Identifying signaling differences between GPCR-induced growth factor receptor transactivation and direct ligand activation

by

Azita Kouchmeshky

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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

Growth factor receptors have significant effects on various normal function of body such as cell proliferation, differentiation and apoptosis. They are also involved in neuronal function and dysfunction, cardiovascular diseases, and malignancies. Recently, multiple G protein-coupled receptors (GPCRs) have been shown to transactivate receptor tyrosine kinases (RTKs). Since both classes of receptors have complicated downstream cascades individually, understanding the signaling differences between GPCR-induced growth factor receptor transactivation and direct ligand activation is an important challenge. To clarifying this phenomenon we investigated the phosphorylation profile and downstream effectors of ligand-activated vs. transactivated PDGFβ receptors. Dopamine receptors (one of the receptors of the GPCRs family) were used to compare the PDGFβ receptor phosphorylation and activity during direct activation and transactivation. Dose-response and time-course data between these two stimuli were evaluated. Furthermore, the phosphorylation site profiles and the intracellular signaling pathways of PDGFβ receptor after direct activation and transactivation were examined. In addition, possible synergic effects between transactivation and direct activation were explored. The results of this project showed that the phosphorylation profile and downstream effectors of ligand activated receptors versus transactivated receptors are different. Our data indicated that transactivation-induced pathways are more involved in survival and proliferation effects compared to ligand activation. This research answered basic questions about transactivation phenomena and proposes that these transactivation pathways could be exploited as a therapeutic approach for neurodegenerative diseases.

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Dedication

A special feeling of gratitude to my loving mother, Lili, who has given me dreams to look forward to and love to support my journey in every aspect of my life

To my father, Ali Reza, for being so supportive

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&

To my special aunt and uncle, Parvin & Richard

&

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Abbreviations

5-HT	5-hydroxytryptamine (Serotonin)
AC	Adenylyl cyclase
ADHD	Attention-deficit hyperactivity disorder
ADMA	A disintegrin and metalloproteinase
AMPA	α-amino-3-hydroxy-5-methylisoxzole-4-proprionic acid
APP	Amyloid precursor protein
ASK-1	Apoptosis signal-regulated kinase 1
ATF-2	Activating transcription factor 2
Aβ	Amyloid β
BAD	Bcl-2-associated death (Bcl2 family member)
Bax	Bcl-2-associated X protein
BBB	Blood–brain barrier
Bcl-2	B-cell lymphoma 2
BDNF	Brain neurotrophic factor
Bim	B-cell lymphoma 2 interacting mediator of cell death
Btk	Bruton's tyrosine kinase
CA1	Cornu Ammonis area (a region in the hippocampus anatomy)
CalB	Calcium/lipid-binding domain
cAMP	Cyclic adenosine-3́,5́-monophosphate
Cdc42	Cell division control protein 42
CHO cell line	Chinese hamster ovary cell line
CHO-D4 cells	CHO cells expressing dopamine D4 receptors
CNS	Central nervous system
CREB	cAMP response element-binding protein
DAG	Diacylglycerol
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
EC50	Half maximal effective concentration
EGFR	Epithelial growth factor receptor
EPSCs	Excitatory postsynaptic currents
EPSP	Excitatory postsynaptic potentiation
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
FKHRL1	Forkhead transcription factor
FOXO	Forkhead box protein O
GABA	γ-aminobutyric acid

GAP	GTPase-activating protein
GDI	Guanine nucleotide dissociation inhibitor
GDNF	Glial cell line- derived neurotropic factor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GnRH	Gonadotropin-releasing hormone
GPCRs	G-protein-coupled receptors
Grb	Growth factor receptor-bound protein
GRKs	G-protein coupled receptor kinases
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphate hydrolase enzymes
HB-EGF	Heparin-binding EGF-like growth factor
Hck	Homologous proteins which are included in the SFKs family
HEK293	Human embryonic kidney 293
HGF	Hepatocyte growth factor
Ig	Immunoglobulin
IL-1	Interleukin 1
IP	Inositol phosphate
IP3	Inositol 1,4,5-trisphosphate
IP4	Inositol 1,3,4,5-tetrakisphosphate
IP5	Inositol 1,2,3,4,5,6-hexakisphosphate
IPSCs	Inhibitory postsynaptic currents
IPSP	Inhibitory postsynaptic potentiation
JAK	Janus-kinase
JNKs	C-Jun N-terminal kinases
kDa	Kilo Dalton
LMW-PTP	Low molecular weight protein tyrosine phosphatase
LTD	Long term depression
LTP	Long term potentiation
MAPK	Mitogen-activated protein kinases (ERK1/2)
MAPKAPK	MAPK-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase (MEK)
MAPKKK	Mitogen-activated protein kinase kinase kinase (Ras)
MCF-7	Michigan cancer foundation-7 (human breast adenocarcinoma cell line)
MDM2	Mouse double minute 2 homolog
MEK kinase	Mitogen-activated protein kinase kinase
MMPs	Membrane-bound matrix metalloproteinases
m-TOR	Mammalian target of rapamycin (mTOR)
Nacc	Nucleus accumbens

NCCs	Neuronal crest cells
Nck	Non-catalytic region of tyrosine kinase adaptor protein
NF1	Neurofibromin
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NMDARs	N-methyl-D-aspartate receptors
NO	Nitric oxide
NSCs	Neuronal stem cells
NT-4	Neurotrophin-4
NTR	Neurotrophin receptor
Ops	Oligodendrocyte progenitors
PACAP	Pituitary adenylyl cyclase-activating polypeptide
PAE	Porcine aortic endothelial
PAH	Pulmonary artial hypertension
PC12	Pheochromocytoma cells
PDGF	The platelet-derived growth factor (PDGF)
PDK	Phosphoinositide-dependent kinase
PH domain	Pleckstrin homology domain
PI(3,4)P2	Phosphatidylinositol (3,4)-bisphosphate /PtdIns(3,4)P2
PI(3,4,5)P3	Phosphatidylinositol (3,4,5)-triphosphate /PtdIns(3,4,5)P3
PI(3,5)P2	Phosphatidylinositol (3,5)-bisphosphate /PtdIns(3,5)P2
PI(4,5)P2	Phosphatidylinositol (4,5)-bisphosphate
PI3K (PI3-kinase)	Phosphatidylinositide 3-kinases
PI3P	Phosphatidylinositol 3-phosphate/PtdIns3P
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phosphoinositide phospholipase C
PNS	Peripheral nervous system (PNS)
PP2	Protein phosphatase 2
PP2A	Protein phosphatase 2A
PTB	Phosphotyrosine binding
PtdIns(3,4)P2	Phosphatidylinositol (3,4)-bisphosphate /PtdIns(3,4)P2
PtdIns(3,4,5)P3	Phosphatidylinositol (3,4,5)-triphosphate /PtdIns(3,4,5)P3
PtdIns(4,5)P2	Phosphatidylinositol (4,5)-bisphosphate
PtdIns/PI	Phosphatidylinositol
PTEN	Tumor suppressor protein
PTP1D	Protein tyrosine phosphatases 1D (another name for SHP-2)
PTP2C	Protein tyrosine phosphatases 2C (another name for SHP-2)
PTPs	Protein tyrosine phosphatases
RalGDS	Ral guanine nucleotide dissociation stimulator

RasGAP	GTPase activating protein of Ras
RGL	Ras-related molecule Ral
RTK	Receptor tyrosine kinase
SAPK	Stress activated protein kinase
Ser	Serine
SFK	Src-Family protein kinases (SFK)
SGZ	Subgranular zone
SH2	Src homology 2
SH3	Src homology 3
SHP-2	Src homology 2 domain (SH2)-containing tyrosine phosphatase
SH-PTP2	Src homology 2 domain (SH2)-containg protein tyrosine phosphatases (SHP-2)
SH-PTP3	Src homology 2 domain (SH2)-containg protein tyrosine phosphatases (SHP-2)
SMCs	Smooth muscle cells
Sos	Son of sevenless
SRDT	Second-receptor-downstream-transstimulation
SRIT	Second-receptor-intracellular-transstimulation
SSV	Simian sarcoma virus
STAT	Signal transducer and activator of transcription, or signal transduction and transcription
SVZ	Subventricular zone
tBA	Tissue plasminogene activator
TGF-B	Transforming growth factor beta
TGF-α	Transforming growth factor alpha
Thr	Threonine
TM	Transmembrane
TMPS	Triple-membrane-passing-signalling
TNF	Tumor necrosis factor
Trk receptor	Tropomyosin related kinase receptor
Tyr	Tyrosine
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
vSMCs	Vascular smooth muscle cells

Chapter 1
Introduction

1.1 Cell-to-cell communication

Cell-to-cell communication in a multicellular organism is essential for life. This communication leads to various physiological activities that are important in proliferation, differentiation, migration, and apoptosis of cells. Different signal transduction pathways are involved to balance and maintain these activities. Different classes of receptors regulate signal transduction: transmembrane receptors with extracellular, transmembrane, and intracellular domains; intracellular receptors such as steroid/thyroid hormone receptors; and peripheral membrane proteins, with a loose attachment to the cell membrane and without any attachment into the lipid bilayer membrane. Extracellular signaling molecules such as neurotransmitters, growth factors, hormones, and cytokines transfer their signal into the cells. Five transmembrane signaling mechanisms for transferring signal from outside the cells to inside the cells are described [Figure 1.1]: intracellular receptors for lipid-soluble molecules, ligand-regulated transmembrane enzymes, ligand-regulated tyrosine kinase, ligand- and voltage-gated channels, G-proteins and second messengers [1]. The cell surface receptors and their downstream cascades are of interest since they play key roles in modulating cell physiology.

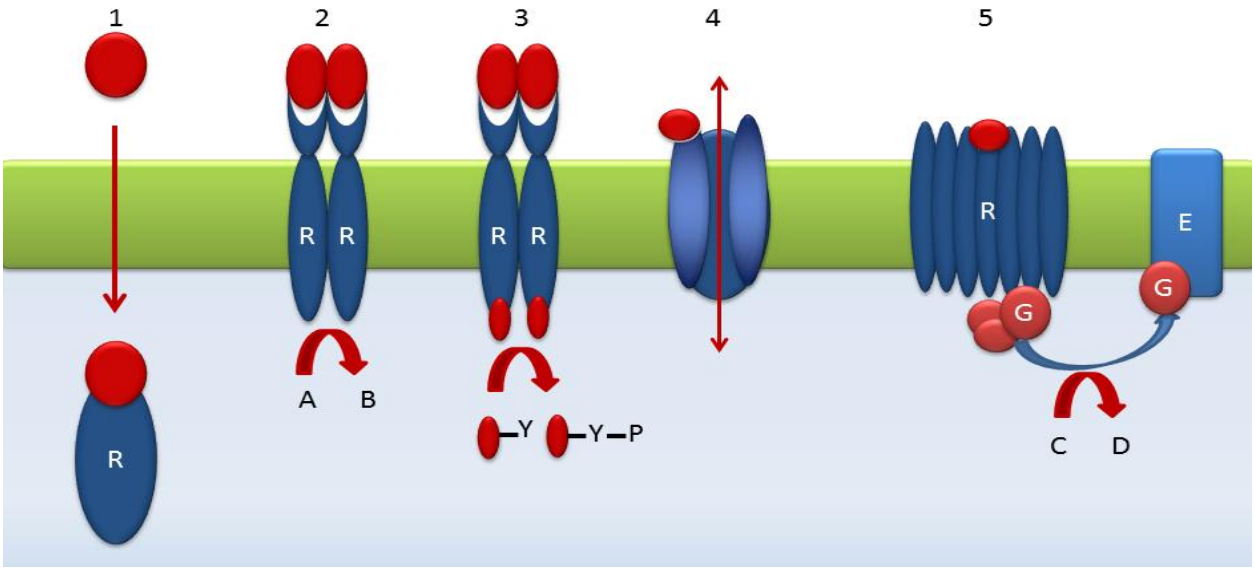

Figure 1.1 Transmembrane signaling mechanisms

1. Intracellular receptors stimulate or inhibit specific gene transcription after activation and translocation to the nucleus. 2. Receptor tyrosine kinase 3. Cytokine receptors are transmembrane receptors that ligand binding results in activating of Janus-kinase (JAK) family associated with the receptor. These kinases phosphorylate tyrosine residues on the cytoplasmic part of the receptors and consequently activate STATs that results in gene transcription. 4. ligand-gated channels act by controlling the flow of the ions through ion channel such as N-methyl-D-aspartate (NMDA) receptors and GABA receptors 5.G-protein coupled receptors [1].

1.2 G-protein-coupled receptors (GPCRs)

Guanine nucleotide binding protein-coupled receptors, or G-protein-coupled receptors (GPCRs), comprise a large family of receptors known as seven-transmembrane domain receptors or serpentine receptors because they pass and wave through the cell membrane seven times. These receptors are the largest family of cell surface receptors, and their genes constitute 5% of the human genome [2]. They also represent the largest classes of current drug targets [3]. GPCRs mediate the majority of cellular responses to chemokines, neurotransmitters including dopamine and serotonin (5-HT), hormones, lipids, and sensory stimuli such as light [3]. GPCRs contain an extracellular N-terminal domain, a 7 α-helice transmembrane region (TM-I to TM-VII), and an intracellular C-terminal domain. The transmembrane region is connected by three intracellular (IL1-2-3) and three extracellular (EL1-2-3) loops. The intracellular domain contains several serine and threonine residues, and their hydroxyl (OH) groups can be phosphorylated.

Intracellular domain phosphorylation may regulate activity, intracellular signaling, and receptor desensitization. The G-proteins themselves interact with the loop between transmembrane V and VI in the cytoplasmic portion of the receptor.

The heterotrimeric G-proteins are composed of α, β, and γ subunits. In humans, sixteen Gα genes encode 23 known Gα isoforms [2]. G protein heterotrimers are identified by the α subunit. Based on sequence similarity, Gα proteins are divided into four classes including $G\alpha_{(s/olf)}$, $G\alpha_{(i1/i2/i3/o/t\text{-}cone/gust/z)}$, $G\alpha_{(q/11/14/16)}$, and $G\alpha_{(12/13)}$ [4,5]. Six Gβ, and eleven Gγ have also been identified [6] and this leads to various combination of Gβγ [2]. The Gα subunit binds to GDP and Gβγ. The Gβγ complex facilitates the association of Gα to the intracellular part of the GPCR. In addition, Gβγ inhibits the release of GDP from Gα and acts as a guanine nucleotide dissociation inhibitor (GDI) [2].

Since GPCRs do not have intrinsic enzymatic activity, binding of a ligand to the extracellular domain of GPCR triggers a conformational change in the receptors; this transduces the ligand's message mechanically to the heterotrimeric G-proteins on the intracellular side of the protein and leads to different downstream signaling cascades [7]. Ligand binding to the receptor acts as a guanine nucleotide exchange factor (GEF) because it promotes the release of GDP and the binding of GTP on the Gα subunit. GTP binding promotes the dissociation of Gα from Gβγ, and then, both GTP-Gα and Gβγ can activate effectors and alter second messenger levels including cyclic adenosine-3,5-monophosphate (cAMP), calcium, or phosphoinositides [1]. Each Gα and Gβγ subunits have specific pathways they activate and regulate [Table 1.1]. The intrinsic guanosine triphosphatase (GTPase) activity of the Gα subunit inactivates Gα by hydrolysing GTP back to GDP. Inactive GDP-Gα re-associates with Gβγ complex and the receptor [8]. This is the primary mechanism for making GPCR activation a transient process.

Often, stimulation of GPCRs results in a rapid loss of subsequent responsiveness. G-protein coupled receptor kinases (GRKs) can regulate the activation of GPCRs [9]; activated receptors stimulate GRKs and in turn GRKs phosphorylate serine residues of the carboxyl terminal of the

GPCRs [9]. Phosphoserines increase affinity of the receptor to β-arrestin proteins. β-arrestins attenuate the ability of the receptor to complex with $G\alpha_s$ and decreases receptor responses to agonists; a process called desensitization [10]. Dissociation of the agonist from the receptor terminates GRK activity, and phosphatases can reverse the desensitization [9]. For some GPCRs, β-arrestins speed up receptor endocytosis [10]. Endocytosis of receptors concomitant with dephosphorylation of the receptors by phosphatase results in dephosphorylated receptors on endosomes that may be recycled back to the membrane [9,10]. However, excessive exposure of the receptors to their ligand leads to endocytosis, transfer to lysosomes, and degradation of the receptors. Thus, endocytosis can restore or attenuate cellular responses to the agonist by rapid recycling or degradation of the receptor, respectively [9,10], depending on duration of the receptor exposure to ligand, the number of ligands available for activation of receptors, the particular receptors that are activated, and the affinity of β-arrestins for phosphorylated receptors [10]. Recently, researchers have suggested that GPCR endocytosis is not only a mechanism for signal desensitization, but also a mechanism for additional signal transduction pathways [11].

Two of the three major intracellular signaling pathways have been described: the classical pathway, ligand binding to the receptor stimulates various G-protein-effectors [7] and the β-arrestin pathway, the phosphorylation of the C-terminal domain of the GPCR via GRKs stimulates ligand-regulating scaffolds through β-arrestin and modulates GPCRs function. A third pathway, the *transactivation* pathway, involves the activation of GPCRs to increase receptor tyrosine kinase (RTK) signaling [8]. The focus of this thesis is to examine the scope of this novel GPCR signaling mechanism, termed transactivation, where the binding of the ligand to GPCRs activates downstream cascades that ultimately lead to the activation of receptor tyrosine kinases (RTKs) [8].

Table 1.1 Gα subunits and Gβγ specific pathways

	Type	*Pathways*	*References*
1	$G_{\alpha s}$	Stimulates AC pathway and cAMP production	[12]
2	$G\alpha_{i/o}$	Inhibits AC and cAMP production	[13]
3	$G\alpha_{q}$, $G\alpha_{11,}$ $G\alpha_{14}$, $G\alpha_{16}$	Stimulate PLC pathway	[14]
4	$G\alpha_{olf}$ $G\alpha_{gust}$	Stimulate odorant and testant GPCRs respectively	[15-17]
5	$G\alpha_{t}$	Control phototransduction	[17]
6	$G\alpha_{12}$,$G\alpha_{13}$	Regulate G-protein RhoA and stimulate PDZ-Rho guanine-nucleotide exchange factors (PDZ-RhoGEF)	[2]
7	Gβγ-complex	1. Inhibits the release of GDP from Gα and acts as a guanine nucleotide dissociation inhibitor (GDI) 2. Regulate K^+ and Ca^{+2} channels. 3. Regulate kinase and small G-protein including Phosphoinositide-3́ kinase-γ (PI3́Kγ) 4.Other various regulation pathways have been considered for downstream activation of Gβγ	[2]

1.2.1 Dopamine and dopamine receptors

Dopamine (3-hydroxytyramine) is a metabolite of the amino acid tyrosine. Dopamine itself is a precursor for other neurotransmitter such as noradrenaline/norepinephrine and adrenaline/epinephrine [18]. Because the dopaminergic system regulates major mammalian basic brain functions, it is associated with many neurologic and psychiatric disorders. Dysregulation of the dopaminergic signal system leads to several pathological and physiological abnormalities such as Parkinson's disease, schizophrenia, Huntington's disease, Tourette's syndrome, depression, and attention-deficit hyperactivity disorder (ADHD) [18-20]. Additionally, an irregular increase in dopamine signal transduction is involved in drug misuse and addiction [21].

Dopamine receptors are GPCRs that control and modulate different intracellular signal transduction pathways. Dopamine exerts its effects via subsequent downstream intracellular cascades such as activating and inhibiting the adenylyl cyclase (AC) pathway and cAMP production or stimulating protein kinase C (PKC) signaling. Dopamine receptors form two different families: D_1-like receptors (D_1 and D_5) and D_2-like receptors (D_2, D_3, and D_4). These

two families interact with various intracellular downstream cascades; have different regulatory mechanisms, neuronal networks localizations, evolutionary origins, and structures [22]. These two classes are primarily differentiated by their effects on adenylyl cyclase [23]. $G\alpha_{s/olf}$ is activated by D_1-family dopamine receptors to stimulate cAMP production via adenylyl cyclase, whereas $G\alpha_{i/o}$ couples to the D_2-family dopamine receptors and inhibits adenylyl cyclase activity [18,22]. In addition to the inhibition of adenylyl cyclase, D_2-family dopamine receptors also activate the Na^+/H^+ exchanger, open K^+ channels, increase Cl^- influx, change neuronal morphology, promote mitogenesis, induce the release of arachidonic acid, increase phosphoinositide hydrolysis, inhibit Ca^{+2} channel, and inhibit apoptosis [24]. Blocking D_2-family dopamine receptors is of interest for antipsychotic drug development [20], whereas dopamine D_2/D_3 receptor agonists are widely used in Parkinson's disease.

1.2.2 Serotonin and serotonin receptors

The monoamine neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) is involved in a wide variety of physiological effects in the CNS, the peripheral nervous system (PNS), and in nonneuronal tissues. It plays roles in the regulation of mood, cognition, perception, memory, aggression, feeding behaviour, sleep and wake cycles, pain, and anxiety [25,26]. Mammals express seven different receptor subtypes (5-HT1–5-HT7) and many subtypes have multiple isoforms. All serotonin receptor subtypes are GPCRs except 5-HT3, which is a ligand-gated ion channel permeable to calcium, sodium, and potassium [26]. 5-HT4, 5-HT6, and 5-HT7 are coupled to $G\alpha_s$; 5-HT1 and 5-HT5 are coupled to $G\alpha_{i/o}$; and 5-HT2 is coupled to $G\alpha_q$ [27].

1.3 Receptor tyrosine kinases (RTKs)

Receptor tyrosine kinases (RTKs), also called growth factor receptors, bind protein growth factor ligands and initiate a variety of intracellular signaling pathways. RTKs consist of an extracellular Ig-ligand-binding domain, a single transmembrane domain, and an intracellular tyrosine kinase domain. Ligand binding to extracellular domains initiates a dimerization process and triggers cross-autophosphorylation of different intracellular tyrosine residues via the intrinsic tyrosine kinase activity of RTKs. These phosphorylation sites become docking sites for adaptor

molecules that in turn activate different downstream intracellular cascades that include RAS/MAPK/ERK, PI3-kinase/Akt, and PLCγ pathways [28]. Several signaling cascades downstream of RTKs ultimately exert phenotypic responses including cell growth, proliferation, differentiation, and survival.

1.3.1 Platelet-derived growth factors and their receptors

The platelet-derived growth factor (PDGF) was first purified from platelets. It plays a key role in stimulating growth and migration in platelets, fibroblasts, smooth muscle cells, neurons, and several other cell types. PDGF also has various physiological effects such as augmentation of immune responses and immune regulation, inflammation, tumorigenesis, and wound healing [29-31]. In the central nervous system (CNS) it exerts neurotrophic effects. Specifically, it has important neurotrophic effects in regulating neuronal proliferation and growth [30] as well as in stimulating and modulating neurogenesis and synaptogenesis [32]. The dimeric polypeptide molecules of the PDGF ligand family consist of five heterodimeric and homodimeric subtypes: PDGF-AA, PDGF-BB, PDGF-AB [33], PDGF-CC [34], and PDGF-DD [35]. Since the endogenous expression of PDGF-B and PDGF-A rarely overlaps, the presence of PDGF-AB is infrequent, and it has only been found in human platelets [36]. PDGF-A and PDGF-B have pro-domain on their N-terminus; intracellular furin or proprotein convertases removes this pro-domain. This process is important for ligand binding ability [36]. PDGF-C and PDGF-D are not similarly processed and they are secreted as latent ligands. Tissue plasminogen activator (tPA) proteolytically dissociates the growth factor domain of PDGF-C and PDGF-D from the CUB-domain in extracellular space in order to activate them in plasma and tissue [37].

All PDGF subtypes have a conserved homology domain that is involved in dimer formation through a disulfide bridge. The mature form of PDGF-A and PDGF-B consists of 100 amino acids with a 60% similar amino acid sequence and eight conserved cysteine residues in each chain. The second and fourth cysteines are involved in intermolecular disulfide bonds, and the rest are involved in intramolecular disulfide bonds [38,39]. The vascular endothelial growth factor (VEGF) is related both structurally and functionally to PDGF [37]. Previously, scientists

believed that each growth factor family had an affinity to bind with its own receptors; however, recent studies have shown that VEGF-A can bind and signal through PDGF receptors in bone-marrow-derived mesenchymal stem cells [40,41]. There is also similarity in the protein structure of PDGF-BB with VEGF, NGF, and TGF-B.

The five dimeric PDGF isoforms induce various intracellular signaling cascades via two cell surface RTKs: PDGFα receptors and PDGFβ receptors [28]. Post-translational modification (glycosylation) is required for cell surface expression of both PDGFα receptors and PDGFβ receptors [143,177]. Three different types of receptor homo- or hetero-dimerization may occur after ligand binding to the receptor based on the ligand configuration and the receptor expression [42]. Dimerization of the receptor via its ligands leads to cross-autophosphorylation of different intracellular tyrosine residues and activation of the receptor. Most of the phosphorylation sites of PDGF receptors are identified outside the kinase region; except Tyr857 that is placed in the kinase domain of the PDGFβ receptors [43]. These phosphorylation sites become docking sites for mediators that lead to different downstream intracellular cascades. Each of the five PDGF isoforms has a specific affinity to each of PDGF receptors: PDGF-AA, -BB, -AB, and -CC bind to PDGFα receptors, whereas PDGF-BB (high affinity) and PDGF-DD (low affinity) can bind to PDGFβ receptors. PDGFαβ receptors heterodimers bind PDGF-BB, -AB, -CC, and -DD [44] [Figure 1.2].

PDGF receptors consist of five extracellular immunoglobulin-like (Ig-like) domains and an intracellular tyrosine kinase domain [36]. The extracellular domain is separated from intracellular domain by a transmembrane domain. The three outermost Ig domains are involved in ligand binding, and a direct receptor-receptor interaction occurs in the fourth immunoglobulin domain [28]. Although ligand binding to the receptor is essential for receptor dimerization, at a high level of receptor expression, dimerization and autophosphorylation of the receptor may occur independent of ligand binding, i.e. PDGF receptor may spontaneously dimerize at high local concentrations [29]. The intracellular domain of the receptor consists of three parts: a juxtamembrane domain without enzyme activity, a tyrosine kinase domain, and a C-terminal

domain. The juxtamembrane domain and tyrosine kinase domain in PDGFα receptors and PDGFβ receptors share 80% similarity in their amino acids sequence; the kinase domain is divided to two separated parts by the insertion of almost 100 amino acids called the kinase insert [43]. In contrast, the extracellular domain and C-terminus domain share just 30% amino acid similarity.

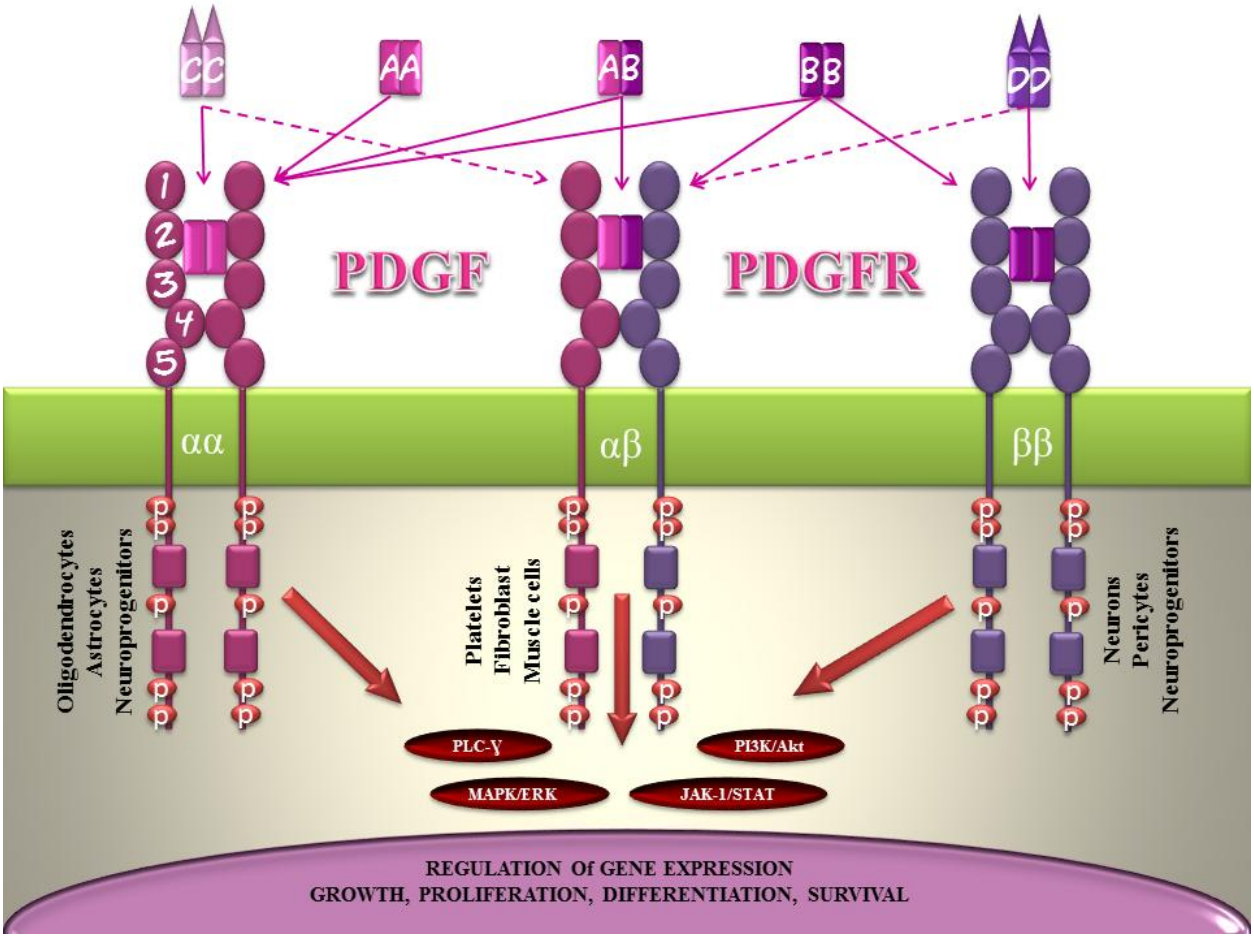

Figure 1.2 The PDGF-PDGF receptor interaction in vitro

There is a lack of evidence for interaction between some of the PDGF-PDGF receptors in vivo. The current in vivo evidence supports only the interaction between PDGF-AA and PDGF-CC with PDGFα receptors, and PDGF-BB and PDGF-DD with PDGFβ receptors [36].

1.3.1.1 PDGF and PDGF receptor expression patterns

PDGF and PDGF receptor genes are located on different chromosomes, and they have independent transcriptional regulation. PDGF is secreted as a paracrine growth factor but in tumors may act as an autocrine factor. Expression of PDGF is dependent on different stimuli such as, growth factors, hypoxia, inflammation, cytokines, and thrombin. Expression of PDGF receptors is also dynamic; TGF-α, estrogen, fibroblast growth factor 2 (FGF-2), lipopolysaccharide, tumor necrosis factor, and interleukin-1 (IL-1) can induce receptor expression [36,42]. The expression of PDGF and PDGF receptors is modulated temporally during development [36].

PDGFs and VEGFs play a key role in hematopoietic development, neurogenesis, and neuroprotection [36]. PDGF-A is produced in neurons and glial cells in the central nervous system [45], and PDGF-B is expressed exclusively in the vascular endothelial cells and megakaryocytes, and in the nervous system where it is produced in the soma, principal dendrites, and axons of the neuron [46]. PDGF-A has mitogenic functions for glial cells and regulates oligodendrocyte development. PDGF-B, but not PDGF-A, promotes neurite outgrowth, neuronal survival, and gene expression in human dopaminergic neurons during embryonic development and, to a lesser extent, in adult brain cells [30]. PDGF-A and PDGF-C are produced in muscle, epithelial cells, and neuronal progenitors. There is not sufficient evidence for PDGF-D expression; however, it has been shown in a certain location of fibroblasts and SMCs [36].

PDGFα receptors and PDGFβ receptors are expressed in CNS and throughout many peripheral tissue types [30,36]. PDGFα receptors are expressed in glial cells but not neurons, oligodendrocyte progenitors (OPs), mesenchymal progenitors in the lungs, intestine, and skin; whereas, PDGFβ receptors are expressed predominantly in neurons, pericytes, mesenchyme, and vascular SMCs (vSMCs) [47,48]. PDGF receptors have been shown to exert neurotrophic effects on both the dopaminergic and GABAergic neurons [49]. Furthermore, they have modulatory effects on N-methyl-D-aspartate receptors (NMDARs), which are important in learning, memory and synaptic plasticity, i.e. long-term potentiation (LTP) or depression (LTD) [49]. The

expression patterns of PDGFα and PDGFβ receptors are associated with different responses; transcriptional regulation of these receptor genes prevents redundancy of their expression. PDGFα receptor knock-out animals showed the normal phenotypes because PDGFβ receptors can substitute PDGFα receptor signaling. However, in the reverse knock-out animals, PDGFα receptors can only partially compensate for the lack of PDGFβ receptors. Thus, the specificity of PDGF receptors signaling is dependent on cell type, specific expression of the receptors, and the distinct downstream cascades of each of these receptors [50]. In the following sections, we will focus on PDGF-BB and PDGFβ receptors because PDGFβ receptors are expressed mainly in neuron cells, whereas PDGFRα is expressed mainly in support cells.

1.3.1.2 Downstream pathways of PDGF receptors

PDGF ligand binding to their receptors induces dimerization that brings the receptor kinases close to each other and leads to auto- and trans-phosphorylation of intracellular tyrosine residues of PDGF receptors. The first three Ig-like domains contribute to ligand binding and the fourth Ig-like domain is essential for dimerization of the receptors. There is a debate whether the dimerization of the receptors is sufficient to activate the receptors or if additional conformational changes of the receptors by ligands are required. The review by Jiang and Hunter highlighted that unliganded dimer receptors can be formed, but the ligand binding is essential for full activation [51]. Autophosphorylation of the receptor regulates the catalytic activity of the receptor intracellular kinase domain. The auto-phosphorylation sites in PDGF receptors belong to two different groups. The first group is tyrosine residues located at the activation loop of the kinase domain, and the phosphorylation of these tyrosine residues increases the kinase activity. In PDGFβ receptors, the tyrosine that is phosphorylated in the kinase domain is tyrosine 857 and its mutation results in lower kinase activity of the PDGFβ receptor [52]. Tyr857 has an important autoinhibitory role in its unphosphorylated state for PDGFβ receptors. Binding of the ligand to the receptor results in conformational changes in that area of the receptor and allows for intrinsic kinase activity to phosphorylate Tyr857 and unlock the kinase, thereby resulting in full enzymatic activity [53]. The second group of tyrosine residues are located outside the kinase domains in the kinase insert; phosphorylation of these tyrosine residues result in docking sites for

a number of known effectors containing Src homology 2 (SH2) domains, phosphotyrosine binding (PTB) domains, and pleckstrin homology (PH) domains [28].

There are 15 tyrosine residues in the intracellular, non-catalytic part of PDGFβ receptors, 11 of these 15 tyrosine residues are autophosphorylation sites for the receptors [28]. Specificity for phosphotyrosine residues in the recognition and binding of SH2 domains is achieved by specific amino acids surrounding them [54]. These phosphorylated tyrosine residues can bind to various signal transduction effectors. Some tyrosines on the receptor are phosphorylated by kinases other than the receptor itself such as Tyr934 in PDGFβ receptors that is phosphorylated by Src [Table 1.2].

Tyrosine phosphorylation sites of PDGFβ receptors have two responsibilities: controlling the activity of the kinase and producing docking sites for signal transduction molecules, such as effectors or adaptor proteins. Protein-protein interactions and protein-lipid interactions play a key role in simulating the downstream signaling cascades of PDGF receptors. Important domains for mediating the activation of downstream effectors include SH2 and PTB domains that recognize phosphorylated tyrosine residues, PH domains that recognize membrane phospholipid residues, SH3 and PDZ domains that recognize proline-rich regions, and C-terminal-specific sequences [28]. To increase the strength of an effector interaction, two domains may interact with two distinct sites of this effector.

SH2 domains are the most important domains for recognizing phosphotyrosine residues. Proteins containing SH2 domains can be separated into three different groups: First, molecules with intrinsic enzyme activity including the Src family of tyrosine kinase, the tyrosine phosphatase SHP-2, phospholipase Cγ (PLCγ), and the GTPase activating protein (GAP) for Ras. Second, molecules acting as adaptor proteins without enzyme activity including Shc, Grb7, Grb2, Nck, Crk, the p85 regulatory subunit of phosphatidylinositol 3 kinase (PI3-kinase). Third, molecules that act as transcription factors such as signal transducers and activators of transcription (Stats) [54].

Table 1.2 Tyrosine phosphorylation sites of PDGFβ receptors and their physiological effects

Tyrosine Phosphorylation sites	*The effector*	*The physiological role*	*References*
Tyr579	Src Shc Stat5	**Src:** Mitogenicity, cell cycle progression to the S phase, *DNA synthesis*, possible negative regulation of cytoskeletal rearrangements and cellular movement **Shc:** Grb2/Ras/MAPK pathway **Stat5:** Gene expression related to proliferation, differentiation and apoptosis	[28,53,55]
Tyr581	Src Stat5	**Src:** Mitogenicity, cell cycle progression to the S phase, DNA synthesis, possible negative regulation of cytoskeletal rearrangements and cellular movement that are induced via PDGF **Stat5:** Gene expression related to proliferation, differentiation and apoptosis	[28,55]
Tyr716	Grb2 Grb7	**Grb2**-Sos/Ras/MAPK pathway **Grb7:** Involved in migration of neuronal cells in embryo	[28,53,58]
Tyr740	PI3-kinase Shc	**PI3-kinase:**Akt/PKB pathway **Shc:** Grb2/Ras/MAPK pathway	[28,53,56]
Tyr751	PI3-kinase Shc Nck	**PI3-kinase:** Akt/PKB pathway **Shc:** Grb2/Ras/MAPK pathway **Nck:** Proliferation (overexpression of Nck lead to transformation)	[28,53,56]
Tyr763	SHP-2	**SHP-2:** Negatively: dephosphorylates autophosphorylation site Tyr740/Tyr751/Tyr771 Positively: Grb2/Ras/MAPK pathway, Src/MAPK pathway	[28]
Tyr771	GTPase of Ras Shc	**GAP :**Negative regulation of Ras **Shc:** Grb2/Ras/MAPK pathway	[28,53]
Tyr775	Grb2 Grb7 Stat5	**Grb2**-Sos/Ras/MAPK pathway **Grb7:** Involved in migration of neuronal cells in embryo **Stat5:** Activation and translocation to nucleus leads to gene expression related to proliferation, differentiation and apoptosis	[28,58]
Tyr857		Regulation of receptor Kinase Activity (allows the access of PLCγ1 and GAP proteins and ATP to the active site of the receptor)	[28,52]
Tyr934		Phosphorylated by Src, negatively regulate PDGF receptor-induced motility by PLCγ pathway	[53,57]
Tyr1009	SHP-2 PLCγ1	**SHP-2:** Negatively: dephosphorylates autophosphorylation site Tyr740/Tyr751/Tyr771 Positively: Grb2/Ras/MAPK pathway,Src/MAPK pathway **PLCγ1:** Ca^{+2} signaling, mitagenicity and in some cell migration, inhibition of NMDA and $GABA_A$ receptors	[28,53]
Tyr1021	PLCγ1	**PLCγ1:** Ca^{+2} signaling, mitagenicity and in some cell migration, inhibition of NMDA and $GABA_A$ receptors	[53]

These interactions and processes initiate various signaling downstream pathways regulating cell growth, differentiation, migration, and survival [28]. Stimulation of PDGFβ receptors by PDGF-BB leads to an array of temporally regulated cellular responses that include: changes in cytoplasmic pH and an influx of intracellular Ca^{+2} [43], cytoskeleton rearrangement, actin stress fiber rearrangement, and cell membrane process formation [43], proliferation, differentiation, migration, cell survival, and specific patterns of gene regulation [43].

1.3.1.2.1 Ras/MAPK/ERK pathway

A group of serine/threonine kinases that stimulate proliferation, differentiation, apoptosis, and long-term potentiation is called the mitogen-activated protein kinase (MAPK) pathway. Typically, the MAPK pathway begins with the binding of the growth factor ligands to the PDGF receptors. This results in the docking of an SH2 domain-containing protein called growth factor receptor-bound protein 2 (Grb-2) that can bind directly or through additional adaptor proteins to the phosphorylated sites of the activated receptor. Grb2, a 24 kDa protein, is the major adaptor protein in growth factor receptor activation of Ras. It contains one SH2 domain that directly binds to phosphorylated Tyr716 and Tyr775 residues of the PDGFβ receptor. Grb2 can also bind to the PDGFβ receptor indirectly via other tyrosine phosphorylated proteins bound to PDGFβ receptor, such as the adaptor protein Shc or the tyrosine phosphatase SHP-2 [58]. SHP-2 binds to phosphorylated Tyr763 and Tyr1009, whereas Shc binds to several phosphorylated sites of PDGFβ receptor [59,60].

The guanine nucleotide exchange factor (GEF), is called Son of Sevenless (Sos), can bind to the two SH3 domains of the Grb-2 protein. Activated Sos has the ability to bind to the membrane-bound protein Ras and catalyzes its activation by promoting GDP for GTP exchange. This exchange leads to the activation of Ras that results in the stimulation of several downstream proteins. Ras proteins are essential components of signaling pathways that regulate cellular proliferation, differentiation, and apoptosis. Human Ras protein has four isoforms that show different biological functions and each of these Ras homologs participates in distinct signal transduction pathway [61]. GTP_{ase}-activating proteins such as GAPs; p120GAP, and

neurofibromin/NF1-GTPase, catalyze GTP hydrolysis are the effectors for inactivating Ras [62]. Activated Ras binds to effector proteins that eventually initiates downstream signaling cascades that results in programmed responses of the cell [61]. The cytosolic serine/threonine-protein kinase Raf is the best-characterized effector protein for Ras; other putative effectors include PI3-kinase [62], RalGDS (a GEF for the Ras-related molecule Ral), RGL (a RalGDS-related molecule), MEK kinase, protein kinase C (PKC), Bcl-2, and JNK (the latter two are important in Ras regulation of apoptosis) [63,64]. Raf phosphorylates and activates dual-specificity kinase MAPK kinase, also called MEK [65]. Activated MEK phosphorylates extracellular signal-regulated kinases (ERK1/2). Finally, activation of ERK1/2 results in stimulation of various cytoplasmic proteins including cytoplasmic phospholipase A_2. The dimerized form of activated ERK1/2 can translocate into the nucleus, where the complex phosphorylates and activates transcription factors to induce gene transcription [66,67].

The MAPK pathway can be divided into three different pathways in mammals: extracellular signal-regulated kinase ERK1/2, which has an important role in cell growth and differentiation [Figure 1.3]; C-Jun N-terminal kinases (JNKs) or stress activated protein kinase (SAPK), which plays a key role in cell transformation, apoptosis and stress responses; and finally, p38 MAPK, which has the similar functions as SAPK/JNKs [68]. Activation of JNKs by stress and inflammatory stimuli, cytokines, or UV light leads to phosphorylation of the transcription factor c-Jun at its N-terminal serine residue. The p38 pathway activates the serine/threonine-kinase MAPK-activated protein kinase (MAPKAPK) 2 and the transcription factor ATF-2. Ras/MAPK pathway regulation by negative feedback is essential for avoiding excessive and abnormal cell responses.

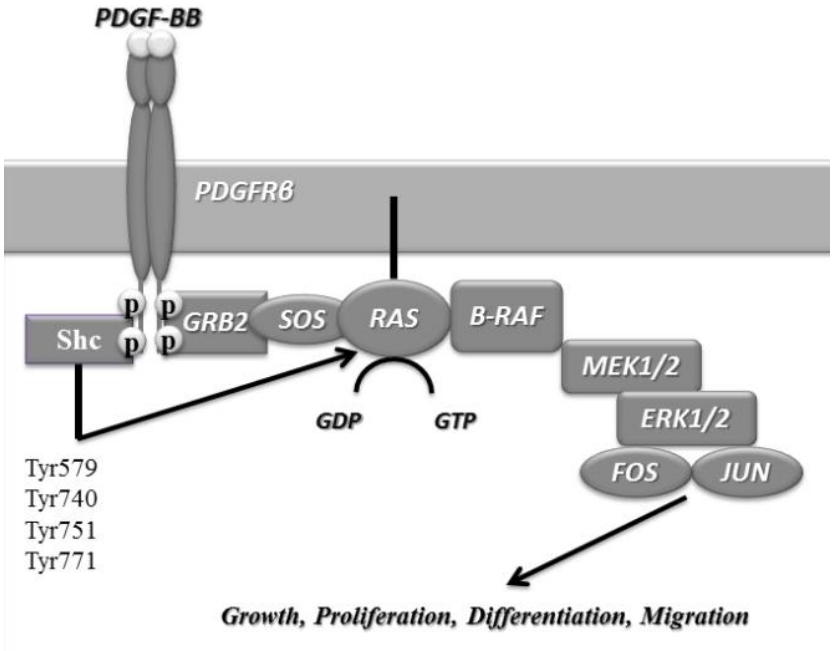

Figure 1.3 The classical pathway of MAPK, ERK1/2 pathway

1.3.1.2.2 Src-family kinases

Src-Family protein kinases (SFK) have been described as oncogenic proteins. They modulate various cellular functions such as proliferation, differentiation, survival, cell morphology, cell motility, and adhesion. Members of the Src family kinase belong to the tyrosine kinase family with molecular masses ranging from 53 kDa to 64 kDa. They have six different domains consisting of an N-terminal membrane association domain (14-carbon myristoyl group attached to an SH4 domain); an SH2 domain; an SH3 domain, a tyrosine kinase domain (catalytic domain or the SH1 domain); a C-terminal negative regulatory domain; and the unique segment (with the greatest sequence diversity and with multiple serine/threonine phosphorylation sites) [Figure 1.4]. Ten homologous proteins are included in the SFKs family: Src, Blk, Hck, Fyn, Fgr, Lck, Lyn, Yes, Yrk, and Frk subfamily proteins. Two important phosphorylation sites on Src family kinases are Tyr416 that promotes SFK activity when phosphorylated and Tyr527 inhibits SFK activity.

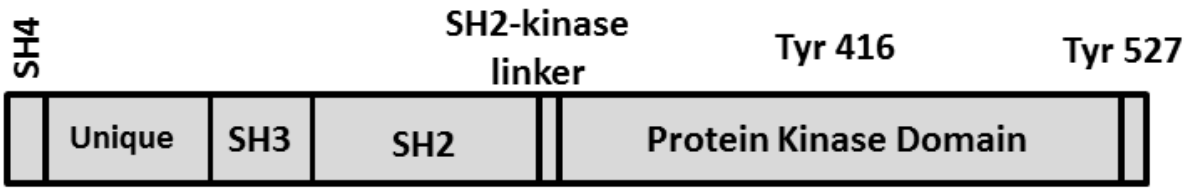

Figure 1.4 Structure of Src family kinase

Src has a key role in many downstream signaling cascades of RTKs including PDGF receptors. Tyr579 and Tyr581 are the docking sites for Src on PDGFβ receptors and Tyr572 and Tyr574 mediate binding of the Src to the PDGFα receptors [28,55]. Provocation of cell cycle progression is one of critical functions of PDGF receptors through Src. Entry into S phase from G phase is part of this process. In addition, Src plays a major role in DNA synthesis that is induced by PDGF receptors [69]. Furthermore, Src has a negative regulatory effect on PDGF receptors-induced motility responses [57]

1.3.1.2.3 PI3-kinase pathway

Phosphoinositide 3-kinases (PI3-kinases) consist of group of lipid kinases that have the ability to phosphorylate the inositol ring of phosphatidylinositol (PtdIns or PI) at their 3-OH position and produce various 3-phosphorylated phosphoinositide such as PI3P, PI(3,4)P_2, PI(3,5)P2, and PI(3,4,5)P3, which in turn recruit specific signaling proteins containing phosphoinositide-binding domains to the cellular membrane [Figure 1.5]. PI3-kinases have two subunits: catalytic and regulatory. PI3-kinases are divided to three classes based on their structure, regulation, and lipid-binding specificity [70]. Class I_A (which is downstream signaling pathway for RTKs) consists of p110 as a catalytic subunit and the p85 as a regulatory subunit. Phosphorylated Tyr740 and Tyr751 are the docking sites on the PDGFβ receptor for p85 subunit of PI3-kinase [56] and this binding leads to activation of the catalytic subunit. In addition to RTKs, other molecules such as Src tyrosine kinases, focal adhesion kinase (FAK), Ras, Rho, and Cdc42 can bind to class I_A PI3-kinase to promote its activation. PI3-kinase downstream effectors include protein kinase B (PKB), also called Akt. Activation of the Akt/PKB by PI3-kinase results in the translocation of Akt to the plasma membrane. Akt also is activated by the 3-phosphoinositide-dependent kinase 1

(PDK1), which is also activated by PI3-kinase. Akt activation protects cells from apoptosis and promotes cell survival [71]. Others downstream effectors that are activated by PI3-kinase include the small GTPases of the Rho family, Bruton's tyrosine kinase (Btk), members of protein kinase C (PKC) family (PKCδ,ε,ζ), PLCγ, c-Jun and N-terminal kinase (JNK), and p70S6 kinase [28,72].

PI3-kinase pathways may have oncogenic effects in various tumors, and the overexpression of Akt pathway is observed in some types of cancers [73]. Akt activation leads to phosphorylation of BAD (Bcl2 family member), and suppression of apoptosis, which is a key phenotype of several malignancies. The lack of the tumor suppressor protein (PTEN) that has lipid phosphatase activity and can dephosphorylate PI3-kinase phospholipid products has also been shown in many human malignancies [73]. PDGF receptors can promote cell growth and motility via PI3-kinase pathway [74]. There are two different chemotactic waves of PI3-kinase activation by PDGF, early (5-30 minutes) and late (3-7 hours). The early wave is needed for membrane ruffling and chemotaxis**,** and the late wave of PI3-kinase activity is required for cell-cycle progression via PDGF-dependent DNA synthesis [74].

1.3.1.2.4 PLC pathway

The phospholipase C (PLC) pathway is stimulated by various extracellular signals. Activated-PLC enzymes hydrolyze cell membrane phospholipids PIP2 (phosphatidylinositol-4,5-bisphosphate) to generate two second messengers: diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). DAG acts to stimulate the protein kinase C family (PKC). IP3 stimulates the intracellular Ca^{+2} release by binding to the IP3 receptors on intracellular Ca^{+2} storage sites of the cells such as the endoplasmic reticulum (ER). This intracellular process results in the release of Ca^{+2} stored within the ER and activation of calcium-dependent proteins. Released Ca^{+2} and DAG cooperate to stimulate protein kinase C (PKC) enzymes associated with the plasma membrane [75] [Figure 1.5]. Further phosphorylation of IP3 produces inositol 1,3,4,5-tetrakisphosphate; IP4 is a regulatory kinase which regulates IP3 metabolism, compete with IP3 to bind PH domain of target proteins, and restores intracellular Ca^{+2} back into its storage sites. IP5 forms are involved

in the modulation of cell differentiation and calcium hemostasis [76]. DAG can be converted to phosphotidic acid, which also has its own biological functions.

PLC isoforms in mammals are divided to three different groups: PLCβ, PLCγ, and PLCδ. All of them have X and Y regions that consist of the catalytic domain and the PH region in the N-terminus. PLCγ isoforms have one SH3 domain, two SH2 domains, and an additional PH domain. PLCγ also has a calcium/lipid-binding domain (CalB) that interacts with Ca^{+2} and phospholipids. RTKs and other tyrosine kinase-dependent signaling receptors, non-receptor protein tyrosine kinases (PTKs), such as B cell antigen receptor (BCR) and T cell antigen receptor (TCR) can activate PLCγ isoforms via their SH2 domains [77,78]. PLCγ binds to phosphorylated tyrosine residues of activated RTKs by its SH2 domains. The phosphorylated PLCγ1 detaches from the receptor tyrosine kinase, and translocates to the cell membrane [79]. Translocation of PLCγ1 is dependent on phosphatidylinositol-3,4,5-trisphosphate that is produced by PI3-kinase, and binds the PH domain of PLCγ1[80].

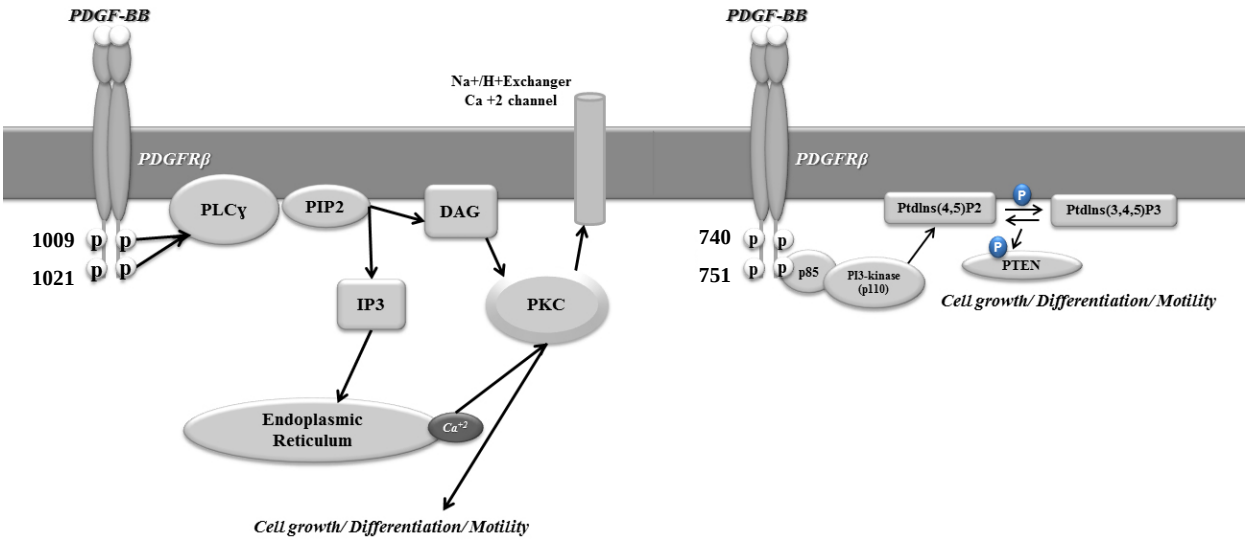

Figure 1.5 PLCγ1/PKC and PI3-kinase pathways

PLCγ1 regulates various cellular functions including mitogenesis, differentiation, chemotaxis, and transformation. The deletion of PLCγ1 in mice results in a lethal phenotype due to global growth inhibition of all organs of the embryo [81]. Both PLCγ and PI3-kinase pathways stimulate chemotaxis and mitogenicity in response to PDGF-BB. Each of these pathways can compensate for the other's absence to promote the effect of PDGF-BB on cell chemotaxis and mitogenicity [81,82,83]. In addition, PLCγ and PI3-kinase both activate the Na^{+}/H^{+} exchanger via Ca^{+2}/PKC pathway after activation of PDGFβ receptors [28,84].

1.3.1.2.5 Protein tyrosine phosphatase

Protein tyrosine phosphatases (PTPs), which dephosphorylate phosphotyrosines, often oppose the activity of tyrosine kinases. Equilibria between phosphorylation and dephosphorylation of tyrosine residues is an essential regulating mechanism for various downstream cascades of RTKs. SHP-2 and low molecular weight phosphatase are two PTPs that are identified for regulating PDGF receptors. SHP-2, also known as PTP1D, PTP2C, SH-PTP2, SH-PTP3 or Syp, is a protein tyrosine phosphatase (PTP) that contains two SH2 domains. Tyr1009 can bind to SH2 domains of SHP-2 and this results in both positive and negative functions downstream of PDGFβ receptors [85]. Phosphatase activity of SHP-2 can dephosphorylate the autophosphorylated tyrosines of the PDGFβ receptors as well as their substrates [28]. For example, SHP-2 can dephosphorylate phospho-Tyr740, a PI3-kinase binding site, phospho-Tyr751, a Nck and PI3-kinase binding site, and phospho-Tyr771, a GTPase-activating protein (GAP) binding site, whereas phospho-Tyr1009 and phospho-Tyr1021 are not putative substrates for the phosphatase activity of SHP-2 (86). After binding to Tyr1009, SHP-2 can dephosphorylate and bind to Grb2, which leads to Ras activation. In addition, SHP-2 can stimulate the MAPK pathway independent of Ras, probably via Src activation [28,58,59]. Therefore, SHP-2 has both inhibitory regulatory effects with respect to PDGFβ receptor signaling.

The low molecular weight protein tyrosine phosphatase (LMW-PTP) is another phosphatase that regulates PDGF receptor activation. It decreases PDGFβ receptor tyrosine phosphorylation and

reduces the mitogenic effects of PDGF-BB by regulating the Src and STAT pathways, which results in modification in the G_1 cell cycle phase progression via changing in c-fos and c-myc expression [87].

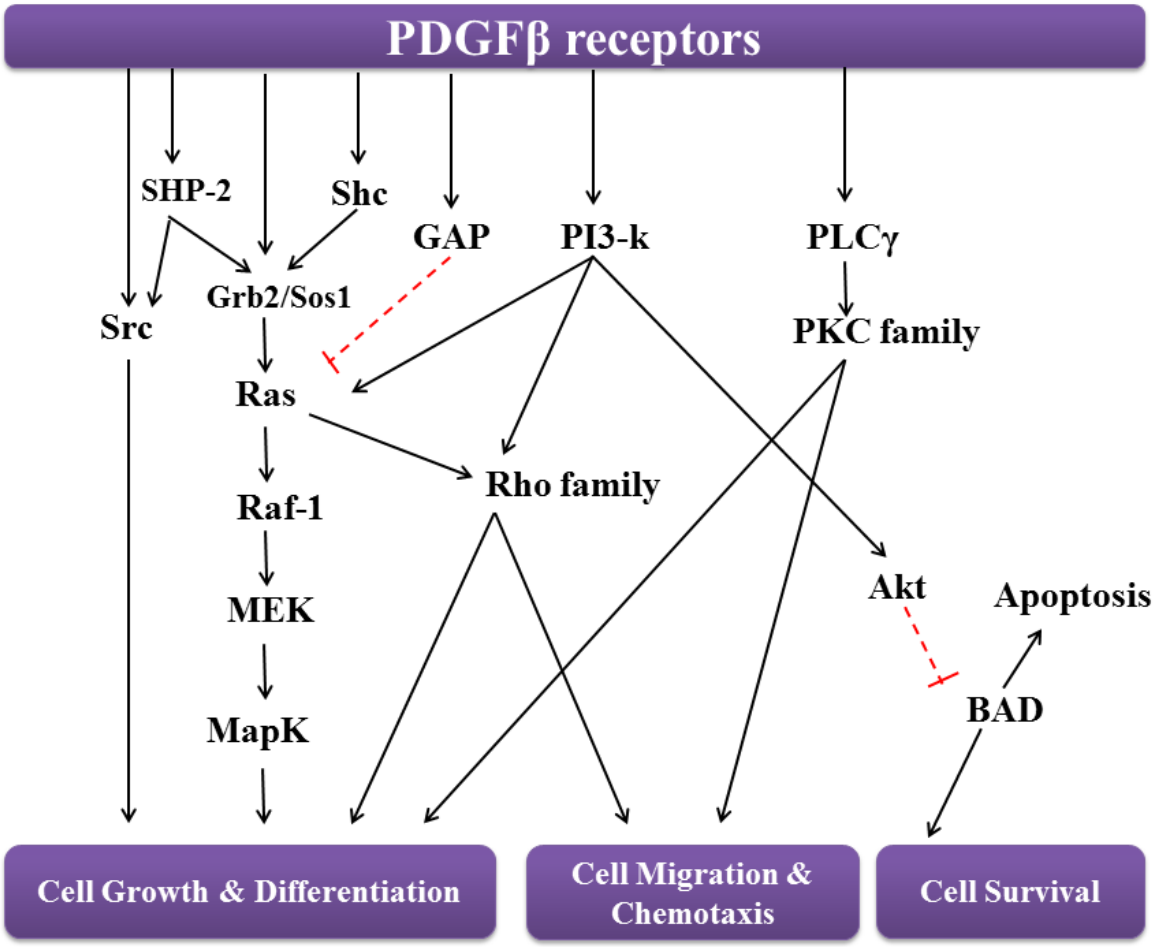

Figure 1.6 Cross-talk between PDGFβ receptors pathways

Each of signaling pathways of PDGF receptors is not an isolated linear pathway, whereas there is an association among all the PDGF receptors pathways. The combination of these pathways results in cell proliferation, differentiation, motility, and survival [1].

1.3.1.3 Cellular functions of PDGF and PDGF receptors

PDGFs are dimers of polypeptide chains that are linked by disulfide bonds and belong to the mitogen family. They have multi-faceted regulatory functions and play crucial roles in cell growth, differentiation, proliferation, and migration during embryonic development as well as in adult cellular homeostasis. PDGF-AB makes up approximately 70% of the PDGF produced in platelets and the remaining consist of PDGF-BB; with trace amounts of PDGF-AA [88] [Table 1.3].

1.3.1.3.1 Proliferation and differentiation

When activated, both PDGFα and PDGFβ receptors induce cell proliferation with measurable cell division observed as early as 8-16 hours after treatment. Cell type variations and variability in expression levels of the receptors make it difficult to determine which of the α or β receptors has a greater association with the promotion of mitogenesis [89]. Compared to homodimers, heterodimeric PDGFαβ receptors have been shown to induce the most efficient mitogenic response [90]. The Ras/MAPK pathway is one of the important pathways for PDGF-induced mitogenicity [91]. The adaptor protein Nck, PI3-kinase, PLCγ, c-Src, and sphinogosine1-1 phosphate are important Ras/MAPK-independent pathways for PDGF-induced mitogenesis [92-94]. The mitogenic characteristics of Ras/MAPK-independent pathways depend on the cell type. Overactivation of various components of these pathways may result in transformation. PDGF receptors have several negative regulatory control mechanisms that prevent excessive cell growth, which could lead to malignancy. The GTPase activating protein of Ras (RasGAP), the Cbl proto-oncogene, the low molecular weight protein tyrosine phosphatase (LMW-PTP), the SH2 domain containing tyrosine phosphatase SHP-2, and integrins are some of the negative regulators for the mitogenic responses to PDGF receptor activation [95,96]. The adaptor protein Nck has a key role in mitogenic signaling downstream of PDGFβ receptors after binding to phosphorylated Tyr751. Nck is involved in JNK serine/threonine kinase activation. In contrast to PDGFβ receptor mitogenic effects, several pathways downstream of PDGFβ receptors have been found to play key roles in differentiation of neuronal cell line PC12. Activation of MAPK, PLCγ/PKC, and Src are important for differentiation effect of PDGFβ receptors depending on the cell type [28]. PLCγ is important for full mitogenic responses of PDGF receptors in some cells such as vascular smooth muscle cells [82].

In conclusion, proliferation and differentiation responses of PDGFβ receptors are regulated via various cellular transduction pathways.

1.3.1.3.2 Motility

PDGF and PDGF receptors have an important role in cytoskeletal rearrangements and migration. Rearrangement of the actin cytoskeleton via loss of stress fibers, the appearance of edge ruffles, and in some cases the formation of circular membrane ruffles, leads to cell movement [89]. Cells that express PDGFβ receptors are able to migrate chemotactically toward higher concentration gradients of PDGF-BB [97]. However, PDGFα receptor-expressing cells do not have this ability [89]. PDGF receptors induce two different types of cell movements: directed migration (chemotaxis) and random migration (chemokinesis) [43]. Chemotaxis is important for embryonic development and wound healing. The physiological role of chemokinesis remains unknown. PI3-kinase is involved in cell motility [98] and mutation of Tyr740 and Tyr751 impairs chemotactic response of PDGFβ receptors to PDGF and the formation of edge ruffles is extinguished; however it does not have any effect on chemokinesis [56,99]. In addition, PLCγ1/PKC, and SHP-2 are important for chemotaxis whereas Ras inhibits chemotaxic response of cells to PDGF [97]. Other downstream pathways of PDGFβ receptors can also regulate motility response. In the CHO cell line, mutation of Tyr1021 (the binding site of PDGFβ receptors for PLCγ1) results in impairment of chemotaxis after PDGF-BB treatment [98]. However, in the same cell line, mutation of Tyr771 (the binding site for RasGAP) results in an elevation in migratory responses [98]. Interestingly, in the porcine aortic endothelial (PAE) cells, similar mutations did not affect cell migratory responses toward PDGF-BB [43,56]. Thus, it seems that the cell type is crucial with respect to migratory response related to downstream pathways of PDGFβ receptors. Regulating chemotactic capacity of the cell in response to PDGFβ receptor stimulation seems to be important in the treatment of various malignancies and cancers. In particular, PI3-kinase's proliferative and migratory effects could depend on the type of catalytic p110 subunit expressed or differences in downstream effectors [43].

1.3.1.3.3 Survival

PDGF plays a pivotal role in preventing cells from apoptosis, also called programmed cell death. The anti-apoptotic effects of PDGF and PDGF receptors in PC12 cells were dependent on the PI3-kinase pathway after Tyr740 and Tyr751phosphorylation. In fibroblasts and neuronal cells, Akt, activated downstream of PI3-kinase, showed significant anti-apoptotic effects [100]. The PI3-kinase/Akt pathway is a proto-oncoprotein with the ability to inhibit apoptosis; activated Akt phosphorylates BAD, a member of BCL-2 family, resulting in the suppression of apoptosis (dephosphorylated BAD has the ability to bind to BCL_{XL} and to promote apoptosis) [28]. Interestingly, Ras has multiple effects on apoptosis depending on how it is activated. Ras has anti-apoptotic effects through activation of PI3-kinase and has pro-apoptotic effects through stimulation of Raf/MAPK/ERK1,2 pathway [30].

1.3.1.3.4 Gene regulation and expression

PDGF receptors play a key role in gene regulation. Early growth response genes are expressed after PDGF receptor stimulation. Transcriptional factors fos, myc, JE, and KC are examples of these genes with the latter two encoding monocyte and neutrophil chemoattractants, respectively [43]. STATs seem to be at least partially responsible for PDGF receptor-induced transcriptional responses. Stimulation of PDGFβ receptors by PDGF-BB results in a transient upregulation of both mRNA transcripts and protein levels of PDGFα and PDGFβ receptors [101].

Table 1.3 Summarized cellular effects of PDGF and PDGFβ receptors

Cellular effect of PDGFβ receptor	*Effect, Downstream pathways, Intermediate effectors or adaptor proteins*			*Tyrosine Phosphorylation sites*
Proliferation	Ras/MAPK pathway	Direct activation of Grb2-Sos		Tyr716/Tyr775
		Indirect activation of Grb2-Sos	Shc	Tyr579/Tyr740/ Tyr751/Tyr771
			SHP-2	Tyr763/Tyr1009
	Ras/MAPK-independent pathway	Src		Tyr579/Tyr581
		PLCγ		Tyr1009/ Tyr1021
		PI3-kinase		Tyr740/Tyr751
		Nck		Tyr751
Differentiation		Ras/MAPK		Tyr716/Tyr775
		PLCγ/PKC		Tyr1009/ Tyr1021
		Src		Tyr579/Tyr581
Motility and Cell Migration	Chemotaxis	PI3-kinase PLCγ/PKC SHP-2	+	Tyr740/Tyr751 Tyr1009/ Tyr1021 Tyr763/Tyr1009
		RasGAP Src/ PLCγ	-	Tyr771 Tyr934
	Chemokinesis	N/A		N/A
Survival	Anti-apoptotic	PI3-kinase/Akt/PKB PI3-kinase/Ras	+	Tyr740/Tyr751 N/A
	Apoptosis	Ras/MAPK	-	Tyr716/Tyr775
	Neuronal protection	PLC/NMDAR Current		1021/1009

1.3.1.4 Physiological role of PDGF and PDGF receptors

1.3.1.4.1 Embryonic development

PDGF receptors have key roles in the development of neural crest cells (NCCs). Both PDGFα receptors and PDGFβ receptors have complementary roles during cardiac NCCs development. PDGFα receptors are also important in non-neuronal and PDGFβ receptors are important in neuronal cardiac NCCs development [102]. PDGF-A and PDGFα receptors play key roles in proliferation and migration of several other organs during embryonic development including

lung, intestine, skin, testis, kidney, eye, CNS, axial skeleton, and teeth [36]. PDGF-B and PDGFβ receptors are important in cardiovascular system, kidney, CNS (particularly the cerebral microcirculation and BBB development), and hematopoietic development [36,204].

1.3.1.4.2 Neuroprotection, neurogenesis, and synaptogenesis

Since the 1960s, our view of neurogenesis has changed dramatically [103]. Previously, it was thought that neuronal production and proliferation ended when development was completed [104]. However, recent studies have demonstrated that the neuronal stem cells (NSCs) in the subventricular zone (SVZ) or dentate gyrus subgranular zone (DG-SGZ) are responsible for maintenance and regeneration of the nervous system [32,104]. This finding has become the focus of intense research in neurodegenerative disease, neurological disorders, and neuronal injury.

It is now accepted that not only can neurons regenerate in the adult brain, but that brain injury can induce neurogenesis. Brain regions in patients with epilepsy, ischemic stroke, Alzheimer's, Huntington's, and Parkinson's disease all show an increase in neurogenesis [32]. The importance of neurogenesis and synaptogenesis in the pathophysiology and treatment of neurological disorders and neurodegenerative diseases such as Alzheimer's and Parkinson's disease highlights the potential for new therapies to target these processes. In early neurodegenerative diseases and in psychiatric disorders, both neurogenesis and synaptogenesis may be significantly impaired, possibly due to a reduction or dysfunction in neurotrophic factors and their receptors. Strategies aimed at increasing neurogenesis may be useful to several CNS disorders.

The term "neurotrophic factor" is often limited to nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4): endogenous soluble proteins that modulate growth, proliferation, differentiation, survival, repair, neurogenesis, synaptogenesis, and synaptic plasticity in neurons [105]. They exert their function through two distinct classes of neurotrophin receptors: p75 neurotrophin receptor ($p75^{NTR}$), which belongs to the tumor necrosis factor (TNF) receptor superfamily and is a transmembrane glycoprotein receptor; and tropomyosin related kinase receptors (TrkA, B, and C) [106,107].

Although the PDGF system is generally thought to function primarily in the periphery, its signaling may also have a significant effect on neuronal activity [48]. PDGF-BB has neurotrophic effects on neurogenesis, neuronal growth and differentiation, survival, synaptogenesis, and synaptic modulation. Importantly, PDGF is neuroprotective against several neurotoxic insults such as ischemia, infection, and various toxins [108,109]. Administration of other growth factors, EGF and FGF, to aged rat brain (*in vivo*) resulted in a 5-fold increase in neurogenesis compared to control, demonstrating the ability of aged brain to response to exogenous growth factor in order to replace neurons which are damaged or lost [110].

PDGF signaling pathways may also regulate specific ion channels in neurons. Glutamate, the major excitatory neurotransmitter in the CNS, exerts its action via N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methylisoxzole-4-proprionic acid (AMPA) receptors, and kainate receptors [111,112]. NMDA receptors have a major role in excitatory synaptic transmission, neuronal development, neuronal physiology, long term potentiation (LTP), and neuronal plasticity [112,113]. On the other hand, dysregulation in NMDA receptor activity is associated with neurological diseases such as disorders due to acute excitotoxic insults (e.g. traumatic brain injury and ischemic brain stroke) as well as diseases associated with chronic neurodegeneration such as Parkinson's, Alzheimer's, and Huntington's, prion disorders, and diseases resulting from hyperexcitability or sensitization of neurons such as neuropathic pain and seizure disorders [112,111]. In contrast, neurodevelopmental and neuropsychiatric disorders such as schizophrenia and autism are associated with hypoactivity of NMDA receptors [112,111]. Thus, NMDA receptors have a paradox in their function; they can both have a neuronal protective effects and neuronal toxicity effects. Excessive NMDA receptor activity due to the high concentration of the glutamate results in neurotoxicity, yet learning and memory in hippocampus are dependent on LTP at CA1 synapses; CA1-LTP is dependent on influx of Ca^{+2} through NMDA receptors [114]. Activation of NMDA receptors in the rat dentate gyrus decreases neurogenesis, whereas acute treatment of rat dentate gyrus by antagonists of NMDA receptors results in an increase in overall density of neurons [115].

PDGFβ receptor activation has a long lasting inhibitory effects on NMDA currents CA1 pyramidal hippocampal neurons which is relevant to the overactivation of NMDA receptors that leading to neurotoxicity [116,114]. PDGF-BB pretreatment of PDGF also regulates γ-aminobutyric acid ($GABA_A$) receptors, which have inhibitory effects on synaptic transmission in the CNS and are the major inhibitory ion-channel receptors in the CNS. In CA1 pyramidal hippocampal neurons, PDGF-BB and PDGFβ receptors cause a long lasting inhibition of NMDA receptor-dependent excitatory postsynaptic currents (EPSCs) and $GABA_A$-dependent inhibitory postsynaptic currents (IPSCs) [49,116,117]. Both the inhibition of NMDA and $GABA_A$ receptor currents are dependent on Tyr1021 phosphorylation and the activation of PLCγ. EGF and BDNF receptors, in contrast to PDGF receptors, cause a long lasting stimulation on excitatory synaptic transmission [114].

1.3.1.4.3 Wound healing

Wound healing is a complex orchestrated cascade that tissues use to repair themselves after injury. Various processes including platelet aggregation, inflammation, cell proliferation, cell migration, and extracellular matrix production are involved. PDGF-BB and PDGFβ receptors are one of the growth factor systems that are involved in regulation of wound healing [36,118]. Platelets release PDGF-BB in the blood clot after the injury. PDGF attracts neutrophils and macrophage to the site of injury; PDGF-BB also attracts fibroblast and stimulates fibroblast and smooth muscle cell proliferation. In addition, PDGF stimulates macrophages to release fibroblast growth factors resulting in production of extracellular matrix scaffolds. Furthermore, PDGF has a role in wound contraction. In normal tissue the expression of PDGFβ receptors is low, whereas the expression of PDGFβ receptors is upregulated in damaged tissues, highlighting the function of PDGF and PDGF receptors in the wound healing process. Recombinant PDGF-BB has therapeutic effects on decubitus ulcers and increases the wound healing process in diabetic patients [36].

1.3.1.4.4 PDGF and PDGF receptors in diseases

Despite numerous roles identified for the PDGF system during development, much less is known about the physiological function of PDGF signaling in developed animals, particularly in neurons. Overexpression/hyperactivity of PDGF ligands can lead to several pathological conditions and diseases and PDGF inhibitors have been used as a choice for cancer therapy [36,44]. In general, the diseases that PDGF and PDGF receptors are involved into could be separated to five categories: malignancies, vascular disorders, fibrosis, inflammation, and polymorphisms.

Malignancies

V-sis gene, simian sarcoma virus (SSV) oncogene, has been found to have a close homology with human PDGF-B gene [44,120]. Subsequent studies revealed that PDGF-B (c-sis) is a proto-oncogene [121]. Many tumor cell lines express and secrete PDGF-A [122]. Further studies have demonstrated that autocrine/paracrine secretion of PDGF stimulates a malignant brain tumor of glial cell origin called glioma via PDGFα receptors, whereas PDGF-B autocrine loops stimulates the vasculature via PDGFβ receptors [44,123]. PDGF and PDGF receptors are expressed mostly in glioblastomas; however, robust expression of PDGF and PDGF receptors has been found in other malignant tumors as well [36]. Autocrine activation of PDGF receptors is important in the genesis of gliomas and sarcomas, whereas paracrine activation of PDGF receptors is seen in many other types of tumors including mammary, colorectal, and lung carcinoma. Both autocrine and paracrine PDGF activation increase tumor cell growth and stroma activity including accelerating VEGF expression and angiogenesis [34]. Truncated PDGFβ receptors (i.e. dummy receptors), receptor antibodies, kinase inhibitors, dominant negative ligands, aptamers, or antibodies against ligands have all been investigated for use in cancer therapy to counteract the overexpression and overactivation of the receptors and their cognate ligands [44].

Vascular disorders

PDGF-B and PDGFβ receptors promote the migration and proliferation of smooth muscle cells leading to intimal thickening and atherosclerosis. In contrast, PDGF-A and PDGFα receptors

inhibit this migration and regulate the smooth muscle cell activity in the arterial wall [124,125]. Upregulated gene expression and production of PDGFs and PDGF receptors result in pulmonary artery vascular smooth muscle cell (vSMC) hyperplasia, a hallmark of pulmonary arterial hypertension (PAH) [36]. Overexpression of PDGF-B and PDGFβ receptors have roles in ischemic retinopathies including proliferative vitreoretinopathy, choroidal neovascularization, and proliferative diabetic retinopathy [36].

Fibrosis

Overactivation/overexpression of PDGFα/PDGFβ/PDGFαβ receptors have been found in several fibrosis such as myelofibrosis (bone marrow fibrosis), liver fibrosis, dermal fibrosis (scleroderma), renal fibrosis, cardiac fibrosis, and pulmonary fibrosis [36,126-129].

Inflammation

Robust expression of PDGF-B and PDGFβ receptors is observed in inflamed joints in patients with rheumatoid arthritis [36,130]. In addition, glomerulonephritis and glomerulosclerosis that are inflammatory kidney disorders are associated with over expression of PDGF-B and PDGFβ receptors resulting in massive mesangial cell proliferation and glomerular filtering destruction. In both of these inflammatory disorders autocrine and paracrine PDGF-B secretion and PDGFβ receptors over activation are responsible for the malfunction [36,131].

Polymorphisms

Single nucleotide polymorphisms in PDGF and PDGF receptor genes have been reported. Overexpression of PDGF-A and overactivation of PDGFα receptors are associated with neural tube defects and gene polymorphism. Spina bifida, anencephaly, and childhood asthma are related to PDGFα receptor promoter polymorphism [36].

1.4 Transactivation

Although GPCRs and RTKs were assumed to have entirely separate signaling cascades and functions, in the past decades it has come to light that there are several intricate interactions between these two receptor families. RTKs can be activated directly by their ligands or can be transactivated through intracellular signaling cascades via cross-talk with other receptors such as GPCRs; this process was first described by Daub, H. *et al.* 1996 and coined the term transactivation [132]. Transactivation pathways may contribute to the proliferative activity of many GPCRs. In other words, transactivation is cross-talk between two heterologous receptors in which the activation of the second receptor is a consequence of the primary activation of the first receptor. Classically, GPCRs are the initiating receptor, and RTKs are the second receptor [133]. Two different models have been described for RTK transactivation by GPCRs. The first model is the ligand-dependent model: upon activation of a GPCR, an intracellular signaling cascade is initiated that ultimately results in the extracellular release of the endogenous RTK ligand by cleavage and shedding of membrane-associated precursors via metalloproteinases of the ADAM family [7] [Figure 1.7]. Metalloproteinases belonging to the ADAM family such as membrane-bound matrix metalloproteinases (MMPs) cleave and shed RTK ligands that in turn stimulate their cognate receptors [134]. Subsequently, receptor activation proceeds in a manner similar to normally-activated receptor [135]. The most well-known example of this transactivation model is the transactivation of epithelial growth factor (EGF) receptors [136]. For example, the gonadotropin-releasing hormone (GnRH) receptor transactivates EGFR through a heparin-binding EGF-like growth factor (HB-EGF) mechanism [137]. Triple-membrane-passing-signaling (TMPS) is a new term for this kind of transactivation; since ligand-dependent transactivation consists of three transmembrane signaling events including GPCR activation (extra to intracellular); subsequent membrane metalloproteinase activation (intra to extracellular); followed by activation of RTKs (extra to intracellular). An important implication of this type of transactivation is that nearby cells may be exposed to the released RTK ligand. In the other word, the released growth factors can either activate the cognate receptors on the same cells or on the adjacent cells.

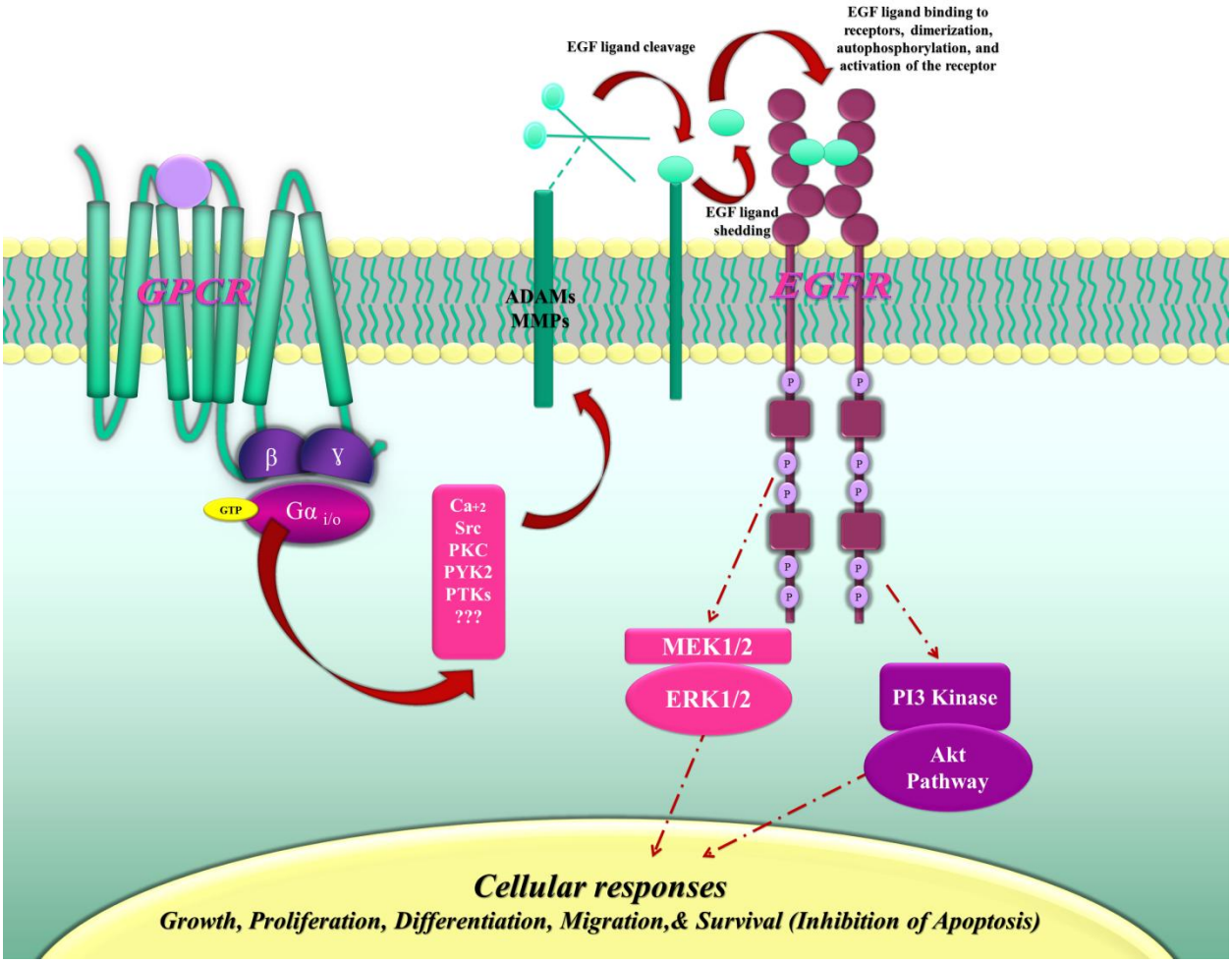

Figure 1.7 Triple-membrane-passing-signaling (TMPS) transactivation

The other type of transactivation is ligand-independent. In this form of transactivation, the activation of the receptor is entirely intracellular and independent of ligand. Thus, the transactivation of RTKs by GPCRs is limited to the cells that express both receptors [138] [Figure 1.8]. For example, dopamine D_2 receptor activation results in the transactivation of PDGFβ receptor even *in the absence* of the PDGF ligand, i.e., it involves only intracellular signaling pathways [24,42]. Pituitary adenylyl cyclase-activating polypeptide (PACAP) transactivates TrkA neurotrophin receptors in a similar, ligand-independent manner [139].

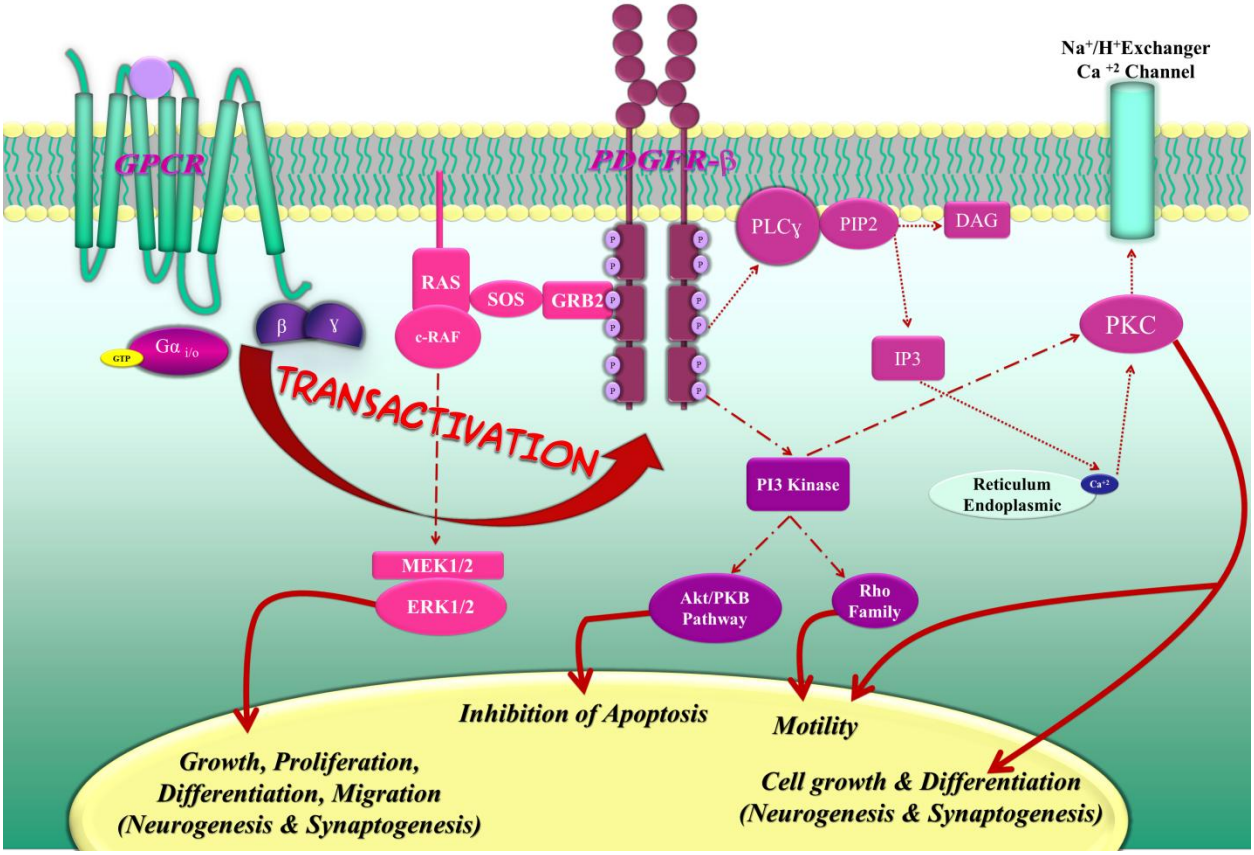

Figure 1.8 Ligand-independent transactivation

1.5 Rationale

In the past two decades, elucidating pathways for different types of cross-communication between receptors has become an area of intense research. Proliferation and differentiation are important in many physiological and pathophysiological processes including cancer. In the brain, neurogenesis and synaptogenesis are keys to our understanding and treatment of neurodegenerative and psychiatric disorders. Although different studies on various cells have shown that GPCRs are able to transactivate PDGFβ receptors, the importance of this phenomenon remains unclear. The main questions are: Why do cells in the body require transactivation at all since PDGFβ receptors can be activated directly by their ligands? Why do cells have two similar, if not identical, pathways to regulate and activate PDGFβ receptors? What

is the relevance of PDGFβ receptors transactivation by GPCRs in disease? Can GPCR ligands substitute for RTK ligands to achieve similar downstream effects such as neuroprotection?

Before we even begin understanding the physiological relevance of GPCR-induced RTK transactivation, we need a better understanding of the differences and/or similarities between direct RTK activation by ligand versus GPCR-induced transactivation of RTKs. These crucial findings will provide direction to future experiments on transactivation pathways in several model systems and the potential physiological, pathological, and therapeutic implications for these pathways. In this project we considered whether transactivation results in the activation of the same downstream cascades as direct ligand activation and whether transactivation activates the PDGFβ receptor to the same degree as direct activation.

1.6 Objectives

A complete understanding of transactivation between GPCRs and RTKs reveals a novel way for regulating cell signaling in both diseased and healthy systems. Many researchers have proposed that the proliferative and pro-survival effects of GPCRs are associated with transactivation of RTKs. This means that many outcomes of GPCR signaling depend on the transactivation. Since this phenomena is intricate and vague, current studies are focused on clarifying the downstream mechanism involved in transactivation. The first null hypothesis stated is that the phosphorylation profile of ligand-activated versus transactivated PDGFβ receptors will be identical. The second null hypothesis is that no differences between downstream cascades of PDGFβ receptors after direct activation and transactivation will be observed. Addressing these two hypotheses may help for future understanding of signaling differences between GPCR-induced growth factor receptor transactivation and direct ligand activation; and, physiological relevance of GPCR-induced RTK transactivation.

Chapter 2

Materials and Methods

2.1 Materials

Summarized table of components and antibodies were used for this project represented in Table 2.1 and Table 2.2.

Table 2.1 Materials

Components	Source
Acrylamide 30% (37.5:1)	Fisher Bio Reagents
Bicinchoninic acid (BCA) protein assay kit	Thermo
Beta-glycerophosphate	MP Bio
β-mercaptoethanol	Fisher Bio Reagents
Bromophenol blue	Sigma
Bovine serum albumin (BSA)	Fisher Bio Reagents
Butanol (1-butanol) 99%	Fisher Bio Reagents
Cell culture media (DMEM and Ham's F12 in a 1:1 ratio)	Fisher #SH20361
Chemiluminescent Substrate	Millipore
Dithiothreitol (DTT)	Fisher Bio Reagents
Dopamine (3,4-dihydroxyphenethylamine hydrochloride)/ Dopamine receptor agonist	Sigma
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
Ethylene diamine tetraacetic acid (EDTA)	Fisher Bio Reagents
Ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA)	Sigma
Ethyl alcohol 95%	Fisher Bio Reagents
Fetal bovine serum (FBS), sterile	Fisher Bio Reagents
Glycerol 99.5%	Sigma
Glycine	MP Biio
Halt protease inhibitor cocktail, EDTA free	Fisher Bio Reagents
Hydrochloric acid 6N	Fisher Bio Reagents
Ladder (EZ-Run™) pre-stained rec protein ladder	Fisher Bio Reagents
Methanol	Fisher Bio Reagents
Nitrocellulose membrane	Fisher Bio Reagents
Non-fat dry milk	Fisher Bio Reagents
Phosphate buffered saline (PBS)	Sigma
PDGF-BB 50ug/mL/PDGFβ receptor agonist	Sigma
100 U/mL penicillin, and 100 µg/mL streptomycin	Hyclone
(−)-Quinpirole hydrochloride ≥98% (HPLC), solid/ Dopamine $D_{2/3}$ agonist	Sigma ALDRISH
S(−)-Raclopride (+)-tartrate salt >97%, solid/ Dopamine $D_{2/3}$ antagonist	Sigma ALDRISH
Sodium dodecyl sulfate (SDS)	Fisher Bio Reagents
Serotonin (5-HT; 5-hydroxytryptamine hydrochloride)/ Serotonin receptor agonist	Cedarlane
Sodium chloride	Sigma
Sodium orthovanadate (Na_3VO_4)	MP Bio
Sodium pyrophosphate,99%	Acros

Components	Source
Tetramethylethylenediamine (TEMED)	BIO-RAD
Tris Base	Fisher Bio Reagents
Triton-X100	MP Bio
Trypsin 0.25%	Sigma
Tween 20	Fisher Bio Reagents
8-hydroxy-DPAT(8-hydroxy-2-dipropylaminotetralin hydrobromide)/ 5HT1A/5HT7 agonist	Cedarlane

Table 2.2 Antibodies

Antibodies against	Dilution	Source	
β-actin	1:800	Mouse	Santa Cruz
PDGFβ receptor	1:800	Rabbit	Santa Cruz
PDGFβ receptor phosphorylation 1021	1:500	Rabbit	Santa Cruz & Cell Signaling
PDGFβ receptor phosphorylation 1009	1:500	Rabbit	Cell Signaling Antibody sampler kit
PDGFβ receptor phosphorylation 740	1:500	Rabbit	Cell Signaling Antibody sampler kit
PDGFβ receptor phosphorylation 751	1:500	Rabbit	Cell Signaling Antibody sampler kit
PDGFβ receptor phosphorylation 771	1:500	Rabbit	Cell Signaling Antibody sampler kit
ERK1/2	1:1000	Rabbit	Cedarlan
phospho-ERK1/2	1:1000	Rabbit	Cedarlan
Phosphor-Akt (Ser473)	1:1000	Rabbit	Cell Signaling Antibody sampler kit
Phosphor-Akt (Ser308)	1:1000	Rabbit	Cell Signaling Antibody sampler kit
Akt	1:1000	Rabbit	Cell Signaling Antibody sampler kit
Phospho-c-Raf (Ser259)	1:1000	Rabbit	Cell Signaling Antibody sampler kit
Phospho-GSK-3β (Ser9)	1:1000	Rabbit	Cell Signaling Antibody sampler kit
Phospho-PTEN (Ser380)	1:1000	Rabbit	Cell Signaling Antibody sampler kit
Phospho-PDK1 (Ser241)	1:1000	Rabbit	Cell Signaling Antibody sampler kit
PLCγ1	1:1000	Rabbit	Cell Signaling Antibody sampler kit
Phospho-PLCγ1(Tyr783)	1:1000	Rabbit	Cell Signaling Antibody sampler kit
PLCγ2	1:1000	Rabbit	Cell Signaling Antibody sampler kit
Phospho- PLCγ2 (Tyr1217)	1:1000	Rabbit	Cell Signaling Antibody sampler kit
Phospho- PLCγ2 (Tyr759)	1:1000	Rabbit	Cell Signaling Antibody sampler kit
Goat anti-rabbit HRP secondary antibody	1:10000	Goat-anti-rabbit	Thermo
Goat-anti mouse HRP secondary antibody	1:5000	Goat-ant-mouse	Thermo

2.2 Methods

The SH-SY5Y human neuroblastoma cell line is a third subcloned cell line of SK-N-SH cells. The SK-N-SH cell line was established in 1970 from a bone marrow biopsy of a four-year-old neuroblastoma female patient with sympathetic adrenergic ganglia origin. Since 1980, SH-SY5Y cell line has been widely used in experimental neurological studies because it expresses many biochemical and functional neuronal properties such as exhibiting tyrosine and dopamine-β-

hydroxylases, the norepinephrine (and serotonin) transporter, as well as opioid, muscarinic, dopamine, and growth factor receptors. Therefore, the SH-SY5Y cell line is often used in experiments on metabolism, analysis of neuronal differentiation, neuronal function related to neurodegenerative disorders, neurotoxicity and neuroprotection [140]. We also have extensive experience in the lab with using SH-SY5Y cells for transactivation studies [141,142].

SH-SY5Y cells were obtained from Dr. Shilpa Buch (University of Nebraska Medical Centre in 2009). Cells were cultured in a complete growth media consisting of DMEM with Ham's F12 in a 1:1 ratio supplemented with 10% fetal bovine serum (Thermo Fisher), and 1% penicillin/streptomycin at a temperature of 37°C, under 5% CO_2 and 98% air. The media was changed every two-three days.

2.2.1 Treatment, cell lysis, protein assay

For cell treatment, quinpirole, D_2-family dopamine receptor agonist; 3,4-dihydroxyphenethylamine hydrochloride (dopamine); 5-hydroxytryptamine hydrochloride (serotonin); 8-OH-DPAT, a 5HT1A and 5HT7 receptor agonist; raclopride, a dopamine $D_{2/3}$ receptor antagonist; and PDGF-BB , a ligand for PDGFβ receptors, were used. Cells were incubated with drugs for various time periods depending on the specific experiment. For studies that used a receptor antagonist, cells were pre-incubated for 5 minutes prior to agonist treatment. After drug treatment, cells were washed with ice-cold phosphate buffered saline (PBS) with pH 7.4. PBS is a balanced salt solution for maintaining pH, regulating osmotic balance, and providing water and essential inorganic ions for short-term cell incubation. PBS was drained and the ice-old lysis buffer [20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 30 mM sodium pyrophosphate, 1 mM betaglycerophosphate, 1 mM sodium orthovanadate (Na_3VO_4), 1% triton and 1% Halt protease and phosphotase inhibitor (Thermo)] [Table 2.3] was used in order to lyse the cells. 80 µL of lysis buffer was used for each 2 cm well. Cells were scraped and sheared by forcing them through 26 gauge needles for lysis. Insoluble material of the lysates was pelleted via centrifugation at 14000 x g for 20 minutes at 4 ˚C, and supernatants collected.

Table 2.3 Lysis buffer components

Lysis buffer	A lysis buffer breaks the cell membrane in order to release the cell contents and proteins. It improves protein stability and keeps them in the solution [20 mM Tris-HCl pH 7.5,150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 30 mM sodium pyrophosphate, 1 mM betaglycerophosphate, 1 mM sodium orthovanadate (Na_3VO_4), 1% triton and 1% Halt Protease and phosphotase inhibitor (Thermo)]
Tris-HCl	Tris-Hcl regulates the acidity and osmolarity of the solution
NaCl	NaCl maintains ionic strength of medium
EDTA **(Ethylene diamine tetraacetic acid)**	EDTA and EGTA are preservatives and chelators for divalent cations; they can reduce the activity of proteolytic enzymes that require calcium and magnesium ions as cofactors. Therefore, they preserve the proteins of digestion via proteolytic enzymes. EDTA inhibits the metalloproteases that require Mg^{2+} and Mn^{2+}
EGTA **(Ethylene glycol tetraacetic acid)**	EGTA, the same as EDTA, reduces oxidation damage, chelates metal ions, and inhibits the metalloproteases that require Ca^{2+}
Sodium pyrophosphate	Sodium pyrophosphate irreversibly inhibits dephosphorylation of proteins (Ser/Thr phosphatases inhibitor)
Beta-glycerophosphate	Beta-glycerophosphate irreversibly inhibits dephosphorylation of proteins (Ser/Thr phosphatases inhibitor)
Sodium orthovanadate (Na_3VO_4)	Na_3VO_4 irreversibly inhibits dephosphorylation of proteins (Tyr and alkaline phosphatases inhibitor)
Triton	Triton is a detergent, and it lyses cell membranes, solubilizes membrane proteins and lipids, and controls protein crystallization
Halt Protease	Halt blocks or inactivates endogenous proteolytic and phospholytic enzymes, which are released from cells during cell lysis and degrade proteins of interest and their activation states. Usually, it serine/threonine and tyrosine phosphatases and prevents protein degradation and simultaneously preserves phosphorylation.

After lysis, the total protein concentration of samples was determined by using a bicinchoninic acid (BCA) protein acid kit (Thermo, Fisher). In 96-well plates, various concentrations of bovine serum albumin (BSA) were used by diluting BSA with Milli-Q water and the solubilization buffer [20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM ethylene diamine tetraacetic acid EDTA, 1 mM EGTA, 30 mM sodium pyrophosphate, 1 mM betaglycerophosphate, 1 mM sodium

orthovanadate and 1% triton] [Table 2.4] for making a standard curve. For samples, 30 µL of Milli-Q water and 8 µL of solubilization buffer were mixed with 2 µL of each sample in duplicate. 300 µL of Working Reagent A:B in a 50:1 ratio (Thermo Fisher) was added to each well cell and incubated for 30 minutes at 37˚C. Absorbance was measured by Spectra Max M5 Multi-Mode Micro plate Reader (Molecular Devices, Sunnyvale, CA) at 562 nm. For calculating the protein concentration of each sample, first, the average of blank absorbance measurement in standard was subtracted from the measurements of all other standards and all samples replicates. The standard curve was prepared by plotting the average blank-corrected data for each BSA standard versus its concentration in µg/mL. The standard curve was used for determining the protein concentration of each sample.

Table 2.4 Solubilization buffer components

Solubilization buffer	A solubilization buffer breaks interactions associated with protein aggregation including disulfide bonds, hydrogen bonds, van der Waals forces, ionic interactions, and hydrophobic interactions. These interactions lead to protein aggregation and precipitation that result in artifacts or sample loss. A good solubilization is important for a successful electrophoretic separation process. [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM ethylene diamine tetraacetic acid EDTA, 1 mM EGTA, 30 mM sodium pyrophosphate, 1 mM betaglycerophosphate, 1 mM sodium orthovanadate and 1% triton]
Tris-HCl	Tris-Hcl regulates the acidity and osmolarity of the solution .As both pH and ionic strength influence protein stability and solubility, choosing an appropriate buffer is important. The choice of buffer and pH influences the results of protein solubilization.
NaCl	NaCl maintains ionic strength of medium
EDTA **(Ethylene diamine tetraacetic acid)**	EDTA reduces oxidation damage, chelates metal ions, and inhibits the metalloproteases that require $Mg^{2+}$and Mn^{2+}
EGTA **(Ethylene glycol tetraacetic acid)**	EGTA reduces oxidation damage, chelates metal ions, and inhibits the metalloproteases that require Ca^{2+}
Sodium pyrophosphate	Sodium pyrophosphate is a Ser/Thr phosphatases inhibitor
Beta-glycerophosphate	Beta-glycerophosphate is a Ser/Thr phosphatases inhibitor
Sodium orthovanadate (Na_3VO_4)	Na_3VO_4 is a Tyr and alkaline phosphatases inhibitor
Triton	Triton is a detergent and, it lyses cell membrane, solubilizes membrane proteins and lipids, and controls protein crystallization

2.2.2 Western blotting

Western blotting or immunoblotting is a qualitative and semiquantitative protein analysis technique. It works via antibody-antigen interaction that helps to identify a target protein in the complex protein mixture. For our experiments, western blotting was used to separate proteins based on their molecular weights. Western blotting consists of three phases: electrophoresis, transferring, and immunoblotting.

2.2.2.1 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) is a process for separating and identifying the macromolecule target such as proteins and peptides in a mixture of macromolecules solution. The process was based on a protein mass-charge ratio separation. We used 8% acrylamide gels, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), containing resolving gel (8%) and stacking gel (4%).

2.2.2.1.1 Preparation of SDS-PAGE gel

SDS-PAGE gel is created via the polymerization of two compounds, acrylamide and *N*,*N*′-methylene bisacrylamide. The polymerization of gel begins with the addition of ammonium persulfate (APS) and tetramethylethylenediamine (TEMED). APS induces the polymerization of acrylamide and bisacrylamide monomers and then decomposes to form free radicals; TEMED promotes the polymerization by stabilizing the free radicals and acting as an oxygen scavenger, since acrylamide and bisacrylamide are nonreactive by themselves. Bisacrylamide acts as a cross-linking agent. These components make a neutral hydrophilic gel that has a three dimensional structure that consists of hydrocarbons cross-linked with methylene groups. The pore size of the gel depends on the size of the protein of interest. Acrylamide alone forms linear polymers, and using bisacrylamide helps make crosslinks among polyacrylamide chains. The pore size of the gel is based on the total amount of acrylamide and the amount of cross-linker. The higher the acrylamide concentration and the higher the ratio of bisacrylamide to acrylamide, the lower the pore size of the gel and the lower the electrophoretic mobility. By varying the total

monomer concentration (which is called %T) and weight percentage of cross linker, (which is called %C), the pore size of the gel can be obtained based on the size of the protein of interest. The higher %T indicates the higher polymer-to-water ratio and the smaller pores.

For casting gels, we assembled the gel glasses on the gel stand appropriately. Resolving gel (8%) was prepared by mixing the appropriate quantity of components of resolving gel [Resolving gel (8%): Milli-Q water 2.43 mL, Tris HCl 1 M (pH 8.8) 3 mL, acrylamide: bisacrylamide 30:0.8% w/v 2 mL, SDS 20% 38 μL, APS 10% 36 μL, TEMED 5 μL]. The resolving gel solution was poured in to the space between gel glasses immediately. The solution was poured within 2-3 cm of the top of the glasses. The top of the gel was covered by water-saturated n-butanol. After one hour, the resolving gel solution was prepared by mixing the components of stacking gel [Stacking gel (4%): Milli-Q water 3.6 mL, Tris HCl 1 M (pH 6.8) 630 μL, acrylamide: bisacrylamide 30:0.8% w/v 660 μL, SDS 20% 25 μL, APS 10% 25 μL, TEMED 5 μL]. The n-butanol on the top of the resolving gel was drained and the remaining was decanted with filter paper. The stacking gel solution was poured on top of the resolving gel and the comb was inserted between two glasses. After 15-20 minutes the gel polymerization was done and the gel was ready for using. The comb was carefully removed and the gel was used for electrophoresis.

2.2.2.1.2 Preparation of samples, sample loading and gel running

After correcting the volume of the samples to account for variation in protein concentrations, samples were diluted in the 3× loading buffer [Table 2.5], and heated at 90-95 °C for 5 minutes. The 3× loading buffer means the mixture of one volume of the sample with 2 volume of the 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 dithiothreitol DTT, 5% v/v β-mercaptoethanol]. After assembling the electrophoresis apparatus, running buffer [25 mM Tris base at pH 8.3, 0.1% w/v SDS, 190 mM Glycine, Milli-Q water] [Table 2.6] was poured to the electrophoresis apparatus. The running buffer must cover all top of the gel(s) in the electrophoresis apparatus. The samples were loaded to the well-cells of the gel and appropriate ladder as a molecular weight marker was used in the first well-cell of the gel. A total of 25-40 μg proteins per well were loaded. After loading, gel

was run at 60 V for 30-40 minutes until the blue dye passed the stacking gel. The voltage was increase after passing stacking to 100-120 V for approximately 60 minutes.

Table 2.5 Loading buffer components

Loading buffer	Antibodies usually recognize a specific part of the protein called epitope. In the 3D conformation structure of the protein, this part can be inaccessible. To enable the access of the antibody to the recognition site of protein, unfolding or denaturing the protein is essential. A loading buffer is used for this purpose [240 mM Tris-HCl at pH 6.8, 6% w/v SDS, 30% v/v glycerol, 0.02% w/v bromophenol blue, 50 mM dithiothreitol DTT, 5% v/v β-mercaptoethanol]
Tris-HCl	Tris-HCl works as a buffer to maintain the appropriate pH
Sodium dodecyl sulfate (SDS) **Dithiothreitol (DTT)** **β-mercaptoethanol** **Heating at 90-95°C**	Actual denaturation of the samples happens via SDS, DTT, and heat. SDS, an ionic detergent with an anionic head group and lipophilic tail, binds to proteins non covalently, adds negative charge to the amino acids, and breaks up the secondary and the tertiary dimensional structure of the proteins. Indeed, SDS can denature proteins molecules without damaging or breaking peptide bonds. Covalent and non-covalent linkages to other molecules and disulfate bonds preserve the quaternary structure of the proteins. To complete denaturation, heating can shake up the molecules and allows SDS to bind to the hydrophobic regions that are tightly associated with lipids. The covalent disulfate bonds that are related to the sulfhydryl (-SH) group of the amino acid cysteine, the last part of the quaternary structure of the proteins, will be reduced and denatured by DDT. β-mercaptoethanol reduces disulfide bonds as well and disrupts protein cross-links. At the end the process, the samples are left in primary structure only, and in the presence of SDS, the intrinsic protein charges are masked; thus, during SDS-PAGE, the rate of protein migration is determined by their mass. Then, the SDS-bound proteins migrate through the gel from the negative charged electrode toward the positive charged electrode. Therefore, proteins with less molecular weight move quickly through the gel compared to proteins with higher molecular weight.
Glycerol	Glycerol helps the samples sink easily into the wells of the gel and makes the sample more dense than the sample buffer; therefore the samples will remain in the bottom of the wells rather than float.
Bromophenol Blue	Bromophenol blue, a tracking dye, helps determine how far the separation has progressed.

Table 2.6 Running buffer components

Running buffer	A running buffer helps proteins to migrate through electrophoresis gel [25 mM Tris base at pH 8.3, 0.1% w/v SDS, 190 mM Glycine, Milli-Q water]
Tris-HCl	Tris-HCl works as a buffer to maintain the appropriate pH
Sodium dodecyl sulfate (SDS)	SDS is an ionic detergent
Glycine	SDS-PAGE gel is composed of resolving gel that is buffered with Tris and adjusted to pH 8.8, and the stacking gel is adjusted to pH 6.8 by the Tris buffer. In addition, a running buffer is buffered by Tris and adjusted to pH 8.3. Glycine is a weak acid with the ability to be presented in two different states: uncharged and charged. Glycine at low pH is protonated and uncharged, and at higher pH is negatively charged. Starting to run a gel at a constant voltage is forces glycine ions in the running buffer to transfer from the cathode toward samples and the stacking gel. The stacking gel has lower pH, and passing the glycine through it uncharges and slows down the glycine. Furthermore, chloride ions available in the stacking gel and samples move away from the cathode as well. Thus, high electrical resistance happens in the stacking part of the gel. This electrical field forces the negatively charged proteins in the samples to move through the stacking gel just behind the chloride ions and in the tight bands. Thus, the result of moving the electrical zone is the migration of the protein from the stacking gel to the resolving gel. The migration of the proteins in the resolving gel depends on molecular weight. In the resolving gel, the pH goes up and the glycine becomes charged. Therefore, because of the pore and sieving characteristics of the resolving gel, the mobility of the glycine goes up and the mobility of proteins goes down. Glycine passes the proteins, and the proteins pass the resistance zone and move at their own pace.

2.2.2.2 Transferring

Wet electrophoretic transferring was used for moving the proteins from gel to the synthetic membrane. Immobilized proteins on the membrane then become accessible to interactions with antibodies. Nitrocellulose membrane (Thermo) was used for transferring. In wet transferring, the membrane and gel are sandwiched between sponges and filter papers,

(sponge/paper/gel/membrane/paper/sponge) and are kept together by a nonconducting cassette and submerged in the tank full of ice cold transfer buffer [3.0285 gr Tris-HCl, 14.2595 gr glycine, 200 mL Methanol, 800 mL Milli-Q-Water] [Table 2.7]. The membrane is placed close to the positive electrode and the gel is paced close to the negative electrode. After applying electricity, the negatively-charged proteins move toward the positively-charged electrode. The membrane binds with high affinity to the proteins and they stop migrating once bound. The transferring time was varied from 90 minutes to 3 hours depending on the primary antibody that was going to be used. The voltage for 90 minutes was 100 V, and for 3 hours 70 V.

Table 2.7 Transferring buffer

Transfer Buffer	A transfer buffer is used for moving the proteins from gel to the synthetic membrane [3.0285 gr Tris-HCl ,14.2595 gr Glycine, 200 mL Methanol, 800 mL Milli-Q-Water]
Tris-Base	Tris-Hcl works as a buffer to maintain the appropriate pH
Glycine	Glycine is a weak acid with the ability to be presented in two different states: uncharged and charged. Glycine at low pH is protonated and uncharged, and at higher pH is negatively charged. It pushes the protein forward onto the membrane through repulsive force.
Methanol	Methanol dissociated SDS from the sample proteins and increases protein absorption on to membrane

2.2.2.3 Immunoblotting

After transferring, the membranes were blocked for an hour in blocking buffer [5% non-fat milk in Tris-buffered saline solution with 0.1% Tween 20 (TBS-T)] [Table 2.8] at room temperature. Then, membranes were incubated overnight at 4°C with the primary antibody (diluted in the blocking buffer). Membranes were washed with TBS-T three times for 10 minutes each. Then, they were incubated with the secondary antibody conjugated to horse radish peroxidase (HRP) for one hour at room temperature. Membranes were washed again with TBS-T three times for 10 minutes each. Finally, western chemiluminescent substrate (Millipore, Billerica, MA) was

applied for 5 minutes in order to visualize proteins. Images of protein bands were taken using the Kodak Image Station 4000 MM Pro Imaging Station. Protein bands were identified by their molecular weights by comparing them with a standard ladder (fisher BioReagents EZ-Run^{TM} Pre-Stained Rec Protein Ladder). The net intensity of each protein band was assessed by Kodak Molecular Imaging software. The protein band intensities were normalized to the control band (vehicle or no treatment), and each phosphorylation band intensity after normalization to control was normalized to its total protein band expression intensity. β-actin served as a loading control for normalization. If necessary, membranes were stripped by stripping buffer (1.5% w/v glycine, 0.1% w/v SDS, 1% v/v Tween 20 at pH 2) and additional primary antibodies were used for blotting. One limitation of the current work is that the primary antibodies for different phosphorylation sites at PDGFβ receptors might cross-react with protein tyrosine kinases or receptor tyrosine kinases when highly overexpressed.

Table 2.8 Blotting procedure

Blotting process	[5% non-fat milk in Tris-buffered saline solution with 0.1% Tween 20 (TBS-T)]
Blocking	A nitrocellulose membrane has an affinity for proteins. To prevent non-specific binding of antibodies during blotting process, it is important to block the rest of membrane.
Incubation (primary antibody)	Primary antibody is an antibody that recognize the target protein
Washing	Washing is essential in order to get rid of unbound reagents and antibodies. It reduces background noise and increases the signal/noise ratio.
Incubation (secondary antibody)	As primary antibody is not detectable directly, the tagged secondary antibody, which is linked to an enzyme, is used for detecting the primary antibody. The choice in choosing the secondary antibody is based on the species that the primary antibody was obtained from.
Washing	Insufficient washing leads to high background noise and excessive washing results in attenuation in sensitivity due to the elution of the antibody or antigen
Incubation (substrate)	The enzyme on the secondary antibody drives a reaction Substrate in order to visualize proteins.
Imaging	Kodak 4000MM Pro Imaging Station
Stripping	(1.5% w/v glycine, 0.1% w/v SDS, 1% v/v Tween 20 at pH 2)
Washing	Washing is for getting rid of unbound reagents

2.2.3 Statistical analysis

Statistical analysis of the data was calculated using the Prism GraphPad program. For drawing graphs we used same program. Data were analyzed by one-way ANOVA, time, drug dose, and drug effects were variable factors. For statistical tests, significance level was set to $\alpha = 0.05$.

Chapter 3

Results

In this chapter, the main focus is to clarify the potential differences between activation, phosphorylation, and downstream pathway of PDGFβ receptors after direct ligand activation compared to transactivation.

3.1 Comparison between transactivation of PDGFβ receptors by various agonists of GPCRs and direct activation of PDGFβ receptors

To evaluate any differences between the magnitude of the change on the phosphorylation state of PDGFβ receptors (Tyr1021) after direct activation and transactivation, SH-SY5Y cells were treated with quinpirole (10 μM, Q), dopamine (100 nM, D), serotonin (100 nM, 5-HT), 8-DPAT (10 nM, PAT), and PDGF (1 ng/mL, P) for 10 minutes. To compare the effect of transactivation with direct activation on downstream effectors of PDGFβ receptors, the fold changes on pERK1/2 were also evaluated. The results were analyzed for phospho-PDGFβ receptor at Tyr1021 and phospho-ERK1/2 immunoreactivity as a phosphorylation site and downstream effector of PDGF receptors respectively. The activation of PDGFβ receptors by 1 ng/mL PDGF-BB increased the phosphorylation state of the receptor almost 100-fold, whereas application of the agonists of GPCRs were able to increase the phosphorylation states by only 2-3 fold [Figure 3.1]. Interestingly, PDGF-BB did not increase ERK1/2 phosphorylation to a significantly greater extent than GPCR agonists. Application of GPCR agonists resulted in a 2-3 fold increase in phosphorylation state of ERK1/2 compared to direct activation of PDGFβ receptors that showed almost 4-fold increases in phosphorylation state of ERK1/2 [Figure 3.2]. The dopamine D_4 receptors stimulate ERK1/2 through transactivation of PDGFβ receptors [143]. Thus, activation of ERK1/2 via quinpirole is associated with downstream of PDGFβ receptors.

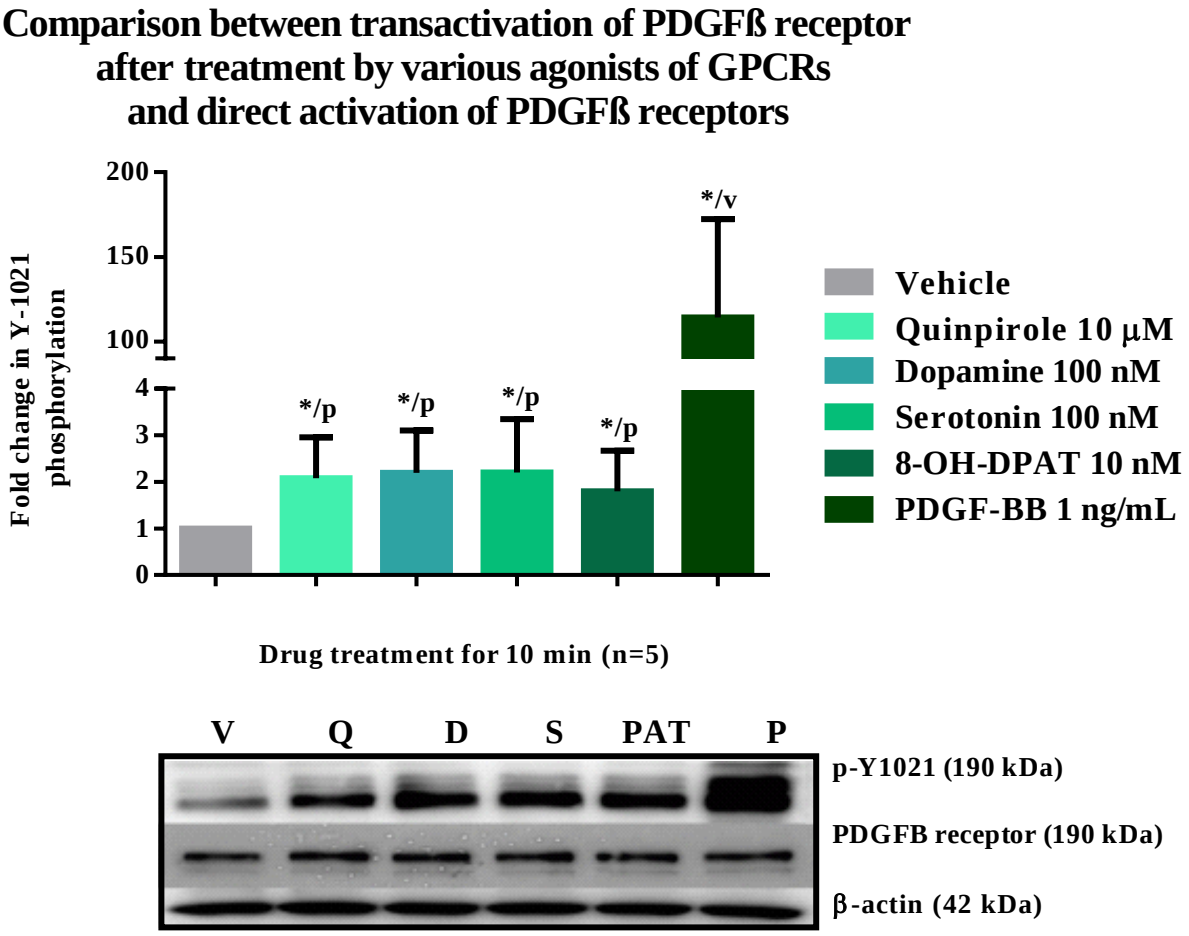

Figure 3.1 Comparison between transactivation and direct activation of PDGFβ receptors

Transactivation of PDGFβ receptors via various GPCR agonists for 10 minutes showed almost same amount of increase in phosphorylation states of p-Y1021 that is 2-3 fold greater compared to non-treated-cells. However, direct activation of PDGFβ receptors showed almost 100 fold increase in phosphorylation state. The data were normalized to the non-treated-cells (vehicle) as a control to deduct the constitutive activity of the receptors, and then were normalized to total PDGFβ receptors expression. β-Actin served as a loading control. The data represent the mean ± standard error of the mean (S.E.M.) for five independent experiments. */V shows statistically significant result with $p < 0.05$ compared to non-treated-cells; */P shows statistically significant results with $p < 0.05$ compared to direct activation of the PDGFβ receptor with PDGF-BB. One-way ANOVA were used for statistical calculation.

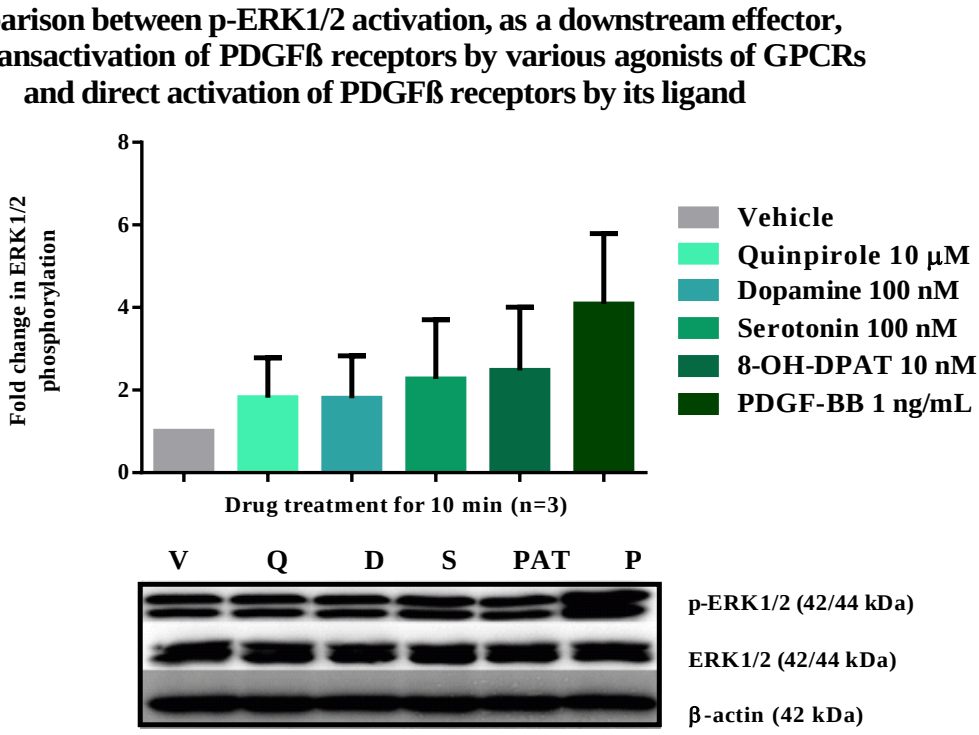

Figure 3.2 Comparison between downstream effector of PDGFβ receptors activation after direct activation and transactivation

Despite the phosphorylation profile of ligand-activated versus transactivated PDGFβ receptor that showed a statistically significant difference, comparison between ERK1/2 activation for transactivation and direct activation did not show any significant differences in results. The data were normalized to the non-treated-cells (vehicle) as a control to deduct the constitutive activity of the receptors, and then were normalized to total protein ERK1/2 expression for downstream effector as a second control. β-Actin served as a loading control. The data represent the mean ± standard error of the mean (S.E.M.) for five independent experiments.

3.2 Time-course for quinpirole transactivation of the PDGFβ receptor

To evaluate the incubation time for maximal dopamine D_2 receptor transactivation of the PDGFβ receptor in SH-SY5Y cells, the cells were treated with quinpirole (10 µM), dopamine (100 nM), and PDGF-BB (1 ng/mL) from 0 to 30 minutes. Cell treatment by quinpirole for 0 to 30 minutes represented sustained phosphorylation of the PDGFβ receptor at Tyr1021 over the time course, with a slight increase (but not significant) between 10 and 30 minutes; 10 minutes was picked as a best treatment time since our project was based on acute treatment of the cells [Figure 3.3B]. The time-course graph for dopamine revealed maximal phosphorylation between 15 and 30

minutes [Figure 3.3C] and 20 minutes for PDGF-BB [Figure 3.3A]. Application of PDGF-BB (1 ng/mL) to SH-SY5Y cells resulted in a rapid increase in PDGFβ receptor phosphorylation at Tyr1021 until minutes 20 followed by a decrease in phosphorylation.

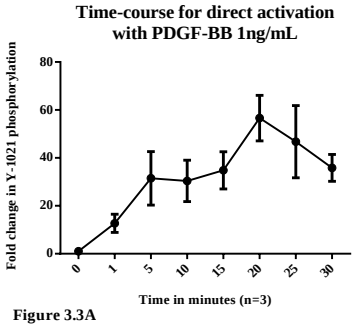

Figure 3.3A

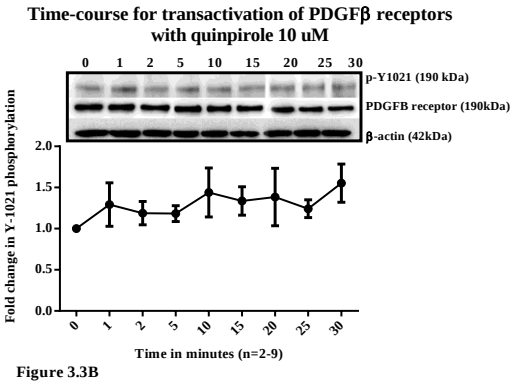

Figure 3.3B

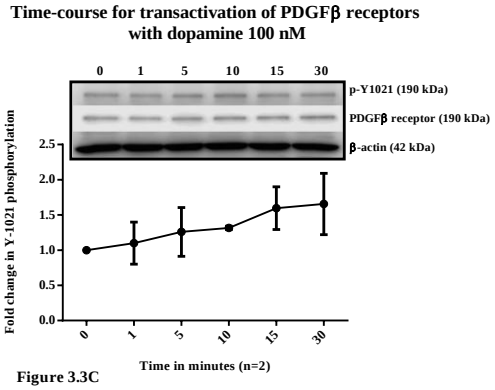

Figure 3.3C

Figure 3.3 A, B, C. Time course comparing transactivation and direct activation of PDGFβ receptors

Representative graphs and blots for activation of phospho-PDGFβ receptor Tyr1021 with quinpirole, dopamine, and PDGF-BB are shown in Figure 3.3A, 3.3B, and 3.3C. The graph in Fig 3.3A shows the time-course for direct activation of PDGFβ receptor via PDGF-BB by evaluating the fold change in phospho-PDGFβ receptor at Tyr1021. Fig 3.3B represents the time-course for transactivation of PDGFβ receptors via quinpirole by evaluating the fold change in phospho-PDGFβ receptor at Tyr1021. Time-course for transactivation of PDGFβ receptors via dopamine by evaluating the fold change in phospho-PDGFβ receptor at Tyr1021 is shown in Fig 3.3C. The results for the time course were analyzed for phospho-PDGFβ receptor Tyr1021 immunoreactivity. The data were normalized to the non–treated-cells (vehicle) as a control and to total PDGFβ receptor expression. β-Actin served as a loading control. The data represent the mean ± standard deviation (S.D.) for two to nine independent experiments for quinpirole, two independent experiments for dopamine, and three independent experiments for PDGF-BB 1 ng/mL.

3.3 Raclopride blocks transactivation of PDGFβ receptor by quinpirole

The dopamine $D_{2/3}$ receptor antagonist, raclopride, was used to investigate whether quinpirole exerts its effect via dopamine $D_{2/3}$ or D_4 receptors to transactivate PDGFβ receptors. SH-SY5Y

cells were preincubated with raclopride (500 nM) for 5 minutes; then the cells were treated by quinpirole (10 μM) or PDGF-BB (0.1 ng/mL) for 10 minutes [Figure 3.4]. Raclopride blocked quinpirole, but not PDGF-BB-induced phosphorylation. At a concentration of 500 nM, raclopride shows some selectivity for dopamine $D_{2/3}$ receptors versus dopamine D_4 receptors [144]. Thus, these data suggest that quinpirole is acting through dopamine $D_{2/3}$ receptors to transactivate PDGFβ receptors in SH-SY5Y cells [Figure 3.5].

SH-SY5Y cell line	Preincubation by raclopride 500nM (5min)	Treatment for 10 min		
		Quinpirole 10μM	Raclopride 500nM	PDGR-BB 0.1ng/ml
Vehicle	-	-	-	-
Q	-	+	-	-
R+Q	+	+	-	-
R	-	-	+	-
R+P	+	-	-	+
P	-	-	-	+

Figure 3.4 Procedure of cell treatments by raclopride

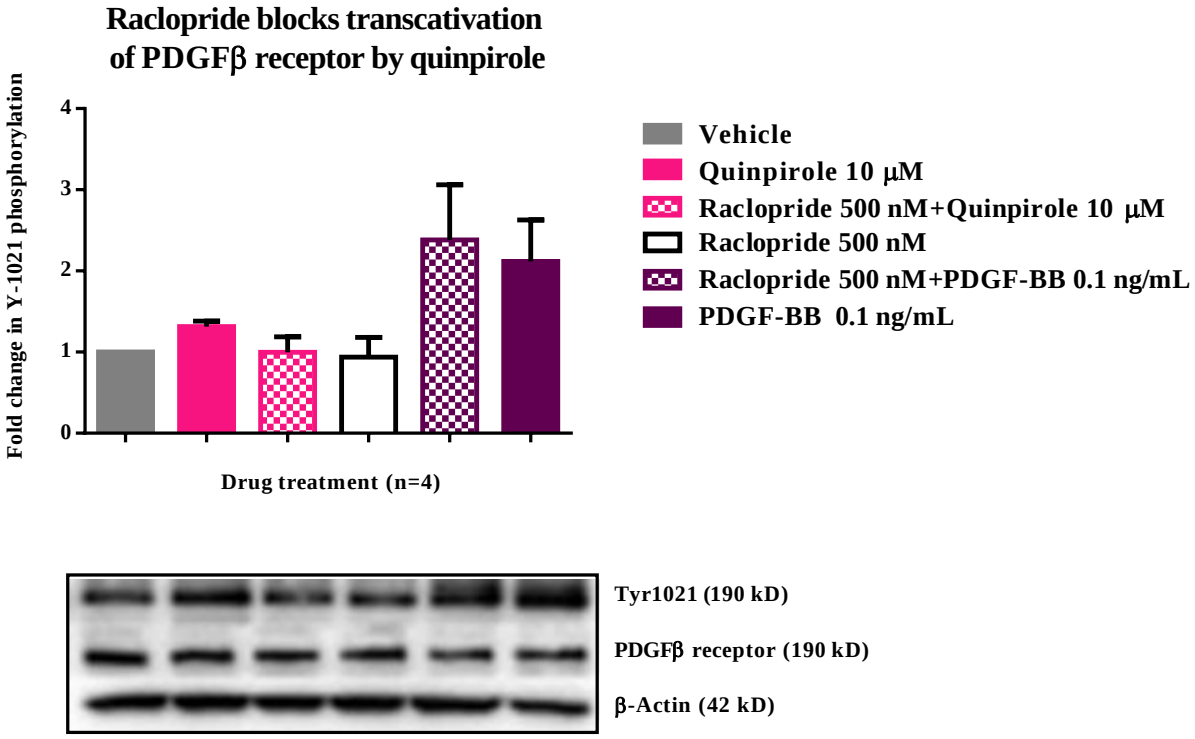

Figure 3.5 Raclopride blocks the transactivation of PDGFβ receptors via quinpirole

Preincubation of the SH-SY5Y cells by raclopride for 5 min blocked dopamine D_2/D_3 receptors and inhibited transactivation of the PDGFβ receptors via quinpirole. Raclopride inhibited quinpirole-induced Tyr1021 phosphorylation. There was no significant effect when raclopride was administered alone. The results were analyzed for phospho-Tyr1021 as a phosphorylation site of the PDGFβ receptors. Cells were lysed after treatment and were evaluated by western blotting. The data were normalized to the non-treated-cells (vehicle) as a control and to total PDGFβ receptors expression for phosphorylation site as a second control. β-Actin served as a loading control. The data represent mean ± S.E.M. for four independent experiments. The data for transactivation and direct activation did not show any statistically significant results on fold change of phospho-PDGFβ receptor Tyr1021with $p < 0.05$. One-way ANOVA was used for statistical calculation.

3.4 Dose-response curve for quinpirole transactivation

To evaluate the effect of increasing the concentration of quinpirole on Tyr1021 phosphorylation, SH-SY5Y cells were treated with quinpirole (100 nM – 100 μM) for 10 minutes. As expected, the phosphorylation at Tyr1021 is concentration-dependent. Quinpirole increased the phospho-PDGFβ receptor Tyr1021 state in SH-SY5Y cells with the half maximal effective concentration

(EC_{50}) of 5.3 μM. In D293 cells, EC_{50} for quinpirole activating D_2-family dopamine receptors was 0.57 nM [145] [Figure 3.6].

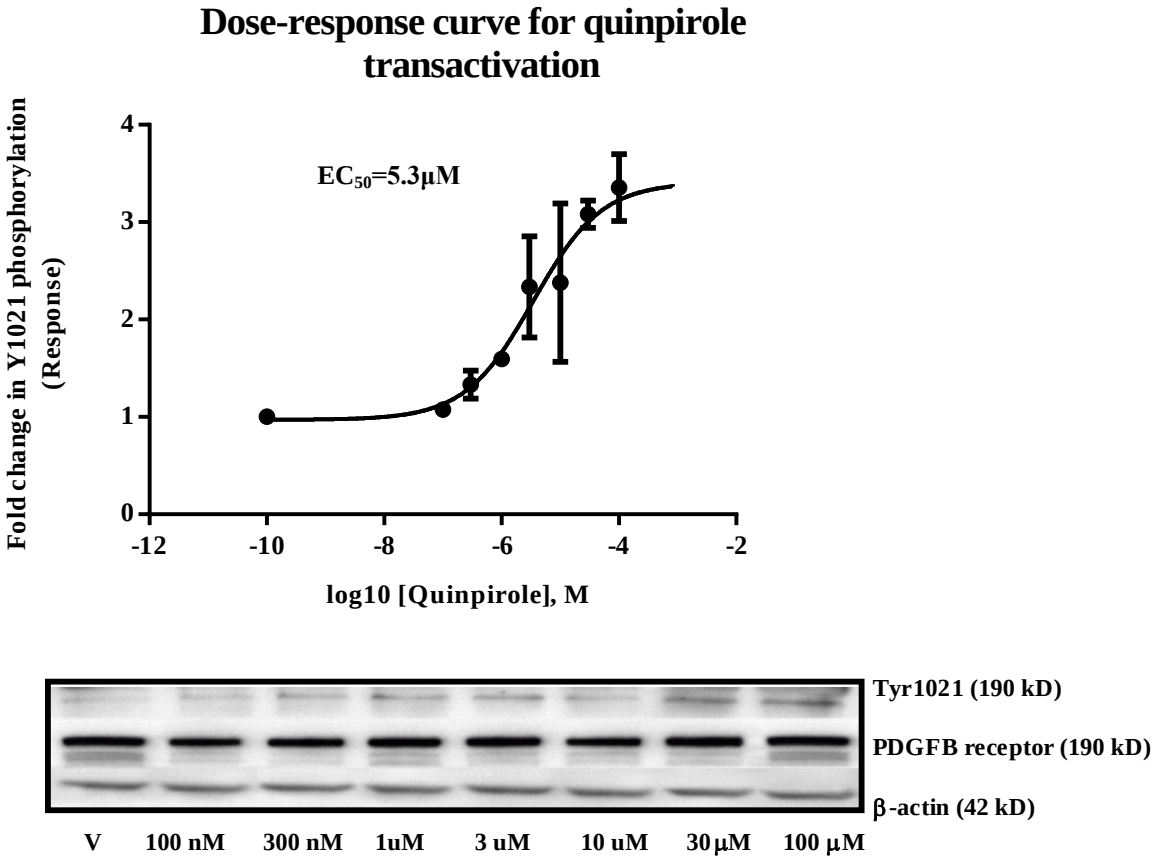

Figure 3.6 Dose-response curve for PDGFβ receptor transactivation via quinpirole

The dose-response curve was made by treating cells via various concentration of quinpirole from lower dose to higher dose and evaluating the amount of fold change in phospho-PDGFβ receptor Tyr1021, in order to find the best dose for treating cells. Cells were lysed after treatment and were evaluated by western blotting. The results for dose-response were analyzed for phospho-Tyr1021 immunoreactivity. The data were normalized to the non-treated-cells (vehicle) as a control and to total PDGFβ receptors expression. β-Actin served as a loading control. The data represent the mean ± standard deviation (S.D.) for three independent experiments.

3.5 Does ligand-activation versus transactivation of the PDGFβ receptor produce different tyrosine phosphorylation profiles

To evaluate whether the phosphorylation profile of ligand-activated versus transactivated PDGFβ receptor is similar, SH-SY5Y cells were treated with 10 μM quinpirole, 100 nM serotonin, 1 ng/mL PDGF-BB, or 0.1 ng/mL PDGF-BB for 10 minutes. Transactivation of PDGFβ receptor via quinpirole resulted in statistically significant differences on phospho-Tyr1021, phospho-Tyr771, and phospho-Tyr740 when compared to direct activation of PDGFβ receptors with 1 ng/mL PDGF-BB ($p < 0.05$)[Figure 3.7A]. Direct activation of PDGFβ receptors resulted in statistically significant increases in the phosphorylation states of Tyr1021, Tyr771, and Tyr740 compared to vehicle (with $p < 0.05$) [Figure 3.7A]. Transactivation of the PDGFβ receptor by quinpirole resulted in statistically significant difference on phospho-Tyr771 and phospho-Tyr751 ($p < 0.05$) compared to direct activation of PDGFβ receptors by 0.1 ng/mL PDGF-BB [Figure 3.7B]. Similar results were observed after the application of serotonin at Tyr771 and Tyr740 [Figure 3.7C].

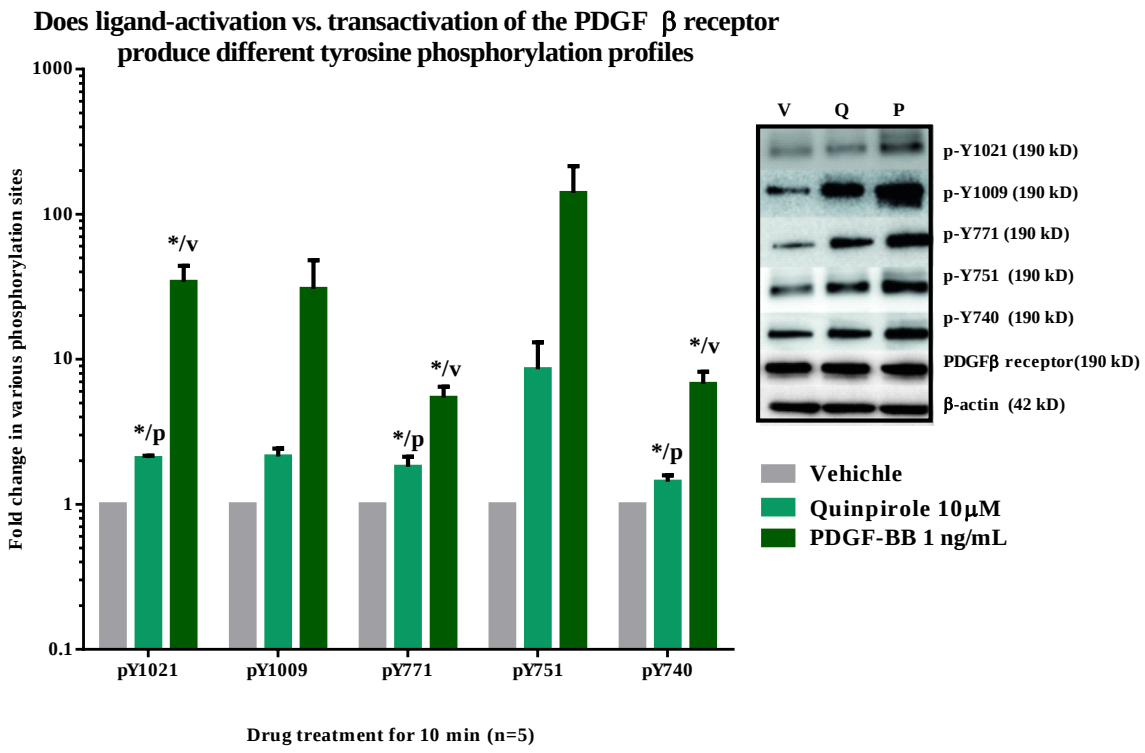

Fig. 3.7A

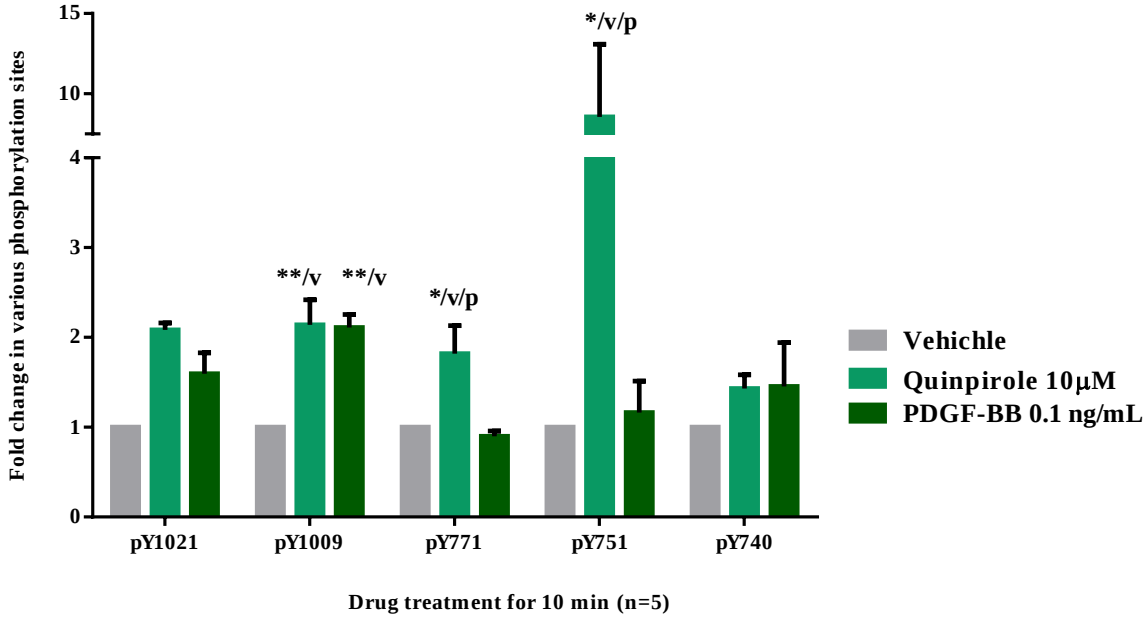

Fig. 3.7B

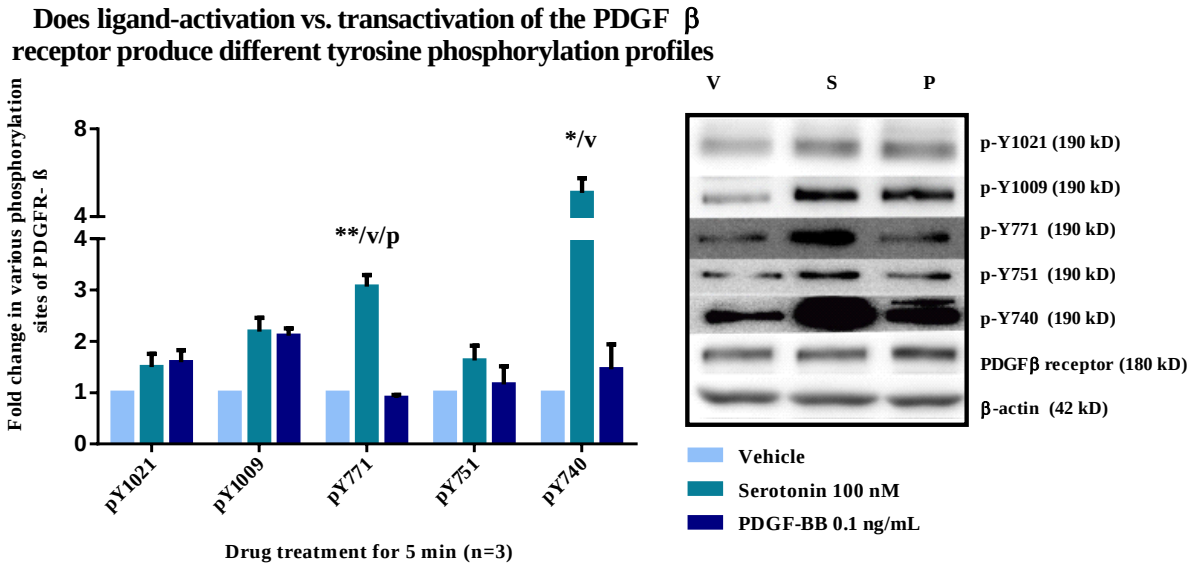

Fig. 3.7C

Figure 3.7 A,B,C. Dose ligand-activation versus transactivation of PDGFβ receptor produce different tyrosine phosphorylation profiles.

Representative graphs and blots for different PDGFβ receptor phosphorylation sites after transactivation by quinpirole and serotonin are shown above. In Fig 3.7.A the data represent the mean ± S.E.M for five independent experiments comparing direct activation by 1 ng/mL PDGF-BB and transactivation by 10 μM quinpirole. In Fig 3.7.B the data represent the mean ± S.E.M for five independent experiments comparing direct activation by 0.1 ng/mL PDGF-BB and transactivation by 10 μM quinpirole. In Fig 3.7.C the data represent the mean ± S.E.M for three independent experiments comparing direct activation 0.1 ng/mL PDGF-BB and transactivation by 100 nM serotonin. Cells were lysed after treatment and were evaluated by western blotting. The results were analyzed for phospho-PDGFβ receptor 1021 (pY1021), 1009 (pY1009), 740 (pY740), 771 (pY771), and 751 (pY751) immunoreactivity; the data were normalized to the non-treated-cells (vehicle) as a control and to total PDGFβ receptors expression. β-Actin served as a loading control.*/V shows statistically significant results with $p < 0.05$ compared to non-treated-cells; */P shows statistically significant result with $p < 0.05$ compared to direct activation of the PDGFβ receptor with 1 ng/mL PDGF-BB. One-way ANOVA were used for statistical calculation.

3.6 Infra-additive effects between direct activation by PDGF-BB and transactivation by serotonin

We have examined and compared direct stimulation of the PDGFβ receptor by PDGF-BB with transactivated receptor by GPCR agonists. In order to examine whether co-treatment with a direct and a transactivation stimulus would synergistically increase PDGFβ receptor phosphorylation, SH-SY5Y cells were treated with serotonin (100 nM), PDGF-BB (0.1 ng/mL), or both for 5 minutes. Cells were lysed after treatment and were evaluated by western blotting. The data showed that co-treatment with 5-HT and PDGF-BB actually reduced the level of phosphorylation of Tyr740 and 771 compared to 5-HT treatment alone [Figure 3.8]. In other words, co-incubation was infra-additive rather than additive or synergistic. Akt is activated downstream of PDGFβ receptors. We analyzed p-Akt473 and p-Akt308 immunoreactivity as a measure of Akt activation after treatment with serotonin (100 nM), or PDGF-BB (0.1 ng/mL), or both. Despite the results with PDGFβ receptor phosphorylation, no significant increases in Akt phosphorylation were observed in any treatment conditions [Figure 3.9].

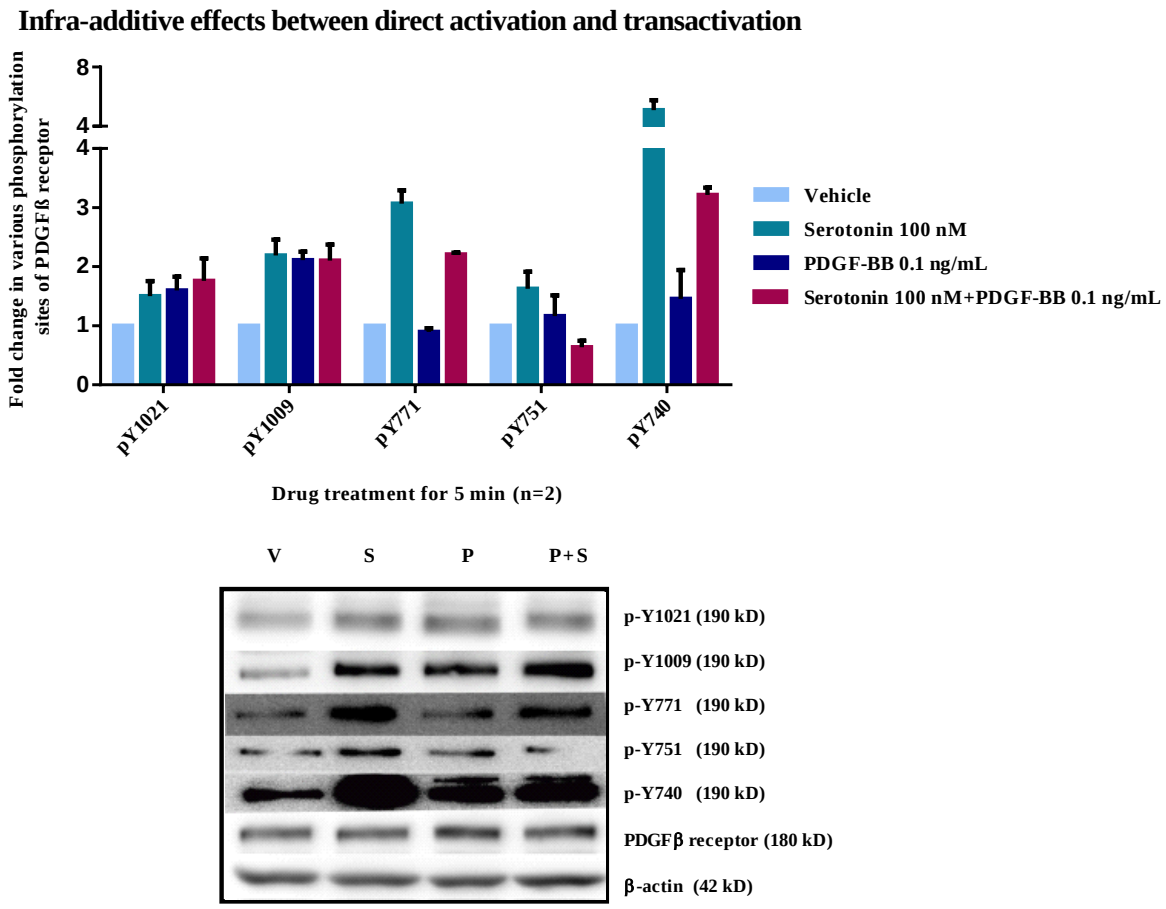

Figure 3.8 Infra-additive effects between direct activation and transactivation, and its effect on fold change in various phosphorylation sites of PDGFβ receptors

The results were analyzed for phospho-PDGFβ receptor 1021 (pY1021), 1009 (pY1009), 740 (pY740), 771 (pY771), and 751 (pY751) immunoreactivity; the data were normalized to the non-treated-cells (vehicle) as a control and to total PDGFβ receptors expression. β-Actin served as a loading control. The data represent the mean ± S.E.M. for two independent experiments. Co-treatment with 5-HT and PDGF-BB reduced the level of phosphorylation of Tyr740 and 771 compared to 5-HT treatment alone.

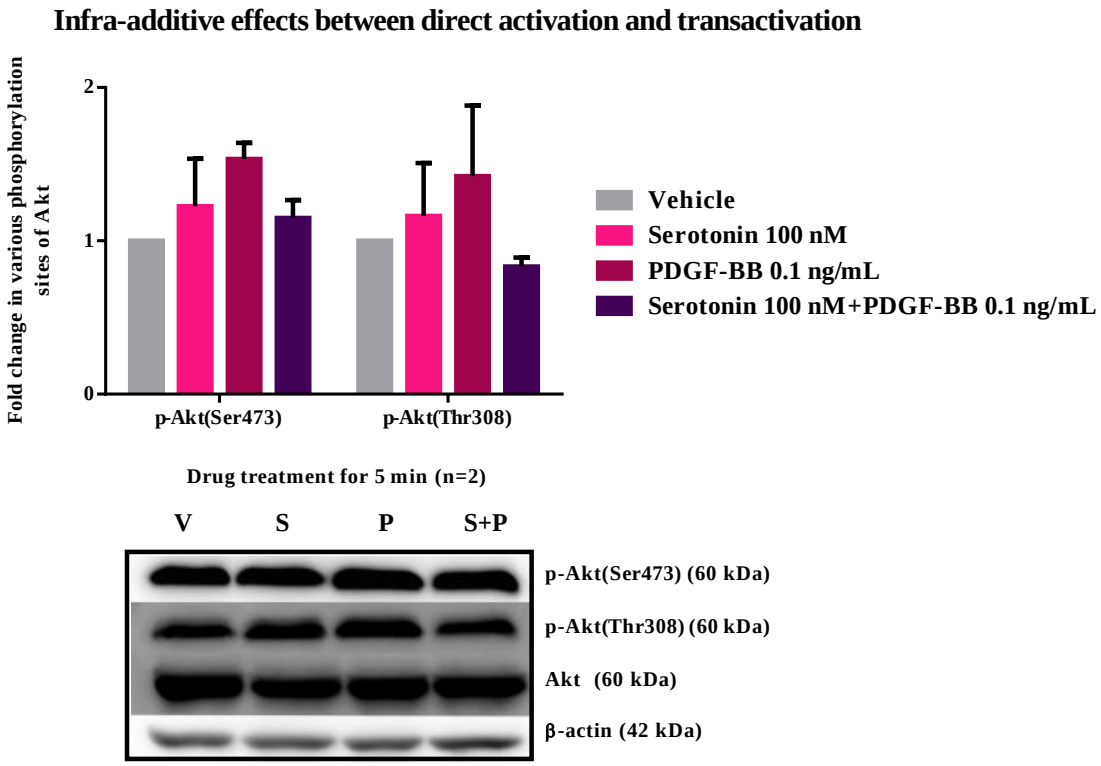

Figure 3.9 Infra-additive effects between direct activation and transactivation, and its effect on Akt phosphorylation as a downstream effector of PDGFβ receptors

The results were analyzed for phospho-Akt (Ser473) and (Thr308) immunoreactivity. The data were normalized to the no-treated-cells (vehicle) as a control and to total Akt protein expression. β-Actin served as a loading control. There was attenuation in phosphorylation of Akt at Ser473 and Thr308 after application of two agonists together. The data represent the mean ± S.E.M. for two independent experiments.

3.7 Downstream effectors activation by quinpirole and PDGF-BB

Quinpirole treatment of SH-SY5Y cells results in the transactivation of PDGFβ receptors and the activation of several downstream signaling cascades. To evaluate the difference between the fold changes in the phosphorylation states of various effectors in downstream of PDGFβ receptors (Tyr1021), cells were treated with quinpirole (10 μM) or PDGF (1 ng/mL) for 10 minutes. Western blots were performed with phospho-ERK1/2 [Figure 3.10], phospho-Akt (Thr308) (Thr473) [Figure 3.11], phospho-c-Raf (Ser259) [Figure 3.12], phospho-PDK1 (Ser241) [Figure 3.13], phospho-PTEN (Ser380) [Figure 3.14], phospho-GSK (Ser9) [Figure 3.15], and phosphor-PLCγ1 (Tyr783) [Figure 3.16].

Our second null hypothesis was that there are no differences between downstream cascades of PDGFβ receptors after direct activation and transactivation. To evaluate this hypothesis the fold changes in the phosphorylation states of various effectors in downstream of PDGFβ receptors were examined. The fold change of p-ERK1/2 after direct activation compared to transactivation of PDGFβ receptors showed greater activation; however the differences were not statistically significant. There was almost 3-fold change in direct activation compared to transactivation [Figure 3.10]. The fold change increase in p-Akt (Thr308) and p-Akt (Ser473), as a part of PI3-kinase/Akt pathway, compared to vehicle is greater for direct activation than transactivation of PDGFβ receptors and there were almost 2-fold changes in direct activation compared to transactivation for both phosphorylation sites [Figure 3.11].

Phospho-c-Raf (Ser259), activated downstream of both MAPK/ERK1/2 and PI3-kinase/Akt pathways, showed a greater increase after transactivation compared to direct activation of PDGFβ receptors [Figure 3.12]. Phospho-c-Raf (Ser259) has a negative regulatory effect on both pathways. The p-PDK1 (Ser241), effector in PI3-kinase/Akt pathway, also showed a greater increase after transactivation compared to direct activation of PDGFβ receptors. There was no significant difference between non-treated cells and direct activation in phosphorylation of PDK1 [Figure 3.13]. PDK1 is responsible for phosphorylation of Akt at Thr308. PTEN, negative regulatory effector of PI3-kinase/Akt pathway, showed almost the same fold change in PTEN (Ser380) phosphorylation after direct activation and transactivation of PDGFβ receptors [Figure 3.14]. GSK-3β, effector in PI3-kinase/Akt pathway, showed slightly more fold change in its Ser9 phosphorylation site after direct activation of PDGFβ receptors via quinpirole compared to transactivation [Figure 3.15]. Activation of Akt has an inhibitory effect on GSK-3β phosphorylation. The phospho-PLCγ1, downstream for PLC pathway, showed a significant fold change after both direct activation and transactivation of PDGFβ receptors compared to non-treated-cells [Figure 3.16]. Thus, based on our results, activation of downstream cascades of PDGFβ receptors after direct activation and transactivation are not identical.

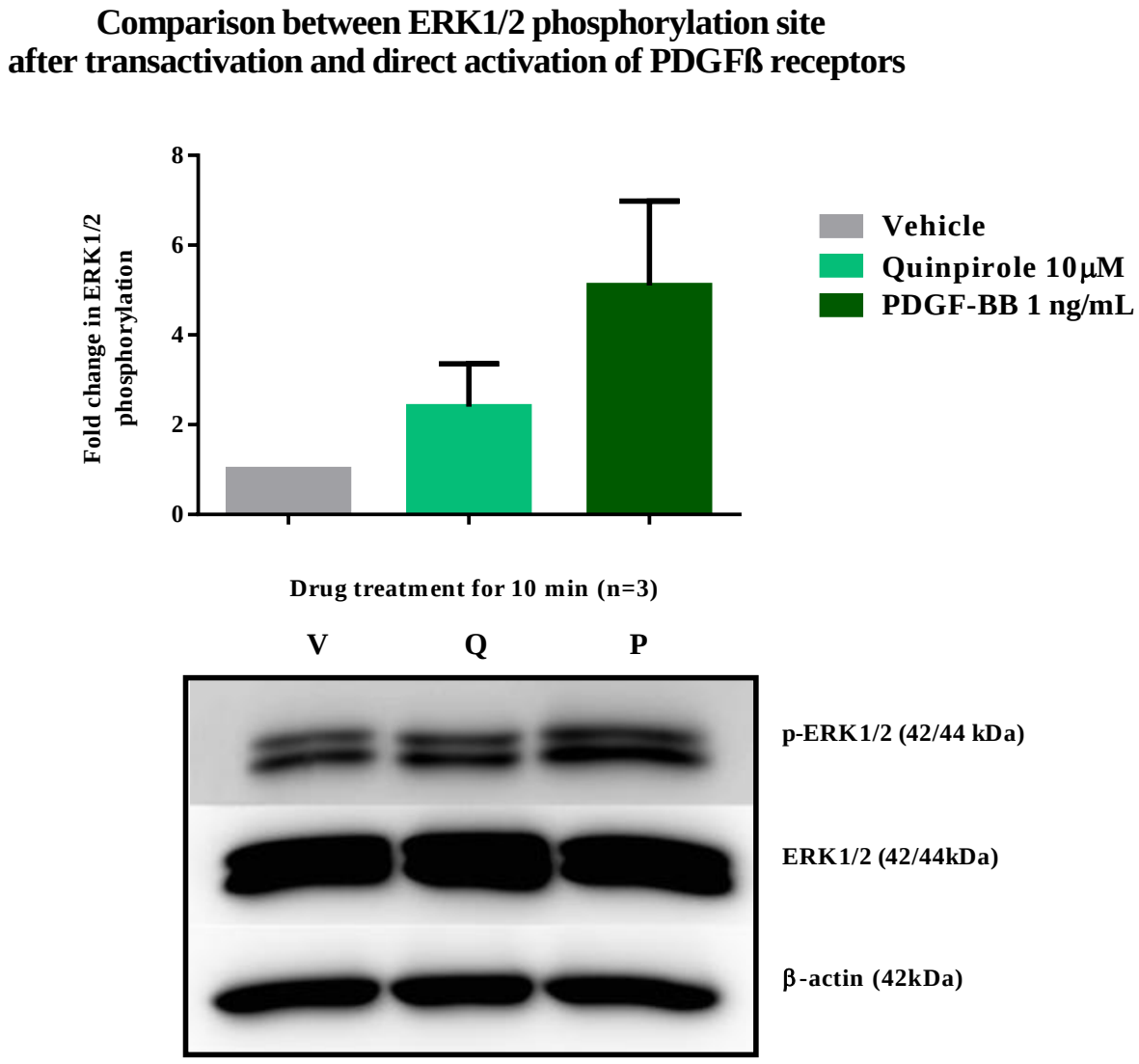

Figure 3.10 Comparison between ERK1/2 fold change of phosphorylation site after transactivation and direct activation

The p-ERK1/2 fold change after direct activation compared to transactivation of PDGFβ receptors showed more activation; however they did not show statistically significant differences with $p < 0.05$.The data were normalized to the non-treated-cells (vehicle) as a control and to total protein expression for ERK1/2. Then, β-Actin served as a loading control. The data represent the mean ± S.E.M. for two independent experiments. One-way ANOVA was used for statistical calculation.

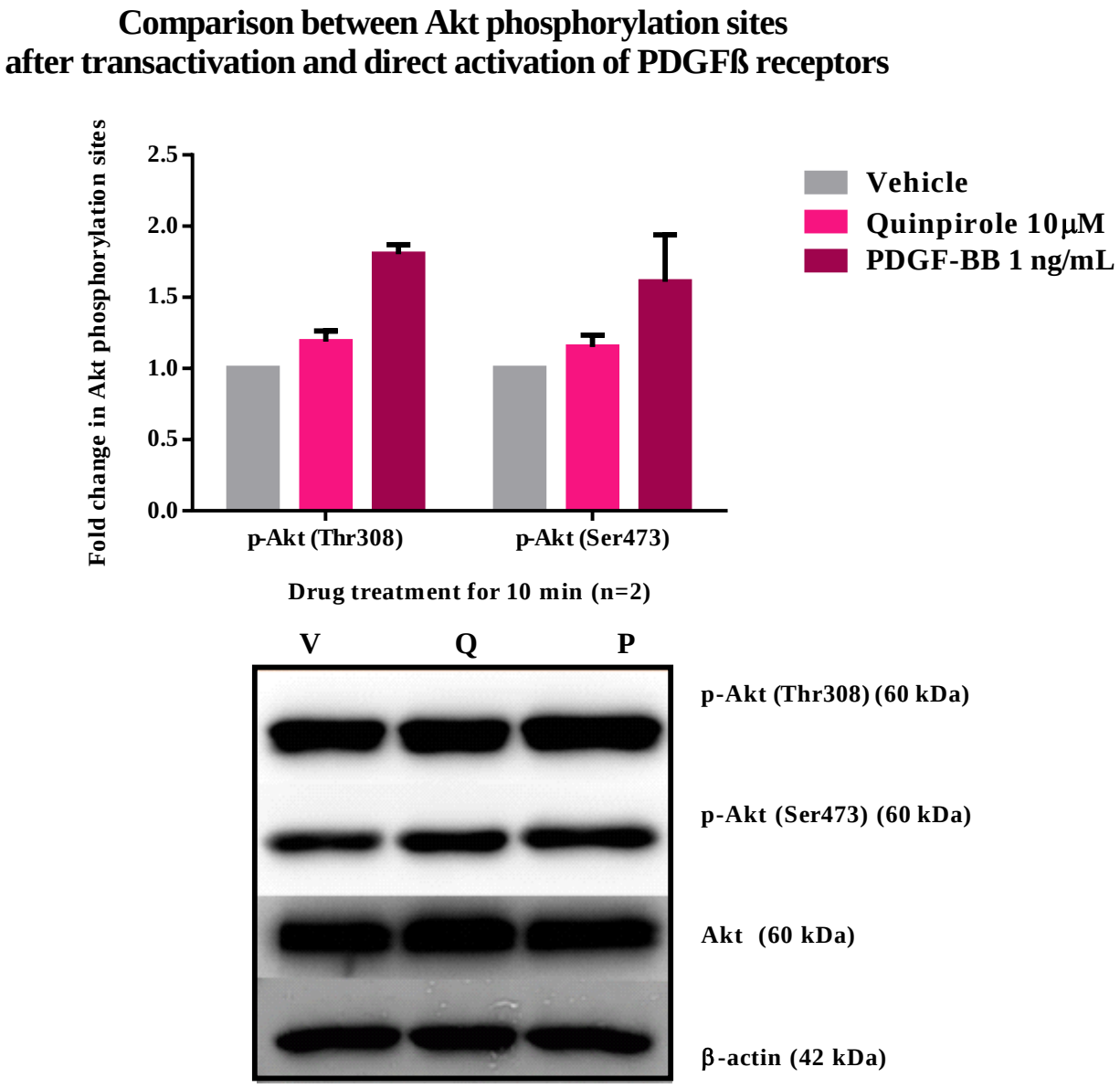

Figure 3.11 Comparison between fold change of Akt phosphorylation sites after transactivation and direct activation

The fold change increase in the p-Akt (Thr308) and p-Akt (Ser473) compared to vehicle is more in direct activation than transactivation of PDGFβ receptors. The data were normalized to the non-treated-cells (vehicle) as a control and to total protein expression for Akt. β-Actin served as a loading control. The data represent the mean ± S.E.M. for two independent experiments.

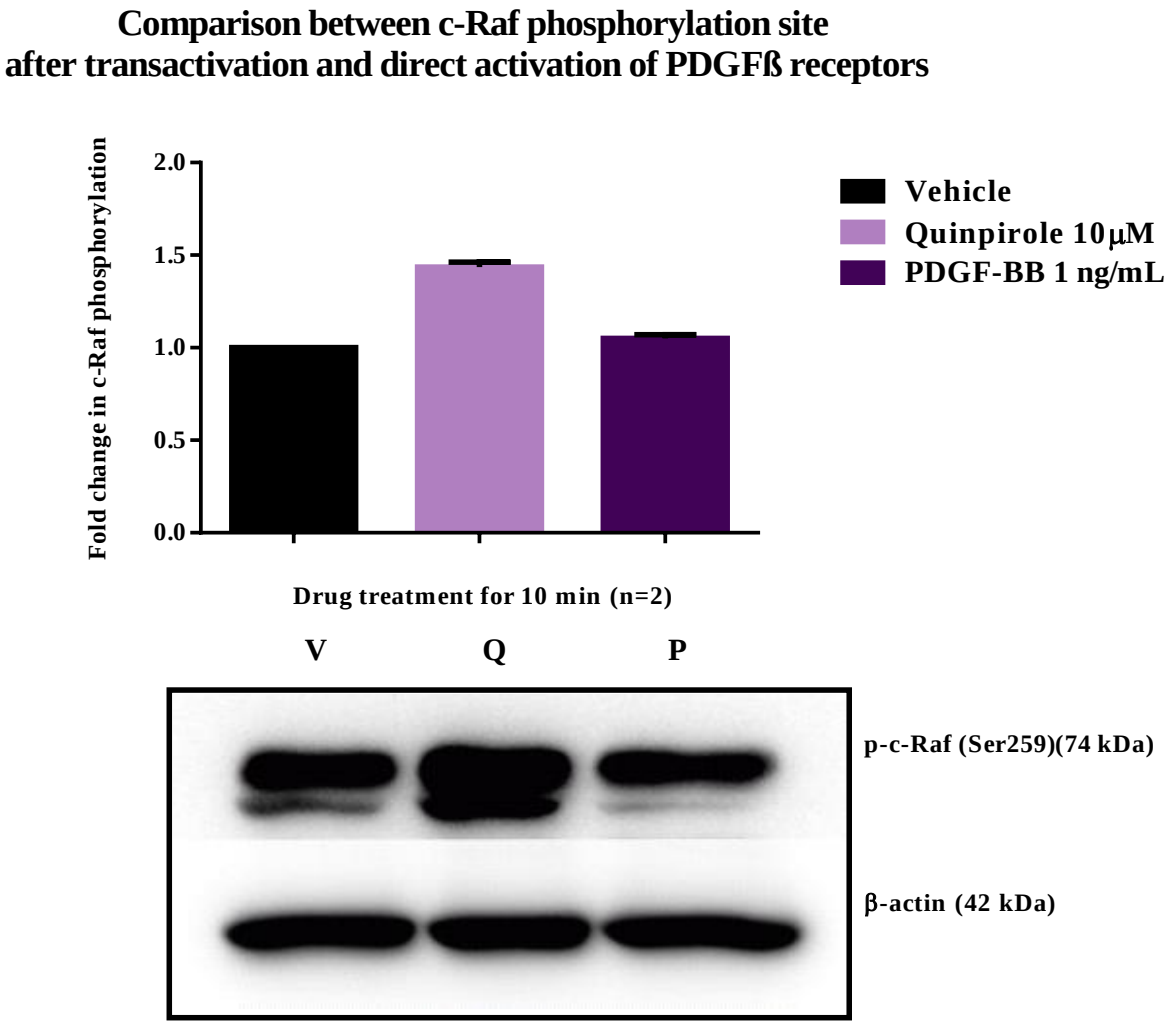

Figure 3.12 Comparison between fold change of phosphorylation of c-Raf at Ser259 after transactivation and direct activation

The phospho-c-Raf (Ser259) as a phosphorylation site of c-Raf showed more increase in fold change after transactivation compared to direct activation of PDGFβ receptors. There were significant difference between phosphorylation of c-Raf after transactivation compared to direct activation and no-treated-cells. There was not any significant difference between non-treated-cells and direct activation in phosphorylation of c-Raf The data were normalized to the non-treated-cells (vehicle) as a control. Then, β-Actin served as a loading control. The data represent the mean ± S.E.M. for two independent experiments.

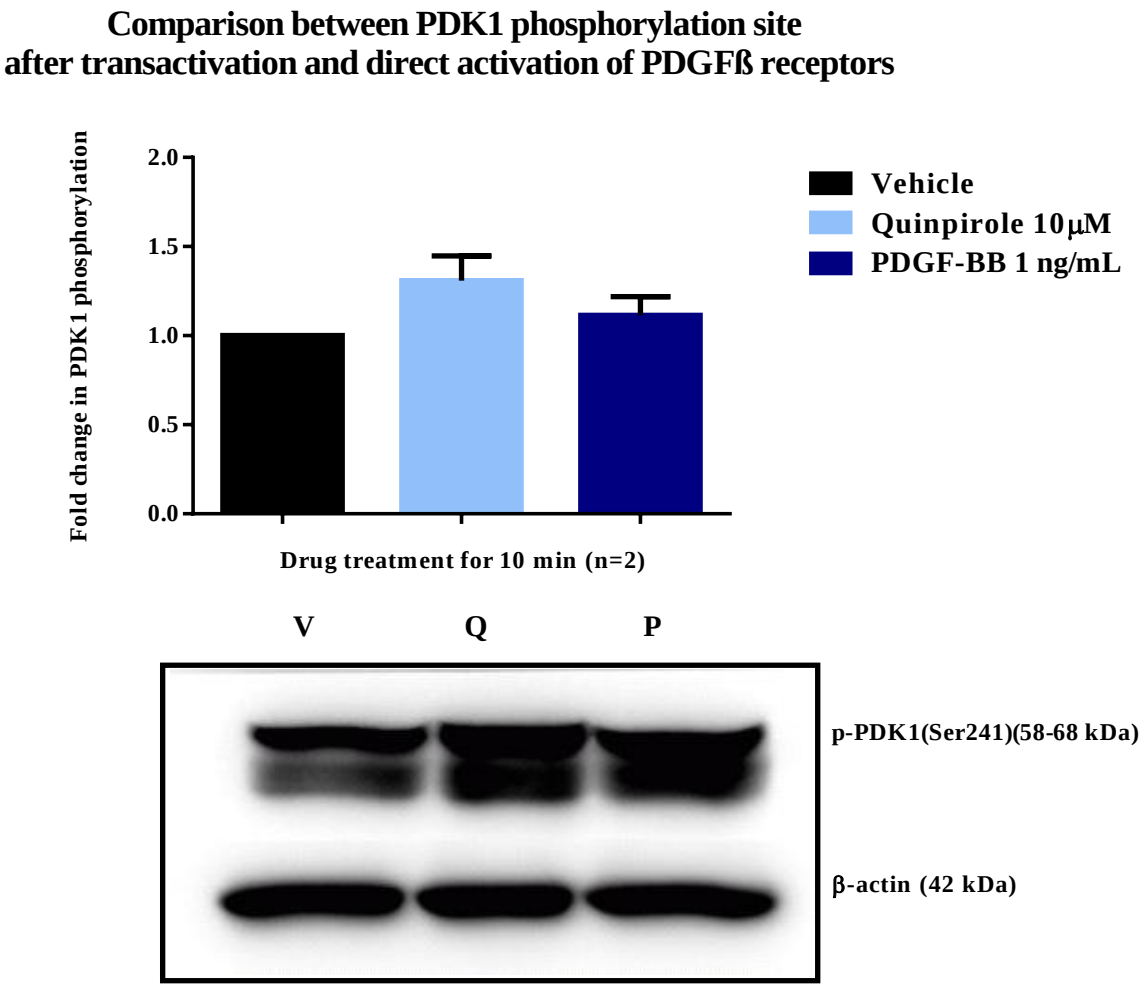

Figure 3.13 Comparison between fold change in PDK1 phosphorylation after transactivation and direct activation

The p-PDK1 (Ser241) as a phosphorylation site of PDK1 showed more increase in fold change after transactivation compared to direct activation of PDGFβ receptors. The data were normalized to the non-treated-cells (vehicle) as a control. Then, β-Actin served as a loading control. The data represent the mean ± S.E.M. for two independent experiments.

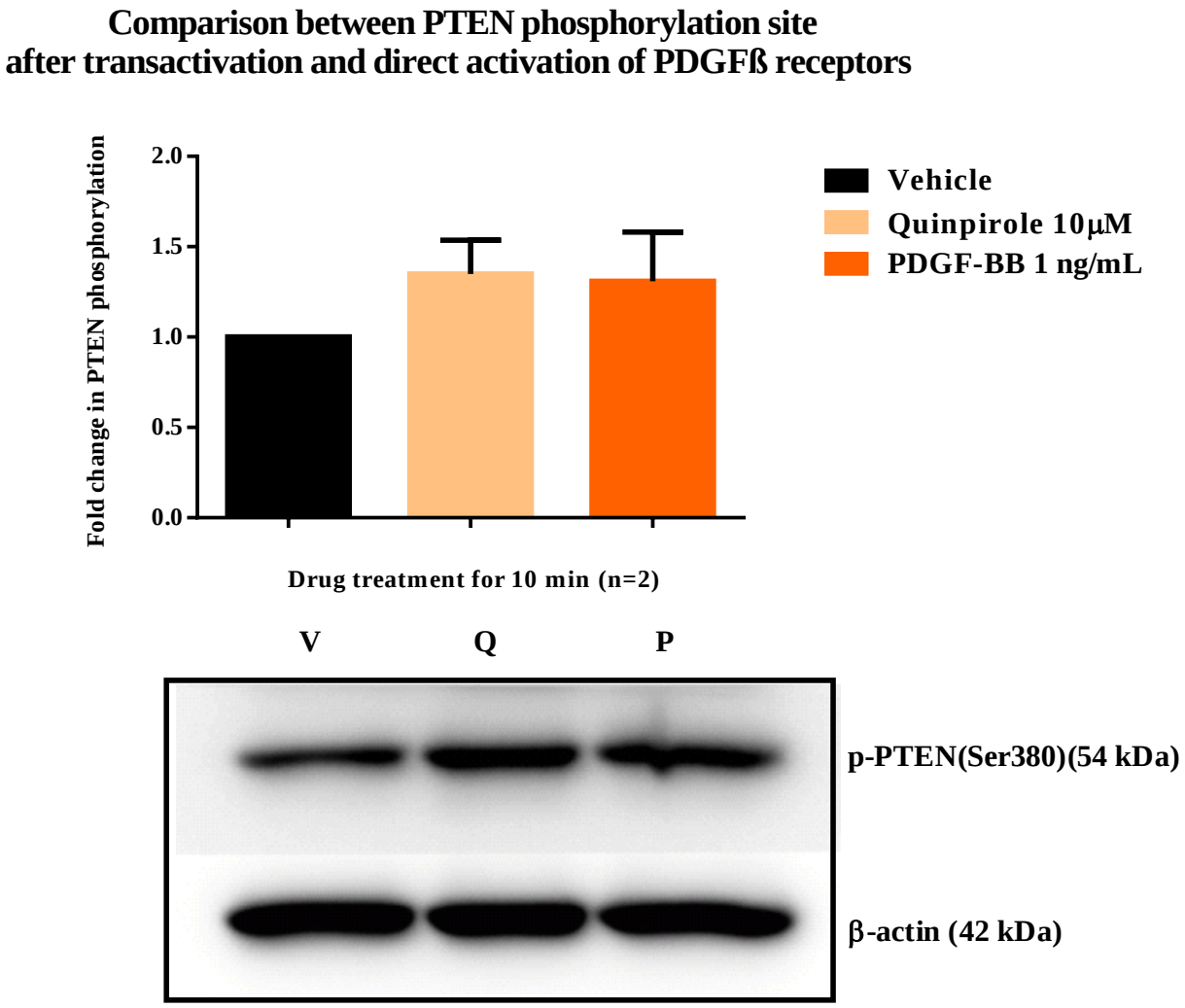

Figure 3.14 Comparison between fold change in phosphorylation of PTEN at Ser380 after transactivation and direct activation

PTEN showed almost the same fold change in PTEN (Ser380) phosphorylation after direct activation and transactivation of PDGFβ receptors. The data were normalized to the non-treated-cells (vehicle) as a control. Then, β-Actin served as a loading control. The data represent the mean ± S.E.M. for two independent experiments.

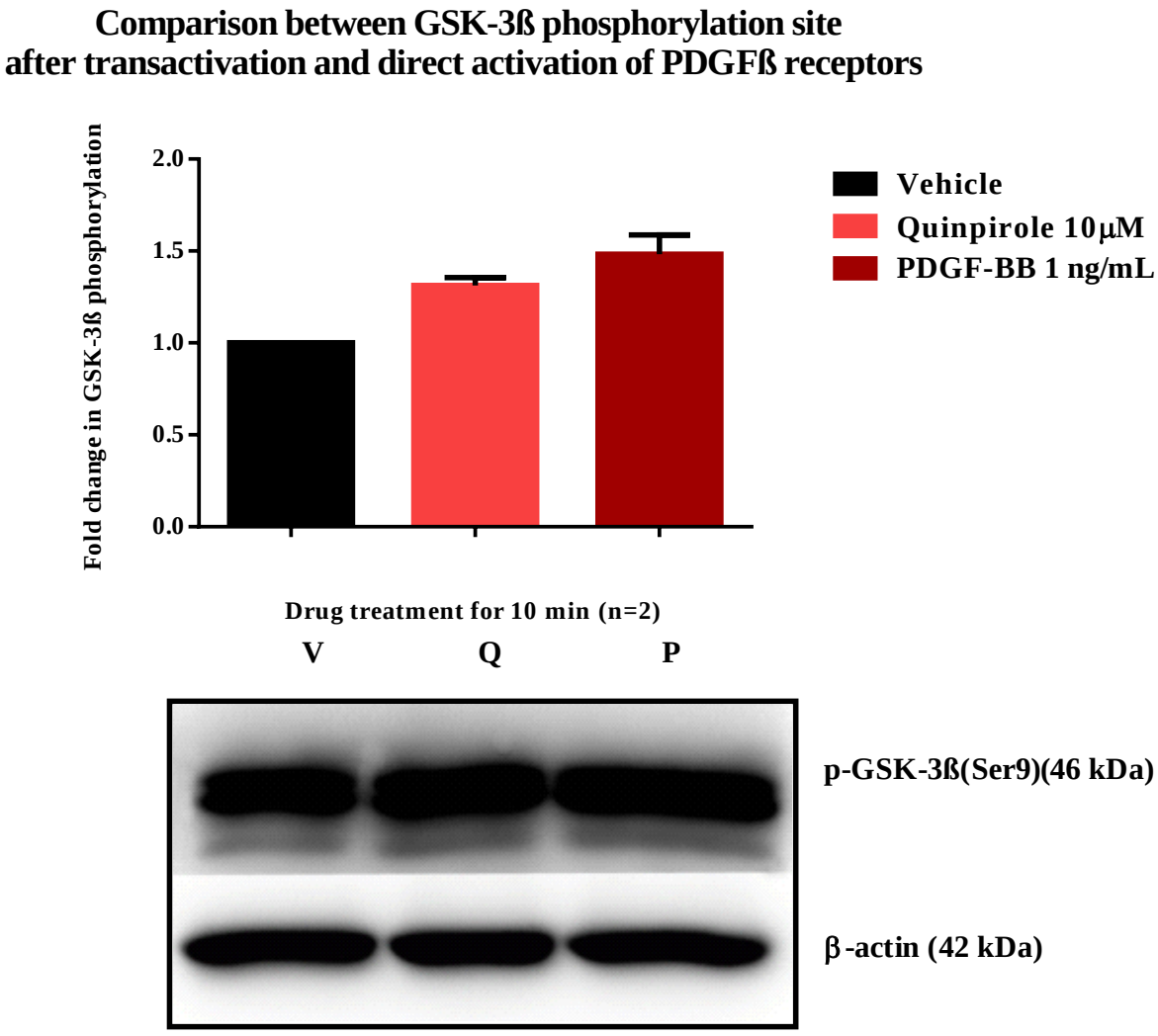

Figure 3.15 Comparison between fold change in phosphorylation of GSK-3β at Ser9 after transactivation of and direct

GSK-3β showed slightly fold change increase (almost 0.5-fold) in its Ser9 phosphorylation site after transactivation of PDGFβ receptors via quinpirole compared to vehicle. The data were normalized to the non-treated-cells (vehicle) as a control. Then, β-Actin served as a loading control. The data represent the mean ± S.E.M. for two independent experiments.

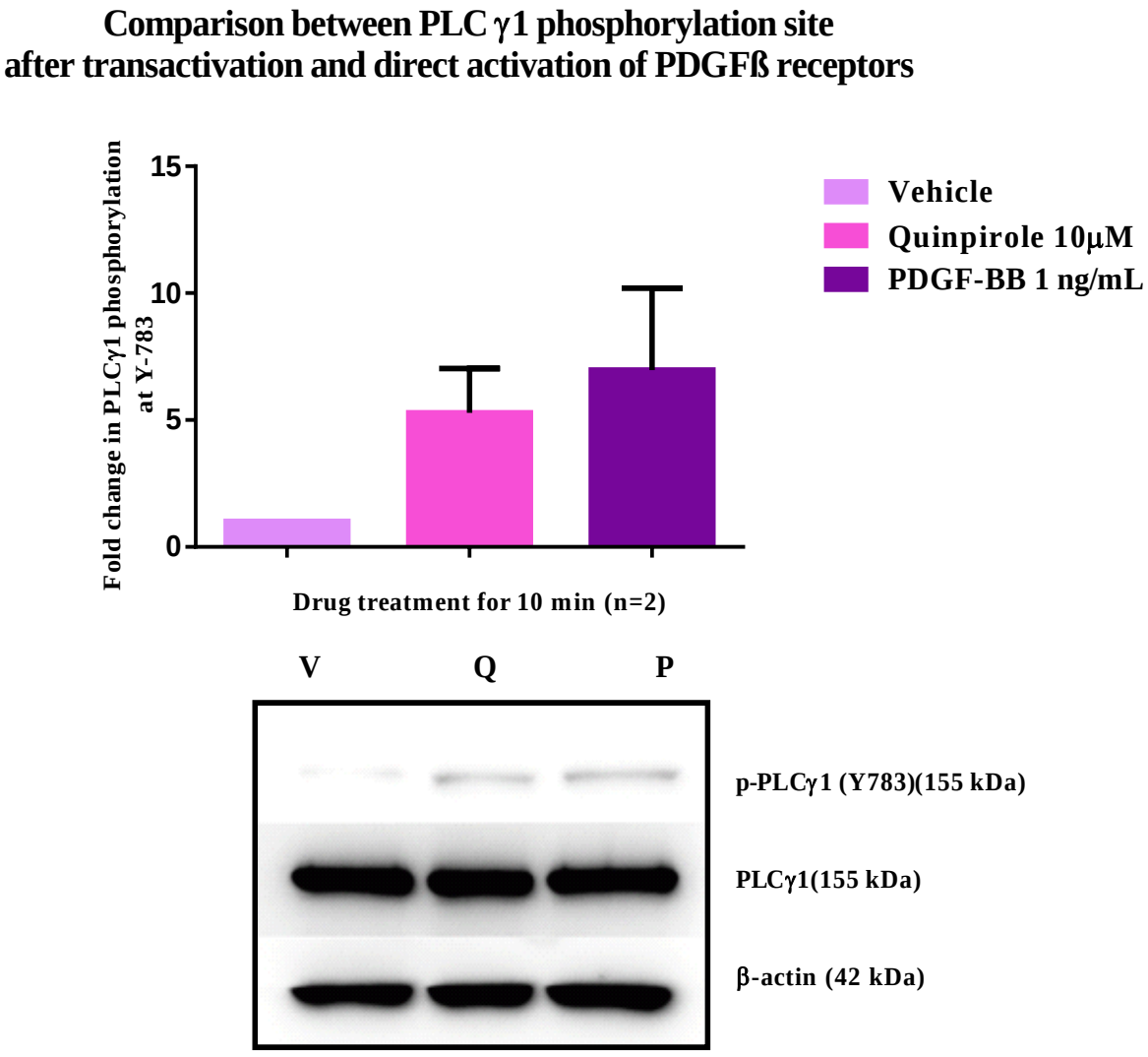

Figure 3.16 Comparison between PLCγ1 fold change of phosphorylation site after transactivation and direct activation

The p-PLCγ1 showed a significant increase in fold change after direct activation and transactivation of PDGFβ receptors compared to no-treated-cells. The data were normalized to the non-treated-cells (vehicle) as a control and to total protein expression for PLCγ1. Then, β-Actin served as a loading. The data represent the mean ± S.E.M. for two independent experiments.

Chapter 4

Discussion

4.1 Comparison between transactivation and direct activation

Application of several GPCR agonists and PDGF-BB induced a significant increase in phosphorylation of the PDGFβ receptors at Tyr1021 in SH-SY5Y cell line compared to the basal level. Application of 0.1 to 1 ng/mL of PDGF-BB induced 10 to 100-fold increase in Tyr1021 phosphorylation, whereas treatment of cells with GPCR agonists increased the phosphorylation state of Tyr1021 only 2-3 fold via transactivation of PDGFβ receptors [Figure 3.1]. However, despite significant differences in the phosphorylation level of the receptor, application of GPCR agonists compared to application of PDGF-BB did not show significant differences in inducing ERK1/2 phosphorylation: PDGF-BB only increased ERK phosphorylation ~ 2-fold more than the D_2-family dopamine receptor agonist, quinpirole [Figure 3.2]. Thus, the higher phosphorylation of the receptor does not necessarily correlate with higher activation of the downstream effectors. In addition, other specific phosphorylation sites on PDGFβ receptors have the ability to induce MAPK/ERK activation, (see section 1.3.1.2.1).

Several papers previously demonstrated the transactivation of PDGFβ receptors via dopamine receptors in various cells [24,143,146-150]. The ability of raclopride to block quinpirole-induced phosphorylation of the PDGFβ suggests that its effects are mediated via D_2-family dopamine receptors [Figure 3.5]. The EC_{50} for quinpirole for activating D_2-family dopamine receptors is 0.57 nM in D293 cells stably expressing the dopamine D_2 receptors and in rat brain [145]. Our dose-response curve showed that EC_{50} for quinpirole to transactivate PDGFβ receptors is approximately 5.3 μM [Figure 3.6]. Thus, the concentration of quinpirole required to transactivate PDGFβ receptors is nearly 10000-fold higher in our model.

Direct activation of PDGFβ receptors by PDGF-BB increases Tyr1021 phosphorylation in a time dependent manner. After 20 minutes, the phosphorylation levels began to drop, likely due to the

action of tyrosine phosphatases and the early stages of receptor downregulation. In contrast, transactivated receptors showed a stable amount of phosphorylation at Tyr1021 over time after application of quinpirole or dopamine. This result could be due to the relatively weaker and continuous/prolonged level of transactivation on PDGFβ receptors in SH-SY5Y cells. Alternatively, the consistent amount of phosphorylation (over time) induced by transactivation could be due to the absence of same regulatory proteins and phosphatases responsible for regulating direct activation. It could be possible that transactivation stimulates its own regulatory proteins and phosphatases, but they have different characteristics compared to direct activation. Furthermore, despite the fact that downregulation of PDGFβ receptors after prolonged or excessive exposure to the ligand can attenuate the activity of the receptors, recent studies have shown that the desensitization and internalization of GPCRs not only are the process for terminating receptor activity, but are themselves signaling events [151,205]. Therefore, the acute transactivation of the PDGFβ receptors over 30 minutes did not show the high peak and attenuation in phosphorylation since transactivation is not dependent on downregulation of the PDGFβ receptors, and because internalization of the GPCRs may not terminate their activity [Figure 3.3].

4.1.1 Phosphorylation of PDGFβ receptors by transactivation at tyrosine residues

The intracellular domain of PDGF receptor consists of several tyrosine phosphorylation sites that can stimulate different downstream cascades when phosphorylated. PDGF receptors tyrosine phosphorylation sites act as docking sites for recruitment of signal transduction proteins that contain SH2 domains [152]. Five different phosphorylation sites containing Tyr740, Tyr751, Tyr771, Tyr1009, and Tyr1021 were examined to compare the effect of transactivation versus direct activation of PDGFβ receptor phosphorylation sites. These five phosphorylation sites are crucial sites in activating major downstream cascades of PDGFβ receptors. Our data showed that the phosphorylation profile of ligand-activated versus transactivated PDGFβ receptors is different and that direct activation of the PDGFβ receptor (1 ng/mL PDGF-BB) increases the level of total tyrosine phosphorylation to a greater extent than quinpirole (10 μM) at all sites [Figure 3.7A].

PDGF is a polypeptide that was purified first from human platelets [28]; it is available in alpha-granules of circulating blood platelets. Usually, it is detectable at high concentrations in blood clots. The amount of PDGF in unclotted blood is less than 1 ng/mL, whereas the amount of PDGF in clotted blood serum is about 15 ng/mL [153]. In order to increase the physiological relevance of our ligand-activated versus transactivated PDGFβ receptor comparison, we used the lowest concentration of PDGF-BB possible. Previous data from our lab indicated that the lowest concentration of PDGF-BB that could increase phosphorylation of PDGFβ receptors (at Tyr1021) was between 0.05 and 0.1 ng/mL [154]. We repeated our experiment comparing the phosphorylation profile of ligand-activated versus transactivated PDGFβ receptors with an application of 0.1 ng/mL PDGF-BB to SH-SY5Y cells [Figure 3.7B,C]. A comparison between the phosphorylation profile of serotonin (100 nM) or quinpirole (10 μM)-transactivated PDGFβ receptors versus PDGF-BB (0.1 ng/mL)-activated PDGFβ receptors revealed different phosphorylation patterns for each phosphorylation site. Quinpirole resulted in significantly higher changes in the phosphorylation of PDGFβ receptors at Tyr751 and Tyr771 compared to ligand-activated PDGFβ receptors; however, there were no significant differences in phosphorylation of PDGFβ receptors at Tyr740, Tyr1009, and Tyr1021 [Figure 3.7B]. On the other hand, serotonin increased the phosphorylation of PDGFβ receptors at Tyr740 and Tyr771 to a greater extent than ligand-activated PDGFβ receptors; however, there was no significant change in the phosphorylation of PDGFβ receptors at Tyr751, Tyr1009, and Tyr1021 [Figure 3.7C]. In conclusion, the increase in PDGFβ receptor phosphorylation at Tyr740, Tyr751, Tyr771, Tyr1020, and Tyr1009 after application of quinpirole (10 μM) was less than the increase by PDGF-BB (1 ng/mL) [Figure 3.7A]; however, quinpirole (10 μM) can increase PDGFβ receptor phosphorylation state at Tyr1021, Tyr751, and Tyr771 to a greater extent than PDGF-BB (0.1 ng/mL) [Figure 3.7B], and serotonin (100 nM) can increase PDGFβ receptor phosphorylation state at Tyr740, Tyr751, and Tyr771 to a greater extent than PDGF-BB (0.1 ng/mL) [Figure 3.7C]. The table below shows the phosphorylation profiles for the PDGFβ receptor after different ligands were applied. Interestingly, even using different concentrations of PDGF-BB itself resulted in different phosphorylation profiles.

PDGF-BB (1 ng/mL)	**Y751>Y1009=Y1021>Y740>Y771**
PDGF-BB (0.1 ng/mL)	**Y1009>Y1021>Y740>Y751>Y771**
Quinpirole (10 μM)	**Y751> Y1009=Y1021>Y771>Y740**
Serotonin (100 nM)	**Y740> Y771>Y1009>Y1021>Y751**

Previous studies showed that, in CHO-D_4 cells, dopamine (1 μM) increase the total tyrosine phosphorylation level of PDGFβ receptor less than PDGF-BB (10 ng/mL) [155]; and Tyr716, Tyr740, Tyr751, and Tyr1021 showed a greater phosphorylation following application of PDGF-BB (10 ng/mL) compared to dopamine (1 μM) [155]. Interestingly, Tyr857, the phosphorylation site involved in catalytic activity of the kinase, was not phosphorylated following transactivation in CHO-D_4 cells [155]. Tyr857 has a primary role in tyrosine kinase activity of PDGFβ receptors *in vitro*, but not *in vivo* [156,52]. The existence of a kinase(s) that could compensate for the lack of intrinsic receptor kinase activity may explain why PDGFβ receptors can be phosphorylated without Tyr857 phosphorylation [156]. Phosphorylation of Tyr857 keeps PDGFβ receptor activated even after dissociation of PDGF-BB [52]. Mutation in Tyr857, resulting in low or no kinase activity, caused a significant elevation in phosphorylation of PDGFβ receptors at Tyr771 compared to wild-type receptors and non-treated-cells [156]. Therefore, the fact that quinpirole (10 μM) and serotonin (100 nM) increased the phosphorylation state of Tyr771 to a greater extent than PDGF-BB (0.1 ng/mL) could be because they caused no phosphorylation of Tyr857 after transactivation compared to direct activation of the PDGFβ receptors.

PDGFβ receptors with mutations of Tyr857 are unable to induce full phosphorylation and activation of SHP-2, Akt, and ERK1/2 in porcine aortic endothelial (PAE) cells [52]. This results in decreased cell proliferation and increased cell apoptosis; however cells with mutation in Tyr857 showed considerable residual ability for PDGF-stimulated DNA synthesis, almost 50% compared to wild type receptors. Compared to PDGF-BB (1 ng/mL), serotonin (100 nM) elevated the phosphorylation state of Tyr740 and Tyr751, which are the docking sites for PI3-kinase/Akt, to a greater extent. Tyr1009 showed the same level of phosphorylation following

quinpirole or serotonin application in SH-SY5Y cell line compared to the application of PDGF-BB (0.1 ng/mL). Because SHP-2 binds to phosphorylated Tyr763 and Tyr1009 [28,59], phosphorylation of Tyr763 might be affected by transactivation that leads to decrease in activation of SHP-2.

One reason why the higher phosphorylation states on PDGFβ receptor did not result in the higher activation of downstream effectors could be due to the activation of Tyr857. Mutations in Tyr857 result in an impairment of the phosphorylation and activation of SHP-2 [156], which selectively dephosphorylates Tyr740, Tyr751, and Tyr771 on PDGFβ receptors [28]. However, dephosphorylation activity of SHP-2 is not a switch-off event; indeed, it seems that SHP-2 acts as a regulatory effector that intensifies the receptor signals by keeping the tyrosine phosphorylation sites at a high level of dynamic turnover [59]. Thus, PDGFβ receptor signal transduction is dependent not only on the level of tyrosine phosphorylation but also on the degree of dynamic turnover of phosphorylation sites and regulatory effectors. Since Tyr857 was not phosphorylated following transactivation, this fact could explain the importance of both tyrosine kinases and phosphatases in PDGFβ receptor downstream signalling pathways. In addition, another compensating kinase could regulate transactivation of PDGFβ receptors rather than identified regulatory mechanisms for direct activation.

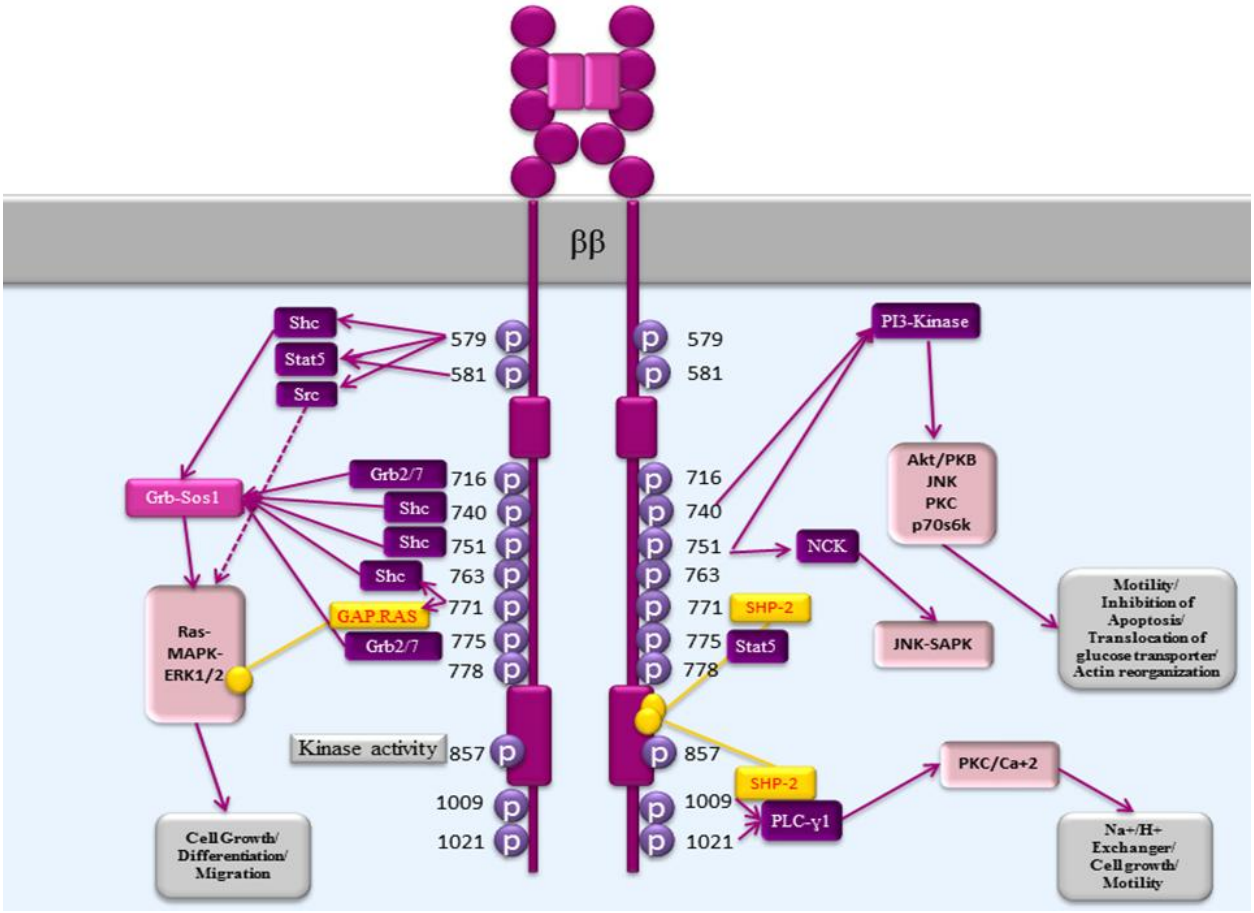

Figure 4.1 Phosphorylation sites on PDGFβ receptors and downstream pathways

These data suggest that not only does GPCR-induced transactivation result in a different phosphorylation profile of the PDGF receptor compared to direct activation, even different concentrations of PDGF-BB (0.1 versus 1 ng/mL) can induce different phosphorylation profiles.

4.1.2 Infra-additive effects between serotonin receptors and PDGFβ receptors

In the "synergy experiment" we found that in SH-SY5Y cells, application of low concentration of quinpirole and PDGF-BB the same time did not show any difference in phosphorylation site of Tyr1021 and Tyr1009. However, concomitant application of quinpirole and PDGF-BB increased the phosphorylation site in Tyr740 and Tyr771 compared to direct activation but decreased it compared to transactivation. Interestingly, this same treatment decreased the

phosphorylation of Tyr751 to a level lower than each of treatment separately as was in fact below the control (untreated) cells. It could be because of activation of Tyr857 and SHP-2 in result of direct activation of PDGFβ receptors after concomitant application of GPCR agonist and PDGFβ receptor agonist. Tyr740 and Tyr751 are the docking sites for PI3-kinase/Akt pathway; the decrease of the phosphorylation in these Tyr residues after concomitant application of drugs results in decrease in phosphorylation state on Akt at Thr308 and Ser473. The table below shows the effector protein profiles for the PDGFβ receptor after direct activation (PDGF-BB 1 ng/mL) versus transactivation (quinpirole 10 μM) of the PDGFβ receptors.

p-ERK1/2	**PDGF-BB > Quinpirole > non-treated-cells**
p-Akt(Thr308)	**PDGF-BB > Quinpirole > non-treated-cells**
p-Akt(Ser473)	**PDGF-BB > Quinpirole > non-treated-cells**
p-c-Raf(Ser259)	**Quinpirole > PDGF-BB > non-treated-cells**
p-PDK1(Ser241)	**Quinpirole > PDGF-BB > non-treated-cells**
p-PTEN(Ser380)	**Quinpirole > PDGF-BB > non-treated-cells**
p-GSK-3β(Ser9)	**PDGF-BB > Quinpirole > non-treated-cells**
p-PLCγ1(Y783)	**PDGF-BB > Quinpirole > non-treated-cells**

4.1.3 MAPK/ERK pathway and transactivation

Since the activation of the downstream pathways of PDGFβ receptors following application of PDGF-BB (0.1 ng/mL) was not sufficient to significantly increase ERK1/2 phosphorylation, 1 ng/mL PDGF-BB was used. Phosph-Tyr740, -Tyr751, and -Tyr771 are the main binding sites for Shc that results in activation of the Ras/MAPK pathway and the phosphorylation of all of these sites was elevated after the application of serotonin or quinpirole. However, the increase in phospho-Tyr771, which is a docking site for GAP, also has a negative regulatory effect on Ras-ERK1/2 pathway [28,53]. The net phosphorylation of ERK1/2 is based on the net phosphorylation of the receptor as well as the decrease in the activation of SHP-2 and phosphorylation of Tyr857. Other pathways independent of PDGFβ receptors might also be

involved in ERK1/2 activation. In addition, elevation in phosphorylation of PDGFβ receptor at Tyr771 after application of serotonin and quinpirole compared to direct activation [Figure 3.7B,C] results in the activation of GTPase for Ras and a decrease in ERK1/2 activation. The adaptor proteins Nck can bind to Sos after activating by phosphorylated Tyr751 in PDGFβ receptor, which could be a substitute pathway for activation of Ras/MAPK/ERK pathway [157]. Thus, a robust phosphorylation of Tyr751 after transactivation could be another alternative pathway in stimulating MAPK/ERK pathway.

It is possible that despite apparent similarities between direct ligand activated versus transactivated PDGFβ receptor with respect to ERK1/2 phosphorylation, each signaling mechanism may use different pathways to reach ERK. Raf plays a key role in controlling ERK1/2 activation [158]. The Raf family consists of three isoforms: a-Raf, b-Raf, and c-Raf. Raf kinases are substrates for Ras after activation by PDGFβ receptors. Binding of Ras-GTP to the active site of Raf results in conformational changes and recruits Raf to the cell membrane, which changes its phosphorylation pattern and stimulates its kinase activity [158]. Ser43, Ser621, and Ser259 are phosphorylation sites on Raf, all of which negatively regulates Raf kinase activity when phosphorylated [160]. Positive regulation of c-Raf is depends on protein phosphatase 2 (PP2), which is serine/threonine phosphatase [160]. Dephosphorylation of Ser259 is associated with Raf activation and occurs as it associates with the cell membrane. Ser621 phosphorylation also has a regulatory effect on kinase activity and membrane translocation of Raf [160]. Throughout the activation of Raf, Ser259 remains dephosphorylated, but Ser621 is phosphorylated quickly [160]. c-Raf (Raf-1) is normally a cytoplasmic inactive kinase bound to adaptor protein 14-3-3 and c-Raf activation involves protein 14-3-3 dissociation, Ser259 dephosphorylation, and c-Raf recruitment to the cell membrane. Raf activation then leads to MEK phosphorylation and activation, which in turn phosphorylates and activates ERK1/2. The biological response of c-Raf/ERK1/2 pathway is associated with the strength and duration of the activation [161,162].

Compared to ligand activated receptors, transactivated PFGFβ receptors resulted in a higher phosphorylation of c-Raf at Ser259. This result lets us speculate that quinpirole could activate the ERK1/2 to a reduced extent than direct activation of the PDGFβ receptors. Our results for ERK1/2 activation after application of quinpirole and PDGF-BB supported this claim: it showed that PDGF-BB (1 ng/mL) induced phosphorylation and activation of ERK1/2 to a greater extent than quinpirole [Figure 3.10]. Interestingly, the phosphorylation c-Raf at Ser259 after application of quinpirole is higher than in non-treated cells (baseline), whereas the phosphorylation of Ser259 after direct activation is almost at the same level of baseline [Figure 3.12].

In addition, we must consider that the activation of MAPK/ERK pathway involves multiple pathways including direct PDGFβ receptor-Grb2-Sos complexes, PDGFβ receptor-Shc-Grb2-Sos complexes, PDGFβ receptor-SHP-2-Grb2-Sos complexes, as well as Raf- and Ras-independent pathways [161,162]. For a better understanding of the effect of transactivation on ERK1/2, an evaluation of each of these pathways and their association with downstream cascades of dopamine receptors or PDGFβ receptors is required.

Ras/MAPK pathway	Direct activation of Grb2-Sos	Grb2	Tyr716/Tyr775
	Indirect activation of Grb2-Sos	Shc	Tyr579/Tyr740/ Tyr751/Tyr771
		SHP-2	Tyr763/Tyr1009

Several papers have examined the phosphorylation of PDGFβ receptors and downstream effectors (primarily ERK1/2, but also Akt) after transactivation [24,155,163,164]. However, it is important to consider ERK1/2 as a downstream effector of GPCRs as well. So, the question remains whether ERK1/2 activation after quinpirole application is due exclusively to PDGFβ receptor transactivation or additional, RTK-independent pathways. Thus, for comparing the increase of ERK1/2 phosphorylation in direct activation and transactivation, ruling out the effect of GPCRs on ERK phosphorylation is important. The most direct way of achieving this is to measure ERK1/2 phosphorylation after GPCR agonist treatment in the presence of RTK inhibitors. Transactivation of PDGFβ receptors by dopamine D_4 receptors activates ERK1/2, in

hippocampal neurons and fibroblasts [24,143,146-150]. Recently, our lab showed that there might be another possible parallel mechanism for ERK1/2 activation through GPCR-induced PDGF receptor transactivation [165]; indeed, this study and other studies suggested that functional oxidase might be necessary for transactivation [206]. Thus, NADPH oxidase components were introduced to stimulate ERK1/2 activation in a parallel mechanism with transactivation of PDGF receptors [165]. PDGFβ receptor transactivation also suppresses NMDA receptor activity [114]. Oak et al. used an *in vitro* Elk1 (ETS domain-containing protein) kinase assay to evaluate activation of ERK1/2 after transactivation [24]. Tyrphostin A9, a receptor tyrosine kinase inhibitor, and AG1295, a selective PDGF receptor tyrosine kinase inhibitor, both blocked activation of ERK1/2 via dopamine D_4 receptors [24].

All D_2-family dopamine receptors are thought to couple to $G\alpha_{i/o}$. However, differences within the class of receptors have been observed. Inhibiting Gβγ subunits prevents dopamine D_4 receptors regulating ERK1/2 pathway but not dopamine D_2 receptors [24]. In addition, dopamine D_4 receptors and dopamine D_2 receptors are coupled to the different Gα subunits [144]. Thus, transactivation mechanism(s) may not be the same between all dopamine receptor subtypes [144]. In addition, various cell types and different initiating GPCRs for transactivating PDGF receptors might use similar but distinct mechanisms to stimulate transactivation. As our lab previously suggested, 5-HT could transactivate both PDGFβ receptors (and TrkB) receptors through increase in intracellular calcium, activation of PKC, and Src. Activation of PKC and Src results in activation of NADPH oxidase subunits which in turn produces ROS. The production of ROS results in oxidation and inactivation of phosphotyrosine phosphatases, resulting in an inhibiting dephosphorylation of PDGFβ receptors and TrkB receptors [165,206].

4.1.4 PI3-kinase/Akt pathway and transactivation

Phosphorylation of PI3-kinase at p85 subunit leads to phosphorylation of catalytic subunit p110. Once activated, PI3-kinase phosphorylates PtdIns(4,5)P2 and produces a novel phosphoinositide PtdIns(3,4,5)P3, which in turn binds to PH domains of PDK1 and Akt/PKB. PtdIns(3,4,5)P3, which is anchored to the membrane, translocates Akt to the plasma membrane where Thr308 is

phosphorylated by PDK1. The phosphatase and tensin homolog (PTEN) dephosphorylates and converts PtdIns(3,4,5)P3 to PtdIns(4,5)P2 and acts as an off-switch in the Akt pathway. Full activation of Akt requires phosphorylation at both Thr308 and Ser473 and it is the mammalian target of rapamycin (mTOR) that phosphorylates Akt at Ser473. Active Akt phosphorylates many downstream targets, resulting in proliferation, cell migration and adhesion, protein synthesis, survival, insulin action, neuroprotection, and NO synthesis. Akt inhibits cell-cycle inhibitors in order to promote proliferation. Akt promotes cell survival by phosphorylation and inhibition of pro-apoptotic proteins such as Bad, Bax, Bim, MDM2, and FOXO1/3/4. This process, in conjunction with signaling through PLC/PKC and MAPK/ERK pathways, promotes a protective effect against apoptosis [208,209].

Tyr740 and Tyr751 are the primary tyrosine residues of PDGFβ receptors that are directly involved in the activation of PI3-kinase [28]. The phosphorylation of both tyrosines showed robust phosphorylation after application of serotonin compared to PDGF-BB [Figure 3.7C]. The application of quinpirole also increased the phosphorylation of PDGFβ receptors at Tyr751 to a greater extent than PDGF-BB (0.1 ng/mL) and at Tyr740 to the same level as PDGF-BB [Figure 3.7B]. However, increasing the concentration of PDGF-BB to 1 ng/mL increased the phosphorylation of PDGFβ receptors at Tyr740 and Tyr751 to a greater extent than quinpirole [Figure 3.7A]. These data is compatible with our results on Akt phosphorylation after direct activation and transactivation [Figure 3.11].

PDK1 (Ser241) phosphorylation showed a higher increase following transactivation compared to direct activation; however this difference was not significant [Figure 3.13]. The fold change in phosphorylation of PTEN was similar following direct activation or transactivation. Even though the fold change in phosphorylation of Akt was greater during direct activation compared to transactivation [Figure 3.11], the activation of the negative regulatory effector, PTEN, of Akt showed the same strength in direct activation and transactivation. Thus, the regulatory system

seems to be different in direct activation compared to transactivation: direct activation results in greater phosphorylation on Akt.

Once activated, the PI3-kinase pathway phosphorylates Akt at Thr308 and Ser473 via PDK1 and PDK2/rictor-mTOR, respectively [166]. Akt, (also called PKB) is a serine/threonine protein kinase belonging to the adenine-guanine-cytosine (AGC) kinases family. Thr308 is located in the kinase domain, and Ser473 is located in the regulatory domain. The Akt pathway is important for cell growth, proliferation, and cell survival. Akt is activated downstream of both GPCRs and RTKs. Phosphorylation at Thr308 and Ser473 was increased following application of quinpirole compared to control [Figure 3.11] and the application of PDGF-BB (1 ng/mL) increased phosphorylation states at both Thr308 and Ser473 to a greater extent than transactivation by quinpirole [Figure 3.11].

The primary question is how much of Akt pathway activation belongs to downstream pathways of PDGFβ receptors and how much belongs to downstream pathways of D_2-family dopamine receptor. Dopamine $D_{2/3}$ receptors induce the activation of Akt shortly after application of dopamine in the dorsal striatum of rat [167]. In the dorsal stratum and NAcc, preincubation with raclopride inhibits phosphorylation of Akt (Ser473 and Thr308), and GSK3α/β at Ser9; suggesting that dopamine D_2 receptors are involved in Akt activation [167]. Activation of Akt leads to phosphorylation and inactivation of GSK3α/β at Ser21 and Ser9. Prolonged stimulation of dopamine D_2 receptors by dopamine or quinpirole leads to an attenuation in Akt activity, especially at the level of Thr308 phosphorylation, likely due to receptor binding and desensitization by β-arrestin2 and phosphatase A2 activity [167]. Inhibition of Akt results in GSK3 activation in the mouse striatum, rat frontal lobe, and zebrafish brain [168,169]. Modulation of cAMP does not have any effect on Akt pathway [169]; however, dopamine D_2 receptor-dependent β-arrestin regulates the activation of Akt/GSK3 [166]. The serine/threonine protein phosphatase 2A (PP2A) inactivates Akt pathway by dephosphorylating Thr308 and Ser473 [170].

Our results indicated an increase in the phosphorylation of Akt at both Thr308 and Ser473 following direct activation or transactivation [Figure 3.11]. In addition, a similar fold-increase in the phosphorylation state of GSK3β after application of PDGF-BB and quinpirole was observed [Figure 3.15]. The difference between previous observations and our experiments could be due to the duration of experiments. Our experiments were based on acute activation of the receptor (usually from 5 to 10 minutes); however, the other experiments were based on longer time periods (usually more than 30 minutes). Downstream cascades of GPCR-mediated by G-protein have a fast onset and a short duration because of receptor desensitization. However, β-arrestin signaling has a slower onset and a prolonged duration [166]. Furthermore, other studies examining the phosphorylation and activation of Akt in CHO cells expressing human dopamine D_2 and D_3 receptors after short-term (5-10 minutes) exposure to dopamine or quinpirole showed similar results to ours [171] [Figure 4.2].

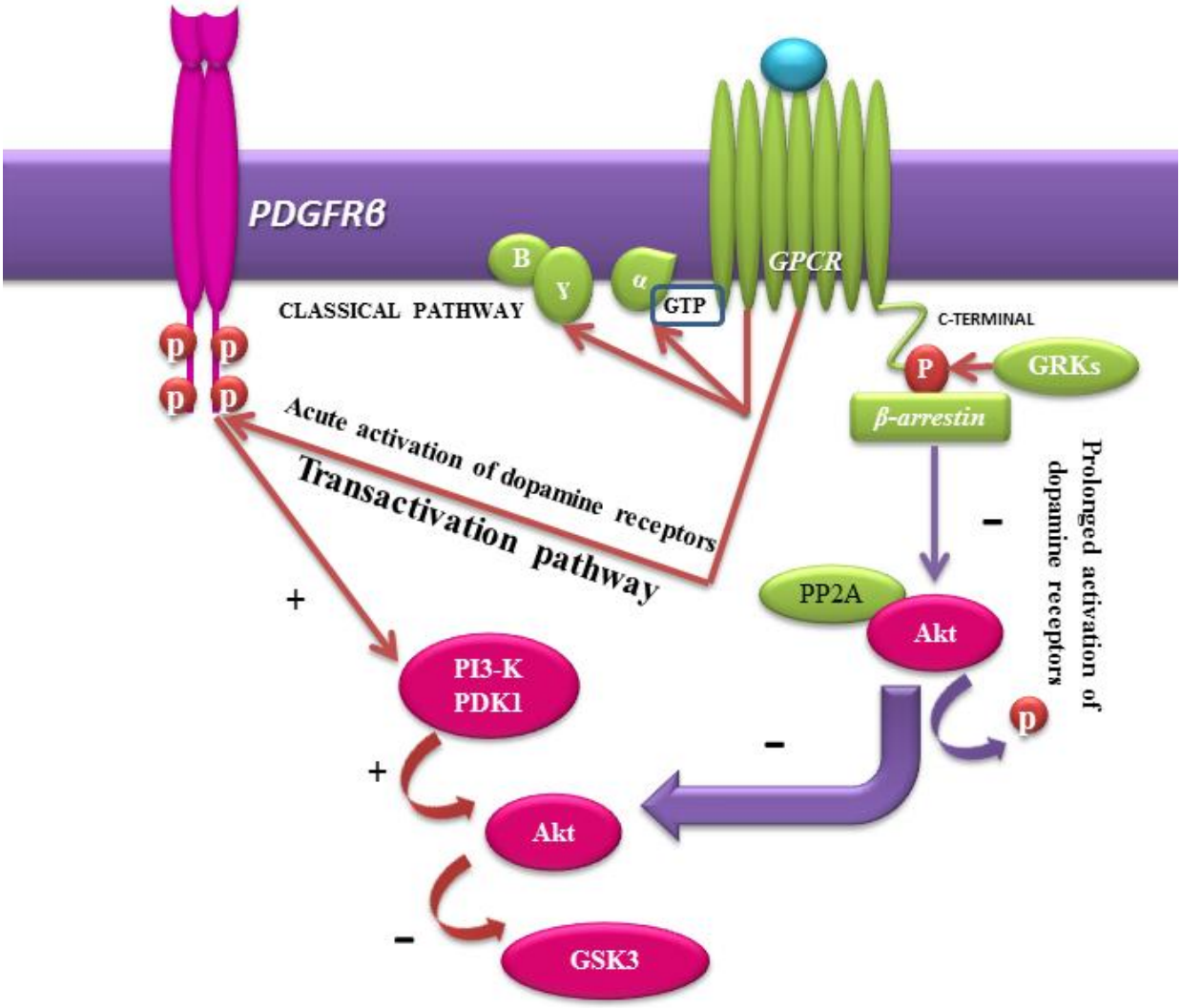

Figure 4.2 Activation and inhibition of Akt pathway

Raf is an intermediate effector in signal transduction pathways that promote survival and protect cells from apoptosis. In the Raf/MAPK/ERK pathway, phosphorylation of Ser259 regulates the kinase activity of Raf. Ser259 phosphorylation results in inactivation of Raf because it promotes Raf binding to 14-3-3 proteins. Akt directly phosphorylates Raf at Ser259 and creates a binding site for a negative regulator of Raf and decreases the kinase activity of Raf [160]. In addition, Akt, by phosphorylation of Bad and FKHRL1 (forkhead transcription factor) indirectly stimulates the binding of 14-3-3 protein to Raf, again leading to its inactivation [162]. Raf

promotes anti-apoptotic effects by binding to Bcl-2, stimulating BAD phosphorylation, activating PI3-kinase, and inhibiting apoptosis signal-regulated kinase 1 (ASK-1). Raf kinase, located at plasma membrane activates ERK signaling, and mitochondrial Raf regulates anti-apoptotic pathways [162]. This cross-talk between downstream pathways alters the biological responses of cells from cell-growth to proliferation in MCF-7 cells; moreover, it can modulate differentiation [172]. On the other hand, ERK1/2 stimulates mTOR leading to phosphorylation of Akt at Ser473 [173]. An increase in phosphorylation of c-Raf at Ser259 following transactivation compared to direct activation of PDGFβ receptors was observed [Figure 3.12], whereas phosphorylation and activation in Akt following transactivation are less than direct activation. Thus, transactivation is a cross-talk not only between two pathways (ERK and Akt) but between two receptors (dopamine D_2 receptor and PDGFβ receptor). The presence of other regulatory pathways and parallel downstream pathways that are activated through transactivation phenomena could elucidate more pieces of transactivation puzzle.

4.1.5 PLCγ1 pathway and transactivation

PLCγ1 binds to PDGFβ receptors at Tyr1021 (with high affinity) and Tyr1009 (with low affinity) to activate downstream signaling pathways that lead to cell growth, migration, and other pathways triggered by calcium signaling [28,174]. There exists different expression patterns for PLCγ isoforms in the human body: PLCγ1 is distributed in almost all cells, whereas PLCγ2 is mainly expressed in spleen and thymus [175]. In examining PLCγ phosphorylation as a marker for PLCγ activation, we observed no phosphorylation of phospho-PLCγ2 (Tyr1217) or phospho-PLCγ2 (Tyr759), despite the fact that we detected the presence of PLCγ2 in SH-SY5Y cell line. These results could be due to PLCγ2 activation by non-receptor protein tyrosine kinases, such as BCR and TCR.

Significant increases in the phosphorylation of PLCγ1 at Tyr783 were observed in both ligand-activated and transactivated receptors compared to baseline [Figure 3.16]. The PLCγ1 pathway plays a major role in mitogenicity and cell migration. Activation of this pathway also promotes

neuroprotection by regulating intracellular Ca^{+2} signaling and via the inhibition of NMDA and $GABA_A$ receptors [116].

4.1.6 Other important results

PDGFβ receptor has two pools of receptors [143] and both of them are expressed in significant quantities. One pool is glycosylated and plasma membrane-localized and is ~180 kDa [176]. The other is intracellular, non-glycosylated, immature PDGFβ receptor (~140 kDa) in humans [177]. The plasma membrane receptors bind to PDGF-BB and may undergo internalization and degradation [178,179]. Downregulated PDGF receptor remains phosphorylated for less than two hours after ligand activation. Dopamine D_4 receptors have been shown to transactivate both cell membrane-localized mature PDGFβ receptors and intracellular immature PDGFβ receptors, whereas classical ligand activation can activate only surface receptors [143,180]. In fact, after application of quinpirole, increased phosphorylation of intracellular immature PDGFβ receptors at Tyr1021 to a greater extent than in non-treated cells was observed [Figure 4.3].

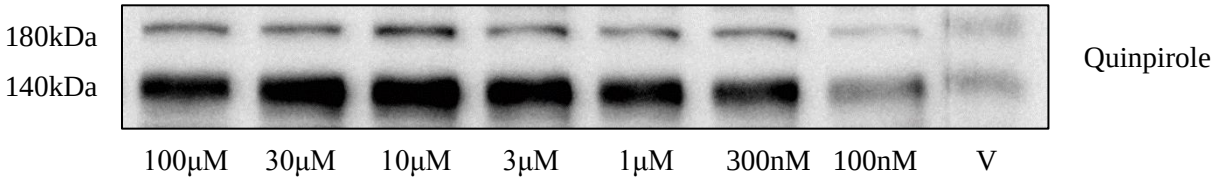

Figure 4.3 Phosphorylation of intracellular immature PDGFβ receptors at Tyr1021

Incubating cells with the glycosylation inhibitor tunicamycin overnight can prevent n-glycosylation and results in a disappearance of surface localized mature PDGFβ receptors [24,143] and is a useful tool for comparing the phosphorylation profile and downstream effector activation of mature versus immature receptors after transactivation.

4.1.7 Potential physiological relevance of transactivation to neurodegenerative diseases and psychiatric disorders

In the last decade, the study of neurological disorders has revealed the importance of neurogenesis and synaptogenesis in the pathophysiology and treatment of neurodegenerative

diseases such as Alzheimer's and Parkinson's disease. PDGF is an important neurotrophic factor for regulating neuronal proliferation and growth as well as the stimulation and regulation of neurogenesis and synaptogenesis. In neurodegenerative and psychiatric illness, both neurogenesis and synaptogenesis can be significantly impaired. In addition, a high level of angiogenesis has been found in tissues with nutrient-deprived conditions due to regulate oxidative metabolism upon replacement of nutrients and oxygen [181]. Interestingly, tissues with high levels of metabolic activity have low level of angiogenesis due to consumption of fatty acids for energy [181].

The application of neurotrophic factors improves neuronal function in *in vivo* models of Alzheimer's disease [32,182] and can also rescue motor neurons and increase motor performance in Parkinson's models [183]. Although these studies demonstrate the effectiveness of using neurotrophic factors as therapeutic tools to treat neurodegenerative diseases, there are several barriers that prevent the use of neurotrophins as a treatment strategy: 1) they do not passively diffuse across the blood-brain barrier (BBB), 2) they need to be administered parenterally, and 3) at high concentrations they may promote unregulated cell growth [184,185]. Unlike large neurotrophic factors that do not cross the BBB, small organic molecules are used frequently to target GPCRs in the brain without the risk of glioma and other serious adverse reactions. Finding small molecules that could transactivate PDGFβ receptors could be a major step toward a more effective and safer therapy for neurodegenerative diseases.

Other neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), are expressed mainly in the hippocampus and cortex and stimulate the survival of cholinergic neurons in the central nervous system. Because the hippocampus and cortex are important for memory and cognition, promoting their activity through transactivation may not only help neuron survival but also increase synaptic function. Transactivation of PDGFβ receptors reduces neuronal excitotoxicity by inhibiting NMDA receptors [144,191]. The neuroprotective effects of PDGFβ receptors depend on the inhibition of NMDA receptors and the induction of pro-survival genes via GSK3β and PI3-kinase/Akt

pathway [186]. PDGF also exerts neurotrophic effects on dopaminergic and GABAergic systems.

4.2 Semantics: distinguishing receptor transactivation & gene transactivation

GPCR-induced transactivation of PDGF receptors is a complex process and depends on various factors, including the cell type, the cellular environment, the availability of downstream effectors and proteins, the type and class of GPCRs, and the type of RTKs. Cross-communication between GPCRs and RTKs is initiated via GPCR ligands result in stimulation of either the upstream or the downstream pathways of RTKs. However cross-talk between GPCRs and RTKs is not a one way phenomenon. Indeed, transactivation of RTKs by GPCRs sometimes results in the recruitment of effectors and scaffold proteins that belong to downstream pathways of RTKs.

Whenever the term transactivation is searched in databases such PubMed, most articles are about gene transactivation, which means the induction of gene expression. This term was first introduced by Thomas, R. 1970 [187]. On the other hand, RTK transactivation is considered one type of cross-talk between receptors. Usually, the terms "receptor transactivation" or "RTK transactivation" is used for GPCR-induced transactivation of RTKs; this process was first described by Daub, H. *et al.* 1996 [132]. However, even within the term "RTK transactivation", at least two common mechanisms are described: ligand-dependent and ligand-independent transactivation. In ligand-dependent transactivation, the activation of the GPCR results in the extracellular release of the growth factor ligand and subsequent direct activation of the RTK. This model is also called triple-membrane-passing-signaling (TMPS). Ligand-independent transactivation is the type of pathway discussed in this work. Finding a specific name for each of these processes would help future research and provide clarity. As a suggestion we could consider "gene transinduction" instead of "transactivation" for gene and "cross-talk" as a general term for the communication between two receptors. We could use "TMPS" for the ligand-dependent model of GPCR-induced RTKs transactivation and "Second-Receptor-Downstream-Transstimulation" (SRDT) or "Second-Receptor-Intracellular-Transstimulation" (SRIT) for ligand-independent model of GPCR-induced RTKs transactivation.

4.3 Fundamental questions (and potential answers) about transactivation

1. ***Why do cells have two similar pathways to regulate and activate RTK receptors?***

Previously, it was assumed that receptors acted in isolation, depending on their own ligands to transduce extracellular signals into the cells. Over the past decades evidence points to intricate network interaction between various families of receptors and their downstream cascades. For example, protein kinases identified with one specific pathway may have the ability to phosphorylate other proteins associated with other downstream signaling pathways. RTK transactivation gives the opportunity to the cells to integrate diverse signals from different receptors and provides intricate network control over modulatory process of the cell functions including cell growth, differentiation, proliferation, and survival. Transactivation could be an opportunity for the cells to modulate a distinct stimulus into multiple pathways by taking advantage of downstream pathways of different receptors. In addition, when neurotrophin levels in CNS are reduced, mechanisms such as transactivation could compensate for survival and growth activity of neurotrophins, and this process could be one of the reasons for the existence of transactivation in the body [199].

2. ***Why do cells in the body require transactivation at all since RTKs can be activated by their ligands directly?***

Our results and other have demonstrated that transactivation may result in neuroprotection by activating pathways downstream of PDGF receptors. These neuroprotective effects involve the PI3-kinase/Akt pathway and PLC/NMDA pathway.

Another reason for existence of transactivation could be because GPCR activation may be achievable on shorter time scales than activation of RTKs directly, particularly if *de novo* synthesis of growth factor is required. For example, transactivation of PDGFβ receptors by angiotensin II (Ang II) type 1 receptors (AT1R), type 2 receptors (AT2R), and β2 adrenergic receptors, can induce rapid activation of ERK1/2 which in turn regulates biophysical hemostasis. Phospho-ERK1/2 is also one of the immediate downstream components for EGF receptors. In

cardiogenic shock, the renin-angiotensin system must react within seconds to control the blood pressure and could also stimulate the downstream pathways of several RTKs over a very short time frame [188].

The signal strength and duration of PDGF receptors are regulated by relatively rapid desensitization and downregulation of the receptors. Exposure of the PDGF receptor (or other RTKs) to high concentrations of ligand or long term exposure to ligand results in endocytosis of the receptors from the cell surface and subsequent degradation of the receptors and their bound ligands. This will reduce the total number of the cell surface receptors until they can be replenished by new receptor protein synthesis. Not all RTK internalization leads to signal termination. For example, EGF receptors may retain their ability to initiate signaling pathways even after internalization [189**].** Similarly, it was believed that endocytosis of GPCRs led to signal termination; however, recent studies have shown some signaling pathways associated with GPCRs actually depend on endocytosis for maximal signaling. For example, activation of ERK/MAPK via the GPCR agonists, lysophosphatidic acid (LPA) and isoproterenol, requires endocytosis. In addition, PI3-kinase/Akt signaling by LPA requires activation of both membrane receptors and endosomal compartment receptors [190]. Indeed, GPCR endocytosis is not only a mechanism for signal desensitization, but also a mechanism for additional signal transduction pathways specially initiating transactivation. Interestingly, transactivation of PDGFβ receptors via dopamine receptors increases the phosphorylation and activity of both cell-surface localized, mature glycosylated PDGFβ receptors and intracellular, immature PDGFβ receptors [143,180]. Thus, downregulation, desensitization, or blocking the extracellular region of PDGF receptors at the cell surface might not prevent transactivation and signaling downstream of the PDGFβ receptor [148]. Ligand-induced activation of PDGFβ receptors requires receptor dimerization; Chi *et al.* suggested that PI3-kinase might be an intermediate effector for activating intracellular, monomeric PDGFβ receptors through transactivation. These findings suggest that dimerization is not required for PDGFβ receptor activation. Monomeric PDGFβ receptors can signal to downstream pathways such as the ERK1/2 pathway [155].

Transactivation provides a mechanism in the body for receptors located remotely from other receptors to exert regulatory effects. For example, dopamine at low concentration (< 50 μM) can potentiate NMDA receptor-mediated responses via activation of dopamine D_1 receptors and dopamine at high concentration (> 50 μM) can depress NMDA receptor-mediated responses by activation of dopamine D_2 receptors [114]. Although, it is improbable that synaptically released endogenous dopamine can reach excitatory glutamatergic synapses at a high enough concentration to exert inhibitory effects, the ability of dopamine receptors to transactivate PDGF receptors allows for an alternative route to regulate glutamatergic synapses. Transactivation of PDGFβ receptors by dopamine $D_{2/4}$ receptors inhibits NMDA receptor currents in a Ca^{+2}/calmodulin-dependent manner [114]. The ability of dopamine $D_{2/4}$ receptors induce transactivation of PDGFβ receptors provides an opportunity for dopaminergic system to regulate excitatory synapses that are located remotely from them [114]. Thus, GPCR-induced transactivation of PDGFβ receptors may play a major role in the acute modulation of the synaptic transmission.

3. What is the relevance of RTK transactivation by GPCRs in disease?

Making a link between specific GPCR agonist(s) and the downstream signaling pathways of the PDGF receptor might help to identify specific pathological responses that could lead to the novel therapeutic targets. Since dopamine, GABA [117], and NMDA receptors [116] are targets for PDGF and PDGF receptors as a neurotrophic agent, it can be a therapeutic choice in treatment of neurodegenerative diseases, especially Parkinson's and Alzheimer's diseases. In experimental animal models of Alzheimer's, Parkinson's, Huntington's disease, and stroke, robust expression in PDGF and PDGF receptors in the lesioned of brain is reported [186].

Regulating NMDA receptor currents is one way for inhibiting neurotoxicity in various neurological disorders. The acute excitotoxicity of the NMDA receptors due to the high concentration of glutamate immediately after ischemic and injury of the brain last for a short period of time [112]. Thus, NMDA receptor antagonists need to have a short half-life in order to

act as neuroprotective agents [112]. Currently, commercial NMDA receptor antagonists are unlikely to have this characteristic; however, unlike direct NMDA receptor antagonists, drugs with anti-NMDA receptor current effects may be better tolerated [112]. Normal physiological activation of NMDA receptors occurs during strong postsynaptic depolarization; however, moderate long-lasting depolarization results in chronic excitotoxic NMDA receptor activation [112]. A major need exist for NMDA receptor antagonists that provide protection against excitotoxicity but leave normal physiological function of NMDA receptors intact. PDGF-BB and PDGFβ receptors cause a long lasting inhibition of NMDA receptor-dependent EPSPs and $GABA_A$-dependent IPSCs [49,116,117]. PDGF-BB has a selective inhibitory effect on NR2B- and not NR2A-containing NMDA receptor currents. PDGF-BB application reduces the expression and phosphorylation state of NR2B subunits. Thus, the neuroprotective effect of PDGF-BB on hippocampal neuron associated with inhibiting NR2B subunits of NMDA receptors [191]**.** Long-term treatment of 5-HT7 receptors leads to upregulation of PDGFβ receptors, which in turn provide a sufficient protection against NMDA receptor excitotoxicity in isolated hippocampal neurons [192]. Upregulation of PDGF ligands and PDGF receptors have been found in such neurological disorders associated with areas of neuronal cell death and excitotoxicity [116]. Thus, upregulation could promote to the neuroprotective effects of PDGFs and PDGF receptors via inhibiting Ca^{+2} influxes by NMDA receptors.

Neurogenesis has been found to continue in the brain post-developmentally. Adult neurogenesis is restricted to dentate gyrus in hippocampus and olfactory bulb [193]. Other parts of the brain such as cortex do not have this ability [194]. Neurogenesis has a major role in maintaining the normal function of the hippocampus including learning and memory. Certain stimuli may increase neurogenesis including stress, ischemia, trauma, hypoxia, and neurodegenerative disorders [194]. Therefore, neurogenesis might serve as a neuroprotective mechanism in the brain. Recent studies demonstrated that the neuroprotection effect of neurogenesis in neurodegenerative disorders depends on the stage of the disease [194]. Four distinct stages are introduced for adult neurogenesis: proliferation from progenitor or stem cells, differentiation into immature neurons, development into mature neurons, and migration into neuronal network

including axon or dendrite targeting or synaptic integrating [194], or more simply, proliferation, differentiation, neuronal generation, and survival/functional neurogenesis [195]. At the early stage of neurodegeneration all the four stages of neurogenesis are increased. At the late stage of neurodegeneration the first three stages of neurogenesis are enhanced, however, the last stage is significantly impaired [194]. Thus, the effect of neurogenesis in neurodegenerative disorders is neurogenic stage-specific.

Much of the evidence for neurogenesis in the context of neurodegrative diseases comes from the study of Alzheimer's disease. Abnormal proteolytic production of amyloid precursor protein (APP) results in formation of Aβ, which in turn promotes synaptic dysfunction and neuronal death. Massive accumulation of Aβ leads to formation of plaques [196]. Aβ is involved in initiating a neurodegenerative cascade that includes overactivation of glutamate and voltage-sensitive calcium channels, leading to death of neuronal progenitor cells [196]. A significant increase in PDGF-BB has been observed in the brain of patient with Alzheimer disease [197]. Previously, our lab investigated whether the application of PDGF-BB could induce neuroprotective effects against Aβ-induced neuronal cell death; however, it failed to reverse the neurotoxicity induced by Aβ [154]. On the other hand, non-receptor tyrosine kinase Src and the small G-protein Rac1, downstream of PDGFβ receptor, can induce Aβ generation. So, the robust expression of PDGF-BB and PDGFβ receptors in neurodegenerative disorders might be further neuronal damage. However, transactivation of the PDGF receptors might be useful to transiently produce neuroprotection and pro-survival effects without inducing aberrant neurogenesis and degradation.

Therapies for improving recovery after stroke are called neurorestorative therapies. Neurorestorative effects promote neurogenesis, angiogenesis, and neuronal and synaptic plasticity [198]. Growth factors such as hepatocyte growth factor (HGF) and glial cell line-derived neurotropic factor (GDNF) cause these effects. In addition, HGF has antifibrotic effects, which contribute to decreasing the formation of glial scarring [198]. However, these and other growth factors do not readily cross the blood-brain barrier. The administration small GPCR

agonists that transactivate PDGFβ receptors or other growth factor receptors could be a major step toward boosting *in situ* RTK activity and could provide an effective therapy in human stroke.

4. Can GPCR ligands substitute for RTK ligands to achieve similar downstream effects such as neuroprotection?

Despite the significant difference between phosphorylation profiles of the receptor between direct ligand and transactivated PDGF receptors, the activation level (as measured by specific phosphorylation sites) of downstream effectors were similar. This suggests that GPCR ligand-induced transactivation could fully substitute for direct RTK activation. In addition, one important fact about the transactivation of PDGF receptors by GPCRs is that, because the pathways are intracellular, only the cells that express both the GPCR and the RTK would experience the effects of transactivation. For example, transactivation of PDGF receptors by D_2-family dopamine receptor regulates NMDA currents. This process in the hippocampus depends on dopamine D_4 receptors and in prefrontal cortex depends on dopamine D_2 and D_3 receptors [144].

To conclude, transactivation of RTKs via GPCRs is one mechanism to convey proliferative signals to cells. In the brain, transactivation of neurotrophic receptors (Trk receptors) via GPCRs leads to neuronal survival responses and neuronal differentiation (i.e., not proliferation) [199]. Transactivation could be a therapeutic approach to enhance adult neurogenesis in the early stages of Alzheimer disease to postpone or inhibit the neurodegenerative progression. In addition, transactivation could have a neuroprotective effects against NMDA excitotoxicity that occurs during stroke. Furthermore, the advantages of GPCR downstream cascades such as dopamine in parallel getting benefits of the neuroprotective effects of PDGF receptors by transactivation might be a useful approach in treatment of neurological disorders and neurodegenerative diseases.

Overall characteristics of GPCR-induced RTKs transactivation

Various RTKs have similar downstream cascades, but what makes them unique involves the cell itself and the receptor involved. For example, Trk receptors, RTKs are activated via neurotrophins, are expressed primarily in CNS. Although activation of this receptor by neurotrophins results in neuronal differentiation, when Trk is ectopically expressed and activated in fibroblasts, it also increases proliferation similar to when the endogenous fibroblast growth factor receptors (FGFRs) are activated [200]. FGFs can induce proliferation in fibroblasts, but in CNS, they can mediate growth arrest and differentiation [201]. Thus, the same RTK can exert different effects depending on the cell type. In transactivation, the cell type and the distinct GPCRs and RTKs expressed will ultimately determine the physiological responses. As a whole, transactivation pathways described in the literature have several common intracellular signaling intermediates including phosphatidylinositol 3-kinases (PI3-kinase), Src kinases, calcium, and protein kinase (PKC). However, there are several differences reported in different pathways as well. For example, transactivation of the PDGF receptor via the dopamine D_4 receptor is dependent on $G_{\beta\gamma}$ but for dopamine D_2 receptor is independent of $G_{\beta\gamma}$ [24] and thromboxane A2 receptor transactivation of the EGF receptor is dependent on cross-talk between G_q and G_i [202]. In CHO cells, transactivation of RTKs by GPCRs is dependent on G_i or $G_{\beta\gamma}$ subunits [203].

Despite the results of this project, some important facts about signaling differences and similarities of PDGFβ receptors after direct activation compared to transactivated receptors must be considered. The fold change in phosphorylation of ERK1/2 and Akt, which are an important consideration for transactivation studies, must be evaluated precisely. It is important to consider whether activation of these PDGFβ receptor downstream effectors is independent of the downstream effects of GPCRs after transactivation. For this reason, ruling out the effect of GPCRs on ERK1/2 and Akt phosphorylation is essential. Using RTK selective kinase inhibitors is the most direct way to measure the dependency of ERK1/2 and Akt phosphorylation on GPCR activation. Another important fact that must be considered about transactivation is the specific responses of the cell to the distinct agonist of GPCR. In this case, the ligand for GPCRs, the specific GPCRs and RTKs express on the cell, and the intermediate effector and available

adaptor proteins could result in specific responses. For instance, in the SH-SY5Y cell line and in the CA1 hippocampus the transactivation of PDGFβ receptors by dopamine agonist such as quinpirole results in neuroprotection by regulating NMDA receptor currents; survival by activating Akt pathway and inhibiting GSK3β pathway; and proliferation by stimulating MAPK/ERK1,2 pathway. However, in the GH3 cell line (a rat pituitary epithelial-like tumor cell line), the application of quinpirole could results in anti-proliferative and apoptosis effects [207]; probably because of dopamine D_2 receptor-induced transactivation of transforming growth factor-β (TGF-β) receptors which are associated with scaffold proteins containing effector proteins related to p38 MAPK pathway involving in oxidative stress and cell apoptosis.

Because different phosphorylation profiles result in different downstream activation pathways, evaluation of all the Tyr phosphorylation residues of PDGFβ receptors for each specific GPCR agonist is essential for better understanding of the signaling profile after transactivation. Furthermore, the data from this project showed that neither phosphorylation profile nor downstream effectors of ligand-activated receptors versus transactivated receptors were similar. Since transactivation is initiated following activation of GPCRs by their ligands, regulatory effector proteins associated with GPCRs signaling could not only modulate GPCR downstream activity but might regulate transactivation mechanism as well. Understanding the exact regulatory mechanisms and regulatory effector molecules which are associated with transactivation modulation will help for better resolving the puzzles of this phenomenon. Moreover, disturbing the n-glycosylation linkage of the receptors and reduction of cell surface receptors could help to evaluate the effect of immature PDGFβ receptor on downstream effector proteins, which is another important fact in identifying signaling differences and similarities of direct activation compared to transactivation. Overcoming these limitations can help us to get a clearer overall conclusion for *in vitro* signaling differences and similarities in GPCR-induced transactivation of PDGFβ receptor and direct ligand activation.

Chapter 5

Conclusion

The results of this project showed that neither phosphorylation profile nor downstream effectors of ligand-activated receptors versus transactivated receptors were identical. Our data indicated that transactivation may result in RTK activation directed towards survival and proliferation pathways. These findings generally fit with previous work suggesting that transactivation of RTKs by GPCRs may be a mechanism for promoting cell survival.

To conclude, the process of transactivation seems to be distinct from one receptor to another receptor. Any given receptor can exist in distinct conformational changes after associating with specific ligands that may determine which downstream cascades of the receptor are going to be activated and which are not. Phosphorylation of the RTK domain and subsequent protein-protein activation and interactions trigger downstream pathways in order to transmit signals to the nucleus. Various possible combinations of transactivation could exist depending on the cell type, the kind of GPCRs which are activated, the kind of RTKs to be activated, the relative abundance of RTKs in the cell, and the intermediate effectors that are available. Taken together, transactivation exists for integrating the complex signaling network between GPCRs and RTKs. It is a regulatory pathway for modulating downstream cascades of the both receptors since transactivation between GPCRs and RTK is a two-way interaction and both of the receptors can transactivate the other receptor type [133]. Furthermore, transactivation could control the activation of downstream cascades of RTKs by phosphorylating specific tyrosine residues. Thus, understanding the exact pathway for various transactivations and the physiological relevance for these could be helpful to treat various diseases.

Future directions

Evaluating ligand-activated versus transactivated receptor by examining the tyrosine phosphorylation profile of each has provided very useful information. Since PDGFβ receptors also contain serine residues, investigating the phosphorylation of these sites and their association to downstream effectors of PDGF receptor is another important fact to be considered. For a better understanding of the effect of transactivation on the ERK1/2 and PI3 kinase/Akt pathways, an evaluation of all these pathways and their association with downstream cascades of dopamine receptors or PDGFβ receptors must be clarified. In addition, it remains unknown whether allosteric modulators for GPCRs will affect transactivation pathways in a similar manner as they affect classical G protein signaling pathways. Furthermore, using allosteric activators for GPCRs and evaluating their effect on transactivation might be result in using lower concentration of GPCR agonist for transactivation of RTKs. Finally, using high throughput screening may allow for the identification of specific GPCR ligands that preferentially promote transactivation.

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