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The cross talk between TrkB and NMDA receptors through RasGrf1

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Graduate Program in Biochemistry
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Abstract

Introduction: Brain-derived neurotrophic factor (BDNF) facilitates neuronal differentiation by activation of the TrkB receptor-tyrosine-kinase. BDNF/TrkB also modulates the activity of the excitatory *N*-methyl-D-aspartate neurotransmitter receptor (NMDAR), thereby also regulating neural plasticity and LTP/LTD, two forms of synaptic plasticity that contribute to the storage of information inside the brain. How TrkB cross-talks to modulate NMDAR function is not known, although our working model involves the RasGrf1 signaling molecule, a nucleotide exchange factor for Ras/Rac-GTPases, which interacts with both receptors. In response to TrkB activation, Ras-Grf1 is tyrosine phosphorylated and mediates neurite outgrowth in PC12 cells. RasGrf1 also binds constitutively to the NMDAR to stimulate long-term-depression (LTD) in primary neurons. We hypothesize that TrkB activation of Ras-Grf1 will increase neuronal outgrowth in TrkB expressing PC12 derived cells (TrkB-B5) and BDNF will uncouple RasGrf1 from the NMDAR thereby facilitating a decrease in LTD and an increase in long-term-potentiation (LTP) in primary neurons.

Method: PC12 (TrkB-B5) cells were transfected with appropriate plasmids, treated with BDNF (1ng/ml) and the percentage of neurite outgrowth was determined. Brain tissue slices from P30 mice were stimulated with either BDNF (100 ng/ml), NMDA (100 µM) or co-stimulated with both. Slices were then lysed and the protein interactions were assayed by immunoprecipitation and western blotting.

Results: We found that RasGrf1 expression significantly (P-value <0.05) increases neurite outgrowth in cell culture, in response to BDNF, which was dependent on the activation of both Ras and Rac. Although Tyr^{1048} and Tyr^{1062} appeared to be crucial sites of tyrosine phosphorylation on RasGrf1 for neurotrophin mediated neurite outgrowth in cell culture, tyrosine phosphorylation of RasGrf1 could not be detected in neural tissue slices in response to BDNF. Furthermore, we found a direct interaction between RasGrf1 and TrkB receptor in response to BDNF treatment in slice cultures. In addition, BDNF stimulated the tyrosine phosphorylation of the NR2B subunit of the NMDA receptor at residue Tyr^{1472}, which facilitates receptor retention at the cell surface, and also stimulated a dissociation of RasGrf1 from the NMDA receptor.

Conclusion: My results strongly suggest that BDNF stimulation changes NMDAR signaling via TrkB activation and that this is mediated by RasGrf1. Future studies using RasGrf1 knockout mice will further address BDNF dependent changes in the activation of individual signalling molecules, and hippocampal culture studies from wild-type/RasGrf1 knock-out mice will address whether changes in BDNF-induced neuronal dendritic growth and spine formation are dependent on RasGrf1.

Keywords: RasGrf1, NMDA receptor, TrkB, NR2B subunit, neurotrophins, neurite outgrowth, BDNF, NMDA, Ras, Rac, LTP, LTD, synaptic plasticity.

The Co-Authorship Statement

This thesis contains some previous un-published data performed by Kim Robinson from her thesis entitled, "The role of RasGrf1 in Trk receptor signaling" in 2008. These are clearly indicated bellow and inside each figure legend.

In chapter 2: Figures 2.1, 2.2 (C, D and E), 2.3 (D, E, F and G) and 2.4

In chapter 3: Figures 3.2 and 3.3

In chapter 4: Figures 4.1 and 4.3 (A, B and C)

All other experiments have been performed by Asghar Talebian. Chapter 2 of this thesis has also been previously published co-authored by Asghar Talebian, Kim Robinson-Brookes, James I. S. MacDonald and Susan O. Meakin, entitled **"Ras Guanine nucleotide Releasing Factor 1 (RasGrf1) enhancement of Trk Receptor mediated Neurite Outgrowth requires Activation of both H-Ras and Rac"**, and published in the Journal of Molecular Neuroscience. 2012 Jun 29.

Acknowledgement

Hereby, I would like to express my appreciation firstly to my supervisor, Dr. Susan Meakin for giving me this opportunity in this program. Secondly I appreciate my advisory committee; Dr. David Litchfield and Dr. Michael Poulter for their advice throughout the entire program. Finally I would like to acknowledge Dr. James MacDonald for his great technical advice throughout my graduate period and also all members of Dr. Meakin's laboratory for providing a friendly and comfortable lab-environment and atmosphere to achieve this research project.

On a personal note, I would also like to thank my parents for their consistent support as well as my wife, Mahnaz, for all of her encouragements especially during the final stages of my thesis.

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List of Abbreviations, Symbols, Nomenclature

AMPA   alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA   analysis of variance

BDNF   brain derived neurotrophic factor

BSA   bovine serum albumin

cAMP   cyclic adenosine monophosphate

CA   constitutively active

CA3-CA1   Cornu Ammonis areas of hippocampus

$CaCl_2$ calcium chloride

CaMKII   calcium calmodulin dependent kinase II

CaMKIV   calcium calmodulin dependent kinase IV

CaMKK   calcium calmodulin dependent kinase Kinases

CC   coiled coil (motif of RasGrf1) and cysteine-rich clusters (on Trk)

CDB   cyclin destruction box

CDC25   cell division cycle 25

Cdk5   Cyclin-dependent kinase 5

CRIB   cdc42/rac interactive binding

CRM   cysteine rich motif

DAG   diacylglycerol

DD   death domain

DH   Db1 homology

DMEM   Dulbecco's modified eagle medium

DN   Dominant negative

DNA   deoxyribonucleic acid

DRG   dorsal root ganglia

DTT   dithiothreitol

ECL   enhanced chemiluminnescence

EDTA   ethylene diamine tetraacetic acid

EFP   elongation factor 1a promoter

EGFP   enhanced green fluorescent protein

Erk   extracellular signal-related kinase

FBS   fetal bovine serum

FRS2   fibroblast growth factor receptor substrate 2

Gab-1   GRB-associated binder-1 adapter

GAPs   GTPase Activating Proteins

GDP   guanine nucleotide diphosphate

GEFs   guanine nucleotide exchange factors

Grb2   growth factor receptor-bound protein 2

GPCR   G-protein coupled receptors

GST   glutathione S-transferase

GTP   guanine nucleotide triphosphate

HA   hemmaglutinin

HEK293   human embryonic kidney 293 cells

HK   HIKE domain

HIKE His^{507}-Glu^{518} motif on TrkA (**HIK**RQDIILKW**E)**

HRP   horseradish peroxidise

IB	immunoblotting
Ig-D	immunoglobin-like domains
IgG	immunoglobulin G
IHC	immunohistochemically
IQ	ilimaquinone
KCl	potassium chloride
KD	kinase domain
KO	knock out
LB	Luria broth
LRM	leucine rich motif
LTD	long term depression
LTP	long term potentiation
MAPK	mitogen activated protein kinase
Mek	MAPK/Erk kinases kinase
$MgCl_2$	magnesium chloride
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
Na_2HPO_4	sodium phosphate
$NaVO_4$	sodium orthovanadate
ND	neural domain
NGF	nerve growth factor
NMDAR	N-methyl-d-aspartate receptor
NR2A	n-methyl-d-aspartate receptor 2A subunit

NR2B	n-methyl-d-aspartate receptor 2B subunit
NT-3	neurotrophin 3
NT-4/5	neurotrophin 4/5
NTR	neurotrophins receptor
PAK	p21 activated kinase
p75 NTR	p75 neurotrophin receptor
PBS	phosphate buffered saline
PC12	pheochromocytoma cells
PDPK	phosphatidylinositide-dependent kinases
PDZ	postsynaptic density-95-discs large-zona occludens-1
PEST	proline, glutamic acid, serine and threonine domain
P30	post-natal 30 (age)
PCR	polymerase chain reaction
PH	pleckstrin homology
PI3K	phosphotidylinositol-3 kinase
PKA	protein kinase A
PKC	protein kinase C
PLCγ	phospholipase Cγ
PMSF	phenylmethanesulphonylflouride
PSD-95	post-synaptic density protein 95
PTB	phosphotyrosine binding
PVDF	polyvinylidene fluoride
RasGrf1	Ras Guanine releasing factor 1

RasGrf2   Ras Guanine releasing factor2

REM   Ras exchange motif

RBD   Raf1-binding domain

RNA   ribonucleic acid

RT-PCR   real time-polymerase chain reaction

SCS   supplemented calf serum

SDS-PAGE   sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Shc   Collagen homology domain

SH2   Src homology 2 (domain)

si-RNA   small interference- ribonucleic acid

SOS   son of sevenless

STP   Short Term Potentiation

TBS-T   Tris buffered saline-tween-20

TPA   tissue plasminogen activator

Trk   Tropomyosin related kinase

WCL   whole cell lysate

WT   wild type

Chapter 1

Cross-talk between Trk and NMDA receptors

1.1 Neurotrophins and the receptors termed tropomyosin-related kinase (Trk)

Neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4/5 (NT 4/5), belong to a family of closely related growth factors (Meakin, 2000; Reichardt, 2006). Structurally, these proteins are homodimers with molecular weights of ~26 kDa and consist of 118-129 amino acid subunits (McDonald et al., 1991; Robinson et al., 1995; Butte et al., 1998). They are derived from a precursor protein so called pre-proneurotrophin, which consists of about 250 residues with a signal sequence and a prodomain followed by the mature neurotrophin sequence (Lessmann and Brigadski, 2009).

NGF was discovered in the 1950s as an essential growth factor in the developing nervous system (Levi-Montalcini, 1987; Shooter, 2001) while BDNF, as the second neurotrophin to be identified, was originally purified from brain lysates and was found to have high amino acid sequence homology to NGF (Barde et al., 1982). Subsequently, other members of the neurotrophin family were isolated using RT-PCR techniques and primer sequences conserved between NGF and BDNF (Barde et al., 1982; Berkemeier et al., 1991). The neurotrophins exert their biological activity by binding to and activating a low affinity receptor termed, p75, and one or more members of the high affinity tropomyosin-related kinase (Trk) family of receptors. *trkA* was originally identified as a transforming oncogene from a human colon tumor as a rearrangement between a gene encoding an intracellular tyrosine kinase domain with an extracellular sequence from a non-muscle tropomyosin gene and was so called the tropomyosin-related kinase (trk).

Subsequently, *trkB* and *trkC* were discovered based on high sequence homology to trkA (Martin-Zanca et al., 1986; Klein et al., 1989).

Structurally, Trk receptors contain two cysteine clusters, with three leucine-rich motifs in between, followed by two immunoglobulin-like domains in the extracellular region (Figure 1.1), a transmembrane region and an intracellular tyrosine kinase domain with 5 effective tyrosine residues. All reigons in the extracellular domains are contributed directly or indirectly in ligand-Trk binding although the immunoglobulin-like domains are the major interfaces in this precess. Besides, from an evolutionary perspective, Trk receptors (A, B and C) are highly conserved in both intracellular and extracellular domains among vertebrates (Benito-Gutierrez et al., 2006). In comparison, the p75 receptor contains four cysteine-rich domains on the extracellular side as well as an intracellular death domain similar to that first described in tumor necrosis factor receptor (NTR) family members (Liepinsh et al., 1997; Chao and Hempstead, 1995; Huang and Reichardt., 2003; He and Garcia, 2004). Although p75 shares no sequence homology to Trk, the three-dimensional structure of its extracellular domain has been shown to form a binding site for the NGF dimer involving all four cysteine-rich repeats (He and Garcia, 2004).

The two different receptor classes of p75 and Trk have distinct preferences for the two forms of ligands; namely, pro-neurotrophins for $p75^{NTR}$ and mature neurotrophins for Trk receptors. In this respect, NGF binds TrkA, BDNF binds TrkB, NT-3 binds TrkC, and NT-4/5 binds both TrkB and TrkC. In addition to binding Trk receptors with high affinity (nM), all mature neurotrophins can bind with low affinity to p75 (μM). In

contrast, all pro-neurotrophins bind with high affinity to p75 (Figure 1.1) (Meakin, 2000; Reichardt, 2006; Frade and Barde, 1998).

A general consequence of Trk receptor tyrosine kinase activation includes cell survival, neuronal growth and differentiation, as well as a specific role of TrkB in modulating synaptic plasticity in the central nervous system (Meakin, 2000; Reichardt, 2006). In contrast, neurotrophin-mediated activation of p75^{NTR} initiates signaling pathways leading to apoptosis (Lee et al., 2001; Song et al., 2002). However, recent studies have also shown some roles for Trk receptor signaling outside the nervous system including cardiac development, neovascularization and in the immune system (Donovan et al., 2000; Lin et al., 2000; Coppola et al., 2004; Kermani et al., 2005).
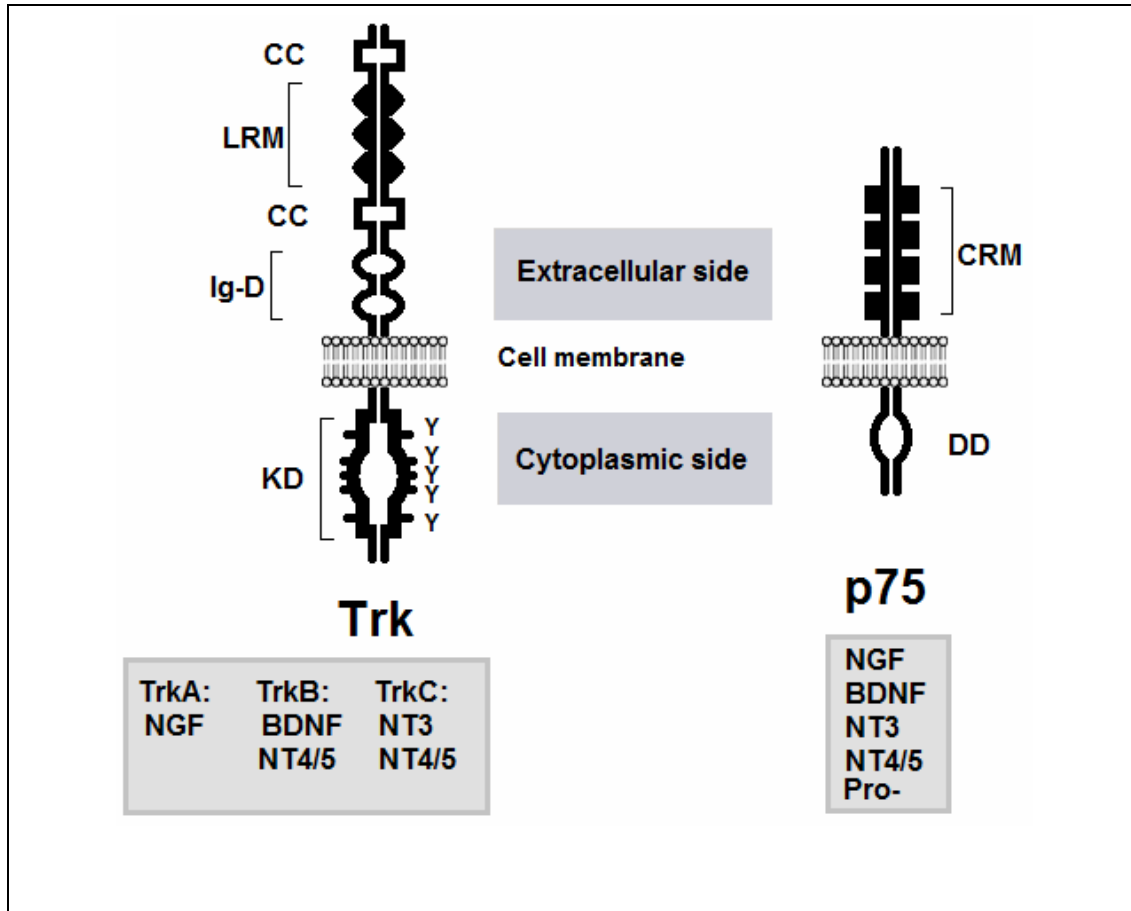
Figure 1.1. A schematic diagram of the neurotrophin receptor subtypes p75^{NTR} and the receptor tyrosine kinase family of Trk receptors. Tropomyosin related kinases (Trk), including TrkA, B and C, are receptors for a family of growth factors including NGF which binds TrkA, BDNF and NT 4/5 which bind TrkB and NT3 which binds TrkC. Moreover, all neurotrophins bind p75 receptor with low affinity while all pro-neurotrophins bind p75 with high affinity. Structurally, Trks have an extracellular region containing 2 cysteine-rich clusters (CC), leucine rich motifs (LRM) and immunoglobin-like domains (Ig-D), and also an intracellular tyrosine kinase domain (KD) with 5 tyrosine residues which can be auto-phosphorylated upon binding to the ligand and dimerization. These phosphorylation sites on TrkB include Tyr^{516} Tyr^{702}, Tyr^{706}, Tyr^{707} and Tyr^{817} which are equivalent to Tyr^{499}, Tyr^{679}, Tyr^{683}, Tyr^{684}, and Tyr^{794} on TrkA. In addition, the p75^{NTR} consists of four cysteine rich motifs (CRM) as wells as an intracellular death domain (DD).

1.2 Neurotrophins and Trk expression

The expression pattern and localization of neurotrophins and their receptors varies depending on regions within the peripheral and central nervous systems. Table 1 summarizes the pattern of neurotrophins and their receptor expression in different tissues of the nervous system (Korsching and Thoenen, 1983; Heumann et al., 1984; Davies et al., 1987; Heumann et al., 1987; Korsching, 1993; Eide et al., 1993; Avila et al., 1993; Ernfors et al., 1992; Knusel et al., 1991; Hyman et al., 1991; Ernfors et al., 1990). Note that generally in the hippocampus, TrkB receptors are localized in the axons, nerve terminals and dendritic spines of glutamatergic neurons (Drake et al., 1999). In particular, although TrkA is not expressed inside the hippocampus, NGF is expressed and secreted in the hippocampus to activate TrkA receptors in the termini of cholinergic neurons projecting from the basal forebrain (Meakin, 2000). Once action potentials are propagated at the nerve terminal, which results in the opening of voltage-gated channels on the presynaptic membrane and fusion machinery are activated , secretory vesicles of neurotrasmitter are released in either regulative or constitutive pathways. NGF is secreted predominately in a constitutive way specifically in hippocampal neurons while BDNF transcription and secretion are regulated in an activity-dependent manner (Mowla et al., 1999; Farhadi et al., 2000; Lessmann et al., 2003; Lu, 2003). Neuronal activity remarkably increases BDNF expression in hippocampal neurons, and its release in the presynaptic membrane in response to an increase in calcium concentration (Patterson et al., 1992; Dragunow et al., 1993; Kuczewski et al., 2009). Activity-dependent secretion of BDNF appears to be important for normal hippocampal function as a mutation in

BDNF prodomain (Val ^{66} to Met) impairs BDNF trafficking and increases the susceptibility to neuropsychiatric disorders (Egan et al., 2003; Hariri et al., 2003).

Table 1.1. Neurotrophin and Trk expression pattern in various areas of the nervous system.

Tissues	NGF	TrkA	BDNF	TrkB	NT3	TrkC
Dorsal Root Ganglia (DRG)	+	+	+	+		+
Sympathetic Ganglia		+				
Motor Neurons			++	+		
Cerebral Cortex			+	+	+	+
Striatum		+				
Hippocampus	+		+	+	+	+
Cholinergic Neurons		+				
GABAergic Neurons				+		
Dopaminergic Neurons				+		
Cerebellum				+	+	+
Hypothalamus				+		+

1.3 Neurotrophin-mediated Trk activation

Neurotrophins initiate their signal transduction through activation of their receptors, Trk or p75^{NTR}. In case for Trk, once neurotrophins bind, the receptor tyrosine kinase is dimerized, which results in tyrosine-autophosphorylation in the intracellular kinase domain. The major sites of phosphorylation on rat TrkA include tyrosine residues 499, 679, 683, 684, and 794. While tyrosine residues 679, 683 and 684 in the kinase domain remain in an open conformation in the inactive receptor, these phosphorylation sites form a stable activation loop by conformational change upon phosphorylation which subsequently, with further phosphorylation of tyrosine 499 and 794, serve as docking sites for intermediate signaling (adaptor) molecules leading to activation of distinct pathways and diverse physiological responses (Hanks et al., 1988; Ibanez et al., 1993; Heldin, 1995; Hubbard, 1997).

One such adaptor molecule is the Src homology 2 (SH2)-domain containing protein Shc (ShcA, B and C) which binds to phosphorylated tyrosine at position Y^{499} by its phosphotyrosine binding (PTB) domain, and another adaptor molecule is Fibroblast growth factor Receptor Substrate 2 (FRS2) which competes with Shc to bind at this site (Liu and Meakin, 2002; Stephens et al., 1994; Meakin et al., 1999). Adaptor proteins are intermediate signaling molecules that mediate specific protein-protein interactions without intrinsic enzymatic activity. In addition, Pleckstrin homology (PH) or Src homology 2 (SH2) domain containing proteins also bind to other tyrosine sites on Trk and activate Trk-mediated signaling pathways. Another major signaling protein is phospholipase Cγ-1 (PLCγ-1) which binds to phosphorylated tyrosine at position Y^{794},

activation of which results in an increase in intracellular Ca^{2+} concentration (Minichiello, 2009).

Through these adaptor molecules, Trk receptors are able to activate three major signaling pathways (Figure 1.2) including the Ras-mitogen activated protein kinase (Ras-MAPK) pathway, the phosphotidylinositol-3 kinase (PI3K)-Akt pathway, and the PLCγ-1 pathway (Barbacid, 1994; Huang and Reichardt, 2003; Reichardt, 2006; Minichiello, 2009).

In the Ras-MAPK pathway, phosphorylated Trk at position 516 of TrkB (Kavanaugh and Williams, 1994), or analogous sites on TrkA and TrkC, mediate Shc activation and recruitment of the adaptor protein Grb2 (growth factor receptor-bound protein 2) and the SOS (son of sevenless) guanine nucleotide exchange factor, leading to activation of Ras-GTPase and the kinase c-Raf-1 (English and Sweatt, 1997; Reichardt, 2006; Minichiello, 2009). Subsequently, associated Mek1/2 kinases are activated resulting in a transient activation of Erk1/2 MAP kinases which underlie proliferation and cell cycle progression (English and Sweatt, 1997; Reichardt, 2006; Minichiello, 2009). In contrast, Trk-mediated FRS2 activation recruits the adaptor protein Crk, the C3G nucleotide exchange factor leading to activation of Rap-1 GTPase, and the kinase b-Raf which subsequently results in prolonged activation of Erk1/2 MAP kinase underling cell cycle arrest and differentiation (Kao et al., 2001; Wu et al., 2001; Egan et al., 1993).

Through the PI3K-Akt pathway, phosphotidylinositol-3 kinase is activated in either Ras-independent or Ras-dependent pathways. In the Ras-independent pathway, the Gab1 (GRB-associated binder-1 adapter) is recruited to TrkB indirectly through Grb2, and in turn binds to PI3K. Upon this association, PI3K is able to convert

phosphatidylinositol (PtdIns) into phosphatidylinositol 3-phosphate (PI3P), and in the presence of PI3P, the 3-phosphatidylinositide-dependent protein kinases (PDPK-1&2) are able to activate the Akt protein kinase (or protein kinase B). Akt can prevent cell death or apoptosis by phosphorylation (inactivation) of pro-apoptotic proteins and activating growth promoting substrates that underlie cell survival (Yuan and Yankner, 2000; Brunet et al., 1999; Yuan et al., 2003; Sini et al., 2004; Huang and Reichardt., 2003; Minichiello, 2009).

In contrast, in the phospholipase Cγ-1 pathway, Trk-mediated activation of PLCγ-1 hydrolyses phosphotidylinositol 4,5-biphosphate (PtdIns 4,5-P_2) to generate inositol 1,4,5-triphosphate (Ins 1,4,5-P_3) and diacylglycerol (DAG). $InsP_3$ promotes the release of Ca^{2+} from the endoplasmic reticulum, while DAG stimulates protein kinase C (PKC) activation at the cell surface (Zirrgiebel et al., 1995; Roback et al., 1995; Patapoutian and Reichardt., 2001; Huang and Reichardt., 2003; Minichiello, 2009). Increases in Ca^{2+} levels result in the activation of a wide variety of signaling proteins underling synaptic plasticity including protein kinase A (PKA) and calcium/calmodulin-dependent kinases (CaMKII, CaMKK and CaMKIV) (Huang and Reichardt., 2003; Minichiello, 2009). Collectively, it appears that by three different major pathways mentioned above, Trk receptors are able to finally activate four different protein kinases upstream of MAP-kinases, including PKA (cAMP-dependent protein kinases), PKB (PI3K-dependent protein kinases), PKC (DAG-dependent protein kinases), and Ca^{2+}-dependent protein kinases.
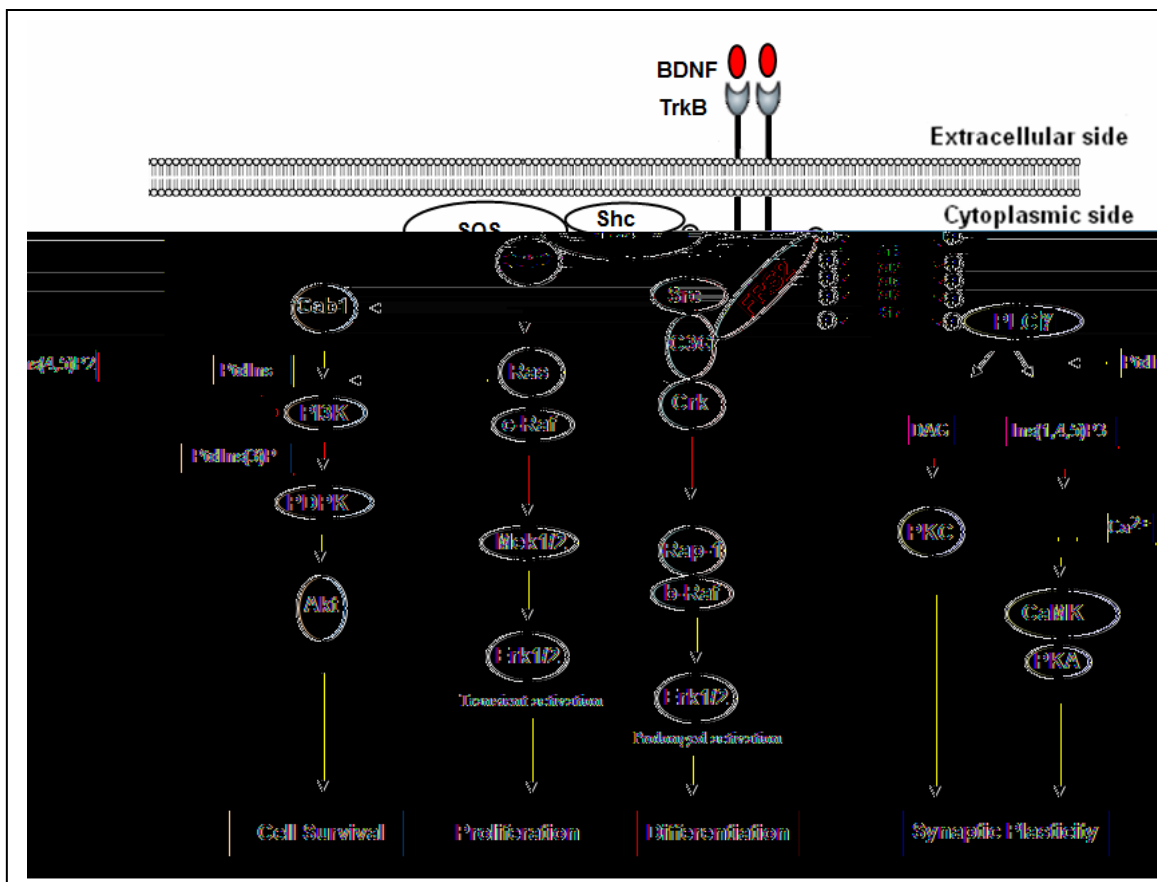
Figure 1.2. A schematic diagram of three major Trk-mediated signaling pathways.

1) In Ras-Erk pathway (white color), tyrosine phosphorylation of Trk at position 516 (Y^{499} on TrkA) activates the Shc adaptor protein which in turn recruit Sos and Grb2, leading to activation of Ras and transient activation of Erk1/2 underlying cell proliferation. Moreover, FRS2 (blue color) competes with Shc to couple Trk to Rap-1 which leads to sustained activation of Erk1/2 underlying neural differentiation. 2). PI3K pathway (grey color) acts indirectly through Grb2 downstream of TrkB to recruit Gab1 or directly through Ras. In response to this association, PI3K activates Akt leading to cell survival. 3) In the PLCγ-1 pathway (yellow color), phospholipase Cγ-1 binds to phosphorylated tyrosine at position 817 (Y^{794} on TrkA) resulting in an increase in intracellular Ca^{2+} concentration, which in turn results in the activation of a wide variety of signaling proteins underling synaptic plasticity. RasGrf1

1.4 Trk and clinical disorders

Several isoforms of TrkA, B and C have been reported in different studies, some of which are associated with clinical disorders either due to changes in the expression level or a mutation in both receptors and neurotrophins. For instance, a decrease in BDNF levels were reported in the postmortem brain of Parkinson's patients (Nagatsu and Sawada, 2007), and in the limbic structure of patients with depression, epilepsy and bipolar disorder (Kozisek et al., 2008; Ren and Dubner, 2007). These group of patients exhibit atrophy in the hippocampus and prefrontal cortex in which treatment with anti-depressants increases BDNF expression and its function, resulting in limitation of the side effects and atrophy (Monteggia et al., 2007). A mutation in BDNF ($V^{66}M$) increases the susceptibility of patients to neuropsychiatric disorders with memory impairment due to deficits in BDNF transport and decreases in BDNF secretion levels (Chen et al., 2006; Bath and Lee, 2006; Egan et al., 2003). As such, BDNF has been suggested as a potential therapeutic agent for neurogolical and psychiatric disorders such as Alzheimer's, Parkinson's, Huntington's, Stroke, Spinal cord injury, and also to regulate metabolic disorders such as Obesity and Diabetes due to a high expression of BDNF in the ventromedial hypothalamus which regulates appetite and food intake (Nagahara and Tuszynski, 2011). In addition, TrkB has been suggested as a therapeutic target for several types of human cancers such as neuroblastoma, prostate and pancreatic adenocarcinomas, and liver metastases (Desmet and Peeper, 2006). Recent studies have also reported a correlation between Alzheimer's disease and impairment in NGF transport from the hippocampus and neocortex to cholinergic neurons of the basal forebrain (Schindowski et al., 2008) as well as mutations in the *trkB* gene (Vepsalainen et al., 2005).

Moreover, alternative splicing of TrkA generates an isoform with an additional 6 amino acid residues in the proximal region of the extracellular domain. This isoform gains the additional ability to bind NT3 and being predominantly expressed in neuronal cells while TrkA lacking this additional amino acid fragment binds specifically to NGF (Barker et al., 1993). Truncated TrkB and TrkC isoforms, lacking a functional motif in the intracellular domain, have been also described as being able to inhibit full length Trk perhaps by forming non-functional heterodimers with full length subunits (Middlemas et al., 1991; Tsoulfas et al., 1993; Eide et al., 1996; Palko et al., 1999; Rose et al., 2003). A dominant mutation on TrkB in an activation loop tyrosine ($Y^{722}C$) has also been observed in patients with severe early onset hyperphagia (excessive appetite) as well as being associated with a severe impairment in learning and memory (Yeo et al., 2004). Moreover, an alternative splicing isoform of TrkC containing additional 14-39 amino acids residues in the intracellular kinase domain has also been reported to reduce the ability of the receptor to activate downstream signaling adaptors such as Shc and PLCγ (Reichardt, 2006; Barbacid, 1994). Trk receptors have also been shown to co-ordinate and co-operate with other receptors including G protein-coupled receptors (GPCR) (Weise et al., 2007; Lee and Chao, 2001; Lee et al., 2002a&b; Rajagopal et al., 2004), AMPA receptors (Wu et al., 2004; Caldeira et al., 2007) as well as NMDA receptors (Kang and Schuman, 2000; Amaral and Pozzo-Miller, 2007; Wu et al., 2004; Xu et al., 2006).

1.5 Neurotrophins and Trk regulation

Several mechanisms regulate neurotrophins and Trk expression, secretion and their function. Neurotrophins are regulated by membrane insertion, through intra and

extracellular trafficking, and by changes in protease activity (Thoenen and Barde, 1980). Since some proteases convert pro-neurotrophins to mature neurotrophins, and pro-neurotrophins have high affinity binding to $p75^{NTR}$ to activate signaling pathways leading to apoptosis (Lee et al., 2001), low protease activity can regulate mature neurotrophin levels. In fact, it has been shown that mature BDNF facilitates hippocampal long term potentiation (LTP) through TrkB while pro-BDNF promotes long term depression (LTD) through $p75^{NTR}$ (Patterson et al., 1996; Minichiello et al., 2002), and several studies suggest that enhanced pro-neurotrophin levels following brain injury may increase neuronal loss due to binding and activation of $p75^{NTR}$ (Fahnestock et al., 2001; Harrington et al., 2004; Pedraza et al., 2005).

Trk is localized intracellularly inside membranous vesicles in the absence of signals. However, signals such as cAMP, electrical activity or calcium increase the insertion of Trk into the plasma membrane (Barker et al., 2002; Heerssen and Segal, 2002). The ligand-activated Trk complex is internalized through clathrin coated pits (Valdez et al., 2005) while its extracellular part is bound to ligand and intracellular side associated with a number of signaling molecules such as PLCγ, PI3K or Ras-MAP kinase (Grimes et al., 1996; Howe et al., 2001). These endosomal vesicles are then transported retrogradely to the cell soma where they activate other Trk-dependent substrates (Wu et al., 2001; York et al., 2000; Delcroix et al., 2003; Huang et al., 1999; Huang and Reichardt, 2003).

1.6 NMDA receptor

The N-methyl-D-aspartate (NMDA) receptor is an ionotropic channel (Figure 1.3A) that upon activation by glutamate *in vivo* or NMDA stimulation *in vitro*, allows the flow of Na^+, and to a lesser extent Ca^{2+}, into the cell as well as K^+ out of the cell.

It is a heterotetrameric complex predominately made up of two obligatory NR1 subunits which bind to glycine and two modulatory NR2 subunits which bind to glutamate and control the electrophysiological properties of the NMDA receptor. Recently, growing evidence suggests that there are also glycine-binding NR3 subunits in the structure of NMDA receptors. NR1 subunits consist of eight different subtypes alternatively spliced from a single gene. NR2 subunits consist of four different A-D subtypes while NR3 subunits consist of two different A and B subtypes and together with NR2, they are encoded by six separate genes (Kutsuwada et al., 1992; Monyer et al., 1992; Paoletti, and Neyton, 2007).

While NMDA receptors contribute to both LTP and LTD, several studies suggest that NR2A subunits of the NMDA receptor promote LTP whereas NR2B subunits contribute to LTD (Li et al, 2006; Kollen et al., 2008; Liu et al., 2004; Massey et al., 2004).

The cell surface localization of the NMDA receptor is regulated through clathrin-mediated endocytosis in which the ^{1472}YEKL internalization motif on the NR2B subunit serves a pivotal role in this process. Phosphorylation of tyrosine 1472 (Tyr^{1472}) of the YEKL motif by Src family kinases uncouples the NMDA receptor from clathrin-mediated internalization and increases the retention and activity of the receptor at the cell surface (Nakazawa et al., 2001; Prybylowski et al., 2005).
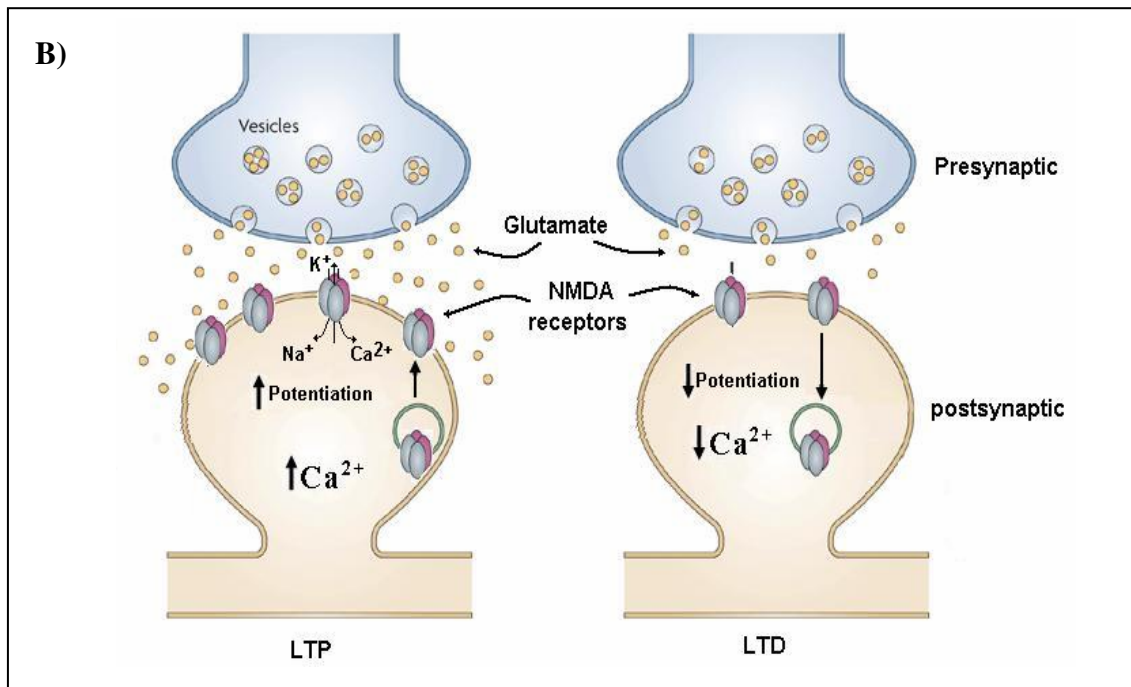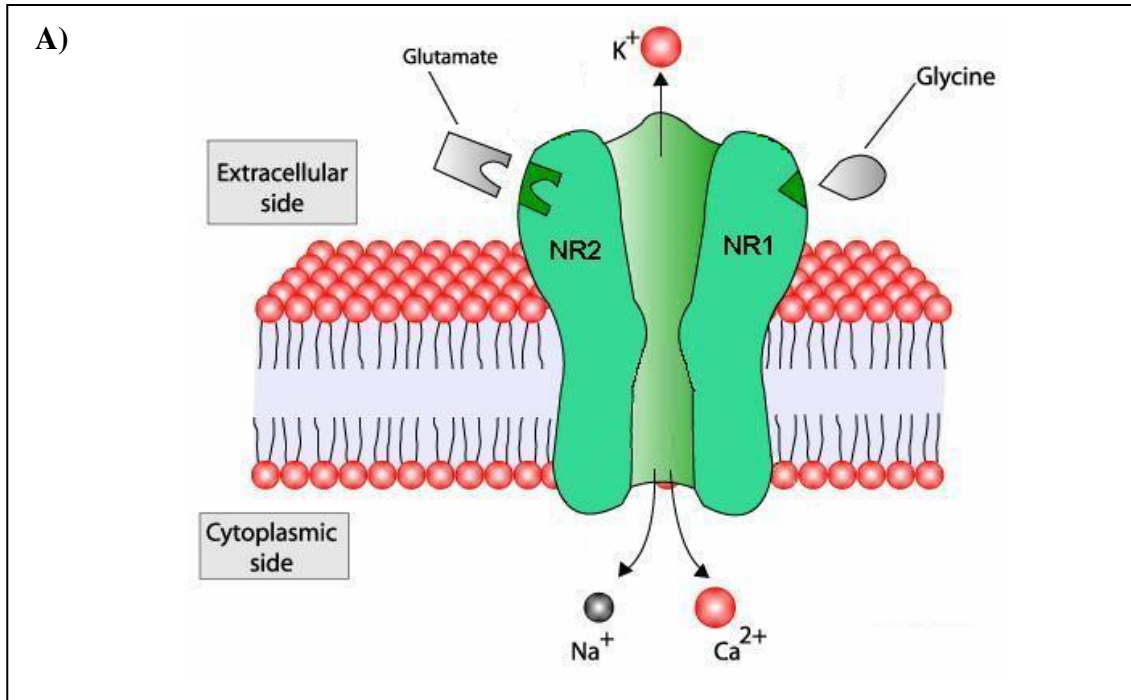
Figure 1.3. A schematic diagram of the NMDA receptor and physiological events during neurite outgrowth, Long Term Potentiation and Long Term Depression. A) The NMDA receptor is an ionotropic channel that upon activation allows the flow of Na^+ and small amounts of Ca^{2+} ions into the cell and K^+ out of the cell. It is a heterotetrameric complex made up of two obligatory NR1 and two modulatory NR2 subunits. It has been shown that RasGrf1 binds to the NR2B subunit of NMDA receptor (Krapivinsky et al., 2003). **B)** The major physiological events that occur during LTP and LTD. In LTP, there is an increase in the postsynaptic transmission of ions such as Na^+, K^+ and Ca^{2+} rather than weakening of postsynaptic transmission during LTD. LTP can be induced with a high-frequency stimulation through receptors present on the surface of the postsynaptic membrane, rather than a low frequency stimulation that stimulates LTD. LTP is an outcome of an increase in the activity of the receptors either by increasing the number of receptors on the postsynaptic cell surface or by increasing presynaptic factors, whereas LTD is an outcome of a decrease in the activity of these same receptors. Ca^{2+} entry above threshold is associated with LTP and low Ca^{2+} influx leads to LTD.

1.7 Long Term Potentiation (LTP) and long Term Depression (LTD)

LTP and LTD are two forms of synaptic plasticity that contribute to the storage of information during different types of learning and memory inside the brain, particularly in the hippocampus area of the brain (Table 2) (Lau and Zukin, 2007; Lu et al., 2008; Lynch, 2004). LTP lasts for hours up until days and involves gene expression and new protein synthesis, and it is in contrast to Short Term Potentiation (STP) which lasts seconds up until minutes involving the interaction between existing proteins and is independent of gene expression and protein synthesis (Figure 1.3B) (Lynch, 2004; Lu et al., 2008; Minichiello, 2009).

LTP can be induced with high-frequency stimulation of receptors present on the surface of the postsynaptic membrane (Figure 1.3B), which results in an increase in the postsynaptic transmission of ions such as Na^+, K^+ and Ca^{2+}. In contrast, LTD is an outcome of low frequency stimulation, a decrease in the activity of the NMDA receptor, and weakening of postsynaptic transmission. The outcome of both LTP and LTD are changes in intracellular Ca^{2+} concentrations, as Ca^{2+} entry above a threshold is associated with LTP and a modest increase in Ca^{2+} level leads to LTD (Lu et al., 2008; Minichiello, 2009). LTP represents improved communication between two neurons and is mediated by the excitatory neurotransmitter glutamate *in vivo* or NMDA in *in vitro* studies.

LTP is observed not only in the hippocampus, but also in other neural structures including the cerebral cortex, cerebellum or amygdala (Bauer et al., 2002; Nakazawa et al., 2006; Minichiello, 2009). In general, LTP can be dependent or independent on NMDA receptors. However, the NMDA receptor-dependent is the most widely studied

model of LTP in the CA3-CA1 (Cornu Ammonis) area of the hippocampus (Lisman, 2003).

Table 1.2. Different types of learning and memory in various areas of the brain.

Types of learning/memory	Brain area involved
Spatial memory	Hippocampus, Parahippocampus, Subiculum, Cortex, Temporal cortex, Area 47, Posterior parietal cortex
Emotional memory	Amygdala
Recognition memory	Hippocampus, Temporal lobe
Working memory	Hippocampus, Prefrontal cortex
Motor skills	Striatum, Cerebellum
Sensory (visual, auditory, tactile)	Various cortical area
Classical conditioning	Cerebellum
Habituation	Basal ganglia

1.8 BDNF-mediated TrkB activation and neural plasticity

The TrkB receptor is expressed widely inside the brain including the cortex and hippocampus and in addition to its well known effects on neuronal outgrowth and differentiation, TrkB activation is crucial in various aspect of synaptic plasticity in the entire brain including the cortex, hippocampus, cerebellum and amygdala (Carvalho et al., 2008; Lu et al., 2008). TrkB receptors have been found on both presynaptic and postsynaptic membranes of the nerve terminal and dendritic spines, and BDNF-mediated TrkB receptors have been suggested to modulate dendritic branches and spine formation

during neural plasticity within cortical, cerebellar and hippocampal synapses (Amaral et al., 2007; Minichiello, 2009).

BDNF plays a role in synaptic transmission on the postsynaptic membrane in addition to its well known effects on neuronal outgrowth and differentiation (Tyler et al., 2002). Although the mechanism by which BDNF regulates NMDA receptor-mediated neural plasticity is poorly understood, it appears that by indirectly modulating NMDA receptor activity, BDNF helps to regulate two important processes of learning and memory, namely LTP and LTD (Xu et al., 2006; Kang et al., 1997; Akaneya et al., 1997; Lessmann and Heumann, 1998; Levine et al., 1998; Levine and Kolb, 2000; Li et al., 1998). The evidence for a role of BDNF-mediated TrkB activation in LTP comes from the fact that any deletion in the *bdnf* or *trkB* genes, or *in vivo* blocking of BDNF binding to TrkB and pre-treating hippocampal slices with anti-TrkB antiserum, can either impair or significantly reduce the induction of LTP (Minichiello, 2009). Further studies have also suggested that NMDA receptor activity can increase BDNF expression leading to further TrkB-mediated regulation of NMDA receptors during synaptic plasticity (Caldeira et al., 2007).

BDNF activates TrkB receptors, which upon activation in turn modulates the tyrosine phosphorylation status of the NR2B subunits of NMDA receptors and increases the retention of these receptors on the postsynaptic cell surface (Nakazawa et al., 2001; Prybylowski et al., 2005). As a result of prolonged surface retention of the NMDA receptor, and the ability to respond to L-glutamate, the intracellular calcium levels increase in the postsynaptic neuron (Minichiello, 2009; Lin et al., 1998; Levine et al., 1998; Levine and Kolb, 2000). Increases in Ca^{2+} influx in postsynaptic neurons finally

influences the physiological properties and trafficking of the postsynaptic glutamate receptors such as the NMDA receptor through phosphorylation/ dephosphorylation, and results in the activation of a number of signaling molecules including PKC, PKA and the calcium-sensitive calmodulin-activated kinases (CaMKI, II and IV) (Ghosh and Greenberg, 1995; West et al., 2001; Minichiello, 2009), leading to initiation of signaling cascades that underlie neuronal growth, development and synaptic plasticity (Li et al., 2006; Minichiello, 2009).

Among these kinases, PKA and CaMK have been suggested to phosphorylate the alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) type of glutamate receptor that is involved primarily in the postsynaptic membrane depolarization and the maintenance of LTP, and also the NMDA receptor which is implicated in the induction or initiation of LTP (Lynch, 2004; Soderling and Derkach, 2000; Song and Huganir, 2002; Malenka and Bear, 2004). Furthermore, they activate a Ras-guanine nucleotide releasing factor termed RasGrf1 (Farnsworth et al., 1995) through either serine phosphorylation at residue 916 (S^{916}) on RasGrf1 (Schmitt et al., 2005) or unknown sites of tyrosine-phosphorylation by the non-receptor tyrosine kinases Src (Kiyono et al., 2000a) and Ack1 (Kiyono et al., 2000b).

1.9 RasGrf1 is a common substrate of both TrkB and NMDA receptors

RasGrfs belong to a family of guanine nucleotide exchange factors (GEFs), other members of which include Sos (Simon et al., 1991; Ferrari et al., 1994) and C3G (Knudsen et al., 1994; Tanaka et al., 1994). RasGrf is present in two forms. One form is RasGrf1 (140 kDa with 1262 amino acids) which is highly expressed in the nervous system (Zippel et al., 1997). The other form is RasGrf2 (130 kDa with 1189 amino

acids), which shows a high degree of sequence homology (80%) to RasGrf1, and is expressed ubiquitously, but is also present within the mature brain (Fam et al., 1997; Anborgh et al., 1999).

The expression pattern and localization of RasGrf1 has been extensively studied in the rat brain. The full length RasGrf1 protein is expressed in mature neurons of the rat brain and spinal cord, but not in glial cells. The hippocampus, amygdala, thalamus, hypothalamus, cortex, striatum, cerebellum, and retina are the major sites of full length RasGrf1 protein expression (Itier et al., 1998; Shou et al., 1992; Wei et al., 1993; Zippel et al., 1997; Fernandez-Medarde et al., 2009), and later sub-cellular fractionation studies have indicated the presence of RasGrf1 in post-synaptic densities and its localization in the dendrites and soma of neuronal cells (Zippel et al., 1997; Sturani et al., 1997). Outside the nervous system, RasGrf1 is also expressed in lower levels in pancreatic β-cells suggesting that it might be involved in normal pancreas development, glucose haemostasis and Diabetes (Itier et al., 1998; Font de Mora et al., 2003; Hoffmann and Spengler, 2012).

Mouse RasGrf1 was originally identified (Shou et al., 1992) as a homologue to CDC25 (cell division cycle 25), an activator of Ras-GTPase, in the yeast *S. cerevisiae,* and was initially so called CDC25Mm (Martegani et al., 1992; Wei et al., 1992). Subsequently, a 140 kDa RasGrf1 was isolated from Rat brain homologous to CDC25Mm (Gariboldi et al., 1994), and further study in the mouse brain led to isolation of other naturally occurring splice variants of RasGrf1 including a 58 kDa and 20 kDa truncated products (Ferrari et al., 1994; Arava et al., 1999; Feig, 2011). The 58 kDa isoform is expressed predominantely during embryogenesis while the full length 140kDa-

RasGrf1 is expressed in the mature mouse brain (Ferrari et al., 1994). The small isoform of 20kDa has been identified predominantely in the mouse pancreas (Arava et al., 1999).

Mouse *RasGrf1* is located on chromosome 9 with 26 exons (de la Puente et al., 2002) while *RasGrf2* is located on the long arm of chromosome 13 with 24 exons. Human RasGrf1 is largely similar to mouse RasGrf1 with 82.7% sequence homology. An extra small fragment of full length hRasGrf1 with 1273 amino acids does not affect the function of any conserved domains (Fernandez-Medarde and Santos, 2011). *RasGrf1* is an imprinted gene (preferentially from the paternal allele) which is expressed only after birth in the mouse, rat and human, and its transcription is mostly regulated through methylation and alternative splicing mechanisms (Plass et al., 1996; Kaneda et al., 2004; Fernandez-Medarde and Santos, 2011).

Structurally, RasGrf1 is a large multi-domain protein that is recruited by a diverse range of signaling molecules (Figure 1.4). These domains include a pleckstrin homology (PH) domain in the amino-terminus, a coiled coil (CC) and ilimaquinone (IQ) motif, a Db1 homology domain (DH), a Ras exchange motif (REM), a cyclin destruction box (CDB) and a cell division cycle 25 (CDC25) domain in the C-terminus (Fernandez-Medarde and Santos, 2011).

Among these domains, the PH domain, which is necessary for RasGrf1 localization in the plasma membrane (Buchsbaum et al., 1996), has been suggested being able to bind to phosphoinositides and other types of phospholipids in the membrane (Harlan et al., 1994; Lemmon and Ferguson, 2000; Varnai et al., 2002), also acts as putative phosphotyrosine binding (PTB) domain (Balla, 2005; Cowburn, 1997). RasGrf1 can also bind to βγ subunits of G protein-coupled receptors via the PH1 domain (Touhara

et al., 1994; Shou et al., 1995; Mattingly and Macara, 1996; Zippel et al., 1996) and has also been shown to interact with Trk family receptor tyrosine kinases via their HIKE motifs (Robinson et al., 2005). Coiled coil domains play a role in protein-protein interactions in cooperation with PH1 and IQ domains (Buchsbaum et al., 1996), but no specific function has been reported for this domain.

The IQ domain is capable of interacting with the protein calmodulin in a Ca^{2+}-independent manner. In response to calcium elevation, RasGrf1 is activated by binding of calcium/calmodulin to the IQ motif (Buchsbaum et al., 1996; Farnsworth et al., 1995). Moreover, it has been revealed that a point mutation in the IQ domain, which blocks calmodulin binding, can in fact prevent calcium-induced Ras activation (Buchsbaum et al., 1996). The DH domain binds the Rho family of GTPases, in particular Rac1, and promotes guanine nucleotide exchange activity toward Rac1 (Freshney et al., 1997; Kiyono et al., 1999; Innocenti et al., 1999). It has been suggested that DH domain might contribute to RasGrfs oligomerization (Anborgh et al., 1999), and in association with the adjacent PH2 domain, they can mediate interaction with other intracellular compartments such as microtubules, microtubule-binding proteins (Forlani et al., 2006; Baldassa et al., 2007).

The REM motif plays a role in stabilization of the core CDC25 domain and the CDB or PEST domain is a region between the REM and CDC25 domains which is rich in proline, glutamic acid, serine and threonine amino acids and constitutes a hypothetical target for proteolysis, an additional mechanism to regulate the cellular levels of RasGrf1 protein (Rogers et al., 1986; Baouz, et al., 1997;; Gnesutta et al., 2001). Lastly, the

CDC25 domain facilitates the activation of the Ras-GTPase (Tian and Feig, 2001; Cen et al., 1993; Wei et al., 1994).

A role for GEFs in general, and RasGrfs in particular, is to promote the exchange of GDP for GTP on small GTPase proteins such as Ras and Rac which act as molecular switches of active GTP-bound and inactive GDP-bound states (Tian et al., 2004; Katoh et al., 2000). Initially, a transient GEF-GTPase-GDP complex is formed by binding of GEF to GTPase-GDP which promotes a conformational change in the GTPase structure and release of GDP. Subsequently release of GDP results in formation of a stable GEF-GTPase-GTP complex. Finally, the GEF is released and the activated GTPase can in turn activate various signaling cascades (Tian and Feig, 2001; Consonni et al., 2003). In contrast to GEFs, GTPase Activating Proteins (GAPs) have been suggested to inactivate Ras-GTPases by hydrolyzing GTP bound to GDP (Pamonsinlapatham et al., 2009). Small GTPase proteins serve pivotal roles in linking external messages from cell surface receptors to several downstream signaling cascades underlying a wide variety of cellular processes including cell cycle regulation, cytoskeletal reorganization, neurite outgrowth and differentiation (Crespo and Leon, 2000; Malumbres and Pellicer, 1998; Huang and Reichardt, 2003).

Although several evidences have documented a correlation between RasGrfs action downstream of Trk (MacDonald et al., 1999; Robinson et al., 2005) or G-protein coupled receptors to the activation of Ras and Erk-MAP kinases (Baouz et al., 2001; Mattingly and Macara, 1996; Shou et al., 1995; Zippel et al., 1996) as well as Rac (Kiyono et al., 1999), RasGrfs have also been suggested as a missing link in the regulation of synaptic plasticity (Brambilla et al., 1997). They act as a specific calcium

sensor to transmit signals from the NMDA receptor to the activation of appropriate GTPases and the induction of LTP or LTD (Li et al., 2006). It has been shown that RasGrf1 interacts directly with the NR2B subunit of the NMDA receptor (Krapivinsky et al., 2003), through which RasGrf1 stimulates p38/MAP kinase activation leading to an increase in LTD. In contrast, RasGrf2 interacts with the NR2A subunit of the NMDA receptor and activates Erk/MAP kinase leading to an increase in LTP (Li et al., 2006). Both interactions are observed in a developmental-dependent manner, in particular after postnatal day 20 to 25 in the mouse (Li et al., 2006). It has further been shown that RasGrf1 promotes p38 activation by Rac *in vitro* while RasGrf2 activates Erk-MAP kinase by targeting Ras-GTPases (Buchsbaum et al., 2002; Tian et al., 2004).

Further evidence supporting a role for RasGrf1 in the regulation of synaptic plasticity, growth and development comes from studies in RasGrf1 knockout mice (Brambilla et al., 1997; Giese et al., 2001; Itier et al., 1998; Font de Mora et al., 2003; Clapcott et al., 2003; d'Isa et al., 2011). Although RasGrf1 knockout mice models represent various phenotypes depending on which part of the RasGrf1gene is being targeted, which isoforms are being blocked, and which region of brain inside the central nervous system is being involved (Feig, 2011), it appears that full length RasGrf1 knockout (-/-) results in a severe deficit in amygdala-dependent synaptic plasticity (Brambilla et al., 1997), as well as impairments in hippocampal-dependent plasticity observed as failures to perform hippocampal-dependent behavioral tests such as the Morris water maze, contextual discrimination and social transmission of food preferences (Giese et al., 2001).

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   1 mqkairlndg hvvtlgllaq kdgtrkgyls krsadnpkwq tkwfallqnl lfyfesdssp
  61 rpsglylleg sickrapspk rgtsskesge kqqhyftvnf sndsqktlel rtedakdcde
 121 wvaaiarasy kilateheal mqkylhllqv vetektvakq lrqqledgev eierlktevt
 181 itnlikdndr iqssnkagsa ddedsdikki kkvqsflrgw lcrrkwknii qdyirsphad
 241 smrkrnqvvf smleaeaeyv qqlhilvnnf lrplrmaass kkppithddv ssiflnseti
 301 mflhqifyqg lkarisswpt lvladlfdil lpmlniyqef vrnhqyslqi lahckqnrdf
 361 dkllkqyeak pdceertlet fltypmfqip ryiltlhell ahtphehver nsldyakskl
 421 eelsrimhde vsetenirkn laiermiteg ceilldtsqt fvrqgslmqm slseksksr
 481 grlgslstkk egerqcflfs khliictrgs ggklhltkng vislidctll depenlddea
 541 kgagpeiehl efkigvepkd slpftvilva strqekaawt sdiiqcvdni rcnglmmnaf
 601 eenskvtvpq miksdaslyc ddvdirfskt mnsckvlqir yasverller ltdlrflsid
 661 flntflhsyr vftnamvvld kliniyrkpm saiparslel lfssshnakl lygdapkspr
 721 asrkfssppp laigtsspsr rrklslnipi itggkalela slgcssdsya nihspispfg
 781 kttldtgklc masslpktpe eidvpatipe kpgelsasrk hssdvlkees eddqnhsded
 841 ntevspvksp ptpksflnrt itefpffnyn ngilmttcrd lvdnnrstls atsafaiata
 901 ganegpsnke vfrrmslant gfssdqrnid kefvirraat nrvlnvlrhw vtkhtqdfdt
 961 ddtlkyrvic fleevmhdpd lltqerkaaa niirtltlee tteqhsmlee vilmtegvkt
1021 epfenhpale iaeqltlldh lvfksipyee ffgqgwmkae kyertpyimk ttkhfnhvsn
1081 fiaseiirne disarasaie kwvavadicr clhnynavle itssinrsai frlkktwlkv
1141 skqtkslldk lqklvssdgr fknlreslrn cdppcvpylg myltdlvfie egtpnytedg
1201 lvnfskmrmi shiireirqf qqttykidpq pkviqyllde sfmldeesly esslliepkl
1261 pt
```

Figure 1.4. A Schematic diagram of the RasGrf1 protein. Mouse RasGrf1, a 140kDa protein with 1262 amino acids consist of several binding domains including a binding domain to interact with TrkB (PH1; pleckstrin domain), and binding area with NR2B subunit (amino acids 714-913), coiled-coil motif (CC), ilimaquinone (IQ) to bind calmodulin, Db1 homology domain (DH) to activate Rac, the second pleckstrin domain (PH2), Ras exchange motif (REM), cycline destruction box (CDB), and catalytic domain (CDC25) to activate Ras. S^{916} is the site for RasGrf1-phosphorylation by PKA, the W^{1056} on RasGrf1 has been suggested as binding site for GDP-Ras, and the sequences area of amino acids 714-913 have been described as neural domain (ND) which binds NR2B subunit of NMDA receptor. The lower panel represents an amino acids sequence of mouse RasGrf1 with S^{916} shown in red color.

1.10 Rationale (see Figure 1.5)

1. The ability of RasGrf1 to increase neurite outgrowth has been studied in the PC12 cells which express endogenous TrkA (Robinson et al., 2005; Yang and Mattingly, 2006). Yet the ability of RasGrf1 to induce neurite growth in BDNF-mediated TrkB-expressing cells, and whether Ras and/or Rac activation is required has not been determined. Furthermore, while RasGrf1 is tyrosine phosphorylated in response to neurotrophin stimulation in transfected cells in culture, the sites of phosphorylation and whether they are essential to RasGrf1 activation has not been determined.

2. Previous data in our lab suggested that RasGrf1 is tyrosine phosphorylated in Human embryonic kidney 293T (HEK293T) and NGF stimulated PC12 cells as well as PC12-derived cells over-expressing TrkB. Consequently, the binding sites on both Trk and RasGrf1 have been identified, being the HIKE domain located after tyrosine 516 (amino acids 531-542) on TrkB which binds the N-terminal PH1 domain on RasGrf1 (Robinson et al., 2005). However, the phosphorylation-dependent interaction of TrkB and RasGrf1 has yet to be observed in primary neurons. It was also not determined whether TrkB directly interacts with RasGrf1 or if this interaction could be mediated indirectly through other scaffolding molecules. Scaffolding proteins are known as crucial regulators of many key signaling pathways; they interact or bind to other members of a signaling pathway to retain them into their complex. In this regard, post-synaptic density protein 95 (PSD95) is the intermediate candidate protein. PSD95 is a PDZ (postsynaptic density-95-discs large-zona occludens-1) domain containing protein that facilitates the phosphorylation of NR2B by Src family kinases (Tezuka et al., 1999; Zhang et al., 2008). PSD95 co-immunoprecipitates with TrkB and its trafficking to dendrites is facilitated by

BDNF (Yoshii and Constantine-Paton, 2007; Ji et al., 2005). Furthermore, RasGrf1 is enriched in post-synaptic densities and previously unpublished data in our lab showed that PSD95 co-immunoprecipitates with NR2B in response to NMDA treatment.

3. RasGrf1 has been identified as a novel interactor of both Trk (MacDonald et al., 1999), where it facilitates neurite outgrowth in response to low doses of NGF stimulation in PC12 cells (Robinson et al., 2005), and the NMDA receptor, where it facilitates LTD by activating p38-MAP kinase (Buchsbaum et al., 2002; Li et al., 2006), and LTP through Erk-MAP kinase activation (Li et al., 2006). However, the potential of RasGrf1, as a downstream signaling molecule, in facilitating a potential cross-talk mechanism between the TrkB and NMDA receptors is unknown.

Thus, the present study has been designed to address the potential of RasGrf1 to modulate both TrkB and the NMDA receptors and identify novel cross-talk mechanisms by which the biological responses to BDNF are regulated. We have initially determined the RasGrf1-induced neurite outgrowth in response to both NGF and BDNF in neuronal-like cells as an *in vitro* model for primary neurons. We also examined the domain(s) and site(s) of RasGrf1 tyrosine phosphorylation in response to BDNF stimulation in these cells. Finally, we determined the interaction between both TrkB and NMDA receptors with RasGrf1 in cortical/hippocampal slices, in response to BDNF and NMDA stimulation, and provide a potential mechanism of how BDNF activation of TrkB facilitates changes in NMDA receptor signaling.
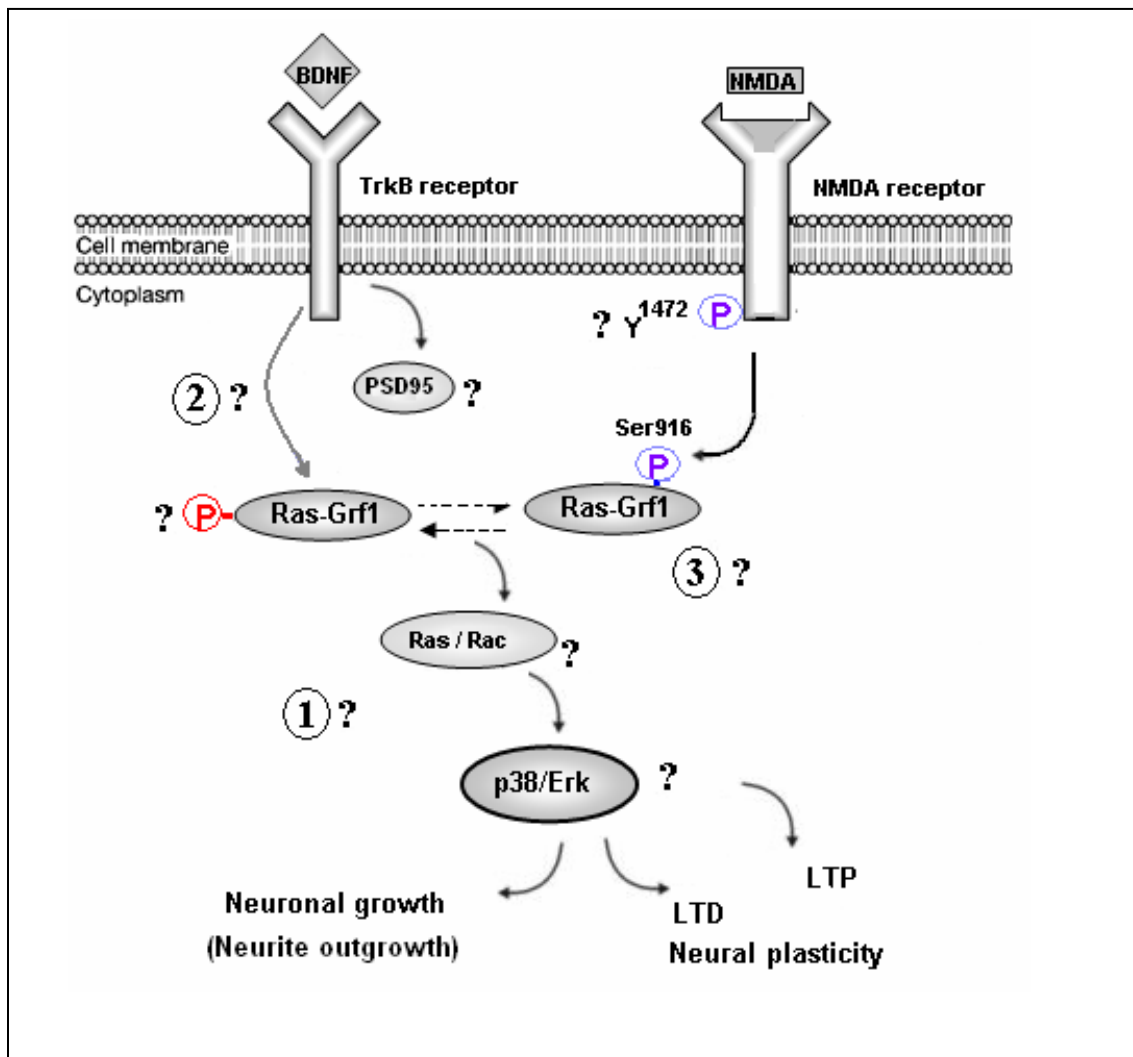
Figure 1.5. A schematic diagram to depict the rationales of this study. The question marks show signaling molecules or mechanisms involved in the process which remain to be investigated in this study. 1) The role of RasGrf1 in neurite outgrowth, the sites of tyrosine phosphorylation and its possible downstream Ras and/or Rac-GTPase activation. 2) Possible interaction of TrkB with RasGrf1. This interaction might be directly or indirectly through PSD95. 3) RasGrf1 signaling pathway as a candidate to mediate cross-talk between TrkB and NMDA receptors during synaptic plasticity, and also RasGrf1-mediated downstream of Rac/Ras GTPases and p38/Erk MAP kinases activation.

1.11 Hypothesis and Objectives

The general hypothesis of my thesis is that BDNF stimulates the recruitment of RasGrf1 to TrkB thereby uncoupling RasGrf1 from the NMDA receptor and decreasing the potential of LTD in favour of LTP. Secondly, the ability of RasGrf1 to increase BDNF-dependent neuronal/dendritic growth may provide a second mechanism to potentiate synaptic connections and facilitate the process of LTP. The specific objectives of this project are as follows:

1. To determine whether the activation of RasGrf1 will increase neurite growth in neuronal-like cells in a Ras and/or Rac-dependent manner.

2. To identify the site(s) of tyrosine phosphorylation on RasGrf1 upon BDNF stimulation.

3. To determine if BDNF stimulation couples RasGrf1 to TrkB and increases Erk-MAP kinase activation.

4. To determine if BDNF stimulation uncouples RasGrf1 from the NMDA receptor, decreases NMDA-induced p38-MAP kinase activation to decrease LTD in favour of LTP.

References

Akaneya Y., Tsumoto T., Kinoshita S., and Hatanaka H. (1997) Brain-derived neurotrophic factor enhances long-term potentiation in rat visual cortex. J. Neurosci. 17, 6707–6716.

Amaral M.D., and Pozzo-Miller L. (2007) TRPC3 channels are necessary for brain-derived neurotrophic factor to activate a nonselective cationic current and to induce dendritic spine formation. J. Neurosci. 27, 5179-89.

Anborgh P. H., Qian X., Papageorge A. G., Vass W. C., DeClue J. E., and Lowy D. R. (1999) Ras-Specific Exchange Factor GRF: Oligomerization through its Dbl Homology Domain and Calcium-Dependent Activation of Raf. Mol. Cell. Biol. 19, 4611-4622.

Arava Y., Seger R., and Walker M.D. (1999) GRFbeta, a novel regulator of calcium signaling, is expressed in pancreatic beta cells and brain. J. Biol. Chem. 274, 24449–24452.

Avila M.A., Varela-Nieto I., Romero G., Mato J.M., Giraldez F., Van De Water T.R., and Represa J. (1993) Brain-derived neurotrophic factor and neurotrophin-3 support the survival and neuritogenesis response of developing cochleovestibular ganglion neurons. Dev Biol. 159, 266-75.

Baldassa S., Gnesutta N., Fascio U., Sturani E., and Zippel R. (2007) SCLIP, a Microtubule-destabilizing Factor, Interacts with RasGRF1 and Inhibits Its Ability to Promote Rac Activation and Neurite Outgrowth. J. Biol. Chem. 282, 2333-2345.

Balla T. (2005) Inositol-lipid binding motifs: signal integrators through protein-lipid and protein-protein interactions. J. Cell. Sci. 118, 2093-104.

Barbacid M. (1994)The Trk family of neurotrophin receptors. J. Neurobiol. 25, 1386-403.

Barde Y.A., Edgar D., and Thoenen H. (1982) Purification of a new neurotrophic factor from mammalian brain. EMBO. J. 1, 549-53.

Barker P.A., Hussain N.K., and McPherson P.S. (2002) Retrograde signaling by the neurotrophins follows a well-worn trk. Trends. Neurosci. 25, 379-81.

Barker P.A., Lomen-Hoerth C., Gensch E.M., Meakin S.O., Glass D.J., and Shooter E.M. (1993) Tissue-specific alternative splicing generates two isoforms of the trkA receptor. J. Biol. Chem. 268, 15150-7.

Bath K.G., and Lee F.S. (2006) Variant BDNF (Val66Met) impact on brain structure and function. Cogn. Affect. Behav. Neurosci. 6, 79-85.

Baouz S., Jacquet E., Accorsi K., Hountondji C., Balestrini M., Zippel R., Sturani E., and Parmeggiani A. (2001) Sites of phosphorylation by protein kinase A in CDC25Mm/GRF1, a guanine nucleotide exchange factor for Ras. J. Biol. Chem. 276, 1742-9.

Baouz S., Jacquet E., Bernardi A., and Parmeggiani A. (1997) The N-terminal moiety of CDC25Mm, a GDP/GTP exchange factor of Ras proteins, controls the activity of the catalytic domain: modulation by calmodulin and calpain. J. Biol. Chem. 272, 6671–6676.

Bauer E.P., Schafe G.E., and LeDoux J.E. (2002) NMDA receptors and L-type voltage-gated calcium channels contribute to long-term potentiation and different components of fear memory formation in the lateral amygdala. J. Neurosci. 22, 5239-49.

Benito-Gutiérrez E., Garcia-Fernàndez J., and Comella J.X. (2006) Origin and evolution of the Trk family of neurotrophic receptors. Mol. Cell. Neurosci. 31, 179-92.

Berkemeier L.R., Winslow J.W., Kaplan D.R., Nikolics K., Goeddel D.V., and Rosenthal A. (1991) Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. Neuron. 7, 857-66.

Brunet A., Bonni A., Zigmond M.J., Lin M.Z., Juo P., Hu L.S., Anderson M.J., Arden K.C., Blenis J., and Greenberg M.E. (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell. 96, 857-68.

Buchsbaum R., Telliez J. B., Goonesekera S., and Feig L. A. (1996) The N-Terminal Peckstrin, Coiled-Coil and IQ Domains of the Exchange Factor Ras-GRF act cooperatively to Facilitate Activation by Calcium. Mol. Cell. Biol. 16, 4888-4896.

Buchsbaum R. J., Connolly B. A., and Feig L. A. (2002) Interaction of Rac exchange factors Tiam1 and RasGrf1 with a scaffold for the p38 mitogen-activated protein kinase cascade. Mol Cell Biol. 22, 4073-4085.

Brambilla R., Gnesutta N., Minichiello L., White G., Roylance A.J., Herron C.E., Ramsey M., Wolfer D.P., Cestari V., Rossi-Arnaud C., Grant S.G., Chapman P.F., Lipp H.P., Sturani E., and Klein R. (1997) A role for the Ras signalling pathway in synaptic transmission and long-term memory. Nature. 390, 281-6.

Butte M.J., Hwang P.K., Mobley W.C., and Fletterick R. J. (1998) Crystal structure of neurotrophin-3 homodimer shows distinct regions are used to bind its receptors. Biochemistry 37, 16 846–52.

Caldeira M.V., Melo C.V., Pereira D.B., Carvalho R., Correia S.S., Backos D.S., Carvalho A.L., Esteban J.A., and Duarte C.B. (2007) Brain-derived neurotrophic factor regulates the expression and synaptic delivery of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor subunits in hippocampal neurons.J Biol Chem. 282, 12619-28.

Carvalho A.L., Caldeira M.V., Santos S.D., Duarte C.B. (2008) Role of the brain-derived neurotrophic factor at glutamatergic synapses. Br. J. Pharmacol. 153, 310-24.

Cen H., Papageorge A.G., Vass W.C., Zhang K.E., and Lowy DR. (1993) Regulated and constitutive activity by CDC25Mm (GRF), a Ras-specific exchange factor. Mol. Cell. Biol. 13, 7718–7724.

Chakrabarti K., Lin R., Schiller N. I., Wang Y., Koubi D., Fan Y.-X., Rudkin B. B., Johnson G. R., and Schiller M. R. (2005) Critical Role for Kalirin in Nerve Growth Factor Signaling through TrkA. Mol. Cell. Biol. 25, 5106-5118.

Chao M.V., and Hempstead B.L. (1995) p75 and Trk: a two-receptor system. Trends. Neurosci. 1995 18, 321-6.

Chen Z.Y., Jing D., Bath K.G., Ieraci A., Khan T., Siao C.J., Herrera D.G., Toth M., Yang C., McEwen B.S., Hempstead B.L., and Lee F.S. (2006) Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. Science. 314, 140-3.

Clapcott S.J., Peters J., Orban P.C., Brambilla R., and Graham C.F. (2003) Two ENU-induced mutations in Rasgrf1 and early mouse growth retardation, Mamm. Genome 14, 495–505.

Consonni R., Arosio I., Recca T., Longhi R., Colombo G., and Vanoni M. (2003) Structure determination and dynamics of peptides overlapping the catalytic hairpin of the Ras-specific GEF Cdc25(Mm). Biochemistry. 42, 12154-62.

Coppola V., Barrick C.A., Southon E.A., Celeste A., Wang K., Chen B., Haddad el-B., Yin J., Nussenzweig A., Subramaniam A., and Tessarollo L. (2004) Ablation of TrkA function in the immune system causes B cell abnormalities. Development. 131, 5185-95.

Cowburn D. (1997) Peptide recognition by PTB and PDZ domains. Curr. Opin. Struct. Biol. 7, 835-8.

Crespo P., and Leon J. (2000). Ras proteins in the control of the cell cycle and cell differentiation. Cell. Mol. Life Sci. 57, 1613–1636.

Davies AM, Bandtlow C, Heumann R, Korsching S, Rohrer H, and Thoenen H. (1987) Timing and site of nerve growth factor synthesis in developing skin in relation to innervation and expression of the receptor. Nature. 326, 353-8.

de la Puente A., Hall J., Wu Y.Z., Leone G., Peters J., Yoon B.J., Soloway P., and Plass C. (2002) Structural characterization of Rasgrf1 and a novel linked imprinted locus. Gene 291, 287–297.

Delcroix J.D., Valletta J.S., Wu C., Hunt S.J., Kowal A.S., and Mobley W.C. (2003) NGF signaling in sensory neurons: evidence that early endosomes carry NGF retrograde signals. Neuron. 39, 69-84.

Desmet C.J., and Peeper D.S. (2006) The neurotrophic receptor TrkB: a drug target in anti-cancer therapy? Cell. Mol. Life. Sci. 63, 755-9.

d'Isa R., Clapcote S.J., Voikar V., Wolfer D.P., Giese K.P., Brambilla R., and Fasano S. (2011) Mice Lacking Ras-GRF1 Show Contextual Fear Conditioning but not Spatial Memory Impairments: Convergent Evidence from Two Independently Generated Mouse Mutant Lines. Front. Behav. Neurosci. 5, 78.

Donovan M.J., Lin M.I., Wiegn P., Ringstedt T., Kraemer R., Hahn R., Wang S., Ibañez C.F., Rafii S., and Hempstead B.L. (2000) Brain derived neurotrophic factor is an endothelial cell survival factor required for intramyocardial vessel stabilization. Development. 127, 4531-40.

Dragunow M., Beilharz E., Mason B., Lawlor P., Abraham W., and Gluckman P. (1993) Brain-derived neurotrophic factor expression after long-term potentiation. Neurosci. Lett. 160, 232–236.

Drake C.T., Milner T.A., and Patterson S. L. (1999) Ultrastructural localization of full-length trkB immunoreactivity in rat hippocampus suggests multiple roles in modulating activity-dependent synaptic plasticity. J. Neurosci. 19, 8009–8026.

Egan S.E., Giddings B.W., Brooks M.W., Buday L., Sizeland A.M., and Weinberg R.A. (1993) Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. Nature. 363, 45-51.

Egan M.F., Kojima M., Callicott J.H., Goldberg T.E., Kolachana B.S., Bertolino A., Zaitsev E., Gold B., Goldman D., Dean M., Lu B., and Weinberger D.R. (2003) The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell. 112, 257-69.

Eide F.F., Lowenstein D.H., and Reichardt LF. (1993) Neurotrophins and their receptors--current concepts and implications for neurologic disease. Exp. Neurol. 121, 200-14.

Eide F.F., Vining E.R., Eide B.L., Zang K., Wang X.Y., and Reichardt L.F. (1996) Naturally occurring truncated trkB receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling. J. Neurosci. 16, 3123-9.

English J.D., and Sweatt J.D. (1997) A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. J. Biol. Chem. 272, 19103-6.

Ernfors P., Merlio J.P., and Persson H. (1992) Cells Expressing mRNA for Neurotrophins and their Receptors During Embryonic Rat Development. Eur. J. Neurosci. 4, 1140-1158.

Ernfors P., Ibáñez C.F., Ebendal T., Olson L., and Persson H. (1990) Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: developmental and topographical expression in the brain. Proc. Natl. Acad. Sci. U S A. 87, 5454-8.

Fahnestock M., Michalski B., Xu B., and Coughlin M.D. (2001) The precursor pro-nerve growth factor is the predominant form of nerve growth factor in brain and is increased in Alzheimer's disease. Mol. Cell. Neurosci. 18, 210-20.

Fam N.P., Fan W.T., Wang Z., Zhang L.J., Chen Z., Moran M.F. (1997) Cloning and characterization of Ras-GRF2, a novel guanine nucleotide exchange factor for Ras. Mol. Cell. Biol. 17, 1396–1406.

Farhadi H.F., Mowla S.J., Petrecca K., Morris S.J., Seidah N.G., and Murphy R.A. (2000) Neurotrophin-3 sorts to the constitutive secretory pathway of hippocampal neurons and is diverted to the regulated secretory pathway by coexpression with brain-derived neurotrophic factor. J. Neurosci. 20, 4059-68.

Farnsworth C. L., Freshney N. W., Rosen L. B., Ghosh A., Greenberg M. E., and Feig L. A. (1995) Calcium activation of Ras mediated by neuronal exchange factor Ras-GRF. Nature. 376, 524-527.

Feig L.A. (2011) Regulation of Neuronal Function by Ras-GRF Exchange Factors. Genes. Cancer. 2, 306-19.

Fernandez-Medarde A., Barhoum R., Riquelme R., Porteros A., Nunez A., de Luis A., de Las Rivas J., de la Villa P., Varela-Nieto I., and Santos E. (2009) RasGRF1 disruption causes retinal photoreception defects and associated transcriptomic alterations, J. Neurochem. 110, 641–652.

Fernández-Medarde A, and Santos E. (2011) The RasGrf family of mammalian guanine nucleotide exchange factors. Biochim. Biophys. Acta. 1815, 170-88.

Ferrari C., Zippel R., Martegani E., Gnesutta N., Carrera V., and Sturani E. (1994). Expression of two different products of CDC25Mm, a mammalian Ras activator, during development of mouse brain. Exp. Cell Res. 210:353–357.

Font de Mora J., Esteban L.M., Burks D.J., Núñez A., Garcés C., García-Barrado M.J., Iglesias-Osma M.C., Moratinos J., Ward J.M., and Santos E. (2003) Ras-GRF1 signaling is required for normal beta-cell development and glucose homeostasis. EMBO. J. 22, 3039-49.

Forlani G., Baldassa S., Lavagni P., Sturani E., and Zippel R. (2006) The guanine nucleotide exchange factor RasGRF1 directly binds microtubules via DHPH2-mediated interaction. FEBS. J. 273, 2127-38.

Frade J.M., and Barde Y.A. (1998) Nerve growth factor: two receptors, multiple functions. Bioessays 20, 137–145.

Freshney N.W., Goonesekera S.D., and Feig L.A. (1997) Activation of the exchange factor Ras-GRF by calcium requires an intact Dbl homology domain. FEBS Lett. 407, 111-5.

Gariboldi M., Sturani E., Canzian F., De Gregorio L., Manenti G., Dragani T.A., and Pierotti M.A. (1994) Genetic mapping of the mouse CDC25Mm gene, a ras-specific guanine nucleotide-releasing factor, to chromosome 9. Genomics. 21, 451-3.

Ghosh A., and Greenberg M.E. (1995) Calcium signaling in neurons: molecular mechanisms and cellular consequences. Science, 268, 239–247.

Giese K. P., Friedman E., Telliez J. B., Fedorov N. B., Wines M., Feig L. A., and Silva A. J. (2001) Hippocampus dependent learning and memory is impaired in mice lacking the Ras guanine nucleotide releasing factor 1 (RasGrf1). Neuropharmacology. 41, 791-800.

Gnesutta N., Ceriani M., Innocenti M., Mauri I., Zippel R., Sturani E., Borgonovo B., Berruti G., and Martegani E. (2001) Cloning and characterization of mouse UBPy, a deubiquitinating enzyme that interacts with the ras guanine nucleotide exchange factor CDC25(Mm)/Ras-GRF1. J. Biol. Chem. 276, 39448–39454.

Grimes M.L., Zhou J., Beattie E.C., Yuen E.C., Hall D.E., Valletta J.S., Topp K.S., LaVail J.H., Bunnett N.W., and Mobley W.C. (1996) Endocytosis of activated TrkA: evidence that nerve growth factor induces formation of signalling endosomes. J. Neurosci. 16, 7950–7964.

Hanks S.K., Quinn A.M., and Hunter T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science. 241, 42-52.

Hariri A.R., Goldberg T.E., Mattay V.S., Kolachana B.S., Callicott J.H., Egan M.F., and Weinberger D.R. (2003) Brain-derived neurotrophic factor val66met polymorphism affects human memory-related hippocampal activity and predicts memory performance. J. Neurosci. 23, 6690-4.

Harlan J.E., Hajduk P.J., Yoon H.S., and Fesik S.W. (1994) Pleckstrin homology domains bind to phosphatidylinositol-4, 5-bisphosphate. Nature. 371, 168–170.

Harrington A.W., Leiner B., Blechschmitt C., Arevalo J.C., Lee R., Mörl K., Meyer M., Hempstead B.L., Yoon S.O., and Giehl K.M. (2004) Secreted proNGF is a pathophysiological death-inducing ligand after adult CNS injury. Proc. Natl. Acad. Sci. U S A. 101, 6226-30.

He X.L., and Garcia K.C. (2004) Structure of nerve growth factor complexed with the shared neurotrophin receptor p75. Science. 304, 870-5.

Heerssen H.M., and Segal R.A. (2002) Location, location, location: a spatial view of neurotrophin signal transduction.Trends. Neurosci. 25, 160-5.

Heldin C.H. (1995) Dimerization of cell surface receptors in signal transduction. Cell. 80, 213-23.

Heumann R., Lindholm D., Bandtlow C., Meyer M., Radeke M.J., Misko T.P., Shooter E., and Thoenen H. (1987) Differential regulation of mRNA encoding nerve growth factor and its receptor in rat sciatic nerve during development, degeneration, and regeneration: role of macrophages. Proc. Natl. Acad. Sci. U S A. 84, 8735-9.

Heumann R., Schwab M., Merkl R., and Thoenen H. (1984) Nerve growth factor-mediated induction of choline acetyltransferase in PC12 cells: evaluation of the site of action of nerve growth factor and the involvement of lysosomal degradation products of nerve growth factor. J. Neurosci. 4, 3039-50.

Hoffmann A., and Spengler D. (2012) Transient Neonatal Diabetes Mellitus Gene Zac1 Impairs Insulin Secretion in Mice through Rasgrf1. Mol. Cell. Biol. 32, 2549-60.

Howe C.L., Valletta J.S., Rusnak A.S., and Mobley W.C. (2001) NGF signalling from clathrin-coated vesicles. Evidence that signaling endosomes serve as a platform for the Ras-MAPK pathway. Neuron. 32, 801–814.

Huang C.S., Zhou J., Feng A.K., Lynch C.C., Klumperman J., DeArmond S.J., and Mobley W.C. (1999) Nerve growth factor signaling in caveolae-like domains at the plasma membrane. J. Biol. Chem. 274, 36707-14.

Huang E. J., and Reichardt L. F. (2003) Trk Receptors: Roles in Neuronal Signal Transduction. Annual Review of Biochemistry. 72, 609-642.

Hubbard S.R. (1997) Crystal structure of the activated insulin receptor tyrosine kinase in complex with peptide substrate and ATP analog. EMBO. J. 16, 5572-81.

Hyman C., Hofer M., Barde Y.A., Juhasz M., Yancopoulos G.D., Squinto S.P., Lindsay R.M. (1991) BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. Nature. 350, 230-2.

Ibanez C.F., Ilag L.L., Murray-Rust J., and Persson H. (1993) An extended surface of binding to Trk tyrosine kinase receptors in NGF and BDNF allows the engineering of a multifunctional pan-neurotrophin. EMBO. J. 12, 2281-93.

Innocenti M., Zippel R., Brambilla R., and Sturani E. (1999) CDC25(Mm)/Ras-GRF1 regulates both Ras and Rac signaling pathways. FEBS. Lett. 460, 357–362.

Itier J.M., Tremp G.L., Leonard J.F., Multon M.C., Ret G., Schweighoffer F., Tocque B., Bluet-Pajot M.T., Cormier V., and Dautry F. (1998) Imprinted gene in postnatal growth role. Nature 393, 125–126.

Ji Y., Pang P.T., Feng L., and Lu B. (2005) Cyclic AMP controls BDNF-induced TrkB phosphorylation and dendritic spine formation in mature hippocampal neurons. Nat. Neurosci. 8, 164-72.

Kaneda M, Okano M., Hata K., Sado T., Tsujimoto N., Li E., and Sasaki H., (2004) Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. Nature 429, 900–903.

Kang H., and Schuman E.M. (2000) Intracellular Ca(2+) signaling is required for neurotrophin-induced potentiation in the adult rat hippocampus. Neurosci. Lett. 282, 141-4.

Kang H., Welcher A.A., Shelton D., and Schuman E.M. (1997) Neurotrophins and time: Different roles for TrkB signaling in hippocampal long-term potentiation. Neuron. 19, 653–664.

Kao S., Jaiswal R. K., Kolch W., and Landreth G. E. (2001) Identification of the Mechanisms Regulating the Differential Activation of the MAPK Cascade by Epidermal Growth Factor and Nerve Growth Factor in PC12 cells. J. Biol. Chem. 276, 18169-18177.

Katoh H., Yasui H., Yamaguchi Y., Aoki J., Fujita H., Mori K., and Negishi M. (2000) Small GTPase RhoG is a key regulator for neurite outgrowth in PC12 cells. Mol. Cell. Biol. 20, 7378-87.

Kavanaugh W.M., and Williams L.T. (1994) An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. Science. 266, 1862–1865.

Kermani P., Rafii D., Jin D.K., Whitlock P., Schaffer W., Chiang A., Vincent L., Friedrich M., Shido K., Hackett N.R., Crystal R.G., Rafii S., and Hempstead B.L. (2005) Neurotrophins promote revascularization by local recruitment of TrkB+ endothelial cells and systemic mobilization of hematopoietic progenitors. J. Clin. Invest. 115, 653-63.

Kiyono M., Satoh T., and Kaziro y. (1999) G protein beta gamma subunit-dependent Rac-guanine nucleotide exchange activity of Ras-GRF1/CDC25(Mm). Proc. Natl. acad. Sci. U S A. 96, 4826-31.

Kiyono M., Kaziro Y., and Satoh T. (2000a) Induction of Rac-Guanine Nucleotide Exchange Activity of Ras-GRF1/CDC25Mm following Phosphorylation by the Nonreceptor Tyrosine Kinase Src. J. Biol. Chem. 275, 5441-5446.

Kiyono M., Kato J., Kataoka T., Kaziro Y., and Satoh T. (2000b) Stimulation of Ras Guanine Nucleotide Exchange Activity of Ras-GRF1/CDC25Mm upon Tyrosine Phosphorylation by the Cdc42-regulated Kinase ACK1. J. Biol. Chem. 275, 29788-29793.

Klein R., Parada L.F., Coulier F., and Barbacid M. (1989) trkB, a novel tyrosine protein kinase receptor expressed during mouse neural development. EMBO. J. 8, 3701-9.

Knudsen B.S., Feller S.M., and Hanafusa H. (1994) Four proline-rich sequences of theguanine-nucleotide exchange factor C3G bind with unique specificity to the first Src homology 3 domain of Crk. J. Biol. Chem. 269, 32781–32787.

Knusel B., Winslow J.W., Rosenthal A., Burton L.E., Seid D.P., Nikolics K., and Hefti F. (1991) Promotion of central cholinergic and dopaminergic neuron differentiation by brain-derived neurotrophic factor but not neurotrophin 3. Proc. Natl. Acad. Sci. U S A. 88, 961-5.

Kollen M., Dutar P., and Jouvenceau A. (2008) The magnitude of hippocampal long term depression depends on the synaptic location of activated NR2-containing N-methyl-D-aspartate receptors. Neuroscience. 154, 1308-17.

Korsching S., and Thoenen H. (1983) Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: correlation with density of sympathetic innervation. Proc. Natl. Acad. Sci. U S A. 80, 3513-6.

Korsching S. (1993) The neurotrophic factor concept: a reexamination. J Neurosci. 13, 2739-48.

Kozisek M.E., Middlemas D., and Bylund D.B. (2008) Brain-derived neurotrophic factor and its receptor tropomyosin-related kinase B in the mechanism of action of antidepressant therapies. Pharmacol. Ther. 117, 30-51.

Krapivinsky G., Krapivinsky L., Manasian Y., Ivanov A., Tyzio R., Pellegrino C., Ben-Ari Y., Clapham D.E., and Medina I. (2003) The NMDA receptor is coupled to the ERK pathway by a direct interaction between NR2B and RasGRF1. Neuron 40, 775–784.

Kuczewski N., Porcher C., Lessmann V., Medina I., and Gaiarsa J.L. (2009) Activity-dependent dendritic release of BDNF and biological consequences. Mol. Neurobiol.39, 37–49.

Kutsuwada T., Kashiwabuchi N., Mori H., Sakimura K., Kushiya E., Araki K., Meguro H., Masaki H., Kumanishi T., Arakawa M., et al. (1992) Molecular diversity of the NMDA receptor channel. Nature. 358, 36-41.

Lau C.G., and Zukin R.S. (2007) NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders. Nat. Rev. Neurosci. 8, 413-26.

Lee F.S., and Chao M.V. (2001) Activation of Trk neurotrophin receptors in the absence of neurotrophins. Proc. Natl. Acad. Sci. U S A. 98, 3555-60.

Lee R., Kermani P., Teng K.K. and Hempstead B.L. (2001) Regulation of cell survival by secreted proneurotrophins. Science. 294,1945-8.

Lee F.S., Rajagopal R., and Chao M.V. (2002a). Distinctive features of Trk neurotrophin receptor transactivation by G protein-coupled receptors. Cytokine. Growth. Factor. Rev. 13, 11-7.

Lee F.S., Rajagopal R., Kim A.H., Chang P.C., and Chao M.V. (2002b) Activation of Trk neurotrophin receptor signaling by pituitary adenylate cyclase-activating polypeptides. J. Biol. Chem. 277, 9096-102.

Lemmon M.A., and Ferguson K.M. (2000) Signal-dependent membrane targeting by pleckstrin homology (PH) domains, Biochem. J. 350, 1–18.

Lessmann V., and Brigadski T. (2009) Mechanisms, locations, and kinetics of synaptic BDNF secretion: an update. Neurosci Res. 65:11-22.

Lessmann V., and Heumann R. (1998) Modulation of unitary glutamatergic synapses by neurotrophin-4/5 or brain-derived neurotrophic factor in hippocampal microcultures: presynaptic enhancement depends on pre-established paired-pulse facilitation. Neuroscience 86, 399– 413.

Lessmann V., Gottmann K., and Malcangio M. (2003) Neurotrophin secretion: current facts and future prospects. Prog. Neurobiol. 69, 341–374.

Levi-Montalcini R. (1987) The nerve growth factor 35 years later. Science. 237, 1154-62.

Levine E.S., and Kolb J.E. (2000) Brain-derived neurotrophic factor increases activity of NR2B- containing N-methyl-D-aspartate receptors in excised patches from hippocampal neurons. J. Neurosci. Res. 62, 357–362.

Levine E.S., Crozier R.A., Black I.B., and Plummer M.R. (1998) Brain-derived neurotrophic factor modulates hippocampal synaptic transmission by increasing N-methyl-D-aspartic acid receptor activity. Proc. Natl. Acad. Sci. USA. 95, 10235–10239.

Li Y.X., Zhang Y., Lester H.A., Schuman E.M., and Davidson N. (1998) Enhancement of neurotransmitter release induced by brain-derived neurotrophic factor in cultured hippocampal neurons. J. Neurosci. 18, 10231–10240.

Li S., Tian X., Hartley D.M., and Feig L.A. (2006). Distinct Roles for Ras-Guanine Nucleotide-Releasing Factor1 (Ras-GRF1) and Ras-GRF2 in the Induction of Long-Term Potentiation and Long-Term Depression. The Journal of Neuroscience. 26, 1721–1729.

Liepinsh E., Ilag L.L., Otting G., and Ibáñez C.F. (1997) NMR structure of the death domain of the p75 neurotrophin receptor. EMBO. J. 16, 4999-5005.

Lin M.I., Das I., Schwartz G.M., Tsoulfas P., Mikawa T., and Hempstead B.L. (2000) Trk C receptor signaling regulates cardiac myocyte proliferation during early heart development in vivo. Dev. Biol. 226, 180-91.

Lin S.Y., Wu K., Levine E.S., Mount H.T., Suen P.C., and Black I.B. (1998) BDNF acutely increases tyrosine phosphorylation of the NMDA receptor subunit 2B in cortical and hippocampal postsynaptic densities. Brain. Res. Mol. Brain Res. 55, 20–27.

Lisman J. (2003) Long-term potentiation: outstanding questions and attempted synthesis. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 358, 829-42.

Liu H.Y., and Meakin S.O. (2002) ShcB and ShcC activation by the Trk family of receptor tyrosine kinases. J. Biol. Chem. 277, 26046-56.

Liu X.B., Murray K.D., and Jones E.G. (2004) Switching of NMDA receptor 2A and 2B subunits at thalamic and cortical synapses during early postnatal development. J. Neurosci. 24, 8885-95.

Lu B. (2003) BDNF and activity-dependent synaptic modulation. Learn. Mem. 10, 86–98.

Lu Y., Christian K., and Lu B. (2008) BDNF: a key regulator for protein synthesis-dependent LTP and long-term memory. Neurobiol. Learn. Mem. 89, 312-23.

Lynch M.A. (2004) Long-term potentiation and memory. Physiol. Rev. 84, 87-136.

MacDonald J.I., Verdi J.M., and Meakin S.O. (1999) Activity-dependent interaction of the intracellular domain of rat trkA with intermediate filament proteins, the beta-6 proteasomal subunit, Ras-GRF1, and the p162 subunit of eIF3. J. Mol. Neurosci. 13, 141-58.

Malenka R.C., and Bear M.F. (2004) LTP and LTD: an embarrassment of riches. Neuron. 44, 5-21.

Malumbres M., and Pellicer A. (1998) Ras pathways to cell cycle control and cell transformation. Front. Biosci. 3, d887–d912.

Martegani E., Vanoni M., Zippel R., Coccetti P., Brambilla R., Ferrari C., Sturani E., and Alberghina L. (1992) Cloning by functional complementation of a mouse cDNA encoding a homologue of CDC25, a Saccharomyces cerevisiae RAS activator. EMBO. J. 11, 2151-7.

Martin-Zanca D., Hughes S.H., and Barbacid M. (1986) A human oncogene formed by the fusion of truncated tropomyosin and protein tyrosine kinase sequences. Nature. 319, 743-8.

Massey P.V., Johnson B.E., Moult P.R., Auberson Y.P., Brown M.W., Molnar E., Collingridge G.L., and Bashir Z.I. (2004) Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. J. Neurosci. 24, 7821-8.

Mattingly M., and Macara I. (1996) Phosphorylation-dependent activation of the Ras-GRF/CDC25Mm exchange factor by muscarinic receptors and G-protein beta-gamma subunits. Nature. 382, 268-272.

McDonald N.Q., Lapatto R., Murray-Rust J., Gunning J., Wlodawer A., and Blundell T.L. (1991) New protein fold revealed by a 2.3-A resolution crystal structure of nerve growth factor. Nature. 354, 411-4.

Meakin S. O. (2000) Nerve Growth Factor Receptors and Mechanisms of Intracellular Signal Transduction. Recent Research Developments in Neurochemistry. 3, 75-91.

Meakin S. O., MacDonald J. I. S., Gryz E. A., Kubu C. J., and Verdi J. M. (1999) The Signaling Adapter Protein FRS-2 Competes with Shc for binding to TrkA: A Model for Discriminating Proliferation and Differentiation. J. Biol. Chem. 274, 9861-9870.

Middlemas D.S., Lindberg R.A., and Hunter T. (1991) trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. Mol. Cell. Biol. 11, 143-53.

Minichiello L. (2009) TrkB signalling pathways in LTP and learning. Nat. Rev. Neurosci. 10, 850-60.

Minichiello L., Calella A.M., Medina D.L., Bonhoeffer T., Klein R., and Korte M. (2002) Mechanism of TrkB-mediated hippocampal long-term potentiation. Neuron. 36, 121-37.

Monteggia L.M., Luikart B., Barrot M., Theobold D., Malkovska I., Nef S., Parada L.F., and Nestler E.J. (2007) Brain-derived neurotrophic factor conditional knockouts show gender differences in depression-related behaviors. Biol. Psychiatry. 61, 187-97.

Monyer H., Sprengel R., Schoepfer R., Herb A., Higuchi M., Lomeli H., Burnashev N., Sakmann B., and Seeburg P.H. (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science. 256, 1217-21.

Mowla S.J., Pareek S., Farhadi H.F., Petrecca K., Fawcett J.P., Seidah N.G., Morris S.J., Sossin W.S., and Murphy R.A. (1999) Differential sorting of nerve growth factor and brain-derived neurotrophic factor in hippocampal neurons. J. Neurosci. 19, 2069-80.

Nagahara A.H., and Tuszynski M.H. (2011) Potential therapeutic uses of BDNF in neurological and psychiatric disorders. Nat. Rev. Drug. Discov. 10, 209-19.

Nagatsu T., and Sawada M. (2007) Biochemistry of postmortem brains in Parkinson's disease: historical overview and future prospects. J. Neural. Transm. Suppl. 113-20.

Nakazawa T., Komai S., Tezuka T., Hisatsune C., Umemori H., Semba K., Mishina M., Manabe T., and Yamamoto T. (2001). Characterization of Fyn-mediated tyrosine phosphorylation sites on GluR _ 2(NR2B) subunit of the N-methyl-D-aspartate receptor. J. Biol. Chem. 276, 693–699.

Nakazawa T., Komai S., Watabe A.M., Kiyama Y., Fukaya M., Arima-Yoshida F., Horai R., Sudo K., Ebine K., Delawary M., Goto J., Umemori H., Tezuka T., Iwakura Y., Watanabe M., Yamamoto T., and Manabe T. (2006) NR2B tyrosine phosphorylation modulates fear learning as well as amygdaloid synaptic plasticity. EMBO. J. 25, 2867-77.

Palko M.E., Coppola V., and Tessarollo L. (1999) Evidence for a role of truncated trkC receptor isoforms in mouse development. J. Neurosci. 19, 775-82.

Pamonsinlapatham P., Hadj-Slimane R., Lepelletier Y., Allain B., Toccafondi M., Garbay C., and Raynaud F. (2009) P120-Ras GTPase activating protein (RasGAP): a multi-interacting protein in downstream signaling. Biochimie. 91, 320-8.

Paoletti P., and Neyton J. (2007) NMDA receptor subunits: function and pharmacology. Curr. Opin. Pharmacol. 7, 39-47.

Patapoutian A., and Reichardt L.F. (2001) Trk receptor: Mediators of neurotrophin action. Curr. Opin.Neurobiol. 11, 272-80.

Patterson S.L., Abel T., Deuel T.A., Martin K.C., Rose J.C., and Kandel E.R. (1996) Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. Neuron. 16, 1137-45.

Patterson S.L., Grover L.M., Schwartzkroin P.A., and Bothwell M. (1992) Neurotrophin expression in rat hippocampal slices: a stimulus paradigm inducing LTP in CA1 evokes increases in BDNF and NT-3 mRNAs. Neuron. 9, 1081–1088.

Pedraza C.E., Podlesniy P., Vidal N., Arévalo J.C., Lee R., Hempstead B., Ferrer I., Iglesias M., and Espinet C. (2005) Pro-NGF isolated from the human brain affected by Alzheimer's disease induces neuronal apoptosis mediated by p75NTR. Am. J. Pathol. 166, 533-43.

Plass C., Shibata H., Kalcheva I., Mullins L., Kotelevtseva N., Mullins J., Kato R., Sasaki H., Hirotsune S., Okazaki Y., Held W.A., Hayashizaki Y., and Chapman V.M. (1996) Identification of Grf1 on mouse chromosome 9 as an imprinted gene by RLGS-M. Nat. Genet. 14, 106–109.

Prybylowski K., Chang K., Sans N., Kan L., Vicini S., and Wenthold R.J. (2005) The synaptic localization of NR2B-containing NMDA receptors is controlled by interactions with PDZ proteins and AP-2. Neuron. 47, 845– 857.

Rajagopal R., Chen Z.Y., Lee F.S., and Chao M.V. (2004) Transactivation of Trk neurotrophin receptors by G-protein-coupled receptor ligands occurs on intracellular membranes. J. Neurosci. 24, 6650-8.

Reichardt L. F. (2006) Neurotrophin-regulated signaling pathways. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 361, 1545-1564.

Ren K., and Dubner R.. (2007) Pain facilitation and activity-dependent plasticity in pain modulatory circuitry: role of BDNF-TrkB signaling and NMDA receptors. Mol. Neurobiol. 35, 224-35.

Roback J.D., Marsh H.N., Downen M., Palfrey H.C. and Wainer B.H. (1995) BDNF-activated signal transduction in rat cortical glial cells. Eur. J. Neurosci. 7, 849–862.

Robinson K. N., Manto K., Buchsbaum R. J., MacDonald J. I. S., and Meakin S. O. (2005) Neurotrophin-dependent Tyrosine Phosphorylation of Ras Guanine-Releasing Factor 1 and Associated Neurite Outgrowth is Dependent on the HIKE Domain of TrkA. J. Biol. Chem. 280, 225-235.

Robinson R.C., Radziejewski C., Stuart D.I., and Jones E.Y. (1995) Structure of the brain-derived neurotrophic factor/neurotrophin 3 heterodimer. Biochemistry 34, 4139–4146.

Rogers S., Wells R., and Rechsteiner M. (1986) Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis, Science. 234, 364–368.

Rose C.R., Blum R., Pichler B., Lepier A., Kafitz K.W., and Konnerth A. (2003) Truncated TrkB-T1 mediates neurotrophin-evoked calcium signalling in glia cells. Nature. 426, 74-8.

Schindowski K., Belarbi K., and Buée L. (2008) Neurotrophic factors in Alzheimer's disease: role of axonal transport. Genes. Brain. Behav. 7, 43-56.

Schmitt J.M., Guire E.S., Saneyoshi T., and Soderling T.R. (2005) Calmodulin-Dependent Kinase Kinase/Calmodulin Kinase I Activity Gates Extracellular-Regulated Kinase-Dependent Long-Term Potentiation The Journal of Neuroscience. 25, 1281–1290.

Shooter E.M. (2001) Early days of the nerve growth factor proteins. Annu. Rev. Neurosci. 24, 601-29.

Shou C., Farnsworth C.L., Neel B.G., and Feig L.A. (1992) Molecular cloning of cDNAs encoding a guanine-nucleotide-releasing factor for Ras p21. Nature 358, 351–354.

Shou C., Wurmser A., Suen K.L., Barbacid M., Feig L.A., and Ling K. (1995) Differential response of the Ras exchange factor, Ras-GRF to tyrosine kinase and G protein mediated signals. Oncogene 10, 1887–1893.

Simon M.A., Bowtell D.D.L., Dodson G.S., Laverty T.R., and Rubin G.M. (1991) Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. Cell. 67, 701–716.

Sini P., Cannas A., Koleske A.J., Di Fiore P.P., and Scita G. (2004) Abl-dependent tyrosine phosphorylation of Sos-1 mediates growth-factor-induced Rac activation. Nat. Cell. Biol. 6, 268-74.

Soderling T.R., and Derkach V.A. (2000) Postsynaptic protein phosphorylation and LTP. Trends. Neurosci. 23, 75– 80.

Song I., and Huganir R.L. (2002) Regulation of AMPA receptors during synaptic plasticity. Trends. Neurosci. 25, 578 –588.

Stephens R.M., Loeb D.M., Copeland T.D., Pawson T., Greene L.A., and Kaplan D.R. (1994) Trk receptors use redundant signal transduction pathways involving SHC and PLC-gamma 1 to mediate NGF responses. Neuron. 12, 691-705.

Sturani E., Abbondio A., Branduardi P., Ferrari C., Zippel R., Martegani E., Vanoni M., and Denis-Donini S. (1997) The Ras Guanine nucleotide Exchange Factor CDC25Mm is present at the synaptic junction. Exp. Cell. Res. 235, 117-23.

Tanaka S., Morishita T., Hashimoto Y., Hattori S., Nakamura S., Shibuya M., Matuoka K., Takenawa T., Kurata T., Nagashima K., et al. (1994) C3G, a guanine nucleotide-releasing protein expressed ubiquitously, binds to the Src homology 3 domains of CRK and GRB2/ASH proteins. Proc. Natl. Acad. Sci. U S A. 91, 3443–3447.

Tezuka T., Umemori H., Akiyama T., Nakanishi S., and Yamamoto T. (1999) PSD-95 promotes Fyn-mediated tyrosine phosphorylation of the N-methyl-D-aspartate receptor subunit NR2A. Proc. Natl. Acad. Sci. USA. 96, 435– 440.

Thoenen H., and Barde Y.A. (1980) Physiology of nerve growth factor. Physiol. Rev. 60, 1284-335.

Tian X., and Feig L.A. (2001) Basis for signaling specificity difference between Sos and Ras-GRF guanine nucleotide exchange factors. J. Biol. Chem. 276, 47248-56.

Tian X., Gotoh T., Tsuji K., Lo EH., Huang S., and Feig L.A. (2004) Developmentally regulated role for Ras-GRFs in coupling NMDA glutamate receptors to Ras, Erk, and CREB. EMBO. J. 23, 1567–1575.

Touhara K., Inglese J., Pitcher J., Shaw G., and Lefkowitz R.J. (1994) Binding of G protein beta gamma-subunits to pleckstrin homology domains. J. Biol. Chem. 264, 10217–10220.

Tsoulfas P., Soppet D., Escandon E., Tessarollo L., Mendoza-Ramirez J.L., Rosenthal A., Nikolics K., and Parada L.F. (1993) The rat trkC locus encodes multiple neurogenic receptors that exhibit differential response to neurotrophin-3 in PC12 cells. Neuron. 10, 975-90.

Tyler W.J, Alonso M., Bramham C.R., and Pozzo-Miller L.D. (2002) From acquisition to consolidation: on the role of brain-derived neurotrophic factor signaling in hippocampal-dependent learning. Learn. Mem. 9, 224-37.

Valdez G., Akmentin W., Philippidou P., Kuruvilla R., Ginty D.D., and Halegoua S. (2005) Pincher-mediated macroendocytosis underlies retrograde signaling by neurotrophin receptors. J Neurosci. 25, 5236-47.

Varnai P., Lin X., Lee S.B., Tuymetova G., Bondeva T., Spät A., Rhee S.G., Hajnóczky G., and Balla T. (2002) Inositol lipid binding and membrane localization of isolated pleckstrin homology (PH) domains. Studies on the PH domains of phospholipase C delta 1 and p130. J. Biol. Chem. 277, 27412-22.

Vepsalainen S., Castren E., Helisalmi S., Iivonen S., Mannermaa A., Lehtovirta M., Hänninen T., Soininen H., and Hiltunen M. (2005) Genetic analysis of BDNF and TrkB gene polymorphisms in Alzheimer's disease. J. Neurol. 252, 423-8.

Wei W., Das B., Park W., and Broek D. (1994) Cloning and analysis of human cDNAs encoding a 140-kDa brain guanine nucleotide-exchange factor, Cdc25GEF, which regulates the function of Ras. Gene 151, 279–284.

Wei W., Schreiber S.S., Baudry M., Tocco G., and Broek D. (1993) Localization of the cellular expression pattern of cdc25NEF and Ras in the juvenile rat brain. Brain Res. Mol. Brain Res. 19, 339–344.

Wei W., Mosteller R.D., Sanyal P., Gonzales E., McKinney D., Dasgupta C., Li P., Liu B.X., and Broek D. (1992) Identification of a mammalian gene structurally and functionally related to the CDC25 gene of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA. 89, 7100–7104.

West A.E., Chen W.G., Dalva M.B., Dolmetsch R.E., Kornhauser J.M., Shaywitz A.J., Takasu M.A., Tao X., and Greenberg M.E. (2001) Calcium regulation of neuronal gene expression. Proc. Natl. Acad. Sci. USA 98, 11024-31.

Wiese S., Jablonka S., Holtmann B., Orel N., Rajagopal R., Chao M.V., and Sendtner M. (2007) Adenosine receptor A2A-R contributes to motoneuron survival by transactivating the tyrosine kinase receptor TrkB. Proc. Natl. Acad. Sci. U S A. 104, 17210-5.

Wu C., Lai C.F., and Mobley W.C. (2001) Nerve growth factor activates persistent Rap1 signaling in endosomes. J. Neurosci. 21, 5406-16.

Wu K., Len G.W., McAuliffe G., Ma C., Tai J.P., Xu F., and Black I.B. (2004) Brain-derived neurotrophic factor acutely enhances tyrosine phosphorylation of the AMPA receptor subunit GluR1 via NMDA receptor-dependent mechanisms. Brain Res. Mol. Brain. Res. 130, 178-86.

Xu F., Plummer M.R., Len G.W., Nakazawa T., Yamamoto T., Black I.B., and Wu K. (2006) Brain-derived neurotrophic factor rapidly increases NMDA receptor channel activity through Fyn-mediated phosphorylation. Brain. Res. 1121, 22-34.

Yang H., and Mattingly R. R. (2006) The Ras-GRF1 Exchange Factor Coordinates Activation of H-Ras and Rac1 to Control Neuronal Morphology. Mol. Biol. Cell. 17, 2177-2189.

Yeo G.S., Connie Hung C.C., Rochford J., Keogh J., Gray J., Sivaramakrishnan S., O'Rahilly S., and Farooqi I.S. (2004) A de novo mutation affecting human TrkB associated with severe obesity and developmental delay. Nat. Neurosci. 7, 1187–1189.

York R.D., Molliver D.C., Grewal S.S., Stenberg P.E., McCleskey E.W., and Stork P.J. (2000) Role of phosphoinositide 3-kinase and endocytosis in nerve growth factor-induced extracellular signal-regulated kinase activation via Ras and Rap1. Mol. Cell. Biol. 20, 8069-83.

Yoshii A., and Constantine-Paton M. (2007) BDNF induces transport of PSD-95 to dendrites through PI3K-AKT signaling after NMDA receptor activation. Nat. Neurosci. 10, 702-11.

Yuan J., and Yankner B.A. (2000) Apoptosis in the nervous system. Nature. 407, 802-9.

Yuan J., Lipinski M., and Degterev A. (2003) Diversity in the mechanisms of neuronal cell death. Neuron. 40, 401-13.

Zhang S., Edelmann L., Liu J., Crandall J.E., and Morabito M.A. (2008) Cdk5 Regulates the Phosphorylation of Tyrosine 1472 NR2B and the Surface Expression of NMDA Receptors. The Journal of Neuroscience. 28, 415– 424.

Zippel R., Orecchia S., Sturani E., and Martegani E. (1996) The brain specific Ras exchange factor CDC25 Mm: modulation of its activity through Gi-protein-mediated signals. Oncogene. 12, 2697–2703.

Zippel R., Gnesutta N., Matus-Leibovitch N., Mancinelli E., Saya D., Vogel Z., Sturani E., Renata Z., Nerina G., Noa M. L., Enzo M., Daniella S., Zvi V., and Emmapaola S. (1997) Ras-Grf, the activator of Ras, is expressed preferentially in mature neurons of the central nervous system. Brain Res. Mol. Brain Res. 48, 140-144.

Zirrgiebel U., Ohga Y., Carter B., Berninger B., Inagaki N., Thoenen H., and Lindholm D. (1995) Characterization of TrkB receptor-mediated signaling pathways in rat cerebellar granule neurons: involvement of protein kinase C in neuronal survival. J. Neurochem. 65, 2241–2250.

Chapter 2

Ras Guanine nucleotide Releasing Factor 1 (RasGrf1) enhancement of Trk Receptor-mediated Neurite Outgrowth requires Activation of both H-Ras and Rac

2.1 Introduction

The neurotrophin receptor tyrosine kinases, TrkA, B and C, activate diverse signaling pathways stimulating distinct cellular responses depending on the cellular context. The ligands for the Trk receptors are a family of related proteins termed neurotrophins and these include nerve growth factor (NGF) which binds TrkA, brain-derived neurotrophic factor (BDNF) which binds TrkB and neurotrophin-3 (NT-3) which binds primarily to TrkC (Meakin, 2000; Reichardt, 2006). Upon neurotrophin binding, Trk receptors dimerize and undergo a conformational change leading to their activation and subsequent trans-phosphorylation on five intracellular tyrosine residues. These sites then recruit intracellular signaling molecules essential to signal propagation. Specifically, the signaling adaptors Shc and FRS2 competitively bind to Y^{499} while PLCγ1 binds to Y^{794} (Meakin, 2000; Reichardt, 2006; Meakin et al., 1999). In all cases, receptor bound molecules become tyrosine phosphorylated which leads to either changes in intrinsic enzymatic activity and/or alters the recruitment/activation of subsequent molecules (Meakin et al., 1999).

Recently, we reported the recruitment of the brain-specific Ras-guanine nucleotide releasing factor, RasGrf1, to activated TrkA and demonstrated that this process facilitates neuronal process formation in PC12 cells (Robinson et al., 2005), a noradrenergic cell line derived from a rat pheochromocytoma used extensively to investigate mechanisms of

cell survival and differentiation downstream of growth factors and their receptors (Greene and Tischler, 1976; Greene, 1978; Vaudry et al., 2002). Herein, we have addressed the mechanism regulating the enhanced neurite outgrowth *via* small GTPases of the Ras and Rho-family (Cdc42, Rac and RhoA) (Ridley et al., 1992; Minden et al., 1995) which are known to serve roles in neuronal growth and differentiation (Nobes and Tolkovsky, 1995). TrkA activation of Rac can be stimulated by one of several mechanisms including PI3K (Soltoff et al., 1992; Wennstrom et al., 1994; Rodriguez-Viciana et al., 1997), Ras and/or guanine nucleotide exchange factors. This leads to inactivation of RhoA, and the release of stress fiber formation, thereby permitting the re-organization of actin that is essential to neurite outgrowth (Huang and Reichardt, 2003). The specific guanine exchange factors that link NGF stimulated TrkA to Rac activation include Kalirin (Chakrabarti et al., 2005) as well as RasGrf1 (Robinson et al., 2005), a neuronal protein that is predominantly expressed in the mature brain and is essential to learning and memory (Zippel et al., 1997; Giese et al., 2001). RasGrf1 is activated in response to both tyrosine phosphorylation from non-receptor tyrosine kinases (Src, Ack1), as well as serine/threonine phosphorylation downstream of protein kinase A (Farnsworth et al., 1995; Mattingly and Macara, 1996; Kiyono et al., 2000a; Kiyono et al., 2000b). Upon activation, RasGrf1 facilitates the exchange of GDP/GTP on Rac through its Dbl homology (DH) domain and/or Ras through its C-terminal Cdc25 domain. Although the sites of Src-dependent tyrosine phosphorylation on RasGrf1 are not known, this results in the activation of Rac (Kiyono et al., 2000a). In contrast, Ack-dependent tyrosine phosphorylation of RasGrf1 stimulates the activation of Ras (Kiyono et al., 2000b).

In terms of investigating the signaling properties of RasGrf1, previous research has shown that its co-expression with H-Ras in PC12 cells promotes constitutive neurite outgrowth and cell soma expansion through the activation of both Ras and Rac (Yang and Mattingly, 2006). Similarly Baldassa et al., (2007) reported that over-expression of RasGrf1 in PC12 cells also stimulated constitutive neurite outgrowth and they suggested that this was due to activation of Rac, not Ras, based on transfection studies in HEK cells (Baldassa et al., 2007). In both cases, these studies addressed RasGrf1's effect on constitutive neurite outgrowth. In contrast, we have examined mechanisms of RasGrf1-induced neurite outgrowth in response to both NGF and BDNF. Herein, we demonstrate that neurotrophin-dependent neurite outgrowth in PC12 and TrkB-expressing nnr5 cells (TrkB-B5) requires activation of both Ras and Rac. Moreover, we demonstrate that co-expression of RasGrf1 with H-Ras in both PC12 and TrkB-B5 cells, compensate and mask the phenotype of function blocking RasGrf1 mutants. Lastly, we demonstrate that the co-expression of Trk and RasGrf1 stimulates Rac activation in HEK cells consistent with the data of Baldassa et al., (2007). However, comparable studies performed in PC12 cells reveals NGF-dependent activation of Ras, not Rac. These studies clarify the mechanism by which RasGrf1 facilitates neurite outgrowth in response to neurotrophin stimulation and demonstrate that RasGrf1 can stimulate different GTPases depending on cell type.

2.2 Materials and Methods

2.2.1 Reagents. Antibodies to RasGrf1, Cdc42, GST-HRP, Trk (C-14), H-Ras, K-Ras, and N-Ras, were from Santa Cruz. Anti-HA was from Roche. H-89 dihydrochloride (B1427), and Mouse monoclonal β-actin antibody was from Sigma. Anti-

phosphotyrosine (p-tyr-100) and anti-Akt (#9272) were from Cell Signaling. Anti-pan Ras and anti-Rac1 antibodies were from Transduction Labs. Rabbit antibodies to the carboxyl-terminal 14 residues of TrkA (1478) were prepared and affinity purified using standard techniques (Robinson, 2008). HRP-coupled goat anti-mouse and goat anti-rabbit secondary antibodies were from The Jackson Laboratories. NGF was from Harlan Products for Bioscience and human recombinant BDNF was from R&D Systems. The DC Protein Assay Kit was from Bio-Rad.

2.2.2 Plasmids. The following plasmids have been previously described: full-length mouse RasGrf1 under control of the human elongation factor 1a promoter (pEFP-RasGrf1) (Anborgh et al., 1999); pCMX-rat TrkA (wild-type and kinase-dead (KD) [$K^{547}A$]) and rat TrkB (Meakin et al., 1999), TrkA-S10 HIKE domain mutant (Robinson et al., 2005). pEGFP was from Clontech. Dominant negative (DN) pcDNA-HA-Rac-N17, constitutively active (CA) pcDNA-HA-Rac-$G^{12}V$, pcDNA-HA-Rac, pEYFP-DN-Ras, pEYFP-CA-Ras ($G^{12}V$) were gifts of S.G. Ferguson (Robarts Research Institute). Dominant-negative pcDNA-Cdc42-$T^{17}N$, CA-Cdc42-$Q^{61}L$ and wild type Cdc42 (in pcDNA) were gifts from R. Cerione (Cornell University, Ithaca, NewYork). pcDNA-HA-H-Ras was the gift from J. Keller (Vanderbilt University Medical Center, Nashville, TN). pEFP-RasGrf1-$W^{1056}E$ was from L. Alberghini (University of Milano, Milan, Italy) and the RasGrf1 DH minus mutant (pMT3-PH1-IQ-Cat) was from L. Feig (Tufts University School of Medicine, Boston, MA). The RasGrf1 $S^{916}A$ mutant was prepared using site-directed mutagenesis and the mutation verified by DNA sequencing. pGex2T-cRaf-RBD was from C. Hermann (Max Planck Institute for Molecular Physiology, Dortmund, Germany) and pGex-PAK-CRIB was the gift of G. Pickering (Robarts Research

Institute). The si-RNAs against Ras (sense: CCACUAUAGAGGAUUCCUACCGGAA , anti-sense: UUCCGGUAGGAAUCCUCUAUAGUGG) and Rac (sense: UUUGACAG CACCGAUCUCUUUCGCC , anti-sense: GGCGAAAGAGAUCGGUGCUGUCAAA) were from Invitrogen.

2.2.3 Cell Lines. Human embryonic kidney 293T (HEK 293T) cells were cultured under standard conditions in DMEM with 5% supplemented calf serum (SCS) and 5% FBS (Hyclone) with 50 μg/ml gentamycin sulfate (Sigma). PC12 rat adrenal pheochromocytoma cells were maintained in DMEM with 5% SCS and 5% horse serum (Hyclone), while TrkB-B5 (nnR5 cells stably over-expressing HA-tagged TrkB receptors) have been described previously (Meakin, 2000; Meakin and MacDonald, 1998). These were cultured in 5% SCS and 5% horse serum in the presence of 100 μg/ml G418 and 50 μg/ml gentamycin sulfate.

2.2.4 Immunoprecipitations and Western Blots. Transfections were performed following standard calcium phosphate (HEK) or Lipofectamine 2000 (PC12 or TrkB-B5 cells; Invitrogen) techniques using a DNA to Lipofectamine ratio of 1:2. Basically, 1.5 x 10^6 cells (100 mm dish) were co-transfected with 0.5-5 μg of each indicated plasmid. Lysates were prepared in NP40 lysis buffer (1% Nonidet P-40, 137 mM NaCl, 20 mM Tris, 0.5 mM EDTA, pH 8.0) containing 1 mM PMSF, 1 mM sodium orthovanadate (NaVO$_4$), 10 μg/ml aprotinin and 10 μg/ml leupeptin and assayed by immunoprecipitation. Lysates containing 0.5 to 3.5 mg of protein were immunoprecipitated with antibodies (0.5 μg anti-Trk1478, or 1 μg anti-RasGrf1), in addition to 5 μl of washed Pansorbin (Calbiochem), at 4 ^{o}C overnight. After washing, bound proteins were resolved on 6 or 12% SDS-PAGE and transferred to polyvinylidene

fluoride (PVDF) membranes. Blocking prior to primary antibody incubation was performed for 1 h at room temperature in 10 ml of 10% milk powder and Tris buffered saline with tween-20 (TBS-T). Westerns were blotted in primary antibody at 4 $^\circ$C for 16 h and then washed for 1 h in TBS-T. Westerns were then blotted in 10 ml 10% milk powder and secondary antibody for 1 h at room temperature. Following washes in TBS-T for 1 h, westerns were exposed to enhanced chemiluminescence (ECL) reagents and developed.

Primary antibody dilutions are as follows: anti-RasGrf1 1:10,000; anti-Cdc42 1:2000; anti-GST-HRP 1:2000; anti-Trk (1478) (1:20,000), anti-pTyr100 (1:10,000); anti-pan Ras (1:5000); H-Ras (1:5000); K-Ras (1:5000); N-Ras (1:5000) and anti-Rac1 (1:5000), anti-Akt (1:5000), anti-actin (1:20000), and anti-HA (1:5000). HRP-coupled secondary antibodies were used at 1:10,000.

2.2.5 Neurite Response Assay and Analysis of Cell Morphology. Cells were plated onto 50 μg/ml poly-D-lysine (Sigma) coated 30 or 100 mm dishes and transfected with pEGFP (0.5 μg) plus Empty plasmid (1 μg), pcDNA-HA-Ras (0.5 μg), pEFP-RasGrf1 (1 μg), pEFP-RasGrf1-S^{916}A (1 μg), pcDNA-RasGrf1-W^{1056}E and/or pMT3-PH1-IQ-Cat (1 μg) with transfection efficiencies of 70 – 90% (Lipofectamine 2000). Fresh media and non-saturating levels of NGF or BDNF (1 ng/ml) were added at 24 h intervals on each of 4 successive days. Inhibitor H89 (50 μM) was added before stimulation when indicated. On each day (from day one at 24 h after transfection to day four), the percentage of EGFP transfected cells (> 10 randomly selected fields and at least 200 cells) with neurite length greater than 2 cell bodies diameter as well as cells that had a flattened, expanded morphology with a width greater than 5 cell body lengths were counted using an inverted

fluorescent microscope. Three independent neurite outgrowth experiments (n=3) were performed and the percentage of EGFP-transfected cells extending neurites, between samples, was statistically analyzed by One way-ANOVA with Post-Tukey test and paired student t-test. TrkA and RasGrf1 expression and tyrosine phosphorylation was confirmed by immunoprecipitation of 500 μg to 3.5 mg of protein, SDS-PAGE and western blotting following the counts performed on day four. The relative densities were then statistically analysed by paired student t-test for significant differences between samples (n=3). For epifluorescence microscopy, cells were visualized for EGFP on inverted microscopes (1 x 70 [Olympus]; Diaphot 300 [Nikon]) at a magnification of 200X.

2.2.6 GTPase binding Assays. pGex2T-PAK-CRIB, pGex2T and GST-Raf1-RBD were grown in 50 ml Luria broth (LB) with 50 μg/ml ampicillin for 16 h at 37 °C, added to 500 ml of LB with 50 μg/ml ampicillin and grown to an OD_{600} of 0.8 to 1.0. Cultures were induced with IPTG (0.2 mg/ml) for 2 h at 37 °C, centrifuged at 5000 rpm for 10 min (4 °C), re-suspended in 10 ml 1X PBS and frozen at -80 °C. Pellets were re-suspended in 20 ml of re-suspension buffer (25 mM Tris-Cl pH 7.5, 5 mM EDTA pH 8.0, 150 mM NaCl, 1 mM PMSF, 1 μg/ml leupeptin) and the bacteria lysed by two passages through a pre-chilled French press at 20,000 psi. Triton X-100 was added to a final concentration of 1% and the sample rotated for 30 min at 4 °C. The sample was then centrifuged at 14,000 rpm for 10 min at 4 °C. Washed glutathione agarose (500 μl) (Sigma) was added to the supernatant and the mixtures incubated for 16 h at 4 °C followed by three washes with 10 ml 1X PBS and re-suspended in 250 μl interaction buffer (20 mM Hepes, 150 mM NaCl, 0.05% NP40, 10% glycerol, 1 mM PMSF, 1 μg/ml leupeptin). Laemmli sample buffer containing 100 mM DTT was added, samples heated at 65 °C for 10 min and then

separated by 12% SDS-PAGE along with known quantities of BSA to estimate the amount of GST proteins isolated. HEK cells were transiently transfected with 0.5 - 5 μg DNA using the calcium phosphate transfection method and PC12 cells were transfected with Lipofectamine 2000. Cells were allowed to express protein for 48 h before stimulation with NGF or BDNF (100 ng/ml) for 5 min. A control plate that was serum-starved was washed 3X with 1X PBS 24 hours before stimulation and serum-free media added. Before stimulation, all plates were washed 3X with PBS at 1 h time points before stimulation and incubated in serum-free media. Following stimulation, cells were incubated for 5 min at 37 °C, followed by a wash with 10 ml PBS. Cells were lysed in 500 μl interaction buffer for 2 min and lysates centrifuged at 10,000 rpm for 10 min at 4 °C. Protein concentrations were evaluated with the DC Protein Assay Kit (BioRad). GST, GST-PAK-CRIB or GSTRaf1- RBD (approximately 30 μg) was added to 500 μg of each lysate and incubated at 4 °C for 16 h. Samples were pelleted at 14,000 rpm and washed twice with NP-40 buffer. Laemmli sample buffer with100 mM DTT was added and samples heated at 70 °C for 10 min. Proteins were analyzed on 12% SDS-PAGE and blotted with anti-GST-HRP (1:5000), anti-Ras (1:2000) or anti-Rac (1:2000).

2.3 Results

2.3.1 Trk-dependent RasGrf1 activation facilitates GTP exchange of Rac in HEK cells.

As RasGrf1 is a dual specificity guanine exchange factor for both Ras and Rac, we examined which GTPase is preferentially activated downstream of Trk and RasGrf1. In particular, RasGrf1 has been shown to activate H-Ras through its Cdc25 domain and Rac1 through its DH domain. We initially used HEK 293T cells, co-transfected with

TrkA and RasGrf1 and stimulated with NGF, due to their ease of high efficiency transfection. GST fused to the CRIB domain of p21-activated kinase (PAK) was used to pull activated GTP bound Rac1 from transfected lysates (Baldassa et al., 2007; Small et al., 2006) while GST fused to the Ras binding domain (RBD) of Raf1 was used to pull activated Ras (Arozarena et al., 2000). As shown in Figure 2.1 (A), CA-Rac1 (lane 3) was pulled out by GST PAKCRIB, while a DN-Rac1 (lane 2) showed no binding to GST-PAK-CRIB. GST alone was used as a control and failed to interact with Rac1 (Figure 2.1A). An additional control of cells grown under serum-free conditions 24 h before stimulation with NGF, showed no basal level of Rac activation (lane 1). While RasGrf1, with or without the kinase-dead TrkA, stimulates a basal level of Rac1 activation (lanes 6 and 7), there is an increase in the activation of Rac1 in the presence of wild type, NGF-stimulated TrkA (lane 8). The TrkA-HIKE domain mutant (HK) has previously been shown to not interact with or phosphorylate RasGrf1 (Robinson et al., 2005) and was therefore used to test the specificity of this increase in Rac activation. Accordingly, in the presence of this mutant there was a decrease in RasGrf1 activation of Rac1 compared to cells expressing wild-type TrkA alone (compare lane 9 to lane 6). These data indicate that Rac1 is being activated via TrkA and RasGrf1 in HEK cells. As shown in Figure 2.1B, RasGrf1 and TrkA are expressed consistently and RasGrf1 is tyrosine phosphorylated upon stimulation with NGF by wild-type TrkA but not the HIKE (HK) domain mutant.

As RasGrf1 is also a guanine exchange factor for Ras, HEK cells transfected with TrkA, RasGrf1 and wild-type, CA or DN-H-Ras were also evaluated for activation of Ras through interaction with the Raf1-Ras RBD fused to GST which binds only active, GTP-bound Ras. As shown in Figure 2.1C, CA-Ras fused to yellow fluorescent protein (YFP-

RAS-CA) bound to Raf1-RBD-GST as expected (Figure 2.1C, lane 3), while a dominant-negative construct (YFP-RAS-DN) did not (lane 2). No detectable basal level of Ras activation was observed in cells that were serum-starved for 24 h prior to stimulation (lane 1) or in cells maintained in serum during this period (lane 4). Unexpectedly under these conditions, TrkA alone did not show a detectable level of Ras activation in the presence of NGF (lane 5). RasGrf1 did however show a high basal ability to activate Ras in the absence of activated TrkA (lane 6) and in the presence of kinase-dead TrkA (lane 7). However, cells transfected with WT-TrkA and RasGrf1 showed a decrease in active GTP-bound Ras (lane 8). To determine whether the loss of interaction between TrkA and RasGrf1 would abolish this decrease, cells were transfected with the TrkA-HIKE domain mutant instead of wild-type TrkA. These cells showed activation of Ras comparable to the TrkA-kinase dead and RasGrf1 transfected cells (lane 9). Thus co-transfection of TrkA and RasGrf1 leads to a decrease in Ras activation in HEK cells potentially due to a switch in activity toward another GTPase, such as Rac (Figure 2.1A) or due to a re-localization away from H-Ras upon TrkA binding. Evaluation of RasGrf1 phosphorylation by TrkA is shown in Figure 2.1D, lane 8, and its lack of phosphorylation by kinase-dead TrkA (KD) and the TrkA-HIKE (HK) mutants are shown in lanes 7 and 9, respectively.

While RasGrf1 can be activated downstream of the Rho family GTPase Cdc42, via the tyrosine kinase ACK1 (Kiyono et al., 2000b), Cdc42 can in turn be activated by DH domain containing guanine exchange factors (Baldassa et al., 2007). For these reasons, Trk-activated RasGrf1 was also evaluated for the potential activation of Cdc42. However, as shown in Figure 2.1E, we detected no endogenous activated Cdc42, the only GTP-

bound Cdc42 detected being in cells transfected with CA-Cdc42 (lane 3). There appears to be no activation downstream of TrkA or RasGrf1 singly or together (lanes 5, 8). GST alone did not interact with Cdc42. Expression and phosphorylation of TrkA and RasGrf1 in the presence of NGF are shown in Figure 2.1F.

2.3.2 Analysis of RasGrf1 mutants, and H-Ras, in NGF-induced neurite outgrowth in PC12 cells.

We have previously evaluated the response of PC12 cells to NGF-stimulated TrkA and RasGrf1 and have found that these cells have enhanced neurite outgrowth in the presence of low doses of neurotrophins (Robinson et al., 2005). The enhanced neurite outgrowth is likely mediated through a GTPase such as Ras or Rac1, both of which regulate differentiation in PC12 cells. In addition, RasGrf1 over-expression has also been reported to constitutively stimulate the flattening and enlargement of the cell soma in PC12 cells in the presence of co-transfected H-Ras (Yang and Mattingly, 2006). Yang and Mattingly (2006) reported that the expanded cell body required RasGrf1 activation of Rac1 and that this was dependent on the co-expression and activation of H-Ras. To determine if NGF stimulation potentiated similar changes in the cellular morphology of the cell soma, we co-transfected cells with RasGrf1 and H-Ras and evaluated changes in both neurite outgrowth and soma size in response to NGF. As shown in Figure 2.2A, we similarly observed both phenotypes in cells co-transfected with RasGrf1 and H-Ras. Quantification of these phenotypes revealed that cells co-transfected with H-Ras and RasGrf1 expressed a higher basal neurite outgrowth (21.6%) response that was approximately 3-fold higher than cells expressing H-Ras alone (7%) and that addition of NGF stimulated a minor additional increase (27.3%) in neurite outgrowth (Figure 2.2B).

Figure 2.2C indicates the levels of Trk and RasGrf1 tyrosine phosphorylation and expression. With respect to changes in cell soma size, we observed that co-transfection of RasGrf1 and H-Ras also increased the number of cells with increased basal soma size to nearly 20% of the total population as compared to control cells transfected with GFP alone (4%). However, there was no significant increase in the percentage of cells showing an enhanced soma size upon stimulation by NGF in either controls or cells co-expressing H-Ras and RasGrf1. The levels of Trk and RasGrf1 expression and tyrosine phosphorylation are shown in Figure 2.2E.

To determine whether activation of either Ras, Rac or both facilitate NGF-induced neurite outgrowth, PC12 cells were transfected with H-Ras and either wild-type RasGrf1, a ($W^{1056}E$) RasGrf1 mutant which binds GDP-Ras but does not facilitate exchange to GTP on Ras (Vanoni et al., 1999), as well as a RasGrf1 ($S^{916}A$) mutant, the site of protein kinase A phosphorylation reported to be required for maximal induction of Ras-dependent neurite outgrowth in PC12 cells (Yang et al., 2003; Baouz et al., 2001) (Figure 2.3A). Importantly, the $W^{1056}E$ mutant does not affect the ability of RasGrf1 to activate Rac (Vanoni et al., 1999). Cells were stimulated with low levels of NGF (1 ng/ml) for four days following transfection and then evaluated for neurites longer than two cell bodies in length. As observed in Figure 2.3, RasGrf1 transfected cells showed levels of basal neurite outgrowth in the presence of co-transfected H-Ras (23.27% ± 0.37) which is only somewhat increased in response to NGF (27.1% ± 0.89) (Figure 2.3B). Interestingly, we found that expression of the RasGrf1 ($S^{916}A$) mutant did not affect either basal levels of neurite outgrowth (22.93% ± 2.28) or those stimulated by NGF (25.93% ± 3.08) (Figure 2.3B). Cells transfected with the RasGrf1 ($W^{1056}E$) mutant exhibited a reduction

in neurite outgrowth (17.67% ± 0.65), albeit levels were still higher than cells transfected with controls, and we observed a small insignificant increase (20.73% ± 2.3) in neurite outgrowth in response to NGF. Analysis of phosphorylation and expression levels indicated that wild-type RasGrf1, RasGrf1 ($W^{1056}E$) and the RasGrf1 ($S^{916}A$) mutant were expressed and phosphorylated at similar levels by NGF-activated TrkA (Figure 2.3C).

To further determine whether PKA facilitates RasGrf1-mediated neurite outgrowth in response to NGF, via other phosphorylation sites and/or mechanisms, PC12 cells were co-transfected with RasGrf1 and H-Ras and then stimulated with NGF in the presence or absence of the PKA inhibitor, H89. As shown in Figure 2.3D, we found that application of H89 did not lead to a decrease in NGF/RasGrf1-mediated outgrowth suggesting that PKA is not important for TrkA-dependent activation of RasGrf1 or enhanced neurite outgrowth in PC12 cells under these conditions. Notably, H89 did not affect tyrosine phosphorylation levels of either Trk or RasGrf1 stimulated by NGF (Figure 2.3E).

Since the Ras/Rac activation assays in HEK cells suggested that TrkA activation of RasGrf1 resulted in activation of Rac, we next evaluated the potential that RasGrf1 activation of Rac is involved in mediating NGF-induced neurite outgrowth in PC12 cells. Accordingly, cells were co-transfected with H-Ras and either wild-type RasGrf1 or a mutant RasGrf1 protein that consists of the PH1 domain, coiled-coil domain and the IQ domain fused directly to the Cdc25 domain (PH1-IQ-Cat). This mutant is missing the second PH domain and the Dbl homology domain required for binding and facilitating GTP exchange of Rac (Buchsbaum et al., 1996). Importantly, this mutant does not affect the activation of H-Ras (Yang and Mattingly, 2006). Cells transfected with EGFP showed

very low levels of outgrowth after four days, which was slightly above 5% when stimulated with NGF (Figure 2.3F). Cells transfected with the PH1-IQ-Cat RasGrf1 mutant showed nearly a two-fold increase in the number of cells with neurites in the absence of NGF stimulation. Previous studies using the PH1-IQ-Cat mutant revealed that it has a slight increase in the basal activity toward Ras, although the reasons for this were unclear (Buchsbaum et al., 1996). Interestingly, however, we found that this mutant stimulated neurite outgrowth in the presence of NGF and while the levels were decreased compared to wild-type RasGrf1, the difference was not statistically different (Figure 2.3F). The levels of phosphorylation and expression of TrkA, RasGrf1 and PH1-IQ-Cat are shown in Figure 2.3G. Taken together, these studies suggest that RasGrf1 mediates outgrowth downstream of the Trk receptor through activation of Ras, not Rac1, in transfected PC12 cells.

2.3.3 Trk activation of RasGrf1 facilitates GTP exchange of Ras in PC12 cells.

The reduction in neurite outgrowth of the RasGrf1 mutant ($W^{1056}E$) (dominant-negative for Ras activation) suggested that in PC12 cells, RasGrf1 might be an activator of Ras instead of Rac. However, this was in contrast to our observations in HEK cells in which we observed an increase in Rac activation but a decrease in the activation of Ras downstream of TrkA and RasGrf1 (Figure 2.1). Thus, we performed a GTP binding assay on lysates from PC12 cells transfected with wild-type H-Ras and RasGrf1. In these studies, RasGrf1 transfected cells showed low levels of basal activation of Ras in the absence of stimulation (Figure 2.4A, lane 7). This agrees with our observations of neurite outgrowth following co-transfection with H-Ras and RasGrf1 (Figure 2.3). However, PC12 cells transfected with RasGrf1 and H-Ras, and stimulated with NGF, showed a

considerable enhancement of active GTP-bound Ras (Figure 2.4A, lane 8). This activation was comparable to that seen in the positive control lane which expressed CA-Ras (lane 4). This also agrees with our neurite outgrowth studies in PC12 cells, but contrasts with the decreased Ras activation observed in HEK cells suggesting that there may be different mechanisms of NGF/TrkA and RasGrf1 signaling occur in the two cell types. NGF-dependent Trk and RasGrf1 phosphorylation and expression levels of both proteins are shown in Figure 2.4B.

2.3.4 Trk activation of RasGrf1 does not stimulate increased Rac activation in PC12 cells.

Yang and Mattingly (2006) indicated that Ras activation by RasGrf1 promotes neurite extension in PC12 cells, but also increases RasGrf1 activity toward Rac. Given this data and the Rac activation observed in HEK cells, a GTP binding assay for Rac1 was also performed in PC12 cells. PC12 cells were co-transfected with RasGrf1 plus WT Rac1 as well as CA or DN forms of Rac1 as positive and negative controls. As shown in Figure 2.4C, the negative control (DN-Rac1) did not interact with GST-PAK-CRIB (lane 3) while the CA-Rac1 did (lane 4) relative to GST alone. We observed a small increase in Rac activation in NGF stimulated, un-transfected cells (lane 6) and a larger increase in un-stimulated cells following transfection with RasGrf1 (lane 7) suggesting that RasGrf1 over-expression can intrinsically activate Rac. However, we found no discernable increase in Rac1 activation in RasGrf1 transfected cells following NGF stimulation above the effect of RasGrf1 alone (lane 8). Whole cell lysates were evaluated to verify expression of Rac1 as well as to verify expression of RasGrf1 and TrkA and their tyrosine phosphorylation status upon NGF-stimulation (Figure 2.4D).

2.3.5 PC12 and TrkB-B5 cells express endogenous H-Ras.

Our initial experiments indicated that RasGrf1 stimulates NGF-induced neurite outgrowth in PC12 cells via a Ras, but not Rac-dependent mechanism (Figure 2.3), consistent with our subsequent observation that NGF stimulates an increase in RasGrf1-dependent activation of Ras in PC12 cells (Figure 2.4). However, we were puzzled with the observation that the dominant negative RasGrf1 mutant ($W^{1056}E$) continued to show high basal levels of neurite outgrowth in PC12 cells (Figure 2.3) suggesting that either another NGF-dependent pathway was also stimulating neurite outgrowth and/or that the over-expression of H-Ras was compensating for a loss of function phenotype. The original decision to co-transfect RasGrf1 mutants with H-Ras was based on the observation by Yang and Mattingly that PC12 cells don't express endogenous H-Ras (Yang and Mattingly, 2006). However, when testing our PC12 and TrkB-B5 cells, we found that they do express endogenous H-Ras, but not K-Ras or N-Ras, to levels comparable to those observed in brain (Figure 2.5). Thus, to determine whether H-Ras over-expression was overcoming the effect of one or more of the RasGrf1 mutants, all mutants were re-tested for their ability to support neurotrophin-induced neurite outgrowth in the absence of ectopic H-Ras expression. Since RasGrf1 is primarily expressed in the brain and is co-expressed predominantly with TrkB, these studies were addressed (as described below) in TrkB-B5 cells. We previously generated this cell line from the PC12-derived cell line, nnR5, that do not express TrkA (Greene and Tischler, 1976), that we engineered to stably over-express TrkB (Meakin and MacDonald, 1998).

2.3.6 BDNF activation of TrkB also stimulates RasGrf1-dependent enhancement of neurite outgrowth *via* both Ras and Rac.

Consistent with our observation that RasGrf1 facilitates NGF-dependent neurite outgrowth in PC12 cells, we found that BDNF-dependent TrkB activation of neurite outgrowth is also significantly enhanced. As shown in Figure 2.6, RasGrf1 expression stimulates an enhancement of BDNF-induced neurite outgrowth compared to un-stimulated cells and un-transfected cells stimulated with BDNF. We then assayed the RasGrf1 mutants for changes in BDNF-induced neurite outgrowth, in the absence of H-Ras co-expression, and as shown in Figure 2.6, both the $W^{1056}E$ and PH-IQ-CAT mutants showed a reduction in BDNF-induced neurite outgrowth while the $S^{916}A$ mutant still appeared to support significant neurite outgrowth. These observations were next quantified in triplicate experiments in the absence and presence of exogenous H-Ras expression. As shown in Figure 2.7A, we find that wild type RasGrf1 and the $S^{196}A$ mutant show a basal level of neurite outgrowth (11.13% ± 1.87 and 9.86% ± 0.88 respectively) in the absence of BDNF and a significant increase in the percentage of cells showing neurite outgrowth (20.13% ± 2.48 and 16.67% ± 1.68 respectively) in presence of BDNF. Wild type RasGrf1 and $S^{916}A$ mutant also showed an approximately 2-fold increase in the average neurite length (220 µm and 247 µm) relative to negative control of empty plasmid (110 µm) (Figure 2.7B). These results are consistent with our data in Figure 2.3 demonstrating that phosphorylation of Ser^{916} is not essential to neurotrophin-induced activation of RasGrf1. However, we found that both the $W^{1056}E$ and PH-IQ-CAT RasGrf1 mutants significantly reduced BDNF-induced neurite outgrowth to levels comparable to negative controls (Figure 2.7A). Interestingly, when cells were co-

transfected with H-Ras (Figure 2.7D), we found that only the PH-IQ-CAT RasGrf1 mutant showed a decrease in BDNF-induced neurite outgrowth relative to WT RasGrf1, to the levels were still significantly greater than controls. These observations are consistent with the results shown above indicating that over-expression of H-Ras masks the phenotype of the RasGrf1 mutations. Levels of RasGrf1 and transfected H-Ras expression are shown relative to endogenous Akt or actin as loading controls (Figures 2.7C, E, F). Collectively, these results indicate that RasGrf1 can also act downstream of TrkB receptors to enhance BDNF-induced neurite outgrowth.

2.3.7 Both Ras and Rac si-RNAs block BDNF-induced neurite outgrowth in TrkB cells.

To further determine whether Ras, Rac or both facilitate BDNF-induced neurite outgrowth, TrkB-B5 cells were transfected with wild-type RasGrf1 in presence of si-RNAs against either Ras or Rac1, and the percentage of neurite outgrowth and the expression level of Ras/Rac1 were determined. As shown in Figure 2.8A, while wild type RasGrf1 significantly (26%) increased neurite outgrowth compared to the negative control of empty plasmid (12%), both si-RNAs against Ras and Rac1 decreased neurite outgrowth (3.7% and 4.3% respectively) relative to wild type RasGrf1. RasGrf1 co-transfection with H-Ras also increased neurite outgrowth significantly as expected. However, it was included in this experiment as a positive control for si-Ras plasmid. These results further support the notion that the activation of both Ras and Rac are required for neurotrophin-induced neurite outgrowth. The expression levels of Ras and Rac are shown in Figure 2.8B verifying that the level of Ras and Rac expression are reduced by the si-RNAs against Ras and Rac.
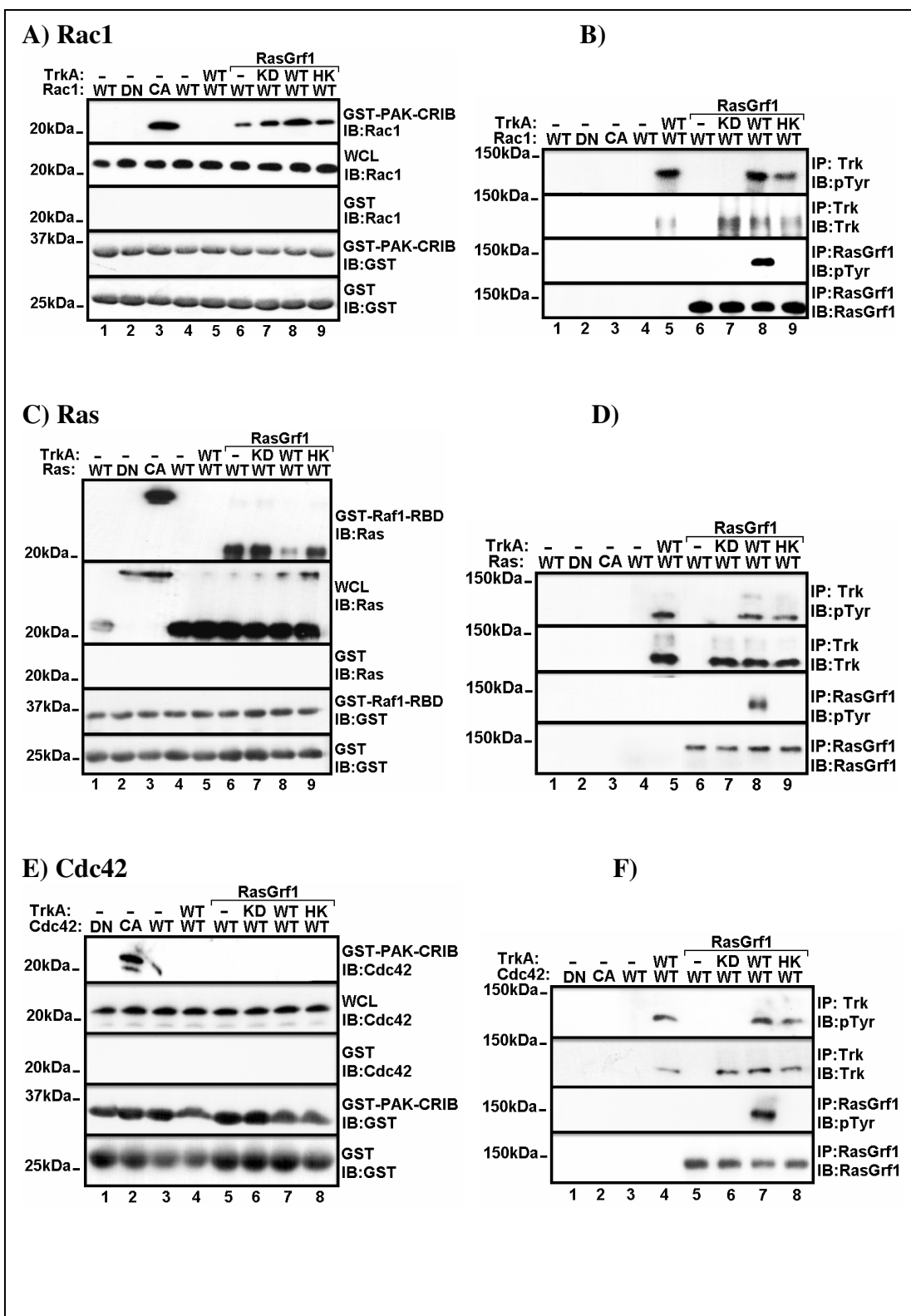
Figure 2.1. Rac/Ras/Cdc42-GTPase pull down assay in transfected HEK cells. Cells were transfected with wild type RasGrf1plus CA, DN or WT GTPases as well as kinase dead (KD), HIKE domain mutated (HK) or WT TrkA. Forty-eight hours post-transfection, cells were serum starved for 1 h, stimulated with 100 ng/ml of NGF for 5 min, lysed and activated GTPases precipitated with indicated GST fusion proteins. Samples were immunoprecipitated (IP) and assayed for phosphorylation assessment by Western blotting (IB) (Robinson, 2008) (n=3). **A)** Rac-GTPase activity. Lane 1 represents a negative control plate in which cells were starved 24 h. **B)** Phosphorylation assessment of RasGrf1 and Trk during Rac-GTPase activity. **C)** Ras-GTPase activity. **D)** Phosphorylation assessment of RasGrf1 and Trk. **E)** Cdc42-GTPase activity. **F)** Phosphorylation assessment of RasGrf1 and Trk.

A)

B) +H.Ras

C)

D) +H.Ras

E)

Figure 2.2. Neurite outgrowth assay of wild type RasGrf1 in PC12 cells. PC12 cells were transfected with EGFP plus Empty-plasmid or WT-RasGrf1, in presence of H-Ras, treated with either NGF (1 ng/ml) for 4 consecutive days or left unstimulated. The percentage of cells with neurite length greater than two cell bodies were counted and statistically analyzed. Samples were immunoprecipitated (IP), separated by SDS-PAGE, and immunoblotted (IB) with indicated antibodies. Three independent neurite outgrowth experiments were performomed (n=3). * indicates statistical significance (P-value<0.05) of WT-RasGrf1 relative to the Empty plasmid. **A)** NGF treated PC12 cells co-transfected with WT-RasGrf1 and H-Ras. Both neurite extension (A1) and soma expansion (A2) are shown. Scale bar is 10µm. **B)** PC12 cells were co-transfected with WT RasGrf1 and H-Ras and neurite extension assessed in -/+ NGF stimulation. **C)** NGF induces tyrosine phosphorylation of both Trk and RasGrf1. RasGrf1 band appeared as expected at ≈140kDa (Robinson, 2008). **D)** Percentage of PC12 cells co-expressing WT RasGrf1 and H-Ras in -/+ NGF stimulation displaying enhanced soma expansion (with expansion > 5-cell body) (Robinson, 2008). **E)** Tyrosine phosphorylation assessment for Trk and RasGrf1during soma expansion. RasGrf1 band appeared as expected at ≈140kDa (Robinson, 2008).

A)
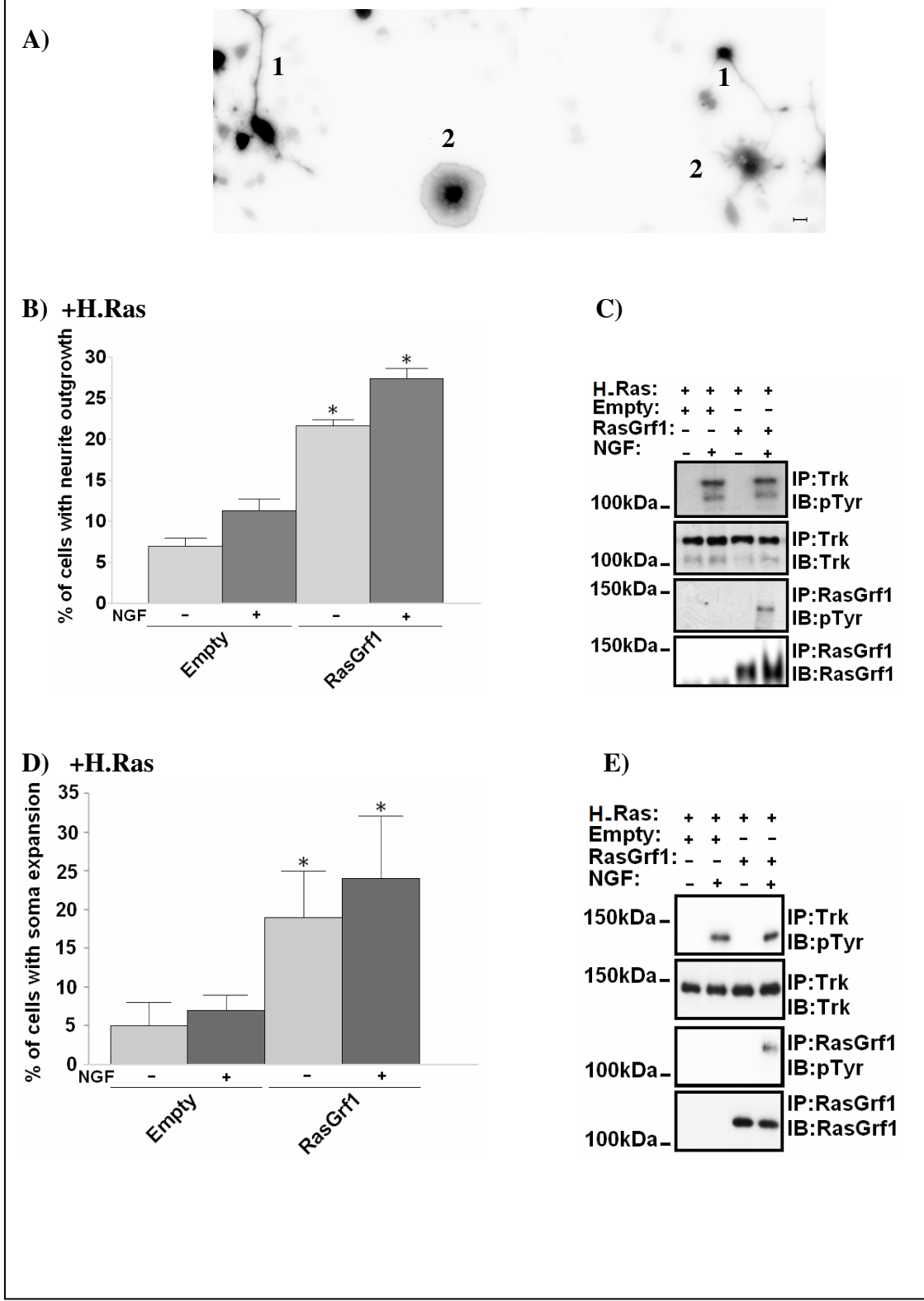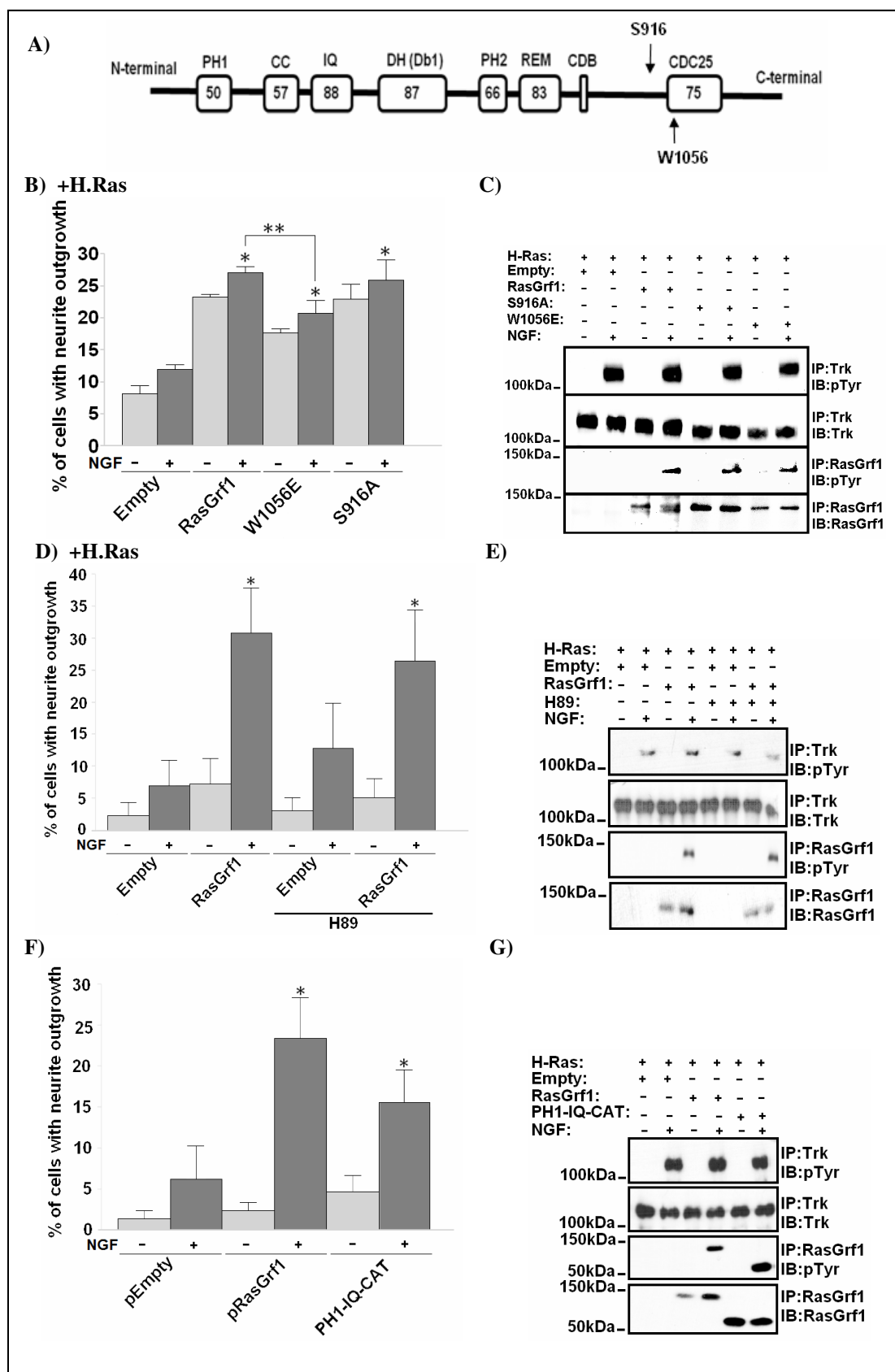
B) +H.Ras

C)

D) +H.Ras

E)

F)

G)

Figure 2.3. Neurite outgrowth assay of RasGrf1-mutants in PC12 cells. PC12 cells were transfected with EGFP plus either empty-plasmid or WT-RasGrf1 and RasGrf1-mutants in the presence of H-Ras. Cells were treated with either NGF (1 ng/ml) for 4 consecutive days or left unstimulated and the percentage of cells with neurite length greater than two cell bodies determined. Samples were immunoprecipitated and analyzed by SDS-PAGE/Western blot with the indicated antibodies (n=3). * indicates significant increase in neurite outgrowth (P-value<0.05) of indicated construct in the presence of NGF relative to Empty plasmid. **A)** Schematic diagram of RasGrf1. RasGrf1 is a multi-domain protein including PH1(Pleckstrin homology, 50 amino acids), coiled-coil motif (CC, 57 amino acids), CaMK binding site (IQ, 88 amino acids), Db1 homology domain (DH, 87 amino acids), pleckstrin homology domain-2 (PH2, 66 amino acids), Ras exchange motif (REM, 83 amino acids, cyclin destruction box (CDB) and catalytic domain (Cdc25, 75 amino acids). In addition, $S^{916}A$, a PKA phosphorylation site on RasGrf1, and the $W^{1056}E$ mutation are indicated. **B)** Percentage of cells with neurites greater than two cell bodies in PC12 cells expressing $W^{1056}E$ and $S^{916}A$ RasGrf1 mutants. ** indicates significant decrease of $W^{1056}E$ relative to WT-RasGrf1. **C)** Phosphorylation assessment for $W^{1056}E$ and $S^{916}A$ mutants. **D)** Percentage cells with neurites greater than two cell bodies following treatment with H89 (Robinson, 2008). **E)** Tyrosine phosphorylation of RasGrf1 in the presence of H89 (Robinson, 2008). **F)** PC12 cells were co-transfected with the PH1-IQ-CAT RasGrf1 mutant and H-Ras -/+ NGF. Data depicts the percentage of cell with neurite length greater than two cell bodies (Robinson, 2008). **G)** Tyrosine phosphorylation of Trk and the PH1-IQ-CAT mutant. The band for this mutant appeared as expected under ≈60kDa (Robinson, 2008).

A) Ras

NGF: – + – – – + RasGrf1 – +
Ras: – – DN CA WT WT WT WT

GST-Raf1-RBD IB:Ras
20kDa

WCL IB:Ras
20kDa

GST IB:Ras
20kDa

GST-Raf1-RBD IB:GST
37kDa

GST IB:GST
25kDa

1 2 3 4 5 6 7 8

B)

NGF: – + – – – + RasGrf1 – +
Ras: – – DN CA WT WT WT WT

IP: Trk IB:pTyr
150kDa

IP:Trk IB:Trk
150kDa

IP:RasGrf1 IB:pTyr
150kDa

IP:RasGrf1 IB:RasGrf1
150kDa

1 2 3 4 5 6 7 8

C) Rac1

NGF: – + – – – + RasGrf1 – +
Rac1: – – DN CA WT WT WT WT

GST-PAK-CRIB IB:Rac1
20kDa

WCL IB:Rac1
20kDa

GST IB:Rac1
20kDa

GST-PAK-CRIB IB:GST
37kDa

GST IB:GST
25kDa

1 2 3 4 5 6 7 8

D)

NGF: – + – – – + RasGrf1 – +
Rac1: – – DN CA WT WT WT WT

IP: Trk IB:pTyr
150kDa

IP:Trk IB:Trk
150kDa

IP:RasGrf1 IB:pTyr
150kDa

IP:RasGrf1 IB:RasGrf1
150kDa

1 2 3 4 5 6 7 8

Figure 2.4. Ras/Rac-GTPase pull down assay in transfected PC12 cells. Cells were transfected with CA, DN, WT Ras or Rac and/or WT-RasGrf1. Forty-eight hours post-transfection, cells were serum starved for 1 h, stimulated with 100 ng/ml of NGF for 5 min, lysed and activated GTPases precipitated with the indicated GST fusion proteins. Samples were also immunoprecipitated (IP) and western blotted (IB) with the indicated antibodies to assess tyrosine phosphorylation (Robinson, 2008) (n=3). **A)** Ras-GTPase activity. The activation of Ras is evident in the presence of RasGrf1 after NGF stimulation (lane 8). **B)** Expression and tyrosine phosphorylation of RasGrf1 and Trk in PC12 cells in -/+ NGF stimulation. **C)** Rac-GTPase activity in transfected PC12 cells. The activation of Rac1 is unchanged in the presence of RasGrf1 after NGF stimulation (lane 8). **D)** Expression and tyrosine phosphorylation of RasGrf1 and Trk in transfected PC12 cells.

WCL

| | TrkB | Brain | PC12 | TrkB | Brain | PC12 | TrkB | Brain | PC12 |

20kDa—

IB: H-Ras IB:K-Ras IB:N-Ras

37kDa—   IB:actin
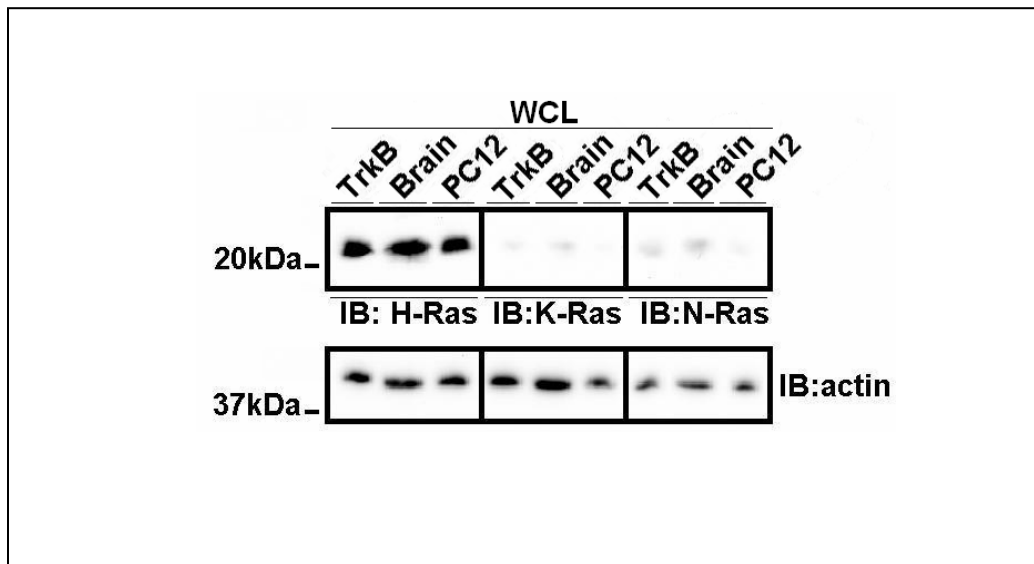
Figure 2.5. Evaluation of endogenous Ras expression in PC12 and TrkB-B5 cells.
Lysates (50 µg) from PC12 and TrkB-B5 cells were immunoblotted (IB) using antibodies against H-Ras, N-Ras and K-Ras. Mouse brain tissue lysate was employed as a positive control and actin (≈43kDa) served as a loading control (n=3).

A) TrkB-B5 cells: WT.RasGrf1 + H.Ras + BDNF
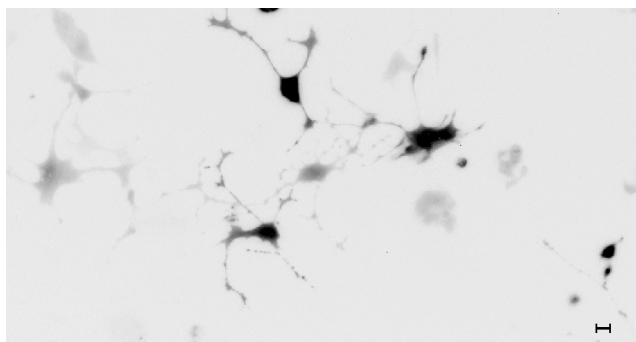
B)

| Empty | Empty + BDNF | WT.RasGrf1 | WT.RasGrf1+ BDNF |

| S916A | S916A + BDNF | W1056E | W1056E +BDNF |

| PH1-IQ-CAT | PH1-IQ-CAT + BDNF |
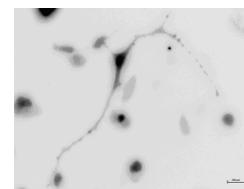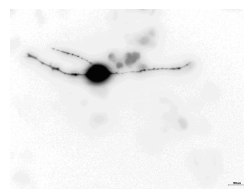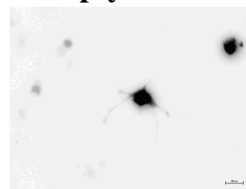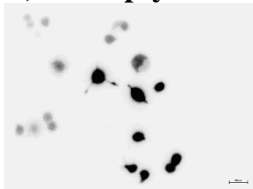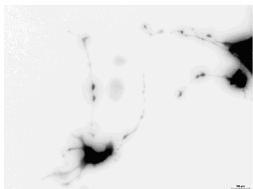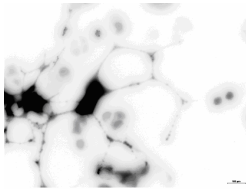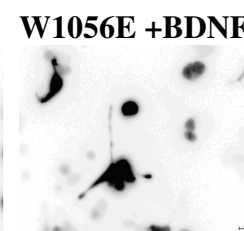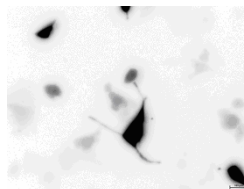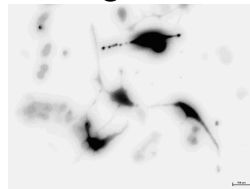
Figure 2.6. The effect of specific functional mutations on RasGrf1 on neurite outgrowth in TrkB expressing PC12-derived cells. TrkB-B5 cells were transfected with EGFP and either empty-plasmid, wild type RasGrf1 or the indicated RasGrf1 mutants, in the absence or presence of co-transfected H-Ras, treated with either BDNF (1 ng/ml) for 4 days or left un-stimulated (n=3). **A)** BDNF stimulated TrkB-B5 cells co-expressing RasGrf1-WT and H-Ras. Scale bar is 10µm. **B)** The effect of various RasGrf1 constructs on neurite outgrowth in TrkB-B5 cells in the presence or absence of BDNF and without co-expression of H-Ras. Scale bar = 10µm.
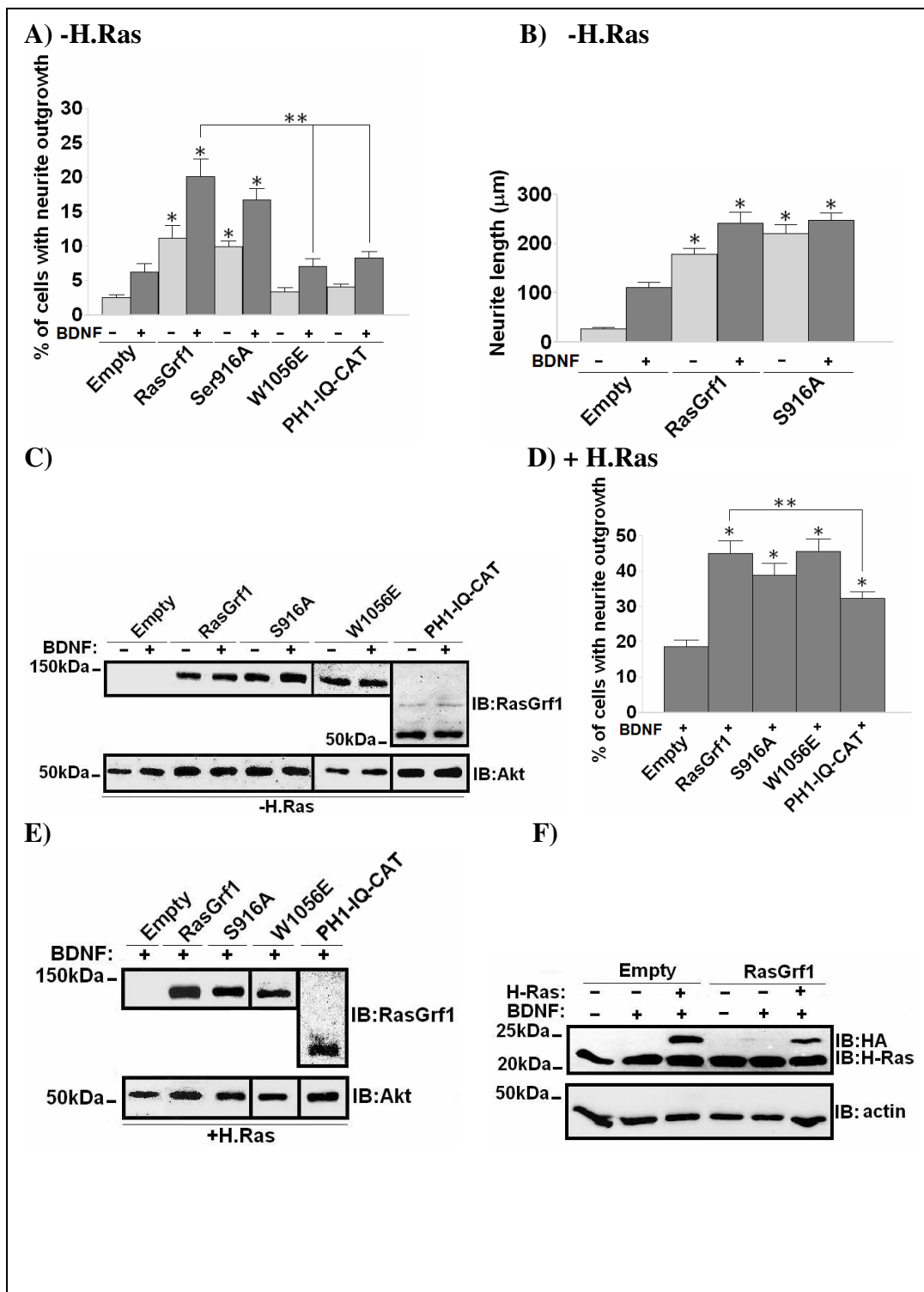
Figure 2.7. The effect of BDNF on neurite outgrowth in TrkB-B5 cells expressing WT and mutant forms of RasGrf1. TrkB-B5 cells were co-transfected with EGFP and either empty plasmid, WT-RasGrf1 or the indicated mutants in the absence or presence of H-Ras, and -/+ BDNF (1 ng/ml) for 4 consecutive days, and the percentage of cells with neurites more than two cell bodies determined. (n=3). * indicates a significant increase in neurite outgrowth (P-value<0.05) of indicated construct compared to Empty plasmid. **A)** Expression of WT-RasGrf1 and the $S^{916}A$ mutant resulted in a significant increase in neurite outgrowth response. Expression of neither the $W^{1056}E$, nor the PH1-IQ-CAT mutant influenced neurite outgrowth significantly. ** indicates significant decrease of PH1-IQ-CAT or $W^{1056}E$ relative to WT-RasGrf1. **B)** Expression of both RasGrf1 and $S^{916}A$ mutant significantly increases the average length of neurites relative to controls. **C)** Expression levels of RasGrf1 constructs in transfected TrkB-B5 cells. Each lane represents 50 µg of lysate protein. The PH1-IQ-CAT mutant appeared as expected at ≈60kDa. The blot was re-probed against Akt (≈55kDa) as a loading control. **D)** The percentage of cells with neurites greater than two cell body lengths in TrkB-B5 cells co-expressing H-Ras and the indicated RasGrf1 constructs in -/+ BDNF stimulation. Under the conditions of H-Ras over-expression the $W^{1056}E$ and PH1-IQ-CAT mutants showed a significant increase in neurite outgrowth. **E)** Expression levels of RasGrf1constructs in TrkB-B5 cells co-transfected with H-Ras. **F)** Expression of endogenous (≈22kDa) and co-transfected HA-tagged H-Ras (HA-tagged; ≈24kDa) was determined by SDS-PAGE/western blot. The blot was first probed with an antibody to H-Ras, stripped and re-probed with an antibody against HA. The blot was re-probed against β-actin (≈43kDa) as a loading control.

A)

B)

C)
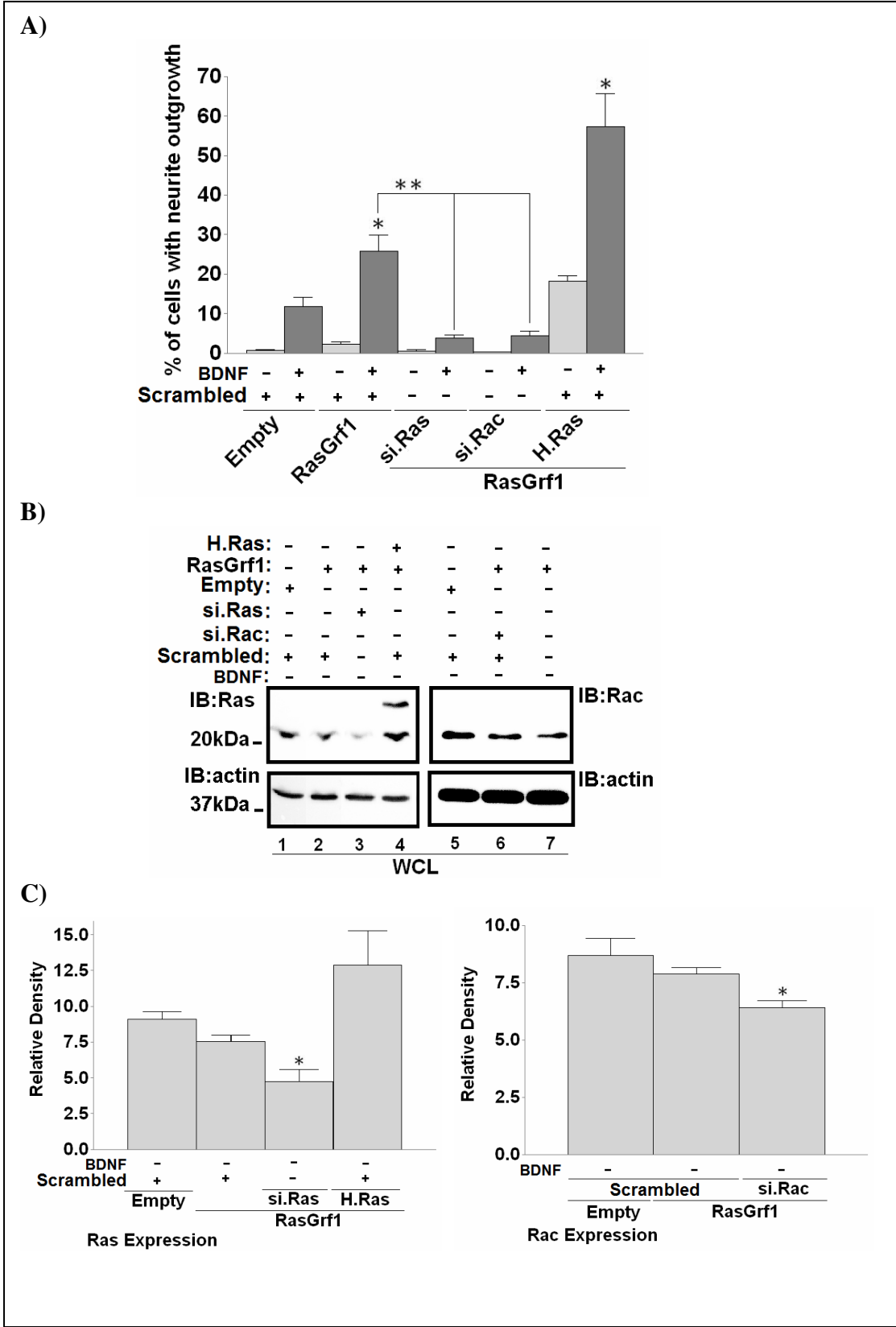
Figure 2.8. The effect of BDNF-induced neurite outgrowth in TrkB-B5 cells expressing si-RNAs against Ras and Rac. TrkB-B5 cells were co-transfected with EGFP and either empty plasmid, or WT-RasGrf1 co-expressing with scrambled-RNA or si-RNA against Ras or Rac, treated with BDNF (1 ng/ml) for 4 consecutive days, or left un-stimulated and the percentage of cells with neurites length greater than two cell bodies diameter were determined (n=3). * indicates a significant increase in neurite outgrowth (P-value<0.05) of indicated construct in the presence of BDNF compared to Empty plasmid, and ** indicates significant decrease of si-Ras/Rac relative to wild type RasGrf1. **A)** Neurite outgrowth assay in TrkB-B5 cells in presence of Ras/Rac si.RNAs in -/+ BDNF stimulation. Both si-RNAs against Ras and Rac reduced neurite outgrowth significantly in comparing to RasGrf1 wild type. RasGrf1 co-expressing with H-Ras was used as a positive control for BDNF-induced neurite outgrowth. **B)** Expression levels of endogenous Ras and Rac in transfected TrkB-B5 cells only in absence of BDNF. Note that the expression level of both Ras and Rac was reduced respectively. Expression of endogenous (≈22kDa) of H.Ras and Rac as well as co-transfected HA-tagged H-Ras (HA-tagged; ≈24kDa, lane 4) as positive control are evident in this blot. The blot was re-probed against β-actin (≈43kDa) as a loading control. **C)** A quantification diagram of the relative intensity for endogenous Ras (left) and Rac (right) in transfected TrkB-B5 cells only in the absence of BDNF (n=3). The blots were normalized to the level of β-actin expression.

2.4 Discussion

In this study, we have addressed whether RasGrf1 activates both Ras and Rac in response to TrkA and TrkB receptors and whether one or both are essential to NGF and BDNF-mediated neurite outgrowth in PC12 and TrkB-B5 cells. We assayed Trk/RasGfr1-mediated activation of Ras and Rac in both HEK cells and PC12 cells and observed a TrkA-dependent increase in the activation of Rac in HEK cells, while activation of Ras in this context was decreased. In contrast, in RasGrf1-transfected PC12 cells, we observed the opposite response and found that NGF-induced the activation of Ras while Rac activity was constitutive and did not appear to change in response to NGF stimulation. By comparison, analysis of RasGrf1 potentiation of neurite outgrowth in both PC12 and TrkB-B5 cells, and the analysis of site-directed mutants in RasGrf1, revealed an essential role for both Ras and Rac in mediating RasGrf1's enhancement of neurotrophin stimulated neurite outgrowth. Our inability to detect NGF stimulation of Rac activity in PC12 cells was surprising given that Rac activity is required for neurite outgrowth. This may either reflect the fact that RasGrf1 was co-transfected with H-Ras in the GTP binding assay or that the kinetics of NGF-induced activation Rac are different than Ras and were not detected at the time point used in assay (5 min). We also found that the neurotrophin and RasGrf1-dependent enhancement of neurite outgrowth is not affected by the site-directed mutant $S^{916}A$ which has been reported as a site of PKA phosphorylation and activation of RasGrf1 signaling in PC12 cells (Baouz et al., 2001) as well as in neurons of the cerebral cortex (Yang et al., 2003). Moreover, the PKA inhibitor, H89, did not affect neurotrophin-induced enhancement of RasGrf1 signaling in the neurite outgrowth assays. Altogether, our results show a prominent role for RasGrf1

activation of both Ras and Rac downstream of neurotrophin-activated Trk receptors that are essential in mediating changes in cellular morphology, specifically, neurite outgrowth.

RasGrf1 has been shown in other studies to be an activator of both Ras and Rac in transfected HEK cells. Specifically, in HEK and Cos-7 cells, RasGrf1 activates Ras downstream of the non-receptor tyrosine kinase ACK1, cAMP-activated PKA, calcium and GPCR activity (Mattingly, 1999; Kiyono et al., 2000b; Yang et al., 2003). Conversely, RasGrf1 activates Rac downstream of the non-receptor tyrosine kinase Src (Kiyono et al., 2000a). While the specific mechanisms involved in regulating the activation of these distinct GTPases in HEK cells have not been determined, it is possible that the assembly of scaffolding complexes facilitated by the Trk receptor or its substrates may also re-direct the activity of RasGrf1 away from Ras following the initial stimulation with NGF. Specifically, RasGrf1 activity toward Rac and p38- MAP kinase, in transfected HEK cells, has recently been shown to depend on the assembly of a complex involving the scaffolding protein IB2/JIP2 (Buchsbaum et al., 2002). Moreover, RasGrf1 has also been shown to interact with a microtubule-destabilizing factor SCLIP (SCG10-like protein) which inhibits its ability to promote Rac activation and neurite outgrowth (Baldassa et al., 2007).

RasGrf1 has also been shown to preferentially activate H-Ras but not N or K-Ras (Jones and Jacksonet., 1998). Different Ras subtypes are specific to differentiative or proliferative pathways. In particular, H-Ras, but not K-Ras, signaling through the Raf/MEK/MAPK pathway requires endocytosis and endocytotic recycling (Porat-Shliom et al., 2008). Other Ras types also play specific roles in PC12 differentiation. For

example, M-Ras generates sustained activation of Erk and neurite outgrowth in an NGF-dependent manner (Sun et al., 2006) and RasGrf1 also interacts with M-Ras (Quilliam et al., 1999), which is an important protein for the development of dendritic spines in primary neuronal cultures (Harvey et al., 2008). RasGrf1 is a neuronal protein and we have seen that it alters cellular morphology in response to NGF activated TrkA in PC12 cells (Zippel et al., 1997; Robinson et al., 2005) as well as BDNF-activated TrkB (this study). These studies suggest that within primary neurons, TrkB-activated RasGrf1 will serve a role in altering cellular morphology such as branching or dendritic spine density through BDNF-dependent activation of RasGrf1 and its subsequent activation of either H- or M-Ras.

The data presented here has noted some important differences in several published PC12 studies regarding the importance and expression levels of H-Ras and its effect on RasGrf1-mediated differentiation. Yang and Mattingly (2006) did not find detectable levels of endogenous H-Ras in their PC12 cell line and did not observe differentiation in the absence of over-expression of transfected H-Ras (Baldassa et al., 2007). In contrast, in our studies and those by others (Baldassa et al., 2007), we both observed endogenous expression of H-Ras and that over-expression of RasGrf1 itself could induce a basal level of neurite outgrowth in the absence of NGF. Moreover, we found that co-expression of H-Ras with RasGrf1 in PC12 cells stimulated the basal neurite outgrowth response and completely eliminated the ability to detect an essential role for Rac in neurotrophin-induced neurite outgrowth. While Baldassa et al., (2007) observed a specific requirement for Rac activation by RasGrf1 in mediating constitutive neurite extension in PC12 cells, Yang and Mattingly (2006) observed a requirement for H-Ras co-expression and

activation and reported no role for Rac in this process. Instead, they suggested that the expansion in soma size in co-transfected cells was dependent on Rac activity (Yang and Mattingly, 2006). While we also observed an expansion in soma size in cells co-transfected with RasGrf1 and H-Ras, this was not observed in either PC12 or TrkB-B5 cells expressing RasGrf1 alone, in either the absence or presence of neurotrophin stimulation. Thus, the expanded 10-fold increase in cell soma size may reflect an artifact of H-Ras over-expression. In fact, under conditions of high levels of Ras expression, some researchers have reported that Ras is able to directly bind and activate B-Raf and small G proteins associated with differentiation (Kao et al., 2001).

Importantly, our study focused on NGF and BDNF-dependent RasGrf1 enhancement of neurite outgrowth. The fact that RasGrf1 specifically alters neuritic growth in response to both NGF and BDNF, through the activation of specific Ras family GTPases, suggests a potential role for RasGrf1 in mediating Trk-dependent structural changes underlying the formation of synapses or dendritic spines in neuronal cells. Such functions would further contribute to our understanding of how TrkB and RasGrf1, both essential proteins for learning and memory, might be mediating aspects of synaptic plasticity within the mature brain.

References

Anborgh P. H., Qian X., Papageorge A. G., Vass W. C., DeClue J. E., and Lowy D. R. (1999) Ras-Specific Exchange Factor GRF: Oligomerization through its Dbl Homology Domain and Calcium-Dependent Activation of Raf. Mol. Cell. Biol. 19, 4611-4622.

Arozarena I., Aaronson D. S., Matallanas D., Sanz V., Ajenjo N., Tenbaum S. P., Teramoto H., Ighishi T., Zabala J. C., Gutkind J. S., and Crespo P. (2000) The Rho Family GTPase Cdc42 Regulates the Activation of Ras/MAP Kinase by the Exchange Factor Ras-GRF. J. Biol. Chem. 275, 26441-26448.

Baldassa S., Gnesutta N., Fascio U., Sturani E., and Zippel R. (2007) SCLIP, a Microtubule-destabilizing Factor, Interacts with RasGRF1 and Inhibits Its Ability to Promote Rac Activation and Neurite Outgrowth. J. Biol. Chem. 282, 2333-2345.

Baouz S., Jacquet E., Accorsi K., Hountondji C., Balestrini M., Zippel R., Sturani E., and Parmeggiani A. (2001) Sites of Phosphorylation by Protein Kinase A in CDC25Mm/GRF1, a Guanine Nucleotide Exchange Factor for Ras. J. Biol. Chem. 276, 1742-1749.

Buchsbaum R., Telliez J. B., Goonesekera S., and Feig L. A. (1996) The N-Terminal Peckstrin, Coiled-Coil and IQ Domains of the Exchange Factor Ras-GRF act cooperatively to Facilitate Activation by Calcium. Mol. Cell. Biol. 16, 4888-4896.

Buchsbaum R. J., Connolly B. A., and Feig L. A. (2002) Interaction of Rac exchange factors Tiam1 and RasGrf1 with a scaffold for the p38 mitogen-activated protein kinase cascade. Mol Cell Biol. 22, 4073-4085.

Chakrabarti K., Lin R., Schiller N. I., Wang Y., Koubi D., Fan Y.-X., Rudkin B. B., Johnson G. R., and Schiller M. R. (2005) Critical Role for Kalirin in Nerve Growth Factor Signaling through TrkA. Mol. Cell. Biol. 25, 5106-5118.

Farnsworth C. L., Freshney N. W., Rosen L. B., Ghosh A., Greenberg M. E., and Feig L. A. (1995) Calcium activation of Ras mediated by neuronal exchange factor Ras-GRF. Nature. 376, 524-527.

Giese K. P., Friedman E., Telliez J. B., Fedorov N. B., Wines M., Feig L. A., and Silva A. J. (2001) Hippocampus dependent learning and memory is impaired in mice lacking the Ras guanine nucleotide releasing factor 1 (RasGrf1). Neuropharmacology. 41, 791-800.

Greene L. A. (1978) Nerve growth factor prevents the death and stimulates the neuronal differentiation of clonal PC12 pheochromocytoma cells in serum-free medium. J. Cell Biol. 78, 747-755.

Greene L. A., and Tischler A. S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA. 73, 2424-2428.

Harvey C. D., Yasuda R., Zhong H., and Svoboda K. (2008) The Spread of Ras Activity Triggered by Activation of a Single Dendritic Spine. Science. 321, 136-140.

Huang E. J., and Reichardt L. F. (2003) Trk Receptors: Roles in Neuronal Signal Transduction. Annual Review of Biochemistry. 72, 609-642.

Jones M. K., and Jackson J. H. (1998) Ras-GRF Activates Ha-Ras, but Not N-Ras or K-Ras 4B, Proteinin Vivo. Journal of Biological Chemistry. 273, 1782-1787.

Kao S., Jaiswal R. K., Kolch W., and Landreth G. E. (2001) Identification of the Mechanisms Regulating the Differential Activation of the MAPK Cascade by Epidermal Growth Factor and Nerve Growth Factor in PC12 cells. J. Biol. Chem. 276, 18169-18177.

Kiyono M., Kaziro Y., and Satoh T. (2000a) Induction of Rac-Guanine Nucleotide Exchange Activity of Ras-GRF1/CDC25Mm following Phosphorylation by the Nonreceptor Tyrosine Kinase Src. J. Biol. Chem. 275, 5441-5446.

Kiyono M., Kato J., Kataoka T., Kaziro Y., and Satoh T. (2000b) Stimulation of Ras Guanine Nucleotide Exchange Activity of Ras-GRF1/CDC25Mm upon Tyrosine Phosphorylation by the Cdc42-regulated Kinase ACK1. J. Biol. Chem. 275, 29788-29793.

Mattingly M., and Macara I. (1996) Phosphorylation-dependent activation of the Ras-GRF/CDC25Mm exchange factor by muscarinic receptors and G-protein beta-gamma subunits. Nature. 382, 268-272.

Mattingly R. R. (1999) Phosphorylation of Serine 916 of Ras-GRF1 Contributes to the Activation of Exchange Factor Activity by Muscarinic Receptors. Journal of Biological Chemistry. 274, 37379-37384.

Meakin S. O. (2000) Nerve Growth Factor Receptors and Mechanisms of Intracellular Signal Transduction. Recent Research Developments in Neurochemistry. 3, 75-91.

Meakin S. O., and MacDonald J. I. S. (1998) A Novel Juxtamembrane Deletion in Rat TrkA Blocks Differentiative but not Mitogenic Cell Signalling in Response to Nerve Growth Factor. J. Neurochem. 71, 1875-1888.

Meakin S. O., MacDonald J. I. S., Gryz E. A., Kubu C. J., and Verdi J. M. (1999) The Signaling Adapter Protein FRS-2 Competes with Shc for binding to TrkA: A Model for Discriminating Proliferation and Differentiation. J. Biol. Chem. 274, 9861-9870.

Minden A., Lin A., Claret F. X., Abo A., and Karin M. (1995) Selective activationof the JNK signaling cascade and c-Jun transcriptional activity by the small GTPase Rac and Cdc42Hs. Cell. 81, 1147-1157.

Nobes C. D., and Tolkovsky A. M. (1995) Neutralizing Anti-p21ras Fabs Suppress Rat Sympathetic Neuron Survival Induced by NGF, LIF, CNTF and cAMP. Eur. J. Neurosci. 7, 344-350.

Porat-Shliom N., Kloog Y., and Donaldson J. G. (2008) A Unique Platform for H-Ras Signaling Involving Clathrin-independent Endocytosis. Mol. Biol. Cell. 19, 765-775.

Quilliam L. A., Castro A. F., Rogers-Graham K. S., Martin C. B., Der C. J., and Bi C. (1999) M-Ras/R-Ras3, a Transforming Ras Protein Regulated by Sos1, GRF1, and p120 Ras GTPase-activating Protein, Interacts with the Putative Ras Effector AF6. Journal of Biological Chemistry. 274, 23850-23857.

Reichardt L. F. (2006) Neurotrophin-regulated signaling pathways. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 361, 1545-1564.

Ridley A. J., Paterson H. F., Johnston C. L., Diekmann D., and Hall A. (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell. 70, 401-410.

Robinson K. N., Manto K., Buchsbaum R. J., MacDonald J. I. S., and Meakin S. O. (2005) Neurotrophin-dependent Tyrosine Phosphorylation of Ras Guanine-Releasing Factor 1 and Associated Neurite Outgrowth is Dependent on the HIKE Domain of TrkA. J. Biol. Chem. 280, 225-235.

Robinson K., 2008. The role of RasGrf1 in Trk receptor signaling. Chapter 3 and 4. The Uniersity of Western Ontario, London, ON.

Rodriguez-Viciana P., Warne P. H., Khwaja A., Marte B. M., Pappin D., Das P., Waterfield M. D., Ridley A., and Downward J. (1997) Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. Cell. 89, 457-467.

Small T. W., Bolender Z., Bueno C., O'Neil C., Nong Z., Rushlow W., Rajakumar N., Kandel C., Strong J., Madrenas J., and Pickering J. G. (2006) Wilms' Tumor 1-Associating Protein Regulates the Proliferation of Vascular Smooth Muscle Cells. Circ Res. 99, 1338-1346.

Soltoff S. P., Rabin S. L., Cantley L. C., and Kaplan D. R. (1992) Nerve growth factor promotes the activation of phosphatidylinositol 3-kinase and its association with the trk tyrosine kinase. J. Biol. Chem. 267, 17472-17477.

Sun P., Watanabe H., Takano K., Yokoyama T., Fujisawa J.-i., and Endo T. (2006) Sustained activation of M-Ras induced by nerve growth factor is essential for neuronal differentiation of PC12 cells. Genes to Cells. 11, 1097-1113.

Vanoni M., Bertini R., Sacco E., Fontanella L., Rieppi M., Colombo S., Martegani E., Carrera V., Moroni A., Bizzarri C., Sabbatini V., Cattozzo M., Colagrande A., and Alberghina L. (1999) Characterization and Properties of Dominant-negative Mutants of the Ras-Specific Guanine Nucleotide Exhange Factor CDC25^{Mm}. J. Biol. Chem. 274, 36656-36662.

Vaudry D., Stork P. J., Lazarovici P., and Eiden L. E. (2002) Signaling pathways for PC12 cell differentiation: making the right connections. Science. 296, 1648-1649.

Wennstrom S., Hawkins P., Cooke F., Hara K., Yonezawa K., Kasuga M., Jackson T., Claesson-Welsh L., and Stephens L. (1994) Activation of phosphoinositide 3-kinase is required for PDGF-stimulated membrane ruffling. Current Biology. 4, 385-393.

Yang H., Cooley D., Legakis J., Qingyuan G., Andrade R., and Mattingly R. (2003) Phosphorylation of the RasGrf1 Exchange Factor at Ser916/898 Reveals Activation of Ras Signaling in the Cerebral Cortex. J. Biol. Chem. 278, 13278-13285.

Yang H., and Mattingly R. R. (2006) The Ras-GRF1 Exchange Factor Coordinates Activation of H-Ras and Rac1 to Control Neuronal Morphology. Mol. Biol. Cell. 17, 2177-2189.

Zippel R., Gnesutta N., Matus-Leibovitch N., Mancinelli E., Saya D., Vogel Z., Sturani E., Renata Z., Nerina G., Noa M. L., Enzo M., Daniella S., Zvi V., and Emmapaola S. (1997) Ras-Grf, the activator of Ras, is expressed preferentially in mature neurons of the central nervous system. Brain Res. Mol. Brain Res. 48, 140-144.

Chapter 3

A novel site of tyrosine phosphorylation on RasGrf1 guanine nucleotide releasing factor

3.1 Introduction

Neurotrophins including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4/5 (NT4/5) initiate their biological functions in part by binding to the Trk family of receptor tyrosinse kinases (Meakin, 2000; Reichardt, 2006). Upon neurotrophin binding, the two receptor subunits dimerize, resulting in tyrosine-autophosphorylation in the intracellular kinase domain. The major sites of phosphorylation on rat TrkA include tyrosine 499, 679, 683, 684, and 794. Tyrosine residues 679, 683 and 684 form part of the activation loop of the kinase while tyrosine 499 and 794 serve as docking sites for intermediate signaling (adaptor) molecules (Hanks et al., 1988; Ibanez, 1993; Heldin, 1995; Hubbard, 1997), including Collagen homology domain (Shc) family proteins and Fibroblast growth factor Receptor Substrate 2 (FRS2) both of which compete to bind Tyr^{499} and phospholipase Cγ-1 (PLCγ-1) which binds to Tyr^{794} (Stephens et al., 1994; Meakin et al., 1999; Minichiello, 2009). Through these adaptor molecules, Trk is able to activate three major signaling pathways including the Ras-mitogen activated protein kinase (Ras-MAPK) pathway underlying cell proliferation and differentiation, the phosphotidylinositol-3 kinase (PI3K)-Akt pathway promoting cell survival, and the phospholipase Cγ-1-Ca^{2+} pathway leading to synaptic plasticity (Reichardt, 2006; Minichiello, 2009).

Although several previous studies suggest that Sos exchange factor couples tyrosine kinase signaling pathway to the activation of Ras, and brain-specific guanine

nucleotide exchange factor, RasGrf1, couples G-protein signals to the activation of Ras (Buday and Downward, 1993; Egan et al., 1993; Shou et al., 1995; Zippel et al., 1996; Mattingly and Macara, 1996; Mattingly, 1999), a large body of evidence have indicated that Trk can also recruit RasGrf1 to promote neurite outgrowth and differentiation in PC12 cells (MacDonald et al., 1999; Ciccarelli et al., 2000; Robinson et al., 2005; Yang et al., 2003; Yang and Mattingly, 2006).

Ras guanine nucleotide exchange factors, namely RasGrfs (Shou et al., 1992), previously known as CDC25Mm (Martegani et al., 1992; Wei et al., 1992) belong to a family of guanine nucleotide exchange factors (GEFs), and present in two forms of 140 kDa-RasGrf1 which is highly expressed in the nervous system particularly in the cortex, hippocampus and hypothalamus (Zippel et al., 1997; Sturani et al., 1997), and 130 kDa-RasGrf2 which shows a high sequence homology to RasGrf1 (80%), and is expressed ubiquitously, but is also present within mature brain (Fam et al., 1997; Anborgh et al., 1999).

Structurally, RasGrf1 is a multi-domain protein (Figure 3.1) including a pleckstrin homology (PH) domain in the N-terminus, a coiled coil (CC) and IQ motif, a Db1 homology domain (DH), a REM motif, a cyclin destruction box (CDB) and a cell division cycle 25 (CDC25) domain at the C-terminus. The PH domain is necessary for RasGrf1 localization to the plasma membrane (Buchsbaum et al., 1996), and has been suggested to bind to phosphoinositides and other types of phospholipids in the membrane (Harlan et al., 1994; Lemmon and Ferguson, 2000; Varnai et al., 2002). In addition, the PH domain may also serve as a putative phosphotyrosine binding (PTB) domain (Balla, 2005; Cowburn, 1997), and bind to βγ subunits of G protein-coupled receptors (Touhara

et al., 1994; Shou et al., 1995; Mattingly and Macara, 1996; Zippel et al., 1996). This domain also interacts with receptor tyrosine kinases through its HIKE motif (Robinson et al., 2005). Coiled coil domains play a role in protein-protein interaction in cooperation with PH1 and IQ domains. The IQ domain interacts with calmodulin, and in response to calcium elevation, RasGrf1 is activated by binding of calcium/calmodulin to the N-terminal IQ motif (Buchsbaum et al., 1996; Farnsworth et al., 1995). The DH domain binds the Rho family of GTPases, in particular Rac1, and promotes guanine nucleotide exchange activity toward Rac1 (Freshney et al., 1997; Kiyono et al., 1999; Innocenti et al., 1999). The REM motif plays a role in stabilization of the core CDC25 domain, and CDB (or PEST) is a region rich in proline, glutamic acid, serine and threonine (PEST) amino acids, that constitutes a hypothetical target for proteolysis, an additional mechanism to regulate the cellular level of RasGrf1 protein (Rogers et al., 1986; Baouz, et al., 1997;; Gnesutta et al., 2001). Lastly, the CDC25 domain facilitates the activation of Ras-GTPases (Tian and Feig, 2001; Cen et al., 1993; Wei et al., 1994).

A role for RasGrf1 is to promote the exchange of GDP for GTP on small GTPases such as Ras and Rac, acting as a molecular switch between active GTP-bound and inactive GDP-bound states (Tian et al., 2004; Katoh et al., 2000). Small GTPases have a crucial role to link external messages from the cell surface to several downstream signaling cascades underlying a wide variety of cellular processes including cell cycle regulation, cytoskeletal reorganization, neurite outgrowth and differentiation (Crespo and Leon, 2000; Malumbres and Pellicer, 1998; Huang and Reichardt, 2003).

In response to upstream signaling, RasGrf1 not only undergoes serine/threonine phosphorylation by protein kinase A (Farnsworth et al., 1995; Schmitt et al., 2005;

Mattingly and Macara, 1996), but it has also been documented in several studies that RasGrf1 is tyrosine phosphorylated by the non-receptor tyrosine kinase Src, leading to the activation of Rac (Kiyono et al., 2000a) and Ack1, resulting in activation of Ras (Kiyono et al., 2000b). RasGrf1 has also been shown to be tyrosine phosphorylated by the Trk family of receptor tyrosine kinases (Robinson et al., 2005). However, the sites of tyrosine phosphorylation, whether they are essential to the enhancement of neurite outrgrowth and whether they affect the activation of either Ras or Rac-GTPases have not been addressed.

In this study, we identified novel phosphorylation sites in the CDC25 domain of RasGrf1, using site directed mutagenesis, and determined the level of RasGrf1 tyrosine phosphorylation by these mutants after either NGF or BDNF stimulation. We also examined the ability of these mutants to facilitate NGF-mediated neurite outgrowth in the PC12 cell line, a neuronal-like model system widely used to study the mechanisms of cell differentiation downstream of TrkA receptor as well as in PC12-derived cells, (namely TrkB-B5) over-expressing TrkB receptors.

3.2 Materials and Methods

3.2.1 Reagents. Antibodies to RasGrf1(C-20, sc-224), Trk (C-14), anti-H-Ras and anti-Myc (9E10) were from Santa Cruz. Anti-Rac1 and TrkB antibodies were from BD Transduction Laboratories. Mouse monoclonal β-actin antibody was from Sigma. Anti-phosphotyrosine (p-Tyr-100) was from Cell Signaling. Rabbit antibodies to the carboxyl-terminal 14 residues of TrkA (1478) were prepared and affinity purified using standard techniques (Robinson, 2008). HRP-coupled goat anti-mouse and goat anti-rabbit secondary antibodies were from The Jackson Laboratories. NGF was from Harlan

Products for Bioscience and human recombinant BDNF was from R&D Systems. The DC Protein Assay Kit was from Bio-Rad.

3.2.2 Plasmids. The following plasmids have been previously described: full-length mouse RasGrf1 under control of the human elongation factor 1a promoter (pEFP-RasGrf1) (Anborgh et al., 1999); pCMX-rat TrkA (wild-type) and rat TrkB (Meakin et al., 1999). pEGFP was from Clontech. pcDNA-HA-H-Ras was the gift from J. Keller (Vanderbilt University Medical Center, Nashville, TN).

3.2.3 Cell Lines. HEK 293T cells were cultured under standard conditions in DMEM with 5% supplemented calf serum (SCS), 5% FBS (Hyclone), and 50 μg/ml gentamycin sulfate (Sigma). PC12 rat adrenal pheochromocytoma cells were maintained in DMEM with 5% SCS and 5% horse serum (Hyclone), while TrkB-B5 (nnR5 cells stably over-expressing HA-tagged TrkB receptors) have been described previously (Meakin, 2000; Meakin and MacDonald, 1998). These were cultured in 5% SCS and 5% horse serum in the presence of 100 μg/ml G418 and 50 μg/ml gentamycin sulfate.

3.2.4 Immunoprecipitations and Western Blots. Transfections were performed following standard calcium phosphate (HEK) or Lipofectamine 2000 (PC12 or TrkB-B5 cells; Invitrogen) techniques using a DNA to Lipofectamine ratio of 1:2. Briefly, 1.5 x 10^6 cells per 100 mm dish were co-transfected with 0.5-5 μg of each indicated plasmid. Lysates were prepared in NP40 lysis buffer (1% Nonidet P-40, 137 mM NaCl, 20 mM Tris, 0.5 mM EDTA, pH 8.0) containing 1 mM PMSF, 1 mM sodium orthovanadate ($NaVO_4$), 10 μg/ml aprotinin and 10 μg/ml leupeptin and assayed by immunoprecipitation. Lysates containing 0.5 to 3.5 mg of protein were immunoprecipitated with antibodies (0.5 μg anti-Trk1478, or 1 μg anti-RasGrf1), in

addition to 10 µl of washed 50% slurry Pansorbin (Calbiochem), at 4 ^{o}C overnight and were washed 3 times with fresh NP-40 lysis buffer on the next day. The bound proteins from immunoprecipitation or whole cell lysate (WCL) were re-suspended in 25 µl Laemmli sample buffer and heated at 65 °C for 10 min. Samples were then resolved on 6-12% SDS-polyacrylamide gels and transferred (5 volts, 1-2 hours) to polyvinylidene flouride membranes (PVDF, Pall Life Sciences). Blocking prior to primary antibody incubation was performed for 1 h at room temperature in 10 ml of 10% milk powder and Tris buffered saline with tween-20 (TBS-T). Western Blots were incubated with primary antibody at 4 ^{o}C for 16 h, and then washed for 1 h in TBS-T. Westerns were then blotted in 10 ml 10% milk powder and secondary antibody for 1 h at room temperature. Following washes in TBS-T for 1 h, westerns were exposed to enhanced chemiluminescence (ECL) reagents and developed. Primary antibody dilutions are as follows: anti-RasGrf1 1:10,000; anti-Trk (1478) (1:20,000), anti-TrkB (BD Transduction Laboratories) (1:2000), anti-Myc (1:2000); anti-pTyr100 (1:10,000); anti-H-Ras (1:5000); and anti-Rac1 (1:5000), anti-actin (1:20000). HRP-coupled secondary antibodies were used at 1:10,000.

3.2.5 Neurite Response Assay and Analysis of Cell Morphology. PC12 or TrkB-B5 Cells were plated onto 50 µg/ml poly-D-lysine (Sigma) coated 30 or 100 mm dishes and transfected with pEGFP (0.5 µg) plus Empty plasmid (1 µg), pcDNA-HA-Ras (0.5 µg), pEFP-RasGrf1 (1 µg) with transfection efficiencies of 70 – 90% (Lipofectamine 2000). Fresh media and non-saturating levels of 1 ng/ml NGF or BDNF (NGF for PC12, BDNF for TrkB-B5) were added at 24 h intervals on each of 4 successive days. On each day (from day one at 24 h after transfection to day four), the percentage of EGFP-expressing

cells in >10 randomly selected fields, at least 200 cells pre field, were scored for neurite outgrowth using an inverted fluorescent microscope (1 x 70 [Olympus]; Diaphot 300 [Nikon]) at a magnification of 200X. The percentages of green cells with a length of neurite greater than 2 cell bodies in diameter were calculated. Three independent neurite outgrowth experiments (n=3) were performed and the percentage of EGFP-transfected cells extending neurites, between samples, was statistically analyzed by One way-ANOVA with Post-Tukey test and paired student t-test. TrkA and RasGrf1 expression and tyrosine phosphorylation was confirmed following the counts performed on day four, by immunoprecipitation of 500 µg to 3.5 mg of protein or loading whole cell lysate on SDS-PAGE gel and western blotting. The relative densities were then statistically analysed by paired student t-test for significant differences between samples (n=3).

3.3 Results

New sites of RasGrf1 tyrosine phosphorylation were identified in this study using site directed mutagenesis in specific RasGrf1 functional domains followed by analysing their effect on neurotrophin-induced neurite outgrowth in PC12 cells or PC12-derived cell lines over-expressing TrkB receptor.

3.3.1 Tyrosine residues Y^{95} and Y^{233} in the PH1-IQ domain on RasGrf1 are sites of tyrosine phosphorylation.

Since the PH1 domain of RasGrf1 (Figure 3.1) was determined to interact with Trk *in vitro* (Ciccarelli et al., 2000; Robinson et al., 2005), and the IQ domain is activated by calmodulin (Buchsbaum et al., 1996; Farnsworth et al., 1995), the PH1-IQ region of RasGrf1 was initially targeted by site directed mutagenesis. Within this region there were a total of 7 potential phosphotyrosine acceptor sites (Tyr^{23}, Tyr^{54}, Tyr^{66}, Tyr^{95}, Tyr^{130},

Tyr^{145}, Tyr^{233}) (Figure 3.2A) and while some residues were predicted to be more likely sites of phosphorylation, a series of combinatorial mutants were generated (Fig 3-2A). Myc-tagged RasGrf1 PH1-IQ domain constructs containing alanine substitution mutations at these residues were then co-transfected with TrkA into HEK cells and the level of tyrosine phosphorylation determined in response to NGF (Robinson, 2008). As shown in Figure 3.2, among the various potential sites of tyrosine phosphorylation on PH-IQ, it appeared the NGF stimulation increased the phosphorylation of RasGrf1 wild type and PHIQ1, but not PHIQ2 and PHIQ3 (Figure 3.2B). Since the only difference between PHIQ2 relative to PHIQ1 is the Y^{233}A on PHIQ2, Y^{233} is suggested as a major site of RasGrf1 tyrosine phosphorylation by NGF-mediated TrkA activation. Moreover, these results indicate that residues Y^{23}, Y^{53} and Y^{145} are not acceptor sites of tyrosine phosphorylation since they were not mutated in the PHIQ2 construct and this construct was not phosphorylated. However, residues $Y^{66, 95, 130}$ could still be sites of additional tyrosine phosphorylation as these sites were mutated in both the PHIQ2&3 constructs, both of which were not phosphorylated. Thus, to ensure whether the level of decrease in phosphorylation in PHIQ2 and PHIQ3 are due to only Y^{233} or potentially to other sites at Y^{66}, Y^{95} and/or Y^{130} , two other mutants were generated in the PH1 domain and tested, namely, Y^{95}A and Y^{130}A (Figure 3.2A). Interestingly, the results of this experiment revealed that the Y^{95}A mutant in the PH1 domain reduced tyrosine phosphorylation levels remarkably, suggesting that Y^{95} is also a target of NGF-dependent phosphorylation of RasGrf1 (Figure 3.2C) (Robinson, 2008).

3.3.2 Neurite outgrowth is independent of RasGrf1 tyrosine phosphorylation in the PH1-IQ domains.

Given the fact that Y^{95} and Y^{233} are the major sites of tyrosine phosphorylation on in the PH1-IQ domains in RasGrf1 in HEK cells, changes in NGF-mediated neurite outgrowth were next evaluated for the RasGrf1-$Y^{95/233}$A double mutant (called RasGrf1-M2) relative to RasGrf1-WT in PC12 cells in the presence of co-transfected H-Ras (Robinson, 2008). To ensure that these phosphorylation sites are functionally relevant, PC12 cells were transfected with either RasGrf1 wild type or RasGrf1-$Y^{95/233}$A mutant and the percentage of cells with neurite outgrowth was assayed after NGF stimulation. As shown in Figure 3.3A, the RasGrf1-$Y^{95/233}$A mutant increased neurite outgrowth significantly (34%) comparable to RasGrf1 wild type (38%) rather than being reduced suggesting that $Y^{95/233}$A sites of tyrosine phosphorylation are not involved in neurite outgrowth. The level of RasGrf1 tyrosine phosphorylation of wild type and $Y^{95/233}$A mutant in PC12 cells was shown in Figure 3.3B. The RasGrf1-$Y^{95/233}$A mutant appears to remain phosphorylated in response to NGF in PC12 cells, comparable to RasGrf1 wild type (compare lane 6 to lane 4).

3.3.3 Tyrosine Y^{1048}A and Y^{1062}A in CDC25 domain are involved in RasGrf1 tyrosine phosphorylation and neurite outgrowth.

Since it is well documented that RasGrf1 activates Ras by its CDC25 domain, we next targeted this region for site-directed mutagenesis. In a study by Vanoni et al., (1999), W^{1056} on RasGrf1 was suggested as a binding site for GDP-Ras-GTPase complex, tempting us to consider that tyrosine residues around this area might be involved in RasGrf1-mediated neurite outgrowth downstream of Trk. Thus, tyrosine residues Y^{1048}

and Y^{1062} were next targeted by site directed mutagenesis as they were predicted to be potential sites of phosphorylation. The prediction has been made based on some criteria such as the optimal protein-protein interaction of RasGrf1 and its cognate binding proteins, the primary group sequences surrounding these phosphoresidues, and spatial configuration for the accessibility of phosphoresidues to be able to predict the location of these phosphorylation sites. This new mutant was made from the previous RasGrf1 triple mutant ($Y^{95/130/233}$A) that had been shown to contain major sites of RasGrf1 tyrosine phosphorylation in the PH1 and IQ domains (Robinson, 2008). Since RasGrf1 is expressed primarily in the brain accordingly with TrkB, neurite outgrowth for RasGrf1-WT and its mutants were further addressed in TrkB-B5 cells. Thus to ensure that these phosphorylation sites, and their effect on neurite outgrowth are functionally relevant, TrkB-B5 cells were transfected with RasGrf1-WT and the RasGrf1-M3/M5 mutants in the absence of co-transfected H-Ras. As mentioned earlier (Chapter 2), the original idea of using H-Ras in PC12 cell study was based on the observation by Yang and Mattingly (Yang and Mattingly, 2006) that PC12 cells don't express sufficient endogenous H-Ras. However, at the time of the current experiments, we discovered that both our PC12 and TrkB-B5 cells do express endogenous H-Ras. As shown in Figure 3.4B, while RasGrf1-WT and $Y^{95/130/233}$A mutant (RasGrf1-M3) demonstrated significant increase (both approximately 16%) in neurite outgrowth in comparison to empty plasmid (4%), the level of neurite outgrowth was reduced significantly by the RasGrf1-$Y^{95/130/233/1048/1062}$A mutant (RasGrf1-M5) (11%) relative to RasGrf1 wild type, implicating Y^{1048} and Y^{1062} are primary sites of tyrosine phosphorylation that are important for neurotrophin-mediated neurite outgrowth. However, since the RasGrf1-M5 mutant still demonstrated neurite

outgrowth that is still significantly increased relative to the negative control (empty plasmid), it is possible that there are potentially additional tyrosine phosphorylation sites, within the DH domain, that are involved in neurite outgrowth. The level of RasGrf1 phosphorylation is shown in Figure 3.4C as well as the relative density in right panel. While RasGrf1-WT is phosphorylated in response to BDNF, the level of tyrosine phosphorylation is significantly (nearly 2-fold) decreased compared to RasGrf1-WT, but still detectable, in the RasGrf1-M5 mutant.

```
Trk binding        CaMK binding   Rac-GEF activity
   area               area           area                        Ras-GEF activity area

                                                          Site of phosphorylation
                                                                  (S916)

N-terminal                                                          ↓                              C-terminal
  1 ━━━ PH1 ━━━ CC ━━ IQ ━━ DH ━━ PH2 ━━ REM ━ CDB ━━━━━━━━━━ CDC25 ━━━ 1262

     23    130  151 178 208 233 244  430 459 588 639 686 798    853    1005  (W1056) 1260

                                               (aa 714-913)
                                              NR2B binding area
```

```
   1 mqkairlndg hvvtlgllaq kdgtrkgyls krsadnpkwq tkwfallqnl lfyfesdssp
  61 rpsglylleg sickrapspk rgtsskesge kqqhyftvnf sndsqktlel rtedakdcde
 121 wvaaiarasy kilateheal mqkylhllqv vetektvakq lrqqledgev eierlktevt
 181 itnlikdndr iqssnkagsa ddedsdikki kkvqsflrgw lcrrkwknii qdyirsphad
 241 smrkrnqvvf smleaeaeyv qqlhilvnnf lrplrmaass kkppithddv ssiflnseti
 301 mflhqifyqg lkarisswpt lvladlfdil lpmlniyqef vrnhqyslqi lahckqnrdf
 361 dkllkqyeak pdceertlet fltypmfqip ryiltlhell ahtphehver nsldyakskl
 421 eelsrimhde vsetenirkn laiermiteg ceilldtsqt fvrqgslmqm slseksksssr
 481 grlgslstkk egerqcflfs khliictrgs ggklhltkng vislidctll depenlddea
 541 kgagpeiehl efkigvepkd slpftvilva strqekaawt sdiiqcvdni rcnglmmnaf
 601 eenskvtvpq miksdaslyc ddvdirfskt mnsckvlqir yasverller ltdlrflsid
 661 flntflhsyr vftnamvvld kliniyrkpm saiparslel lfssshnakl lygdapkspr
 721 asrkfssppp laigtsspsr rrklslnipi itggkalela slgcssdsya nihspispfg
 781 kttldtgklc masslpktpe eidvpatipe kpgelsasrk hssdvlkees eddqnhsded
 841 ntevspvksp ptpksflnrt itefpffnyn ngilmttcrd lvdnnrstls atsafaiata
 901 ganegpsnke vfrrmslant gfssdqrnid kefvirraat nrvlnvlrhw vtkhtqdfdt
 961 ddtlkyrvic fleevmhdpd lltqerkaaa niirtltlee tteqhsmlee vilmtegvkt
1021 epfenhpale iaeqltlldh lvfksipyee ffgqgwmkae kyertpyimk ttkhfnhvsn
1081 fiaseiirne disarasaie kwvavadicr clhnynavle itssinrsai frlkktwlkv
1141 skqtkslldk lqklvssdgr fknlreslrn cdppcvpylg myltdlvfie egtpnytedg
1201 lvnfskmrmi shiireirqf qqttykidpq pkviqyllde sfmldeesly esslliepkl
1261 pt
```

Figure 3.1. A schematic diagrams of RasGrf1 protein. Mouse RasGrf1, a 140kDa protein with 1262 amino acids consists of several binding domains including a binding domain to interact with TrkB (PH1; pleckstrin domain), and a region that binds with the NR2B subunit (amino acids 714-913), coiled-coil motif (CC), ilimaquinone (IQ) domain to bind calmodulin, Db1 homology domain (DH) to activate Rac, the second pleckstrin domain (PH2), Ras exchange motif (REM), cycline destruction box (CDB), and the catalytic domain (CDC25) to activate Ras. S^{916} is the site for RasGrf1-phosphorylation by PKA, the W^{1056} on RasGrf1 has been suggested as binding site for GDP-Ras, and the sequence area encoding amino acids 714-913 have been described as a neural domain (ND) which binds the NR2B subunit of NMDA receptor. The lower panel represents an amino acids sequence of mouse RasGrf1 with S^{916} shown in red color.

A)

RasGrf1-PH1-IQ (aa 1-233)

PHIQ-WT: PH1-IQ (no mutations)
PHIQ1: PH1-IQ-myc Y66A,Y95A,Y130A
PHIQ2: PH1-IQ-myc Y66A, Y95A, Y130A, Y233A
PHIQ3: PH1-IQ-myc Y66A, Y95A, Y130A, Y145A, Y233A
PH1-Y130A
PH1-Y95A
RasGrf1-Y95/233A mutant (RasGrf1-M2)

B)

C)
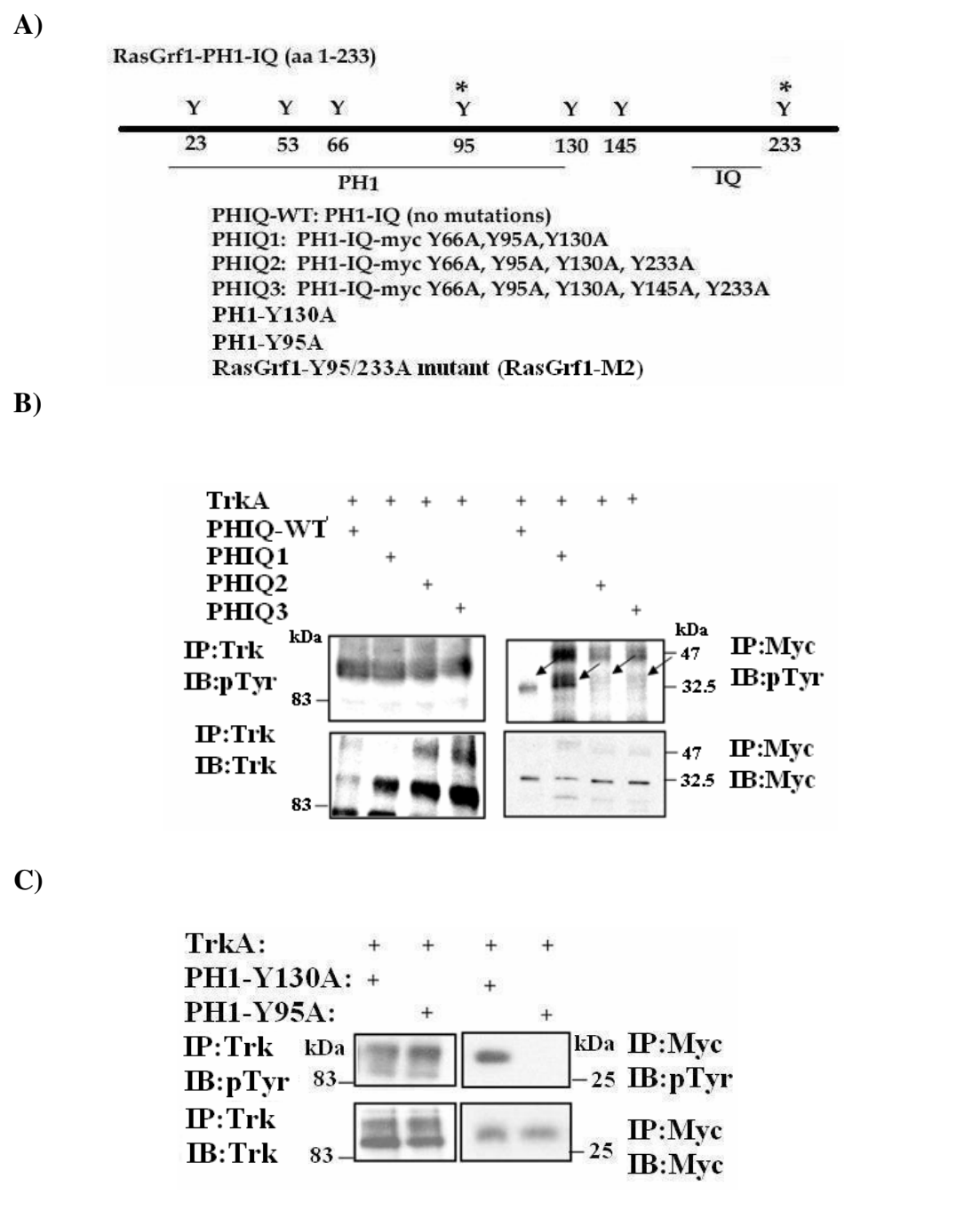
Figure 3.2. Tyrosine phosphorylation analysis of RasGrf1-PHIQ domains mutants in HEK cells. Tyrosine phosphorylation levels of RasGrf1 after site directed mutagenesis of tyrosine (Y) to alanine (A) in the PH1-IQ domain (Robinson, 2008) (n=3). A) A schematic diagram of RasGrf1 presenting the tyrosine mapping in PH1-IQ domains and different provided mutants. RasGrf1 contains 7 tyrosine sites in PH1 to IQ domains including tyrosine 23, 53, 66, 95, 130, 145, and 233. B) The level of tyrosine phosphorylation of RasGrf1 wild type (PHIQ-WT) and its point mutants including PHIQ1, PHIQ2, and PHIQ3 in HEK cells in respond to NGF. Note that Y^{233} involves in RasGrf1 tyrosine phosphorylation. C) The level of tyrosine phosphorylation in PH1-Y^{95}A and PH1-Y^{130}A point mutants. Note that Y^{95} appeared to be a site of tyrosine phosphorylation.

A)
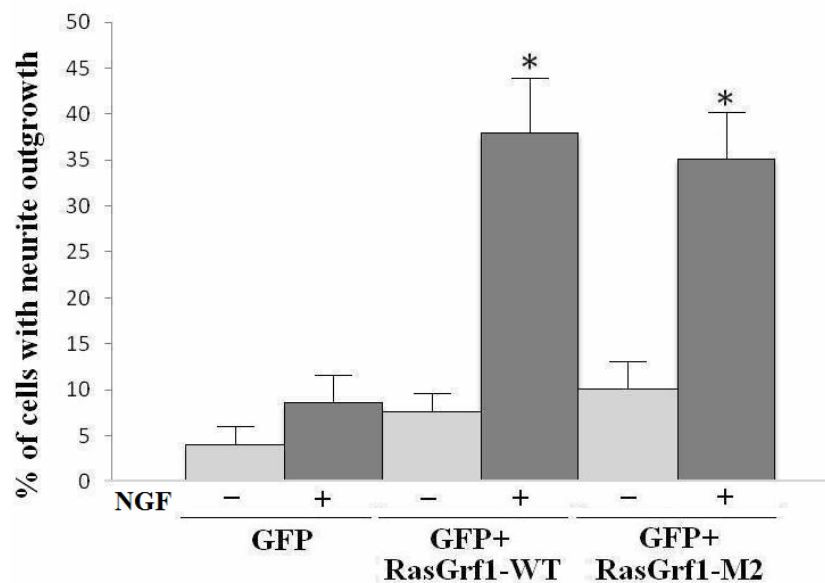
B)
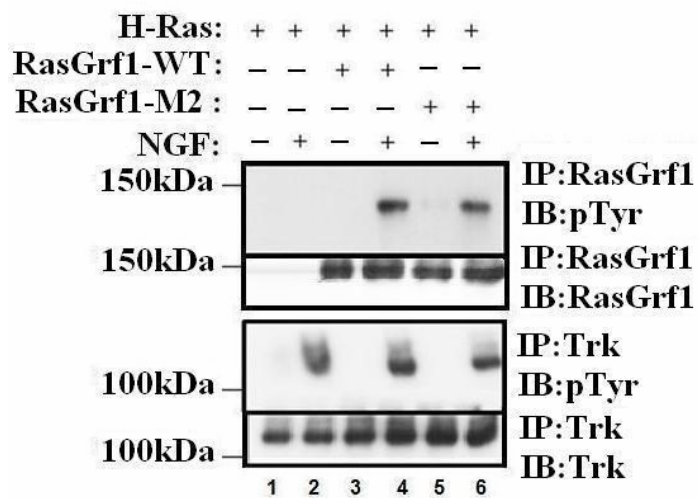
Figure 3.3. Neurite outgrowth assay of RasGrf1 wild type versus Y95/233A mutant in PC12 cells. PC12 cells were transfected with EGFP plus Empty-plasmid or RasGrf1-WT/M2 mutant, in presence of H-Ras, treated with either NGF (1 ng/ml) for 4 consecutive days or left un-stimulated. The percentage of cells with neurite length greater than two cell bodies in diameter were counted and statistically analyzed. Samples were immunoprecipitated (IP), separated by SDS-PAGE, and immunoblotted (IB) with indicated antibodies (n=3). * indicates statistical significance (P-value<0.05) of RasGrf1-WT/M2 in presence of NGF relative to the Empty plasmid (Robinson, 2008). A) NGF-mediated TrkA neurite outgrowth assay of RasGrf1 wild type (RasGrf1.WT) and the RasGrf1-Y$^{95/233}$A mutant in PC12 cells co-transfected with H-Ras. RasGrf1-Y$^{95/233}$A mutant did not reduce neurite outgrowth relative to negative control. B) The level of tyrosine phosphorylation of RasGrf1 wild type and Y$^{95/233}$A mutant in PC12 cells in response to NGF. Note that RasGrf1-M2 appeared to be still phosphorylated.

A)

RasGrf1-CDC25 (aa 1005-1260)
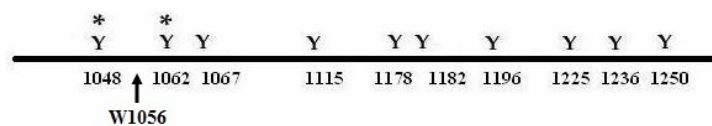
RasGrf1-WT
RasGrf1-Y95/130/233A mutant (RasGrf1-M3)
RasGrf1-Y95/130/233/1048/1062A mutant (RasGrf1-M5)
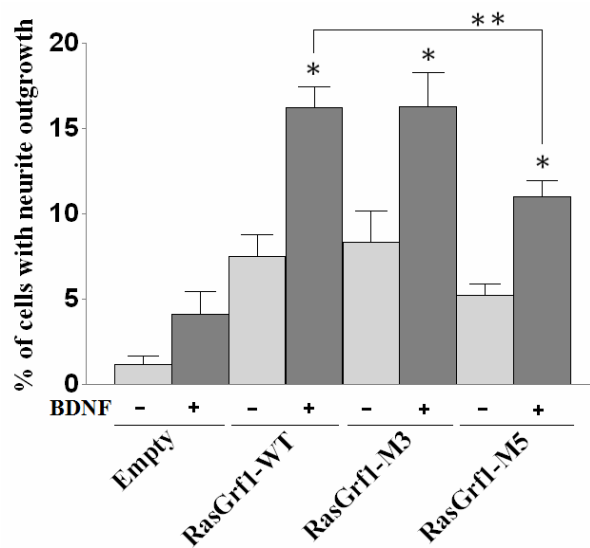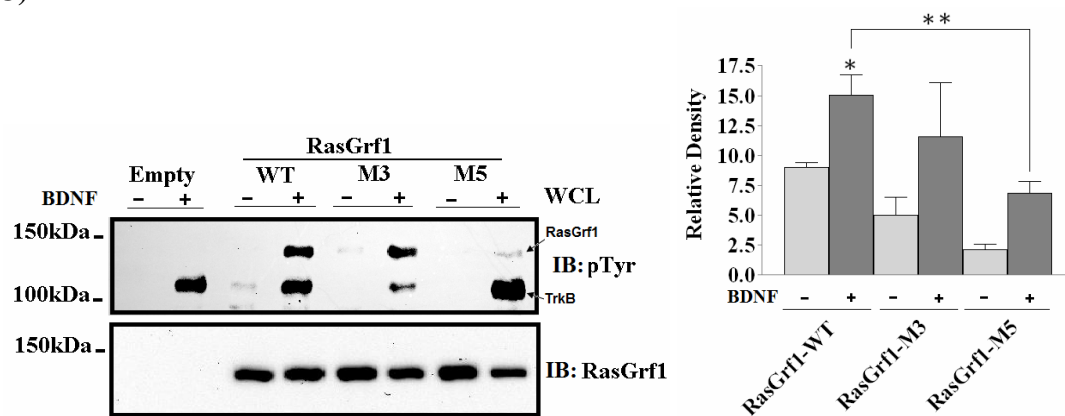
B)

C)

Figure 3.4. Neurite outgrowth assay of RasGrf1 wild type versus RasGrf1-CDC25 domain mutant in TrkB-B5 cells. TrkB-B5 or HEK cells were transfected with EGFP plus Empty-plasmid or RasGrf1-WT/M3/M5 mutants (and TrkB construct for HEK cells only), treated with either BDNF (1 ng/ml) for 4 consecutive days or left un-stimulated. The percentage of cells with neurite length greater than two cell bodies in diameter were counted and statistically analyzed. Samples were immunoprecipitated (IP), separated by SDS-PAGE, and immunoblotted (IB) with indicated antibodies (n=3). * indicates statistical significance (P-value<0.05) of indicated construct in presence of BDNF relative to the Empty plasmid, and ** indicates significant decrease of RasGrf1-M5 relative to wild type RasGrf1. **A)** A schematic diagram of RasGrf1 presenting the tyrosine mapping in CDC25 domain as well as different provided mutants including RasGrf1-Y$^{95/130/233}$A (RasGrf1-M3) mutant in PHIQ domain and RasGrf1-Y$^{95/130/233/1048/1062}$A mutant (RasGrf1-M5). RasGrf1 contains 10 tyrosine sites in CDC25 domain including tyrosine 1048, 1062, 1067, 1115, 1178, 1182, 1196, 1225, 1236, and 1250. **B)** Neurite outgrowth assay of RasGrf1 wild type (RasGrf1-WT) and various point mutants including triple mutant of RasGrf1-M3 and RasGrf1-M5 mutants in TrkB-B5 cells in respond to BDNF. RasGrf1-M5 mutant reduced neurite outgrowth significantly relative to RasGrf1-WT. **C)** The level of tyrosine phosphorylation in RasGrf1-WT and its M3 and M5 mutants in HEK cells. RasGr1-M5 significantly decreased the level of RasGrf1 phosphorylation. A quantification diagram of the relative density for the level of tyrosine phosphorylation is shown in right panel (n=3). The blots were normalized to the level of RasGrf1 protein expression.

3.4 Discussion

In this study we have addressed novel sites of Trk-induced tyrosine phosphorylation in RasGrf1. Although some studies have indicated tyrosine phosphorylation of RasGrf1 (Kiyono et al., 2000a&b) downstream of Trk (Robinson et al., 2005), the specific site(s) of tyrosine phosphorylation on RasGrf1 have not yet been determined. The results here provide evidence about the properties of several tyrosine phosphorylation sites in different domains of RasGrf1 including the PH, IQ and CDC25 domains. These domains have been selected based on a previous study, which demonstrated that PH and IQ domains are involved in both the interaction between Trk and RasGrf1, and in the level of RasGrf1-tyrosine phosphorylation (Robinson et al., 2005). Furthermore, Ras activation (compared to Rac1) has been suggested as a prominent event for neurotrophin-dependent neurite extension downstream of RasGrf1-CDC25 domain (Yang and Mattingly, 2006; Chapter 2).

Firstly, while the PH1 domain of RasGrf1 is the major site of interaction between Trk and RasGrf1 (Ciccarelli et al., 2000; Robinson et al., 2005) and we find that it is in fact tyrosine phosphorylated in response to Trk activation, these sites of phosphorylation are not required during NGF-mediated neurite outgrowth in PC12 cells. The IQ domain of RasGrf1 also did not appear to be a site of tyrosine phosphorylation of RasGrf1 during NGF-mediated neurite outgrowth, although this domain has been indicated to cooperate with the PH domain in the interaction between Trk and RasGrf1 (Robinson et al., 2005). This result was expected as the IQ domain was suggested to bind calcium/calmodulin kinases in response to calcium (Buchsbaum et al., 1996; Farnsworth et al., 1995), by

which RasGrf1 undergoes serine phosphorylation at residue 916 (Farnsworth et al., 1995; Schmitt et al., 2005).

Secondly, we demonstrate that specific tyrosine sites in the CDC25 domain are critical for tyrosine phosphorylation of RasGrf1 during neurotrophin-mediated neurite outgrowth. Specifically, tyrosine 1048 and/or tyrosine 1062 appear to be crucial for neurotrophin-dependent RasGrf1 tyrosine phosphorylation as well as enhanced neurite outgrowth. Further study is required to understand whether one or both tyrosine sites are necessary for RasGrf1 tyrosine phosphorylation. To answer this question, site directed mutagenesis of only $Y^{1048}A$ or $Y^{1062}A$ is recommended for future study. Interestingly, both tyrosine sites are located approximately beside Tryptophan 1056 (W^{1056}), a site that has been indicated to be crucial for binding of RasGrf1 to Ras-GTPase (Vanoni et al., 1999). It is possible that these phosphorylation sites cooperate with W^{1056} to bind and/or activate Ras-GTPases.

Furthermore, in this study, the level of neurite outgrowth in RasGrf1-M5 still appeared to be slightly higher (nearly 2-fold) than negative control, and the level of reduction in tyrosine phosphorylation of RasGrf1-M5 is not completed, suggesting that other tyrosine residues might be involved in RasGrf1 tyrosine phosphorylation to maximize neurotrophin-mediated neurite outgrowth. RasGrf1 is also tyrosine phosphorylated by Src, which leads to the activation Rac (Kiyono et al., 2000a), suggesting that the DH domain of RasGrf1, the domain for Rac activation, might be a site of RasGrf1 tyrosine phosphorylation during neurotrophin-mediated neurite outgrowth. Since only the PH, IQ and CDC25 domains were investigated in this study, tyrosine phosphorylation sites in the DH domain of RasGrf1 remain to be investigated. In this

regard, the DH domain has eight tyrosine sites: Y^{259}, Y^{308}, Y^{337}, Y^{346}, Y^{367}, Y^{384}, Y^{392}, and Y^{415}, two of which (Y^{259} and Y^{415}) have been predicted to be sites of tyrosine phosphorylation and can be targeted by site directed mutagenesis in future studies. It would be also interesting to use this mutant construct to investigate RasGrf1-mediated GTPase activity *in vitro* in PC12 cells and *in vivo* in primary neuronal cultures to understand how tyrosine phosphorylation co-ordinates RasGrf1 toward either Ras or Rac activation.

Collectively, these data suggest a novel site of tyrosine phosphorylation within the CDC25 domain of RasGrf1 during neurotrophin-mediated neurite outgrowth, and that Y^{1048} and/or Y^{1062} are crucial for neurotrophin-mediated neurite outgrowth through RasGrf1 downstream of Trk receptors.

References

Anborgh P.H., Qian X., Papageorge A.G., Vass W.C., DeClue J.E., and Lowy D.R. (1999) Ras-specific exchange factor GRF: oligomerization through its Dbl homology domain and calcium-dependent activation of Raf. Mol. Cell. Biol. 19, 4611-22.

Balla T. (2005) Inositol-lipid binding motifs: signal integrators through protein-lipid and protein-protein interactions. J. Cell. Sci. 118, 2093-104.

Baouz S., Jacquet E., Bernardi A., and Parmeggiani A. (1997) The N-terminal moiety of CDC25Mm, a GDP/GTP exchange factor of Ras proteins, controls the activity of the catalytic domain: modulation by calmodulin and calpain. J. Biol. Chem. 272, 6671–6676.

Buchsbaum R.J., Telliez J.B., Goonesekera S., and Feig L.A. (1996) The N-Terminal Peckstrin, Coiled-Coil and IQ Domains of the Exchange Factor Ras-GRF act cooperatively to Facilitate Activation by Calcium. Mol. Cell. Biol. 16, 4888-4896.

Buday L., and Downward J. (1993) Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. Cell. 73, 611-20.

Cen H., Papageorge A.G., Vass W.C., Zhang K.E., and Lowy DR. (1993) Regulated and constitutive activity by CDC25Mm (GRF), a Ras-specific exchange factor. Mol. Cell. Biol. 13, 7718–7724.

Ciccarelli F.D., Acciarito A., and Alberti S. (2000) Large and diverse numbers of human diseases with HIKE mutations. Hum. Mol. Genet. 9, 1001-7.

Cowburn D. (1997) Peptide recognition by PTB and PDZ domains. Curr. Opin. Struct. Biol. 7, 835-8.

Crespo P., and Leon J. (2000). Ras proteins in the control of the cell cycle and cell differentiation. Cell. Mol. Life Sci. 57, 1613–1636.

Egan S.E., Giddings B.W., Brooks M.W., Buday L., Sizeland A.M., and Weinberg R.A. (1993) Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. Nature. 363, 45-51.

Fam N.P., Fan W.T., Wang Z., Zhang L.J., Chen Z., and Moran M.F. (1997) Cloning and characterization of Ras-GRF2, a novel guanine nucleotide exchange factor for Ras. Mol. Cell. Biol. 17, 1396–1406.

Farnsworth C.L., Freshney N.W., Rosen L.B., Ghosh A., Greenberg M. E., and Feig L.A. (1995) Calcium activation of Ras mediated by neuronal exchange factor Ras-GRF. Nature. 376, 524-527.

Freshney N.W., Goonesekera S.D., and Feig L.A. (1997) Activation of the exchange factor Ras-GRF by calcium requires an intact Dbl homology domain. FEBS Lett. 407, 111-5.

Gnesutta N., Ceriani M., Innocenti M., Mauri I., Zippel R., Sturani E., Borgonovo B., Berruti G., and Martegani E. (2001) Cloning and characterization of mouse UBPy, a deubiquitinating enzyme that interacts with the ras guanine nucleotide exchange factor CDC25(Mm)/Ras-GRF1. J. Biol. Chem. 276, 39448–39454.

Hanks S.K., Quinn A.M., and Hunter T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science. 241, 42-52.

Harlan J.E., Hajduk P.J., Yoon H.S., and Fesik S.W. (1994) Pleckstrin homology domains bind to phosphatidylinositol-4, 5-bisphosphate. Nature. 371, 168–170.

Heldin C.H. (1995) Dimerization of cell surface receptors in signal transduction. Cell. 80, 213-23.

Huang E.J, and Reichardt L.F. (2003) Trk receptors: roles in neuronal signal transduction . Annu. Rev. Biochem. 72, 609–642.

Hubbard S.R. (1997) Crystal structure of the activated insulin receptor tyrosine kinase in complex with peptide substrate and ATP analog. EMBO. J. 16, 5572-81.

Ibanez C.F., Ilag L.L., Murray-Rust J., and Persson H. (1993) An extended surface of binding to Trk tyrosine kinase receptors in NGF and BDNF allows the engineering of a multifunctional pan-neurotrophin. EMBO. J. 12, 2281-93.

Innocenti M., Zippel R., Brambilla R., and Sturani E. (1999) CDC25(Mm)/Ras-GRF1 regulates both Ras and Rac signaling pathways. FEBS. Lett. 460, 357–362.

Katoh H., Yasui H., Yamaguchi Y., Aoki J., Fujita H., Mori K., and Negishi M. (2000) Small GTPase RhoG is a key regulator for neurite outgrowth in PC12 cells. Mol. Cell. Biol. 20, 7378-87.

Kiyono M., Satoh T., and Kaziro y. (1999) G protein beta gamma subunit-dependent Rac-guanine nucleotide exchange activity of Ras-GRF1/CDC25(Mm). Proc. Natl. acad. Sci. U S A. 96, 4826-31.

Kiyono M., Kaziro Y., and Satoh T. (2000a) Induction of Rac-Guanine Nucleotide Exchange Activity of Ras-GRF1/CDC25Mm following Phosphorylation by the Nonreceptor Tyrosine Kinase Src. J. Biol. Chem. 275, 5441-5446.

Kiyono M., Kato J., Kataoka T., Kaziro Y., and Satoh T. (2000b). Stimulation of Ras guanine nucleotideexchange activity of Ras-GRF1/CDC25(Mm) upon tyrosine phosphorylation by the Cdc42-regulated kinase ACK1. J. Biol. Chem. 275, 29788–29793.

Lemmon M.A., and Ferguson K.M. (2000) Signal-dependent membrane targeting by pleckstrin homology (PH) domains, Biochem. J. 350, 1–18.

Malumbres M., and Pellicer A. (1998) Ras pathways to cell cycle control and cell transformation. Front. Biosci. 3, d887–d912.

Martegani E., Vanoni M., Zippel R., Coccetti P., Brambilla R., Ferrari C., Sturani E., and Alberghina L. (1992) Cloning by functional complementation of a mouse cDNA encoding a homologue of CDC25, a Saccharomyces cerevisiae RAS activator. EMBO. J. 11, 2151-7.

Mattingly R.R., and Macara I.G. (1996) Phosphorylation-dependent activation of the Ras-GRF/CDC25Mm exchange factor by muscarinic receptors and G-protein beta-gamma subunits. Nature. 382, 268-272.

Mattingly R.R. (1999) Phosphorylation of Serine 916 of Ras-GRF1 Contributes to the Activation of Exchange Factor Activity by Muscarinic Receptors. Journal of Biological Chemistry. 274, 37379-37384.

MacDonald J.I., Verdi J.M., and Meakin S.O. (1999) Activity-dependent interaction of the intracellular domain of rat trkA with intermediate filament proteins, the beta-6 proteasomal subunit, Ras-GRF1, and the p162 subunit of eIF3. J. Mol. Neurosci. 13, 141-58.

Meakin S.O. (2000) Nerve Growth Factor Receptors and Mechanisms of Intracellular Signal Transduction. Recent Research Developments in Neurochemistry. 3, 75-91.

Meakin S.O., and MacDonald J.I. (1998) A novel juxtamembrane deletion in rat TrkA blocks differentiative but not mitogenic cell signaling in response to nerve growth factor. J. Neurochem. 71, 1875-88.

Meakin S.O., MacDonald J.I.S., Gryz E.A., Kubu C. J., and Verdi J.M. (1999) The Signaling Adapter Protein FRS-2 Competes with Shc for binding to TrkA: A Model for Discriminating Proliferation and Differentiation. J. Biol. Chem. 274, 9861-9870.

Minichiello L. (2009) TrkB signalling pathways in LTP and learning. Nat. Rev. Neurosci. 10, 850-60.

Reichardt L.F. (2006) Neurotrophin-regulated signaling pathways. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 361, 1545-1564.

Robinson K.N., Manto K., Buchsbaum R.J., MacDonald J.I.S., and Meakin S.O. (2005) Neurotrophin-dependent Tyrosine Phosphorylation of Ras Guanine-Releasing Factor 1 and Associated Neurite Outgrowth is Dependent on the HIKE Domain of TrkA. J. Biol. Chem. 280, 225-235.

Robinson K., 2008. The role of RasGrf1 in Trk receptor signaling. Chapter 3 and 4. The Uniersity of Western Ontario, London, ON.

Rogers S., Wells R., and Rechsteiner M. (1986) Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis, Science. 234, 364–368.

Schmitt J.M., Guire E.S., Saneyoshi T., and Soderling T.R. (2005) Calmodulin-Dependent Kinase Kinase/Calmodulin Kinase I Activity Gates Extracellular-Regulated Kinase-Dependent Long-Term Potentiation The Journal of Neuroscience. 25, 1281–1290.

Shou C., Farnsworth C.L., Neel B.G., and Feig L.A. (1992) Molecular cloning of cDNAs encoding a guanine-nucleotide-releasing factor for Ras p21. Nature 358, 351–354.

Shou C., Wurmser A., Suen K.L., Barbacid M., Feig L.A., and Ling K. (1995) Differential response of the Ras exchange factor, Ras-GRF to tyrosine kinase and G protein mediated signals. Oncogene 10, 1887–1893.

Stephens R.M., Loeb D.M., Copeland T.D., Pawson T., Greene L.A., and Kaplan D.R. (1994) Trk receptors use redundant signal transduction pathways involving SHC and PLC-gamma 1 to mediate NGF responses. Neuron. 12, 691-705.

Sturani E., Abbondio A., Branduardi P., Ferrari C., Zippel R., Martegani E., Vanoni M., and Denis-Donini S. (1997) The Ras Guanine nucleotide Exchange Factor CDC25Mm is present at the synaptic junction. Exp. Cell. Res. 235, 117-23.

Tian X., and Feig L.A. (2001) Basis for signaling specificity difference between Sos and Ras-GRF guanine nucleotide exchange factors. J. Biol. Chem. 276, 47248-56.

Tian X., Gotoh T., Tsuji K., Lo EH., Huang S., and Feig L.A. (2004) Developmentally regulated role for Ras-GRFs in coupling NMDA glutamate receptors to Ras, Erk, and CREB. EMBO. J. 23, 1567–1575.

Touhara K., Inglese J., Pitcher J., Shaw G., and Lefkowitz R.J. (1994) Binding of G protein beta gamma-subunits to pleckstrin homology domains. J. Biol. Chem. 264, 10217–10220.

Vanoni M., Bertini R., Sacco E., Fontanella L., Rieppi M., Colombo S., Martegani E., Carrera V., Moroni A., Bizzarri C., Sabbatini V., Cattozzo M., Colagrande A., and Alberghina L. (1999) Characterization and Properties of Dominant-negative Mutants of the Ras-Specific Guanine Nucleotide Exhange Factor CDC25^{Mm}. J. Biol. Chem. 274, 36656-36662.

Varnai P., Lin X., Lee S.B., Tuymetova G., Bondeva T., Spät A., Rhee S.G., Hajnóczky G., and Balla T. (2002) Inositol lipid binding and membrane localization of isolated pleckstrin homology (PH) domains. Studies on the PH domains of phospholipase C delta 1 and p130. J. Biol. Chem. 277, 27412-22.

Wei W., Mosteller R.D., Sanyal P., Gonzales E., McKinney D., Dasgupta C., Li P., Liu B.X., and Broek D. (1992) Identification of a mammalian gene structurally and functionally related to the CDC25 gene of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA. 89, 7100–7104.

Wei W., Das B., Park W., and Broek D. (1994) Cloning and analysis of human cDNAs encoding a 140-kDa brain guanine nucleotide-exchange factor, Cdc25GEF, which regulates the function of Ras. Gene 151, 279–284.

Yang H., and Mattingly R.R. (2006) The Ras-GRF1 Exchange Factor Coordinates Activation of H-Ras and Rac1 to Control Neuronal Morphology. Molecular Biology of the Cell. 17, 2177–2189.

Yang H., Cooley D., Legakis J., Qingyuan G., Andrade R., and Mattingly R. (2003) Phosphorylation of the RasGrf1 Exchange Factor at Ser916/898 Reveals Activation of Ras Signaling in the Cerebral Cortex. J. Biol. Chem. 278, 13278-13285.

Zippel R., Orecchia S., Sturani E., and Martegani E. (1996) The brain specific Ras exchange factor CDC25 Mm: modulation of its activity through Gi-protein-mediated signals. Oncogene. 12, 2697–2703.

Zippel R., Gnesutta N., Matus-Leibovitch N., Mancinelli E., Saya D., Vogel Z., Sturani E., Renata Z., Nerina G., Noa M. L., Enzo M., Daniella S., Zvi V., and Emmapaola S. (1997) Ras-Grf, the activator of Ras, is expressed preferentially in mature neurons of the central nervous system. Brain Res. Mol. Brain Res. 48, 140-144.

Chapter 4

RasGrf1 cross talks between TrkB and NMDA receptors

4.1 Introduction

The high affinity tropomyosin-related kinases (Trk), including TrkA, B and C, are receptors for a family of closely related growth factors collectively termed the neurotrophins. These neurotrophins include nerve growth factor (NGF) which binds TrkA, brain-derived neurotrophic factor (BDNF) and neurotrophin 4 (NT4) which bind TrkB, and neurotrophin 3 (NT3) which binds TrkC respectively (Meakin, 2000; Reichardt, 2006). The Trk receptor tyrosine kinases function primarily in the regulation of pathways involved in neuronal differentiation and survival. Of particular relevance to my thesis, TrkB regulates synapse development and plasticity in the central nervous system in addition to the mechanoreceptive sensory neurons in the peripheral nervous system (Reichardt, 2006; Kaplan and Miller, 2000; Huang and Reichardt, 2001).

The TrkB receptor is expressed widely on both presynaptic and postsynaptic membranes of the nerve terminal and dendritic spines inside the brain including the cortex and hippocampus. Accordingly, in addition to its well known effects on neuronal outgrowth and differentiation, BDNF-mediated TrkB activation is crucial in various aspects of synaptic plasticity and modulates dendritic branches and spine formation in the entire brain including the cortex, hippocampus, cerebellum and amygdala (Carvalho et al., 2008; Lu et al., 2008; Amaral et al., 2007; Minichiello, 2009).

By indirectly modulating the N-methyl-D-aspartate (NMDA) receptor, BDNF contributes in the regulation of the molecular processes of learning and memory namely long-term potentiation (LTP) and long-term depression (LTD) (Wu et al., 2004; Xu et al.,

2006; Kang et al., 1997; Akaneya et al., 1997; Lessmann and Heumann, 1998; Levine and Kolb, 2000; Li et al., 1998). The evidence for a role of BDNF-mediated TrkB activation of LTP comes from the fact that any deletion in the *Bdnf* or *trkB* genes, *in vivo* blocking of BDNF binding to TrkB and/or pre-treating hippocampal slices with TrkB antiserum, has been found to either impair or significantly reduce the induction of LTP (Reichardt, 2006; Minichiello, 2009). LTP can be observed in the hippocampus, and also in other neural structures including the cerebral cortex, cerebellum and amygdala (Bauer et al., 2002; Nakazawa et al., 2006; Minichiello, 2009). Furthermore, LTP is dependent or independent of the NMDA receptor. However, the NMDA receptor-dependent model of LTP has been widely studied in the hippocampal area (Lisman, 2003).

The NMDA receptor is an ionotropic channel that upon activation by *in vivo* glutamate or *in vitro* NMDA, allows the intracellular flow of Na^+, and to a lesser extent Ca^{2+}, as well as extracellular flow of K^+. Structurally, it is a heterotetrameric complex made up of two obligatory NR1 subunits, and two modulatory NR2 (A-D) subunits which control the electrophysiological properties of the NMDA receptor (Kutsuwada et al., 1992; Monyer et al., 1992; Paoletti, and Neyton, 2007; Kohr, 2006). Although the NMDA receptor contributes to both LTP and LTD, several studies suggest that the NR2A subunits of the NMDA receptor contribute to LTP whereas the NR2B subunits promote LTD (Li et al, 2006; Kollen et al., 2008; Liu et al., 2004; Massey et al., 2004).

The cell surface localization of the NMDA receptor is regulated by clathrin-mediated endocytosis in which the [1472]YEKL motif on the NR2B subunit serves a critical role in this internalization. It has been shown that phosphorylation of tyrosine 1472 (Tyr[1472]) in the YEKL motif by Src family kinases uncouples the receptor from clathrin-

mediated endocytosis and increases the cell surface retention and activity of the NMDA receptor (Nakazawa et al., 2001; Prybylowski et al., 2005).

Although the mechanism by which BDNF regulates NMDA receptor-mediated neural plasticity is largely unknown, it appears that BDNF activation of TrkB modulates the tyrosine phosphorylation of the NR2B subunit of the NMDA receptors through which increases the retention of the receptors on the postsynaptic cell surface (Nakazawa et al., 2001; Prybylowski et al., 2005). As a result of prolonged surface retention of the NMDA receptor, and responding to L-glutamate, the intracellular calcium levels increase in the postsynaptic neuron (Minichiello, 2009; Lin et al., 1998; Levine et al., 1998; Levine and Kolb, 2000). Increases in the Ca^{2+} influx in postsynaptic neurons results in the activation of a number of signaling molecules including protein kinase C (PKC), protein kinase A (PKA) and calcium calmodulin-activated kinases (CaMKII and IV) (Ghosh and Greenberg, 1995; West et al., 2001) leading to the initiation of signaling cascades that underlie neuronal development and synaptic plasticity (Li et al., 2006; Minichiello, 2009).

Among these kinases, PKA and CaMKII have been suggested to activate a Ras Guanine nucleotide exchange factor (RasGrf1)-dependent signaling pathway (Farnsworth et al., 1995) through phosphorylation of serine 916 (S^{916}) on RasGrf1 (Schmitt et al., 2005), and tyrosine-phosphorylation, at unknown sites, through non-receptor tyrosine kinases such as Src (Kiyono et al., 2000a) or the Cdc42-GTPase-dependent of the kinase Ack1 (Kiyono et al., 2000b).

Ras-Guanine nucleotide Releasing Factors (RasGrfs) consist of two forms: RasGrf1 (140 kDa) which is highly expressed in the nervous system (Zippel et al., 1997),

and RasGrf2 (130 kDa), which shows a high degree of sequence homology (80%) to RasGrf1, and is expressed ubiquitously, but is also present within the mature brain (Fam et al., 1997; Anborgh et al., 1999). RasGrfs have been suggested to be a missing link in the NMDA receptor regulation of synaptic plasticity. They act as a specific calcium sensor to transmit signals from the NMDA receptor to the activation of appropriate GTPases and the induction of LTP or LTD (Li et al., 2006).

It has been suggested that RasGrf1 interacts directly with the NR2B subunit of the NMDA receptor (Kraivinsky et al., 2003), through which RasGrf1 stimulates p38/MAP kinase activation leading to an increase in LTD. In contrast, RasGrf2 interacts with the NR2A subunit of the NMDA receptor and activates Erk/MAP kinase leading to an increase in LTP. However, both interactions are observed in a developmental-dependent manner, in particular after postnatal day 20 to 25 in the mouse (Li et al., 2006). It has been further shown that RasGrf1 promotes p38 activation by Rac *in vitro* while RasGrf2 activates Erk-MAP kinase by targeting Ras-GTPases (Buchsbaum et al., 2002; Tian et al., 2004).

We have previously identified RasGrf1 as a novel substrate of Trk to facilitate neurite outgrowth in response to low doses of NGF stimulation in PC12 cells and BDNF stimulation in PC12-derived cells overexpressing TrkB namely TrkB-B5 cells (Robinson et al., 2005; Chapter 2). In this study, the potential of RasGrf1 to co-ordinate the activation and function of both TrkB and NMDA receptors was addressed using cortical and hippocampal slices from postnatal-30 adult mice. Furthermore, the interactions between the NR2B subunit and TrkB with RasGrf1 were determined, and the changes in

activation of Erk and p38 MAP kinase were characterized in response to BDNF and/or NMDA stimulation.

4.2 Materials and Methods

4.2.1 Reagents.

The anti-RasGrf1 (C-20, sc-224), anti-RasGrf2 (sc-7591), anti-Trk (sc-11), anti-NMDA NR2B (NMDAε2 sc-1469), anti-NMDA NR2A (NMDAε1 sc-9056), anti-PSD95 (7E3, sc-6926), anti-Shc (sc-28883), normal mouse IgG (sc-2343), normal rabbit IgG (sc- 2027 and normal goat IgG (sc-2028) antibodies were from Santa Cruz. The anti-TrkB mouse antibody #1494 was from (BioVision). Mouse anti-TrkB monoclonal antibody (610102), anti-ShcC (#610643) and anti-RasGrf2 (#610840) antibodies were from Transduction Labs. Rabbit anti-Trk antibodies (Trk1478) to the carboxyl-terminal 14 residues of TrkA were prepared and affinity purified using standard techniques (Robinson, 2008). Anti-phosphotyrosine (p-Tyr-100, #9411S), anti-phospho-p38 MAP Kinase (Thr180/Tyr182, #9211S), anti-p38 MAP Kinase (9212), anti-phospho-Erk p44/42 (Thr202/Tyr204, #9106S) and anti-Erk p44/42 MAP Kinase (#9102) antibodies were from Cell Signaling. HRP-coupled goat anti-mouse, rabbit anti-goat and goat anti-rabbit secondary antibodies were from Jackson Laboratories. Human recombinant BDNF was obtained from R & D Systems and used at a concentration of 100 ng/ml final. NMDA (M3262) was used at a final concentration of 100 μM and was from Sigma. NMDA was used in conjunction with 10 mM KCl to promote depolarization.

4.2.2 Plasmids and Cell Lines.

The plasmids pEFP-RasGrf1 and pEFP-RasGrf2 (Anborgh et al., 1999), pCMX-rat TrkB (wild-type and kinase-dead) have been described previously (Meakin and MacDonald,

1998). HEK 293T cells (Graham et al., 1977) were cultured under standard conditions in Dulbecco's modified Eagle media (DMEM) with 5% supplemented calf serum (SCS) and 5% fetal bovine serum (FBS)(Hyclone) with 50 µg/ml gentamycin sulfate (Sigma). PC12 rat adrenal pheochromocytoma cells were maintained in DMEM with 5% SCS and 5% horse serum (Hyclone). TrkB-B5 [nnR5 cells stably overexpressing HA-tagged TrkB receptors (pCMX-HATrkB)] have been described previously (Meakin and MacDonald, 1998; Meakin, 2000). These cells were cultured in 5% SCS and 5% horse serum in the presence of 100 µg/ml G418 and 50 µg/ml gentamycin sulfate.

4.2.3 Transfections, Immunoprecipitations, Western Blots.

Transfections were performed following standard calcium phosphate (HEK 293T) or lipofectamine 2000 (PC12 cells, Invitrogen) transfection approaches (Jordan et al., 1996). Lipofectamine transfection was performed according to manufacturer's protocol with a ratio of DNA to lipofectamine of 1:2. Basically, 1.5×10^6 cells (100 mm dish) were co-transfected with 0.5-5 µg of each indicated plasmid. Following 48 hrs expression, lysates were prepared in NP40 lysis buffer (1% Nonidet P-40, 137 mM NaCl, 20 mM Tris (pH 8.0), 0.5 mM EDTA) containing 1 mM PMSF, 1 mM sodium orthovanadate ($NaVO_4$), 10 µg/ml aprotinin and 10 µg/ml leupeptin and assayed by immunoprecipitation. Lysates containing 0.5 to 3.5 mg of protein (concentrations determined using the DC Protein Assay Kit from Bio-Rad) were immunoprecipitated with 0.5 µg anti-Trk1478, 1 µg Trk1494 (BioVision) or 1 µg anti-RasGrf1 as indicated, in addition to 5 µl of washed Pansorbin (Calbiochem) or 2 µl γ-bind sepharose (Amersham Pharmacia Biotech) at 4 ºC overnight. After washing three times with NP-40 lysis buffer, bound proteins were re-suspended in 25 µl Laemmli sample buffer and heated at 65 °C for 10 minutes. Samples

were then resolved on 6-12% SDS-polyacrylamide gels and transferred (5 volts, 1-2 hours) to PVDF membranes (Pall Life Sciences). Blocking prior to primary antibody incubation was performed for 1 h at room temperature in 10 ml 10% milk powder and phosphate buffered saline with tween-20 (TBS-T) or 2% BSA (for phospho-antibodies). Westerns were blotted in 10 ml 10% milk powder with primary antibody at 4 ºC for 16 h followed by washing in TBS-T for 30 minutes. Westerns were then incubated in 10 ml 10% milk powder and secondary antibody for 1 h at room temperature. Blots were washed for 30 minutes in TBS-T and exposed to enhanced chemiluminescence reagents (BioRad) and developed.

Primary antibody dilutions for western blotting are as follows: anti-RasGrf1 and anti-RasGrf2 (Santa Cruz) 1:10,000; anti-RasGrf2 (Transduction Labs) (1:5000), anti-Trk(1478) (1:20,000), anti-TrkB 1494 (1:5000), anti-TrkB monoclonal antibody (Transduction Labs) (1:2000), anti-NR2B (1:2000), anti-NR2A (1:2000), anti-PSD95 (1:2000), anti-ShcC (1:2000) and anti-pTyr-100 (1:10,000). anti-phospho-p38 MAP Kinase (1:2000), anti-p38 MAP Kinase (1:2000), anti-phospho-Erk p44/42 MAP Kinase (1:5000) and anti-Erk p44/42 MAP Kinase (1:5000). HRP coupled goat anti-mouse and goat anti-rabbit secondary antibodies were used at 1:10,000 dilution while HRP-coupled rabbit anti-goat was used at 1:5000 dilution.

4.2.4 Neural Slice Analysis.

Post-natal day 30-35 male CD1 mice were killed with O_2/CO_2 and their cortices dissected into the ice-cold-fresh- 95%O_2/5%CO_2 saturated Kreb's Ringer Solution (11.1 mM glucose, 1.1 mM $MgCl_2$, 1 mM Na_2HPO_4, 1.3 mM $CaCl_2$, 25 mM $NaHCO_3$, 120 mM NaCl, 4.7 mM KCl). Cortices were then embedded in 2% agarose and 300 μm coronal

slices were cut with a vibrotome (frequency 8 Hz, speed 4 Hz). Four slices of cortex (10 slices of hippocampus) were placed into each well of a 6 well plate and incubated in 2 ml Kreb's Ringer Solution for 1 h at 37° C with 95% O_2/5%CO_2. Slices were then stimulated for 15 minutes at 37 °C with 100 ng/ml BDNF, 100 μM NMDA with 10 mM KCl. Slices were removed from Kreb's Ringer and immediately lysed in lysis buffer A (10 mM Tris-HCl pH7.5, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM $NaVO_3$). Samples were briefly homogenized with a Polytron (2×30 second) followed by centrifugation at 1400 rpm for 10 minutes at 4° C. The supernatant was removed, transferred to microfuge tubes and centrifuged again for 20 minutes at 4° C, 14,000 rpm (eppendorf Centrifuge 5417R, Hamburg, Germany). A protein assay was performed on each sample using a Bio-Rad Kit to determine the protein concentrations in each lysate. For co-immunoprecipitation in neural slices, prepared lysates (750 μg to 1.5 mg) were pre-cleared with γ-bind sepharose and normal mouse, goat or rabbit IgG (depending on specificity of the primary antibody to be used) for one hour. Following centrifugation at 4 °C, 14,000 rpm, supernatants were immunoprecipitated with anti- NR2B (1 µg), anti-TrkB (2 µg), anti-NR2A (1 µg) or IgG (1 µg) (as a negative control) in LysisA buffer with 2 µl γ- bind sepharose for 16 h at 4 °C. Immunoprecipitations were washed twice in lysis buffer A, re-suspended in Laemmli sample buffer with 100 mM dithiotreitol (DTT) and incubated at 70 °C for 10 minutes. Samples were separated by 6% SDS-PAGE and western blotted with indicated antibodies. For analysis of phosphorylation of Erk and p38 MAP kinase, Laemmli sample buffer with 100 mM DTT was added to whole cell lysates from treated P16 or P30 slices and samples incubated at 70 °C for 10 minutes. Samples were separated by 12% SDS-

PAGE and western blotted as mentioned above. The band intensity was quantified using a BioRad imaging system (ChemiDoc^{TM}). Relative densities were evaluated compared to levels to that in the control lane as indicated in each figure. The means of three separate slice preparation were evaluated. Relative densities were then statistically analysed by paired student t-test for significant differences between two different stimulation conditions.

4.3 Results

A coordination of TrkB and NMDA receptors through the downstream signaling molecule, RasGrf1, was investigated in this study using post-natal day 30 (P30) acute cortical slices after stimulation with either BDNF and/or NMDA. Since several studies have suggested that RasGrf1 expression is regulated developmentally in the mouse brain (Ferrari et al., 1994; Zippel et al., 1997) with a maximum level being expressed in adult mouse cortex (2 months) (Li et al., 2006; Robinson, 2008), this study was performed of an age that was optimal for RasGrf1 expression.

4.3.1 BDNF stimulation disrupts the interaction between NR2B and RasGrf1 in neural slices.

As mentioned earlier, phosphorylation of Tyr^{1472} on the NR2B subunit of the NMDA receptor is necessary to retain the receptors on the cell surface (Nakazawa et al., 2001; Prybylowski et al., 2005; Wu et al., 2007). Moreover, a direct interaction between the NR2B subunit of the NMDA receptor and RasGrf1 has been observed in primary cell culture (Krapivinsky et al., 2003). Thus, the phosphorylation status of NR2B and changes in RasGrf1 binding to NR2B were determined in adult mouse brain slices after stimulation with either BDNF and/or NMDA. As shown in Figure 4.1A, while there is a

basal level of Tyr^{1472} phosphorylation of NR2B under basal conditions (Figure 4.1A, left panel, lane 1), this level of phosphorylation is increased more than 2-fold after BDNF stimulation (lane 2). In comparison, co-stimulation with NMDA does not stimulate Tyr1472 phosphorylation (Figure 4.1A, lane 3) and, in fact, decreases or prevents the levels stimulated by BDNF (Figure 4.1A, left panel, lane 4). The expression level of NR2B was verified by blotting with the anti-NR2B antibody (Figure 4.1A, lower panel). Quantification of the levels of Tyr^{1472} phosphorylation is shown in the right panel (Figure 4.1A).

In addition, a small basal level of interaction between NR2B and RasGrf1 was detected in the absence of BDNF/NMDA stimulation (Figure 4.1B, left, lane 1) relative to negative control levels obtained with IgG (Figure 4.1B, lane 5), and this interaction was significantly increased almost 2-fold in the presence of NMDA (Figure 4.1B, left, lane 3). However, an interaction between RasGrf1 and NR2B was not observed following BDNF stimulation (Figure 4.1B, Lane 2) and the NMDA-induced increase in interaction was lost in the presence of BDNF (Figure 4.1B, left, lane 4). This result suggests that BDNF-mediated TrkB activation may regulate NMDA receptor signaling by stimulating RasGrf1 dissociation from the NR2B subunit. The expression level of NR2B was verified using the anti-NR2B antibody (Figure 4.1B, lower panel), and a quantification of the levels of NR2B-RasGrf1 interaction, normalized to the level of NR2B, is shown in the right panel (Figure 4.1B, right).

Since NR2A subunit of the NMDA receptor interacts with RasGrf2 (Li et al., 2006), and the PH domain of RasGrf1 which mediates binding to Trk (Robinson et al., 2005; Graham et al., 1977) is highly similar to the PH domain of RasGrf2; thus, BDNF-

dependent changes in the interaction between NR2A and RasGrf2 was also tested. As shown in Figure 4.1C, NR2A was co-immunoprecipitated with RasGrf2 in response to NMDA (lane 5) stimulation relative to the negative control of IgG alone (Figure 4.1C, lanes 1 and 2). However, no loss of NMDA-induced interaction was observed in the presence of BDNF (Figure 4.1C, lane 6), which is in contrast to what was observed with NR2B and RasGrf1. These data suggest that BDNF stimulation does not change the interaction between NR2A and RasGrf2, and that BDNF-TrkB modulation of the NMDA receptor is specific to RasGrf1 and NR2B.

4.3.2 BDNF stimulation increases the association of RasGrf1 towards TrkB.

A previous study indicated a direct interaction between Trk and RasGrf1 in transfected HEK293 cells and consequently, the binding sites on both TrkB and RasGrf1 have been mapped (Robinson et al., 2005). Thus, the interaction between Trk and RasGrf1 was also assessed by co-immunoprecipitation of lysates of P30 cortical slices to determine the association of RasGrf1 to TrkB after BDNF stimulation.

As shown in Figure 4.2A, while there is a basal interaction between RasGrf1 and TrkB before stimulation (Figure 4.2A, left, lane 3), relative to negative control of IgG (Figure 4.2A, left, lane 7 and 8), this interaction was significantly increased almost 2.5-fold upon BDNF stimulation (Figure 4.2A, left, lane 4) compared to unstimulated condition, indicating that BDNF stimulation increases RasGrf1 association with TrkB. To further verify the interaction between TrkB and RasGrf1, an immunoprecipitated sample of HEK293 cells, transfected with TrkB and RasGrf1, and stimulated with BDNF was also added to this study (Figure 4.2A, left, lane 1 and 2). The level of TrkB expression was verified using an antibody against TrkB (Figure 4.2A, left lower panel), and a

quantification of the level of interaction is presented in the right panel. Collectively, this data as well as the results from the RasGrf1/NR2B dissociation studies suggest that BDNF stimulation uncouples RasGrf1 from the NMDA receptor toward the TrkB receptor.

The levels of TrkB and RasGrf1 tyrosine phosphorylation were then analyzed in the P30 mouse cortical slices. As shown in Figure 4.2B (left panel), tyrosine phosphorylation of TrkB was increased significantly after BDNF stimulation alone (lane 2), and the site of phosphorylation appeared to be Tyr^{516} on TrkB (Figure 4.2B, middle panel), but not Tyr^{817} (Figure 4.2B, middle panel). The immunoprecipitation of TrkB was verified in all treatment using an anti-TrkB antibody to detect TrkB expression (Figure 4.2B, lower panels). While TrkB was tyrosine phosphorylated in response to BDNF in cortical slices, weak but insignificant levels of RasGrf1 tyrosine phosphorylation were detected in both the presence of BDNF alone or in the presence of BDNF and NMDA (Figure 4.2C).

4.3.3 BDNF stimulation modifies the ShcC adapter downstream of TrkB and PSD-95 downstream of NR2B receptors.

In addition to a direct interaction between TrkB and RasGrf1 in response to BDNF, Shc has also been shown to be tyrosine phosphorylated (Figure 4.3A), and interact with Trk (Figure 4.3B), in the presence of BDNF alone and upon stimulation by both BDNF and NMDA in P30 cortical slices (Figure 4.3B, lanes 2 and 4), but not in the presence of NMDA alone (lane 3) (Robinson, 2008). Furthermore, since RasGrf1 is highly expressed at post-synaptic densities (Zippel et al., 1997) and the post-synaptic density protein 95 (PSD-95) is a major regulator of NMDA receptor signalling, that

facilitates the phosphorylation of NR2B by Src family kinases (van Zundert et al., 2004). Thus, it is likely that NMDA would facilitate an interaction between NR2B and PSD-95. As shown in Figure 4.3C (Robinson, 2008), a basal level of interaction was observed between NR2B and PSD-95 in absence of any treatment (lane 1), but this interaction was remarkably increased in treatment by NMDA alone (lane 3) similar to what observed between RasGrf1 and NR2B (Figure 4.1B, lane 3).

Since we found that the site of BDNF-induced tyrosine phosphorylation on TrkB was tyrosine[516] (Figure 4.2B), the docking site for ShcC (Minichiello, 2009), I addressed whether ShcC might also form a complex with RasGrf1 which binds the adjacent HIKE domain (His[531] to Glu[542]) on TrkB and whether RasGrf1 might form part of a signalling complex downstream of the NMDA receptors. Thus, I tested for direct interactions between RasGrf1 and both PSD-95 and ShcC under basal or stimulated conditions. However, no interaction was observed between RasGrf1 and either ShcC or PSD-95 in response to either BDNF or NMDA stimulation in this study (Figures 4.3D and 4.3E).

4.3.4 BDNF stimulation alters NMDA-mediated Erk and p38-Map Kinase phosphorylation in an age specific manner.

A study by Li et al (2006) suggests that NR2B/RasGrf1 interaction increases the activation of p38, while NR2A/RasGrf2 interaction enhances the activation of Erk 1 and 2 (Li et al., 2006). Thus, the phosphorylation levels of the p38 and Erk MAP-kinases were evaluated in this study, in response to BDNF and/or NMDA.

As shown in Figure 4.4A, a basal level of Erk1/2 was observed under basal conditions (Figure 4.4A, lane 1). Subsequently, a small but significantly higher level of Erk phosphorylation was observed in response to 15 minutes of BDNF stimulation

relative to the unstimulated condition (lane 2). While NMDA treatment did not stimulate a change in levels of Erk phosphorylation relative to controls, a significant decrease in Erk1/2 phosphorylation was observed after treatment with both BDNF/NMDA (Figure 4.4A, lane 4) relative to that observed with BDNF treatment alone. The levels of Erk expression are shown in the lower panel of Figure 4.4A and a quantification of the levels of Erk phosphorylation is shown in the right panel.

With respect to p38, I found basal levels of p38-MAP kinase phosphorylation under both basal conditions and following BDNF stimulation (Figure 4.4B, lane 2). In contrast, I observed a substantial increase (almost 2-fold) in the levels of p38 phosphorylation upon NMDA treatment (lane 3). When slices were co-stimulated with both BDNF/NMDA, there was a slight decrease, that was not statistically significant, in the level of p38-MAP kinase phosphorylation and the levels of phosphorylation were still significantly higher than unstimulated controls (lane 4). Quantification of the levels of p38 phosphorylation, are shown in the right panel. Collectively, these observations suggest that BDNF stimulation modulates the activity of Erk and p38-MAP kinase downstream of NMDA receptor.
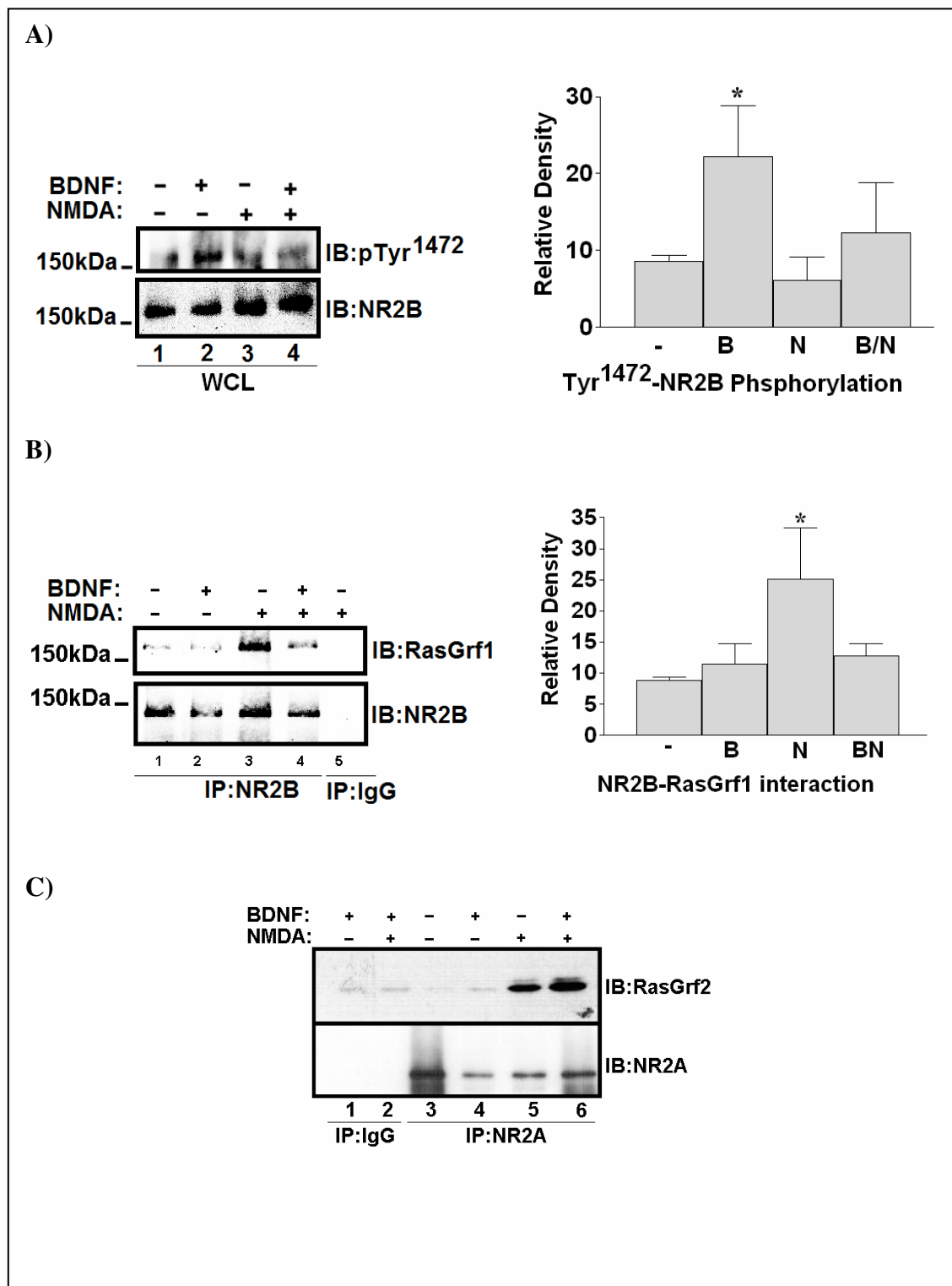
Figure 4.1. The phosphorylation of NR2B and its interaction with RasGrf1 in P30 brain slices. Cortical slices were lysed after BDNF and/or NMDA stimulation, and 3 mg lysates were immunoprecipitated (IP) with the NR2B or NR2A antibodies. Ips or a whole cell lysate (WCL; 100 μg) were analyzed by SDS-PAGE, and blotted (IB) with the indicated antibodies (n=3). * indicates statistical significance (P-value<0.05) of indicated condition relative to unstimulated condition. **A)** Tyrosine[1472] phosphorylation assessment of NR2B subunit in WCL in response to BDNF and/or NMDA (n=3). The level of Tyr[1472] phosphorylation was increased more than 2-fold in the presence of BDNF relative to the unstimulated lane. Relative density of this phosphorylation levels are shown in the right panel. The blots were normalized to the level of NR2B expression. **B)** Interaction between NR2B and RasGrf1 upon BDNF and/or NMDA stimulation in P30 slices. NR2B interaction with Ras-Grf1 was increased almost 2-fold in response to NMDA relative to the unstimulated lane, and BDNF prevents this interaction. Goat immunoglobulin (IgG) was used as a negative control. A quantification of the interaction levels are shown in the right panel. Blots were normalized to the levels of NR2B expression. **C)** The interaction between NR2A and RasGrf2. BDNF stimulation does not change the interaction between RasGrf2 and NR2A (Robinson, 2008).
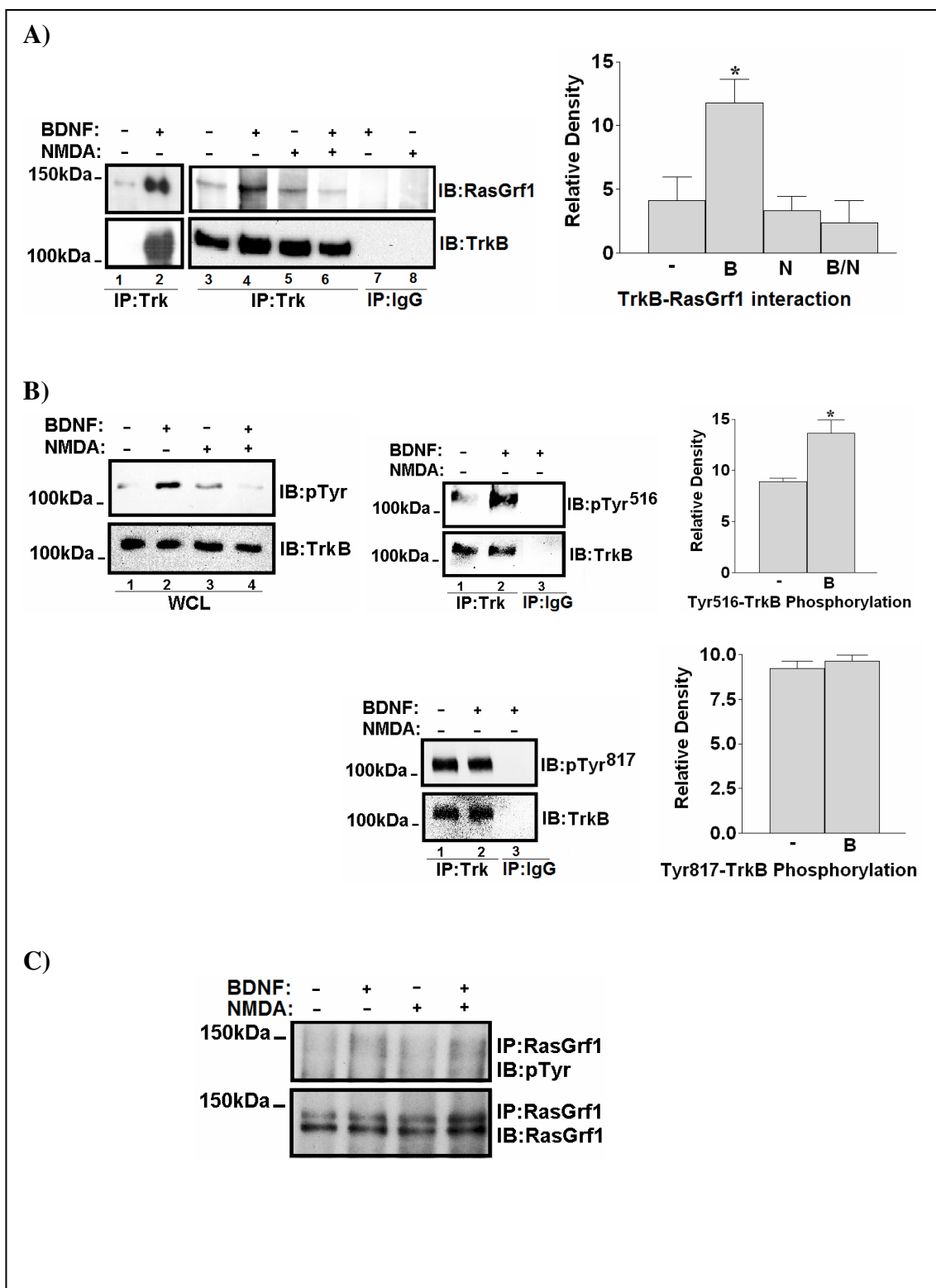
Figure 4.2. The interaction between Trk and RasGrf1 and the level of Trk/RasGrf1 phosphorylation in P30 brain slices. Cortical slices were lysed after BDNF and/or NMDA stimulation, and 2 mg of lysates immunoprecipitated (IP) with Trk (C-14) or RasGrf1 (C-20) antibodies. Ips or a whole cell lysates (WCL; 100 μg) were analyzed by SDS-PAGE, and blotted (IB) with the indicated antibodies (n=3). * indicates statistical significance (P-value<0.05) of indicated condition relative to unstimulated condition. **A)** Interaction between Trk and RasGrf1 (n=3). An enhanced interaction (almost 3-fold) between TrkB and Ras-Grf1 is evident in response to BDNF relative to the unstimulated lane,.Co-stimulation with NMDA decreases this interaction. Rabbit immunoglobulin (IgG) was used as a negative control for immunoprecipitation. A quantification diagram of the interaction between Trk and RasGrf1, normalized to the level of Trk in each lane, is shown in the right panel. **B)** Tyrosine phosphorylation of TrkB. BDNF stimulation increases the tyrosine phosphorylation of tyrosine[516], not tyrosine[817], on TrkB. **C)** Tyrosine phosphorylation of RasGrf1. A weak-insignificant level of tyrosine phosphorylation is detectable in the presence of BDNF and both BDNF/NMDA treatment.
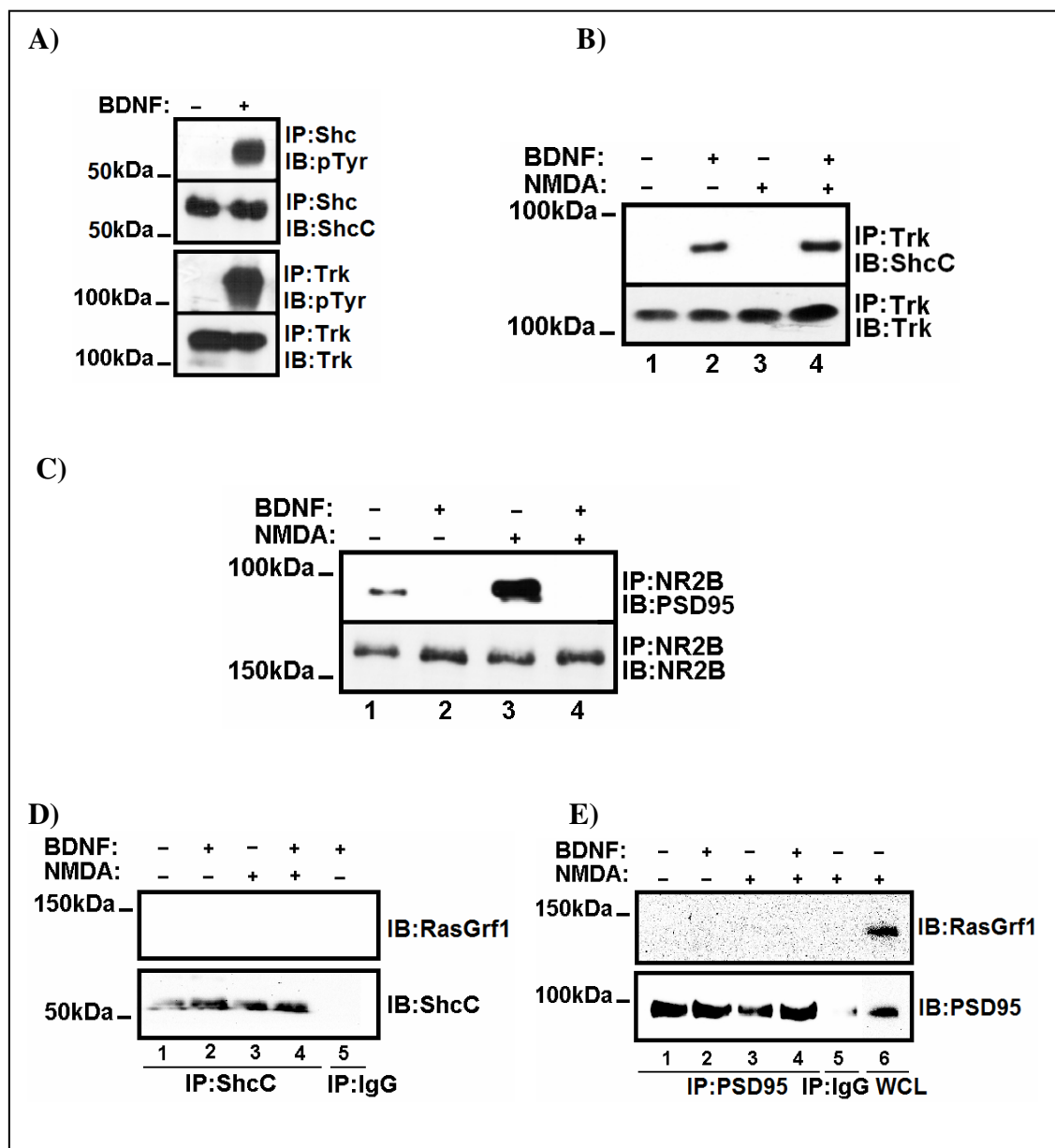
Figure 4.3. Western blot analysis of complex signaling molecules downstream of TrkB and NR2B in P30 mouse brain slices. Cortical slices were lysed after BDNF and/or NMDA stimulation, and immunoprecipitated (IP) with the indicated antibodies. Ips or whole cell lysates (WCL) were analyzed by SDS-PAGE and blotted (IB) with the indicated antibodies (n=3). **A)** The level of ShcC phosphorylation upon BDNF stimulation. ShcC tyrosine phosphorylation is increased in the presence of BDNF (Robinson, 2008). **B)** The interaction between ShcC and TrkB after BDNF and/or NMDA stimulation. BDNF stimulation increases Trk-ShcC interaction (Robinson, 2008). **C)** The interaction between PSD-95 and NR2B after BDNF and/or NMDA stimulation. NMDA stimulation increases the NR2B-PSD-95 association (Robinson, 2008). **D)** The interaction between ShcC and RasGrf1, as being part of a complex, after BDNF and/or NMDA stimulation. No interaction was observed between RasGrf1 and ShcC in any condition. **E)** The interaction between PSD-95 and RasGrf1, as being part of a complex, after BDNF and/or NMDA stimulation. No interaction was observed between RasGrf1 and PSD-95 in any condition.
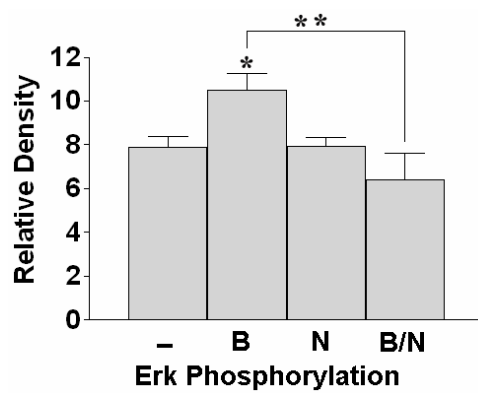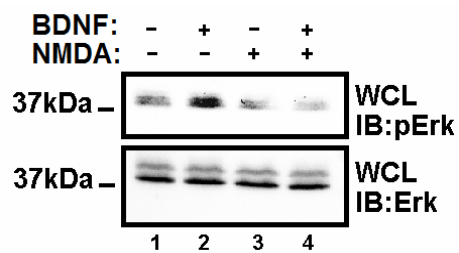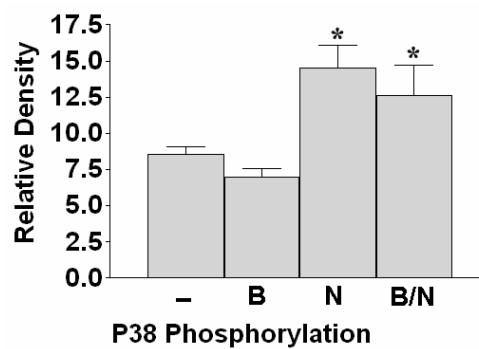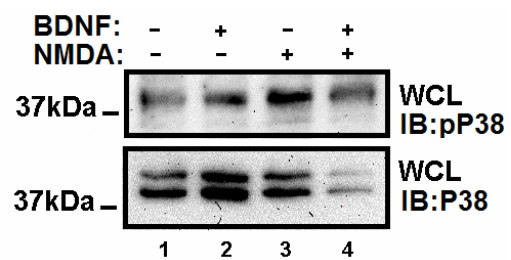
Figure 4.4. The activation of Erk/p38 MAP-kinase activation upon BDNF and/or NMDA stimulation. Cortical slices were lysed after BDNF and/or NMDA stimulation, and a whole cell lysates (WCL; 100 µg) were analyzed by SDS-PAGE, and blotted (IB) with indicated antibodies (n=3). * indicates statistical significance (P-value<0.05) of indicated condition relative to unstimulated. **A)** The phosphorylation of Erk in P30 slices after BDNF and/or NMDA stimulation. The level of Erk phosphorylation is increased significantly after BDNF stimulation alone relative to the unstimulated lane, and was decreased in combination treatment relative to BDNF alone. Quantification of Erk phosphorylation, normalized to levels of Erk expression, is shown in the right panel. ** indicates significant decrease of combination treatment relative to BDNF alone. **B)** The phosphorylation of p38 in P30 slices after BDNF and/or NMDA stimulation. NMDA stimulation alone increased significantly (almost 2-fold) the level of p38 phosphorylation, relative to unstimulated cells, and the level of phosphorylation was decreased in combination treatment. Quantification of p38 phosphorylation, relative to levels of p38 expression, is shown in the right panel.
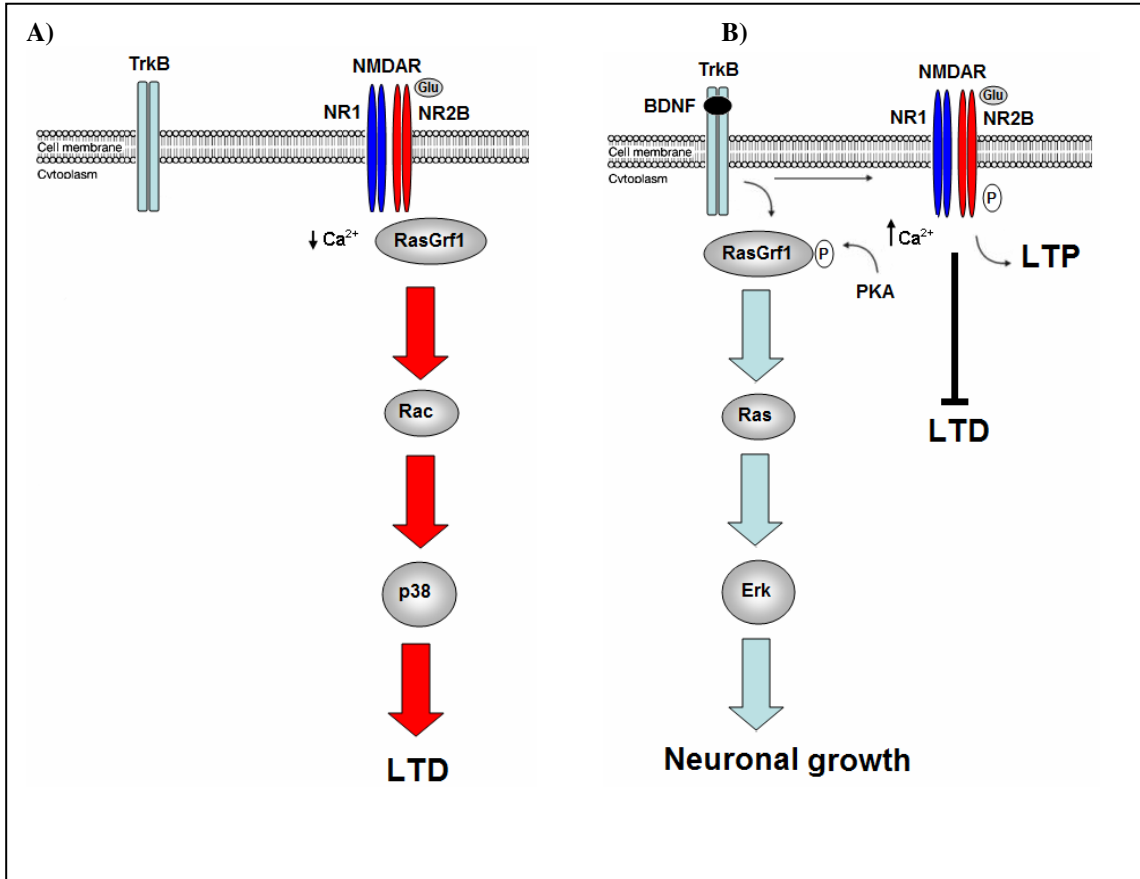
Figure 4.5. A schematic diagram depicting the proposed model of RasGrf1-mediated cross-talk between TrkB and NMDA receptors. Note that intracellular Ca^{2+} is a key determinant in generating LTD or LTP. In the absence of BDNF (A), RasGrf1 binds to the NR2B subunit of the NMDA receptor. Subsequently, p38-MAP kinase is activated predominantly through Rac-GTPases. This activation leads to NMDA receptor internalization, low Ca^{2+} influx and LTD. In the presence of BDNF (B), TrkB is activated, leading to Src-mediated phosphorylation of NMDA receptor on Tyr^{1472}. NR2B is stabilized on cell surface which blocks LTD, consequently intracellular Ca^{2+} levels are increased, result in activation of PKA, and phosphorylation of RasGrf1 at Ser916 with the resultant dissociation of RasGrf1 from the NR2B. This dissociation allows for the RasGrf1 to directly interact with TrkB and mediate the activation of signaling pathways leading to neuritogenesis. Increase in the level of intracellular Ca^{2+} also associate NMDA receptor to LTP on receptor level.

4.4 Discussion

LTP and LTD, two molecular processes contributing in learning and memory formation, are regulated in part by the NMDA receptor in the hippocampus area of the brain, and it has been suggested that BDNF increases both hippocampal presynaptic and postsynaptic transmission by regulating the activity of the NMDA-NR2B receptor (Levine and Kolb, 2000). Through postsynaptic TrkB receptor activation, BDNF increases the NMDAR single-channel-open probability associated with both LTP and/or LTD. In fact, it has been shown that blocking the NR2B subunit of the NMDAR inhibits LTD with no effect on LTP, while NR2A inhibition prevent LTP induction but not LTD (Lu et al., 2008; Liu et al., 2004). In this regard, synaptic plasticity is associated with an increase in MAP kinase family members activated downstream of both NMDA receptors (Levine and Kolb, 2000), and BDNF-mediated TrkB receptors. While several documents have addressed the nature of the cross talk between NMDAR and TrkB, the signalling pathways downstream of these receptors are not fully understood.

Previous results from the Meakin Lab (Robinson, 2008) indicate that RasGrf1 is expressed at very low levels in the neonate, and levels rise developmentally with the maximum expression predominantly in the mature brain (>P30) which was consistent with a previous study (Zippel et al., 1997). Thus, all analyses in this study have been addressed in P30 CD1 mice. Moreover, acute cortical slices including cortex and hippocampus were used in this study as these areas have been suggested to express BDNF and TrkB associated with plasticity, LTP and LTD (Li et al., 2006). Consequently, BDNF stimulation appeared to sustain Tyr^{1472} phosphorylation of the NR2B subunit of the NMDA receptor similar to what has been observed in other studies (Lin et al., 1998;

Levine et al., 1998; Levine and Kolb 2000). As mentioned earlier, the phosphorylation of NR2B-Tyr[1472] is crucial to retain NMDA receptor on cell membrane (Nakazawa et al., 2001; Prybylowski et al., 2005).

Moreover, there was a direct interaction between the NR2B subunit of the NMDA receptor and RasGrf1 in the presence of NMDA in P30 cortical slices which is in agreement with previous studies (Kraivinsky et al., 2003; Li et al., 2006), and the level of interaction was reduced remarkably by the addition of BDNF. *In vitro* studies in TrkB-B5 cells further verified that NMDA treatment increases the association of NR2B with RasGrf1 similar to that observed using brain slices (Robinson, 2008).

A relevant interaction was also observed between TrkB and RasGrf1 in P30 cortical slices in response to BDNF stimulation, which is consistent with previous observations in HEK293 cells (Robinson et al., 2005). BDNF stimulation increased tyrosine phosphorylation of the TrkB receptor at Tyr[516], equivalent to Tyr[499] on TrkA, which is the docking site for the ShcC adaptor protein (Minichiello, 2009). Since this site is close to the HIKE domain (His[507]-Glu[518]) on TrkA, the binding site of RasGrf1, the question arose as to whether ShcC might be interacting with RasGrf1. While a direct interaction between TrkB and ShcC was observed after BDNF stimulation, no direct interaction between ShcC and RasGrf1 was observed.

Furthermore, an increase in interaction between NR2B and PSD-95 was also observed upon NMDA treatment (Robinson, 2008), and a loss of this interaction occurred following the addition of BDNF similar to what was observed between NR2B and RasGrf1, suggesting that RasGrf1 might be part of a complex with the PSD-95

intermediate signalling molecule. However, when interactions between PSD-95 with RasGrf1 were examined, no interactions were observed.

While both NGF and BDNF have been shown to stimulate tyrosine phosphorylation of RasGrf1 in transfected cells in culture (Robinson et al., 2005; Chapter 2), I was unable to detect sufficient levels of RasGrf1 tyrosine phosphorylation in this study, even by using 20 mg of lysates (data not shown). This data suggests that RasGrf1 either is not phosphorylated *in vivo*, opposite to what we have observed *in vitro*, or that the levels of phosphorylation are stoichiometrically low and can only be detected in other conditions such as using higher levels of lysate, specifically examining post synaptic density fractions or using a more specific antibody in future studies.

In this study, the phosphorylation of the Erk/MAP kinase after BDNF treatment alone was increased significantly compared to that observed in untreated conditions while the level of p38 phosphorylation was increased significantly after NMDA stimulation . In fact, this data suggests that interaction between RasGrf1 and TrkB has been associated with an increase in Erk-MAP kinase phosphorylation while interaction between RasGrf1 and NR2B has been associated with an increase in p38-MAP kinase phosphorylation. This data is not in agreement to what observed in the study by Krapivinsky et al., (2003) in which an interaction between RasGrf1 and NR2B has been associated with increased in Erk phosphoryation. In contrast, the results presented here agree with the study by Li et al (2006) in which the interaction between RasGrf1 and NR2B specifically activated p38 MAP Kinase but not Erk associated with increases in long-term depression, while the association of RasGrf2 with NR2A facilitates Erk activation with the result of promoting LTP (Li et al., 2006).

Furthermore, in terms of regulating the balance between Erk and p38-MAP kinase phosphorylation by either BDNF or NMDA, it appears that the NMDA- mediated NR2B activation shows a stronger influence than the BDNF-mediated TrkB activation as the combination treatments in Erk-MAP kinase phosphorylation, adding NMDA to BDNF significantly blocked Erk phosphorylation while in p38-MAP kinase phosphorylation assessment, adding BDNF to NMDA only slightly decreased p38 phosphorylation. These observations suggest that RasGrf1 might initiate part of a signalling pathway upstream of Erk and that some other signalling molecules with distinct consequences rather than RasGrf1-mediated neuronal growth might be also involved in Erk phosphorylation. Since RasGrf2 associates with NR2A to facilitate the activation of Erk (Li et al., 2006) and since the interaction between NR2A and RasGrf2 was unaffected by BDNF, I expected to observe a slight increase in the level of Erk activation. While this additive effect was not observed under the conditions used, it is possible that it is transitory or may be revealed in another time point or by performing more careful analyses of the kinetics of Erk activation in slice cultures. Moreover, the distinct role(s) of RasGrf1 and RasGrf2 in mediating Erk activation need to be further investigated, and future studies using slices from RasGrf1 and RasGrf2 knockout mice could verify distinct roles of each in regulating in Erk activation.

This study presents important data demonstrating that signalling cross-talk occurs in response to BDNF and NMDA stimulation within cortical and hippocampal slices of the brain (see proposed model in Figure 4.5). While BDNF recruits and couples both ShcC and RasGrf1 to the TrkB receptor, the later of which is uncoupled from the NR2B subunit of the NMDA receptor, co-stimulation with NMDA uncouples RasGrf1 from

TrkB but does not affect TrkB binding to ShcC. Conversely, while NMDA stimulates NR2B binding to PSD-95, co-stimulation with BDNF uncouples this interaction. The evaluation of co-ordination between TrkB, NR2B and RasGrf1 in specific regions, such as postsynaptic density, may further clarify the nature of this signalling pattern.

Since RasGrf1 increases neurite outgrowth in PC12 cells upon NGF and BDNF stimulation, and given the fact that TrkB regulates dendritic spine density and synapse formation, the evaluation of hippocampal neurons from RasGrf1 knockout mice in the presence of BDNF may identify a potential role of RasGrf1 and TrkB in neurons to modulate potential synaptic morphology. It would be interesting to investigate if BDNF and/or NMDA stimulation affects dendritic spine density in wild-type versus RasGrf1 knockout hippocampal cultures to address whether NR2B activation of p38-MAP kinase and LTD are associated with a loss of dendritic spines. Furthermore, RasGrf1-siRNA could be used to knock down RasGrf1 expression in hippocampal cell cultures in the absence and presence of BDNF and evaluate dendritic spine density.

Collectively, a novel signalling pathway was identified in this study within the mature brain where BDNF-mediated TrkB activation modifies NMDA receptor signalling pathway through RasGrf1 to link synaptic plasticity to neuronal differentiation.

References

Akaneya Y., Tsumoto T., Kinoshita S., and Hatanaka H. (1997) Brain-derived neurotrophic factor enhances long-term potentiation in rat visual cortex. J. Neurosci. 17, 6707–6716.

Amaral M.D., Chapleau C.A., and Pozzo-Miller L. (2007) Transient receptor potential channels as novel effectors of brain-derived neurotrophic factor signaling: potential implications for Rett syndrome. Pharmacol. Ther. 113, 394-409.

Anborgh P.H., Qian X., Papageorge A.G., Vass W.C., DeClue J.E., and Lowy D.R. (1999) Ras-specific exchange factor GRF: oligomerization through its Dbl homology domain and calcium-dependent activation of Raf. Mol. Cell. Biol. 19, 4611-22.

Bauer E.P., Schafe G.E., and LeDoux J.E. (2002) NMDA receptors and L-type voltage-gated calcium channels contribute to long-term potentiation and different components of fear memory formation in the lateral amygdala. J. Neurosci. 22, 5239-49.

Buchsbaum R.J., Connolly B.A., and Feig L.A. (2002) Interaction of Rac exchange factors Tiam1 and Ras-GRF1 with a scaffold for the p38 mitogenactivated protein kinase cascade. Mol. Cell. Biol. 22, 4073–4085.

Carvalho A.L., Caldeira M.V., Santos S.D., Duarte C.B. (2008) Role of the brain-derived neurotrophic factor at glutamatergic synapses. Br. J. Pharmacol. 153, 310-24.

Fam N.P., Fan W.T., Wang Z., Zhang L.J., Chen Z., and Moran M.F. (1997) Cloning and characterization of Ras-GRF2, a novel guanine nucleotide exchange factor for Ras. Mol. Cell. Biol. 17, 1396–1406.

Farnsworth C.L., Freshney N.W., Rosen L.B., Ghosh A., Greenberg M. E., and Feig L.A. (1995) Calcium activation of Ras mediated by neuronal exchange factor Ras-GRF. Nature. 376, 524-527.

Ferrari C., Zippel R., Martegani E., Gnesutta N., Carrera V., and Sturani E. (1994). Expression of two different products of CDC25Mm, a mammalian Ras activator, during development of mouse brain. Exp. Cell Res. 210:353–357.

Ghosh A., and Greenberg M.E. (1995) Calcium signaling in neurons: molecular mechanisms and cellular consequences. Science, 268, 239–247.

Graham FL, Smiley J., Russell WC, and Nairn R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. 36, 59-74.

Huang E.J., and Reichardt L.F. (2001) Neurotrophins: roles in neuronal development and function. Annu. Rev. Neurosci. 24, 677-736.

Jordan M., Schallhorn A., and Wurm FM. (1996) Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. Nucleic. Acids. Res. 24, 596-601

Kang H., Welcher A.A., Shelton D., and Schuman E.M. (1997) Neurotrophins and time: Different roles for TrkB signaling in hippocampal long-term potentiation. Neuron. 19, 653–664.

Kaplan D.R., and Miller F.D. (2000) Neurotrophin signal transduction in the nervous system. Curr. Opin. Neurobiol. 10, 381-91.

Kiyono M., Kaziro Y., and Satoh T. (2000a) Induction of Rac-Guanine Nucleotide Exchange Activity of Ras-GRF1/CDC25Mm following Phosphorylation by the Nonreceptor Tyrosine Kinase Src. J. Biol. Chem. 275, 5441-5446.

Kiyono M., Kato J., Kataoka T., Kaziro Y., and Satoh T. (2000b). Stimulation of Ras guanine nucleotideexchange activity of Ras-GRF1/CDC25(Mm) upon tyrosine phosphorylation by the Cdc42-regulated kinase ACK1. J. Biol. Chem. 275, 29788–29793.

Kohr G. (2006) NMDA receptor function: subunit composition versus spatial distribution.Cell Tissue Res 326, 439-446.

Kollen M., Dutar P., and Jouvenceau A. (2008) The magnitude of hippocampal long term depression depends on the synaptic location of activated NR2-containing N-methyl-D-aspartate receptors. Neuroscience. 154, 1308-17.

Krapivinsky G., Krapivinsky L., Manasian Y., Ivanov A., Tyzio R., Pellegrino C., Ben-Ari Y., Clapham D.E., and Medina I. (2003) The NMDA receptor is coupled to the ERK pathway by a direct interaction between NR2B and RasGRF1. Neuron 40, 775–784.

Kutsuwada T., Kashiwabuchi N., Mori H., Sakimura K., Kushiya E., Araki K., Meguro H., Masaki H., Kumanishi T., Arakawa M., et al. (1992) Molecular diversity of the NMDA receptor channel. Nature. 358, 36-41.

Lessmann V., and Heumann R. (1998) Modulation of unitary glutamatergic synapses by neurotrophin-4/5 or brain-derived neurotrophic factor in hippocampal microcultures: presynaptic enhancement depends on pre-established paired-pulse facilitation. Neuroscience 86, 399– 413.

Levine E.S., Crozier R.A., Black I.B., and Plummer M.R. (1998) Brain-derived neurotrophic factor modulates hippocampal synaptic transmission by increasing N-methyl-D-aspartic acid receptor activity. Proc. Natl. Acad. Sci. USA. 95, 10235–10239.

Levine E.S., and Kolb J.E. (2000) Brain-derived neurotrophic factor increases activity of NR2B- containing N-methyl-D-aspartate receptors in excised patches from hippocampal neurons. J. Neurosci. Res. 62, 357–362.

Li S., Tian X., Hartley D.M., and Feig L.A. (2006). Distinct Roles for Ras-Guanine Nucleotide-Releasing Factor1 (Ras-GRF1) and Ras-GRF2 in the Induction of Long-Term Potentiation and Long-Term Depression. The Journal of Neuroscience. 26, 1721–1729.

Li Y.X., Zhang Y., Lester H.A., Schuman E.M., and Davidson N. (1998) Enhancement of neurotransmitter release induced by brain-derived neurotrophic factor in cultured hippocampal neurons. J. Neurosci. 18, 10231–10240.

Lin S.Y., Wu K., Levine E.S., Mount H.T., Suen P.C., and Black I.B. (1998) BDNF acutely increases tyrosine phosphorylation of the NMDA receptor subunit 2B in cortical and hippocampal postsynaptic densities. Brain. Res. Mol. Brain Res. 55, 20–27.

Lisman J. (2003) Long-term potentiation: outstanding questions and attempted synthesis. Philos. Trans. R. Soc. Lond. B. Biol. Sci.358, 829-42.

Liu X.B., Murray K.D., and Jones E.G. (2004) Switching of NMDA receptor 2A and 2B subunits at thalamic and cortical synapses during early postnatal development. J. Neurosci. 24, 8885-95.

Lu Y., Christian K., and Lu B. (2008) BDNF: a key regulator for protein synthesis-dependent LTP and long-term memory. Neurobiol. Learn. Mem. 89, 312-23.

Massey P.V., Johnson B.E., Moult P.R., Auberson Y.P., Brown M.W., Molnar E., Collingridge G.L., and Bashir Z.I. (2004) Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. J. Neurosci. 24, 7821-8.

Meakin S.O., and MacDonald J.I. (1998) A novel juxtamembrane deletion in rat TrkA blocks differentiative but not mitogenic cell signaling in response to nerve growth factor. J. Neurochem. 71, 1875-88.

Meakin S.O. (2000) Nerve Growth Factor Receptors and Mechanisms of Intracellular Signal Transduction. Recent Research Developments in Neurochemistry. 3, 75-91.

Minichiello L. (2009) TrkB signalling pathways in LTP and learning. Nat. Rev. Neurosci. 10, 850-60.

Monyer H., Sprengel R., Schoepfer R., Herb A., Higuchi M., Lomeli H., Burnashev N., Sakmann B., and Seeburg P.H. (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science. 256, 1217-21.

Nakazawa T., Komai S., Tezuka T., Hisatsune C., Umemori H., Semba K., Mishina M., Manabe T., and Yamamoto T. (2001). Characterization of Fyn-mediated tyrosine phosphorylation sites on GluR _ 2(NR2B) subunit of the N-methyl-D-aspartate receptor. J. Biol. Chem. 276, 693–699.

Nakazawa T., Komai S., Watabe A.M., Kiyama Y., Fukaya M., Arima-Yoshida F., Horai R., Sudo K., Ebine K., Delaary M., Goto J., Umemori H., Tezuka T., Iwakura Y., Watanabe M., Yamamoto T., and Manabe T. (2006) NR2B tyrosine phosphorylation modulates fear learning as well as amygdaloid synaptic plasticity. EMBO. J. 25, 2867-77.

Paoletti P., and Neyton J. (2007) NMDA receptor subunits: function and pharmacology. Curr. Opin. Pharmacol. 7, 39-47.

Prybylowski K., Chang K., Sans N., Kan L., Vicini S., and Wenthold R.J. (2005) The synaptic localization of NR2B-containing NMDA receptors is controlled by interactions with PDZ proteins and AP-2. Neuron. 47, 845– 857.

Reichardt L.F. (2006) Neurotrophin-regulated signaling pathways. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 361, 1545-1564.

Robinson K.N., Manto K., Buchsbaum R.J., MacDonald J.I.S., and Meakin S.O. (2005) Neurotrophin-dependent Tyrosine Phosphorylation of Ras Guanine-Releasing Factor 1 and Associated Neurite Outgrowth is Dependent on the HIKE Domain of TrkA. J. Biol. Chem. 280, 225-235.

Robinson K., 2008. The role of RasGrf1 in Trk receptor signaling. Chapter 3 and 4. The Uniersity of Western Ontario, London, ON.

Schmitt J.M., Guire E.S., Saneyoshi T., and Soderling T.R. (2005) Calmodulin-Dependent Kinase Kinase/Calmodulin Kinase I Activity Gates Extracellular-Regulated Kinase-Dependent Long-Term Potentiation The Journal of Neuroscience. 25, 1281–1290.

Tian X., Gotoh T., Tsuji K., Lo EH., Huang S., and Feig L.A. (2004) Developmentally regulated role for Ras-GRFs in coupling NMDA glutamate receptors to Ras, Erk, and CREB. EMBO. J. 23, 1567–1575.

van Zundert B., Yoshii A., and Constantine-Paton M. (2004) Receptor compartmentalization and trafficking at glutamate synapses: a developmental proposal. Trends. Neurosci. 27, 428-37.

West A.E., Chen W.G., Dalva M.B., Dolmetsch R.E., Kornhauser J.M., Shaywitz A.J., Takasu M.A., Tao X., and Greenberg M.E. (2001) Calcium regulation of neuronal gene expression. Proc. Natl. Acad. Sci. USA 98, 11024-31.

Wu H.Y., Hsu F.C., Gleichman A.J., Baconguis I., Coulter D.A., and Lynch D.R. (2007) Fyn-mediated phosphorylation of NR2B Tyr-1336 controls calpain-mediated NR2B cleavage in neurons and heterologous systems. J. Biol. Chem. 282, 20075-87.

Wu K., Len G.W., McAuliffe G., Ma C., Tai J.P., Xu F., and Black I.B. (2004) Brain-derived neurotrophic factor acutely enhances tyrosine phosphorylation of the AMPA receptor subunit GluR1 via NMDA receptor-dependent mechanisms. Brain Res. Mol. Brain. Res. 130, 178-86.

Xu F., Plummer M.R., Len G.W., Nakazawa T., Yamamoto T., Black I.B., and Wu K. (2006) Brain-derived neurotrophic factor rapidly increases NMDA receptor channel activity through Fyn-mediated phosphorylation. Brain. Res. 1121, 22-34.

Zippel R., Gnesutta N., Matus-Leibovitch N., Mancinelli E., Saya D., Vogel Z., Sturani E., Renata Z., Nerina G., Noa M. L., Enzo M., Daniella S., Zvi V., and Emmapaola S. (1997) Ras-Grf, the activator of Ras, is expressed preferentially in mature neurons of the central nervous system. Brain Res. Mol. Brain Res. 48, 140-144.

Chapter 5

General Discussion and Conclusions

The mechanism of coordination between TrkB and NMDA receptor through RasGrf1 has been addressed in this study and the possible signaling molecules downstream of RasGrf1 were investigated.

Initially to understand a role for RasGrf1 in cell differentiation, RasGrf1-mediated neurite outgrowth was determined in an *in vitro* neuronal-like model, PC12 cells and TrkB receptor over-expressing cells. It appeared that RasGrf1 activates both Ras and Rac during neurotrophin-mediated neurite outgrowth (Chapter 2). By using several domain/site- directed mutants of RasGrf1 in a comparison study including NGF-mediated neurite outgrowth in PC12 cells versus BDNF-mediated in TrkB-B5 cells, it has been revealed that *both* Ras and Rac-GTPases are essential in neurotrophin-mediated enhancement of neurite outgrowth through RasGrf1. This notion is consistent with other studies in which RasGrf1 activates Ras in HEK cells downstream of the non-receptor tyrosine kinase ACK1, cAMP-activated PKA, calcium and GPCR activity (Mattingly, 1999; Kiyono et al., 2000b; Yang et al., 2003). Conversely, RasGrf1 activates Rac downstream of the non-receptor tyrosine kinase Src (Kiyono et al., 2000a). Furthermore, Baldassa et al., (2007) have shown that RasGrf1 interacts with a microtubule-destabilizing factor SCLIP (SCG10-like protein) which inhibits its ability to promote Rac activation and neurite outgrowth (Baldassa et al., 2007).

It has been denoted in this study to take extreme precaution regarding the expression of endogenous H-Ras while using the PC12 cell line, as the level of endogenous H-Ras could explain some differences in the results of various studies performed in PC12 cells.

For instance, Yang and Mattingly (2006) reported no detectable levels of endogenous H-Ras expression in their PC12 cell line and they found a requirement for H-Ras co-expression in order to stimulate neurite extension. Moreover, they reported no role for Rac in this process; rather, they have suggested that the expansion in soma size in co-transfected cells was dependent on Rac activity (Yang and Mattingly, 2006). In contrast, in this study and others (Baldassa et al., 2007), the level of endogenous H-Ras was detectable and it was sufficient to induce neurite outgrowth in presence of RasGrf1 expression. Consistently, Baldassa et al., (2007) observed a specific requirement for Rac activation by RasGrf1 in mediating constitutive neurite extension in PC12 cells. In addition, in this study, I found that the co-expression of H-Ras with RasGrf1 in PC12 cells highly induced neurite outgrowth, and could completely eliminate the ability to detect Rac activity in neurotrophin-induced neurite outgrowth. In fact, some researchers have also reported that Ras is able to bind and activate b-Raf and small G proteins associated with differentiation under conditions of high levels of Ras expression (Kao et al., 2001).

In addition to the evidence provided here to indicate RasGrf1 regulation of Ras family GTPase activation, some essential sites of RasGrf1 tyrosine phosphorylation for neurite outgrowth were also evaluated (Chapter 3). Using site directed mutagenesis targeting of several domains/sites of RasGrf1, a novel site of tyrosine phosphorylation was identified in the CDC25 domain. While 2 sites of Trk-induced tyrosine phosphorylation were identified in the PH1 and IQ domains of RasGrf1 (Tyr[95] and Tyr[233]), these were not essential to support NGF-induced neurite outgrowth in PC12 cells. In instead, Tyrosine 1048 and/or tyrosine 1062 in the CDC25 domain appeared to be

crucial for RasGrf1 phosphorylation and to support neurotrophin-mediated neurite outgrowth. Further studies are required to understand whether one or both tyrosine sites are necessary for RasGrf1 tyrosine phosphorylation. Further study is also required to test other tyrosine sites within CDC25 domain and the Rac activation domain (DH domain) which might be involved in RasGrf1 tyrosine phosphorylation for neurotrophin-mediated neurite outgrowth.

Collectively, our study focused on both NGF and BDNF-dependent RasGrf1 enhancement of neurite outgrowth through specific Ras family GTPases to advance our understanding about a potential role for RasGrf1 in mediating Trk-dependent synaptic plasticity. Synaptic plasticity is regulated in part by NMDA receptors in the hippocampal area of brain, where BDNF-mediated TrkB receptor activation increases both presynaptic and postsynaptic transmission and regulates the activity of the NMDA-NR2B receptor (Levine and Kolb, 2000) although the mechanism of the coordination between two receptors and the downstream signalling pathways have not been fully understood.

Consistent with the studies suggesting that BDNF increases phosphorylation of the NMDA receptor (Lin et al., 1998; Levine et al., 1998; Levine and Kolb, 2000), we have provided evidence showing that in cortical slices (Chapter 4), BDNF stimulation increases tyrosine[1472] phosphorylation of the NR2B subunit of NMDA receptor, the site of which is crucial to retain NMDA receptors at the cell membrane (Nakazawa et al., 2001; Prybylowski et al., 2005).

On one hand, a direct interaction between the NR2B subunit and RasGrf1 has been observed in the presence of NMDA in both P30 cortical slices and TrkB-B5 cells (Robinson, 2008) which is in agreement with previous studies (Li et al., 2006; Kraivinsky

et al., 2003), and the level of interaction was reduced remarkably by addition of BDNF, suggesting that NMDA stimulation couples RasGrf1 to the NR2B subunit of the NMDA receptor while BDNF stimulation uncouples RasGrf1 from the NR2B subunit. On the other hand, a novel interaction was also observed between TrkB and RasGrf1 upon BDNF treatment in P30 cortical slices, which is consistent with similar observation in HEK293 cells (Robinson et al., 2005). Yet, no discernible detectable level of RasGrf1 phosphorylation was observed in this interaction, suggesting that RasGrf1 either is not phosphorylated *in vivo* at all, opposite to what we have observed *in vitro*, or that the level of phosphorylation, or its relative stoichiometry, is lower than what we can detect with the currently available antibodies. Thus, to provide a more enriched lysate for the detection of RasGrf1 tyrosine-phosphorylation, a post synaptic density preparation is recommended for future studies. Altogether, these observations suggest that BDNF stimulation associates RasGrf1 with the Trk receptor while dissociating it from the NR2B subunit. BDNF stimulation increased tyrosine phosphorylation of the TrkB receptor on Tyr^{516}, the site which is equivalent to Tyr^{499} on TrkA and serves as a docking site for the ShcC adaptor protein (Minichiello, 2009). This notion was further verified by a observing a direct interaction between TrkB and ShcC after BDNF stimulation with a loss of this interaction after NMDA stimulation (Robinson, 2008).

The interaction between NR2B subunit and PSD-95 was also increased upon NMDA treatment (Robinson, 2008), and a loss of this interaction by the addition of BDNF was observed, similar to what was observed between the NR2B and RasGrf1, suggesting that RasGrf1 might be part of a complex with PSD95 intermediate signalling molecule. However, when RasGrf1 co-interaction with PSD95 intermediate signalling

proteins was tested, no interaction was detected between RasGrf1 and PSD95 under the condition in our study. Although it cannot be ruled out that there might be an interaction between RasGrf1 and PSD95 under other conditions such as in post-synaptic density preparation, which needs to be further addressed in future studies.

To evaluate the activation of Erk/p38 MAP kinases downstream of RasGrf1, this study revealed that the phosphorylation of Erk MAP kinase was increased after 15 min BDNF treatment while NMDA treatment increased the phosphorylation of p38 MAP kinase. Although this observation is not in agreement with what was observed in the study by Krapivinsky et al., (2003), in which the interaction between RasGrf1-NR2B was associated with preferentially increased levels of Erk phosphoryation (Krapivinsky et al., 2003), this result did agree with the study by Li et al., (2006). They showed that the interaction between RasGrf1 and the NR2B predominantely activated p38 MAP Kinase resulting in increases in long-term depression while the association of RasGrf2 with NR2A facilitates Erk activation with the result of promoting LTP (Li et al., 2006). However, RasGrf1 and RasGrf2 may activate Erk through distinct pathways which need to be further investigated in future studies. Application of RasGrf1 and RasGrf2 knockout mice for the purpose of slice preparation could verify distinct roles of both RasGrf1 and RasGrf2 in the regulation of Erk activation in future studies.

The coupling of NMDA receptor to Erk in the study by Krapivinsky et al., (2003) could be due to several reasons including whether the study had been performed in cultures or slices, the age of the mouse, and the specific region of the brain studied, all of which have been shown to affect the activation of signalling pathways downstream of the NMDA receptor (Tovar and Westbrook, 1999). Collectively this data suggests that

NMDA stimulation couples RasGrf1 to the NR2B subunit of the NMDA receptor to activate p38 MAP kinases while BDNF stimulation couples RasGrf1 to TrkB to activate Erk MAP kinases.

To further verify RasGrf1-mediated coordination of Trk and NMDA receptors, it would be interesting to perform some inhibition experiments in future studies. For instance, since elevation in intracellular calcium promotes RasGrf1 activation as an intracellular calcium sensor, and it is involved in regulating the prolonged activation of the NMDA receptor, brain slices could be treated with an intracellular calcium chelator, BAPTA-AM, to determine if BDNF-induced RasGrf1 dissociation from the NR2B subunit of the NMDA receptor is affected, as well as whether BDNF/NMDA induces changes in Erk and/or p38 MAP kinase activation. Moreover, in this regard, the application of the NR2B inhibitor (Ifenprodil), Erk inhibition (by MEK inhibitor; U0126) or P38 inhibitor (SB230580) could also further clarify the changes in Erk or p38 MAP kinase activation in the RasGrf1-mediated coordination of Trk and NMDA receptor.

To further address the role of RasGrf1 mediation of neurite outgrowth and neural plasticity, one of the most important regulators of RasGrf1 to study is Cyclin-dependent kinase 5, Cdk5. Cdk5 is a member of the cyclin-dependent kinase family. It is a proline-directed serine/threonine kinase and crucial to the proper modulation of neurite formation and structure, neurite outgrowth and neuronal migration during brain development as well as learning and memory (Zhange et al., 2008). It has been shown that Cdk5 interacts with Trk and NMDA receptors as well as with RasGrf1. In fact, Cdk5 has been shown to phosphorylate RasGrf1 resulting in RasGrf1 degradation (Kesavapany et al., 2006). TrkB also interacts with Cdk5 as a substrate through which BDNF stimulation enhances Cdk5

activity resulting in dendritic growth (Cheung et al., 2007). In addition, Cdk5 regulates the activity of the NMDA receptor by decreasing NR2B phosphorylation and increasing receptor internalization (Zhange et al., 2008). How TrkB and NMDA receptors, as well as RasGrf1, may regulate Cdk5 activity and function is another question to be investigated in a future study. In this regard, brain slices can be treated with a Cdk5 inhibitor, Roscovitine, before stimulation with BDNF/NMDA and assay changes in the level of RasGrf1 expression, its phosphorylation status, possible interaction with the TrkB/NR2B as well as changes in Erk/p38 MAP activities under these conditions. In addition, the localization study of RasGrf1 with either TrkB or the NR2B in specific regions of brain, such as postsynaptic density, may further clarify the nature of the signalling pattern down stream of both receptors.

Using RasGrf1 knockout mice would also extensively clarify the RasGrf1 modulation of TrkB-NR2B during neuronal growth and synaptic plasticity. RasGrf1 knockout (-/-) mice exibit a severe deficit in amygdala-dependent synaptic plasticity with high synaptic activity in amygdala and hippocampal neurons (Brambilla et al., 1997), as well as impaired hippocampal-dependent plasticity evidenced as failures in performing hippocampal-dependent behavioral tests such as the Morris water maze, contextual discrimination and social transmission of food preferences (Giese et al., 2001). Thus, it would be interesting to perform hippocampal cell cultures from wild-type versus RasGrf1 knockout mice to clarify whether BDNF-mediated Trk activation affects dendritic spine density, and whether NMDA-mediated NR2B activation are associated with a loss of dendritic spines. The activation of Rac and/or Ras-GTPases, as well as p38 and/or Erk-MAP kinases downstream of RasGrf1 can also be compared in both wild type and

RasGrf1 knockout mice. This experiment can be examined either in intact cells or cells transfected with RasGrf1 plasmids, including RasGrf1-wild type, RasGrf1-DH domain mutant lacking binding site for Rac, and RasGrf1-CDC25 domain mutant lacking binding site for Ras. In this regard, as Post-natal day 3-5 is the optimum age in which RasGrf1 is expressed optimally, post-natal day 3-5 hippocampal cells from RasGrf1 knockout (-/-) and wild type mice could be cultured in the presence and absence of BDNF/NMDA, and dendrite spines assessed by immunohistochemically (IHC) using the dendritic spine marker, microtubule associated protein-2 (MAP-2), tubulin (axon marker), or Drebrin- an actin binding protein, and changes in cell morphology, including the length, count, size and intensity of dendrites could be determined statistically.

Furthermore, a study by Li et al (2006) suggested that the interaction between RasGrf1 and NR2B specifically activated p38 MAP Kinase, but not Erk with increases in LTD while the association of RasGrf2 with NR2A facilitates Erk activation that results in the promotion of LTP. However, RasGrf1 and RasGrf2 may activate Erk through distinct pathways which need to be further investigated in a future study. In this respect, slice preparations from RasGrf1 knockout mice could aid in verifying a distinct role for both RasGrf1 and RasGrf2 in Erk activation.

Altogether, in this study two novel signalling pathways have been identified. First, that RasGrf1 facilitates neurotrophin-dependent neurite outgrowth *via* both Ras and Rac and that this requires tyrosine phosphorylation of RasGrf1 within the Cdc25 domain. Secondly, I have identified a mechanism whereby BDNF-mediated activation of TrkB may regulate the process of learning and memory. Specifically, within the mature brain I demonstrate that BDNF-mediated TrkB activation can modify NMDA receptor signalling

by specifically uncoupling RasGrf1 from NR2B while not affecting the association of RasGrf2 with NR2A. Since RasGrf1 signaling via NR2B is associated with LTP, this provides a potentially important mechanism by which TrkB may be facilitating the process of learning and memory. In addition to decreasing the potential of NMDA signaling to result in LTD, which may indirectly facilitate a potential increase in LTP, the potential for RasGrf1 to facilitate TrkB-mediated dendritic growth and spine formation may provide another means by which TrkB signaling may facilitate the process of learning and memory (see the proposed model in Figure 4-5).

References

Baldassa S., Gnesutta N., Fascio U., Sturani E., and Zippel R. (2007) SCLIP, a Microtubule-destabilizing Factor, Interacts with RasGRF1 and Inhibits Its Ability to Promote Rac Activation and Neurite Outgrowth. J. Biol. Chem. 282, 2333-2345.

Brambilla R., Gnesutta N., Minichiello L., White G., Roylance A.J., Herron C.E., Ramsey M., Wolfer D.P., Cestari V., Rossi-Arnaud C., Grant S.G., Chapman P.F., Lipp H.P., Sturani E., and Klein R. (1997) A role for the Ras signalling pathway in synaptic transmission and long-term memory. Nature. 390, 281-6.

Cheung Z.H., Chin W.H., Chen Y, Ng Y.P., and Ip N.Y. (2007) Cdk5 Is Involved in BDNF- Stimulated Dendritic Growth in Hippocampal Neurons. PLoS. Biol. 5, e63

Giese K.P., Friedman E., Telliez J.B., Fedorov N.B., Wines M., Feig L.A., and Silva A.J. (2001) Hippocampus dependent learning and memory is impaired in mice lacking the Ras guanine nucleotide releasing factor 1 (RasGrf1). Neuropharmacology. 41, 791-800.

Kao S., Jaiswal R.K., Kolch W., and Landreth G.E. (2001) Identification of the mechanisms regulating the differential activation of the mapk cascade by epidermal growth factor and nerve growth factor in PC12 cells. J. Biol. Chem. 276, 18169-77.

Kesavapany S., Pareek T.K., Zheng Y.L., Amin N., Gutkind J.S., Ma W., Kulkarni A.B., Grant P., and Pant H.C. (2006) Neuronal Nuclear Organization Is Controlled by Cyclin-Dependent Kinase 5 Phosphorylation of Ras Guanine Nucleotide Releasing Factor-1. Neurosignals. 15,157–173.

Kiyono M., Kaziro Y.and Satoh T. (2000a) Induction of Rac-Guanine Nucleotide Exchange Activity of Ras-GRF1/CDC25Mm following Phosphorylation by the Nonreceptor Tyrosine Kinase Src. J. Biol. Chem. 275, 5441-5446.

Kiyono M., Kato J., Kataoka T., Kaziro Y., and Satoh T. (2000b). Stimulation of Ras guanine nucleotideexchange activity of Ras-GRF1/CDC25(Mm) upon tyrosine phosphorylation by the Cdc42-regulated kinase ACK1. J. Biol. Chem. 275, 29788–29793.

Krapivinsky G., Krapivinsky L., Manasian Y., Ivanov A., Tyzio R., Pellegrino C., Ben-Ari Y., Clapham D.E., and Medina I. (2003) The NMDA receptor is coupled to the ERK pathway by a direct interaction between NR2B and RasGRF1. Neuron 40, 775–784.

Levine E.S., Crozier R.A., Black I.B., and Plummer M.R. (1998) Brain-derived neurotrophic factor modulates hippocampal synaptic transmission by increasing N-methyl-D-aspartic acid receptor activity. Proc. Natl. Acad. Sci. USA. 95, 10235–10239.

Levine E.S., and Kolb J.E. (2000) Brain-derived neurotrophic factor increases activity of NR2B- containing N-methyl-D-aspartate receptors in excised patches from hippocampal neurons. J. Neurosci. Res. 62, 357–362.

Li S., Tian X., Hartley D.M., and Feig L.A. (2006). Distinct Roles for Ras-Guanine Nucleotide-Releasing Factor1 (Ras-GRF1) and Ras-GRF2 in the Induction of Long-Term Potentiation and Long-Term Depression. The Journal of Neuroscience. 26, 1721–1729.

Lin S.Y., Wu K., Levine E.S., Mount H.T., Suen P.C., and Black I.B. (1998) BDNF acutely increases tyrosine phosphorylation of the NMDA receptor subunit 2B in cortical and hippocampal postsynaptic densities. Brain. Res. Mol. Brain Res. 55, 20–27.

Mattingly R.R. (1999) Phosphorylation of Serine 916 of Ras-GRF1 Contributes to the Activation of Exchange Factor Activity by Muscarinic Receptors. Journal of Biological Chemistry. 274, 37379-37384.

Minichiello L. (2009) TrkB signalling pathways in LTP and learning. Nat. Rev. Neurosci. 10, 850-60.

Nakazawa T., Komai S., Tezuka T., Hisatsune C., Umemori H., Semba K., Mishina M., Manabe T., and Yamamoto T. (2001). Characterization of Fyn-mediated tyrosine phosphorylation sites on GluR _ 2(NR2B) subunit of the N-methyl-D-aspartate receptor. J. Biol. Chem. 276, 693–699.

Prybylowski K., Chang K., Sans N., Kan L., Vicini S., and Wenthold R.J. (2005) The synaptic localization of NR2B-containing NMDA receptors is controlled by interactions with PDZ proteins and AP-2. Neuron. 47, 845– 857.

Robinson K.N., Manto K., Buchsbaum R.J., MacDonald J.I.S., and Meakin S.O. (2005) Neurotrophin-dependent Tyrosine Phosphorylation of Ras Guanine-Releasing Factor 1 and Associated Neurite Outgrowth is Dependent on the HIKE Domain of TrkA. J. Biol. Chem. 280, 225-235.

Robinson K., 2008. The role of RasGrf1 in Trk receptor signaling. Chapter 3 and 4. The Uniersity of Western Ontario, London, ON.

Tovar K.R., and Westbrook G.L. (1999) The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. J. Neurosci. 19, 4180-8.

Yang H., Cooley D., Legakis J., Qingyuan G., Andrade R., and Mattingly R. (2003) Phosphorylation of the RasGrf1 Exchange Factor at Ser916/898 Reveals Activation of Ras Signaling in the Cerebral Cortex. J. Biol. Chem. 278, 13278-13285.

Yang H., and Mattingly R.R. (2006) The Ras-GRF1 Exchange Factor Coordinates Activation of H-Ras and Rac1 to Control Neuronal Morphology. Molecular Biology of the Cell. 17, 2177–2189.

Zhang S., Edelmann L., Liu J., Crandall J.E., and Morabito M.A. (2008) Cdk5 Regulates the Phosphorylation of Tyrosine 1472 NR2B and the Surface Expression of NMDA Receptors. The Journal of Neuroscience. 28, 415– 424.

Western

June 18, 2009

This is the Original Approval for this protocol
A Full Protocol submission will be required in 2013

Dear Dr. Meakin:

Your Animal Use Protocol form entitled:
'Neurotrophin Signaling in Genetically Modified Mice
Funding Agency NSERC - Grant #BCGB; CIHR - Grant #RRT6

has been approved by the University Council on Animal Care. This approval is valid from **June 18, 2009 to June 30, 2010.** The protocol number for this project is **#2009-048 and replaces #2005-010 which has expired.**.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED FOR 4 Years

Mouse ID# 1	CD1 Charles River # 022	Male/Female > 2 months	1400
	CD1	Male/Female P3 - P30	1200
Mouse ID# 2	ShcC -/- CD1/ICR	Male/Female > 2 months	1000
	ShcC -/- CD1/ICR	P30	600
Mouse ID# 3	C57/Bl6 x 129 (B6129) Taconic	Male/Female > 2 months	1400
	C57/Bl6 x 129 (B6129)	P30	1200
Mouse ID# 4	C57/Bl6 x 129 Ras Grf1-/-	Male/Female > 2 months	400
	C57/Bl6 x 129 Ras Grf1-/-	P30	400
Mouse ID# 5, 6	Flox-FRS2 (C57/BL6x129) Flox-FRS3 (C57/BL6x129)	Male/Female > 2 months	1400 1400
Mouse ID# 7, 8	Driver Line: (1) CMV-Cre (Balb/c) Jackson Labs # 3465	Male/Female > 2 months	400
	(2) Foxg1-Cre (B6129) Jackson Labs #6084	Male/Female > 2 months	400
Mouse ID#	CMV-Cre x Flox-FRS3	E15-P30	400
Mouse	Foxg1-Cre x Flox-FRS3	E15-P30	400

The **University** *of* **Western Ontario**
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ID#				
	Foxg1-Cre x Flox-FRS2		E15-P30	400

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

c.c. Approved Protocol   - S. Meakin, M. Pickering, W. Lagerwerf
      Approval Letter   - S. Meakin, M. Pickering, W. Lagerwerf

AUSPC

From:	Dr. Susan Meakin
Sent:	Thursday, June 18, 2009 11:16 AM
To:	'AUSPC'
Subject:	RE: 2009-048 Meakin New Animal Protocol

Added to final protocol.

The room # for euthanizing animals is #1271.
It is also used by the Barrier.

Thanks
Susan

From: AUSPC
Sent: Wednesday, June 17, 2009 1:47 PM
To: 'Dr. Susan Meakin'
Subject: 2009-048 Meakin New Animal Protocol

Dr. Meakin:

Your new animal protocol " Neurotrophin Signaling in Transgenic Mice and Normal Rats" was reviewed at the recent meeting of the Animal Use Subcommittee (AUS) and approved pending an acceptable response to the following question:

1. **Section I. Euthanasia indicates that animals will be euthanized using CO@, but we are unaware of a CO_2 chamber in Robarts – please clarify.**

   I would be grateful if you could send your response to auspc@uwo.ca as this greatly facilitates our process.

Thank you for your assistance.

AUS Administration

Curriculum Vitae

Educations:

2008 - 2012   PhD Candidate, Biochemistry program, The University of Western
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Publications:

Asghar Talebian, Kim Robinson-Brookes, James I. S. MacDonald and Susan O. Meakin. Ras Guanine nucleotide Releasing Factor 1 (RasGrf1) enhancement of Trk Receptor mediated Neurite Outgrowth requires Activation of both H-Ras and Rac. Journal of Molecular Neuroscience. 2012 Jun 29. PubMed

Sazonova O, James KA, McCudden CR, Segal D, **Talebian A**, Wagner GF. Stanniocalcin-1 secretion and receptor regulation in kidney cells. Am J Physiol Renal Physiol 2008; 294(4): 788-94. PubMed

M.R. Sadeghi, **A. Talebian**, M.Jalali, M.Jeddi Tehrani, M.M.Akhondi. An ELISA method for detection of antisperm antibody. Immunology 2004, International Proceeding, Vol ISBN 88-7587-069-1, Publisher Medimond S.r.l., pp 231-237.

A.Talebian, M. Sedghazar, N. Amir-jannati, M.R. Sadeghi. Effect of time on antisperm antibody production in vasectomized males. Immunology 2004, International Proceeding, Vol ISBN 88-7587-069-1, Publisher Medimond S.r.l., pp 419-423.