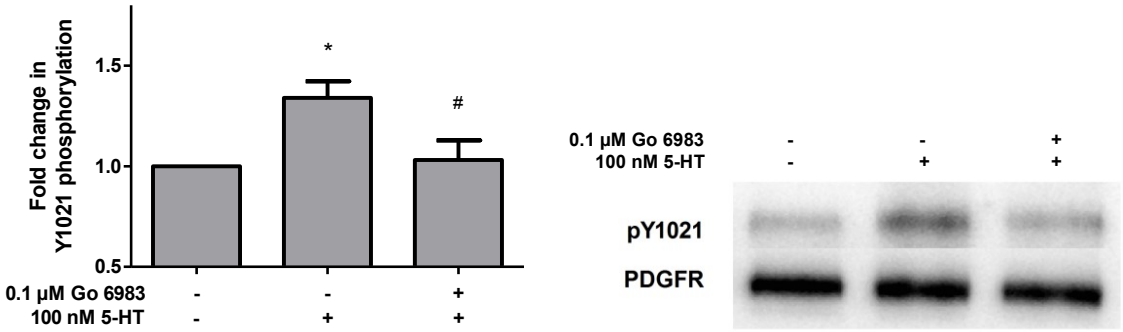


Figure 3.31. PDGFβ receptor transactivation is sensitive to PKC inhibition.
SH-SY5Y cell cultures were pretreated with vehicle or 0.1 µM of the PKC inhibitor Go 6983 for 45 min followed by treatment with vehicle or 100 nM 5-HT for 5 min. Following drug treatments, cell lysates were evaluated by immunoblot analysis as described in Materials and methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 3$).

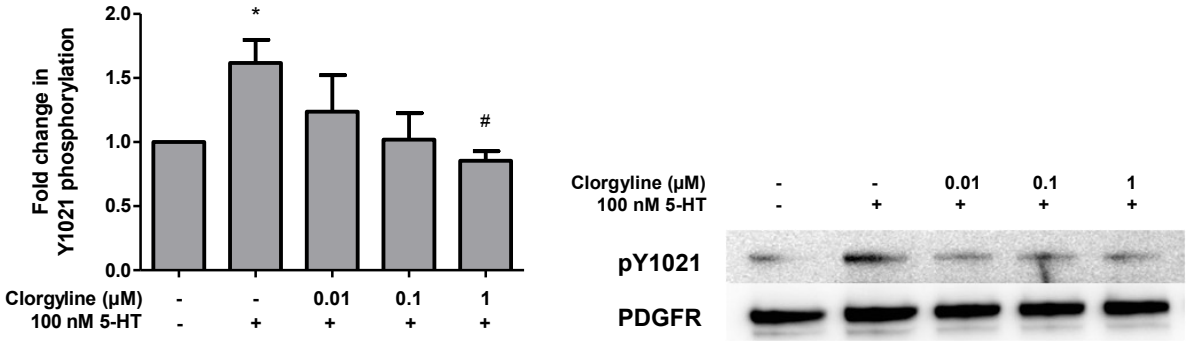


Figure 3.32. PDGFβ receptor transactivation is blocked by clorgyline.
SH-SY5Y cells were treated with vehicle, 100 nM 5-HT for 5 min, with or without a 45 min pretreatment of 0.01, 0.1, or 1 µM clorgyline. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 3$).

3.1.8 5-HT-induced PDGFβ receptor transactivation involves Src-family kinases

Src kinases are known effectors of PDGF receptors [47, 49] as well as GPCRs [161, 162]; however, their role in GPCR-mediated transactivation pathways is still not fully understood. In SH-SY5Y cells, 5-HT application led to activation of Src family kinases as measured by an increase in phosphorylation at Y418 (Figure 3.33), the tyrosine residue associated with kinase activation [163]. The outcome of 5-HT application on Y529, a site attributed to Src deactivation, was also investigated; however, 5-HT did not have any effect on the phosphorylation status of this residue (data not shown). To investigate whether Src family kinases are required for PDGFβ receptor transactivation, cells were incubated with the Src family kinase inhibitor PP2. PP2 (1 μM) attenuated PDGFβ receptor transactivation by 5-HT (Figure 3.34) and 5-CT (Figure 3.35). As controls for PP2, the inactive analog PP3 was tested and did not have any effect on 5-HT-induced PDGFβ receptor transactivation (Figure 3.36), and PP2 was also able to block 5-HT-induced Src phosphorylation at Y418 (Figure 3.37). In addition to blocking PDGFβ receptor phosphorylation, 1 μM of the PLC inhibitor U73122 (Figure 3.38), and 10 μM of the intracellular calcium chelator BAPTA-AM (Figure 3.39), but not the extracellular calcium chelator EGTA (Figure 3.40), attenuated Src phosphorylation at Y418, placing Src activation downstream of calcium signaling in this transactivation pathway. To determine the effect of ROS and NADPH oxidase inhibition on Src phosphorylation, the ROS scavenger *N*-acetyl-L-cysteine and the NADPH oxidase inhibitor apocynin were employed. Neither of these drugs had any effect on Src phosphorylation at Y418 at the concentrations that affected PDGFβ receptor transactivation (Figure 3.41, Figure 3.42), suggesting that Src activity occurs upstream of NAPDH oxidase activation and ROS production (see Figure 3.70).

3.1.9 Effects of PDGF receptor kinase inhibition

The PDGFβ receptor kinase inhibitor AG 1296 blocked PDGF-BB-induced PDGFβ receptor activation (Figure 3.43), as did the dual PDGFβ receptor/Abl kinase inhibitor imatinib (data not shown). These drugs also abrogated 5-HT-induced PDGFβ receptor phosphorylation at Y1021 (Figure 3.44 and Figure 3.45). However, AG 1296 did not prevent Src phosphorylation at Y418 (Figure 3.46), further suggesting that Src activity is a required upstream component for 5-HT-induced PDGFβ receptor transactivation rather than activated by PDGFβ receptor itself in this instance.

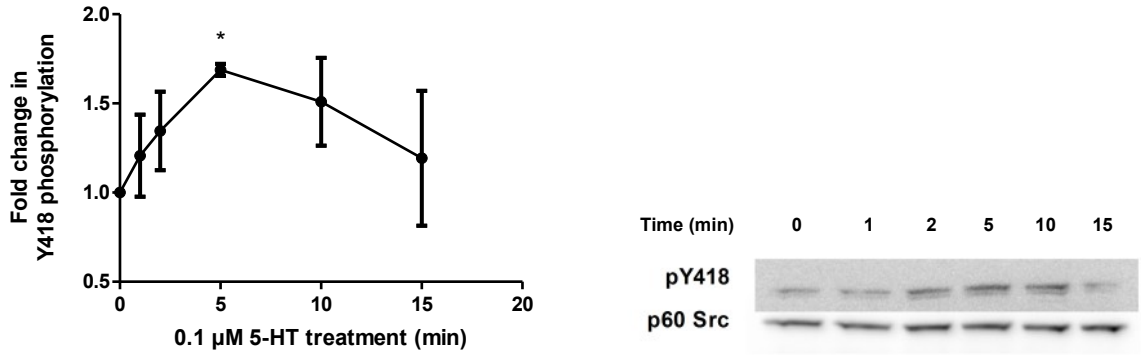


Figure 3.33. 5-HT induces transient Src phosphorylation at Y418.
SH-SY5Y cells were treated with 100 nM 5-HT for 0, 1, 2, 5, 10, or 15 min. Proteins were separated and resolved by SDS-PAGE, transferred to a membrane and probed for p60 Src and phospho-Src Y418. Data were normalized to total p60 Src protein expression and are expressed as the fold change in pY418 immunoreactivity compared to vehicle-treated cells (* = $p<0.05$ compared to vehicle-treated cells; $n = 3$).

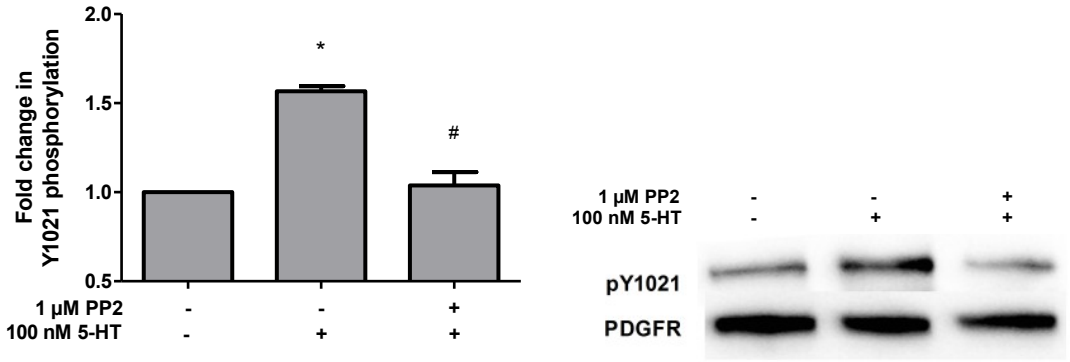


Figure 3.34. 5-HT-induced PDGFβ receptor transactivation is blocked by Src inhibition.
SH-SY5Y cells were treated with vehicle, 100 nM 5-HT for 5 min, with or without pretreatment with 1 µM PP2. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 4$).

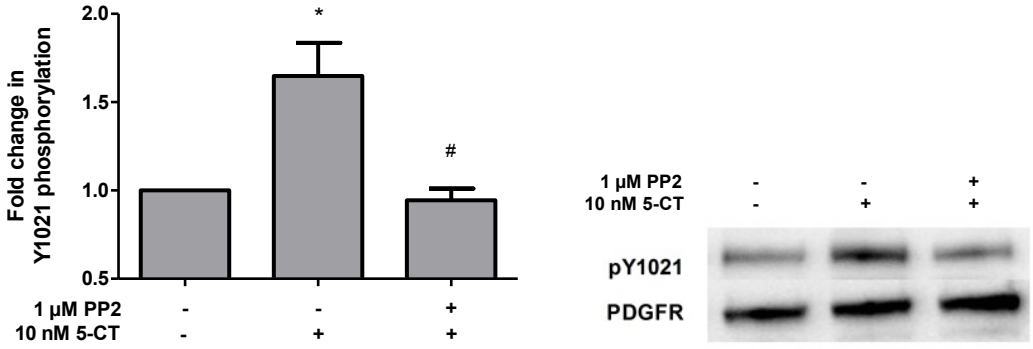


Figure 3.35. 5-CT-induced PDGFβ receptor transactivation is blocked by Src inhibition.
SH-SY5Y cells were treated with vehicle, 10 nM 5-CT for 5 min, with or without pretreatment with 1 µM PP2. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-CT-treated cells; $n = 7$).

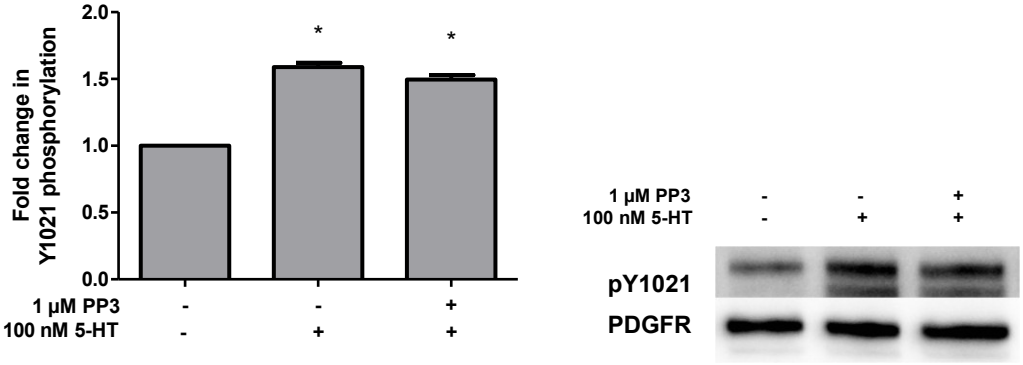


Figure 3.36. 5-HT-induced PDGFβ receptor transactivation is unaffected by the PP2 inactive analog, PP3.
As a control for PP2, SH-SY5Y cells were treated with vehicle, 100 nM 5-HT for 5 min, with or without pretreatment with 1 µM PP3. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 3$).

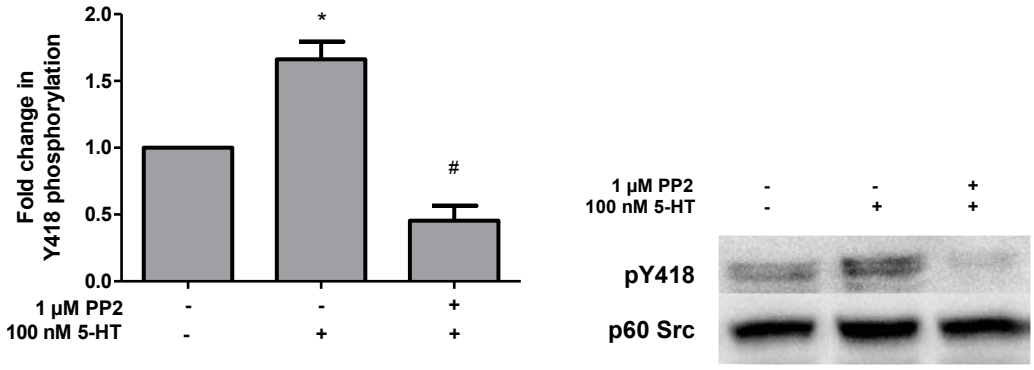


Figure 3.37. PP2 blocks 5-HT-induced Src activation.
SH-SY5Y cells were treated with vehicle, 100 nM 5-HT for 5 min, with or without pretreatment with 1 µM PP2. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total p60 Src protein expression and are expressed as the fold change in phospho-Y418 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-Y418 (pY418) and p60 Src are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 6$).

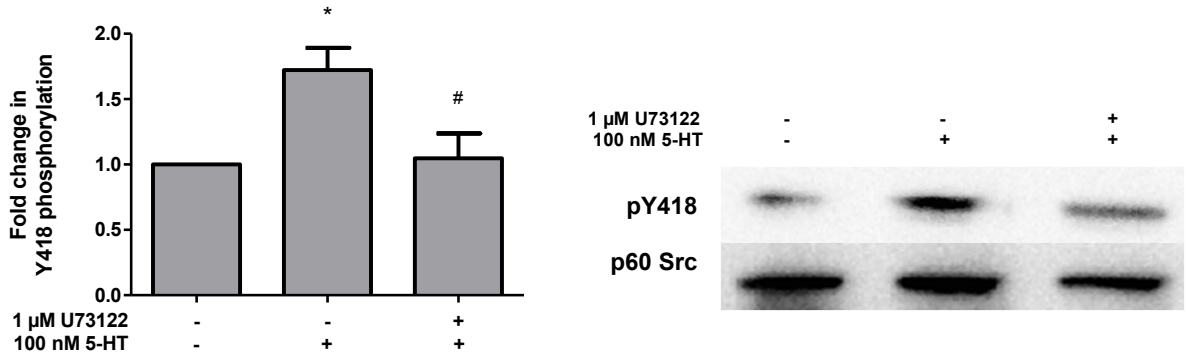


Figure 3.38. The PLC inhibitor U73122 attenuates 5-HT-induced Src activation.
SH-SY5Y cells were treated with vehicle, 100 nM 5-HT for 5 min, with or without pretreatment with 1 µM U73122. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total p60 Src protein expression and are expressed as the fold change in phospho-Y418 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-Y418 (pY418) and p60 Src are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 3$).

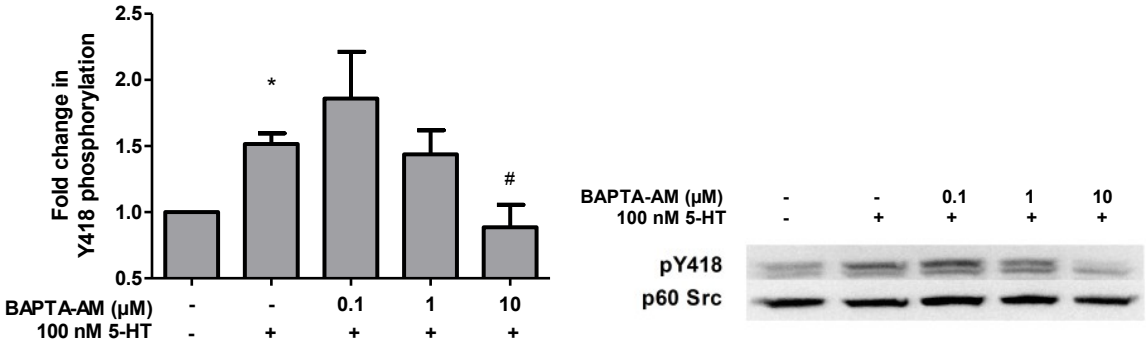


Figure 3.39. 5-HT-induced Src activation is sensitive to BAPTA-AM.
SH-SY5Y cells were treated with vehicle, 100 nM 5-HT for 5 min, with or without 10 min pretreatment with 0.1 to 10 μM BAPTA-AM. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total p60 Src protein expression and are expressed as the fold change in phospho-Y418 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-Y418 (pY418) and p60 Src are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 5$).

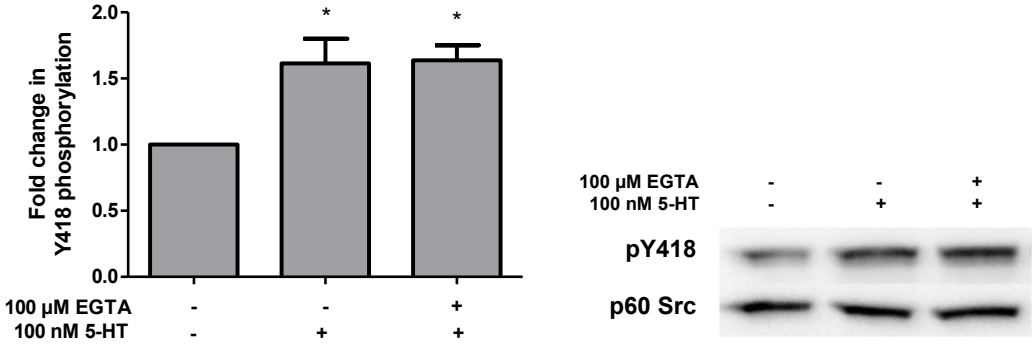


Figure 3.40. EGTA does not block 5-HT-induced Src activation.
SH-SY5Y cells were treated with vehicle, 100 nM 5-HT for 5 min, with or without a 10 min pretreatment of 100 μM EGTA. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total p60 Src protein expression and are expressed as the fold change in phospho-Y418 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-Y418 (pY418) and p60 Src are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 4$).

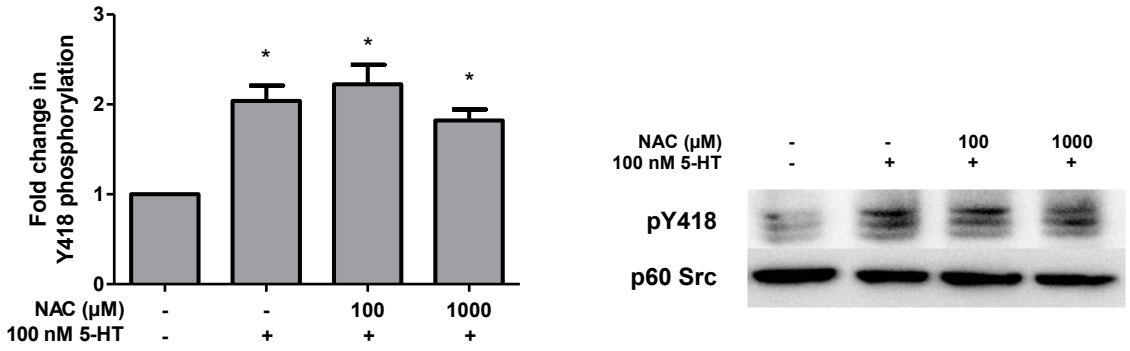


Figure 3.41. *N*-acetyl-L-cysteine does not block 5-HT-induced Src activation.
SH-SY5Y cells were treated with vehicle, 100 nM 5-HT for 5 min, with or without a 45 min pretreatment of 100 or 1000 μM *N*-acetyl-L-cysteine (NAC). Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total p60 Src protein expression and are expressed as the fold change in phospho-Y418 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-Y418 (pY418) and p60 Src are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 3$).

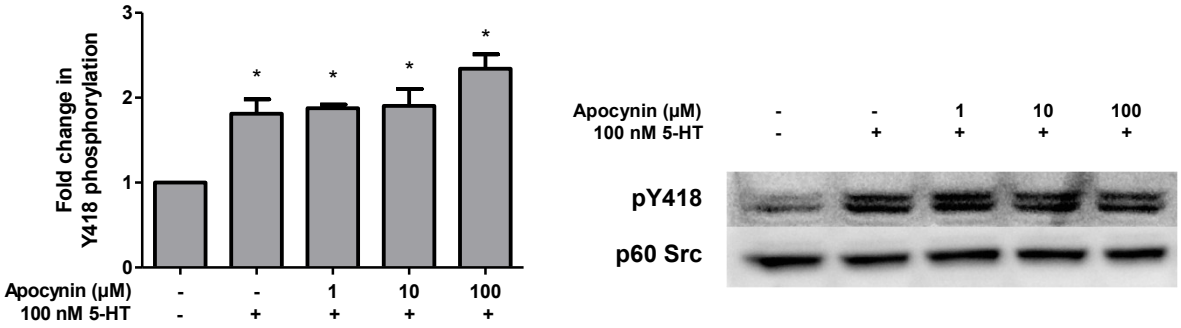


Figure 3.42. Apocynin does not block 5-HT-induced Src activation.
SH-SY5Y cells were treated with vehicle, 100 nM 5-HT for 5 min, with or without a 45 min pretreatment of 1, 10 or 100 μM apocynin, an NADPH oxidase inhibitor. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total p60 Src protein expression and are expressed as the fold change in phospho-Y418 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-Y418 (pY418) and p60 Src are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 3$).

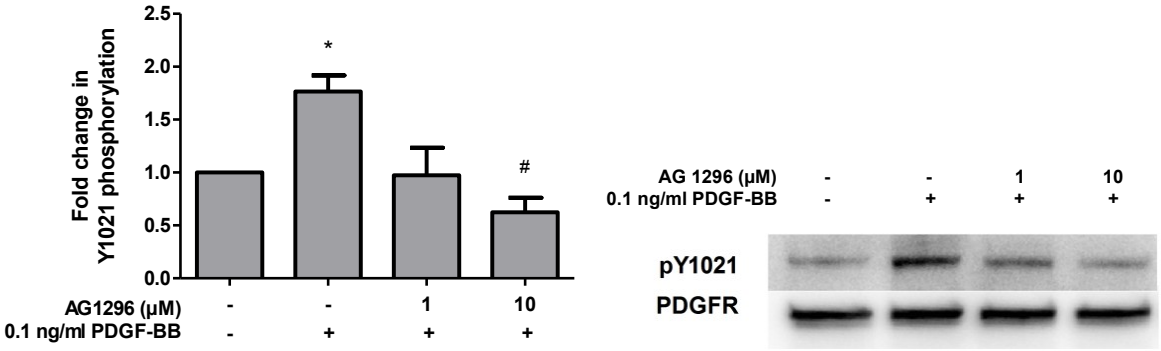


Figure 3.43. PDGFβ receptor activation by PDGF-BB is sensitive to AG 1296.
SH-SY5Y cells were treated for 5 min with vehicle, 0.1 ng/ml PDGF-BB, with or without 1 or 10 μM AG 1296. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with PDGF-BB-treated cells; $n = 4$).

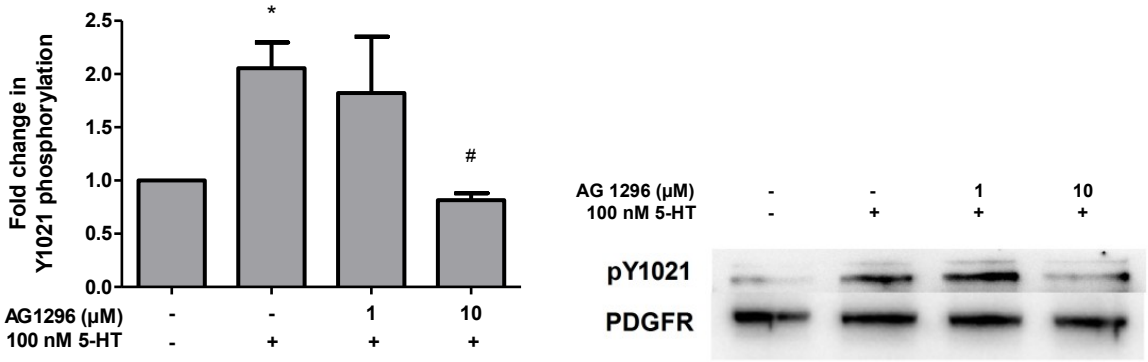


Figure 3.44. PDGFβ receptor transactivation is sensitive to AG 1296.
SH-SY5Y cells were treated for 5 min with vehicle, 100 nM 5-HT, with or without 1 or 10 μM AG 1296. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 3$).

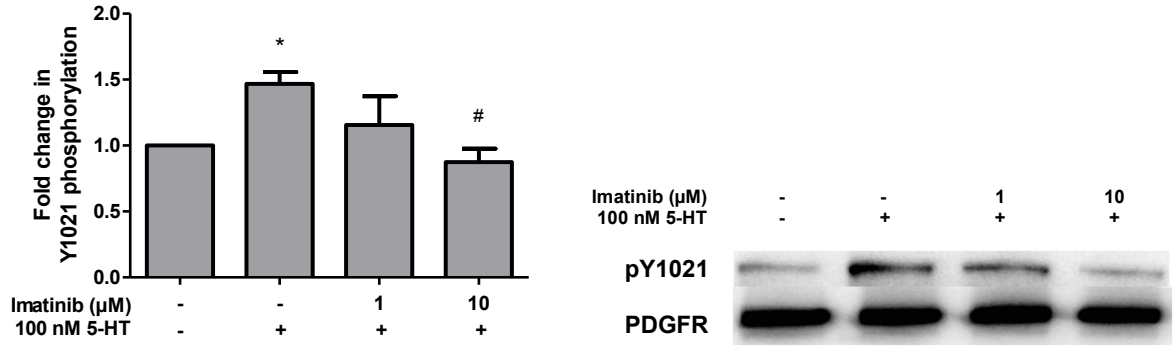


Figure 3.45. PDGFβ receptor transactivation is sensitive to imatinib.
SH-SY5Y cells were treated for 5 min with vehicle, 100 nM 5-HT, with or without 1 or 10 μM imatinib. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 4$).

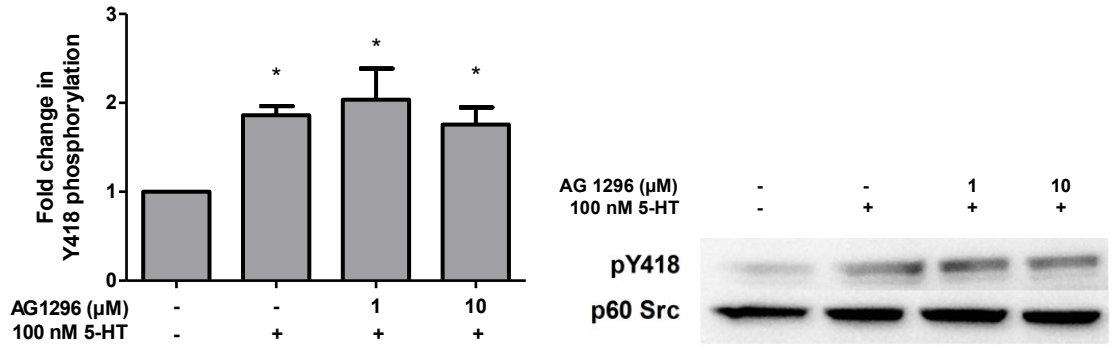


Figure 3.46. 5-HT-induced Src activation is unaffected by PDGF receptor inhibition.
SH-SY5Y cells were treated with vehicle, 100 nM 5-HT for 5 min, with or without pretreatment with 1 or 10 μM AG 1296. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total p60 Src protein expression and are expressed as the fold change in phospho-Y418 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-Y418 (pY418) and p60 Src are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 4$).

3.1.10 5-HT-induced ERK1/2 phosphorylation is not PDGFβ receptor-dependent

ERK1/2 are kinases that promote survival and proliferation [68], and can be acutely activated by both 5-HT and PDGFβ receptor signaling pathways [47, 68]. Originally used as a positive control for 5-HT activity, an analysis of ERK1/2 phosphorylation within the transactivation pathway yielded surprising results. Oak *et al.* [68] demonstrated that D2-class dopamine receptor-induced ERK1/2 phosphorylation *required* transactivated PDGFβ receptors [68]. However, this differs from the results of the present study. In SH-SY5Y cells, while Ptx was only fully blocked 5-HT induced PDGFβ receptor phosphorylation at concentrations greater than 0.1 μg/mL (see Figure 3.15), Ptx blocked 5-HT-induced ERK1/2 phosphorylation at a 100-fold lower concentration (0.001 μg/mL, Figure 3.47). Furthermore, none of 5-CT (Figure 3.48), the 5-HT_7 agonist LP 12 (Figure 3.49) or the 5-HT_{1A} receptor agonist DPAT (data not shown) increased ERK1/2 phosphorylation at any concentration tested despite being able to transactivate the PDGFβ receptor. These results suggest that a 5-HT receptor activated by 5-HT, but not 5-CT, LP 12, or DPAT (i.e. 5-HT receptor subtypes 2-4, or 6), was responsible for ERK1/2 phosphorylation and that there are distinct pathways initiated by 5-HT treatment which lead to PDGFβ receptor and ERK1/2 phosphorylation. Interestingly, in addition to blocking ERK1/2 activation by dopamine and 5-HT (see Figure 3.13 and Figure 3.47), Ptx also partially inhibited PDGF-BB-induced ERK1/2 activation (Figure 3.50).

Downstream of 5-HT receptors, 5-HT-induced ERK1/2 activation was blocked by the PLC inhibitor U73122 (Figure 3.51) and the membrane-permeable calcium chelator BAPTA-AM (Figure 3.52), but not the extracellular chelator EGTA (Figure 3.53). The PKC inhibitor Go 6983 and the Src kinase inhibitor PP2 also attenuated 5-HT-induced ERK1/2 phosphorylation (Figure 3.54 and Figure 3.55). Since these inhibitors blocked ERK1/2 phosphorylation, its activation must occur downstream of calcium signaling, PLC, PKC, and Src activation.

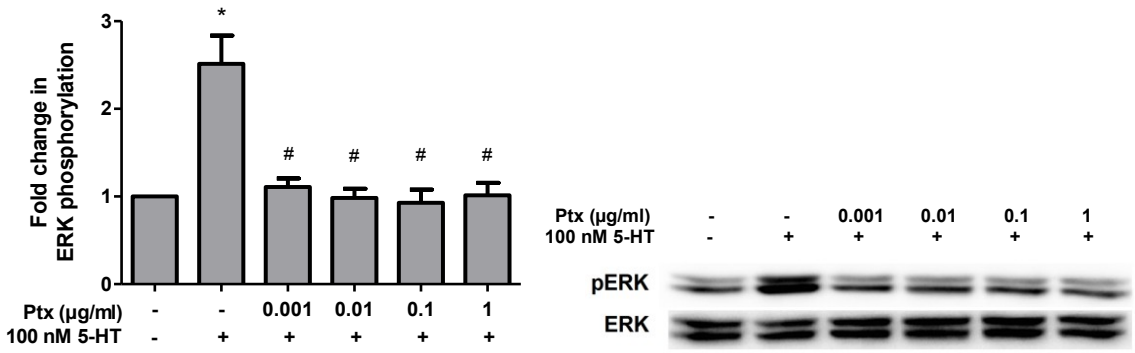


Figure 3.47. Pertussis toxin inhibits 5-HT-induced ERK1/2 phosphorylation.
SH-SY5Y cells were incubated overnight with 0.001 to 1 µg/mL pertussis toxin (Ptx) followed by 5 min treatment with 100 nM 5-HT. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in combined phospho-ERK1/2 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and ERK1/2 are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 5$).

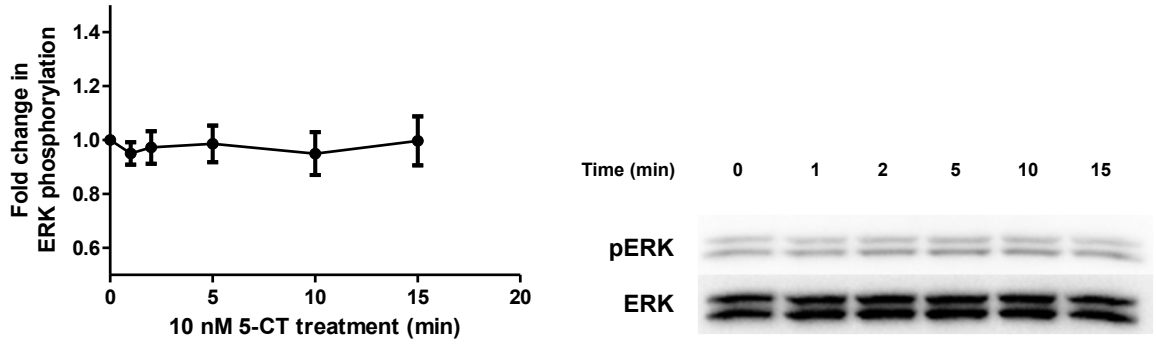


Figure 3.48. 5-CT does not induce ERK1/2 phosphorylation.
SH-SY5Y cells were treated with 10 nM 5-CT for 0, 1, 2, 5, 10, or 15 min. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in combined phospho-ERK1/2 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and ERK1/2 are shown ($n = 6$).

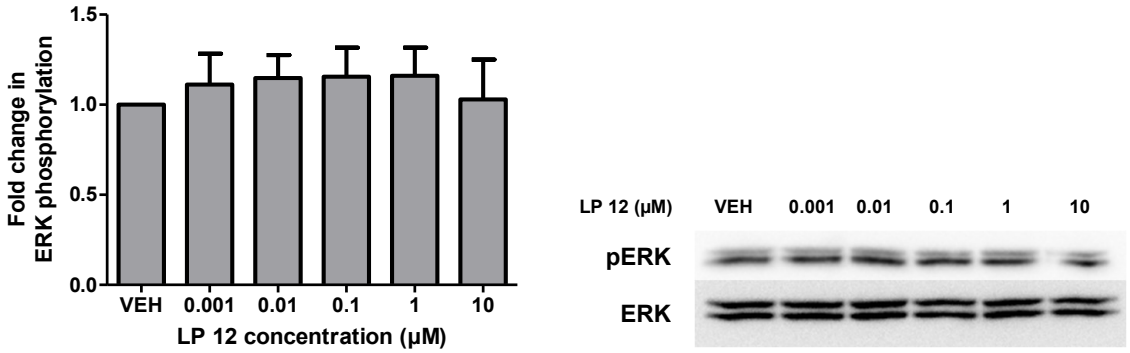


Figure 3.49. LP 12 does not induce ERK1/2 phosphorylation.
SH-SY5Y cells were treated with 0, 0.001, 0.01, 0.1, 1, 10 μM LP 12 for 5 min. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in combined phospho-ERK1/2 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and ERK1/2 are shown (n = 6).

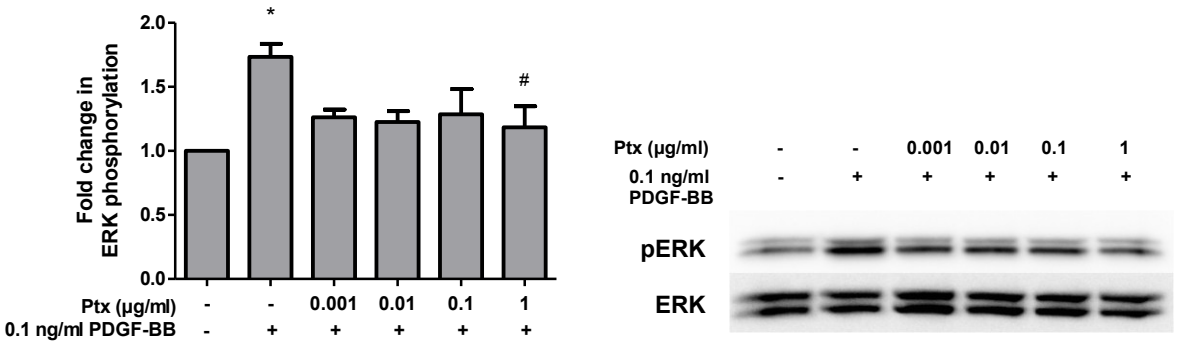


Figure 3.50. Pertussis toxin inhibits PDGF-BB-induced ERK1/2 phosphorylation.
SH-SY5Y cells were incubated overnight with 0.001 to 1 µg/mL pertussis toxin (Ptx) followed by 5 min treatment with 0.1 ng/ml PDGF-BB. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in combined phospho-ERK1/2 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and ERK1/2 are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with PDGF-BB-treated cells; $n = 4$).

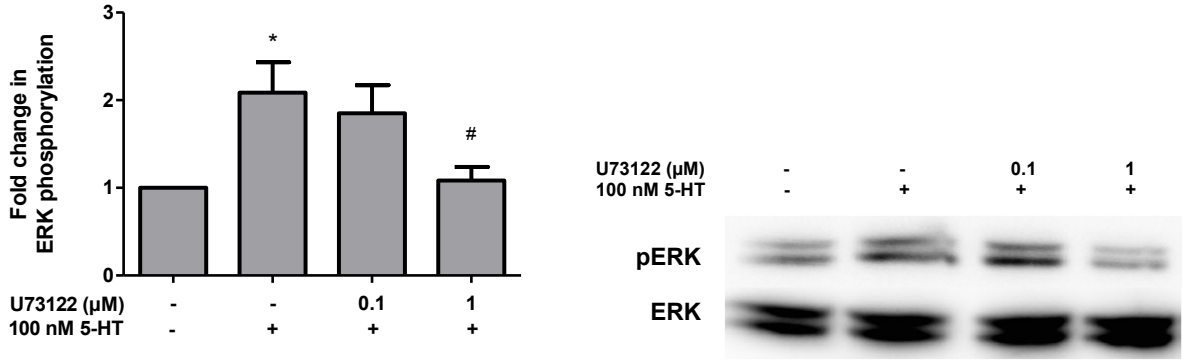


Figure 3.51. ERK1/2 phosphorylation is abrogated by PLC inhibition.
SH-SY5Y cells were treated with vehicle, 100 nM 5-HT for 5 min, with or without pretreatment with 0.1 or 1 µM U73122. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in phospho-ERK1/2 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and ERK1/2 are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 7$).

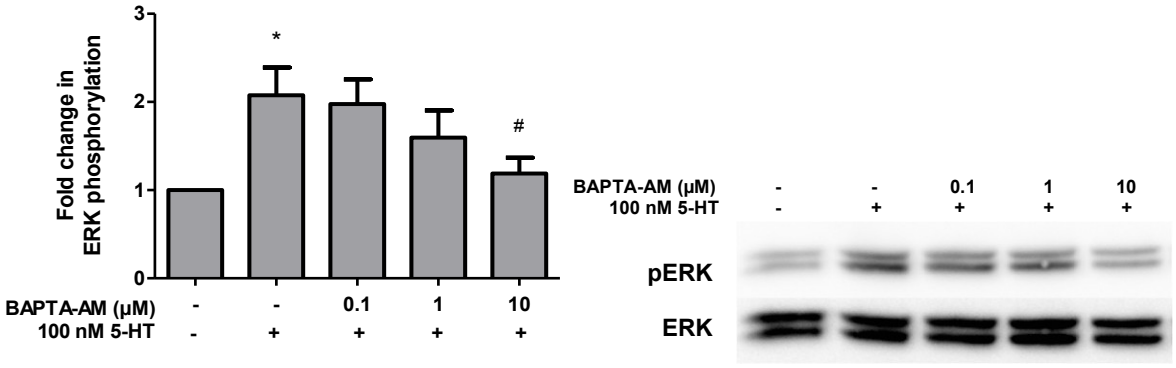


Figure 3.52. BAPTA-AM blocks 5-HT-induced ERK1/2 activation.
SH-SY5Y cells were treated with vehicle, 100 nM 5-HT for 5 min, with or without a 10 min pretreatment of 0.1, 1, or 10 μM BAPTA-AM. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in phospho-ERK1/2 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and ERK1/2 are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 4$).

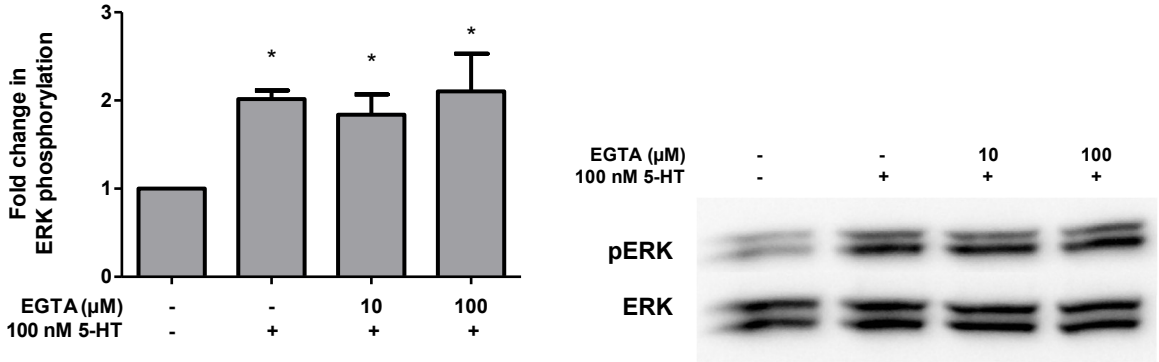


Figure 3.53. EGTA does not block 5-HT-induced ERK1/2 activation.
SH-SY5Y cells were treated with vehicle, 100 nM 5-HT for 5 min, with or without a 10 min pretreatment of 10 or 100 μM EGTA. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in phospho-ERK1/2 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and ERK1/2 are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 5$).

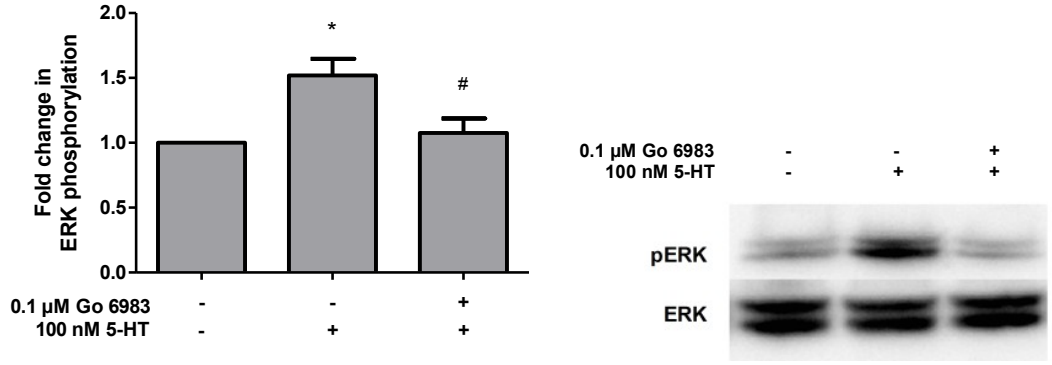


Figure 3.54. 5-HT-induced ERK1/2 phosphorylation is sensitive to PKC inhibition.
SH-SY5Y cells were pretreated with vehicle or 0.1 µM of the PKC inhibitor Go 6983 for 45 min followed by treatment with vehicle or 100 nM 5-HT for 5 min. Following drug treatments, cell lysates were evaluated by Western blot analysis as described in Materials and methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in phospho-ERK immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and total ERK1/2 are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 4$).

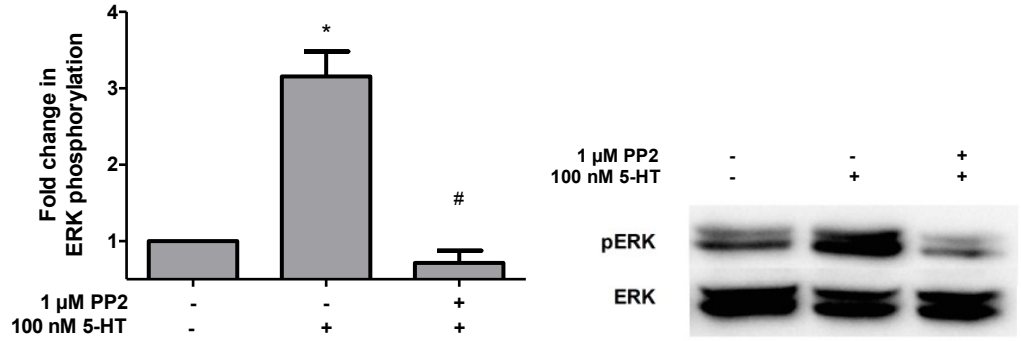


Figure 3.55. ERK1/2 phosphorylation is abrogated by Src inhibition.
SH-SY5Y cells were treated with vehicle, 100 nM 5-HT for 5 min, with or without pretreatment with 1 µM PP2. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in phospho-ERK1/2 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and ERK1/2 are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 3$).

Given the results described above, it was subsequently determined whether 5-HT-induced ERK1/2 phosphorylation involved ROS and NADPH oxidase in a manner similar to PDGFβ receptor transactivation. However, when SH-SY5Y cells were treated with H_2O_2, no significant increase in ERK1/2 phosphorylation was observed (Figure 3.56), suggesting a divergence point in ERK1/2 and PDGFβ receptor signaling prior to ROS production. H_2O_2 treatment also failed to induce ERK1/2 phosphorylation in primary cortical neurons (data not shown). Furthermore, in contrast to its ability to block 5-HT-induced PDGFβ receptor phosphorylation, pretreatment with *N*-acetyl-L-cysteine had no effect on 5-HT-induced ERK1/2 phosphorylation (Figure 3.57). However, the NADPH oxidase inhibitors diphenyleneiodonium chloride and apocynin both blocked 5-HT-induced ERK1/2 phosphorylation (Figure 3.58 and Figure 3.59). This suggests that the divergence point in signaling pathways for 5-HT-induced ERK1/2 and PDGFβ receptor phosphorylation occurs after PKC and at the point of NADPH oxidase, prior to ROS production.

Further corroborating this evidence is the inability of the PDGF receptor kinase inhibitor AG 1296 or the PDGF receptor/Abl kinase inhibitor imatinib to block 5-HT-induced ERK1/2 activation (Figure 3.60 and Figure 3.61), indicating that the modulation of ERK1/2 activity by 5-HT is not PDGFβ receptor-dependent in this system.

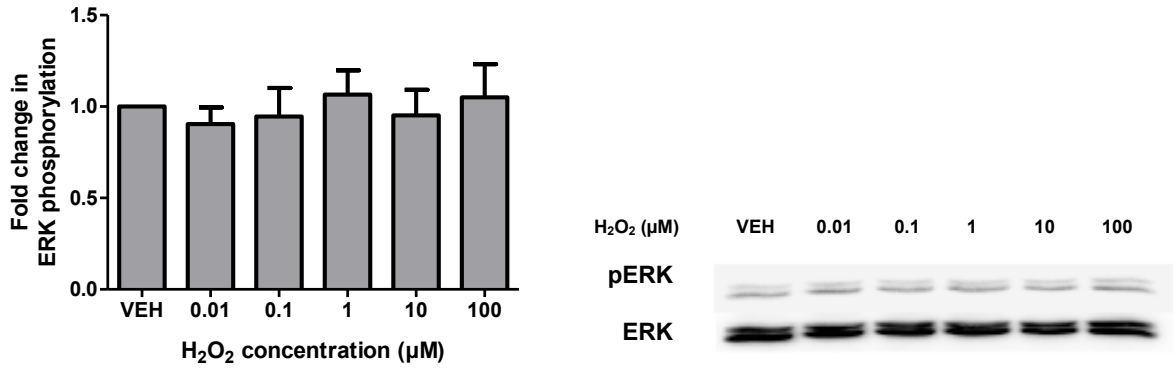


Figure 3.56. H_2O_2 does not induce ERK1/2 phosphorylation in SH-SY5Y cells.
SH-SY5Y cells were treated with 0.01 to 100 μM H_2O_2 for 5 min. Following drug treatments, cell lysates were evaluated by Western blot analysis as described in Materials and methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in phospho-ERK immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and total ERK1/2 are shown. Representative blots for phospho-ERK1/2 (pERK) and ERK1/2 are shown (*n* = 5).

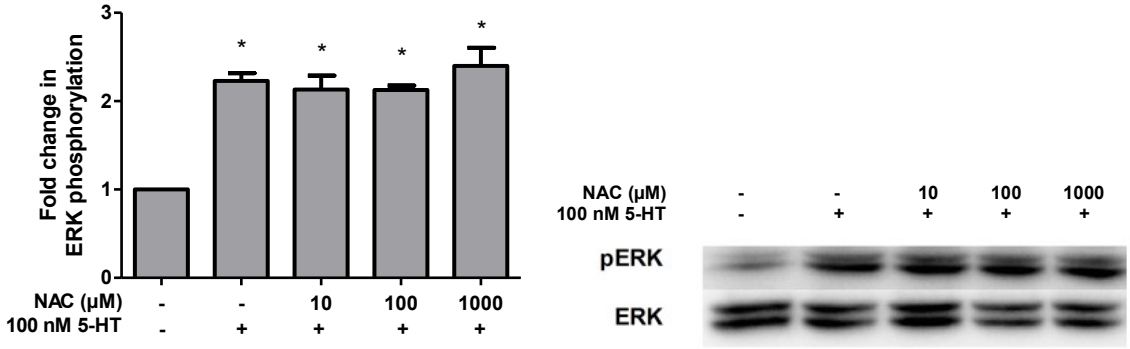


Figure 3.57. Scavenging ROS has no effect on 5-HT-induced ERK1/2 phosphorylation.
SH-SY5Y cells were pretreated with vehicle or 10, 100 or 1000 μM of the ROS scavenger *N*-acetyl-L-cysteine (NAC) for 45 min followed by treatment with vehicle or 100 nM 5-HT for 5 min. Following drug treatments, cell lysates were evaluated by Western blot analysis as described in Materials and methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in phospho-ERK immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and total ERK1/2 are shown (* = $p<0.05$ compared to vehicle-treated cells; *n* = 5).

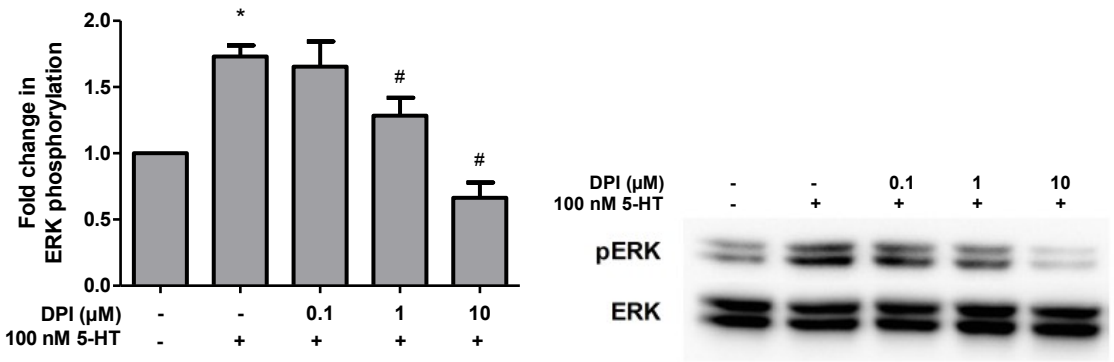


Figure 3.58. Diphenyleneiodonium chloride inhibits 5-HT-induced ERK1/2 phosphorylation.
SH-SY5Y cells were pretreated with vehicle or 0.1, 1 or 10 μM of the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI) for 45 min followed by treatment with vehicle or 100 nM 5-HT for 5 min. Following drug treatments, cell lysates were evaluated by Western blot analysis as described in Materials and methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in phospho-ERK immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and total ERK1/2 are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 4$).

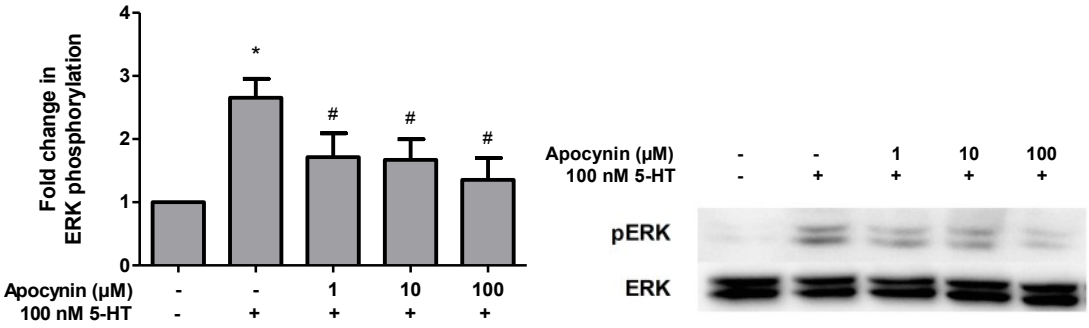


Figure 3.59. Apocynin inhibits 5-HT-induced ERK1/2 phosphorylation.
SH-SY5Y cells were pretreated with vehicle or 1, 10, or 100 μM of the NADPH oxidase inhibitor apocynin for 45 min followed by treatment with vehicle or 100 nM 5-HT for 5 min. Following drug treatments, cell lysates were evaluated by Western blot analysis as described in Materials and methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in phospho-ERK immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and total ERK1/2 are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 7$).

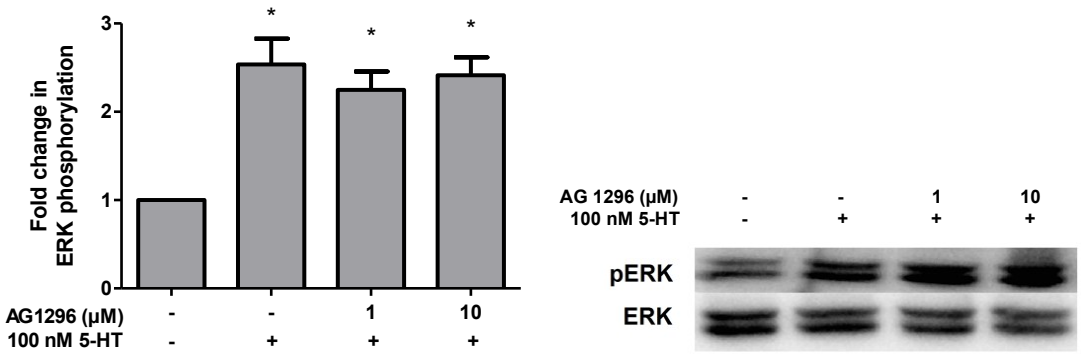


Figure 3.60. 5-HT-mediated ERK1/2 phosphorylation is unaffected by AG 1296.
SH-SY5Y cells were treated with vehicle, 100 nM 5-HT for 5 min, with or without pretreatment with 1 or 10 μM AG 1296. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in phospho-ERK1/2 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and ERK1/2 are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 5$).

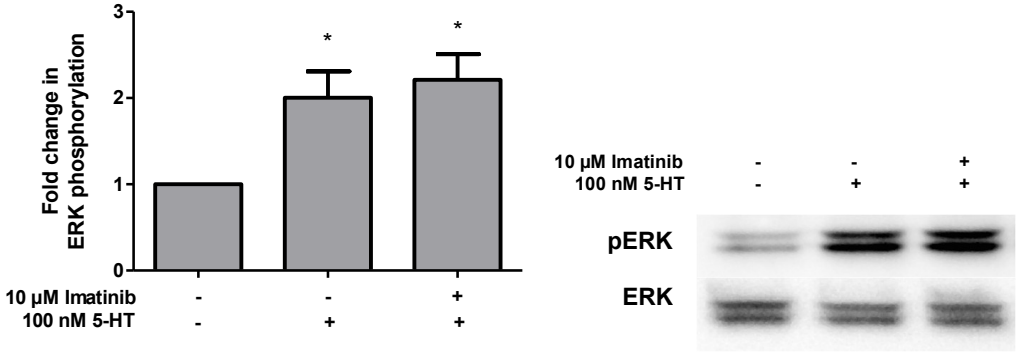


Figure 3.61. 5-HT-mediated ERK1/2 phosphorylation is unaffected by imatinib.
SH-SY5Y cells were treated with vehicle, 100 nM 5-HT for 5 min, with or without pretreatment with10 μM imatinib. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in phospho-ERK1/2 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and ERK1/2 are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 6$).

3.1.11 5-HT can transactivate TrkB receptors via ROS

In addition to PDGF receptors, activation of 5-HT receptors was found to trigger transactivation of fibroblast growth factor and epidermal growth factor receptors [79, 164, 165]. However, it was unknown if 5-HT could transactivate TrkB receptors in neurons (or in any cell type), and whether the enzymes implicated in PDGF receptor transactivation are also involved in TrkB receptor transactivation. Indeed, like PDGFβ receptor, 5-HT induced a transient phosphorylation of TrkB as measured by the PLCγ binding site Y816 with a maximum phosphorylation occurring at 5 min (Figure 3.62). In addition, TrkB showed a similar H_2O_2 dose-response curve with a maximum transactivating concentration of 0.1 μM after 5 min (Figure 3.63), the same conditions used to assess PDGFβ receptor phosphorylation. Not surprisingly, the ROS scavenger *N*-acetyl-L-cysteine blocked 5-HT-induced TrkB transactivation (Figure 3.64). NADPH oxidase was implicated as the source of ROS once more, given that the NADPH oxidase inhibitor diphenyleneiodonium chloride abrogated 5-HT-induced TrkB receptor phosphorylation at 1 and 10 μM (Figure 3.65). Further, like the 5-HT-PDGFβ receptor transactivation pathway (see Figure 3.15), 0.1 μg/ml pertussis toxin blocked 5-HT-induced TrkB phosphorylation (Figure 3.66), suggesting that a $G\alpha_i$-coupled 5-HT receptor was also responsible for initiating these pathways. Finally, although the PDGF receptor kinase inhibitor AG 1296 blocked PDGF receptor transactivation by 5-HT, it did not block TrkB transactivation (Figure 3.67), suggesting that TrkB transactivation was not dependent on prior PDGF receptor activity.

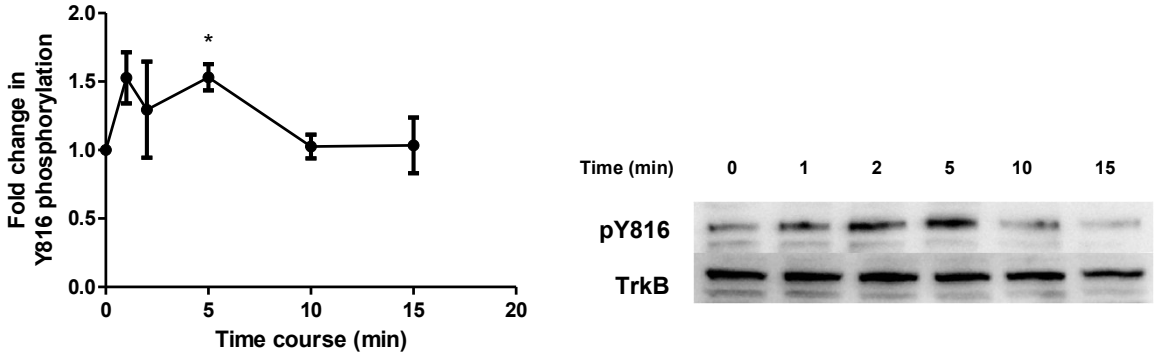


Figure 3.62. 5-HT can transactivate TrkB receptors in SH-SY5Y cells.
SH-SY5Y cells were treated with 0.1 µM 5-HT for 0, 1, 2, 5, 10, 15 min. Following drug treatments, cell lysates were evaluated by Western blot analysis as described in Materials and methods. Data were normalized to total TrkB protein expression and are expressed as the fold change in TrkB phospho-816 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-TrkB Y816 (pY816) and TrkB are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 5$).

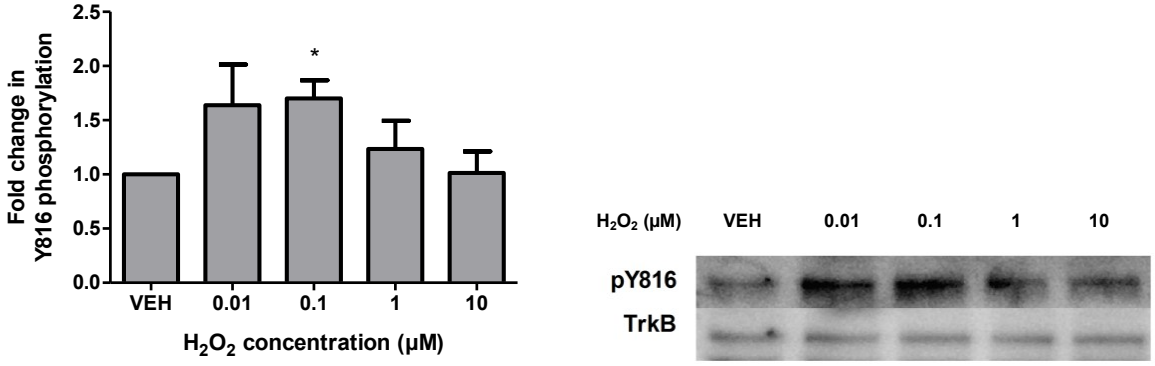


Figure 3.63. H_2O_2 can transactivate TrkB receptors.
SH-SY5Y cells were treated with vehicle (VEH) or 0.01 to 10 µM H_2O_2 for 5 min. Following drug treatments, cell lysates were evaluated by Western blot analysis as described in Materials and methods. Data were normalized to total TrkB protein expression and are expressed as the fold change in TrkB phospho-816 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-TrkB Y816 (pY816) and TrkB are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 6$).

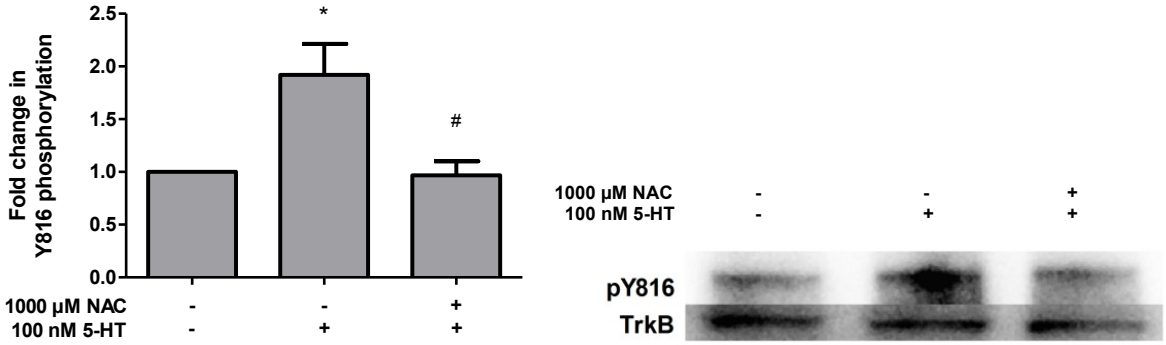


Figure 3.64. ROS mediate 5-HT-induced TrkB phosphorylation.
SH-SY5Y cells were pretreated with vehicle or 1000 µM of the ROS scavenger *N*-acetyl-L-cysteine (NAC) for 45 min followed by treatment with vehicle or 100 nM 5-HT for 5 min. Following drug treatments, cell lysates were evaluated by Western blot analysis as described in Materials and methods. Data were normalized to total TrkB protein expression and are expressed as the fold change in TrkB phospho-816 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-TrkB Y816 (pY816) and TrkB are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 5$).

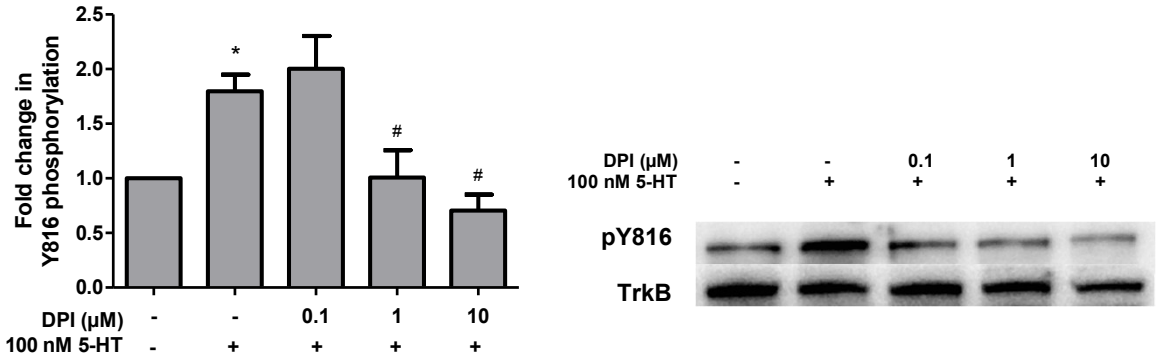


Figure 3.65. NADPH oxidase is implicated in 5-HT-induced TrkB transactivation.
SH-SY5Y cells were pretreated with vehicle or 0.1, 1, or 10 µM of the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI) for 45 min followed by treatment with vehicle or 100 nM 5-HT for 5 min. Following drug treatments, cell lysates were evaluated by Western blot analysis as described in Materials and methods. Data were normalized to total TrkB protein expression and are expressed as the fold change in TrkB phospho-816 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-TrkB Y816 (pY816) and TrkB are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 4$).

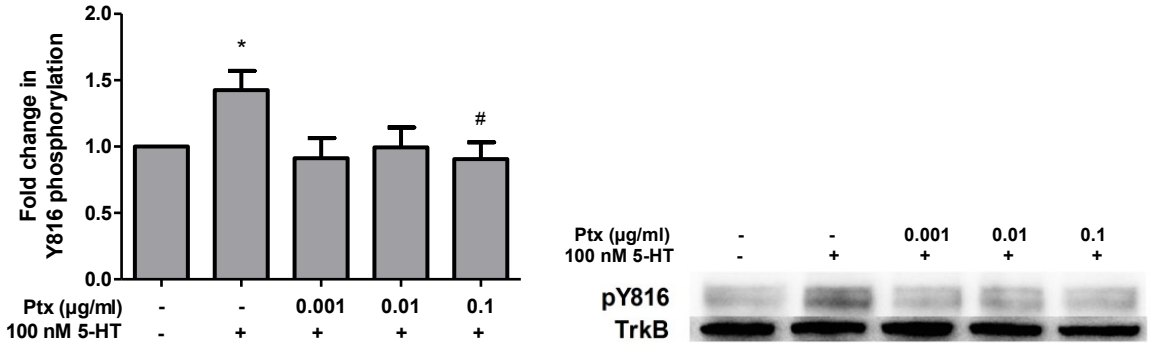


Figure 3.66. 5-HT-induced TrkB phosphorylation is sensitive to pertussis toxin.
SH-SY5Y cells pretreated were incubated overnight with 0.001 to 0.1 µg/mL pertussis toxin (Ptx) followed by 5 min treatment with 0.1 µM 5-HT. Following drug treatments, cell lysates were evaluated by Western blot analysis as described in Materials and methods. Data were normalized to total TrkB protein expression and are expressed as the fold change in TrkB phospho-816 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-TrkB Y816 (pY816) and TrkB are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 6$).

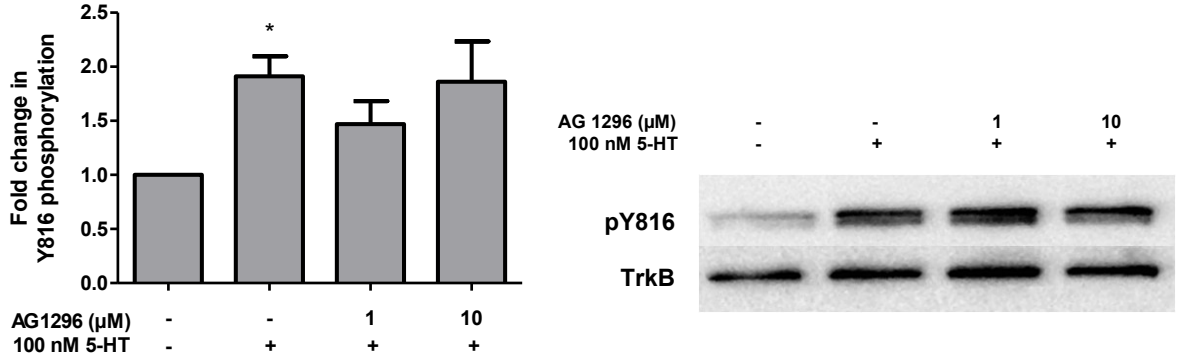


Figure 3.67. 5-HT-induced TrkB phosphorylation is insensitive to the PDGF receptor inhibitor AG 1296.
SH-SY5Y cells pretreated were incubated for 45 min with 0, 1 or 10 µM AG 1296 followed by 5 min treatment with 100 nM 5-HT. Following drug treatments, cell lysates were evaluated by Western blot analysis as described in Materials and methods. Data were normalized to total TrkB protein expression and are expressed as the fold change in TrkB phospho-816 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-TrkB Y816 (pY816) and TrkB are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 5$).

3.2 Discussion

The abovementioned data demonstrate that 5-HT is capable of transactivating PDGFβ receptors via an intracellular signaling pathway involving several enzymes, and this mechanism is not dependent on PDGF-BB. To our knowledge, this is the first study showing transactivation of PDGFβ receptors by 5-HT and 5-HT receptor agonists in neuronal cells, and 5-HT-mediated TrkB transactivation in any cell type.

3.2.1 Direct activation vs. transactivation of PDGFβ receptor

The relative level of 5-HT-induced transactivation of PDGFβ receptors as reflected by changes in phosphorylation differed in several ways compared to direct PDGFβ receptor activation with PDGF-BB ligand. Transactivation of PDGFβ receptors by 5-HT was transient; PDGFβ receptor phosphorylation returned to baseline faster than direct ligand activation, which showed prolonged phosphorylation for longer periods (past 20 min as shown here). In addition, phosphorylation via transactivation was not nearly as robust as compared to direct activation by PDGF-BB ligand. Whereas transactivation increases phosphorylation by 1.5-2 fold, PDGF-BB can increase phosphorylation by 100 fold, likely to the point of receptor/tyrosine phosphorylation saturation (see Figure 3.6 and Figure 3.7). Whether that is physiologically realistic remains to be determined, as a significant amount of PDGF ligand is needed for that level of phosphorylation. It may be more reasonable to suggest that lower concentrations of PDGF-BB such as 0.1 ng/ml are more practical and if so, would result in comparable phosphorylation levels achieved by PDGF-BB-mediated activation and transactivation. Signaling by prolonged direct activation of PDGF receptor ends via internalization and degradation of the receptor [49], whereas transactivated PDGF receptor is not internalized and is presumably rapidly dephosphorylated by the phosphatase SHP-2 [47]. On the other hand, this difference in phosphorylation intensity may be directly related to the physiological responses discussed in later Section 5.2: transactivation of PDGFβ receptor leads to cellular proliferation and in some cases hyperplasia [166, 167], while PDGF-mediated receptor activation can induce further proliferation to the point of fibrosis and tumor formation [48, 50].

3.2.2 Receptors and G proteins initiating transactivation

The ability of pertussis toxin (Ptx) to attenuate 5-HT-induced transactivation of PDGFβ receptors suggests that 5-HT is activating $G\alpha_i$-coupled GPCR(s). This is consistent with several other studies implicating $G\alpha_i$-linked receptors in the transactivation of PDGFβ receptors, including D2-class dopamine receptors [68], lysophosphatidic acid receptors [73], and sphingosine-1-phosphate receptors [72].

Despite this pathway's sensitivity to Ptx, it is intriguing that the PLC inhibitor U73122 and intracellular calcium chelation also blocked 5-HT-induced transactivation of the PDGFβ receptor, as these are signal transduction pathways normally associated with downstream effects of $G\alpha_q$-coupled receptors [168]. Transactivation through $G\alpha_q$-coupled receptors was observed in mouse fibroblasts through the $5\text{-}HT_{2B}$ receptor [79], and was implicated in rat myocytes through the angiotension II receptor type 1 [70]. However, there is precedent for $G\alpha_i$-coupled GPCRs being able to activate PLC [169], possibly via Gβγ subunits [149]. In fact, all five Gβ isoforms can activate at least one PLCβ isoform and increase IP_3 concentrations when complexed with certain (but not all of the twelve) Gγ isoforms [170-172]. Ptx also preferentially inhibits $G\alpha_i$ when it is associated with Gβγ [173]; thus, Gβγ is likely permanently bound to Gα and unable to participate in signaling as well. Therefore, it may indeed be Gβγ subunits that are responsible for PLC activation in transactivation pathways initiated by $G\alpha_i$-coupled receptors.

Notwithstanding these data, the apparent lack of $G\alpha_s$-mediated transactivation examples here and in literature cannot be disregarded. This study showed that the $G\alpha_s$-coupled $5\text{-}HT_7$ receptor agonist LP12 was unable to induce PDGFβ receptor transactivation over a range of concentrations, leading to the conclusion that $5\text{-}HT_7$ receptors do not participate in transactivation signaling. Most other instances of RTK transactivation also indicate that $G\alpha_i$ or $G\alpha_q$-coupled GPCRs initiate transactivation signaling pathways [70, 72, 174]. Thus, it may be the combination of Gα (either $G\alpha_i$ or $G\alpha_q$, and not $G\alpha_s$) and its associated Gβγ isoforms that ultimately determines whether PLC is activated and subsequent transactivation occurs.

Further, with respect to PLC signaling, protein kinase C (PKC) activation was shown to be involved in this transactivation pathway given the sensitivity to the PKC inhibitor Go 6983. The data suggest that transactivation of PDGFβ receptor is dependent on calcium ions and PLC activity, which forms diacylglycerol. Thus, it is likely that conventional PKC isoforms (i.e. PKCα, PKCβ, and/or PKCγ) are involved in transactivation signaling.

3.2.3 Src kinase in transactivation

c-Src is a member of the Src family of non-receptor tyrosine kinases, and was associated with cell proliferation, differentiation and motility [175]. It is activated by several mechanisms including GPCRs: increased c-Src activity was observed or implicated upon 5-HT [61, 79], dopamine [68], sphingosine-1-phosphate [72], and β-adrenergic [174] receptor activation. Other Src family tyrosine kinases are activated by 5-HT as well, including c-Yes and Fyn, but inhibition of these kinases did not affect previous examples of PDGF receptor transactivation [79].

It is clear that c-Src is involved in PDGF receptor transactivation; however, its placement in the signaling cascade has been widely debated in literature. Some studies including the present study situate c-Src before ROS production [152] due to the ability of PP2 to inhibit ERK1/2 phosphorylation and the ROS scavenger *N*-acetyl-L-cysteine being unable to inhibit Src phosphorylation, while some studies set it after ROS production [71]. An interesting idea from Catarzi *et al.* suggests that perhaps c-Src is playing a dual role [152]: acute GPCR activation leads to c-Src activation, followed by ROS production [152]. ROS production then sustains c-Src activity [152], which then may act as an effector of ROS signaling. It is also interesting to note that c-Src binds to PDGF-activated PDGF receptor and signals downstream from it [49], further illustrating the versatile role of c-Src in cellular signaling.

3.2.4 ROS is a signaling mediator in RTK transactivation in this system

There are similarities in the pathways reported for both 5-HT- and ROS-induced transactivation of TrkB and PDGFβ receptor. In both 5-HT- and ROS-mediated signaling pathways, the phosphorylation of these RTKs follows a similar dose response, and achieves a similar maximum fold change (1.5-2 fold) compared to baseline. These data, along with the ability of the ROS scavenger *N*-acetyl-L-cysteine to abrogate transactivation, suggest that ROS is a component of 5-HT-initiated transactivation pathways in neurons. This also adds to the growing list of ROS-dependent GPCR-to-RTK transactivation mechanisms including those initiated by angiotensin II, endothelin, and sphingosine-1-phosphate [69, 71, 72], and likely implicates ROS as a component common to transactivation pathways in general.

However, one segment of the transactivation pathway that remains to be fully elucidated is the mechanism of ROS signaling to RTKs. Low levels of ROS have been shown to be a second messenger capable of participating in intracellular signaling pathways [176]. The consensus within the available literature suggests that ROS have the ability to oxidize catalytic cysteine residues in tyrosine phosphatase enzymes, such as the RTK phosphatase SHP-2, and the result of this oxidization is phosphatase inactivation [76, 177]. These phosphatases possess a microenvironment that lowers the pK_a of the catalytic cysteine residue from the usual value of 8.5 to less than 5.5, sufficient for the thiol group to exist as a thiolate ion at physiological pH and become sensitive to H_2O_2-induced oxidation [176]. This phosphatase inactivation is readily reversible and short-lived [76], which may explain why, if phosphatase inactivation is involved in RTK transactivation, PDGF receptor phosphorylation by transactivation is transient instead of sustained (as seen by PDGF ligand-induced phosphorylation). Besides PDGFβ receptor, SHP-2 is known to associate with epidermal growth factor receptor and insulin receptor [178, 179], and it (or other phosphatases) may represent the general mechanism of dephosphorylating transactivated RTKs. Therefore, activation and inactivation of these phosphatases may function as a "molecular switch" in cell signaling [180], temporarily shifting the balance of phosphorylation-dephosphorylation of RTKs in favor of kinase activity.

Since H_2O_2 was implicated in the transactivation pathway of several RTKs, including PDGFβ and TrkB receptors here, it is conceivable that the physiological relevance of ROS in transactivation may ultimately consist of ROS-mediated phosphorylation of *multiple* RTKs via phosphatase inactivation, rather than ROS being involved in one specific GPCR-to-RTK pathway. If so, and given the apparent generalness of the proposed mechanism of ROS-induced phosphotyrosine phosphatase inhibition, the *sum* of multiple small increases to RTK activation would lead to a greater increase in overall cellular RTK activity. The identification of ROS in transactivation pathways may also indicate an endogenous protective mechanism whereby an initial, mild cell stress and production of ROS protects the cell against subsequent, severe insults (and higher, toxic levels of ROS) by first activating the mitogenic effects of multiple RTKs.

3.2.5 The role of NADPH oxidase in transactivation

While the signaling steps downstream of ROS remain to be categorically confirmed, here it is suggested that the upstream component responsible for ROS generation in transactivation pathways is NADPH oxidase. The NADPH oxidase enzyme is a large, multi-subunit complex (Figure 3.68) that

produces superoxide from oxygen and a donated electron from NADPH [157]. Superoxide dismutases then quickly convert superoxide to H_2O_2 [181]. Although often associated with respiratory burst in phagocytes [181], NADPH oxidase is active in non-phagocytic cells, with some subunits replaced with corresponding non-phagocytic homologs [157]. Among these subunits within this complex is Rac1, a member of the Rho GTPases family, which can be activated by both RTKs and GPCRs, and is required for oxidase activity [182, 183]. Two studies have shown that PKC can activate Rac1 [158, 184] possibly leading to assembly of the oxidase, while other studies demonstrated that PKC activates NADPH oxidase components directly: $gp91^{phox}$/NOX2 (to enhance its association with other NADPH oxidase subunits [185]) and $p47^{phox}$ (required for NADPH oxidase activity [186]). In addition, activated c-Src was shown to increase phosphorylation of the NoxA1 activator protein and to increase NOX1 upregulation, another oxidase subunit, leading to increased NOX1-dependent ROS production [187, 188]. Two different NADPH oxidase inhibitors (diphenyleneiodonium chloride and apocynin) using two different mechanisms [154, 189] were able to abrogate PDGFβ receptor transactivation by 5-HT. Whether ROS formation by NADPH oxidase activity occurs intracellularly or extracellularly is still unclear in non-phagocytic cells; however, some studies show NADPH oxidase assembles and functions in the cytoplasm, possibly in a vesicle or endoplasmic reticulum [190, 191], which would result in intracellular ROS accumulation [192-194].

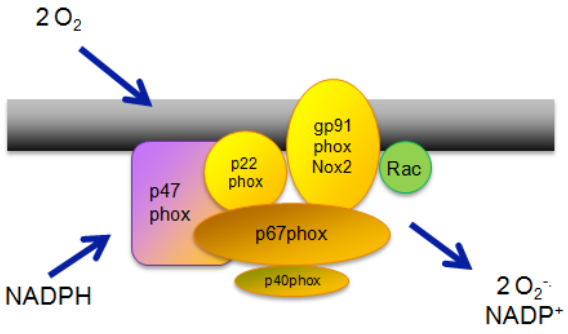


Figure 3.68. The NADPH oxidase complex.
The NADPH oxidase complex is composed of several subunits including the core $p22^{phox}$ and $gp91^{phox}$. Complete assembly is required for oxidase activity, which converts molecular oxygen to superoxide via donated electrons from NADPH. Where NADPH oxidase assembles in non-phagocytes remains to be definitively determined. Adapted from Dusting *et al.*, 2005 [192].

3.2.6 Kinase inhibition abrogates PDGFβ receptor transactivation

Figure 3.44 and Figure 3.45 show that inhibition of PDGF receptor kinase function eliminates receptor transactivation. Both AG 1296 and imatinib function by blocking the ATP binding domain

of the receptor [60, 195]. This is itself a significant result, and suggests that the kinase activity of PDGFβ receptor is responsible for receptor phosphorylation in response to a transactivating stimulus. Had there been no abrogation of 5-HT-induced PDGFβ receptor phosphorylation when these inhibitors were added, it would be reasonable to conclude that a kinase not targeted by these drugs is responsible for the increase in PDGFβ receptor phosphorylation mediated by 5-HT. Interestingly, one study examined the need for PDGF receptor dimerization in transactivation and found that dimerization was not needed for dopamine-mediated PDGF receptor transactivation [196]. If this is also true in the present study, then taken together, these data suggest that the increase in phosphorylation seen in transactivation may simply be an increase in basal phosphorylation mediated by the receptor's own kinase activity in response to a decrease in net phosphatase activity. However, it would be prudent to remain open to the possibility that a yet-unidentified tyrosine kinase that is also a target of these two inhibitors may be responsible for the increase in phosphorylation given that these inhibitors, although relatively specific, do target proteins other than PDGF receptors such as c-kit [197].

3.2.7 The role of ERK1/2 in PDGF receptor transactivation in neuronal cultures

Although the PDGFβ receptor kinase inhibitors AG 1296 and imatinib were able to block 5-HT-induced PDGFβ receptor phosphorylation, they did not block ERK1/2 activation. This led to the initial working hypothesis: since RTKs also signal to ERK1/2, transactivation through ROS production activates multiple RTKs, and these RTKs collectively contribute to activating ERK1/2. Thus, blocking only one RTK (i.e. PDGFβ receptor with AG 1296 or imatinib) would have a negligible effect on overall ERK1/2 activation by RTKs. This would be in line with several findings stating that ERK1/2 phosphorylation was not inhibited by PDGF receptor antagonists when exposed to endothelin in myometrial cells, or when exposed to S1P in smooth muscle or fibroblasts [72, 162, 198].

On the other hand, Oak *et al.* [68] found that dopamine-induced ERK1/2 activation required transactivated PDGFβ receptors [68]. Likewise, endothelin-induced ERK1/2 activation was dependent on PDGFβ receptor activity, as well as on epidermal growth factor (EGF) receptor activation [71]. Therefore, ERK1/2 dependence on PDGF receptor activation may rely partially on the GPCR agonist and partially on the cell types involved – possibly due to a different repertoire of

expressed receptors that mediate these pathways. Further, this study shows that DPAT and 5-CT acting via the $G\alpha_i$-coupled 5-HT_1 (and possibly 5-HT_5) receptors are able to transactivate PDGFβ receptors, yet these drugs do not activate ERK1/2. In light of these data, a hypothesis modification was required, leading to an investigation into the role of ERK1/2 in transactivation events. 5-HT-induced activation of ERK1/2 in SH-SY5Y cells clearly must occur through the activation of additional 5-HT receptor subtypes such as 5-HT_2 receptors [199, 200], and be independent of (or on a parallel pathway to) PDGFβ receptor transactivation in this system.

Further, H_2O_2-induced increases in ERK1/2 phosphorylation were not detected in this study, an observation that contradicts previous work showing that exogenously applied H_2O_2 results in ERK1/2 phosphorylation [201-203]. However, those studies used H_2O_2 concentrations between 0.1 and 2 mM – at least 100-fold higher than the concentrations used here. Lower concentrations may not be sufficient to induce ERK1/2 phosphorylation, possibly because a threshold concentration of ROS needs to accumulate. This is corroborated by the ROS scavenger *N*-acetyl-L-cysteine being able to block TrkB and PDGFβ receptor phosphorylation, but not ERK1/2 phosphorylation induced by 5-HT. Conversely, the NADPH oxidase inhibitors apocynin and diphenyleneiodonium chloride were able to inhibit ERK1/2 activation. These drugs may prevent the assembly of the oxidase or chemically modify the subunits [154, 189], suggesting that the complete, functional oxidase is necessary for PDGFβ receptor transactivation. Since the NADPH oxidase subunit Rac1 was shown to activate MEK and subsequent ERK1/2 activation [158, 204], it is conceivable that these drugs may be inhibiting the activity of subunits such as Rac1 and this prevents both NADPH oxidase function and the separate function (possibly independent of the oxidase) of phosphorylating and activating ERK1/2.

Thus, this study concludes that PDGF receptor transactivation by 5-HT is not a necessary component of ERK1/2 activation in this system. Furthermore, given the lack of ERK1/2 activation by 5-HT receptor agonists that elicit transactivation, ERK1/2 activation may be operating on a parallel mechanistic pathway, with NADPH oxidase components being the last common factor. The differences seen in this and other studies may be the result of different systems used, including cell types, overexpression of certain proteins, and different overall experimental procedures.

3.2.8 The role of TrkB receptors and a possible link to depression

This study has shown for the first time that 5-HT can transactivate TrkB receptors in neuronal cultures. Based on the sensitivity to *N*-acetyl-L-cysteine, this mechanism may also be sensitive to ROS as alluded to previously with PDGFβ receptor transactivation. The notion that 5-HT receptors cross-talk with neurotrophin receptors such as TrkB is of significance as it may provide evidence bridging the two main hypotheses of depression pathology. The monoamine and the neurotrophic hypotheses both state decreased signaling in their respective pathways results in the manifestation of clinical depression [109, 119]. 5-HT activating both serotonergic and neurotrophic signaling pathways would provide a valuable link between these hypotheses: antidepressant-mediated increases in 5-HT neurotransmission normalize deficient signaling pathways by not only activating classical serotonergic signaling transduction but also simultaneously activating neurotrophic signaling via transactivation of TrkB receptors, thus helping to alleviate symptoms in the depressed brain.

Another interesting aspect of neurotrophin signaling in general is the apparent dichotomy of responses. In addition to Trk receptors, neurotrophins also bind the low-affinity neurotrophin receptor (p75) [205]. p75 receptors bind ligand at four extracellular cysteine-rich motifs, and possess both a transmembrane domain and intracellular death domains [82]. Although neurotrophin binding to Trk receptors generally leads to proliferative effects and binding p75 was shown to result in cell survival when associated with Trk receptors, p75 activity is more commonly associated with cell death when segregated from Trk receptors [205-207]. Since certain neurotrophins bind both populations of Trk and p75 receptors [81], there is a risk that exposure to neurotrophins may lead to cell death in the presence of p75. However, with transactivation, the extracellular binding by neurotrophins is bypassed, and only RTKs (including Trk receptors) are transactivated (Figure 3.69). There is no evidence as of yet that p75 receptors, a member of tumor necrosis factor receptor family, can be transactivated via GPCR signaling given their lack of a RTK kinase-like domain [81, 208]. Thus, with transactivation, the proliferative effects of TrkB receptors, and not the apoptotic effects of p75 receptors, would dominate.

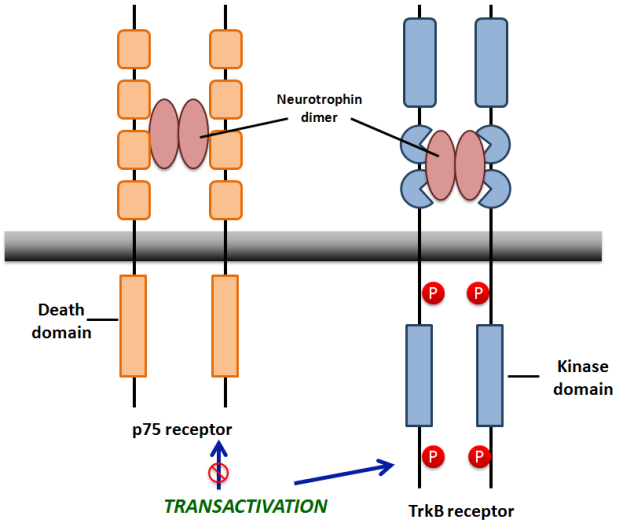


Figure 3.69. TrkB versus p75 receptors. Both TrkB and p75 receptors bind neurotrophins. But whereas the TrkB receptor possesses an intracellular kinase domain that is postulated to phosphorylate the RTK in transactivation pathways, the p75 receptor does not. This receptor has an intracellular death domain similar to TNF family receptors that is not known to participate in transactivation signaling. Adapted from Hempstead, 2002 [209].

3.2.9 Proposed mechanism

Thus, we show that 5-HT can indeed transactivate not only PDGFβ receptors but TrkB receptors as well, and transactivation is dependent on both concentration and exposure time. A diagram of the proposed signaling pathway is presented in Figure 3.70. Transactivation is initiated at $G\alpha_i$- or $G\alpha_q$-coupled GPCRs such as 5-HT_{1A} or 5-HT_2 (a topic of Chapter 4). The signal is dispatched to PLC via the Gα and/or Gβγ subunits [149], which results in intracellular calcium release and activation of PKC. NADPH oxidase subunits assemble to produce ROS, perhaps through PKC or Src-dependent activation of certain NADPH oxidase subunits. Through NADPH oxidase production of ROS or exogenously applied H_2O_2, phosphotyrosine phosphatases become temporarily oxidized and inactivated, and are unable to dephosphorylate RTKs including PDGFβ and TrkB receptors leading to an increase in RTK phosphorylation.

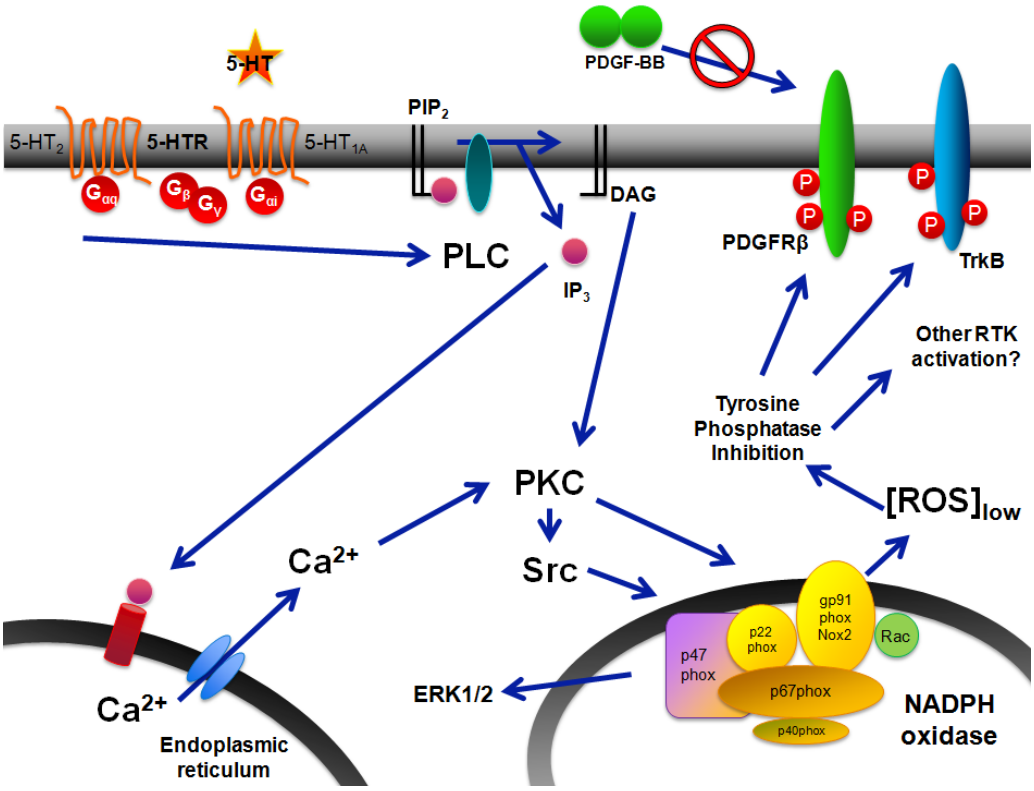


Figure 3.70. Proposed mechanism for transactivation.
A proposed pathway for 5-HT receptor-induced TrkB and PDGF receptor transactivation, based on the ability of pharmacological inhibitors to block activation of the enzymes listed. See text for details. Fluoxetine also binds to 5-HT_2 receptors, and will become an important topic in Chapter 4.

Chapter 4

Fluoxetine-induced transactivation of the platelet-derived growth factor type β receptor reveals a novel heterologous desensitization process

4.1 Results

In this section, the effects of selective serotonin reuptake inhibitors, particularly fluoxetine, on PDGF receptor transactivation were investigated. Interestingly, it was discovered that not only did fluoxetine inhibit 5-HT-induced PDGF receptor transactivation, but it also transactivated the receptor by itself. These results suggest that transactivation events were subject to a desensitization mechanism, distinct from either the GPCR or the PDGF receptor, and are investigated further.

4.1.1 Selective serotonin reuptake inhibitors can acutely block 5-HT-induced PDGFβ receptor transactivation in neuronal cells

It was previously reported that SSRIs can block PDGFβ receptor transactivation in smooth muscle cell cultures [78]. This was a fascinating finding, and we questioned whether this was also the case in neurons, where both 5-HT and SSRIs play a significant role in human brain function, dysfunction and/or antidepressant drug therapy. In SH-SY5Y cells, pretreatment with fluoxetine did indeed block 5-HT-induced PDGFβ receptor transactivation (Figure 4.1), and similarly abrogated 5-HT-induced ERK1/2 phosphorylation as well (Figure 4.2).

As this was an intriguing result, we queried whether the effects of other PDGFβ receptor-activating compounds could be blocked by fluoxetine. Fluoxetine also attenuated 5-CT-induced PDGFβ transactivation (Figure 4.3) but not PDGF-BB ligand-induced receptor activation (Figure 4.4), suggesting that this attenuation was transactivation-specific and did not affect the ability of PDGFβ receptor to be activated directly. To determine if other SSRIs can also block PDGFβ receptor transactivation, citalopram was applied prior to 5-HT treatment and we found that similar to fluoxetine, citalopram blocked 5-HT-induced PDGFβ receptor phosphorylation (Figure 4.5), but not PDGF-BB-induced PDGFβ receptor phosphorylation (Figure 4.6).

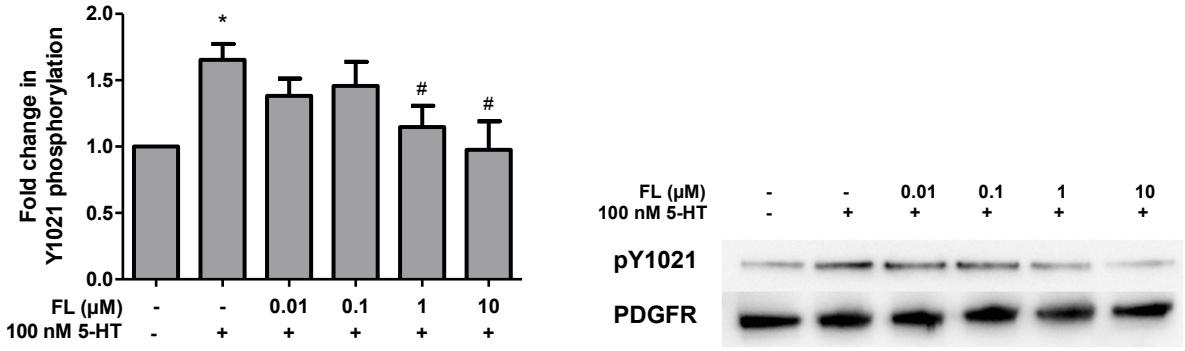


Figure 4.1. Fluoxetine can block 5-HT-induced PDGFβ receptor transactivation.
SH-SY5Y cells were pretreated with 0.01 to 10 μM fluoxetine (FL) for 45 min followed by 5 min treatment with 100 nM 5-HT. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFβ receptor protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 6$).

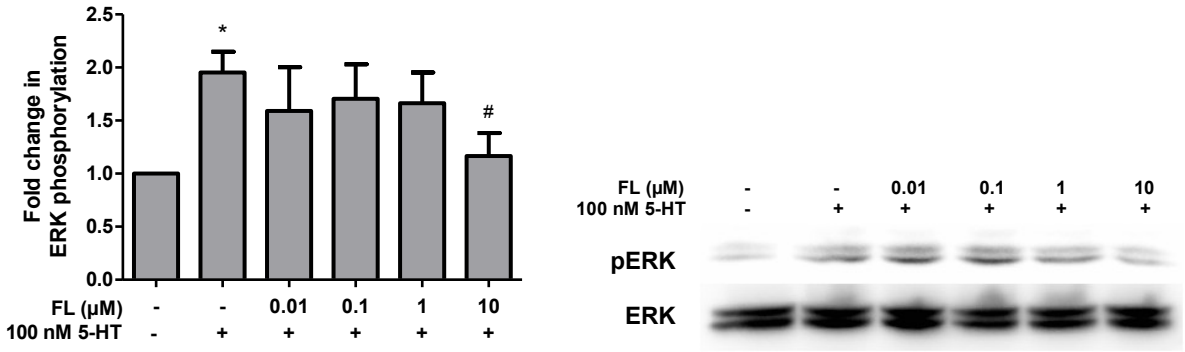


Figure 4.2. Fluoxetine can block 5-HT-induced ERK1/2 phosphorylation.
SH-SY5Y cells were pretreated with 0.01 to 10 μM fluoxetine (FL) for 45 min followed by 5 min treatment with 100 nM 5-HT. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in phospho-ERK1/2 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and ERK1/2 are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 4$).

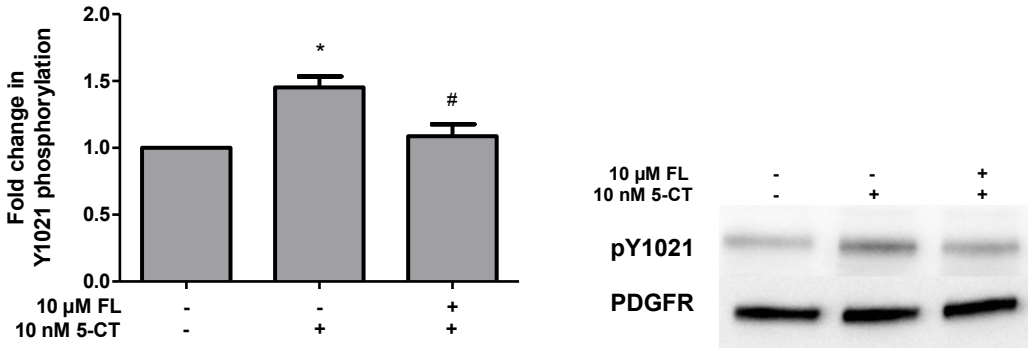


Figure 4.3. Fluoxetine can block 5-CT-induced PDGFβ receptor transactivation.
SH-SY5Y cells were pretreated with 10 µM fluoxetine (FL) for 45 min followed by 5 min treatment with 10 nM 5-CT. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFβ receptor protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-CT-treated cells; $n = 8$).

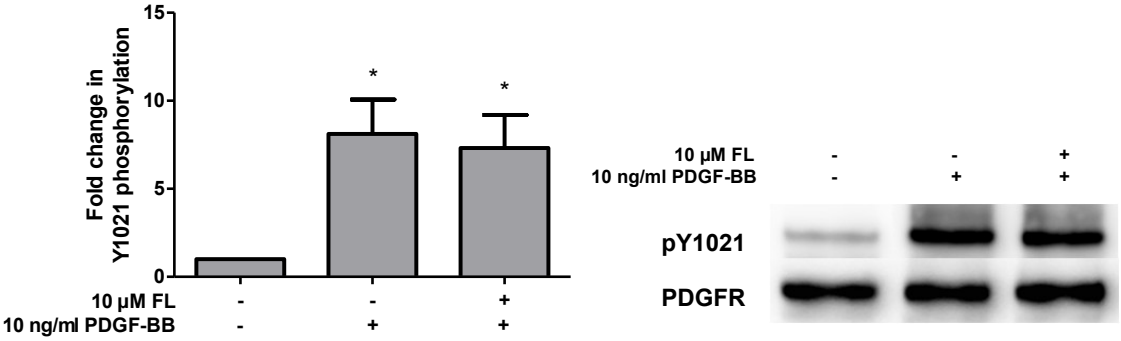


Figure 4.4. Fluoxetine does not affect PDGF-BB-induced PDGFβ receptor activation.
SH-SY5Y cells were pretreated with 10 µM fluoxetine (FL) for 45 min followed by 5 min treatment with 10 ng/ml PDGF-BB. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFβ receptor protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 6$).

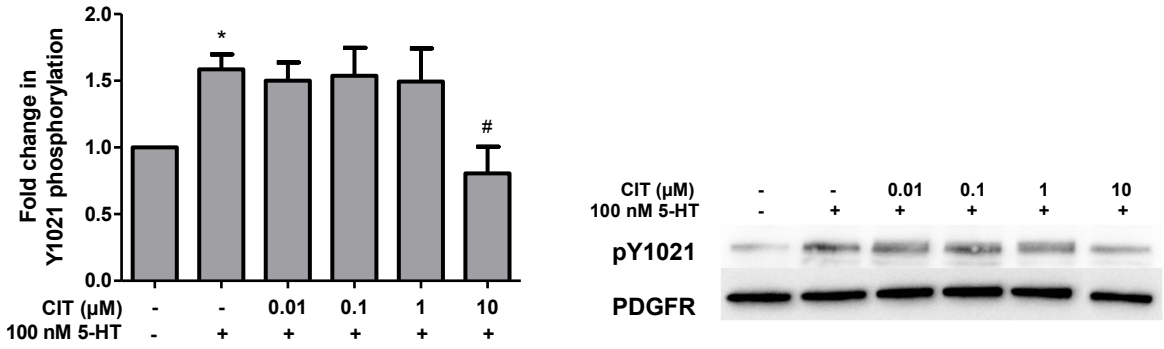


Figure 4.5. Citalopram can block 5-HT-induced PDGFβ receptor transactivation.
SH-SY5Y cells were pretreated with 0.01 to 10 μM citalopram (CIT) for 45 min followed by 5 min treatment with 100 nM 5-HT. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFβ receptor protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; $n = 7$).

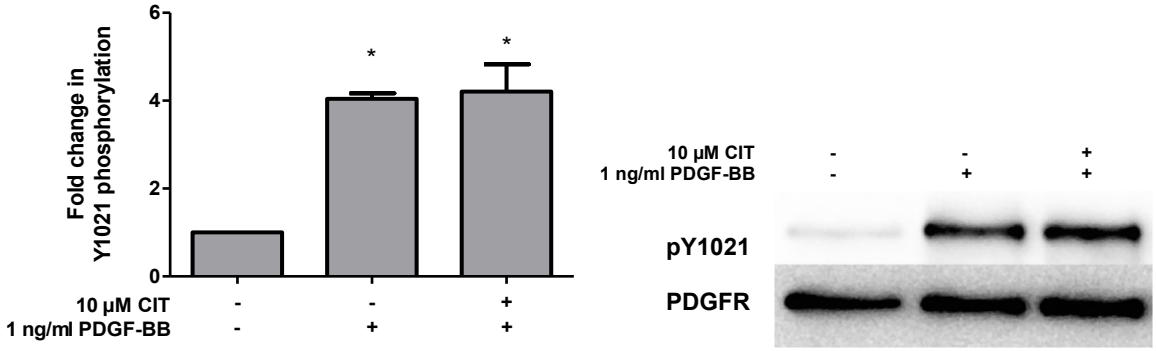


Figure 4.6. Citalopram does not affect PDGF-BB-induced PDGFβ receptor activation.
SH-SY5Y cells were pretreated with 10 μM citalopram (CIT) for 45 min followed by 5 min treatment with 1 ng/ml PDGF-BB. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFβ receptor protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 4$).

Given the effect of the 5-HT transporter (5-HTT) inhibitors fluoxetine and citalopram described above, the 5-HTT was postulated to be involved in this pathway. Fluoxetine binding to the 5-HTT may induce a novel conformational change to the transporter [210]; thus, if 5-HTT and PDGFβ receptors are in direct contact, perhaps a conformational change in 5-HTT relays a mechanically-mediated conformational change in PDGFβ receptors that inhibits transactivation. To investigate this possibility, PDGFβ receptors were immunopreciptated from SH-SY5Y cells under non-denaturing conditions and probed for the presence of 5-HTT that may have also been pulled down. However, while 5-HTT protein was observed in cell lysates, it did not co-immunoprecipitate with PDGFβ receptor (Figure 4.7). Two contaminating bands were detected flanking the position of 5-HTT; but since these bands were also observed in the precleared lanes (containing protein A/G agarose beads, IgG control antibody and vehicle-treated sample), they were dismissed as non-specific binding and inherent to the experimental system.

4.1.2 Long-term SSRI application does not affect 5-HT-induced PDGFβ receptor transactivation

Given the importance of SSRIs in their ability to modify 5-HT signaling in the brain, the effects of a more therapeutically relevant long-term SSRI exposure period was examined in 5-HT signaling to PDGFβ receptor. SSRIs such as fluoxetine are stable in solution and have long *in vivo* half-lives [211, 212]. Overnight exposure of fluoxetine or citalopram failed to affect 5-HT-induced PDGFβ receptor phosphorylation (Figure 4.8 and Figure 4.9), suggesting that the attenuation by SSRIs observed previously was a short-term effect.

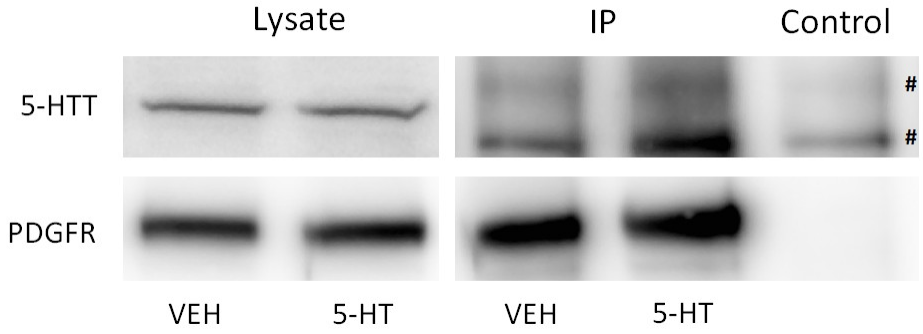


Figure 4.7. PDGFβ and 5-HTT do not associate.
SH-SY5Y cells were treated with either vehicle (VEH) or 100 nM 5-HT for 5 min. Following drug treatments, cell lysates were collected and subjected to immunoprecipitation under non-denaturing conditions. PDGFβ receptor was pulled down, and both immunoprecipitates and lysates were probed for PDGFβ receptors (180 kDa) and 5-HTT (90 kDa). Representative blots are shown. # indicates contaminating bands described in text. The control lane contains the pellet derived from centrifugation of vehicle-treated sample, IgG control (non-specific) antibody and protein A/G-coated agarose beads, showing that PDGFβ receptors or 5-HTT does not non-specifically bind to beads or antibody ($n = 3$).

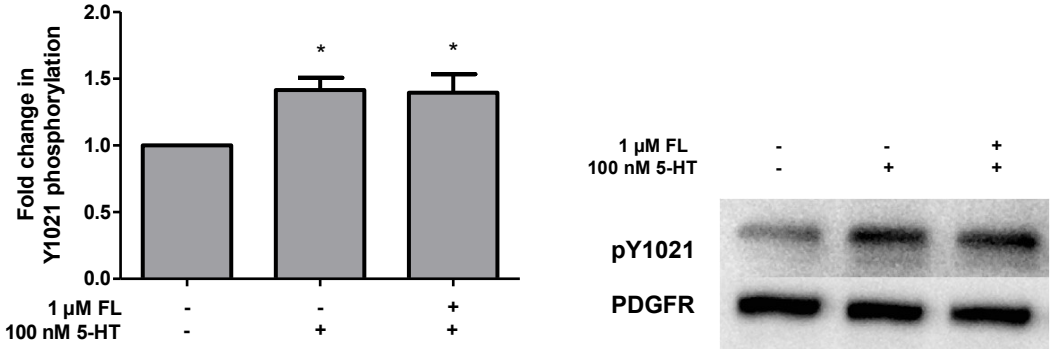


Figure 4.8. Overnight fluoxetine does not affect 5-HT-induced PDGFβ receptor transactivation.
SH-SY5Y cells were pretreated overnight with 1 µM fluoxetine (FL) followed by 5 min treatment with 100 nM 5-HT. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFβ receptor protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 7$).

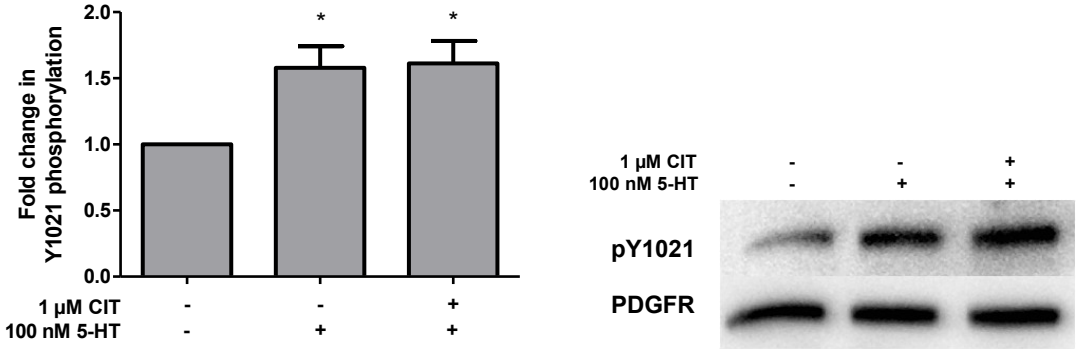


Figure 4.9. Overnight citalopram does not affect 5-HT-induced PDGFβ receptor transactivation.
SH-SY5Y cells were pretreated overnight with 1 µM citalopram (CIT) followed by 5 min treatment with 100 nM 5-HT. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFβ receptor protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 8$).

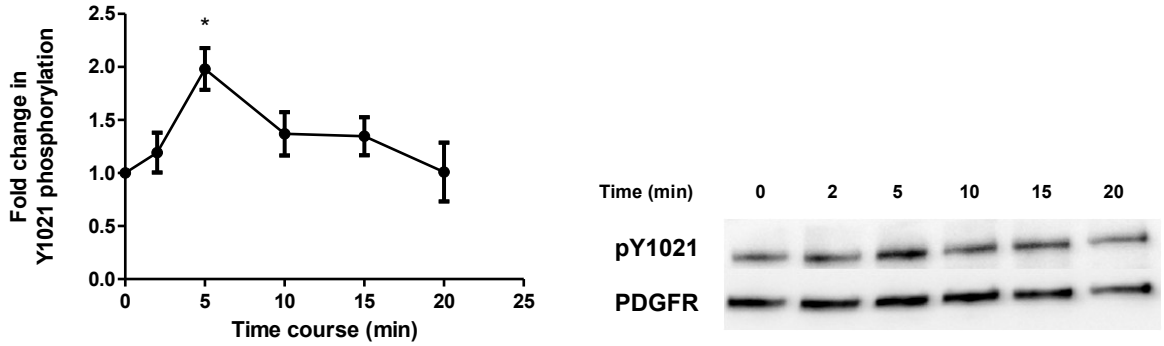


Figure 4.10. Fluoxetine itself can transactivate PDGFβ receptor in SH-SY5Y cells.
SH-SY5Y cells were treated with 10 μM fluoxetine for 0, 2, 5, 10, 15 or 20 min. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFβ receptor protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 4$).

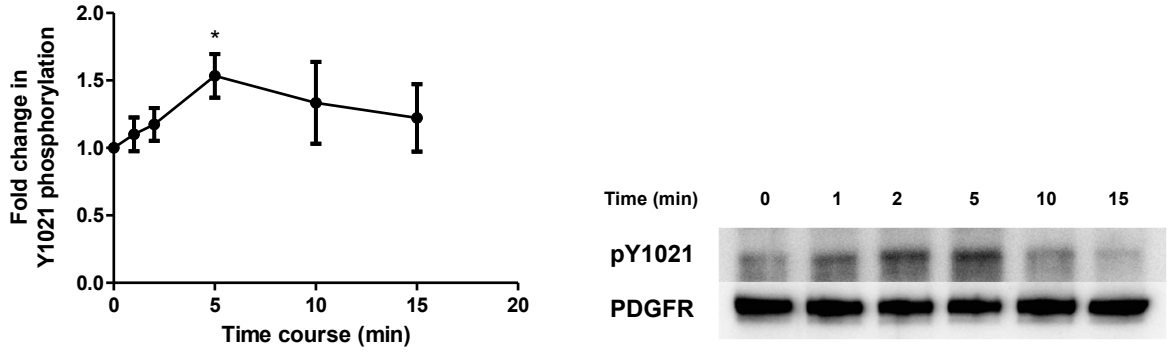


Figure 4.11. Fluoxetine itself can transactivate PDGFβ receptor in primary neurons.
Primary cultured cortical neurons (7-8 DIV) were treated with 10 μM fluoxetine for 0, 1, 2, 5, 10, 15 min. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFβ receptor protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 6$).

4.1.3 Fluoxetine transactivates the PDGFβ receptor through 5-HT_2 receptors

The results in Figure 4.1 demonstrate that 45-min fluoxetine pretreatment blocked 5-HT-induced PDGFβ receptor transactivation and suggested that the 5-HTT was required for 5-HT-induced transactivation of the PDGFβ receptor. However, given the stability of fluoxetine in aqueous solution [212], fluoxetine should have been able to block 5-HT-induced PDGFβ receptor transactivation after 24 h pretreatment (Figure 4.8). Second, fluoxetine prevented PDGFβ receptor transactivation by 5-CT (Figure 4.3) but, unlike 5-HT, whether there is an interaction between 5-CT and 5-HTT is not clear [213]. Third, the K_i of fluoxetine for the 5-HTT is on the order of 1 nM [214]; however, a significant inhibition of transactivation was not observed until the concentration of fluoxetine was 1-10 μM, a 1,000 to 10,000-fold increase over the reported K_i. To determine if perhaps this attenuation phenomenon is related to the relatively high concentration of fluoxetine, the phosphorylation state of the PDGFβ receptor was monitored during the fluoxetine pretreatment period. Surprisingly, it was found that fluoxetine itself transactivated the PDGFβ receptor with maximal phosphorylation at 5 min, followed by a decrease to baseline levels in both SH-SY5Y cells (Figure 4.10) and primary cortical neuron cultures (Figure 4.11). Additionally, a modest but significant increase in ERK1/2 (Figure 4.12) and Src Y418 (Figure 4.13) phosphorylation was noted with fluoxetine application in SH-SY5Y cells, paralleling the PDGFβ receptor phosphorylation time course (Figure 4.10). A fluoxetine dose-response curve in SH-SY5Y cells revealed a significant increase in PDGFβ receptor phosphorylation at both 1 μM and 10 μM (Figure 4.14). Taken together, these data suggest that although fluoxetine is a well-known inhibitor of 5-HTT in the nanomolar concentration range, at higher concentrations fluoxetine may itself be initiating transactivation pathways. To investigate whether other SSRIs can induce PDGFβ receptor phosphorylation in the same manner as fluoxetine, citalopram was added over a 20-min time course (Figure 4.15). Likewise, citalopram was able to acutely cause transactivation with a maximum receptor phosphorylation occurring after 5 min.

Previous reports suggest that at higher concentrations, SSRIs activate 5-HT_2 receptors to induce ERK1/2 activation [215-217]. Since SH-SY5Y cells express 5-HT_2 receptors [218-220], we sought to determine whether fluoxetine-induced PDGFβ receptor transactivation requires these receptors. Cells were pretreated with the 5-HT_2 receptor antagonist LY 272015, which binds 5-HT_{2B} receptors with a K_i on the order of 0.1 nM and 5-HT_{2A} and 5-HT_{2C} receptors with a K_i on the order of 10 nM [221]. A significant abrogation of PDGFβ receptor phosphorylation was not observed at lower concentrations, but there was an inhibition at 5 μM LY 272015 (Figure 4.16). This would suggest that this transactivation pathway is being initiated at either 5-HT_{2A} or 5-HT_{2C} receptors.

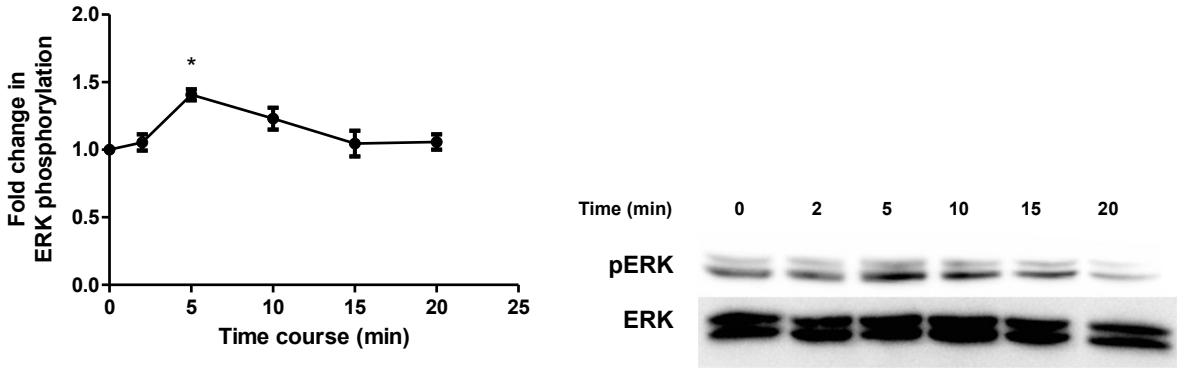


Figure 4.12. Fluoxetine treatment increases ERK1/2 phosphorylation.
SH-SY5Y cells were treated with 10 μM fluoxetine for 0, 2, 5, 10, 15 or 20 min. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in phospho-ERK1/2 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and ERK1/2 are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 5$).

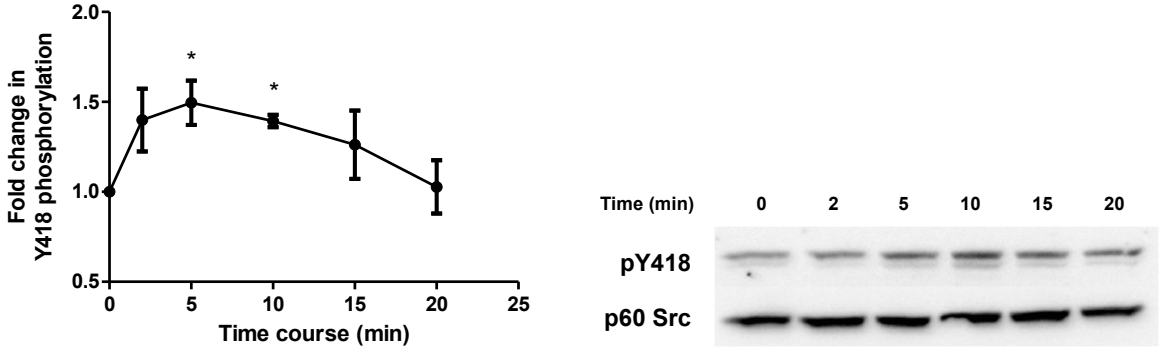


Figure 4.13. Fluoxetine treatment increases Src phosphorylation.
SH-SY5Y cells were treated with 10 μM fluoxetine for 0, 2, 5, 10, 15 or 20 min. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total p60 Src protein expression and are expressed as the fold change in phospho-Y418 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-Y418 (pY418) and p60 Src are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 4$).

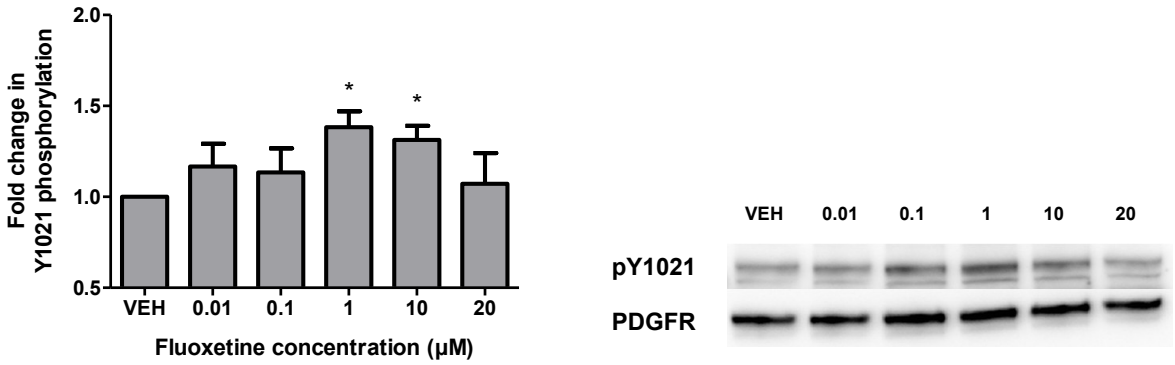


Figure 4.14. Fluoxetine-induced PDGFβ receptor transactivation is concentration-dependent.
SH-SY5Y cells were treated with 0.01, 0.1, 1, 10, or 20 μM fluoxetine for 5 min. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFβ receptor protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 7$).

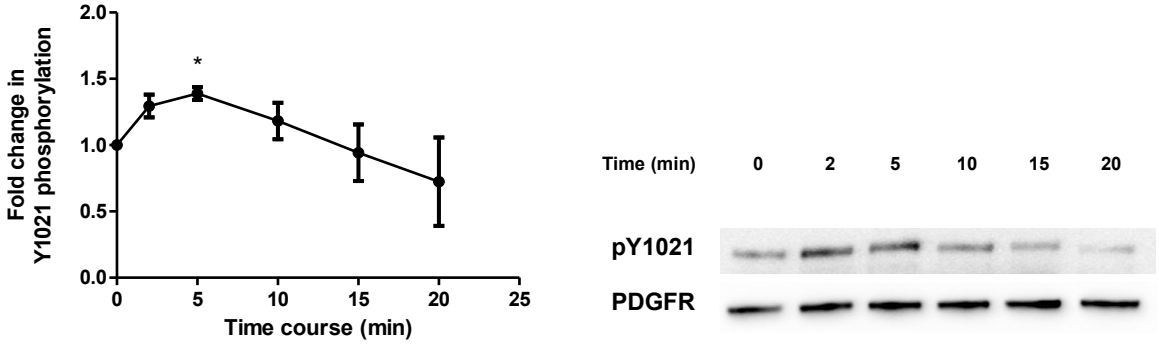


Figure 4.15. Citalopram also transactivates PDGFβ receptors.
SH-SY5Y cells were treated with 10 μM citalopram for 0, 2, 5, 10, 15 or 20 min. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFβ receptor protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 4$).

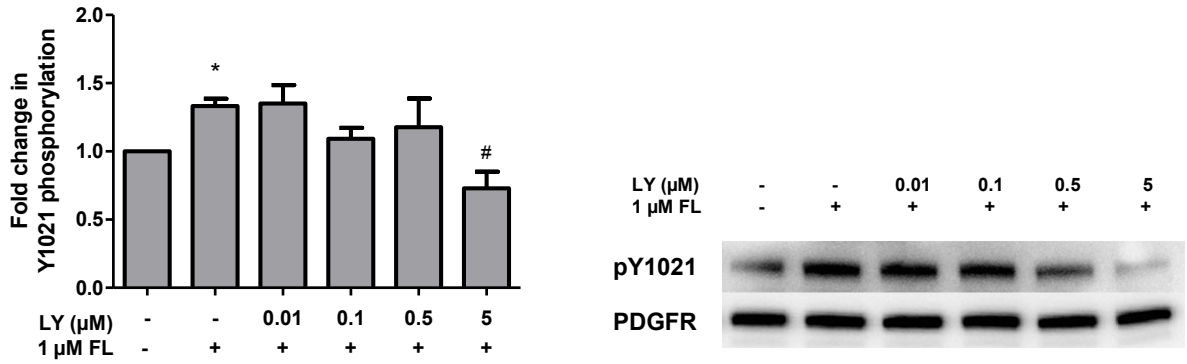


Figure 4.16. Fluoxetine-induced transactivation is initiated at 5-HT$_2$ receptors.
SH-SY5Y cells were pretreated with 0, 0.01, 0.1, 0.5 or 5 μM LY 272015 (LY) followed by treatment with vehicle or 1 μM fluoxetine (FL) for 5 min. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with FL-treated cells; $n = 7$).

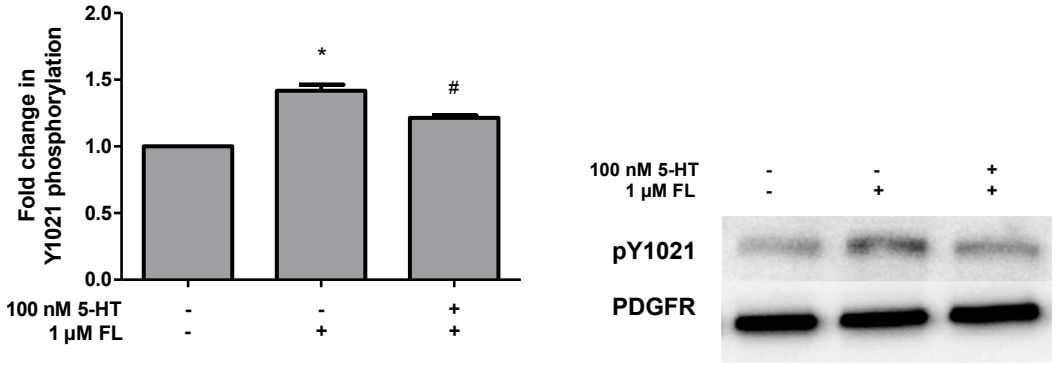


Figure 4.17. Reverse result: 5-HT attenuates fluoxetine-induced PDGFβ receptor transactivation.
SH-SY5Y cells were incubated with 100 nM 5-HT for 45 min followed by 5 min treatment with 1 μM fluoxetine (FL). Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with FL-treated cells; $n = 4$).

4.1.4 Transactivation of PDGFβ receptor is subject to heterologous desensitization

To this point, it has been shown that both 5-HT and fluoxetine can independently transactivate PDGFβ receptors via 5-HT receptor activation. This study also demonstrated that 45-min fluoxetine pretreatment blocked the effects of 5-HT-induced PDGFβ receptor transactivation (see Figure 4.1). To determine if the reverse is true, cells were pretreated with 5-HT for 45 min, followed by 5-min fluoxetine application. Indeed, 5-HT was able to diminish fluoxetine-induced PDGFβ receptor transactivation (Figure 4.17). The ability of one transactivating stimulus to block a transactivation event from another transactivating stimulus may indicate that transactivation pathways are vulnerable to desensitization.

The aforementioned drugs have thus far targeted 5-HT receptors. This study and others have shown that dopamine was also able to transactivate PDGFβ receptors (see Figure 3.14 and [68]). To determine if the desensitization of transactivation could be observed with two distinct GPCR systems, cells were pretreated with 5-HT for 45 min (well past the point where PDGFβ receptor phosphorylation returns to baseline, see Figure 3.2), followed by dopamine application for 2 min. If transactivation inhibition was occurring at the same receptors as the first stimulus (in this case 5-HT receptors), activating dopamine receptors should still cause transactivation. Interestingly, 45-min pretreatment with 5-HT blocked dopamine-induced transactivation (Figure 4.18), suggesting that transactivation desensitization did not occur at the initiating receptor, and that this may be an example of heterologous desensitization. In order to determine if desensitization is continuous or expires after a certain time, we pretreated cell cultures with 5-HT for 1, 2, or 3 h followed by dopamine treatment (Figure 4.19). Interestingly, dopamine-induced transactivation occurred after 3 h in spite of 5-HT pretreatment, but not at earlier time points. Cell cultures treated with 5-HT alone for 1, 2, and 3 h did not show any change in receptor phosphorylation (Figure 4.20). Therefore, although a sustained transactivating stimulus inhibited further PDGFβ receptor transactivation after 45 min, exposure of the initial drug for more than 3 h did not prevent receptor phosphorylation (Figure 4.22), indicating that desensitization lasts no more than 3 h after applying the initial transactivating stimulus.

To ascertain if this regulation occurred at the PDGFβ receptor itself, cells were pretreated with 5-HT, followed by 5-min PDGF-BB – a ligand that directly activates PDGFβ receptor. A concentration of 0.1 ng/ml PDGF-BB was selected to have a comparable level of PDGFβ receptor phosphorylation as observed with GPCR-induced transactivation. However, 5-HT was unable to inhibit PDGF-BB-induced receptor activation (Figure 4.21), suggesting that desensitization did not occur at PDGFβ receptors, but rather occurred at a point within the GPCR-PDGFβ receptor transactivation pathway.

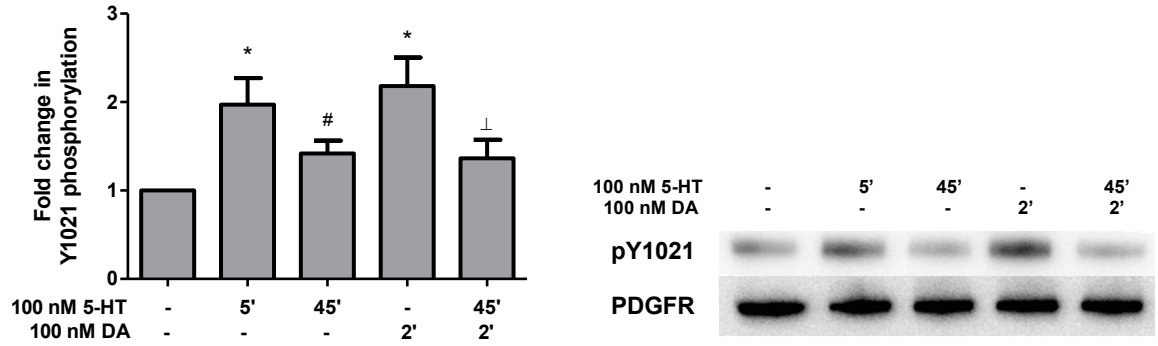


Figure 4.18. PDGFβ receptor transactivation is subject to heterologous desensitization.
SH-SY5Y cells were treated with 100 nM dopamine (DA) for 2 min (bar 4) or with 100 nM 5-HT for 45 min followed by 2 min dopamine treatment (bar 5). As controls relative phosphorylation with vehicle-treated cells (bar 1), with 5 min 5-HT treatment (bar 2) and with 45 min 5-HT treatment (bar 3) are shown. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; ⊥ = $p<0.05$ compared with DA-treated cells; $n = 8$).

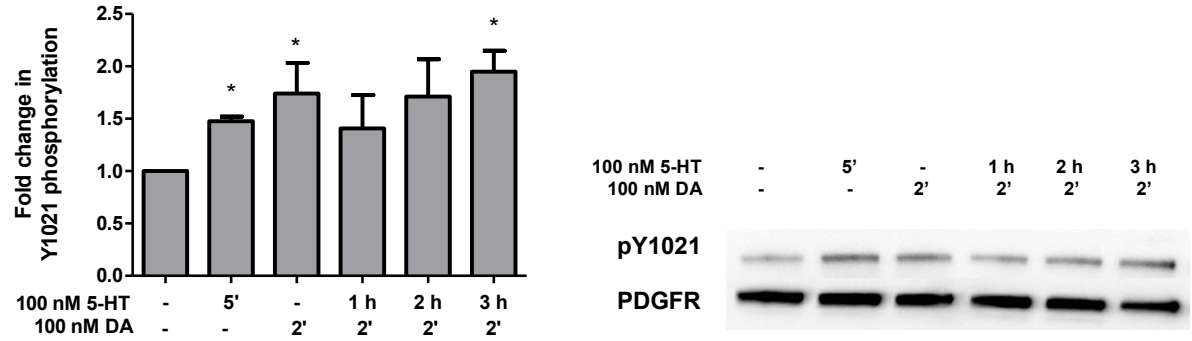


Figure 4.19. PDGFβ receptor desensitization is limited to 3 h.
SH-SY5Y cells were treated with vehicle (bar 1) or 100 nM for 5 min (bar 2). Additional cells were pretreated with 100 nM 5-HT for 0, 1, 2, or 3 h followed by 100 nM dopamine (DA) for 2 min (bar 3-6). Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 6$).

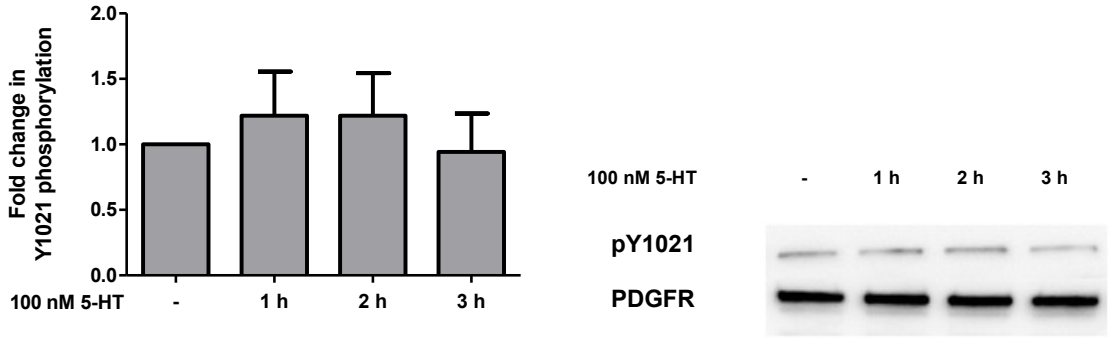


Figure 4.20. Long-term 5-HT treatment does not affect PDGFβ receptor phosphorylation.
As a control for Figure 4.19, SH-SY5Y cells were treated with 100 nM 5-HT for 0, 1, 2, or 3 h. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (n = 6).

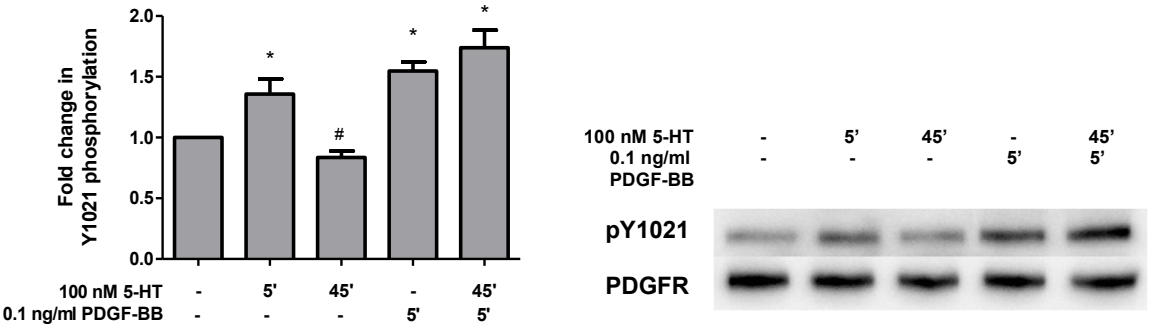


Figure 4.21. PDGFβ receptor activation by PDGF-BB is unaffected by 5-HT pretreatment.
SH-SY5Y cells were treated with 0.1 ng/ml PDGF-BB for 5 min (bar 4) or with 100 nM 5-HT for 45 min followed by 5 min 0.1 ng/ml PDGF-BB (bar 5). As controls, relative phosphorylation with vehicle-treated cells (bar 1), with 5 min 5-HT treatment (bar 2) and with 45 min 5-HT treatment (bar 3) are shown. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared with 5-HT-treated cells; n = 5).

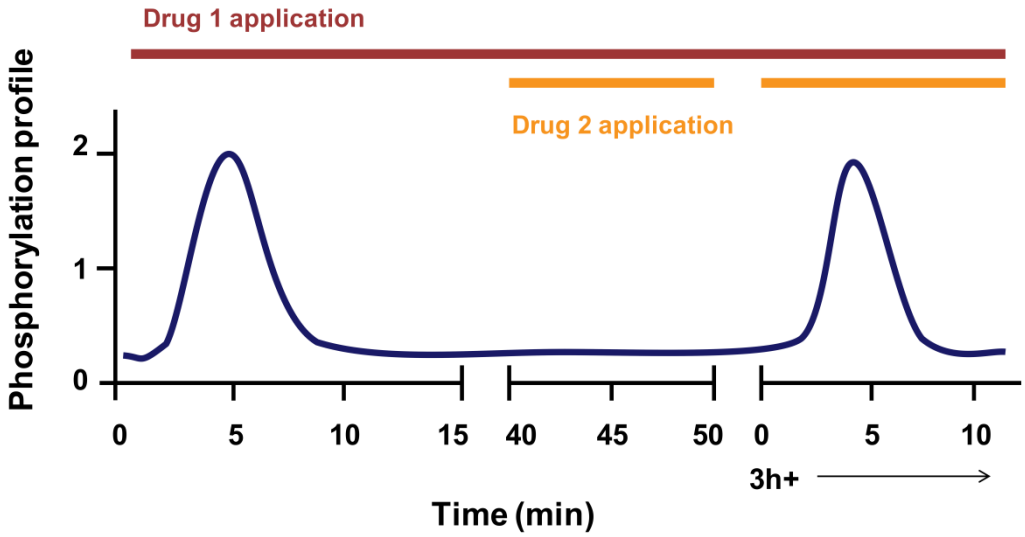


Figure 4.22. Heterologous desensitization in transactivation.
Given two independent drugs that are capable of independently inducing transactivation of PDGFβ receptor, the initial stimulus (Drug 1) acutely causes transactivation with peak phosphorylation taking place after 5 min. Heterologous desensitization occurs after 45 min of the initial drug application where transactivation by a second drug (Drug 2) is occluded. After 3 h of sustained exposure to the first drug (Drug 1), transactivation is again able to occur by the second drug (Drug 2).

4.2 Discussion

In this section, the notion of 5-HT receptor-induced transactivation was built upon by revealing a temporally dependent attenuation of GPCR-mediated transactivation of PDGFβ receptors. It was demonstrated that fluoxetine induced an increase in PDGFβ receptor phosphorylation, likely via activation of 5-HT_2 receptors. To our knowledge, this is the first example of a selective serotonin reuptake inhibitor (SSRI) causing PDGFβ receptor transactivation. Interestingly, this experimental protocol also allowed for the identification of a novel transactivation signaling phenomenon: heterologous desensitization. Transactivation of the PDGFβ receptor by one stimulus was able to prevent a subsequent transactivation signal by another stimulus within a 3 h window.

4.2.1 The 5-HTT hypothesis

The initial hypothesis upon analyzing the abrogation of 5-HT-induced PDGFβ receptor transactivation by fluoxetine (Figure 4.1) was that perhaps the main target of fluoxetine, the serotonin transporter (5-HTT), was involved. Previously, it was demonstrated that the 5-HTT was phosphorylated by PKC at serine/threonine residues, and increasing phosphorylation correlated with a decrease in function [222]. Also, tyrosine phosphorylation was observed on the 5-HTT, and this phosphorylation was necessary for maintaining membrane expression levels and function [223]. The C-terminal region of the 5-HTT also appears important for transport function [21]. However, other than these trafficking and functional roles, it remains to be determined whether the 5-HTT can initiate or directly participate in signal transduction cascades, either through altering its phosphorylation states or by another means. Despite the lack of evidence and the possible high impact of discovering a signaling role for the 5-HTT, the relatively high concentrations of SSRIs required to achieve the abrogation of transactivation (compared to the K_i for 5-HTT) seemed suspicious and warranted further study. Thus, this experimental direction was favored for exploration over the former and yielded satisfactory, if not complex, results.

On a side note, other avenues for disputing 5-HTT involvement were explored. An attempt to knock-down 5-HTT protein using siRNA was attempted; however, inconsistent results were obtained (data not shown). Various times and concentrations of siRNA incubation were attempted, indicating a long half-life of existing 5-HTT protein. The more likely reason was the inability to find the appropriate conditions for optimal transfection of SH-SY5Y cells despite using two different

transfection reagents. However, with overwhelming evidence supporting the fluoxetine-to-5-HT_2 receptor hypothesis as described below, it felt reasonable to conclude that the 5-HTT was not involved in fluoxetine-mediated 5-HT-induced transactivation abrogation. Therefore, this avenue of investigation was suspended.

4.2.2 Involvement of 5-HT_2 receptors

With the high concentrations of fluoxetine needed to abrogate 5-HT-mediated PDGFβ receptor transactivation, further investigation into the action of fluoxetine was warranted. The K_i of fluoxetine for the 5-HTT is 1 nM [214], so a concentration of 10 nM should presumably lead to the vast majority of 5-HTT being bound by fluoxetine. If the 5-HTT was involved, an abrogation in 5-HT-mediated transactivation (or at least a partial one) would be expected to be seen at this concentration. However, this was not the case: fluoxetine was only capable of blocking transactivation at 1-10 μM. Further, when acutely treated with these high concentrations of fluoxetine, this drug was independently able to transactivate PDGFβ receptors, and this signaling was sensitive to the 5-HT_2 antagonist LY 272015. Activation of 5-HT_2 receptor isoforms have been shown to activate ERK1/2 [199, 200, 224], and since ERK1/2 was also activated by fluoxetine application, this drug was likely an agonist for one or more isoforms of 5-HT_2 receptors as previously described [165, 215]. This notion of fluoxetine binding 5-HT receptors is a recently discovered drug effect compared to 5-HTT binding, with studies outlining an affinity of fluoxetine for 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors [165, 214, 216, 225]. Nevertheless, this is the first example of a selective serotonin reuptake inhibitor (SSRI) causing PDGFβ receptor transactivation.

4.2.3 Heterologous desensitization in transactivation

One definition of heterologous desensitization is "activation of one GPCR inhibits signaling from another heterologous GPCR" [226], and was represented by the "refractory period" where transactivation was blocked in the presence of a previously applied agonist. Four pieces of evidence are given to describe this: short-term fluoxetine pretreatment was able to abrogate 5-HT-induced PDGFβ receptor phosphorylation, short-term 5-HT pretreatment was able to block fluoxetine-induced PDGFβ receptor phosphorylation, short-term 5-HT pretreatment could inhibit dopamine-mediated PDGFβ receptor phosphorylation, and all three of these drugs induce PDGFβ phosphorylation when

applied alone. Given that sustained SSRI application prevented 5-HT-induced PDGFβ receptor transactivation at 45 min but not after 3 h or overnight treatment, the time required for the transactivation refractory period to expire is between 45 min and 3 h (Figure 4.22). In all probability, this period would involve a yet-unidentified regulatory enzyme that is preventing further transactivation events, and is common to the transactivation pathways described here (that is, 5-HT-, dopamine- and fluoxetine-mediated pathways). Although fluoxetine and 5-HT may be signaling through the same set of receptors, this refractory period is considered to be heterologous desensitization based on the data showing that dopamine signaling to PDGFβ receptors, which does not occur through 5-HT receptors, is also attenuated by 5-HT. The expiration of this refractory period may involve the cell eventually becoming desensitized to the first transactivating stimulus by phosphorylating and/or internalizing the activated GPCRs [8], thus removing the input of the external stimulus and allowing the signaling enzymes to "reset".

The regulation of transactivation which leads to the inability of a second transactivation event during the refractory period by a second stimulus is not occurring at the initiating GPCRs, nor is it occurring at the PDGFβ receptor. Rather, it must be taking place at an enzyme in the transactivation pathway that has been previously described in this system. ERK1/2 activation was described as branching off the transactivation pathway prior to reactive oxygen species (ROS) production. Given the ability of ERK1/2 to be inhibited by fluoxetine pretreatment (see Figure 4.2), the regulatory enzyme likely occurs upstream of ROS production as well (see Figure 3.70). One potential candidate for transactivation regulation is PKC. This enzyme has been shown to phosphorylate GPCRs at various intracellular serine and threonine residues, which correlates with decreased receptor activity [6]. This is notable for the reason that PKC indiscriminately targets active and inactive GPCRs [6]. For example, 5-HT-induced PKC activation may aide in phosphorylating and desensitizing activated 5-HT receptors, but also inactive dopamine (and possibly other) receptors, as seen in Figure 4.18. In contrast, G protein-coupled receptor kinase (GRK)-induced desensitization requires an active GPCR to initiate phosphorylation [8], and this is a main mechanism of homologous desensitization. While GRK-mediated phosphorylation promotes arrestin binding and induces a conformational change in the GPCR to encourage internalization [227], this may not be the case for PKC-induced phosphorylation of GPCRs. If so, PKC-mediated desensitization that occludes transactivation would result in a greater number of GPCRs remaining on the membrane. These would be more readily available for consequent signal transduction upon eventual dephosphorylation and reactivation of GPCRs after the expiration of the desensitization period.

In vivo, a typical neuron is regularly exposed to neurotransmitters that initiate signaling events, so would there be a desensitizing refractory period as well? Perhaps, but unlike this study, neurotransmitter signaling usually has a mechanism in place for rapidly voiding the synaptic space of neurotransmitter within milliseconds [228] to end the signal: be it a transporter for reuptake as is the case for 5-HT, dopamine, norepinephrine and glutamate, or a hydrolyzing enzyme to deactivate the neurotransmitter as is the case for acetylcholine. Thus, the exposure time of receptors to such a short stimulus is likely insufficient to cause desensitization. However, a potential problem arises when the system is overloaded in the medium term such that transporters or inhibiting enzymes cannot keep up. For example, glutamate receptor overactivity was shown to cause excitotoxicity, while PDGFβ receptor activation is neuroprotective and can mitigate the effects of various insults [229-234]. *In vivo* studies in rats show PDGF receptor activation attenuates hypoxia-mediated apoptosis in brainstem [230], and is also protective against glutamate- and NMDA-induced excitotoxicity in neurons [233-235]. Glutamate also transactivates PDGF receptor (Appendix Figure I), so if a glutamate-induced refractory period occurs in the short to medium term, additional transactivating stimuli may be unable to activate the protective effects of PDGFβ receptor, which may contribute to increased cell damage in this period.

Chapter 5
General discussion, perspectives, and outlooks

The cross-talk between 5-HT receptors and PDGFβ and TrkB receptors adds to an increasing body of knowledge that GPCR and growth factor signaling are not as discrete as once thought. As the concept of RTK transactivation is a relatively recent discovery, many pieces to this puzzle still remain under-investigated, and molecular mechanisms of their proliferative and detrimental effects are just now coming to light. The notion that ROS mediates phosphatase inhibition as a means of increasing RTK phosphorylation may suggest that transactivation is a more global pathway responsible for mitogenic or protective effects against cytotoxic insults, and that this phenomenon is likely not restricted to 5-HT and RTK pathways elucidated here. Given the involvement of $G\alpha_i$ and $G\alpha_q$-linked GPCR activation, studies involving these G protein subtypes may inevitably result in the discovery of further instances of transactivation of RTKs.

In this chapter, potential outcomes of PDGF receptor signaling, and the future directions that this research could take are considered. In particular, we discuss the notion that although PDGFβ receptors are important mediators of various mitogenic signaling pathways and prolonged, direct activation of PDGFβ receptor has been shown to lead to hyperplasia and cancerous growth [49], short-term transactivation could serve as a "back door" to activating the receptor at a sub-hyperplastic level to provide protective and mitogenic effects.

5.1 Other links to depression

One current theory of antidepressant action involves downregulation of 5-HT_1 autoreceptors. In this model, 5-HTT inhibition increases levels of synaptic 5-HT which increasingly binds autoreceptors found on the presynaptic neuron, which results in their desensitization and downregulation over time [116]. Normally, these activated inhibitory autoreceptors prevent further 5-HT release from the presynaptic neuron, but with autoreceptor downregulation, this inhibition is lost and the net effect is a greater amount of 5-HT released presynaptically [18].

On this note, antidepressants such as fluoxetine that bind 5-HT receptors may have a similar mechanism involving 5-HT_2 heteroreceptors. Initially, fluoxetine may be acting as an agonist at these receptors to cause a signaling cascade (possibly including transactivation). With a K_d on the order of

0.1-1 μM for 5-HT_2 receptors [214, 236, 237] in combination with its long *in vivo* half-life of 24-72 h [236], chronic fluoxetine exposure may be building up to sufficient concentrations such that desensitization and downregulation of 5-HT_2 receptors occurs over time. This is in line with current research that suggests that 5-HT_2 receptor activation increases instances of depression in rodents, whereas 5-HT_2 receptor inhibition has antidepressant effects [238-240]. These depressive effects may be the result of excitatory 5-HT_2 receptors present on GABAergic interneurons, which when active, leads to GABA release and binding to GABA receptors on nearby serotonergic neurons, thus preventing their firing [241]. This would result in less 5-HT signaling and consequently, increases in depressive behavior [241]. Theoretically, this mechanism could be halted with fluoxetine-mediated 5-HT_2 receptor desensitization which may lead to an improvement in the symptoms of depression. Thus, it would be of interest to determine the effect of fluoxetine on 5-HT_2 receptor expression in depressed and non-depressed *in vivo* models.

There are also reports of fluoxetine binding 5-HT_{1A} receptors with a K_d on the order of 10 μM [214, 236, 237]. Therefore, it is possible that fluoxetine treatment may result in 5-HT_{1A} autoreceptor inhibition by the fluoxetine itself. While 5-HT may bind to 5-HT_{1A} autoreceptors, 5-HT reuptake (even if repressed by SSRIs) is not 100% inhibited and would still clear 5-HT from the synapse, albeit more slowly. With fluoxetine treatment in which a rapid reuptake or clearance mechanism presumably does not exist, *in vivo* concentrations may gradually build up to adequate concentrations (similar to the proposed mechanism in the above paragraph) such that binding and possible desensitization of 5-HT_{1A} autoreceptors become significant. This would further increase 5-HT neuron firing and, in combination with 5-HTT inhibition, increase synaptic 5-HT levels. Although these micromolar concentrations of fluoxetine seem high, some studies suggest, taking into consideration the long half-life, that these concentrations are still therapeutically relevant [217]. Nuclear magnetic resonance imaging of the human brain have estimated fluoxetine/norfluoxetine concentrations to be as high as 10.7 μg/mL (31 μM) during chronic fluoxetine treatment [216] – more than sufficient to activate the 5-HT receptors described here.

5.2 Physiological responses of PDGF receptor transactivation: Short-term protection but detrimental over the long term?

Physiological cell responses to transactivation are still not well understood; however, there seems to be a general trend demonstrating a survival or proliferative cellular response dependent on PDGF receptor transactivation, which is beneficial in the short term but detrimental in the long term. The available data is limited, although some examples are discussed here. Acute exposure to insults such as cigarette smoke extract or hypoxic conditions induces PDGF receptor transactivation and subsequent activation of cell survival signaling pathways to limit the extent of inflammation- or apoptosis-induced injuries [229-232]. However, pharmaceutical or genetic inhibition of PDGF receptor activity decreases the proliferative/survival effects of this pathway [230, 231]. The exact details of the initiating factor in these transactivation pathways are still unclear, but one study has suggested the involvement of $G\alpha_i$ proteins [242].

On the other hand, long-term PDGF receptor transactivation is linked to disease states. Prolonged angiotensin II and endothelin exposure through GPCRs induces PDGF receptor-dependent cellular responses such as cell migration and proliferation of smooth muscle that contribute to vascular remodeling processes in hypertension [166, 167]. Similarly, long-term exposure to cigarette smoke extract causing PDGF receptor transactivation was linked to COX-2 upregulation in tracheal smooth muscle [243], which may be responsible for lung inflammation and damage seen in smokers. Mice exposed to chronic hypoxia (months) also show a continued increase in PDGF receptor phosphorylation, and development of thicker vascular smooth muscle similar to that reported in pulmonary hypertension [244]. Finally, oxidized forms of the cholesterol-carrying low-density lipoproteins (oxLDLs) have resulted in PDGF receptor transactivation in a ROS-dependent manner [245], which may be responsible for migration of smooth muscle cells into the endothelial layer, the destruction of ECM proteins, and causing plaques to rupture and form thrombi as seen in atherogenesis [246, 247]. Thus, while short-term PDGFβ receptor transactivation appears to initiate cell survival and proliferative pathways, long-term implications of this signaling leads to an overabundance of cell growth that can have implications in these disease etiologies. In the CNS, long-term exposure to transactivating stimuli may have different outcomes in neurons. The majority of neurons exists in a terminally differentiated state and will not re-enter the cell cycle, therefore neuron survival, rather than proliferation, may be the only relevant outcome.

5.2.1 Neuroprotection

Prior studies have shown that PDGF-BB binding to its receptor leads to rapid internalization and degradation of both receptor and ligand [49], whereas 5-HT receptor activation does not appear to result in PDGF receptor internalization, and in some cases, leads to increased receptor expression. This was demonstrated in our previous work which showed that long-term 5-HT and 5-CT application to primary cultured neurons did not down-regulate, but rather *up-regulated* PDGFβ receptors [140], whereas PDGF-BB application led to decreased cell surface expression of these receptors [49]. Additionally, we have unpublished observations suggesting that long-term 5-HT receptor activation also modestly increases TrkB receptor expression. Since growth factor receptor activity is typically proliferative in nature, one consequence of this PDGF receptor up-regulation is increased neuroprotection against NMDA-induced excitotoxicity as seen in primary hippocampal neuron cultures [233]. We believe that the cross-talk between PDGF and NMDA receptors is dependent upon the PDGFβ receptor phosphorylation site Y1021 and subsequent PLCγ activity. This cross-talk may also act via transactivated PDGFβ receptors, which is the rationale for choosing Y1021 as the primary phosphorylation site in this study. Correspondingly, we have preliminary evidence of short-term transactivating agonist application eliciting protection against H_2O_2-induced cell death (Figure 5.1).

Given the difference in potency between growth factors and agonists that evoke transactivation pathways, one might question why these latter agonists would even be considered to be applicable. As therapeutic agents, growth factors and neurotrophic factors are not permeable to the blood-brain barrier (BBB) due to their size, nor would they survive in the gastrointestinal tract if given as an oral preparation. However, many of these small molecules that activate GPCRs (or their precursors) are BBB-permeable, and can be formulated for oral ingestion. Examples include the 5-HT precursor 5-hydroxytryptophan and the dopamine precursor levodopa [248], which are converted to the active molecule within the CNS. The use of GPCR agonists may ultimately result in short-term transactivation pathways and long-term increases in PDGF receptor expression. Using GPCR agonists such as 5-HT to initiate short-term transactivation not only avoids the impermeability problems but also the dangers of hyperplasia and cancer-like conditions associated with exogenous application of growth factors such as PDGF [49]. Although the details of GPCR-induced PDGFβ receptor up-regulation remains to be elucidated (and is currently being investigated by other laboratory members),

this up-regulation of expression and activity through transactivation could serve as a mechanism for sensitizing or priming the cell for relieving present or preventing future neuronal insults.

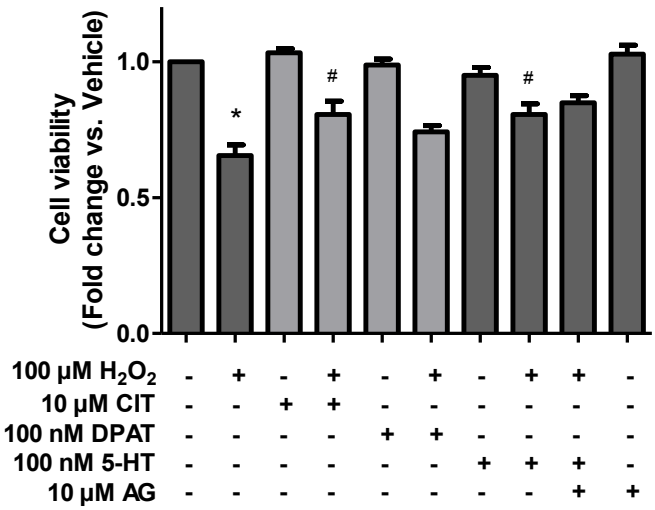


Figure 5.1. 5-HT and citalopram partially protect SH-SY5Y cells from H_2O_2-induced cell death, but AG 1296 has no effect.
Cells were treated with citalopram (CIT) or fluoxetine (FL) or DPAT or 5-HT for 5 min followed by the addition of 100 µM H_2O_2 for 30 min. Cells treated with 5-HT were additionally pretreated with vehicle or the PDGF receptor kinase inhibitor AG 1296 (AG) for 30 min prior to the addition of 5-HT. Media was changed and cultures were returned to the incubator for 48 h prior to adding MTT reagents. Cell viability was assessed by absorbance of the resulting solution at 570 nm. Although DPAT was trending toward modest protective effects, the result was not statistically significant. AG 1296 application did not prevent 5-HT-induced neuroprotection against H_2O_2, which may be expected if multiple RTKs are activated via transactivation. (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ with respect to H_2O_2 (alone)-treated cells.)

5.2.2 Conclusions

While both short-term and long-term transactivation events lead to cell proliferation, excess proliferation including hyperplasia is observed in long-term studies, evocative of PDGF ligand-induced cancerous growths. Could these transactivation pathways then be thought of as "diet" or "lite" RTK activation? Perhaps transactivation signaling and subsequent short-term desensitization has the mechanistic responsibility to filter competing signals from GPCRs, where only one of a group

of successive GPCR input signals is allowed to continue to produce an output, thus streamlining the ultimate cellular response.

In addition to GPCRs, these examples show that PDGF receptors can be activated by oxidative reactions. These examples deviate from the classical definition of transactivation, as the involvement of an activated GPCR is only speculative at this point. It is conceivable that oxidative reactions producing ROS may bypass GPCRs altogether and enter the transactivation pathway at the point of ROS production (see Figure 3.70 on page 86). This may indicate that transactivation pathways are a subset of proliferative oxidative pathways (or vice versa) that all share a common mechanism, including ROS production, and lead to growth factor receptor activation through phosphatase inhibition. These examples also provide evidence for a transactivation-like signaling model, given the involvement of specific enzymes in our proposed mechanism such as Src, NADPH oxidase, ROS, and PKC [231, 243-246]. Although not all of the enzymes that were elucidated in the present study were investigated in these examples, it would not be surprising to discover that additional enzymes may be involved, given the proposed universality of transactivation pathways in general.

5.3 Future Directions

5.3.1 G protein-coupled receptor kinases and PDGFβ receptor phosphorylation

Up to this point, phosphorylation of the PDGF receptor at various *tyrosine* residues has involved activation of the receptor. Besides phosphorylating GPCRs, G protein-coupled receptor kinases (GRKs) are also capable of phosphorylating the PDGF receptor on *serine* residues [249]. Experimental evidence shows that PDGF-induced PDGF receptor phosphorylation on tyrosine residues leads to tyrosine phosphorylation in GRK proteins, which enhances the ability of GRK to phosphorylate the PDGF receptor, but not β_2-adrenergic receptors [249-251]. This also implies GRK is a substrate for activated PDGF receptors [252], or that it is phosphorylated by an effector of PDGF receptor such as c-Src [253]. Serine phosphorylation of PDGF receptors also correlates to a decrease in tyrosine phosphorylation, possibly by the phosphatase SHP-2, and this in combination with the increased serine phosphorylation leads to a deactivated or desensitized receptor [249, 251]. Both GRK2 and GRK5 induced serine phosphorylation on PDGFβ receptors, as described by Freedman and colleagues [249-252, 254]. A suspected PDGFβ receptor phosphorylation site in mouse is S1104, which is also part of a domain that binds NHERF, a protein that facilitates PDGF receptor

dimerization [254, 255]. A S1104A PDGFβ receptor mutant has reduced NHERF binding and is unable to be desensitized, even with GRK2 overexpression [254], further implicating serine phosphorylation as a desensitizing mechanism.

Regulation of RTK activation is typically understood to involve internalization of the receptor-ligand complex. This is followed by either recycling or proteolytic degradation of the receptor [49]. However, now GRKs may also have a role in this process. Nevertheless, the above experiments were performed with PDGF as the activator of PDGFβ receptor. It is unknown if GPCR agonists can also elicit this deactivation effect via a transactivation pathway, nor is it known if NHERF would play a role, especially since PDGF receptor dimerization in transactivation pathways is not required [196]. However, if GPCRs can induce GRK-mediated regulation of transactivated PDGF receptor, this could indicate a much stronger interrelationship between GPCRs and PDGF receptor (and possibly other RTKs) than originally thought. If true, a potential feedback loop emerges: GPCR activation leads to PDGF receptor/RTK transactivation, which in turn enhances GRK activity. Activated GRKs are then available to regulate PDGF receptor/RTK signaling through serine phosphorylation (see Figure 5.2).

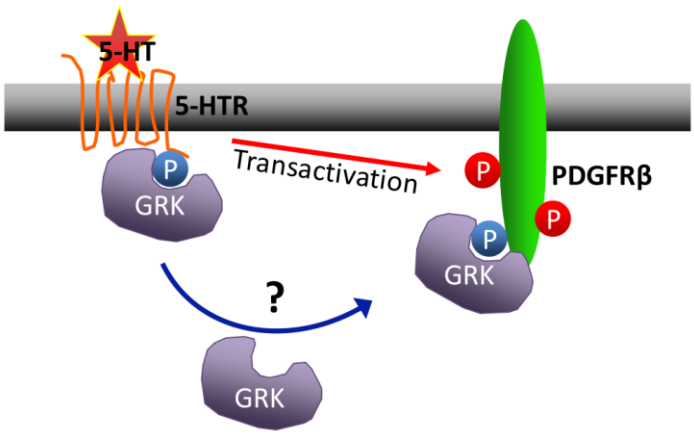


Figure 5.2. Possible feedback mechanism for PDGFβ receptor desensitization.
5-HT activates 5-HT receptors, which in turn initiate tyrosine phosphorylation (red P) of PDGFβ receptors. Active 5-HT receptors are also capable of activating GRKs, which desensitize GPCRs through serine phosphorylation (blue P). GRKs activated by GPCRs may also have the ability to translocate to and phosphorylate PDGFβ receptors on serine residues to terminate transactivation.

5.3.2 Drug targets: Wanted vs. unwanted

This project highlights the inherent weakness in pharmaceutical-based approaches: a given pharmaceutical never binds only the intended target. There is usually at least one other molecular target with which a pharmaceutical can interact in a concentration-dependent fashion. This is very evident in the present study with fluoxetine given its ability to block 5-HT-induced PDGFβ receptor activation. Fluoxetine is designed for and used as a 5-HT transporter inhibitor, and one might conclude based on this evidence that the 5-HTT is involved, were it not for the data suggesting fluoxetine is acting as a 5-HT_2 receptor agonist in this situation. As a control for Figure 4.1, which demonstrated the ability of fluoxetine to block 5-HT-induced PDGFβ receptor phosphorylation, fluoxetine was added to cell cultures in absence of 5-HT for the pretreatment time interval of 45 min. Fluoxetine-treated cells did not show a change in PDGFβ receptor phosphorylation compared to vehicle-treated cells after 45 min (data not shown), because it was well past the point of PDGFβ receptor phosphorylation returning to baseline. The experimental approach examining the effect of short-term fluoxetine treatment was serendipitous and led to an entirely new direction for this project.

In another experiment, the 5-HT_{1A} antagonist WAY 100135 partially blocked 5-HT-induced PDGF receptor activation at 100 nM (Figure 3.17), a concentration at which an inhibition would be expected to occur given the IC_{50} of WAY 100135 to be 15 nM for 5-HT_{1A} receptors [256, 257]. Interestingly, there was not a significant reduction in PDGF receptor activation when 1000 nM WAY 100135 was used yet there was at 100 nM. Although unreported with WAY 100135, a structurally and functionally similar compound and 5-HT_{1A} antagonist, WAY 100635, was later discovered to be a strong dopamine D_4 agonist [258] as well. It is therefore possible that WAY 100135 may also activate other receptors such as the $G\alpha_i$-linked D_4 receptors at higher concentrations, which would increase phosphorylation of PDGF receptor through a transactivation pathway. If true, then this is an identical situation to fluoxetine-mediated transactivation inhibition: WAY 100135 may be transactivating PDGFβ receptor within 5 min but PDGFβ receptor phosphorylation would drop back down to baseline levels within 15 min, as shown with both 5-HT and fluoxetine-mediated transactivation. Subsequent 5-HT application after 45 min would occur in the WAY-induced desensitized period, resulting in no observed PDGFβ receptor phosphorylation. A dose-response and timed-response analysis of WAY 100135 would be of interest to determine if it can transactivate PDGFβ receptor acutely. A positive result may lead to a reevaluation of the involvement of 5-HT_{1A} receptors in this phenomenon.

5.3.3 Other considerations

In addition to the transactivation data elucidated in Chapter 3, there are other mechanistic questions that could be posed. For instance, with concentrations of 5-HT above 100 nM (i.e. 1 μM or 10 μM) in the 5-min dose response experiment (Figure 3.2, page 32), statistically significant increases in PDGFβ receptor phosphorylation were not detected. Was this because transactivation already had taken place and phosphorylation had returned to baseline? For example, a 1 μM 5-HT time course experiment similar to Figure 3.2 may reveal that maximum phosphorylation takes place earlier than 5 min, and along the same lines, a 5-HT concentration of 10 nM may take longer than 5 min to reach maximum phosphorylation (see Figure 5.3). This could determine whether transactivation is ultimately dependent on receptor affinity of the GPCR agonist, or whether a certain agonist concentration threshold is required to initiate transactivation.

We focused primarily on the PDGFβ receptor phosphorylation site Y1021 because it is implicated in another mechanistic pathway that is being investigated in our laboratory. Y1021 is the docking site of PLCγ, which we believe is involved in PDGFβ receptor signaling to NMDA receptors. However, there are several other PDGFβ phosphorylation sites whose activity could differ from Y1021 and should be investigated. These phosphorylation site profiles may also differ with respect to PDGF ligand-induced receptor phosphorylation, and this avenue of research is currently being investigated by other laboratory members.

In section 4.1.4, the notion of heterologous desensitization in transactivation was discussed. Clearly, this line of investigation should be expanded to clarify when desensitization begins and ends, although we do have preliminary evidence that indicates desensitization is limited to no more than 3 h. The mechanism and enzymes involved in the actual desensitization process should also be investigated. Additionally, although we staggered the application of agonists (e.g. we pretreated cells with fluoxetine, with 5-HT applied later), this was only because we thought that fluoxetine was acting solely as a 5-HT transporter antagonist, and required sufficient time for binding before agonist application. In the future, it would be of interest to add agonists simultaneously to determine if heterologous receptor activation can additively or synergistically affect transactivation, or whether phosphorylation levels are limited to a 1.5-2-fold increase.

It was observed in electrophysiology that PDGF-BB application acutely decreased NMDA-evoked peak currents in isolated hippocampal neurons [234], whereas 5-CT application increased NMDA-evoked peak currents [259]. Does this latter result depend on PDGF receptor activity, and if so, do other agents that cause transactivation also modulate NMDA-evoked currents in a PDGF receptor-dependent manner? This could potentially lead to greater evidence supporting a signaling triad between 5-HT receptors (or possibly GPCRs in general), PDGF receptors, and NMDA receptors. In addition, we had observed that direct activation of PDGFβ receptors by PDGF-BB enhanced long-term depression in hippocampal neurons as measured by a decrease in excitatory post-synaptic potential amplitude [234]. If activation of PDGF receptors via transactivation can elicit similar results then this may further our understanding of the cellular relevance of transactivation.

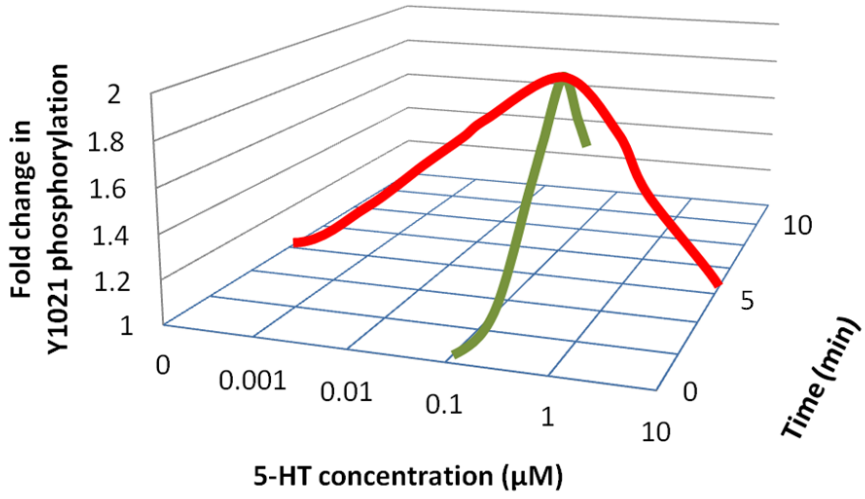


Figure 5.3. **Fold change in phosphorylation of PDGFβ receptor with respect to 5-HT concentration and exposure time.**
Data from the concentration (Figure 3.6) and exposure time (Figure 3.2) to 5-HT are plotted on the same graph, with a phosphorylation maximum and intercept at 0.1 µM, 5 min. One future experiment could determine whether maximum receptor phosphorylation seen in transactivation at a given concentration or exposure time is limited to the maxima created by these two curves. That is, if a different concentration were used in a time course experiment, would the maximum phosphorylation occur at the intercept with the red curve (i.e. at 5 min), or would a different time point yield the maximum phosphorylation? Likewise, if a different exposure time were used in the dose-response experiment, would the maximum phosphorylation occur at the intercept with the green curve (i.e. at 0.1 µM), or would a different concentration yield the maximum phosphorylation?

5.4 Final remarks

The notion of transactivation of RTKs has traditionally been attributed to their activation via GPCRs. Given the recent influx of new data showing PDGF receptor activation by signaling pathways and receptors other than GPCRs (particularly ROS), it would be reasonable to suggest that the definition of transactivation may eventually evolve to include these alternate means of "ligand-independent RTK activation". Certainly, the transactivation of PDGF receptors has shown important mitogenic effects that result in cell survival, proliferation, and disease states in various cell types and may in fact account, at least partly, for these effects and other growth factor-like outcomes.

With the physiological relevance of transactivation still requiring thorough investigation, there are clearly many more questions to be answered. At the very least, these data may indicate that PDGF receptor transactivation is playing a vital role in cell survival in the short term (minutes to hours) by inducing a tolerance to toxic insults, likely via inhibition of apoptosis. Long-term (days to weeks) studies, however, suggest that prolonged growth factor transactivation can lead to abnormal cell proliferation and vascular remodeling as observed in pulmonary hypertension and atherosclerosis. In fact, short-term use of transactivation-inducing agonists may be beneficial in a prophylactic or acute treatment of brain injuries such as hypoxia, oxidative stress, excitotoxicity, and other neurodegenerative diseases. Interestingly, the administration of the dopamine precursor levodopa is widely used as treatment to Parkinson's disease, a disorder that results from loss of dopaminergic neurons in the substantia nigra and manifests various motor deficiencies. Levodopa, which is decarboxylated to dopamine in the CNS, may not only be replacing a critical neurotransmitter but may also be neuroprotective to neurons expressing dopamine receptors via RTK transactivation pathways. An experiment investigating RTK phosphorylation responses to short and chronic levodopa exposure *in vivo*, as well as cognitive outcomes of levodopa versus levodopa plus RTK antagonist-treated conditions would test this hypothesis could provide valuable insight.

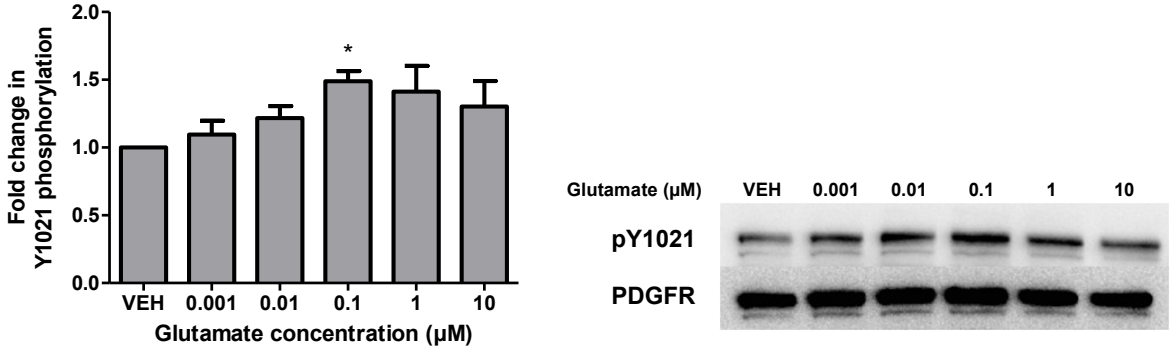
Thus, this study contributes to the already-intricate realm of cellular signal transduction; through our quest of knowledge and discovery, we continue to map out this complex web, teasing out one strand at a time.

Appendix A
Supplementary Data

This appendix contains additional data that may be of interest to the reader. These did not make the final cut and was left on the metaphorical editing room floor. Being one of the first graduate students in the laboratory and starting from scratch necessitated that I have multiple horses in the race in case some experimental directions flopped. These are, however, interesting scientific data that could easily spawn other research projects.

Glutamate induces PDGFβ receptor transactivation

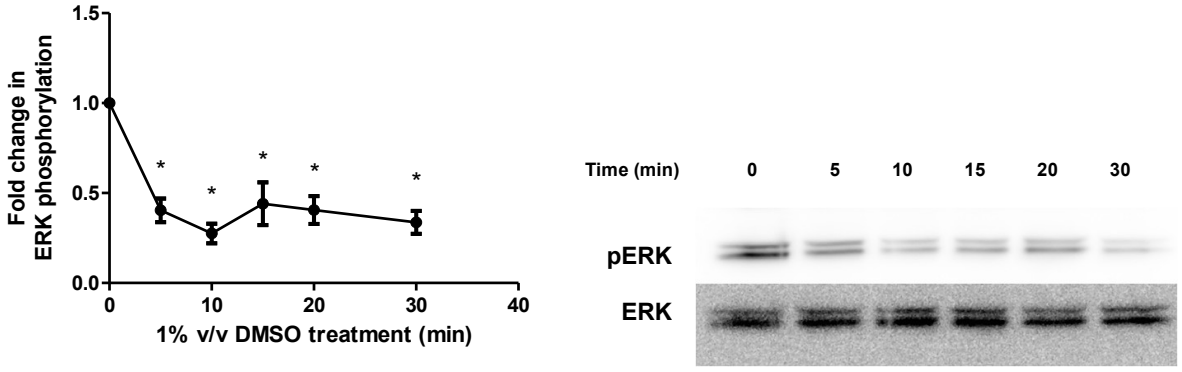
SH-SY5Y cells express metabotropic glutamate receptors [260]. Given that both dopamine [68] and 5-HT were able to activate PDGF receptors through $G\alpha_i$ receptors, we applied glutamate to SH-SY5Y cells and observed a significant increase in Y1021 of PDGF receptor at 100 nM (Appendix Figure I). Metabotropic glutamate receptors are linked primarily to $G\alpha_i$ and some $G\alpha_q$ [261], and thus this $G\alpha_i$-initiated pathway further corroborates the idea that transactivation events are initiated by $G\alpha_i$-coupled GPCRs. This idea should clearly be expanded as glutamate and the time course involved potentially relates to the mechanism of glutamate-induced excitotoxicity, a common pathology that leads to neuron death.



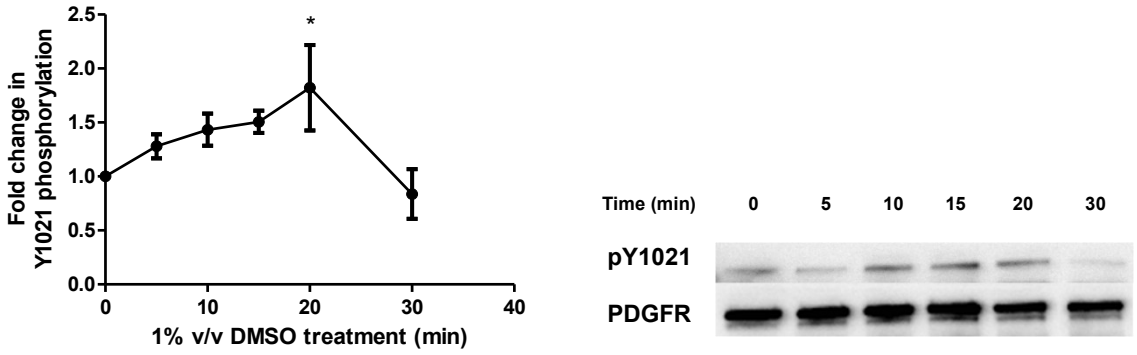
Appendix Figure I. Glutamate is able to transactivate PDGFβ receptors.
SH-SY5Y cells were treated for 5 min with 0 to 10 μM glutamate. Following drug treatments, cell lysates were evaluated by Western blot analysis as described in Materials and methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 6$).

Dimethyl sulfoxide affects basal phosphorylation of multiple proteins

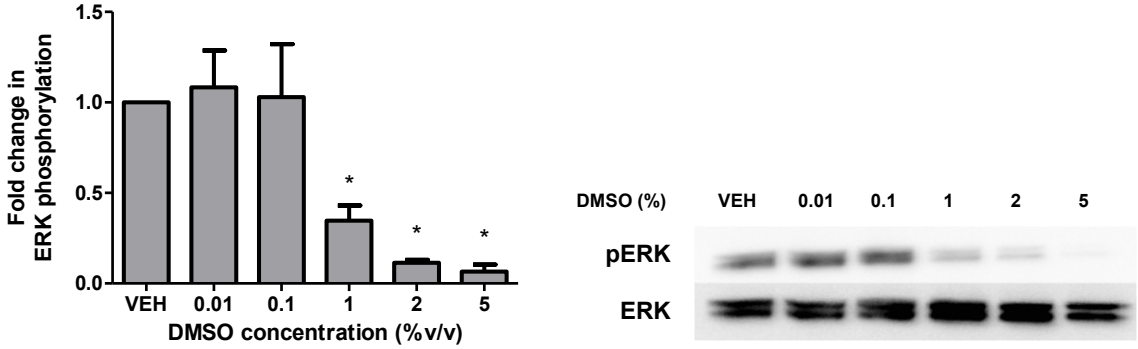
One of the more surprising and interesting results arose from control experiments involving dimethyl sulfoxide (DMSO). Some pharmaceuticals are not water-soluble and require solubilization in DMSO. Since DMSO is able to pass through the plasma membrane [262, 263], it may affect signal transduction. In order to determine the effect of DMSO on SH-SY5Y cells, we applied a1% DMSO solution to cells for 0-30 min and measured the fold-change in baseline phosphorylation of ERK1/2 and PDGFβ receptor Y1021 (Appendix Figure II and Appendix Figure III). We also performed a dose-response analysis of DMSO with 0-5% solutions for 40 min and measured the effect on ERK1/2, PDGFβ receptor Y1021 and Src Y418 (Appendix Figure IV, Appendix Figure V, and Appendix Figure VI). Surprisingly, cells treated with DMSO concentrations of 1% or higher resulted in ERK1/2 phosphorylation being reduced by at least 50% below baseline. Phosphorylation of Src Y418 also showed a decrease in phosphorylation above 1%. PDGFβ receptor Y1021 phosphorylation results were not statistically significant after 5 min, but did show an increase after 20 min at a 1% concentration. Therefore, concentrations of our drug stocks were limited to less than 1% DMSO.



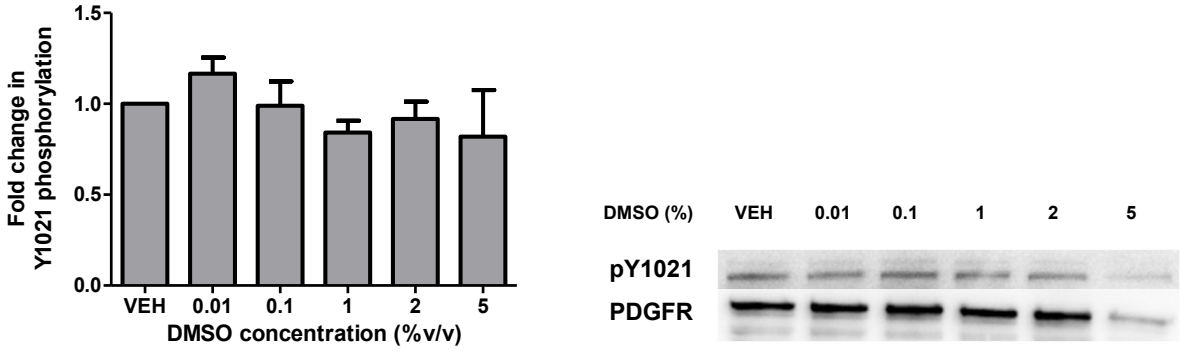
Appendix Figure II. DMSO treatment significantly affects basal ERK 1/2 phosphorylation.
SH-SY5Y cells were treated with 1% (v/v) DMSO for 0, 5, 10, 15, 20, or 30 min. Following drug treatments, cell lysates were evaluated by Western blot analysis as described in Materials and methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in phospho-ERK immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and total ERK1/2 are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 4$).



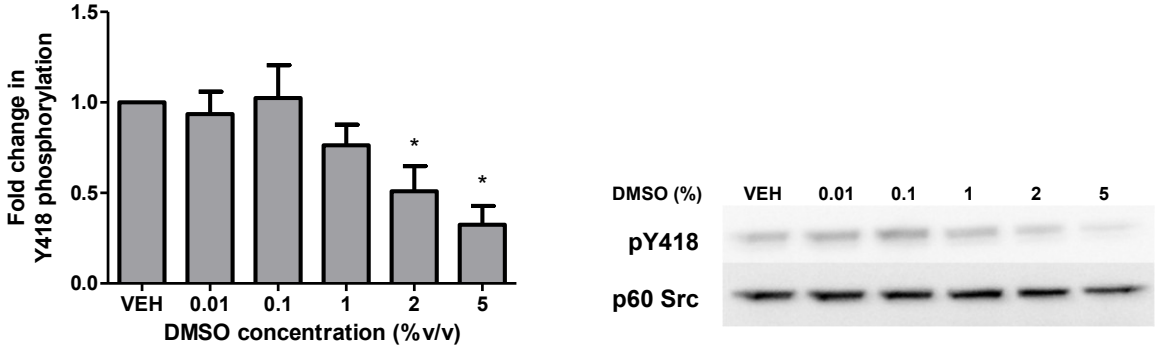
Appendix Figure III. DMSO treatment significantly affects basal PDGFβ receptor phosphorylation.
SH-SY5Y cells were treated with 1% (v/v) DMSO for 0, 5, 10, 15, 20, or 30 min. Following drug treatments, cell lysates were evaluated by Western blot analysis as described in Materials and methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 4$).



Appendix Figure IV. Basal ERK1/2 phosphorylation is affected by DMSO treatment in a concentration-dependent manner.
SH-SY5Y cells were treated with 0, 0.01, 0.1, 1, 2, or 5% (v/v) DMSO for 40 min. Following drug treatments, cell lysates were evaluated by Western blot analysis as described in Materials and methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in phospho-ERK immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and total ERK1/2 are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 5$).



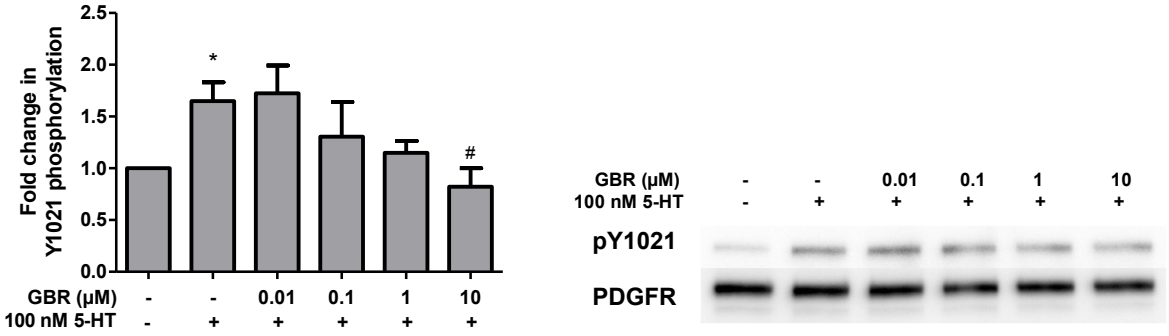
Appendix Figure V. 5-min DMSO treatment does not affect basal PDGFβ receptor phosphorylation.
SH-SY5Y cells were treated with 0, 0.01, 0.1, 1, 2, or 5% (v/v) DMSO for 40 min. Following drug treatments, cell lysates were evaluated by Western blot analysis as described in Materials and methods. Data were normalized to total PDGFRβ protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (n = 3).



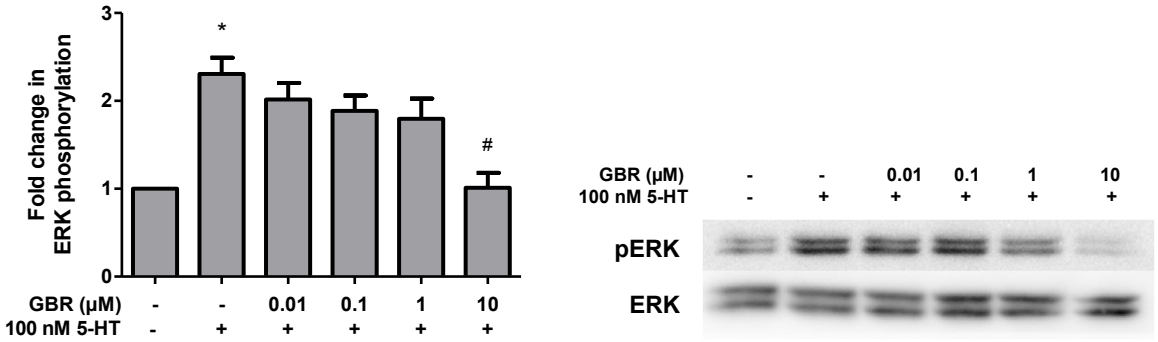
Appendix Figure VI. DMSO treatment affects basal Src phosphorylation.
SH-SY5Y cells were treated with 0, 0.01, 0.1, 1, 2, or 5% (v/v) DMSO for 40 min. Following drug treatments, cell lysates were evaluated by Western blot analysis as described in Materials and methods. Data were normalized to total p60 Src protein expression and are expressed as the fold change in phospho-418 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-Src Y418 (pY418) and p60 Src are shown (* = $p<0.05$ compared to vehicle-treated cells; n = 5).

GBR 12935 also transactivates PDGFβ receptor

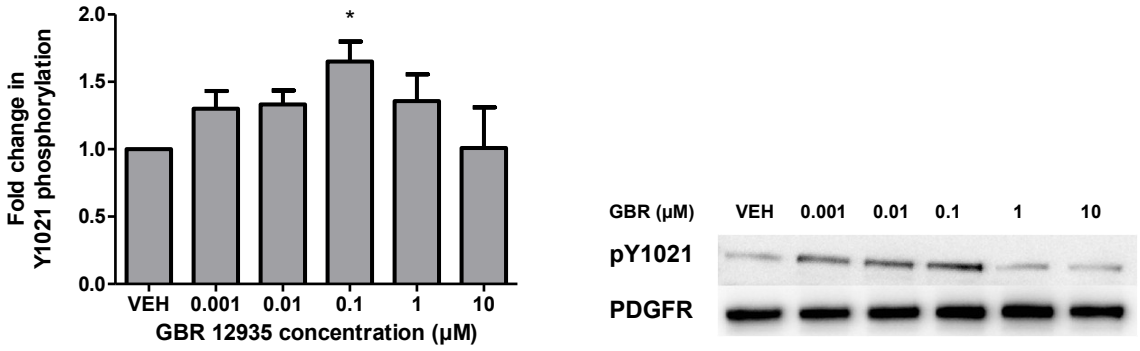
In addition to the 5-HTT inhibitors fluoxetine and citalopram, we also examined the effect of a dopamine/norepinephrine transporter inhibitor, GBR 12935. These experiments were not continued in order to more fully examine the effect of fluoxetine, as this drug is currently on the market and may have had a higher impact should any "interesting" results have emerged. For example, a newly discovered indication for a drug already on the market would require less work/cost as phase I clinical trial safety data has already been done. GBR 12935 is not approved for human administration as of yet. Like fluoxetine and citalopram, GBR 12935 blocked acute 5-HT-induced PDGFβ phosphorylation at Y1021 and ERK1/2 at micromolar concentrations (Appendix Figure VII and Appendix Figure VIII). Also, this led to the determination that GBR 12935 transactivated the PDGFβ receptor by itself, possibly through an unknown GPCR (Appendix Figure IX). Furthermore it was more potent than either fluoxetine or citalopram, requiring only 0.1 μM instead of 1 or 10 μM on a 5-min dose-response curve. Similarly, a 10 μM GBR 12935 time course showed maximum phosphorylation after only 2 min at 10 μM (Appendix Figure X), compared with 5 min for fluoxetine or citalopram.



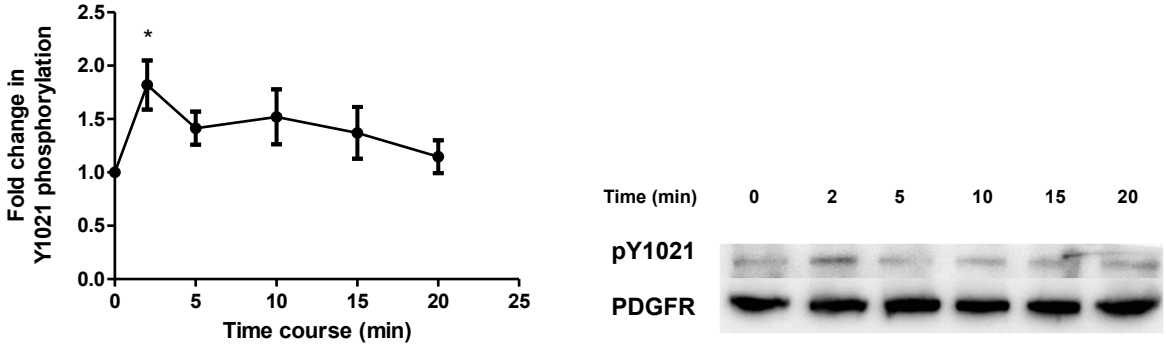
Appendix Figure VII. GBR 12935 can block 5-HT-induced PDGFβ receptor transactivation.
SH-SY5Y cells were pretreated with 0.01 to 10 μM GBR 12935 (GBR) for 45 min followed by 5 min treatment with 100 nM 5-HT. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFβ receptor protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared to 5-HT-treated cells; $n = 5$).



Appendix Figure VIII. GBR 12935 can block 5-HT-induced ERK1/2 phosphorylation.
SH-SY5Y cells were pretreated with 0.01 to 10 μM GBR 12935 (GBR) for 45 min followed by 5 min treatment with 100 nM 5-HT. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total ERK1/2 protein expression and are expressed as the fold change in phospho-ERK1/2 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-ERK1/2 (pERK) and ERK1/2 are shown (* = $p<0.05$ compared to vehicle-treated cells; # = $p<0.05$ compared to 5-HT-treated cells; $n = 8$).



Appendix Figure IX. GBR 12935-induced PDGFβ receptor transactivation is concentration-dependent.
SH-SY5Y cells were treated with 0, 0.01, 0.1, 1, 10 μM GBR 12935 for 5 min. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFβ receptor protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 5$).



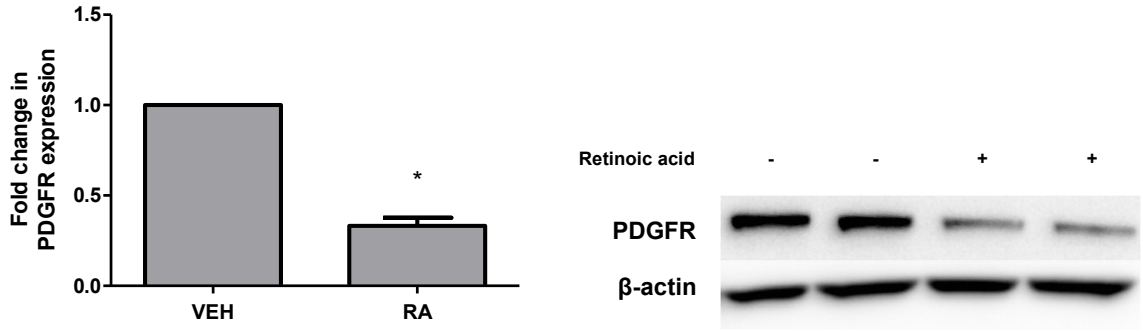
Appendix Figure X. GBR 12935 itself can transactivate PDGFβ receptor.
SH-SY5Y cells were treated with 10 μM GBR 12935 for 0, 2, 5, 10, 15 or 20 min. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFβ receptor protein expression and are expressed as the fold change in phospho-1021 immunoreactivity compared to vehicle-treated cells. Representative blots for phospho-PDGFRβ 1021 (pY1021) and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 6$).

Differentiated SH-SY5Y cells

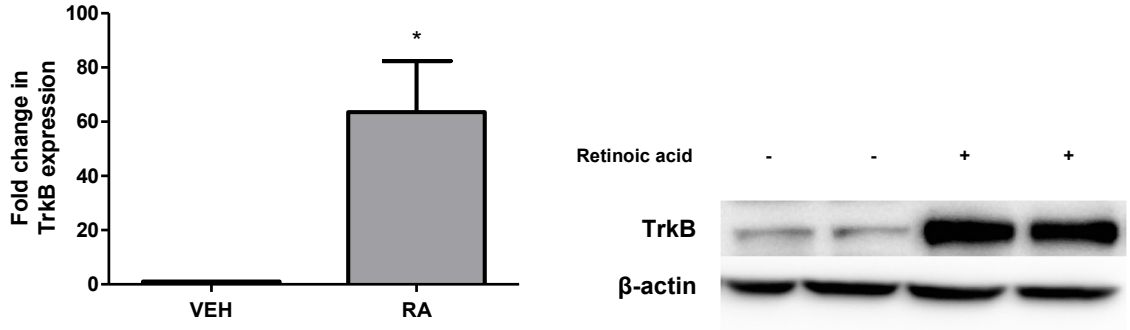
It has been postulated that SH-SY5Y cells can be differentiated into a more neuron-like form through the use of retinoic acid [264]. An attempt to replicate this phenomenon was performed. SH-SY5Y cells were grown to 70% confluency in complete growth media (DMEM/F12, supplemented with 10% FBS). At this point, serum concentration was reduced to 1% and cells were treated with 10 μM retinoic acid or vehicle for 6 days. Media were changed every two days (at 1% FBS) to replenish the retinoic acid. On the seventh day, cells were serum-starved overnight (with no retinoic acid present) prior to further drug treatments. While vehicle-treated cells had approximately doubled in cell number (as determined by microscopic inspection and total protein assay, data not shown), retinoic acid-treated cell numbers remained relatively constant from the point of retinoic acid application and reduced serum conditions. These cells also displayed slightly more outgrowth of neurite-like processes. There were also changes in the expression of two relevant proteins: retinoic acid-treated cells showed an approximately 3-fold decrease in p180 PDGFβ receptor expression (Appendix Figure XI), while expression of p145 TrkB had increased by approximately 60-fold (Appendix Figure XII) as determined by western blotting.

After serum starvation, both retinoic acid- and vehicle-treated cells were subsequently subjected to 100 μM 5-HT or vehicle for 5 min. Despite these changes in PDGFβ and TrkB receptor expression, western blot analyses showed that 5-HT was still capable of transactivating both receptor types with a similar increase in phosphorylation at site Y1021 (Appendix Figure XIII) and Y816 (Appendix Figure XIV), respectively. This would seem to indicate that transactivation is not dependent on total receptor expression or on the level of differentiation of the cell, and may be a universal process.

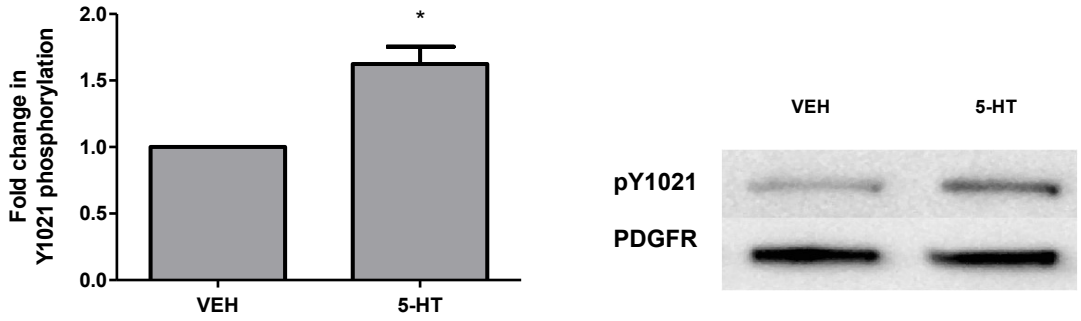
In hindsight, differentiated SH-SY5Y may have been a better platform to use for the transfection experiments, since they do not divide. Knockdown of 5-HTT protein likely failed due to cells growing too rapidly, and thus the knockdown effect was occluded. The other possibility is that the 5-HTT protein has a long half-life.



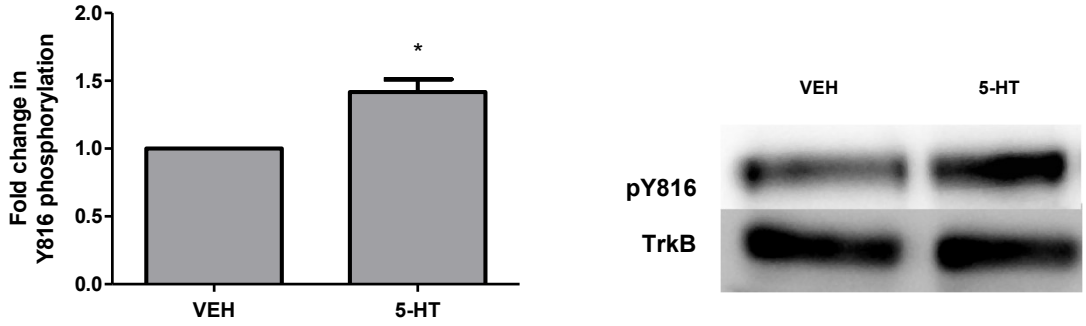
Appendix Figure XI. Differentiated SH-SY5Y cells show a decrease in PDGFβ receptor expression.
SH-SY5Y cells were treated with 10 μM retinoic acid (RA) for 6 days. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to β-actin protein expression and are expressed as the fold change in PDGFβ receptor immunoreactivity compared to vehicle-treated cells. Representative blots for PDGFRβ and β-actin are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 8$).



Appendix Figure XII. Differentiated SH-SY5Y cells show an increase in TrkB receptor expression.
SH-SY5Y cells were treated with 10 μM retinoic acid (RA) for 6 days. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to β-actin protein expression and are expressed as the fold change in TrkB receptor immunoreactivity compared to vehicle-treated cells. Representative blots for TrkB and β-actin are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 6$).



Appendix Figure XIII. 5-HT transactivates PDGFβ receptors in differentiated SH-SY5Y cells.
SH-SY5Y cells were treated with 10 μM retinoic acid for 6 days. Cells were then subjected to 100 μM 5-HT or vehicle for 5 min. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total PDGFβ receptor protein expression and are expressed as the fold change in phospho-Y1021 PDGFβ receptor immunoreactivity compared to vehicle-treated cells. Representative blots for pY1021 and PDGFRβ are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 5$).



Appendix Figure XIV. 5-HT transactivates TrkB receptors in differentiated SH-SY5Y cells.
SH-SY5Y cells were treated with 10 μM retinoic acid for 6 days. Cells were then subjected to 100 μM 5-HT or vehicle for 5 min. Following drug treatments, cells were lysed and lysates were evaluated by Western blot as described in the methods. Data were normalized to total TrkB receptor protein expression and are expressed as the fold change in phospho-Y816 TrkB receptor immunoreactivity compared to vehicle-treated cells. Representative blots for pY816 and TrkB are shown (* = $p<0.05$ compared to vehicle-treated cells; $n = 7$).

Appendix B

Research activity resulting from this program

PUBLICATIONS AND MANUSCRIPTS

Kruk JS, Beazely MA. Platelet-derived growth factor receptor transactivation: Molecular mechanisms and clinical implications. (Review article.) Manuscript in preparation.

Kruk JS, Grimberg N, Kouchmeshky A, Rezkella M, Beazely MA. Transactivation of receptor tyrosine kinases by dopamine receptors. (Book chapter.) Manuscript in preparation.

Liu H, Saffi GT, Vasefi MS, Choi V, **Kruk JS**, Ahmed N, Gondora N, Mielke J, Leonenko Z, Beazely MA. β-amyloid inhibits PDGFβ receptor activation and prevents PDGF-BB-induced neuroprotection. Manuscript in preparation.

Kruk JS, Vasefi MS, Heikkila JJ, Beazely MA. Fluoxetine-induced transactivation of the platelet-derived growth factor type β receptor reveals a novel heterologous desensitization process. Submitted to Molecular and Cellular Neuroscience.

Vasefi MS, Yang K, Li J, **Kruk JS**, Heikkila JJ, Jackson MF, MacDonald JF, Beazely MA. (2013). Acute 5-HT7 receptor activation increases NMDA-evoked currents and differentially alters NMDA receptor subunit phosphorylation and trafficking in hippocampal neurons. Mol Brain 6(1):24.

Kruk JS, Vasefi MS, Heikkila JJ, Beazely MA. (2013). Reactive oxygen species are required for 5-HT-induced transactivation of neuronal platelet-derived growth factor and TrkB receptors, but not for ERK1/2 activation. PLoS One 8(9):e77027.

Vasefi MS, **Kruk JS**, Heikkila JJ, Beazely MA. (2013). 5-HT7 receptor-mediated neuroprotection against NMDA–induced excitotoxicity is PDGFß receptor-dependent. J Neurochem 125(1):26-36.

Kruk JS, Vasefi MS, Liu H, Heikkila JJ, Beazely MA. (2013). 5-HT1A receptors transactivate the platelet-derived growth factor receptor type beta in neuronal cells. Cell Signal. 25(1):133-43.

Vasefi MS, **Kruk JS**, Liu H, Heikkila JJ, Beazely MA. (2012). Activation of 5-HT7 receptors increases neuronal platelet-derived growth factor beta receptor expression. Neurosci Lett 511:65-69.

PRESENTATIONS

Guest lecturer, PHARM 141 - Pharmacology (University of Waterloo School of Pharmacy), 2013
"Antimicrobial drugs and mechanisms of resistance."

Poster, Association of Faculties of Pharmacy: Canadian Pharmacy Education and Research Conference (Niagara-on-the-Lake, ON), 2013
Kruk JS, Beazely MA. "Fluoxetine-induced transactivation of the platelet-derived growth factor type β receptor in serotonergic signaling reveals heterologous desensitization in neurons."

Seminar, University of Waterloo School of Pharmacy Research Seminar Series (Waterloo, ON), 2013
Kruk, JS. "Linking hypotheses of clinical depression: A role for growth factor transactivation?"

Poster, Southern Ontario Neuroscience Association (Waterloo, ON), 2013
Kruk JS, Beazely MA. "Transactivation of platelet-derived growth factor type beta receptors is subject to heterologous desensitization in neuronal cultures."

Seminar, University of Waterloo Department of Biology (Waterloo, ON), 2013
Kruk, JS. "Serotonin-mediated TrkB neurotrophic receptor transactivation in neurons: A possible link to depression?"

Poster, Experimental Biology (Boston, MA), 2013
Beazely MA, **Kruk, JS**. "Serotonin transactivation of PDGFβ receptors results in a heterologous desensitization to subsequent transactivation stimuli."

Poster, The Great Lakes G Protein-Coupled Receptor Retreat (London, ON), 2012
Kruk JS, Vasefi MS, Beazely MA. "Monoamine transporter inhibitors can transactivate the platelet-derived growth factor receptor type beta in neuronal cells."

Poster, Southern Ontario Neuroscience Association (Guelph, ON), 2011
Kruk JS, Beazely MA. "Serotonin transactivates platelet-derived growth factor receptor type beta in neuronal cells."

Seminar, University of Waterloo (Waterloo, ON), 2011
Kruk, JS. "Serotonin transactivates PDGFRβ in the neuronal cell line SH-SY5Y."

Poster, The Great Lakes G Protein-Coupled Receptor Retreat (King City, ON), 2010
Beazely MA, **Kruk JS**, Vasefi SM, and Yang K. "Bidirectional regulation of NMDA receptor signaling by type 7 serotonin receptors."

Appendix C

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Inclusion in a thesis or dissertation	Yes	Yes with full acknowledgement of final article	Yes with full acknowledgement of final article

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Bibliography

1. Audet M and Bouvier M. 2012. Restructuring G-protein- coupled receptor activation. *Cell* 151(1):14-23.

2. Gutkind JS. 1998. The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. *J Biol Chem* 273(4):1839-1842.

3. Hubbard KB and Hepler JR. 2006. Cell signalling diversity of the Gqalpha family of heterotrimeric G proteins. *Cell Signal* 18(2):135-150.

4. Hofmann KP, Scheerer P, Hildebrand PW, Choe HW, Park JH, Heck M, and Ernst OP. 2009. A G protein-coupled receptor at work: the rhodopsin model. *Trends Biochem Sci* 34(11):540-552.

5. Luttrell LM. 2008. Reviews in molecular biology and biotechnology: transmembrane signaling by G protein-coupled receptors. *Mol Biotechnol* 39(3):239-264.

6. Luttrell LM. 2006. Transmembrane signaling by G protein-coupled receptors. *Methods Mol Biol* 332:3-49.

7. Tuteja N. 2009. Signaling through G protein coupled receptors. *Plant Signal Behav* 4(10):942-947.

8. Ferguson SS. 2001. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev* 53(1):1-24.

9. Pitcher JA, Freedman NJ, and Lefkowitz RJ. 1998. G protein-coupled receptor kinases. *Annu Rev Biochem* 67:653-692.

10. Steiner JA, Carneiro AM, and Blakely RD. 2008. Going with the flow: trafficking-dependent and -independent regulation of serotonin transport. *Traffic* 9(9):1393-1402.

11. Monti JM and Jantos H. 2008. The roles of dopamine and serotonin, and of their receptors, in regulating sleep and waking. *Prog Brain Res* 172:625-646.

12. Filip M and Bader M. 2009. Overview on 5-HT receptors and their role in physiology and pathology of the central nervous system. *Pharmacol Rep* 61(5):761-777.

13. Geldenhuys WJ and Van der Schyf CJ. 2009. The serotonin 5-HT6 receptor: a viable drug target for treating cognitive deficits in Alzheimer's disease. *Expert Rev Neurother* 9(7):1073-1085.

14. Abrams JK, Johnson PL, Hollis JH, and Lowry CA. 2004. Anatomic and functional topography of the dorsal raphe nucleus. *Ann N Y Acad Sci* 1018:46-57.

15. Hornung JP. 2003. The human raphe nuclei and the serotonergic system. *J Chem Neuroanat* 26(4):331-343.

16. Michelsen KA, Schmitz C, and Steinbusch HW. 2007. The dorsal raphe nucleus--from silver stainings to a role in depression. *Brain Res Rev* 55(2):329-342.

17. Mohammad-Zadeh LF, Moses L, and Gwaltney-Brant SM. 2008. Serotonin: a review. *J Vet Pharmacol Ther* 31(3):187-199.

18. Elhwuegi AS. 2004. Central monoamines and their role in major depression. *Prog Neuropsychopharmacol Biol Psychiatry* 28(3):435-451.

19. Sudhof TC. 2008. Neurotransmitter release. *Handb Exp Pharmacol* (184):1-21.

20. Kubota N, Kiuchi Y, Nemoto M, Oyamada H, Ohno M, Funahashi H, Shioda S, and Oguchi K. 2001. Regulation of serotonin transporter gene expression in human glial cells by growth factors. *Eur J Pharmacol* 417(1-2):69-76.

21. Larsen MB, Fjorback AW, and Wiborg O. 2006. The C-terminus is critical for the functional expression of the human serotonin transporter. *Biochemistry* 45(4):1331-1337.

22. Nobukuni M, Mochizuki H, Okada S, Kameyama N, Tanaka A, Yamamoto H, Amano T, Seki T, and Sakai N. 2009. The C-terminal region of serotonin transporter is important for its trafficking and glycosylation. *J Pharmacol Sci* 111(4):392-404.

23. Andersen J, Kristensen AS, Bang-Andersen B, and Stromgaard K. 2009. Recent advances in the understanding of the interaction of antidepressant drugs with serotonin and norepinephrine transporters. *Chem Commun (Camb)* (25):3677-3692.

24. Kendler KS, Kuhn JW, Vittum J, Prescott CA, and Riley B. 2005. The interaction of stressful life events and a serotonin transporter polymorphism in the prediction of episodes of major depression: a replication. *Arch Gen Psychiatry* 62(5):529-535.

25. Gerretsen P and Pollock BG. 2008. Pharmacogenetics and the serotonin transporter in late-life depression. *Expert Opin Drug Metab Toxicol* 4(12):1465-1478.

26. Mendlewicz J, Massat I, Souery D, Del-Favero J, Oruc L, Nothen MM, Blackwood D, Muir W, Battersby S, Lerer B, Segman RH, Kaneva R, Serretti A, Lilli R, Lorenzi C, Jakovljevic M, Ivezic S, Rietschel M, Milanova V, and Van Broeckhoven C. 2004. Serotonin transporter 5HTTLPR polymorphism and affective disorders: no evidence of association in a large European multicenter study. *Eur J Hum Genet* 12(5):377-382.

27. Alex KD and Pehek EA. 2007. Pharmacologic mechanisms of serotonergic regulation of dopamine neurotransmission. *Pharmacol Ther* 113(2):296-320.

28. Hannon J and Hoyer D. 2008. Molecular biology of 5-HT receptors. *Behav Brain Res* 195(1):198-213.

29. Lanfumey L and Hamon M. 2004. 5-HT1 receptors. *Curr Drug Targets CNS Neurol Disord* 3(1):1-10.

30. Toth M. 2003. 5-HT1A receptor knockout mouse as a genetic model of anxiety. *Eur J Pharmacol* 463(1-3):177-184.

31. Ramboz S, Oosting R, Amara DA, Kung HF, Blier P, Mendelsohn M, Mann JJ, Brunner D, and Hen R. 1998. Serotonin receptor 1A knockout: an animal model of anxiety-related disorder. *Proc Natl Acad Sci U S A* 95(24):14476-14481.

32. Heisler LK, Chu HM, Brennan TJ, Danao JA, Bajwa P, Parsons LH, and Tecott LH. 1998. Elevated anxiety and antidepressant-like responses in serotonin 5-HT1A receptor mutant mice. *Proc Natl Acad Sci U S A* 95(25):15049-15054.

33. Parks CL, Robinson PS, Sibille E, Shenk T, and Toth M. 1998. Increased anxiety of mice lacking the serotonin1A receptor. *Proc Natl Acad Sci U S A* 95(18):10734-10739.

34. Bert B, Fink H, Rothe J, Walstab J, and Bonisch H. 2008. Learning and memory in 5-HT(1A)-receptor mutant mice. *Behav Brain Res* 195(1):78-85.

35. Cornea-Hebert V, Riad M, Wu C, Singh SK, and Descarries L. 1999. Cellular and subcellular distribution of the serotonin 5-HT2A receptor in the central nervous system of adult rat. *J Comp Neurol* 409(2):187-209.

36. Bowers BJ, Henry MB, Thielen RJ, and McBride WJ. 2000. Serotonin 5-HT(2) receptor stimulation of dopamine release in the posterior but not anterior nucleus accumbens of the rat. *J Neurochem* 75(4):1625-1633.

37. Belvis R, Pagonabarraga J, and Kulisevsky J. 2009. Individual triptan selection in migraine attack therapy. *Recent Pat CNS Drug Discov* 4(1):70-81.

38. Meneses A. 2007. Stimulation of 5-HT1A, 5-HT1B, 5-HT2A/2C, 5-HT3 and 5-HT4 receptors or 5-HT uptake inhibition: short- and long-term memory. *Behav Brain Res* 184(1):81-90.

39. Popa D, Lena C, Fabre V, Prenat C, Gingrich J, Escourrou P, Hamon M, and Adrien J. 2005. Contribution of 5-HT2 receptor subtypes to sleep-wakefulness and respiratory control, and functional adaptations in knock-out mice lacking 5-HT2A receptors. *J Neurosci* 25(49):11231-11238.

40. Kayser V, Elfassi IE, Aubel B, Melfort M, Julius D, Gingrich JA, Hamon M, and Bourgoin S. 2007. Mechanical, thermal and formalin-induced nociception is differentially altered in 5-HT1A-/-, 5-HT1B-/-, 5-HT2A-/-, 5-HT3A-/- and 5-HTT-/- knock-out male mice. *Pain* 130(3):235-248.

41. Filip M, Bubar MJ, and Cunningham KA. 2004. Contribution of serotonin (5-hydroxytryptamine; 5-HT) 5-HT2 receptor subtypes to the hyperlocomotor effects of cocaine: acute and chronic pharmacological analyses. *J Pharmacol Exp Ther* 310(3):1246-1254.

42. Filip M. 2005. Role of serotonin (5-HT)2 receptors in cocaine self-administration and seeking behavior in rats. *Pharmacol Rep* 57(1):35-46.

43. Hoyer D, Hannon JP, and Martin GR. 2002. Molecular, pharmacological and functional diversity of 5-HT receptors. *Pharmacol Biochem Behav* 71(4):533-554.

44. Launay JM, Herve P, Peoc'h K, Tournois C, Callebert J, Nebigil CG, Etienne N, Drouet L, Humbert M, Simonneau G, and Maroteaux L. 2002. Function of the serotonin 5-hydroxytryptamine 2B receptor in pulmonary hypertension. *Nat Med* 8(10):1129-1135.

45. De Deurwaerdere P and Spampinato U. 1999. Role of serotonin(2A) and serotonin(2B/2C) receptor subtypes in the control of accumbal and striatal dopamine release elicited in vivo by dorsal raphe nucleus electrical stimulation. *J Neurochem* 73(3):1033-1042.

46. Tecott LH, Sun LM, Akana SF, Strack AM, Lowenstein DH, Dallman MF, and Julius D. 1995. Eating disorder and epilepsy in mice lacking 5-HT2c serotonin receptors. *Nature* 374(6522):542-546.

47. Heldin CH, Ostman A, and Ronnstrand L. 1998. Signal transduction via platelet-derived growth factor receptors. *Biochim Biophys Acta* 1378(1):F79-113.

48. Andrae J, Gallini R, and Betsholtz C. 2008. Role of platelet-derived growth factors in physiology and medicine. *Genes Dev* 22(10):1276-1312.

49. Heldin CH and Westermark B. 1999. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev* 79(4):1283-1316.

50. Alvarez RH, Kantarjian HM, and Cortes JE. 2006. Biology of platelet-derived growth factor and its involvement in disease. *Mayo Clin Proc* 81(9):1241-1257.

51. Keating MT, Harryman CC, and Williams LT. 1989. Platelet-derived growth factor receptor inducibility is acquired immediately after translation and does not require glycosylation. *J Biol Chem* 264(16):9129-9132.

52. Daniel TO, Milfay DF, Escobedo J, and Williams LT. 1987. Biosynthetic and glycosylation studies of cell surface platelet-derived growth factor receptors. *J Biol Chem* 262(20):9778-9784.

53. Pawson T and Scott JD. 1997. Signaling through scaffold, anchoring, and adaptor proteins. *Science* 278(5346):2075-2080.

54. Mellor P, Furber LA, Nyarko JN, and Anderson DH. 2012. Multiple roles for the p85alpha isoform in the regulation and function of PI3K signalling and receptor trafficking. *Biochem J* 441(1):23-37.

55. Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, and Bilanges B. 2010. The emerging mechanisms of isoform-specific PI3K signalling. *Nat Rev Mol Cell Biol* 11(5):329-341.

56. Vines CM. 2012. Phospholipase C. *Adv Exp Med Biol* 740:235-254.

57. Fukami K, Inanobe S, Kanemaru K, and Nakamura Y. 2010. Phospholipase C is a key enzyme regulating intracellular calcium and modulating the phosphoinositide balance. *Prog Lipid Res* 49(4):429-437.

58. Zeng L, Webster SV, and Newton PM. 2012. The biology of protein kinase C. *Adv Exp Med Biol* 740:639-661.

59. Newton AC. 1995. Protein kinase C: structure, function, and regulation. *J Biol Chem* 270(48):28495-28498.

60. Kovalenko M, Ronnstrand L, Heldin CH, Loubtchenkov M, Gazit A, Levitzki A, and Bohmer FD. 1997. Phosphorylation site-specific inhibition of platelet-derived growth factor beta-receptor autophosphorylation by the receptor blocking tyrphostin AG1296. *Biochemistry* 36(21):6260-6269.

61. Kruk JS, Vasefi MS, Liu H, Heikkila JJ, and Beazely MA. 2013. 5-HT(1A) receptors transactivate the platelet-derived growth factor receptor type beta in neuronal cells. *Cell Signal* 25(1):133-143.

62. Leveen P, Pekny M, Gebre-Medhin S, Swolin B, Larsson E, and Betsholtz C. 1994. Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes Dev* 8(16):1875-1887.

63. Soriano P. 1994. Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. *Genes Dev* 8(16):1888-1896.

64. Lindahl P, Johansson BR, Leveen P, and Betsholtz C. 1997. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* 277(5323):242-245.

65. Bostrom H, Willetts K, Pekny M, Leveen P, Lindahl P, Hedstrand H, Pekna M, Hellstrom M, Gebre-Medhin S, Schalling M, Nilsson M, Kurland S, Tornell J, Heath JK, and Betsholtz C. 1996. PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell* 85(6):863-873.

66. Meltzer EB and Noble PW. 2008. Idiopathic pulmonary fibrosis. *Orphanet J Rare Dis* 3:8.

67. Nister M, Claesson-Welsh L, Eriksson A, Heldin CH, and Westermark B. 1991. Differential expression of platelet-derived growth factor receptors in human malignant glioma cell lines. *J Biol Chem* 266(25):16755-16763.

68. Oak JN, Lavine N, and Van Tol HH. 2001. Dopamine D(4) and D(2L) Receptor Stimulation of the Mitogen-Activated Protein Kinase Pathway Is Dependent on trans-Activation of the Platelet-Derived Growth Factor Receptor. *Mol Pharmacol* 60(1):92-103.

69. Heeneman S, Haendeler J, Saito Y, Ishida M, and Berk BC. 2000. Angiotensin II induces transactivation of two different populations of the platelet-derived growth factor beta receptor. Key role for the p66 adaptor protein Shc. *J Biol Chem* 275(21):15926-15932.

70. Wang C, Wu LL, Liu J, Zhang ZG, Fan D, and Li L. 2008. Crosstalk between angiotensin II and platelet derived growth factor-BB mediated signal pathways in cardiomyocytes. *Chin Med J (Engl)* 121(3):236-240.

71. Gomez Sandoval YH and Anand-Srivastava MB. 2011. Enhanced levels of endogenous endothelin-1 contribute to the over expression of Gialpha protein in vascular smooth muscle cells from SHR: Role of growth factor receptor activation. *Cell Signal* 23(2):354-362.

72. Tanimoto T, Lungu AO, and Berk BC. 2004. Sphingosine 1-phosphate transactivates the platelet-derived growth factor beta receptor and epidermal growth factor receptor in vascular smooth muscle cells. *Circ Res* 94(8):1050-1058.

73. Goppelt-Struebe M, Fickel S, and Reiser CO. 2000. The platelet-derived-growth-factor receptor, not the epidermal-growth-factor receptor, is used by lysophosphatidic acid to activate p42/44 mitogen-activated protein kinase and to induce prostaglandin G/H synthase-2 in mesangial cells. *Biochem J* 345 Pt 2:217-224.

74. Wang L, Cummings R, Zhao Y, Kazlauskas A, Sham JK, Morris A, Georas S, Brindley DN, and Natarajan V. 2003. Involvement of phospholipase D2 in lysophosphatidate-induced transactivation of platelet-derived growth factor receptor-beta in human bronchial epithelial cells. *J Biol Chem* 278(41):39931-39940.

75. McMahon B, Mitchell D, Shattock R, Martin F, Brady HR, and Godson C. 2002. Lipoxin, leukotriene, and PDGF receptors cross-talk to regulate mesangial cell proliferation. *FASEB J* 16(13):1817-1819.

76. Chen CH, Cheng TH, Lin H, Shih NL, Chen YL, Chen YS, Cheng CF, Lian WS, Meng TC, Chiu WT, and Chen JJ. 2006. Reactive oxygen species generation is involved in epidermal growth factor receptor transactivation through the transient oxidization of Src homology 2-containing tyrosine phosphatase in endothelin-1 signaling pathway in rat cardiac fibroblasts. *Mol Pharmacol* 69(4):1347-1355.

77. McCole DF, Keely SJ, Coffey RJ, and Barrett KE. 2002. Transactivation of the epidermal growth factor receptor in colonic epithelial cells by carbachol requires extracellular release of transforming growth factor-alpha. *J Biol Chem* 277(45):42603-42612.

78. Liu Y, Li M, Warburton RR, Hill NS, and Fanburg BL. 2007. The 5-HT transporter transactivates the PDGFbeta receptor in pulmonary artery smooth muscle cells. *FASEB J* 21(11):2725-2734.

79. Nebigil CG, Launay JM, Hickel P, Tournois C, and Maroteaux L. 2000. 5-hydroxytryptamine 2B receptor regulates cell-cycle progression: cross-talk with tyrosine kinase pathways. *Proc Natl Acad Sci U S A* 97(6):2591-2596.

80. Wetzker R and Bohmer FD. 2003. Transactivation joins multiple tracks to the ERK/MAPK cascade. *Nat Rev Mol Cell Biol* 4(8):651-657.

81. Friedman WJ and Greene LA. 1999. Neurotrophin signaling via Trks and p75. *Exp Cell Res* 253(1):131-142.

82. Skaper SD. 2008. The biology of neurotrophins, signalling pathways, and functional peptide mimetics of neurotrophins and their receptors. *CNS Neurol Disord Drug Targets* 7(1):46-62.

83. Ip NY and Yancopoulos GD. 1996. The neurotrophins and CNTF: two families of collaborative neurotrophic factors. *Annu Rev Neurosci* 19:491-515.

84. Lindsay RM and Yancopoulos GD. 1996. GDNF in a bind with known orphan: accessory implicated in new twist. *Neuron* 17(4):571-574.

85. Lai KO, Fu WY, Ip FC, and Ip NY. 1998. Cloning and expression of a novel neurotrophin, NT-7, from carp. *Mol Cell Neurosci* 11(1-2):64-76.

86. Berkemeier LR, Winslow JW, Kaplan DR, Nikolics K, Goeddel DV, and Rosenthal A. 1991. Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. *Neuron* 7(5):857-866.

87. Lee R, Kermani P, Teng KK, and Hempstead BL. 2001. Regulation of cell survival by secreted proneurotrophins. *Science* 294(5548):1945-1948.

88. Butte MJ. 2001. Neurotrophic factor structures reveal clues to evolution, binding, specificity, and receptor activation. *Cell Mol Life Sci* 58(8):1003-1013.

89. Rantamaki T and Castren E. 2008. Targeting TrkB neurotrophin receptor to treat depression. *Expert Opin Ther Targets* 12(6):705-715.

90. Minichiello L. 2009. TrkB signalling pathways in LTP and learning. *Nat Rev Neurosci* 10(12):850-860.

91. Nagappan G and Lu B. 2005. Activity-dependent modulation of the BDNF receptor TrkB: mechanisms and implications. *Trends Neurosci* 28(9):464-471.

92. Lee FS and Chao MV. 2001. Activation of Trk neurotrophin receptors in the absence of neurotrophins. *Proc Natl Acad Sci U S A* 98(6):3555-3560.

93. Rajagopal R and Chao MV. 2006. A role for Fyn in Trk receptor transactivation by G-protein-coupled receptor signaling. *Mol Cell Neurosci* 33(1):36-46.

94. Wiese S, Jablonka S, Holtmann B, Orel N, Rajagopal R, Chao MV, and Sendtner M. 2007. Adenosine receptor A2A-R contributes to motoneuron survival by transactivating the tyrosine kinase receptor TrkB. *Proc Natl Acad Sci U S A* 104(43):17210-17215.

95. Kessler RC, Berglund P, Demler O, Jin R, Merikangas KR, and Walters EE. 2005. Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. *Arch Gen Psychiatry* 62(6):593-602.

96. Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, and Monteggia LM. 2002. Neurobiology of depression. *Neuron* 34(1):13-25.

97. Krishnan V and Nestler EJ. 2008. The molecular neurobiology of depression. *Nature* 455(7215):894-902.

98. Shaffery J, Hoffmann R, and Armitage R. 2003. The neurobiology of depression: perspectives from animal and human sleep studies. *Neuroscientist* 9(1):82-98.

99. Berton O and Nestler EJ. 2006. New approaches to antidepressant drug discovery: beyond monoamines. *Nat Rev Neurosci* 7(2):137-151.

100. Drevets WC. 2001. Neuroimaging and neuropathological studies of depression: implications for the cognitive-emotional features of mood disorders. *Curr Opin Neurobiol* 11(2):240-249.

101. Ressler KJ and Mayberg HS. 2007. Targeting abnormal neural circuits in mood and anxiety disorders: from the laboratory to the clinic. *Nat Neurosci* 10(9):1116-1124.

102. Sheline YI. 2003. Neuroimaging studies of mood disorder effects on the brain. *Biol Psychiatry* 54(3):338-352.

103. Sackeim HA. 2001. The definition and meaning of treatment-resistant depression. *J Clin Psychiatry* 62 Suppl 16:10-17.

104. Holtzheimer PE, 3rd and Nemeroff CB. 2006. Advances in the treatment of depression. *NeuroRx* 3(1):42-56.

105. Youdim MB, Edmondson D, and Tipton KF. 2006. The therapeutic potential of monoamine oxidase inhibitors. *Nat Rev Neurosci* 7(4):295-309.

106. Tuncel M and Ram VC. 2003. Hypertensive emergencies. Etiology and management. *Am J Cardiovasc Drugs* 3(1):21-31.

107. Homberg JR, Schubert D, and Gaspar P. 2010. New perspectives on the neurodevelopmental effects of SSRIs. *Trends Pharmacol Sci* 31(2):60-65.

108. Cipriani A, Brambilla P, Furukawa T, Geddes J, Gregis M, Hotopf M, Malvini L, and Barbui C. 2005. Fluoxetine versus other types of pharmacotherapy for depression. *Cochrane Database Syst Rev* 19(4):CD004185.

109. Hirschfeld RM. 2000. History and evolution of the monoamine hypothesis of depression. *J Clin Psychiatry* 61 Suppl 6:4-6.

110. Schildkraut JJ. 1965. The catecholamine hypothesis of affective disorders: a review of supporting evidence. *Am J Psychiatry* 122(5):509-522.

111. Kuhn R. 1958. The treatment of depressive states with G 22355 (imipramine hydrochloride). *Am J Psychiatry* 115(5):459-464.

112. Henry JP and Scherman D. 1989. Radioligands of the vesicular monoamine transporter and their use as markers of monoamine storage vesicles. *Biochem pharmacol* 38(15):2395-2404.

113. Lee S, Jeong J, Kwak Y, and Park SK. 2010. Depression research: where are we now? *Mol Brain* 3:8.

114. Pineyro G and Blier P. 1999. Autoregulation of serotonin neurons: role in antidepressant drug action. *Pharmacol Rev* 51(3):533-591.

115. Invernizzi R, Bramante M, and Samanin R. 1994. Chronic treatment with citalopram facilitates the effect of a challenge dose on cortical serotonin output: role of presynaptic 5-HT1A receptors. *Eur J Pharmacol* 260(2-3):243-246.

116. Hervas I, Vilaro MT, Romero L, Scorza MC, Mengod G, and Artigas F. 2001. Desensitization of 5-HT(1A) autoreceptors by a low chronic fluoxetine dose effect of the concurrent administration of WAY-100635. *Neuropsychopharmacology* 24(1):11-20.

117. Pompili M, Serafini G, Innamorati M, Moller-Leimkuhler AM, Giupponi G, Girardi P, Tatarelli R, and Lester D. 2010. The hypothalamic-pituitary-adrenal axis and serotonin abnormalities: a selective overview for the implications of suicide prevention. *Eur Arch Psychiatry Clin Neurosci* 260(8):583-600.

118. Arborelius L, Owens MJ, Plotsky PM, and Nemeroff CB. 1999. The role of corticotropin-releasing factor in depression and anxiety disorders. *J Endocrinol* 160(1):1-12.

119. Castren E and Rantamaki T. 2010. The role of BDNF and its receptors in depression and antidepressant drug action: Reactivation of developmental plasticity. *Dev Neurobiol* 70(5):289-297.

120. Duman RS and Monteggia LM. 2006. A neurotrophic model for stress-related mood disorders. *Biol Psychiatry* 59(12):1116-1127.

121. Nibuya M, Morinobu S, and Duman RS. 1995. Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. *J Neurosci* 15(11):7539-7547.

122. Shirayama Y, Chen AC, Nakagawa S, Russell DS, and Duman RS. 2002. Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. *J Neurosci* 22(8):3251-3261.

123. Monteggia LM, Luikart B, Barrot M, Theobold D, Malkovska I, Nef S, Parada LF, and Nestler EJ. 2007. Brain-derived neurotrophic factor conditional knockouts show gender differences in depression-related behaviors. *Biol Psychiatry* 61(2):187-197.

124. Gorski JA, Zeiler SR, Tamowski S, and Jones KR. 2003. Brain-derived neurotrophic factor is required for the maintenance of cortical dendrites. *J Neurosci* 23(17):6856-6865.

125. Eisch AJ, Bolanos CA, de Wit J, Simonak RD, Pudiak CM, Barrot M, Verhaagen J, and Nestler EJ. 2003. Brain-derived neurotrophic factor in the ventral midbrain-nucleus accumbens pathway: a role in depression. *Biol Psychiatry* 54(10):994-1005.

126. Gage FH. 2002. Neurogenesis in the adult brain. *J Neurosci* 22(3):612-613.

127. Dranovsky A and Hen R. 2006. Hippocampal neurogenesis: regulation by stress and antidepressants. *Biol Psychiatry* 59(12):1136-1143.

128. Surget A, Saxe M, Leman S, Ibarguen-Vargas Y, Chalon S, Griebel G, Hen R, and Belzung C. 2008. Drug-dependent requirement of hippocampal neurogenesis in a model of depression and of antidepressant reversal. *Biol Psychiatry* 64(4):293-301.

129. Pittenger C and Duman RS. 2008. Stress, depression, and neuroplasticity: a convergence of mechanisms. *Neuropsychopharmacology* 33(1):88-109.

130. Boldrini M, Underwood MD, Hen R, Rosoklija GB, Dwork AJ, John Mann J, and Arango V. 2009. Antidepressants increase neural progenitor cells in the human hippocampus. *Neuropsychopharmacology* 34(11):2376-2389.

131. Tsankova NM, Berton O, Renthal W, Kumar A, Neve RL, and Nestler EJ. 2006. Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. *Nat Neurosci* 9(4):519-525.

132. Schroeder FA, Lin CL, Crusio WE, and Akbarian S. 2007. Antidepressant-like effects of the histone deacetylase inhibitor, sodium butyrate, in the mouse. *Biol Psychiatry* 62(1):55-64.

133. Tsankova NM, Kumar A, and Nestler EJ. 2004. Histone modifications at gene promoter regions in rat hippocampus after acute and chronic electroconvulsive seizures. *J Neurosci* 24(24):5603-5610.

134. Avery D and Lubrano A. 1979. Depression treated with imipramine and ECT: the DeCarolis study reconsidered. *Am J Psychiatry* 136(4B):559-562.

135. Avery D and Winokur G. 1978. Suicide, attempted suicide, and relapse rates in depression. *Arch Gen Psychiatry* 35(6):749-753.

136. Zielinski RJ, Roose SP, Devanand DP, Woodring S, and Sackeim HA. 1993. Cardiovascular complications of ECT in depressed patients with cardiac disease. *Am J Psychiatry* 150(6):904-909.

137. Fink M. 2001. Convulsive therapy: a review of the first 55 years. *J Affect Disord* 63(1-3):1-15.

138. Rose D, Fleischmann P, Wykes T, Leese M, and Bindman J. 2003. Patients' perspectives on electroconvulsive therapy: systematic review. *BMJ* 326(7403):1363.

139. Schlaepfer TE, Cohen MX, Frick C, Kosel M, Brodesser D, Axmacher N, Joe AY, Kreft M, Lenartz D, and Sturm V. 2008. Deep brain stimulation to reward circuitry alleviates anhedonia in refractory major depression. *Neuropsychopharmacology* 33(2):368-377.

140. Vasefi MS, Kruk JS, Liu H, Heikkila JJ, and Beazely MA. 2012. Activation of 5-HT7 receptors increases neuronal platelet-derived growth factor beta receptor expression. *Neurosci Lett* 511(2):65-69.

141. Locht C. 1999. Molecular aspects of Bordetella pertussis pathogenesis. *Int Microbiol* 2(3):137-144.

142. Levitt ES, Purington LC, and Traynor JR. 2011. Gi/o-coupled receptors compete for signaling to adenylyl cyclase in SH-SY5Y cells and reduce opioid-mediated cAMP overshoot. *Mol Pharmacol* 79(3):461-471.

143. Fujio M, Togo Y, Tomozane H, Kuroita T, Morio Y, Katayama J, and Matsumoto Y. 2000. N-[[1-(2-phenylethyl)pyrrolidin-2-yl]methyl]cyclohexanecarboxamides as selective 5-HT1A receptor agonists. *Bioorg Med Chem Lett* 10(5):509-512.

144. el Ahmad Y, Laurent E, Maillet P, Talab A, Teste JF, Dokhan R, Tran G, and Ollivier R. 1997. New benzocycloalkylpiperazines, potent and selective 5-HT1A receptor ligands. *J Med Chem* 40(6):952-960.

145. Long JS, Natarajan V, Tigyi G, Pyne S, and Pyne NJ. 2006. The functional PDGFbeta receptor-S1P1 receptor signaling complex is involved in regulating migration of mouse embryonic fibroblasts in response to platelet derived growth factor. *Prostaglandins Other Lipid Mediat* 80(1-2):74-80.

146. Waters C, Sambi B, Kong KC, Thompson D, Pitson SM, Pyne S, and Pyne NJ. 2003. Sphingosine 1-phosphate and platelet-derived growth factor (PDGF) act via PDGF beta receptor-sphingosine 1-phosphate receptor complexes in airway smooth muscle cells. *J Biol Chem* 278(8):6282-6290.

147. Tsien RY. 1980. New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry* 19(11):2396-2404.

148. Paschen W, Hotop S, and Aufenberg C. 2003. Loading neurons with BAPTA-AM activates xbp1 processing indicative of induction of endoplasmic reticulum stress. *Cell Calcium* 33(2):83-89.

149. Liu B and Wu D. 2004. Analysis of G protein-mediated activation of phospholipase C in cultured cells. *Methods Mol Biol* 237:99-102.

150. Mikoshiba K. 2011. Role of IP receptor in development. *Cell Calcium* 49(5):331-340.

151. Chen K, Thomas SR, Albano A, Murphy MP, and Keaney JF, Jr. 2004. Mitochondrial function is required for hydrogen peroxide-induced growth factor receptor transactivation and downstream signaling. *J Biol Chem* 279(33):35079-35086.

152. Catarzi S, Giannoni E, Favilli F, Meacci E, Iantomasi T, and Vincenzini MT. 2007. Sphingosine 1-phosphate stimulation of NADPH oxidase activity: relationship with platelet-derived growth factor receptor and c-Src kinase. *Biochim Biophys Acta* 1770(6):872-883.

153. Sun C, Sellers KW, Sumners C, and Raizada MK. 2005. NAD(P)H oxidase inhibition attenuates neuronal chronotropic actions of angiotensin II. *Circ Res* 96(6):659-666.

154. O'Donnell BV, Tew DG, Jones OT, and England PJ. 1993. Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase. *Biochem J* 290 (Pt 1):41-49.

155. Rupin A, Paysant J, Sansilvestri-Morel P, Lembrez N, Lacoste JM, Cordi A, and Verbeuren TJ. 2004. Role of NADPH oxidase-mediated superoxide production in the regulation of E-selectin expression by endothelial cells subjected to anoxia/reoxygenation. *Cardiovasc Res* 63(2):323-330.

156. El-Benna J, Dang PM, and Gougerot-Pocidalo MA. 2008. Priming of the neutrophil NADPH oxidase activation: role of p47phox phosphorylation and NOX2 mobilization to the plasma membrane. *Semin Immunopathol* 30(3):279-289.

157. Sheppard FR, Kelher MR, Moore EE, McLaughlin NJ, Banerjee A, and Silliman CC. 2005. Structural organization of the neutrophil NADPH oxidase: phosphorylation and translocation during priming and activation. *J Leukoc Biol* 78(5):1025-1042.

158. Zhang J, Anastasiadis PZ, Liu Y, Thompson EA, and Fields AP. 2004. Protein kinase C (PKC) betaII induces cell invasion through a Ras/Mek-, PKC iota/Rac 1-dependent signaling pathway. *J Biol Chem* 279(21):22118-22123.

159. Mellor H and Parker PJ. 1998. The extended protein kinase C superfamily. *Biochem J* 332 (Pt 2):281-292.

160. Rendu F, Peoc'h K, Berlin I, Thomas D, and Launay JM. 2011. Smoking related diseases: the central role of monoamine oxidase. *Int J Environ Res Public Health* 8(1):136-147.

161. Liu J, Liao Z, Camden J, Griffin KD, Garrad RC, Santiago-Perez LI, Gonzalez FA, Seye CI, Weisman GA, and Erb L. 2004. Src homology 3 binding sites in the P2Y2 nucleotide receptor interact with Src and regulate activities of Src, proline-rich tyrosine kinase 2, and growth factor receptors. *J Biol Chem* 279(9):8212-8218.

162. Robin P, Boulven I, Desmyter C, Harbon S, and Leiber D. 2002. ET-1 stimulates ERK signaling pathway through sequential activation of PKC and Src in rat myometrial cells. *Am J Physiol Cell Physiol* 283(1):C251-260.

163. Kmiecik TE, Johnson PJ, and Shalloway D. 1988. Regulation by the autophosphorylation site in overexpressed pp60c-src. *Mol Cell Biol* 8(10):4541-4546.

164. Tsuchioka M, Takebayashi M, Hisaoka K, Maeda N, and Nakata Y. 2008. Serotonin (5-HT) induces glial cell line-derived neurotrophic factor (GDNF) mRNA expression via the transactivation of fibroblast growth factor receptor 2 (FGFR2) in rat C6 glioma cells. *J Neurochem* 106(1):244-257.

165. Li B, Zhang S, Zhang H, Nu W, Cai L, Hertz L, and Peng L. 2008. Fluoxetine-mediated 5-HT2B receptor stimulation in astrocytes causes EGF receptor transactivation and ERK phosphorylation. *Psychopharmacology* 201(3):443-458.

166. Kelly DJ, Cox AJ, Gow RM, Zhang Y, Kemp BE, and Gilbert RE. 2004. Platelet-derived growth factor receptor transactivation mediates the trophic effects of angiotensin II in vivo. *Hypertension* 44(2):195-202.

167. Jankov RP, Kantores C, Belcastro R, Yi S, Ridsdale RA, Post M, and Tanswell AK. 2005. A role for platelet-derived growth factor beta-receptor in a newborn rat model of endothelin-mediated pulmonary vascular remodeling. *Am J Physiol Lung Cell Mol Physiol* 288(6):L1162-1170.

168. Jalili T, Takeishi Y, and Walsh RA. 1999. Signal transduction during cardiac hypertrophy: the role of G alpha q, PLC beta I, and PKC. *Cardiovasc Res* 44(1):5-9.

169. Yoon CM, Hong BS, Moon HG, Lim S, Suh PG, Kim YK, Chae CB, and Gho YS. 2008. Sphingosine-1-phosphate promotes lymphangiogenesis by stimulating S1P1/Gi/PLC/Ca2+ signaling pathways. *Blood* 112(4):1129-1138.

170. Dingus J, Wells CA, Campbell L, Cleator JH, Robinson K, and Hildebrandt JD. 2005. G Protein betagamma dimer formation: Gbeta and Ggamma differentially determine efficiency of in vitro dimer formation. *Biochemistry* 44(35):11882-11890.

171. Poon LS, Chan AS, and Wong YH. 2009. Gbeta3 forms distinct dimers with specific Ggamma subunits and preferentially activates the beta3 isoform of phospholipase C. *Cell Signal* 21(5):737-744.

172. Dingus J and Hildebrandt JD. 2012. Synthesis and Assembly of G Protein betagamma Dimers: Comparison of In Vitro and In Vivo Studies. *Subcell Biochem* 63:155-180.

173. Locht C and Antoine R. 1995. A proposed mechanism of ADP-ribosylation catalyzed by the pertussis toxin S1 subunit. *Biochimie* 77(5):333-340.

174. Yano N, Ianus V, Zhao TC, Tseng A, Padbury JF, and Tseng YT. 2007. A novel signaling pathway for beta-adrenergic receptor-mediated activation of phosphoinositide 3-kinase in H9c2 cardiomyocytes. *Am J Physiol Heart Circ Physiol* 293(1):H385-393.

175. Roskoski R, Jr. 2005. Src kinase regulation by phosphorylation and dephosphorylation. *Biochem Biophys Res Commun* 331(1):1-14.

176. Rhee SG, Kang SW, Jeong W, Chang TS, Yang KS, and Woo HA. 2005. Intracellular messenger function of hydrogen peroxide and its regulation by peroxiredoxins. *Curr Opin Cell Biol* 17(2):183-189.

177. Callsen D, Sandau KB, and Brune B. 1999. Nitric oxide and superoxide inhibit platelet-derived growth factor receptor phosphotyrosine phosphatases. *Free Radic Biol Med* 26(11-12):1544-1553.

178. Tomic S, Greiser U, Lammers R, Kharitonenkov A, Imyanitov E, Ullrich A, and Bohmer FD. 1995. Association of SH2 domain protein tyrosine phosphatases with the epidermal growth factor receptor in human tumor cells. Phosphatidic acid activates receptor dephosphorylation by PTP1C. *J Biol Chem* 270(36):21277-21284.

179. Kharitonenkov A, Schnekenburger J, Chen Z, Knyazev P, Ali S, Zwick E, White M, and Ullrich A. 1995. Adapter function of protein-tyrosine phosphatase 1D in insulin receptor/insulin receptor substrate-1 interaction. *J Biol Chem* 270(49):29189-29193.

180. Cattaneo F, Iaccio A, Guerra G, Montagnani S, and Ammendola R. 2011. NADPH-oxidase-dependent reactive oxygen species mediate EGFR transactivation by FPRL1 in WKYMVm-stimulated human lung cancer cells. *Free Radic Biol Med* 51(6):1126-1136.

181. Vignais PV. 2002. The superoxide-generating NADPH oxidase: structural aspects and activation mechanism. *Cell Mol Life Sci* 59(9):1428-1459.

182. Guo D, Zhang JJ, and Huang XY. 2010. A new Rac/PAK/GC/cGMP signaling pathway. *Mol Cell Biochem* 334(1-2):99-103.

183. Bosco EE, Mulloy JC, and Zheng Y. 2009. Rac1 GTPase: a "Rac" of all trades. *Cell Mol Life Sci* 66(3):370-374.

184. George A, Pushkaran S, Li L, An X, Zheng Y, Mohandas N, Joiner CH, and Kalfa TA. 2010. Altered phosphorylation of cytoskeleton proteins in sickle red blood cells: the role of protein kinase C, Rac GTPases, and reactive oxygen species. *Blood Cells Mol Dis* 45(1):41-45.

185. Raad H, Paclet MH, Boussetta T, Kroviarski Y, Morel F, Quinn MT, Gougerot-Pocidalo MA, Dang PM, and El-Benna J. 2009. Regulation of the phagocyte NADPH oxidase activity: phosphorylation of gp91phox/NOX2 by protein kinase C enhances its diaphorase activity and binding to Rac2, p67phox, and p47phox. *FASEB J* 23(4):1011-1022.

186. Fontayne A, Dang PM, Gougerot-Pocidalo MA, and El-Benna J. 2002. Phosphorylation of p47phox sites by PKC alpha, beta II, delta, and zeta: effect on binding to p22phox and on NADPH oxidase activation. *Biochemistry* 41(24):7743-7750.

187. Shi G, Fu Y, Jiang W, Yin A, Feng M, Wu Y, Kawai Y, Miyamori I, and Fan C. 2011. Activation of Src-ATF1 pathway is involved in upregulation of Nox1, a catalytic subunit of NADPH oxidase, by aldosterone. *Endocr J* 58(6):491-499.

188. Gianni D, Taulet N, DerMardirossian C, and Bokoch GM. 2010. c-Src-mediated phosphorylation of NoxA1 and Tks4 induces the reactive oxygen species (ROS)-dependent formation of functional invadopodia in human colon cancer cells. *Mol Biol Cell* 21(23):4287-4298.

189. Stefanska J and Pawliczak R. 2008. Apocynin: molecular aptitudes. *Mediators Inflamm* 2008:106507.

190. Bayraktutan U, Blayney L, and Shah AM. 2000. Molecular characterization and localization of the NAD(P)H oxidase components gp91-phox and p22-phox in endothelial cells. *Arterioscler Thromb Vascular Biol* 20(8):1903-1911.

191. Li JM and Shah AM. 2002. Intracellular localization and preassembly of the NADPH oxidase complex in cultured endothelial cells. *J Biol Chem* 277(22):19952-19960.

192. Dusting GJ, Selemidis S, and Jiang F. 2005. Mechanisms for suppressing NADPH oxidase in the vascular wall. *Mem Inst Oswaldo Cruz* 100 Suppl 1:97-103.

193. Kleniewska P, Piechota A, Skibska B, and Goraca A. 2012. The NADPH oxidase family and its inhibitors. *Arch Immunol Ther Exp* 60(4):277-294.

194. Moldovan L, Moldovan NI, Sohn RH, Parikh SA, and Goldschmidt-Clermont PJ. 2000. Redox changes of cultured endothelial cells and actin dynamics. *Circ Res* 86(5):549-557.

195. Buchdunger E, Zimmermann J, Mett H, Meyer T, Muller M, Druker BJ, and Lydon NB. 1996. Inhibition of the Abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminopyrimidine derivative. *Cancer Res* 56(1):100-104.

196. Chi SS, Vetiska SM, Gill RS, Hsiung MS, Liu F, and Van Tol HH. 2010. Transactivation of PDGFRbeta by dopamine D4 receptor does not require PDGFRbeta dimerization. *Mol Brain* 3:22.

197. Kovalenko M, Gazit A, Bohmer A, Rorsman C, Ronnstrand L, Heldin CH, Waltenberger J, Bohmer FD, and Levitzki A. 1994. Selective platelet-derived growth factor receptor kinase blockers reverse sis-transformation. *Cancer Res* 54(23):6106-6114.

198. Baudhuin LM, Jiang Y, Zaslavsky A, Ishii I, Chun J, and Xu Y. 2004. S1P3-mediated Akt activation and cross-talk with platelet-derived growth factor receptor (PDGFR). *FASEB J* 18(2):341-343.

199. Knauer CS, Campbell JE, Chio CL, and Fitzgerald LW. 2009. Pharmacological characterization of mitogen-activated protein kinase activation by recombinant human 5-HT2C, 5-HT2A, and 5-HT2B receptors. *Naunyn Schmiedebergs Arch Pharmacol* 379(5):461-471.

200. Li B, Zhang S, Li M, Hertz L, and Peng L. 2010. Serotonin increases ERK1/2 phosphorylation in astrocytes by stimulation of 5-HT2B and 5-HT2C receptors. *Neurochem Int* 57(4):432-439.

201. Hu Y, Kang C, Philp RJ, and Li B. 2007. PKC delta phosphorylates p52ShcA at Ser29 to regulate ERK activation in response to H2O2. *Cell Signal* 19(2):410-418.

202. Kim YK, Bae GU, Kang JK, Park JW, Lee EK, Lee HY, Choi WS, Lee HW, and Han JW. 2006. Cooperation of H2O2-mediated ERK activation with Smad pathway in TGF-beta1 induction of p21WAF1/Cip1. *Cell Signal* 18(2):236-243.

203. Mbong N and Anand-Srivastava MB. 2012. Hydrogen peroxide enhances the expression of Gialpha proteins in aortic vascular smooth cells: role of growth factor receptor transactivation. *Am J Physiol Heart Circ Physiol* 302(8):H1591-1602.

204. Eblen ST, Slack JK, Weber MJ, and Catling AD. 2002. Rac-PAK signaling stimulates extracellular signal-regulated kinase (ERK) activation by regulating formation of MEK1-ERK complexes. *Mol Cell Biol* 22(17):6023-6033.

205. Underwood CK and Coulson EJ. 2008. The p75 neurotrophin receptor. *Int J Biochem Cell Biol* 40(9):1664-1668.

206. Yamashita T, Fujitani M, Hata K, Mimura F, and Yamagishi S. 2005. Diverse functions of the p75 neurotrophin receptor. *Anat Sci Int* 80(1):37-41.

207. Roux PP and Barker PA. 2002. Neurotrophin signaling through the p75 neurotrophin receptor. *Prog Neurobiol* 67(3):203-233.

208. Dechant G and Barde YA. 2002. The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system. *Nat Neurosci* 5(11):1131-1136.

209. Hempstead BL. 2002. The many faces of p75NTR. *Curr Opin Neurobiol* 12(3):260-267.

210. Tavoulari S, Forrest LR, and Rudnick G. 2009. Fluoxetine (Prozac) binding to serotonin transporter is modulated by chloride and conformational changes. *J Neurosci* 29(30):9635-9643.

211. Klimek V, Zak-Knapik J, and Mackowiak M. 1994. Effects of repeated treatment with fluoxetine and citalopram, 5-HT uptake inhibitors, on 5-HT1A and 5-HT2 receptors in the rat brain. *J Psychiatry Neurosci* 19(1):63-67.

212. Peterson JA, Risley DS, Anderson PN, and Hostettler KF. 1994. Stability of fluoxetine hydrochloride in fluoxetine solution diluted with common pharmaceutical diluents. *Am J Hosp Pharm* 51(10):1342-1345.

213. Wolfel R and Graefe KH. 1992. Evidence for various tryptamines and related compounds acting as substrates of the platelet 5-hydroxytryptamine transporter. *Naunyn Schmiedebergs Arch Pharmacol* 345(2):129-136.

214. Owens MJ, Morgan WN, Plott SJ, and Nemeroff CB. 1997. Neurotransmitter receptor and transporter binding profile of antidepressants and their metabolites. *J Pharmacol Exp Ther* 283(3):1305-1322.

215. Li B, Zhang S, Li M, Hertz L, and Peng L. 2009. Chronic treatment of astrocytes with therapeutically relevant fluoxetine concentrations enhances cPLA2 expression secondary to 5-HT2B-induced, transactivation-mediated ERK1/2 phosphorylation. *Psychopharmacology* 207(1):1-12.

216. Palvimaki EP, Roth BL, Majasuo H, Laakso A, Kuoppamaki M, Syvalahti E, and Hietala J. 1996. Interactions of selective serotonin reuptake inhibitors with the serotonin 5-HT2c receptor. *Psychopharmacology* 126(3):234-240.

217. Zhang S, Li B, Lovatt D, Xu J, Song D, Goldman SA, Nedergaard M, Hertz L, and Peng L. 2010. 5-HT2B receptors are expressed on astrocytes from brain and in culture and are a chronic target for all five conventional 'serotonin-specific reuptake inhibitors'. *Neuron Glia Biol* 6(2):113-125.

218. Porter RH, Benwell KR, Lamb H, Malcolm CS, Allen NH, Revell DF, Adams DR, and Sheardown MJ. 1999. Functional characterization of agonists at recombinant human 5-HT2A, 5-HT2B and 5-HT2C receptors in CHO-K1 cells. *Br J Pharmacol* 128(1):13-20.

219. Holik AK, Rohm B, Somoza MM, and Somoza V. 2013. N(epsilon)-Carboxymethyllysine (CML), a Maillard reaction product, stimulates serotonin release and activates the receptor for advanced glycation end products (RAGE) in SH-SY5Y cells. *Food Funct* 4(7):1111-1120.

220. Duxon MS, Flanigan TP, Reavley AC, Baxter GS, Blackburn TP, and Fone KC. 1997. Evidence for expression of the 5-hydroxytryptamine-2B receptor protein in the rat central nervous system. *Neuroscience* 76(2):323-329.

221. Audia JE, Evrard DA, Murdoch GR, Droste JJ, Nissen JS, Schenck KW, Fludzinski P, Lucaites VL, Nelson DL, and Cohen ML. 1996. Potent, selective tetrahydro-beta-carboline antagonists of the serotonin 2B (5HT2B) contractile receptor in the rat stomach fundus. *J Med Chem* 39(14):2773-2780.

222. Ramamoorthy S, Giovanetti E, Qian Y, and Blakely RD. 1998. Phosphorylation and regulation of antidepressant-sensitive serotonin transporters. *J Biol Chem* 273(4):2458-2466.

223. Annamalai B, Mannangatti P, Arapulisamy O, Shippenberg TS, Jayanthi LD, and Ramamoorthy S. 2012. Tyrosine phosphorylation of the human serotonin transporter: a role in the transporter stability and function. *Mol Pharmacol* 81(1):73-85.

224. Gooz M, Gooz P, Luttrell LM, and Raymond JR. 2006. 5-HT2A receptor induces ERK phosphorylation and proliferation through ADAM-17 tumor necrosis factor-alpha-converting enzyme (TACE) activation and heparin-bound epidermal growth factor-like growth factor (HB-EGF) shedding in mesangial cells. *J Biol Chem* 281(30):21004-21012.

225. Knight AR, Misra A, Quirk K, Benwell K, Revell D, Kennett G, and Bickerdike M. 2004. Pharmacological characterisation of the agonist radioligand binding site of 5-HT(2A), 5-HT(2B) and 5-HT(2C) receptors. *Naunyn Schmiedebergs Arch Pharmacol* 370(2):114-123.

226. Hosey MM. 1999. What molecular events underlie heterologous desensitization? Focus on "receptor phosphorylation does not mediate cross talk between muscarinic M(3) and bradykinin B(2) receptors". *Am J Physiol* 277(5 Pt 1):C856-858.

227. Steele AD, Szabo I, Bednar F, and Rogers TJ. 2002. Interactions between opioid and chemokine receptors: heterologous desensitization. *Cytokine Growth Factor Rev* 13(3):209-222.

228. Diamond JS. 2005. Deriving the glutamate clearance time course from transporter currents in CA1 hippocampal astrocytes: transmitter uptake gets faster during development. *J Neurosci* 25(11):2906-2916.

229. Chen EY, Mazure NM, Cooper JA, and Giaccia AJ. 2001. Hypoxia activates a platelet-derived growth factor receptor/phosphatidylinositol 3-kinase/Akt pathway that results in glycogen synthase kinase-3 inactivation. *Cancer Res* 61(6):2429-2433.

230. Simakajornboon N, Szerlip NJ, Gozal E, Anonetapipat JW, and Gozal D. 2001. In vivo PDGF beta receptor activation in the dorsocaudal brainstem of the rat prevents hypoxia-induced apoptosis via activation of Akt and BAD. *Brain Res* 895(1-2):111-118.

231. Shih RH, Lee IT, Hsieh HL, Kou YR, and Yang CM. 2010. Cigarette smoke extract induces HO-1 expression in mouse cerebral vascular endothelial cells: involvement of c-Src/NADPH oxidase/PDGFR/JAK2/STAT3 pathway. *J Cell Physiol* 225(3):741-750.

232. Tagawa A, Kaneko T, Shinohara T, Ueda A, Sato T, and Ishigatsubo Y. 2005. Heme oxygenase-1 inhibits cigarette smoke-induced increase in the tracheal mucosal permeability in guinea pigs in vivo. *Inflamm Res* 54(5):229-234.

233. Vasefi MS, Kruk JS, Heikkila JJ, and Beazely MA. 2013. 5-Hydroxytryptamine type 7 receptor neuroprotection against NMDA-induced excitotoxicity is PDGFbeta receptor dependent. *J Neurochem* 125(1):26-36.

234. Beazely MA, Lim A, Li H, Trepanier C, Chen X, Sidhu B, and MacDonald JF. 2009. Platelet-derived growth factor selectively inhibits NR2B-containing N-methyl-D-aspartate receptors in CA1 hippocampal neurons. *J Biol Chem* 284(12):8054-8063.

235. Tseng HC and Dichter MA. 2005. Platelet-derived growth factor-BB pretreatment attenuates excitotoxic death in cultured hippocampal neurons. *Neurobiol Dis* 19(1-2):77-83.

236. Sanchez C and Hyttel J. 1999. Comparison of the effects of antidepressants and their metabolites on reuptake of biogenic amines and on receptor binding. *Cell Mol Neurobiol* 19(4):467-489.

237. Cusack B, Nelson A, and Richelson E. 1994. Binding of antidepressants to human brain receptors: focus on newer generation compounds. *Psychopharmacology* 114(4):559-565.

238. Diaz SL and Maroteaux L. 2011. Implication of 5-HT(2B) receptors in the serotonin syndrome. *Neuropharmacology* 61(3):495-502.

239. Patel JG, Bartoszyk GD, Edwards E, and Ashby CR, Jr. 2004. The highly selective 5-hydroxytryptamine (5-HT)2A receptor antagonist, EMD 281014, significantly increases swimming and decreases immobility in male congenital learned helpless rats in the forced swim test. *Synapse* 52(1):73-75.

240. Strong PV, Greenwood BN, and Fleshner M. 2009. The effects of the selective 5-HT(2C) receptor antagonist SB 242084 on learned helplessness in male Fischer 344 rats. *Psychopharmacology* 203(4):665-675.

241. Quesseveur G, Nguyen HT, Gardier AM, and Guiard BP. 2012. 5-HT2 ligands in the treatment of anxiety and depression. *Expert Opin Investig Drugs* 21(11):1701-1725.

242. Lanner MC, Raper M, Pratt WM, and Rhoades RA. 2005. Heterotrimeric G proteins and the platelet-derived growth factor receptor-beta contribute to hypoxic proliferation of smooth muscle cells. *Am J Respir Cell Mol Biol* 33(4):412-419.

243. Yang CM, Lee IT, Lin CC, Yang YL, Luo SF, Kou YR, and Hsiao LD. 2009. Cigarette smoke extract induces COX-2 expression via a PKCalpha/c-Src/EGFR, PDGFR/PI3K/Akt/NF-kappaB pathway and p300 in tracheal smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 297(5):L892-902.

244. Nisbet RE, Graves AS, Kleinhenz DJ, Rupnow HL, Reed AL, Fan TH, Mitchell PO, Sutliff RL, and Hart CM. 2009. The role of NADPH oxidase in chronic intermittent hypoxia-induced pulmonary hypertension in mice. *Am J Respir Cell Mol Biol* 40(5):601-609.

245. Escargueil-Blanc I, Salvayre R, Vacaresse N, Jurgens G, Darblade B, Arnal JF, Parthasarathy S, and Negre-Salvayre A. 2001. Mildly oxidized LDL induces activation of platelet-derived growth factor beta-receptor pathway. *Circulation* 104(15):1814-1821.

246. Akiba S, Yamaguchi H, Kumazawa S, Oka M, and Sato T. 2007. Suppression of oxidized LDL-induced PDGF receptor beta activation by ginkgo biloba extract reduces MMP-1 production in coronary smooth muscle cells. *J Atheroscler Thromb* 14(5):219-225.

247. Galis ZS and Khatri JJ. 2002. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. *Circ Res* 90(3):251-262.

248. Hardebo JE and Owman C. 1980. Barrier mechanisms for neurotransmitter monoamines and their precursors at the blood-brain interface. *Ann Neurol* 8(1):1-31.

249. Wu JH, Goswami R, Kim LK, Miller WE, Peppel K, and Freedman NJ. 2005. The platelet-derived growth factor receptor-beta phosphorylates and activates G protein-coupled receptor kinase-2. A mechanism for feedback inhibition. *J Biol Chem* 280(35):31027-31035.

250. Cai X, Wu JH, Exum ST, Oppermann M, Premont RT, Shenoy SK, and Freedman NJ. 2009. Reciprocal regulation of the platelet-derived growth factor receptor-beta and G protein-coupled receptor kinase 5 by cross-phosphorylation: effects on catalysis. *Mol Pharmacol* 75(3):626-636.

251. Wu JH, Goswami R, Cai X, Exum ST, Huang X, Zhang L, Brian L, Premont RT, Peppel K, and Freedman NJ. 2006. Regulation of the platelet-derived growth factor receptor-beta by G protein-coupled receptor kinase-5 in vascular smooth muscle cells involves the phosphatase Shp2. *J Biol Chem* 281(49):37758-37772.

252. Freedman NJ, Kim LK, Murray JP, Exum ST, Brian L, Wu JH, and Peppel K. 2002. Phosphorylation of the platelet-derived growth factor receptor-beta and epidermal growth factor receptor by G protein-coupled receptor kinase-2. Mechanisms for selectivity of desensitization. *J Biol Chem* 277(50):48261-48269.

253. Penela P, Ribas C, and Mayor F, Jr. 2003. Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases. *Cell Signal* 15(11):973-981.

254. Hildreth KL, Wu JH, Barak LS, Exum ST, Kim LK, Peppel K, and Freedman NJ. 2004. Phosphorylation of the platelet-derived growth factor receptor-beta by G protein-coupled receptor kinase-2 reduces receptor signaling and interaction with the Na(+)/H(+) exchanger regulatory factor. *J Biol Chem* 279(40):41775-41782.

255. Maudsley S, Zamah AM, Rahman N, Blitzer JT, Luttrell LM, Lefkowitz RJ, and Hall RA. 2000. Platelet-derived growth factor receptor association with Na(+)/H(+) exchanger regulatory factor potentiates receptor activity. *Mol Cell Biol* 20(22):8352-8363.

256. Grace KP, Liu H, and Horner RL. 2012. 5-HT(1A) receptor-responsive pedunculopontine tegmental neurons suppress REM sleep and respiratory motor activity. *J Neurosci* 32(5):1622-1633.

257. Zhuang ZP, Kung MP, Chumpradit S, Mu M, and Kung HF. 1994. Derivatives of 4-(2'-methoxyphenyl)-1-[2'-(N-2"-pyridinyl-p-iodobenzamido)ethyl]pipera zine (p-MPPI) as 5-HT1A ligands. *J Med Chem* 37(26):4572-4575.

258. Chemel BR, Roth BL, Armbruster B, Watts VJ, and Nichols DE. 2006. WAY-100635 is a potent dopamine D4 receptor agonist. *Psychopharmacology* 188(2):244-251.

259. Vasefi MS, Yang K, Li J, Kruk JS, Heikkila JJ, Jackson MF, Macdonald JF, and Beazely MA. 2013. Acute 5-HT7 receptor activation increases NMDA-evoked currents and differentially alters NMDA receptor subunit phosphorylation and trafficking in hippocampal neurons. *Mol Brain* 6(1):24.

260. Naarala J, Nykvist P, Tuomala M, and Savolainen K. 1993. Excitatory amino acid-induced slow biphasic responses of free intracellular calcium in human neuroblastoma cells. *FEBS Lett* 330(2):222-226.

261. Bradley SJ and Challiss RA. 2012. G protein-coupled receptor signalling in astrocytes in health and disease: a focus on metabotropic glutamate receptors. *Biochem Pharmacol* 84(3):249-259.

262. Liu J, Zieger MA, Lakey JR, Woods E, and Critser JK. 1997. Water and DMSO permeability at 22 degrees C, 5 degrees C, and -3 degrees C for human pancreatic islet cells. *Transplant Proc* 29(4):1987.

263. Yu ZW and Quinn PJ. 1994. Dimethyl sulphoxide: a review of its applications in cell biology. *Biosci Rep* 14(6):259-281.

264. Pahlman S, Ruusala AI, Abrahamsson L, Mattsson ME, and Esscher T. 1984. Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbolester-induced differentiation. *Cell Differ* 14(2):135-144.