University of Alberta

Differential Neurotrophic Regulation of Sodium and Calcium Channels in Adult Sympathetic Neurons

by

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Abstract

Electrophysiology studies from our laboratory have examined regulation of ion channels by NGF and LHRH. Our laboratory found that 1) Exposure of BFSG to NGF in defined-serum-free media upregulates Na^+ current as well as both N- and L-type Ca^{2+} currents. 2) LHRH also upregulates Ca^{2+} currents, but does not affect Na^+ current. 3) LHRH appears to increase Ca^{2+} current via the MAPK pathway; 4) this effect may be mediated via PKC and PKA is necessary but not sufficient to affect the transduction process. 5) NGF increases Na^+ currents via the PI3K pathway. The studies presented in this thesis support and extend the above findings. I found that 1) LHRH signals via Ras-MAPK and not by Rap-1 to increase $I_{Ca,N}$; 2) this effect is mediated via PKCβII. The immunoblot studies confirm that 1) PKA is necessary but not sufficient to effect transduction; 2) NGF signals via PI3K to increase I_{Na}.

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List of Abbreviations

AC........................adenylyl cyclase

Ach........................achetylcholine

Akt........................protein kinase B

AP........................action potential

ARTN....................artemin

BDNF....................brain-derived neurotrophic factor

BFSG.....................bullfrog sympathetic ganglia

B-Raf.....................B-type MAPK kinase kinase

Ca^{2+} ....calcium

cAMP....................Adenosine-3', 5'-cyclic mononphosphate

CaaX.....................carboxyl terminal motif with two aliphatic residues

CAM Kinase II.........calmodulin kinase type 2

CDC42...................cell division cycle 42

CNS.......................central nervous system

CREB....................cAMP response element-binding protein

C-terminus..............carboxyl terminal

DAG.....................1, 2-diacyglycerol

DH.......................Dbl-homology

DMSO...................dimethyl sulfoxide

DNA.....................deoxyribonucleic acid

ECL Plus................enhanced chemiluminescence plus

EDTA....................ethylenediamine tetraacetic acid

EGTA.....................ethylene glycol tetraacetic acid

Epsp.......................excitatory post-synaptic potential

ERK.......................extracellular-regulated kinase

FPA124...................PKB inhibitor

FSH.......................follicle-stimulating hormone

G_i...alpha i/o subunit of G-protein

$G_{\alpha 12/13}$.................................alpha 12/13 subunit of G-protein

$G_{q/11}$.....................................alpha q/11 subunit of G-protein

Gs........................alpha s subunit of G-protein

Gβγ.............................beta gamma subunit ofG-protein

GABA....................γ-aminobutyric acid

GAP......................GTPase activating proteins

GDI.......................guanine nucleotide dissociation inhibitor

GDNF...................glial-cell-line-derived neurotrophic factor

GDP......................guansosine diphosphate

GEF......................guanine-nucleotide exchange factor

GFLs.....................(GDNF) family ligands

GFRα.....................type α GDNF family receptor

GFRα1...................type α1 GDNF family receptor

GFRα2...................type α2 GDNF family receptor

GFRα3...................type α3 GDNF family receptor

GFRα4...................type α4 GDNF family receptor

GnRH....................gonadotropin-releasing hormone

GPCR.....................G-protein coupled receptor

GRF.......................guanine-releasing factor

GRK.......................G-protein receptor kinase

GRP.......................Ras guanylnucleotide-releasing protein

GST.......................glutathione S transferase

GST-RBD...............GST fused to Ras binding domain of Raf1

GTP.......................guanosine triphosphate

GTPase.................guanosine triphosphatase

GTP-γ-S.................guanosine triphosphate gamma S

H-89......................N-[2-(p-Bromocinnamylamina)ethyl]-5-

isoquinolinesulfonamide-2HCl

α-HFA...................α-Hydroxyfarnesylphosphonic acid

HPLC.....................high performance liquid chromatography

H-Ras.....................H-type MAPK kinase kinase kinase

I_A......................................A-current

I_{AHP}..................................slow voltage-insensitive calcium-sensitive potassium current

I_C......................................fast voltage-sensitive calcium-sensitive potassium current

I_K......................................delayed rectifier potassium current

I_M......................................slow-activating non-inactivating muscarinic-sensitive

potassium current

IP_3....................................inositol 1, 4, 5-triphosphate

JNK/SAPK.............c-Jun-N-terminal kinase/stress-activated protein kinase

K+........................potassium

kDa……………………..kilo-Dalton

K-Ras………………..K-type MAPK kinase kinase kinase

LH……………………luteinizing hormone

LHRH………………..luteinizing hormone releasing hormone

LHRH-R……………..luteinizing hormone releasing hormone receptor

LY294002……………PI3K inhibitor

MAPK……………….mitogen-activated protein kinase

MEK…………………mitogen-activated protein kinase kinase

Na^+ …………………..sodium

NaCl…………………sodium chloride

Na_2VO_4 ....sodium orthovanadate

NaF…………………..sodium fluoride

NGF…………………nerve growth factor

NH2………………….amino acid terminal

N-Ras………………..N-type MAPK kinase kinase kinase

NRTN.........................neurturin

NT-3…………………neurotrophin-3

NT4/5………………..neurotrophin-4/5

NT-6…………………neurotrophin-6

NT-7…………………neurotrophin-7

p38 MAPK…………...p38 type mitogen-activated protein kinase

p75……………………p75 type neurotrophin receptor

p90-RSK……………..p90- ribosomal S6 kinase

PA……………………perillic acid

Pac1…………………PACAP receptor

PACAP………………pituitary adenylate cyclase-activating polypeptide

PAGE…………………polyacrylamide gel electrophoresis

PC12…………………pheochromocytoma cell 12

PD98059……………2'-amino-3'-methoxyflavone

PDGF…………………platelet-derived growth factor

PDK1…………………3-phophoinositide-dependent kinase

PH……………………pleckstrin-homology

PI……………………phosphoinositol

PI-3-P…………………phosphatidylinositol-3-monophosphate

PI-3, 4-P2……………phosphatidylinositol-3,4-bisphosphate

PI-3, 4, 5-P3…………phosphatidylinositol-3, 4, 5-triphosphate

PI-4-P…………………phosphatidylinositol-4-monophosphate

PI-4, 5-P2……………phosphatidylinositol-4, 5-bisphosphate

PI3K…………………phosphatidylinositide-3-kinase

PI-PLC………………phosphoinositide-specific phospholipase C

PKA…………………protein kinase A

PKB…………………protein kinase B

PKC…………………protein kinase C

PLC…………………phospholipase C

PMA…………………phorbol 12-myristate-13-acetate

4α PMA………………4 alpha phorbol 12-myristate-13-acetate

PSPN.....................persephin

PTB........................phosphotyrosine-binding

PVDF....................'Immobilin' polyvinylidene difluoride

PyK2......................proline-rich tyrosine kinase 2

Raf-1......................MAPK kinase kinase

Rap1......................Repressor activator protein 1

Ras........................MAPK kinase kinase kinase

Rp-cAMPS..............Rp-Adenosine-3', 5'-cyclic mononphosphorothioate triethylamine salt

RTKs.....................receptor tyrosine kinases

SDS......................sodium dodecyl sulfate

SfN ....Society for Neuroscience

SH-2.....................src-homology-2

Sp-cAMPS.............Sp-Adenosine-3',5'-cyclic mononphosphorothioate triethylamine salt

STX......................saxitoxin

TBS......................tris-buffered saline

TGF-β....................transforming growth factor-β

TM.......................transmembrane

7TM......................seven transmembrane

Trk.......................tropomyosine-related kinase

TrkA.....................tropomyosine-related kinase A

$TrkA_{II}$.........................tropomyosine-related kinase A type II

TrkB..................... tropomyosine-related kinase B

Trk C.....................tropomyosine-related kinase C

TTBS.....................tween-20 tris-buffer saline

TTX.......................tetrodotoxin

U73122...................PLC inhibitor

UV........................ultra-violet

VGCC....................voltage-gated calcium channel

Y490......................tyrosine residue 490

Y670......................tyrosine residue 679

Y674......................tyrosine residue 674

Y675......................tyrosine residue 675

Y785......................tyrosine residue 785

Y905......................tyrosine residue 905

Y1015....................tyrosine residue 1015

Y1062....................tyrosine residue 1062

Y1096....................tyrosine residue 1096

Chapter 1

General Introduction

1.1 Introduction

Appropriate neuronal phenotypic expression is essential for proper functioning of the nervous system. Although the genetic makeup of each organism supplies the foundation for the nervous system, correct phenotypic expression of neurons is dependent on exogenous and endogenous signals. Classically neurotrophins are responsible for growth, development, and repair of the nervous system, whereas, neurotransmitters are thought to be primarily responsible for rapid communication between neurons. However, recent work has demonstrated that neurotrophins and neurotransmitters can act similarly in certain systems; hence, the division between their roles is becoming less distinct.

Neurotrophins can be released through an activity-dependent mechanism (Balkowiec and Katz, 2002), which can modulate synaptic transmission as well as having acute non-trophic regulatory effects on ion channels. Acute application of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) has been shown to potentiate synaptic transmission via the Trk receptors (Lohof et al., 1993). Other studies have also demonstrated BDNF having direct regulation of various ion channels and also the release of glutamate (Levine et al., 1998; Takei et al., 1998; Yang et al., 2001). Hence, it appears that neurotrophin has two acute mechanisms of action 1) by regulating ion channels and 2) by regulating synaptic transmission. In addition, neurotrophins can trigger rapid generation of action potentials in a time course that is similar to classical neurotransmitters, further indicating that in certain systems neurotrophins can act similarly to traditional neurotransmitters (Kafitz et al., 1999; Kovalchuk et al., 2004; Blum and Konnerth, 2005).

Since neurotrophin receptors and G-protein coupled receptor (GPCR) are capable of activating comparable pathways, it is possible that GPCR agonists could regulate neuronal survival, growth, and differentiation. Pituitary adenylate cyclase-activating polypeptide (PACAP) has been demonstrated to induce differentiation and neurite outgrowth through an extracellular regulated kinase (Erk)-dependent but Ras-independent manner in pheochromocytoma (PC12) cells (Barrie et al., 1997; Lazarovici et al., 1998). Many neurotransmitters have also been shown to influence multiple aspects of neuronal maturation (Lauder, 1993; Cameron et al., 1998). For instance, γ-aminobutyric acid (GABA) has been shown to signal through the $GABA_B$ receptor to regulate nerve growth cone guidance (Xiang et al., 2002). Moreover, GABA has been shown to influence cell proliferation, dendritic growth, pre-synaptic specialization, and GABA receptor expression in various systems (Wolff et al., 1978; Redburn and Schousboe, 1987; Spoerri, 1988). These events have been attributed to increased L-type voltage-gated calcium channel (VGCC) activity leading to increased intracellular Ca^{2+} level, activating immediate early genes such as: c-fos, jun-B, fos-C, and zif268, which trigger transcriptional events (Murphy et al., 1991). Further evidence for trophic action of neurotransmitters is from GnRH. GnRH is a classical neurotransmitter capable triggering a late slow excitatory post-synaptic potential (late slow epsp) in bullfrog sympathetic ganglia (BFSG) (Jan et al., 1979; Jan et al., 1980a; Jan et al., 1980b; Jan and Jan, 1982). Gonadotropin-releasing hormone also known as luteinizing hormone-releasing-hormone (LHRH) is known as a primary initiator of the hormonal cascade in the reproductive system (King and Millar, 1980). It triggers the synthesis and release of follicle-stimulating hormone and luteinizing hormone

from the anterior pituitary, after its release from the hypothalamus (Cheng and Leung, 2000; Klausen et al., 2002). GnRH also has neuroendocrine and neuromodulatory effects in the peripheral nervous system (Millar et al., 1987).

It is also known that neuronal phenotypes in the adult nervous system are defined by differential morphological, biochemical, and electrical properties. Expression of ion channels on a given neuronal type may change during development, maturation, injury or disease (Jassar et al., 1993; Jassar et al., 1994; Cummins and Waxman 1997; Lhuillier and Dryer 2002; Craner et al., 2002; Martin-Caraballo and Dryer, 2002). Hence, various neurotrophic factors have been studied in adult neurons and in cell lines to examine their critical roles in ion channel expression (Chalazonitis et al., 1987; Levine et al., 1995; Pollock et al., 1990; Pollock and Rane 1996; Fanger et al., 1997). Studies have shown that in B-neurons of adult bullfrog sympathetic ganglia (BFSG), target-derived nerve growth factor (NGF) is responsible for the maintenance of TTX-sensitive and TTX-insensitive sodium currents (I_{Na}) and of N- and L-type calcium currents ($I_{Ca,N}$ and $I_{Ca,L}$) (Lei et al., 1997; Lei et al., 1998; Lei et al., 2001; Petrov et al., 2001). Furthermore, in serum-free, defined medium culture, NGF has been reported to induce increases in I_{Ca} and is mediated through the mitogen-activated protein kinase (MAPK) pathway (Lei et al., 1998), whereas, the transduction mechanism underlying the NGF-induced increases in I_{Na} remains to be unravelled. Interestingly, LHRH, a neurotransmitter released from preganglionic C-fibers in BFSG (Jan et al., 1979; Jan et al., 1980) is also able to regulate functional expression of Ca^{2+} channels (Ford et al., 2003a). Gonadotrophin receptors can couple

through $G_{q/11}$, Gs, or G_i to various downstream substrates including Ras-MAPK (Sim et al., 1995; Naor et al., 1998b) to mediate their effects.

1.2 BFSG organization

All experiments in this thesis have been carried out on bullfrog paravertebral sympathetic ganglia (BFSG). Unlike the mammalian ganglia such as the dorsal root ganglia, which have many different neuronal subtypes (Lewin, 1996), the BFSG system has only two principal types of neurons that are easily identifiable in vitro based on physical size and consistent electrophysiological properties: large, exocrine B-neurons and smaller C-neurons. B-neurons project to targets in the skin and C-neurons project mainly to blood vessels (Adams et al., 1986; Horn et al., 1988; Jassar et al., 1993; Smith, 1994; Ivanoff and Smith, 1995; Jobling and Horn, 1996; Thorne and Horn, 1997).

Preganglionic B- and C- fibers originate from separate neuronal populations in the spinal cord and remain separate through the paravertebral ganglia (Nishi et al., 1965; Dodd and Horn, 1983a; Horn and Stofer, 1988). B-fibers emerge through roots IVth, V^{th}, and VIth, whereas C-fibers emerge through roots VIIth and $VIII^{th}$ (Libet et al., 1968; Horn and Stofer, 1988). Furthermore, adult BFSG neurons can be maintained in a serum-free, defined medium culture system for over 14 days (Lei et al., 1997). A serum-free, defined medium culturing system is free of complicating effects from exogenous neurotrophins allowing for the study of trophic actions of neurotransmitter. In BFSG LHRH is solely released from pre-ganglionic C-fibers (Jan

et al., 1979; Jan et al., 1980b; Jobling and Horn, 1996; Thorne and Horn, 1997) and diffuses to B-neurons, hence, is capable of influencing B-neurons by means of "volume" transmission.

1.3 BFSG B-neuron ion channels

Since the focus of this thesis is on the signalling pathways that may be responsible for Na^+ and Ca^{2+} channel regulation in BFSG, the properties of ion channels that have been characterized by others will be briefly discussed. The properties of voltage-dependent Na^+, Ca^{2+}, and various K^+ channels have been well studied on B-cells of BFSG (Smith 1994).

The fast, voltage-activated Na^+ channel currents that are responsible to the upstroke of an action potential (AP) have been characterized in BFSG cells (Jones, 1987).

Total Na^+ channel currents are comprised of two pharmacological and kinetic components. A large portion of the total current is tetrodotoxin (TTX) and saxitoxin (STX) sensitive that also activates and inactivates rapidly. A smaller portion of the total current is resistant to TTX but sensitive to STX and Cd^{2+}. This also activates rapidly but inactivates 3-folds slower than the larger component (Lei et al., 2001).

Voltage-gated calcium channels (VGCC) in BFSG are well characterized (Jones and Marks, 1989a, b; Jones and Jacobs, 1990; Sala, 1991; Werz et al., 1993). These channels also play critical roles in the generation of AP in BFSG neurons (Pennefather et al., 1985; Smith, 1994). Activation and deactivation of these channels

are rapid (Jones and Marks, 1989a), whereas the inactivation kinetics are slower and more complex (Sala, 1991). VGCC are classified into four types: N-, P/Q-, R-, and L-type (Catterall, 2000). In BFSG, N-type channels carry ~ 90% of the total I_{Ca}, whereas L-type channels carry most of the remaining amount (Jones and Elmslie, 1992). N-, P/Q-, and R-type channels are mainly responsible for Ca^{2+} entry into neurons to induce neurotransmitters release, whereas, L-type channels are mainly responsible for Ca^{2+} entry to initiate gene transcription and endocrine secretion (Catterall, 2000).

The various types of K^+ channels that have been characterized in BFSG are: M-current (I_M); delayed rectifier K^+ current (I_K); fast, voltage-sensitive, Ca^{2+}-sensitive K^+ current (I_C); slow, voltage-insensitive, Ca^{2+}-sensitive K^+ current (I_{AHP}); and A current (I_A). I_M is a slowly activating, non-inactivating, and muscarinic-sensitive K^+ current (Brown and Adams, 1980; Adams et al., 1982a, 1986; Marrion et al., 1991). I_M is inhibited by nucleotides (Groul et al., 1981; Adams et al., 1982b; Tokimasa and Akasu, 1990), Acetylcholine (Ach) and LHRH (Adams and Brown, 1982; Jones et al., 1984; Jones, 1985), adrenoceptor agonists (Akasu, 1988; Selyanko et al., 1990a), and peptidergic agonists (Adams and Brown, 1982; Adams et al., 1982b, 1983; Jones, 1985; Pfaffinger et al., 1988; Bosma and Hille, 1989; Bley and Tsien, 1990). The physiological role of I_M may be to act as a voltage-clamp for cells to resist depolarizing stimulus influence (Adams et al., 1982a); therefore, suppression of I_K will give a powerful excitatory effect. I_K activates at potentials above -25mV and inactivates during long depolarizing events (Adams et al, 1982a) and is blocked by 3, 4-diaminopyridine (Goh et al., 1989). Although the physiological role of I_K is

unclear, one possible role is to control repetitive discharge when other K^+ currents are antagonized (Goh et al., 1989). I_C, as the name suggests is a fast activating, voltage-sensitive, Ca^{2+}-sensitive K^+ current (Adams et al., 1982a), which can be blocked by removal of extracellular Ca^{2+} or addition of Cd^{2+}. The physiological role of I_C is to repolarize action potentials and generate part of the afterhyperpolarization (AHP) (Adams et al., 1982c; Pennefather et al., 1985; Lancaster and Pennefather, 1987). I_{AHP} is a slow, non-inactivating, voltage-insensitive, Ca^{2+} sensitive K^+ current (Marrion et al., 1991), which can be blocked by d-tubocurare and apamin (Goh and Pennefather, 1987). The physiological role of I_{AHP} is to generate the latter portion of an AP afterhyperpolarization (Lei dissertation 1997). I_A is a rapid but transient outward K^+ current (Adams et al, 1982a). Activation of this channel requires the removal of inactivation that normally occurs at resting potential (Lei dissertation 1997). The physiological role of I_A is still unclear to date.

1.4 Neurotrophic factors

Since the aim of this thesis is to examine the effect of NGF on Ca^{2+} and Na^+ channel regulation, basic neurotrophin properties, particularly NGF, as well as its downstream effector PI3K will be discussed, the remaining effectors will be discussed in a later section. A brief discussion of glial-cell-line-derived neurotrophic factor (GDNF), a member of the second family of neurotrophic factors will also be included since studies have shown its involvement in sympathetic neuron survival and regulation.

1.4.1 Neurotrophins

Neurotrophins are a class of secretory proteins that are considered to be critical for neuronal survival, growth, and differentiation. Currently, six neurotrophins have been identified: NGF, BDNF, NT-3, neurotrophin-4/5 (NT4/5), neurotrophin-6 (NT-6), neurotrophin-7 (NT-7) (Hallbook, 1999). NT-6 and NT-7 have only been characterized in fish (Gotz et al., 1994, Nilsson et al., 1998). As to date, there are two known classes of neurotrophin receptors: the p75 receptor, which is non-selective, has equal affinity for all neurotrophins, and the second class Trk (tropomyosine-related kinase) receptor tyrosine kinase, which has high affinity for mature neurotrophins. Neurotrophins elicit their effects by binding to the high affinity Trk receptors that includes (TrkA, TrkB, and Trk C). NGF binds to TrkA, BDNF and NT-4/5 bind to TrkB, and NT-3 binds to TrkC (Chao, 1992). By contrast, all neurotrophins are able to bind competitively to the p75 receptor (Squinto et al., 1991).

Activation of Trk receptors results in dimerization of the receptors leading to autophosphorylation of the cytoplasmic domain tyrosine residues (Y490, Y785, Y670, Y674, Y675) (Stephens et al., 1994) on the receptors (Huang and Reichardt, 2001). Following intracellular tyrosine phosphorylation, adaptor proteins containing phosphotyrosine-binding (PTB) or src-homology-2 (SH-2) motifs are recruited to the receptor (Pawson and Nash, 2000). These adaptor proteins then couple Trk receptors to intracellular signalling cascades that include the Ras/ERK pathway, the phosphatidylinositide-3-kinase (PI3K/Akt) pathway, and the phospholipase C (PLC)-γ1 pathway (Reichardt and Farinas, 1997; Kaplan and Miller, 2000). Shc binding at Y490 acts as an adaptor for recruiting other proteins which lead to activation of the

Ras/Erk or PI3K pathways. PLCγ binds at Y790 where it is activated and mediates its effects (Arevalo and Wu, 2006).

1.4.2 Nerve growth factor (NGF)

NGF, the most studied neurotrophin, was discovered over 50 years ago as a molecule important in regulating neuron survival, development and maintenance of vertebrate nervous systems (Levi-Montalcini and Hamburger, 1951; Levi-Montalcini and Hamburger, 1953). The NGF gene is expressed as two splice variants (Edwards et al., 1986). The mature, biologically active form appears to be similar in all tissues, which consists of 13-kDa polypeptide chains (Sofroniew et al., 2001).

NGF mediates its effects through binding to its receptors, TrkA and p75 (Bothwell, 1995). The TrkA receptor features a single-pass transmembrane domain acting as a receptor for NGF (Loeb et al., 1991). There are two isoforms of TrkA, which differ by six additional amino acids near the extracellular transmembrane domain of one variant ($TrkA_{II}$) (Clary and Reichardt, 1994).The p75 receptor is a transmembrane glycoprotein, which binds all neurotrophins with equal affinity. Actions by p75 receptor are classed into two categories. First, p75 is a Trk co-receptor that can increase or suppress neurotrophin-mediated Trk receptor activity. Second, p75 can activate signalling cascades that can induce apoptosis or promote neuron survival (Casaccia-Bonnefil et al., 1999; Dobrowsky et al., 1994; Friedman and Greene, 1999; Roux and Barker, 2002). Although the signalling cascades triggered by the p75 receptor remain elusive, studies have shown activation of NF-κB, Akt, and JNK pathways by the p75 receptor (Roux and Barker 2002).

NGF signalling is broad based, and dynamically regulated with many intracellular signalling cascades that also converge with signalling from other molecules (Sofroniew et al., 2001). Most neurons depend on BDNF, NT-3 or NT4/5 for survival and maturation; sympathetic, nociceptive, and sensory neurons depend on NGF for survival during their development (Levi-Montalcini, 1987; Lindsay, 1996). Upon maturation these neurons lose their dependence on NGF for survival and utilize NGF to specify and maintain neuronal phenotypes (Lewin et al., 1992; Lewin, 1996; Lindsay, 1996).

The current prevalent theory for NGF signalling is that NGF bound to TrkA at the axon terminal is internalized into signalling endosomes. NGF remains bound to the phosphorylated TrkA in its membranes while retrogradedly transported in the lumen to the cell body, where TrkA activates downstream signalling molecules (reviewed in Campenot and MacInnis, 2003). Other possible retrograde signalling mechanisms have also been suggested: 1) Retrograde transport of phosphorylated TrkA unbound to NGF. 2) Retrograde transport of signalling molecules downstream of phosphorylated TrkA. 3) Serial propagation of phosphorylation among downstream signalling molecules. 4) Serial propagation of ionic fluxes, such as changes in cytosolic Ca^{2+} (Campenot and MacInnis, 2003).

1.4.3 Glial-cell-line-derived neurotrophic factor (GDNF)

GDNF belongs to a second family of neurotrophic factors know as GDNF family ligands (GFLs). Other members of the GFLs are neurturin (NRTN), artemin (ARTN), and persephin (PSPN) (Sariola and Saarma 2003). GFLs have been shown

to maintain several neuronal populations in the CNS such as: midbrain dopamine neurons (Lin et al., 1993) and motoneurons (Henderson et al., 1993). GFLs have also been shown to support the survival and differentiation of peripheral neurons including sympathetic, parasympathetic, enteric, and sensory neurons (reviewed in Sariola and Saarma 2003).

All GFLs signal through receptor tyrosine kinase RET, and the ligand-binding specificity is determined by GFRα proteins. GDNF, NRTN, ARTN, and PSPN specifically bind to GFRα1, GFRα2, GFRα3, and GFRα4 respectively (Airaksinen and Saarma, 2002). GDNF dimer complexes with GFRα1 homodimers which brings two RET molecules together, activating transphosphorylation of specific tyrosine residues (Y905, Y1015, Y1062, and Y1096) (reviewed in Sariola and Saarma, 2003) in their domains (Airaksinen and Saarma, 2002). RET can subsequently activate several intracellular signalling cascades including the MAPK pathway (Kaplan and Miller, 2000; Fisher et al., 2001), the PI3K and PLCγ pathways, as well as the Src-family kinases (Airaksinen and Saarma, 2002). However, studies have also demonstrated that GDNF can signal independently of RET in RET deficient cell lines and primary neurons (Poteryaev et al., 1999; Trupp et al., 1999). Surprisingly, the neurotrophic effect of GDNF in both *in vitro* and *in vivo* experiments, except for motoneurons, requires transforming growth factor-β (TGF-β) (Peterziel et al., 2002). TGF-β is involved in GFRα1 membrane translocation, thus, regulates GDNF signalling (Peterziel et al., 2002). Interestingly, RET phosphorylation increases with postnatal age in sympathetic neurons (Sariola and Saarma, 2003), and NGF has been reported to promote phosphorylation of RET51 via an "inter-receptor-tyrosine-

kinase" signalling mechanism (Tsui-Pierchala et al., 2002). These studies demonstrate that growth factors and their receptors participate in cross-talk that guides development (Sariola and Saarma, 2003).

1.5 Phosphotidylinositide-3-kinase (PI3K)

PI3K, one of the primary effectors of Trk activity by NGF, is crucial for survival of several types of neurons (Huang and Reichardt, 2001). PI3K is a heterodimer comprised of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit (Sofroniew et al., 2001). Activation of PI3K involves binding of the regulatory subunit to activated RTKs directly or via adaptors (Sofroniew et al., 2001). Binding of PI3K to the intracellular domain of RTKs triggers the recruitment of the 110-kDa catalytic subunit to the plasma membrane where it interacts with membrane phosphoinositides (Carter and Downes 1992; Soltoff et al., 1992).

Studies have shown that purified PI3K phosphorylates phosphoinositol (PI), phosphatidylinositol-4-monophosphate (PI-4-P), and phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2), generating phosphatidylinositol-3-monophosphate (PI-3-P), phosphatidylinositol-3,4-bisphosphate (PI-3,4-P2), and phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P3) respectively (Whitman et al., 1988; Auger et al., 1989; Carpenter et al., 1990). Protein kinase B (PKB), also known as Akt, then interacts with the generated PIP_2 or PIP_3 and with the 3-phophoinositide-dependent kinase (PDK1). PDK1 possesses a pleckstrin homology (PH) domain that binds PIP_2 or PIP_3 which is necessary for PDK1 to activate Akt (Alessi et al., 1997a, b; Cohen et al.,

1997; Stephens et al., 1998; Stokoe et al., 1997). Akt/PKB is a primary downstream effector of PI3K. It phosphorylates and controls several proteins that are critical in modulating cell survival (reviewed in Datta et al., 1999; Yuan and Yankner 2000); although not all Akt substrates are involved in cell survival (Huang and Reichardt, 2001). Mechanisms by which PI3K could influence cell functions include: via production of phosphoinositides, via phosphorylation of proteins on serine or threonine through endogenous protein kinase activity, or by serving as an adaptor (Huang and Reichardt, 2001). Cellular functions that PI3K have been shown to be involved with are: inhibition of apoptosis, mitogenesis, vesicle trafficking/secretion, cytoskeleton function regulation, and neurite elongation (Huang and Reichardt, 2001).

Other downstream effectors of PI3K are 1) the Ras/Erk pathway, which is the primary mitogen-conducting pathway 2) the PKC/MEK/Erk pathway 3) the Rac/JNKK/JNK pathway, which contributes partially to the mitogen signal transduction but involved primarily with other cell functions such as stress reaction or cytoskeleton reorganization (review in Krasilnikov 2000).

1.6 Luteinizing hormone releasing hormone (LHRH)

A major focus of this thesis is to examine the mechanism by which LHRH regulates Ca^{2+} channel regulation; therefore, its basic properties will be discussed.

1.6.1 LHRH

Gonadotropin-releasing hormone (GnRH) also known as LHRH is a primary initiator of the hormonal cascade in the reproductive system (King and Millar, 1980). Although hypothalamic LHRH was known to be distinct in structure with a central role in regulating luteinizing hormone (LH) and follicle stimulating hormone (FSH), it is now evident numerous forms exist in vertebrates (King and Millar, 1980).

Within the 23 forms of known LHRH, the most common form is chicken LHRH II, which was initially isolated from chicken brain (Miyamoto et al., 1984). Chicken LHRH II is entirely conserved form fish to human; therefore, may be the earliest evolved form with vital functions (Millar and King, 1987). In addition to their role as a stimulator of luteinizing hormone release, LHRH has numerous other functions in vertebrates (Millar, 2005). These include neuroendocrine, paracrine, autocrine, neuromodulatory, and neurotransmitter functions (Millar et al., 1987; Millar and King, 1987; Emons and Schally, 1994).

As already mentioned, LHRH triggers a late slow excitatory post-synaptic potential (late slow epsp) in BFSG (Jan et al., 1979; Jan et al., 1980a; Jan et al., 1980b; Jan and Jan, 1982). Chicken II GnRH is approximately 100 times and 1000 times more potent than salmon GnRH and other naturally occurring GnRH analogs respectively, in BFSG, which suggests that chicken II-GnRH (chicken II-LHRH) could be the endogenous transmitter that mediates the late slow epsp effect (Jones, 1987b). Features of LHRH receptors are characteristic of GPCRs such as an NH_2-terminal followed by a seven alpha-helical transmembrane domain. Function of the extracellular loops of the seven transmembrane (7TM) domain typically involves

binding with agonists, whereas, the TM domains are involved with conformational changes following receptor activation. The intracellular domains of the receptor are associated G-protein and other molecules that are involved with intracellular signalling (Millar 2005). The major structural difference between mammalian and non-mammalian LHRH receptors is the presence of a carboxyl terminal in non-mammalian LHRH receptors and the absence of this terminal in mammalian LHRH receptors (Millar 2005). This difference may account for β-arrestin independency for internalization of mammalian LHRH receptors (Hislop et al., 2001).

LHRH increases IP_3 production and intracellular Ca^{2+} level in BFSG (Pfaffinger et al., 1988) and its effects are prevented by the PLC inhibitor U73122 (Ford et al., 2004). This suggests the involvement of PLC and the coupling of $G\alpha_{q/11}$ to LHRH-R. $G\alpha_{q/11}$ coupling to GnRH-R has been demonstrated in several systems (Hsieh and Martin, 1992; Stojilkovic and Catt, 1995; Naor et al., 1998). However, other studies have identified $G\alpha_{i/o}$ (Hawes et al., 1993; Imai et al., 1996) and $G\alpha s$ (Hawes et al., 1993; Janovick and Conn, 1993; Kuphal et al., 1994) as the G-proteins mediating the effects of GnRH. A study by Elmslie and colleagues in 1990 showed that acute application of LHRH actually reduced Ca^{2+} currents in a G-protein coupled manner (Elmslie et al., 1990), presumably via $G_{i/o}$. Bley and Tsien in 1990 also showed that LHRH reduced Ca^{2+} currents in a G-protein dependent manner as well as being PKC independent (Bley and Tsien, 1990).

GnRH activates Erk in various cells and cell lines (Mitchell et al., 1994; Sim et al., 1995; Roberson et al., 1995; Sundaresan et al., 1996; Reiss et al., 1997; Mulvaney et al., 1999; Han and Conn, 1999; Naor et al., 2000; Benard et al., 2001;

Klausen et al., 2002). Several lines of evidence have suggested that GnRH activates Erk through PKC and MEK dependent pathways with the possible contributions from tyrosine phosphorylation and Ca^{2+} dependent mechanisms (Sundaresan et al., 1996; Reiss et al., 1997; Call and Wolfe, 1999; Mulvaney et al., 1999; Benard et al., 2001).

Currently, the two suggested pathways by which GnRH could activate Erk are: through activating the PKC-Raf-1 pathway; and through the growth factor transactivation by Src leading to Ras activation pathway (Benard et al., 2001; Klausen et al., 2002). GnRH activation of Erk via the PKC-Raf-1 pathway seems unlikely since B-Raf is the major Raf isoform in neurons and not Raf-1.

1.7 Signal transduction of G-protein coupled receptors

Since one of the aims of this thesis is to examine actions mediated by GPCRs, their properties and mechanisms of action will be discussed.

1.7.1 Properties of G-protein coupled receptors (GPCRs)

Cellular signalling is achieved through many proteins, lipids, ions, peptides, and many other small molecules. Mechanisms by which GPCRs stimulate MAPKs fall into three general categories: activation of classical G-protein effectors such as PKA, PKC, and intracellular calcium; transactivation via classical receptor tyrosine kinases; and signals initiated by direct interaction between β-arrestins and molecules of the MAPK cascade (Luttrell, 2002).

Intracellular propagation of such events often requires that these signals be turned "on" and "off" in order to regulate the intensity and duration of the signals. G-proteins are one of the most ubiquitous of these intracellular switches that alternates between a guanosine triphosphate (GTP)-bound on state and a guansosine diphosphate (GDP)-bound off state. Heterotrimeric G-proteins (G-proteins) are consisted of Gα, Gβ and Gγ subunits in close proximity to the intracellular component of GPCRs. During the "off" state GDP-bound Gα subunits are tightly bound to their cognate Gβγ heterodimer. This union assists Gα localization to the plasma membrane and is crucial for functional coupling to GPCRs. Upon GPCR activation GDP is released from the Gα subunit then GTP binds. The binding of GTP creates conformational changes in the flexible switch regions of Gα, resulting in the dissociation of Gβγ. Free GTP-bound Gα and Gβγ are then able to interact with downstream effector proteins. Hydrolysis of GTP to GDP by the intrinsic guanosine triphosphatase (GTPase) returns Gα to its inactive state. GDP-bound Gα re-associates with Gβγ and terminates all interactions with effector proteins.

Gα subunits are categorized into four primary classes based on preferential regulation of specific classes of effectors: $G_{\alpha s}$, $G_{\alpha i/o}$, $G_{\alpha q/11}$, and $G_{\alpha 12/13}$. All four classes of Gα subunits have well studied downstream effectors. $G_{\alpha s}$ class typically activates adenylyl cyclase (AC). AC stimulation generates cAMP (Birnbaumer, 1992). cAMP is classically known to activate PKA, and it has also been shown to directly activate Ras (Ambrosini et al., 2000; Tsygankova et al., 2000) leading to Erk activation. Furthermore, cAMP can also activate Rap-1 (de Rooij et al., 1998; Kawasaki et al., 1998). However, direct activation of Rap-1 by PKA could also occur

(Vossler et al., 1997; Grewal et al., 2000). Activated Rap-1 then proceeds to activate Raf. Different isoforms of Raf exist in different types of cells, and in neurons B-Raf is predominant. Activation of B-Raf in neurons and PC-12 cells have been demonstrated to activate Erk (Erdhardt et al., 1995; Vossler et al., 1997; Dugan et al., 1999; Grewal et al., 1999; Grewal et al., 2000). Interestingly, in non-neuronal cells Raf-1 is present which is inhibited by the Rap-1-cAMP pathway, hence; Raf-1 activation inhibits ERK (Wu et al., 1993; Cook and McCormick, 1993; Hafner et al., 1994; D'Angelo et al., 1997; Dugan et al., 1999; Impey et al., 1999).

The second class of Gα, $G_{\alpha i/o}$, inhibits AC (Birnbaumer, 1992), as a result can inhibit Erk activation via the mechanism that is opposite of $G_{\alpha s}$. However, $G_{\alpha o}$ has been shown to directly activate B-Raf causing Erk activation (Antonelli et al., 2000). In addition, $G_{\alpha i/o}$ coupled receptor could interact with the $G_{\beta\gamma}$ subunits leading to Erk activation (Pierce et al., 2001; Luttrell, 2002). The pathways that $G_{\beta\gamma}$ subunits could follow to activate Erk include: transactivation of growth factor receptors (Daub et al., 1996; Ferguson, 2003); activation of Ras-GRF, a GEF that activates Ras (Crespo et al., 1994; Koch et al., 1994; Mattingly and Macara, 1996); activation of PI3Kγ (Hawes et al., 1996; Stephens et al., 1997) leading to PKC activation (Takeda et al., 1999) and subsequently, Erk activation.

The third class of G_{α} subunit is the $G_{\alpha q/11}$ class, which activates phosphoinositide-specific phospholipase C (PI-PLC). Upon activation, PI-PLCs will hydrolyze phosphatidylinositol 4, 5-bisphosphate (PIP_2) generating inositol 1,4,5-triphosphate (IP_3) and DAG second messengers (Voet and Voet, 1995). Generation of

IP_3 could elevate intracellular Ca^{2+} levels by releasing intracellular Ca^{2+} from IP_3-sensitive Ca^{2+} stores leading to ERK activation, as mentioned in a previous section.

The $G_{\alpha 12/13}$ class is capable of regulating the small G-protein RhoA family of effectors that have Dbl-homology (DH) and pleckstrin-homology (PH) domains (Worthylake et al., 2000). Although activation of Rho primarily affects cytoskeletal rearrangements, this pathway may contribute addition mechanisms to the activation of the Erk cascade through Rho- dependent PTKs (Naor et al., 2000). $G_{\alpha 12/13}$ can potently induce JNK activation in many cellular contexts (Radhika and Dhanasekaran, 2001) via small GTPases Ras, Rac, Rho, and CDC42 (Goldsmith and Dhanasekaran, 2007). In certain cell types $G_{\alpha 12/13}$ can weakly stimulate Erk1/2 activation in a Ras-dependent manner (Mitsui et al., 1997), whereas in other systems they reduce Erk1/2 activation at the MEKs level (Voyno-Yasenetskaya et al., 1996). In certain cell types $G_{\alpha 12}$ has been shown to inhibit p38 MAPK (Dermott et al., 2004), but $G_{\alpha 13}$ stimulates p38 MAPK (Marinissen et al., 2003). In addition, agonists that stimulate $G_{\alpha 12/13}$-coupled pathways remain unclear (Sugden and Clerk, 1997) although some studies suggest that thrombin and thromboxane A2 signal through $G_{\alpha 12/13}$ in platelets and astrocytoma cells (Offermanns et al., 1994; Aragay et al., 1995).

Currently, there are five known human Gβ and twelve human Gγ subunit genes allowing for many potential combinations of Gβγ dimers. It was previously thought that the roles of Gβγ were to support coupling of Gαβγ to GPCRs and to act as Gα inhibitor with its guanine nucleotide dissociation inhibitor (GDI) activity. It is now recognized that free Gβγ after dissociating from Gαβγ can activate many of its

own effectors (McCudden et al., 2005). It is also known that free Gβγ is capable of regulating small G proteins and kinases. Activation of various GPCRs results in Gβγ mediated activation of MAPKs. Furthermore, it has been demonstrated that Gβγ can regulate certain AC forms, activate PLC-β and PLC-ε, and localize GRK2 and GRK3 to the plasma membrane (Gao and Gilman, 1991; Tang and Gilman, 1991; Boyer et al, 1992; Taussig et al., 1994; Wing et al., 2001). However, the mechanisms that Gβγ interacts with its downstream effectors remain unclear.

Extended application of agonist to GPCRs often results in rapid receptor desensitization. There are two types of desensitization: 1) heterologous desensitization and 2) homologous desensitization. Heterologous desensitization of GPCRs involves phosphorylation of the receptor by PKA and PKC (Benovic et al., 1985; Pitcher et al., 1992). This form of desensitization is non-specific and occurs when any signal activates intracellular kinases, resulting in feedback from second messenger activated kinases impairing receptor activity and inhibiting production of second messengers. The mechanism involves these kinases phosphorylating serine residues on the C-terminus tail of the GPCR causing the receptor to uncouple from the G-proteins. The second form receptor desensitization termed homologous desensitization is more common to agonist-triggered GPCR desensitization. This process involves uncoupling the G-protein from the receptor, and also receptor internalization (Freedman and Lefkowitz, 1996). The mechanism includes several events: phosphorylation of the agonist-bound receptor by G-protein receptor kinases (GRKs), increasing receptor binding to the arrestin family of proteins, thereby

decreasing receptor availability for G-protein coupling (Tilakaratne and Sexton, 2005).

GRKs are comprised of a family of seven serine/threonine protein kinases (Kohout and Lefkowitz, 2003). Five members of the GRKs (GRK1, GRK4, GRK5, GRK6, and GRK7) are constitutively associated with the plasma membrane (Kohout and Lefkowitz, 2003). The other two members (GRK2 and GRK3) are recruited to the membrane after GPCR activation (Kohout and Lefkowitz, 2003). After an agonist binds, GPCR changes conformations which then promote binding of one or more GRKs. GRKs then phosphorylate GPCRs at specific residues within either the third intracellular loop or the carboxyl terminal domains (Ferguson, 2001). GRK phosphorylation of GPCR increases binding affinity of arrestin proteins which inhibit G-protein coupling (Lohse et al., 1990; Lohse et al., 1992). In turn, GRK phosphorylation of GPCR can be positively or negatively modulated by phosphorylation of second messenger kinases (Kohout and Lefkowitz, 2003).

Currently, there are four known arrestin proteins categorized into two groups based on sequence homology and their associated receptors and anatomical allocation (Ferguson, 2001). Members of the first group of arrestins are visual arrestin and cone arrestin (Shinohara et al., 1987; Yamaki et al., 1987; Murakami et al., 1993; Craft et al., 1994). Members of the second group of arrestins are β-arrestins (β-arrestin 1 and β-arrestin 2) (Lohse et al., 1990a; Attramadal et al., 1992). β-arrestins are ubiquitously distributed with predominance in neuronal tissues and spleen (Attramadal et al., 1992). The mechanisms by which arrrestins promote GPCR desensitization includes physical uncoupling of GPCRs from heterotrimeric G-

proteins as well as targeting desensitized GPCRs to endocytosis (Ferguson, 2001). Targeting GPCRs to endocytosis is achieved by linking the receptors to clathrin and AP2 forming clathrin coated pits which will subsequently be internalized (Goodman et al., 1996; Laporte et al., 2000). This process results in reduction of the number of cell surface membrane-bound receptors (Tilakaratne and Sexton, 2005).

1.7.2 Phospholipase C (PLC)

Phospholipase C (PLC), which is categorized into four classes: PLCβ (1-4), PLCγ (1-2), PLCδ, and PLCε (Fukami, 2002) is the primary downstream effector of $G_{q/11}$. PLCβ may be activated by the βγ subunits associated with $G_{q/11}$ or $G_{i/o}$ proteins as well as by $G_{q/11}$ α-subunits (Fukami, 2002). PLCγ may be activated by receptor tyrosine kinases and also by non receptor tyrosine kinases (Fukami, 2002). PLCδ are controlled by Ca^{2+} and PLCε are controlled by Ras proteins (Fukami, 2002). Activated PLC typically hydrolysizes PIP_2 to diacylglycerol (DAG) and inositol-1, 4, 5 - triphosphate (IP_3). The generated IP_3 could lead to elevation of intracellular Ca^{2+} by releasing it from IP_3-sensitive Ca^{2+} stores (Rhee and Choi, 1992). Increased Ca^{2+} levels can trigger CAM Kinase II-induced inhibition of Ras-GAP, thereby indirectly increasing Ras activation and subsequent ERK activation (Chen et al., 1998). Elevated Ca^{2+} levels may activate various Ras-GEF causing Ras activation (Farnsworth et al., 1995). Moreover, Ca^{2+} can trigger protein tyrosine (PyK2) autophosphorylation leading to recruitment of nonreceptor kinase c-Src and activation of Erk in a Ras-dependent manner (Lev et al., 1995; Dikic et al., 1996; Della Rocca et

al., 1999). Production of DAG can activate PKC leading to a PKCα-Raf pathway activation of Erk, which is independent of Ras proteins (Kolch et al., 1993).

1.7.3 Protein kinase C (PKC)

Of mammalian PKC, there are 10 isoforms derived from 9 genes (Ohno and Nishizuka, 2002). These define a serine/threonine family of phosphorylating enzymes (Amadio et al., 2006). PKCs are ubiquitously expressed and influence multiple cellular functions (Amadio et al., 2006). PKCs mediate signals involved in acute processes (ion fluxes and neurotransmitter release), mid-term processes (receptor modulation), and also long-term processes (cell proliferation, synaptic remodelling, and gene expression) (Amadio et al., 2006).

All PKC isoforms have a highly conserved catalytic domain with a highly variable regulatory domain (Popp et al., 2006). Majority of the differences seen in sequence homology, substrate preferences and activators are accounted for by the differences in the regulatory domains (Ohno and Nishizuka, 2002; Newton, 2001). The different isoforms are grouped into three classes: 1) Classical PKC isoforms (α, βI, βII, and γ) have a C1 domain containing a repeat of cysteine-rich motifs that form binding sites for phorbol esters and DAG and a Ca^{2+}-dependent, phospholipids binding C2 domain (Popp et al., 2006). 2) Novel PKC isoforms (δ, ε, η, and θ) possess a C1 domain, a C2 domain that lacks amino acid residues necessary for Ca^{2+} binding. Hence, activation of these isoforms is influenced by DAG and phorbol esters without requiring Ca^{2+} (Popp et al., 2006). 3) Atypical PKC isoforms (ζ and λ) contain only a single cysteine-rich motif and no C2 domain; therefore, activation is

unaffected by DAG, phorbol esters, or Ca^{2+} (Popp et al., 2006). The precise mechanism of action of atypical PKCs remains unclear.

Phosphorylation of several serine/threonine sites in PKC is essential for the full activation of the enzyme in response to different stimulations (Hofmann, 1997; Parekh et al., 2000; Newton, 2003). The three important phosphorylation sites in cPKC and nPKC are: the activation loop site, the turn motif site, and the hydrophobic motif site, with the threonine residue in the activation loop being the most crucial phosphorylation site for PKC kinase activity (Cazaubon et al., 1994; Orr and Newton, 1994; Cenni et al., 2002; Liu et al., 2002).

Interaction with specific activators such as: physiological activator DAG or the analogue phorbol ester (PMA), allows the activation of the enzyme by opening its folded conformation (Amadio et al., 2006). This activation mechanism is related to the translocation of PKCs to different intracellular sites/membranes (Amadio et al., 2006). PKC are known to phosphorylate numerous intracellular proteins and many studies have also reported their involvement in mediating the effects of GnRH (Sundaresan et al., 1996; Bernard et al., 2001; Shah et al., 2003).

1.7.4 Protein kinase A (PKA)

cAMP-dependent protein kinase (PKA) is a well characterized member of the protein kinase superfamily. Structurally, PKA is comprised of an inactive holoenzyme with two fully phosphorylated and an active catalytic subunits embedded within complexing with a dimmer of regulatory subunits (Kim et al., 2006). Activation of cAMP signalling entails binding of an extracellular ligand to a GPCR

and via G-proteins regulates one of many isoforms of adenylyl cyclase which generates cAMP (Tasken and Aandahl, 2004). Although other effectors of cAMP have been reported, the most demonstrated downstream effector system is PKA (Tasken and Aandahl, 2004).

The cAMP-PKA pathway has been implicated in regulating diverse cellular processes such as cell cycle, proliferation, and differentiation, regulation of chromatin condensation/ decondensation, nuclear assembly/disassembly, microtubule dynamics, as well as intracellular transport mechanism and ion fluxes (Tasken and Aandahl, 2004). Upon binding of cAMP to the regulatory unit, the active enzyme is released (Taylor et al., 2005). PKA has been reported to inhibit as well as activate ERKs in various systems (Stork and Schmitt, 2002): 1) PKA can impede Ras-dependent activation of Erks by inhibiting Raf-1 activation (Cook and McCormick, 1993). 2) PKA phosphorylation seems to block Raf-1 activity directly (Mischak et al., 1996). 3) Src can mediate PKA activation of Rap-1 in the activation of Erks (Stork and Schmitt, 2002). 4) In selected neurons, cAMP and PKA can activate Erk via Ras (Ambrosini et al., 2000; Iida et al., 2001).

1.7.5 Mitogen-activated protein kinases (MAPK)

Mitogen-activated protein kinases (MAPK) are a family of serine/threonine protein kinases that transduce extracellular stimuli into intracellular post-translational and transcriptional responses (Seger and Krebs, 1995; Lewis et al., 1998; Widman et al., 1999). In mammalian cells, three MAPK families have been clearly characterized: Erk, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38

kinase (p38) (fig. 1) (Ma and Quirion, 2002). MAPK pathways relay, amplify and integrate extracellular signals from a broad range of stimuli and elicit an appropriate physiological response including cellular proliferation, differentiation, development, inflammatory responses and apoptosis (Zhang and Liu, 2002).

Mitogens, growth factors, and cytokines are known activators of Erk. The Erk subfamily includes Erk1/ Erk2/ Erk3. The biological responses commonly attributed to Erk are cell growth, differentiation, and development (Zhang and Liu, 2002). Although most protein kinases require serine, threonine, or tyrosine residue phosphorylation, Erk activation requires an unusual dual- phosphorylation of serine and threonine residues. This phenomenon could be important in preventing non-specific activation of Erks, which may be potentially oncogenic (Sugden and Clerk, 1997).

The JNK/SAPK subfamily of MAPK has three isoforms (JNK1, 2, and 3). JNK1 and JNK2 are ubiquitously expressed while JNK3 is mainly expressed in neuronal and heart tissues (Nishina et al., 2004). Known activators of JNK include many types of stress such as UV and γ-radiation, inflammatory cytokines, and growth factors (Nishina et al., 2004). JNK activation can lead diverse biological responses such as inflammation, differentiation, growth, and apoptosis (Liu and Lin, 2005).

The p38 MAPK subfamily also responds to a wide range of extracellular stimuli, in particular, to stress factors such as UV radiation, hypoxia, osmotic shock, pro-inflammatory cytokines, and less often growth factors (Raman et al., 2007). Currently, at least four isoforms of p38 are known p38α, p38β, p38γ, and p38δ (Liu and Lin, 2005). Activation of p38 can lead to inflammation, growth, differentiation, and apoptosis (Zhang and Liu, 2002).

1.7.6 Extracellular-regulated kinase (Erk)

Extracellular-regulated kinases 1, 2 (Erk1/2) signal transduction is complex, and is influenced by numerous pathways with immense cross-talk. However, it is also the complexity that allows for regulating subtle differences, fine-tuning, and amplification of signals varying with signals and cell type. The nature of this complexity also creates enormous possibilities for convergence and divergence of signals to and from the Ras-Raf-MEK-Erk pathway (Caunt et al., 2006). Specificity of signals is maintained, at least partially, by two basic mechanisms (Pouyssegur et al., 2002): 1) by scaffolding proteins that assemble specific kinases into a complex (Whitmarsh and Davis, 1998), and 2) by each MAPK members having specified docking sites that ensure correct binding and activation only by the specific kinase (Tanoue et al., 2000). Erk1/2 are distributed throughout the cell, and they are associated with plasma membrane receptors and transporters (Raman et al., 2007).

Various components within the Erk cascade have also been discovered in lipid rafts and caveolae (Furuchi and Anderson, 1998).The classic receptor mediated signalling pathway to Erk activation is via the small GTPase Ras. Small GTPases are low molecular weight (20-24 kDa) monomeric proteins with intrinsic GTP-hydrolysing activity (Bhattacharya et al., 2004). Small GTPases contribute to a wide range of functions such as: regulation of gene expression, cell proliferation, cell migration, cytoskeletal rearrangement, intracellular vesicle trafficking, and protein nucleocytoplasmic transportation (Tilakaratne and Sexton, 2005). Ras is a small monomeric GTP binding protein within a superfamily of over 150 members. The Ras superfamily has 6 subfamilies: Ras, Rho, Ran, Rab, Arf, and Kir/Rad/Rem (Ehrhardt et al., 2002). Within the Ras subfamily there are 13 members categorized into 5

groups: ^{p21}Ras (H-Ras, N-Ras, and K-Ras), R-Ras, M-Ras, Rap, and Ral (Ehrhardt et al., 2002). In order for Ras to be activated it must be translocated from the cytoplasm to the cell membrane where GTP/GDP exchange can take place. Ras is subsequently anchored to the plasma membrane through post-translational isoprenylation (farnesylation or geranylgeranylation), which targets proteins with a CaaX motif that is common in the Ras family members (Casey, 1995). The GTP/GDP exchange process is conducted by guanine-nucleotide exchange factors (GEFs), which target GDP-bound Ras then promote the exchange of GTP for GDP to activate Ras (Ehrhardt et al., 2002). GEF members that activate Ras are SOS1/2, Ras-GRF, Ras-GRP, and Epac 1/2 (Ehrhardt et al., 2002). GEFs are activated by various signals such as phosphorylation, diacyglycerol, Ca^{2+}, and cyclic nucleotides (Ehrhardt et al., 2002). Inactivation of G-protein is achieved by GTPase activating proteins (GAPs), which increase G-proteins GTPase activity (Dovovan et al., 2002).

Once Ras is activated it recruits and activates the serine/threonine MAPK kinase kinase (Raf) at the plasma membrane. Currently, the three known members of the Raf family are: A-Raf, B-Raf, and Raf-1, with B-Raf as the primary isoform in neurons (Marais et al., 1997). Interestingly, Ras binding alone could activate B-Raf but not Raf-A or Raf-1 (Marais et al., 1997). Activated Raf could subsequently activate a threonine/tyrosine MAPK kinase (MEK) that in turn activates Erk (Malumbres and Pellicer, 1998). Activation of Erk by growth factors and other agonists triggers its translocation to the nucleus. This translocation gives Erk1/2 access to nuclear transcription factors and other nuclear proteins that are then phosphorylated or stabilized to give relevant changes in gene expression (Raman et al., 2007) such as cAMP response element-binding protein (CREB), Elk, Ets, Myc,

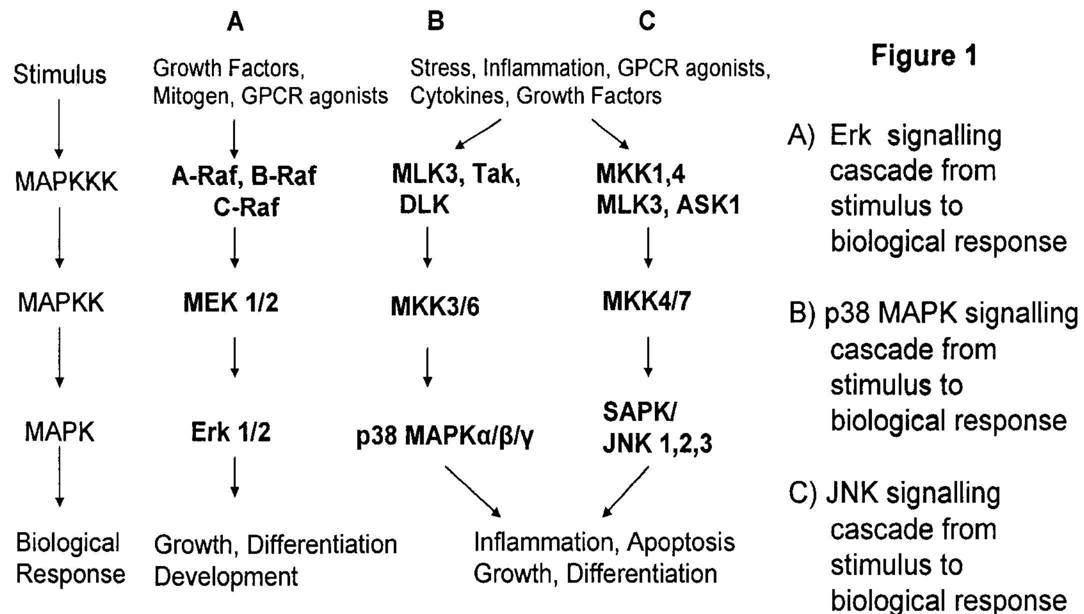
and p90-RSK (ribosomal S6 kinase) that participate in DNA transcription, cell growth, and cell division (Malumbres and Pellicer, 1998).

Statement of Problem

Studies have shown that target-derived NGF is responsible for the maintenance of TTX-sensitive and TTX-insensitive $I_{Na,}$ as well as N- and L-type calcium currents ($I_{Ca,N}$ and $I_{Ca,L}$) in adult BFSG B-neurons(Lei et al., 1997; Lei et al., 1998; Lei et al., 2001; Petrov et al., 2001). The effects of NGF on I_{Ca} in serum-free, defined medium cultures are transduced via the MAPK pathway (Lei et al., 1998), whereas the transduction mechanism that is responsible for NGF-induced increase in I_{Na} remains to be determined. In addition, LHRH has been demonstrated to be capable of regulating functional expression of Ca^{2+} channels (Ford et al., 2003a).

The work in this thesis in conjunction with electrophysiological data (Ford dissertation, 2003) is undertaken to examine 1) whether LHRH-induced increase in I_{Ca} in BFSG occurs via the MAPK pathway similar to NGF 2) how G-protein coupled receptors signal to the MAPK pathway 3) the mechanism by which NGF produces long-term increase in I_{Na} 4) whether LHRH has access to the transduction mechanism by which it can produce long-term increase in I_{Na} in a similar manner as NGF.

Mitogen activated protein kinases (MAPKs)



Modified from **Cell Signaling Technology**

Figure 1. Schematic of mitogen activated protein kinases (MAPKs) cascade. A) Erk cascade **B)** p38 MAPK cascade **C)** SAPK/JNK cascade.

Chapter 2

Methodology

2.1 Methods

2.1.1 Cell culture

The following procedures are adapted and modified from Selyanko et al., 1990 and Lei et al., 1997. Adult bullfrogs (Rana Catesbeiana) (size 10-15cm; body weight 250-350g) purchased from Wards Natural Sciences Ltd. were kept at room temperature. Dissection, isolation, dissociation, and culture procedures were conducted under aseptic conditions. Following pithing, the ventral section of each frog was sterilized with 9% iodine in ethanol. The abdominal muscle was removed to carefully expose the paravertebral sympathetic chain. The VIIIth-Xth paravertebral sympathetic ganglia were removed from both sides of each frog and dissociated with trypsin and collagenase (Selyanko et al., 1990). After enzymatic and mechanical dissociations, the dissociated neurons were resuspended in 3mL of defined, serum-free medium containing 73% Leibovitz's L-15 medium (Gibco) in HPLC grade water supplemented with 1mM $CaCl_2$, 10mM glucose, 10 μM cytosine arabinoside, 100 μg/mL streptomycin and 100 units/mL penicillin at pH 7.2 (Lei et al., 1997). Neurons were plated into 24-well tissue culture plates at 1.2 ganglia/well, each well was then filled with 2mL of L-15 medium. The medium was exchanged the next day and every second day after that unless otherwise stated. Plates were maintained at room temperature (22°C) for 6-7 days.

2.1.2 Chemicals

The following drugs and reagents were from Biomol (Plymouth Meeting, PA), wortmannin, PD98059 (2'-Amino-3'-methoxyflavone), perillic acid (PA), α-Hydroxyfarnesylphosphonic acid (α-HFA), H-89 (N-[2-(p-Bromocinnamylamina)ethyl]-5-isoquinolinesulfonamide-2HCl), Sp- and Rp-cAMPS (-Adenosine-3',5'-cyclic mononphosphorothioate triethylamine salt), PMA (Phorbol 12-myristate-13-acetate) and 4α-PMA . Chicken II Luteinizing Hormone Releasing Hormone (LHRH) and Nerve Growth Factor (NGF) were from Alomone Labs (Jerusalem, Israel). All other reagents were from Sigma-Aldrich (Oakville, Ont., Canada) or Fisher Scientific (Edmonton, AB, Canada). Some of the inhibitors used were exceedingly hydrophobic; therefore, were dissolved in dimethyl sulfoxide (DMSO) to make stock solutions. The stock solutions were then dissolved in culture media to obtain working concentrations. The final DMSO concentration in all cases was < 0.1%.

2.1.3 Morphology analysis

Dissection, isolation, and dissociation were conducted following procedures described in *Tissue Culture* section. The dissociated cells were then redistributed into 2.5ml of medium in each of 20 35-mm polystyrene tissue culture dishes. Dishes were placed in a light proof, humidified chamber and maintained at room temperature (22°C) for 7 days. Dishes were divided into three groups: control, LHRH, and NGF. Control group dishes were maintained in L-15 medium, LHRH group received 100nM LHRH, and NGF group received 200ng/mL NGF. Drug treatments and

medium exchanges were performed the next day and every second day for 7 days. At day 7, pictures of cells from all three groups were captured at 100x magnification, counted, and grouped as having neurites or not having neurites.

2.1.4 Immunoblot analysis

The VIIIth, IXth and Xth paravertebral ganglia were removed from both sides of 2 adult bullfrogs and the neurons dissociated with trypsin and collagenase as described above. The cell suspension was plated into 8 to10-wells of a 24-well dish at a density of 1.2 ganglia/well. The dissociated cells were cultured in L-15 medium for 5-6 days. At day 6, some treatment groups were given L-15 supplemented with 50μM PD98059 for 24 hours to reduce basal phosphorylation of Erk. Incubation with 100nM LHRH began after two 5 min washes in L-15 to remove PD98059. Cells were incubated for 10 min, 1h, or 6h in the presence of the peptide. NGF experiments involved treatment of different groups of cells with 200ng/ml NGF for 15 min, 1h, 6h, and 6 days. BFSG neurons cultured in 24-well dishes were washed with ice cold L-15 medium prior to harvesting. Akt experiments included 4 treatment groups: 100nM LHRH treatment for 10 min; 200ng/ml NGF treatment for 15 min; 1μM wortmannin treatment for 6hrs; and 1μM wortmannin pre-treatment for 6hrs followed by 200ng/ml NGF incubation for 15 min. PKA experiments involved incubation with Sp-cAMP or its inactive form Rp-cAMP for 1hr to activate PKA then Erk phosphorylation was determined. PKC experiments involved incubation with PMA or its inactive form 4α PMA for 1hr to activate PKC then Erk phosphorylation was determined. Procedures for harvesting neurons and electrophoresis were based on

modified method described in Song and Posse de Chaves 2003. These neurons were washed with ice cold washing buffer with 1mM Na_2VO_4 and 1mM NaF to inhibit phosphatase activity. Cells from two wells of the same treatment were harvested with modified Laemmli sample buffer (40 mM Tris-HCl pH 6.8, 0.002% bromophenol blue, 10% glycerol, 1% sodium dodecyl sulfate (SDS) and 4% 2-mercaptoethanol) and boiled for 2 min. Proteins were separated by SDS-PAGE on 10% polyacrylamide containing 0.1% SDS. After electrophoresis, proteins were transferred to 'Immobilin' polyvinylidene difluoride (PVDF; Bio-RAD; Hercules, CA, USA) membranes overnight at 4°C in 25mM Tris (192 mM Glycine, 16% methanol buffer, 0.1% SDS, and pH 8.3). Membranes were blocked for 1h in 0.1% TTBS (Tris Buffered Saline with 0.1% Tween-20) with 5% non-fat milk at room temperature and incubated in the primary antibody overnight at 4°C. The primary antibodies used were: rabbit polyclonal anti-phospho TrkA (Tyr490) (1:1000), anti-phospho p42/44 MAPK (Thr202/Tyr204) (Erk 1/ 2) (1:1000), and anti-phospho Akt (S473) (1:1000) from Cell Signalling Technology (Beverly, MA, USA); anti-pan-Trk polyclonal antibody Trk (C-14) (1:500), and polyclonal anti-Erk 1 (C-16) (1:1000) from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) monoclonal anti-α-tubulin (T9026) (1:2000) from Sigma, Canada and monoclonal anti-β-tubulin (T4026) (1:800) from Sigma, Canada. Membranes underwent three washes in TBS, TTBS, and TBS followed by incubation with the secondary antibody (goat anti rabbit I_{Gg}; Pierce, Brockville, Ont., Canada) (1:2000) in blocking buffer for 1 hr at room temperature. Immunoreactivity was detected using enhanced chemiluminescence ('ECL Plus Detection System' Amersham Biosciences, Piscataway, NJ, USA). Equal protein

loading was checked by probing for the non-phosphorylated forms of Erk 1/2, TrkA, or α/β-tubulin.

To demonstrate that PKC is activated and not downregulated, cells were treated with PMA or 4αPMA for 1hr each day for 6days. After treatments, cells were rinsed with ice cold phosphate buffered saline (with protease inhibitor cocktail, NaF, and Na_2VO_4) and then quickly transferred to homogenization buffer (250 mM sucrose, 2mM EGTA, 1mM EDTA, 20mM Hepes, 100mM NaF, 2mM Na_2VO_4, and protease inhibitor cocktail). Cells were lysed by sonication (3x5 sec) followed by protein assay determination to assess, normalize and load equal amount of protein for ultracentrifugation for 1hr at 100000 rpm at 4°C. The supernatants were saved as the soluble fraction, pellets fractions were rinsed twice with ice cold PBS then resuspended in homogenization buffer with 1% Triton X-100. Electophoresis is performed on 10% SDS-PAGE followed by transfer to PDVF membrane for immunoblotting with antibodies that recognize total PKCα, PKCβ1, PKCγ, PKCδ, PKCε, PKCξ (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Bands were detected by enhanced chemiluminescence.

2.1.5 Ras activation assay

Ras activation experiments were adapted and modified using method described in Herrmann et al 1995. Cell culture density was increased in these experiments to 4 ganglia/well. After treatments, cells were rinsed with ice cold phosphate buffered saline [with protease inhibitor cocktail (Roche Diagnostic #11836153001), NaF, and Na_2VO_4] then immediately harvested and lysed in 300μL

of BOS buffer (50nM Tris-HCl, pH 7.4, 200 mM NaCl, 1% Nonidet P-40, 10% glycerol, 10 mM NaF, 2.5 mM MgCl2, 1 mM EDTA). A sample from each group was reserved for protein assay. Protein assay was performed on the lysates and protein concentration was normalized. Equal amounts of GST-RBD (gift of Dr. Jim Stone, Dept Biochemistry, University of Alberta) construct pre-coupled to glutathione-agarose beads in BOS buffer was added to each concentration normalized lysate group. Samples were incubated for 2hrs at 4°C with gentle shaking to capture activated Ras. Samples were then rinsed 3 times followed by addition of 2X sample buffer and boiling for 5 min to cleave glutathione-agarose beads from GST-RBD constructs with active Ras attached. Equal volumes of GST-RBD with bound active Ras in 2X sample buffer for each experimental group were loaded for gel electrophoresis. Anti-Ras antibody [from Upstate Upstate Cell Signaling Solutions/Millipore Corporation
(Billerica, MA)] was used to detect active Ras across experimental groups and anti-GST antibody (from Abcam, Cambridge, MA) was used as loading control.

2.1.6 Rap-1 activation assay

Procedures were followed according to EZ-Detect RAP-1 activation kit (Pierce Brockville, Ont., Canada). Cell culture density was increased to 4 ganglia/well. Briefly, after treatments, cells were rinsed with ice cold phosphate buffered saline (with protease inhibitor cocktail, NaF, and Na_2VO_4) then immediately harvested and lysed in 300uL of lysis buffer. A sample from each group is reserved

for protein assay. Protein assay was performed on the lysates and protein concentration was normalized. Equal amounts of GST-RalGDS-RBD construct were added to individual SwellGel^{R} (immobilized glutathione disc) and each normalized lysate experimental group was immediately added to each individual SwellGel-GST-RalGDS-RBD construct. Samples were incubated for 1hr at 4°C with gentle shaking to capture activated Rap-1. Samples were then rinsed 3 times followed by addition of 2X sample buffer and boiling for 5 min to cleave glutathione beads from GST-RalGDS-RBD construct with active Rap-1 attached. Equal volumes of GST-RalGDS-RBD with bound active Rap-1 in 2X sample buffer for each experimental group were loaded for gel electrophoresis. Anti-Rap-1 antibody (from Pierce Brockville, Ont., Canada) was used to detect active Rap-1 across experimental groups and anti-GST antibody (from Abcam, Cambridge, MA) was as loading control.

2.2 Quantification

Data from immunoblots were scanned and their densities were quantified using "Un-Scan-it gel" software (Silk Scientific, Orem, UT). Changes in density were assessed relative to loading controls from 3-4 gels for each experiment. For comparison and presentation of data from replicated experiments, relative densities were normalized to the control group in each particular series.

Chapter 3

Results

3.1 Results

3.1.1 Mechanism of LHRH-induced increase of I_{Ca}

It has been reported that I_{Ba} maximum current density in 6d cultured neurons was 118±7 pA/pF (fig 2A). This increased to 158±16 pA/pF ($p < 0.02$) by 34% in cells cultured for 6d with 0.45μM LHRH (fig. 2B) (Ford dissertation, 2003). It has also been shown that this LHRH effect to increase I_{Ba} is not due to a change in the voltage-dependence of activation or inactivation (Ford et al., 2003a). This increase in $I_{Ca,N}$ was shown to be a transcription-dependent event (Ford et al., 2003a). Hence, exposure to LHRH may induce synthesis or affect the trafficking of N-type Ca^{2+} channels. An LHRH-mediated change in I_{Ca} has been reported to involve MEK using the MEK inhibitor PD98059 (10μM) (Alessi et al., 1995). Moreover, this compound, which inhibits the NGF-induced increase in I_{Ba} in BFSG neurons (Lei et al., 1998), also blocked LHRH-induced increase in current density (fig. 2C, 2D; Table 1) (Ford dissertation, 2003). These findings are consistent with the hypothesis that the effect of LHRH on the functional expression of Ca^{2+} channels involves the activation of MAPK via MEK.

If the effect of LHRH is mediated through the MAPK pathway, it should be possible to show the activation of MAPK after 6d exposure to LHRH. Examination of phosphorylation of Erk1/2 of the MAPK family was carried out by immunoblot analysis. All immunoblots figures represent results from at least three replicate experiments, except for figures 6 and 13 which had two replicate experiments. Immunoblot data are presented with representative blots such as figure 3A and as quantified and normalized to controls using the format shown in figure 3B. Although

prolonged exposure to 200ng/mL NGF induced an obvious increase in Erk 1/2 phosphorylation (fig. 3A, lane 2, summarized in fig. 3B), this was not observed in neurons exposed to 6d 100nM LHRH (fig. 3A, lane 3, summarized in fig. 3B). It has been suggested that Erk mediated cellular effects could depend strongly on the temporal pattern of Erk activation, such as, sustained activation of Erk is required for the induction of axonal growth (Huang and Reichardt, 2003). However, it is possible that LHRH may trigger a transient activation of Erk that may signal the surface expression of new Ca^{2+} channels via a transcription-dependent manner (Ford et al., 2003a). As a result, a shorter 10min exposure to LHRH was conducted to test whether LHRH-induced transient activation of Erk. Because basal level of Erk phosphorylation was strong in untreated control neurons, this would intrude with Erk activation detection by short-term LHRH incubation. Therefore; it was necessary to pre-treat neurons with the MEK inhibitor, PD98059 (50μM for 24hrs) prior to LHRH exposure. Under these conditions, 100nM LHRH for 10min induced an obvious increase in Erk phosphorylation (fig. 4A, lane 3, summarized in fig. 4C).

Time course characterization of Erk phosphorylation revealed that maximal LHRH-induced Erk phosphorylation was obtained after 10min exposure, which then returned to basal activation levels after 6h of exposure to LHRH (fig. 4A, lane 5, summarized in fig. 4C). However, NGF induced sustained Erk activation throughout the duration of exposure (fig. 5A, lanes 2-4, summarized in fig. 5C). Anti-total Erk and anti-total TrkA were used as loading controls for the above experiments. No increase in Erk phosphorylation was detected when LHRH was applied in the continued exposure of PD98059 (fig. 6A, lane 4, summarized in fig. 6B).

At least two different small GTPases (Rap-1 and Ras) can contribute to the activation of MAPK by extracellular agonists (Sofroniew et al., 2001). Moreover, Rap-1 has been reported to participate in Ca^{2+} channels regulation by neurotrophins in PC12 cells (Black et al., 2003). Therefore, Ras and Rap-1 pull down assays were performed to test their possible role in the action of LHRH. Figures 7A, lanes 3 and 4, and summary in figure 7B illustrate activation of Ras by both NGF and LHRH, but neither substance activated RAP-1 (fig. 7C, lanes 4 and 5, summarized in fig. 7D). Lane 1 in figures 7A and 7C are positive controls showing Ras and Rap-1 activation by GTP-γ-S.

It is possible that the observed effect of LHRH was mediated by TrkA because Erk 1/2 is a well-defined downstream effector of TrkA, and since ligand binding to GPCR may lead to transactivation of Trk and other growth factors (Rajagopal et al., 2004). This seemed unlikely since LHRH did not trigger TrkA phosphorylation (fig. 4A, lanes 2 to 5, summarized in fig. 4B). On the other hand, 200ng/mL NGF induced clear TrkA phosphorylation (fig. 5A, lanes 2-4, summarized in fig. 5B). Furthermore, treatment with NGF but not with LHRH caused activation of the PI3K/Akt pathway, which is also downstream of TrkA (fig. 8A, lanes 2 and 3, summarized in fig. 8B).

Interestingly, in all of the immunoblot analyses performed both anti-phospho-Erk and anti-total Erk antibodies detected only a single band with molecular weight corresponding to Erk2, although the characteristic doublet was observed in rat neuronal samples performed in parallel (fig. 9, lanes 1 and 2). Since both anti-phospho-Erk and anti-total-Erk were unable to detect the band corresponding to Erk

1, it is likely that frogs do not express Erk1. However, another possibility is that mammalian-origin antibodies have limited cross-reactivity with frog proteins or that amphibian Erk1 has the same molecular weight as amphibian Erk2.

3.1.2 How do LHRH receptors couple to MAPK?

In pituitary cell lines, GnRH receptor signalling to MAPK has been reported to involve PLC and PKC (Reiss et al., 1997; Sim et al., 1995; Naor et al., 2000). Electrophysiological studies using the PKC inhibitor chelerythrin (Herbert et al., 1990) was used to investigate the role of PKC in LHRH-induced potentiation of I_{Ba} (Ford dissertation, 2004). The results suggest that chelerythrine (1μM) prevented LHRH-mediated increases in current density, as I_{Ba} recorded in the presence of chelerythrine plus LHRH was not significantly different from cells cultured for 6d with chelerythrine alone ($P>0.05$) (Table 1) (Ford dissertation, 2003). Antibodies directed against the phosphorylated forms of PKCβII and PKCγ were used to examine activation of PKC by LHRH. These antibodies revealed two bands corresponding to approximately 80 and 90-kDa isoforms and LHRH significantly increased the phosphorylation of phospho-PKC βII (fig. 10A, lane 2, summarized in fig. 10B). These results would be expected if LHRH signalling involves PKC. However, LHRH failed to significantly increase phosphorylation of PKCγ (fig. 10A, lane 2, summarized in fig. 10C).

In certain pituitary cell lines, the actions of GnRH are mediated through PKA (Han and Conn, 1999). Moreover, signalling pathways involving PKA, and/or cAMP are known to affect the Ras-MAPK cascade (Naor et al., 2000; Impey et al., 1998).

These findings along with results from cells treated for 6d with H-89 (PKA inhibitor) (Chijiwa et al., 1990) plus LHRH did not show the increase in current density that was seen with LHRH alone ($P<0.05$) (table 1) (Ford dissertation, 2003). This finding suggests the possibility that PKA is also involved in the LHRH effect. However, the direct activation of PKA with the cAMP analogue Sp-cAMP was not sufficient to increase I_{Ba} density. Current densities recorded from cells treated with Sp-cAMP (57.3 ± 4.2 pA/pF) were no different than current density recorded from cells treated with the enantiomer Rp-cAMP, a competitive inhibitor of the activation of PKA by cAMP (63.8 ± 2.8 pA/pF) ($p>0.05$) (Ford dissertation, 2003). To demonstrate that the lack of electrophysiological effects of the extracellularly-applied Sp-cAMP were not due to penetration failure into the neurons, Erk activation in response to extracellular Sp-cAMP by immunoblotting was measured. The effects of Sp-cAMP were compared with the inactive isomer Rp-cAMP (fig. 11A, lanes 5 and 6, summarized in fig. 11B). The effects of 10μM (concentration used for electrophysiology studies) Sp-cAMP were weaker than that of 80nM (concentration used for electrophysiology studies) PMA (fig. 11A, lanes 5 and 3, summarized in fig. 11B) on ERK activation and 10μM (concentration used for electrophysiology studies) Rp-cAMP was ineffective. This confirms the effectiveness of extracellularly-applied Sp-cAMP. The requirement for PKA activation for the action of LHRH is illustrated further by experiments shown in figures 11C and 11D. This shows the possibility of the competitive cAMP antagonist, Rp-cAMP (80μM, concentration used for electrophysiology studies) attenuating LHRH activation of Erk. These results, taken together, with results from the inhibition of PKA and PKC during stimulation with

LHRH suggest that both pathways (PKA and PKC) are necessary in the regulation of Ca^{2+} channels by LHRH, yet only PKC on its own is sufficient to produce this effect. In other words, PKA may be "necessary but insufficient" to alter the functional expression of Ca^{2+} channels.

A concern with the use of phorbol ester is that prolonged exposure may downregulate rather than activate PKC. Therefore, intermittent application protocol was developed for electrophysiological experiments in hope of promoting sustained PKC activation for the 6d duration experiments (Ford dissertation, 2003). In these experiments BFSG B-cells treated with the phorbol ester PMA at 80nM for 1hr/day over 6 days to activate PKC (Ryves et al., 1991) showed I_{Ba} densities (70.8 ± 5.8 pA/pF) (fig. 2F) that were significantly greater than neurons treated with 4αPMA, the negative control for PMA (55.4 ± 4.3 pA/pF) (fig. 2E) ($p<0.05$). Figure 11A and its summarized data in figure 11B illustrate the effect of time course treatments with 80nM PMA. 10min and 1hr PMA exposure induced obvious phosphorylation of both approximately 80 and 90kDa isoforms of PKCβII (lanes 3 and 4). By contrast, the continuous 24h exposure of 80nM PMA showed an attenuation of PKCβII phosphorylation (fig. 11A and lane 5). However, intermittent application of 80nM PMA as was done for electrophysiological experiments (1hr/d for 6d) failed to convincingly demonstrate increase in PKCβII phosphorylation (fig. 11A and lane 6). The intermittent PMA application protocol did not achieve the sustained PKC activation as previously anticipated. This may be of little consequence as the effect of LHRH on Erk phosphorylation is transient (fig. 4A, lanes 3 to 5, summarized in fig. 4C). Hence, the first hour exposure of PMA to neurons in the electrophysiological

experiments (Ford dissertation, 2003) and the transient activation of PKCβII likely initiated the transcriptional changes responsible for altered functional expression of I_{Ca}. Subsequent applications of PMA likely did not activate PKC but this is irrelevant if the first stimulus initiated the signal for altered channel expression.

3.1.3 Mechanism of NGF-induced increase in I_{Na}

It has been shown that long-term exposure of 200ng/mL to BFSG B-neurons resulted in a doubling of Na^+ current density, and this effect is transcription-dependent and does not reflect changes in activation or inactivation (Lei et al., 2001). Although the PI3K inhibitor, wortmannin failed to prevent LHRH or NGF-induced increases in I_{Ba} (Lei et al., 1998), it was highly effective in inhibiting NGF-induced increases in total I_{Na} (Lu et al., Society for Neuroscience abstract, 2002). Exposure of NGF for 10 days increased peak I_{Na} density from 299.1±36.2 to 447.2±52.5 pA/pF ($p<0.03$) (fig. 12A and 12B) (Ford dissertation 2003). By contrast, NGF failed to affect I_{Na} density in the presence of 1μM wortmannin (peak I_{Na} density in wortmannin 343.5±38.5, in wortmannin + NGF 343.7±49.2 pA/pF) ($p>0.85$) (Ford dissertation, 2003). The effect of NGF was also blocked by the more selective PI3K inhibitor, LY294002 (10μM). Peak I_{Na} in LY294002 was 328.5±35.3 compared to 327.0±33.6 pA/pF ($P<0.95$) in the presence of LY294002 + NGF (Ford dissertation 2003). These findings along with immunoblot results indicating NGF's failure to activate Akt in the presence of wortmannin (fig. 13A, lanes 3 and 4, summarized in fig. 13B) suggest the involvement of the PI3K pathway in the action of NGF on Na^+ channel expression.

3.1.4 Does LHRH activate Akt?

Peak, total I_{Na} density is unaffected by LHRH. In 6d cultures, maximum I_{Na} was 238.8±35.0 pA/pF in 0.45μM LHRH compared with 229.9±1.5 pA/pF ($p>0.8$) for controls (Ford dissertation, 2003). Similarly, for 9d cultures in LHRH, current density in LHRH was 276.1±41.5 pA/pF, and 260.0±35.3 pA/pF ($p>0.75$) for controls (Lu et al., Society for Neuroscience abstract, 2002).

Since NGF is capable of exerting effects via both the MAPK and PI3K pathways (Kaplan and Stephens 1994; Sofroniew et al., 2001), the present data suggest that the PI3K pathway is involved in regulation of I_{Na} and previous data suggest the MAPK pathway is involved in NGF regulation of I_{Ca} (Lei et al., 1998). Since LHRH regulates Ca^{2+} channels but not Na^{+} channels, it may only be able to signal via the MAPK pathway and not through the PI3K pathway. Experiments were performed to examine the effect of LHRH on the phosphorylation of Akt, one of the downstream effectors of PI3K (Sofroniew et al., 2001). Figure 8A, lanes 2 and 3, summarized in figure 8B show that 10min exposure to 100nM LHRH failed to increase Akt phosphorylation, in contrast, a robust increase in Akt phosphorylation was seen after 15 min 200ng/mL NGF exposure. Furthermore, the effect of NGF was attenuated by 6hr pre-treatment to the PI3K inhibitor, wortmannin at 1μM (fig. 13A, lanes 2-4, summarized in fig. 13B).

3.1.5 LHRH effect is independent of neurite outgrowth

It is possible that effects on ion channels simply reflect an overall neurotrophic effect that is cells are healthier with trophic support. In order to

determine if LHRH and NGF effects are independent of neurite outgrowth, morphology of the neurons were examined. In these experiments 28 of 65 control cells (fig. 14A) had neurite outgrowth, 25 of 78 LHRH treated cells (fig. 14A) had neurite outgrowth, and 48 of 56 NGF treated cells (fig. 14A) had neurite outgrowth after 7 days in culture (fig. 14). The lack neurite outgrowth in LHRH treated cells (fig. 14C) when compared to control (fig. 14B) results along with transient effect on Erk activation by LHRH suggest that LHRH regulation of functional expression of Ca^{2+} channels is independent of neurite outgrowth. By contrast, NGF treated cells increased in number of cells sprouted (fig. 14D) along with sustained Erk activation as discussed earlier suggest that effects of NGF on Na^{+} and Ca^{2+} regulation may be contributed by neurite outgrowth.

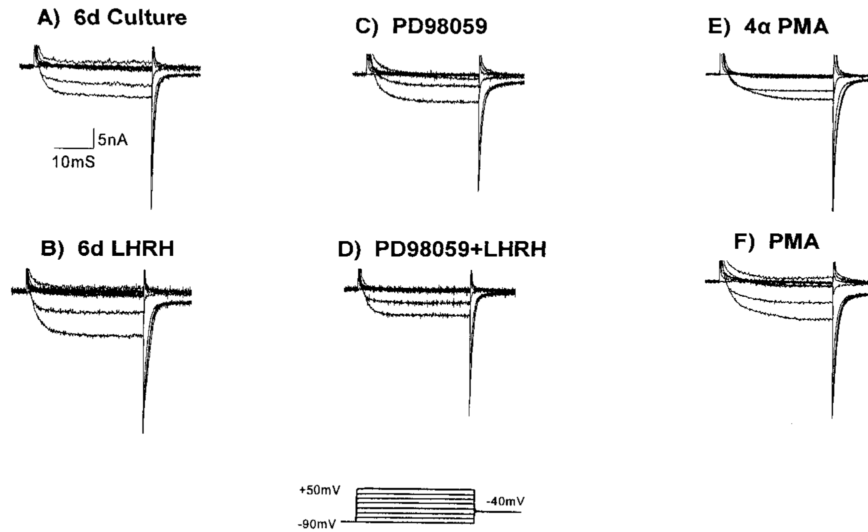
Table 1

	Control I_{Ba} Density (pA/pF) Blank Control	**I_{Ba} Density in LHRH (pA/pF) Positive Control**	**I_{Ba} Density in Inhibitor (pA/pF)**	**I_{Ba} Density in LHRH + Inhibitor (pA/pF)**
PD98059 (10μM)	117.7±6.8 (49)	158.4.±16.0 (35)*a*	112.1±9.9 (10)	129.3±9.1(20)
Chelerythrine (1μM)	117.7±6.8 (49)	175.4.±16.0 (35)*a*	74.0±4.0 (28)	90.8±9.2 (25)
U73122 (20μM)	*67.2±7.3 (23)**	*116.8±16.4 (19)*a*	63.4±4.7 (25)	67.3±5.3 (26)
H-89 (1μM)	117.7±6.8 (49)	175.4.±16.0 (35)*a*	122.6±10.2 (21)	96.0±4.9 (27)
Wortmannin (100nM)	117.7±6.8 (49)	175.4.±16.0 (35)*a*	48.3±6.2 (18)	72.1±8.4 (17)*b*

Modified from Ford Dissertation 2003

Table 1 Data are expressed as means±SEM with n-values in parentheses; statistical tests done with one-way ANOVA. Current densities are maximum values recorded at 0mV. *Control values for U73122 were obtained with the inactive analogue, U73343. *a* $P<0.05$ compared to 'blank' control; *b* $P<0.05$ compared to inhibitor alone. All other data for the blank and positive controls were pooled and obtained from cells in serum-free defined medium. Inhibitors: PD98059, inhibitor of MEK. Chelerythrine, inhibitor of PKC. U73122, inhibitor of PLC. H-89, inhibitor of PKA. PP1, inhibitor of Src kinases. Wortmannin, inhibitor of PI3 kinase.

Figure 2



Modified from Ford Dissertation 2003

Figure 2. Long-term regulation of total I_{Ca} by LHRH and phorbol ester. All recordings are families of I_{Ba} produced in response to 20mV incremental steps from a holding potential of -90mV. **A)** Currents recorded after 6d in defined-medium culture. **B)** Currents recorded after 6d in the presence of 0.45μM LHRH (note larger amplitude compared to **A)**, lower trace is voltage recording from neuron illustrated in upper trace. **C)** Family of I_{Ba}'s recorded after 6d in the presence of the MEK inhibitor PD98059 (10μM). **D)** Currents recorded after 6d in the presence of 0.45μM LHRH plus PD98059 (note similarity in amplitude compared to **C**). **E)** Currents recorded after 6d in the presence of 4α phorbol (80nM). **F)** Currents recorded following 1hr daily exposure to 80nM PMA for 6d (note increased amplitude compared to **E**). Calibration bar in **A** refers to all current traces.

Figure 3

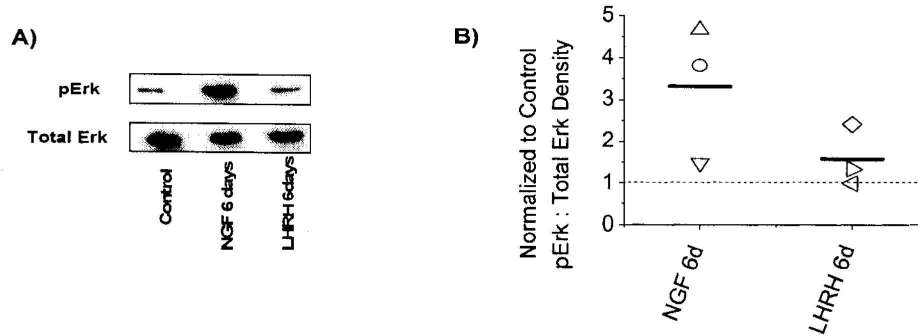


Figure 3. Activation of Erk by 6d exposure to NGF, and lack of Erk activation by 6d exposure to LHRH. The figure shows representative blots as well as quantification of data from 3 replicate experiments. The symbols, (circles, diamonds or triangles) indicate results from individual experiments. The black horizontal bars represent mean values for each experiment. All data were measured in terms of percentage change in blot density relative to loading controls. For further ease of comparison these ratios were normalized to the ratio observed in the control experimental situation. **A)** Effect of long term (6 days) exposure to NGF (200ng/ml) or LHRH (100nM). **B)** Quantification of data from 3 experiments similar to that illustrated in **A.**

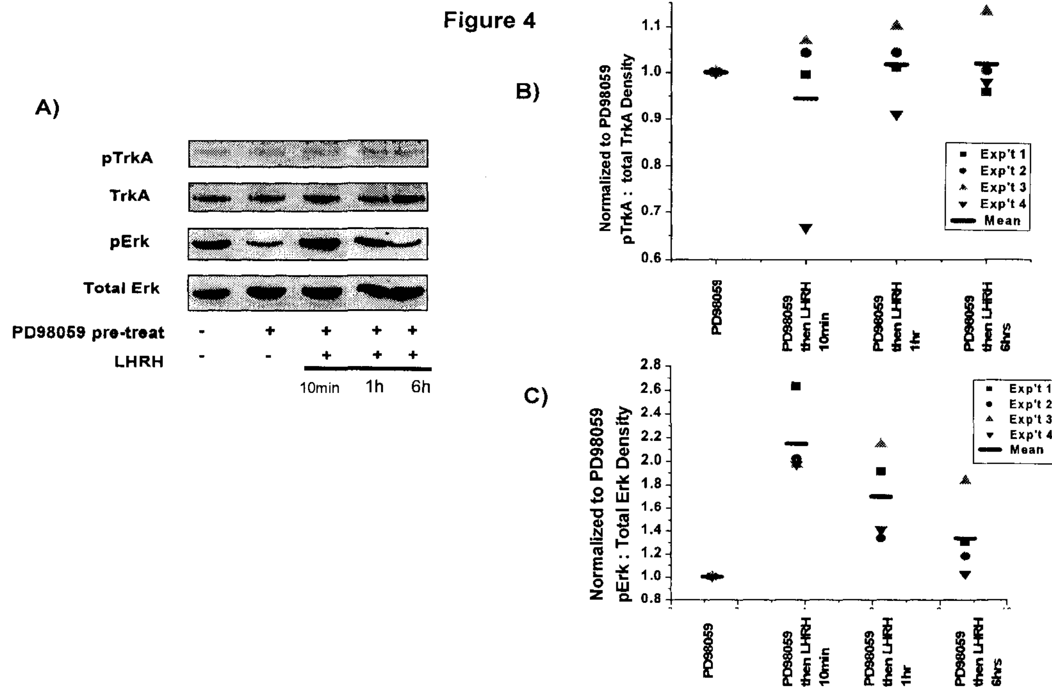


Figure 4. Activation of Erk and lack of activation of TrkA by LHRH. The symbols, (circles, diamonds or triangles) indicate results from individual experiments. The black horizontal bars represent mean values for each experiment. All data were measured in terms of percentage change in gel density relative to loading controls. **A)** Neurons were incubated without or with 50 μM PD98059 for 24h. At the end of the incubation, cultures as indicated were given 100 nM LHRH for times as specified. **B) and C)** Quantification of data from 4 experiments similar to that illustrated in **A** to illustrate LHRH activation of Erk but not TrkA.

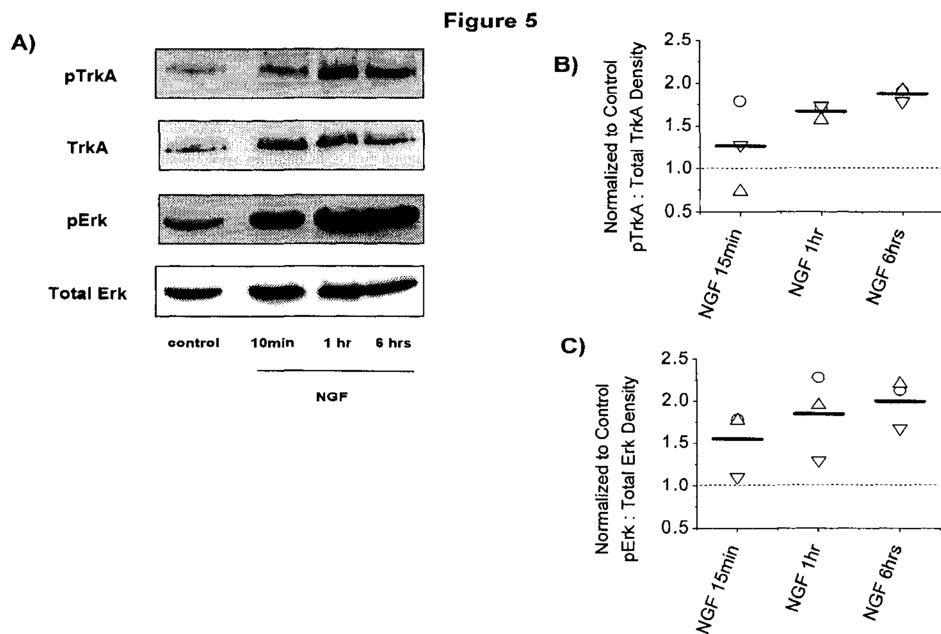


Figure 5. Sustained activation of Erk and TrkA by NGF. The symbols, (circles, diamonds or triangles) indicate results from individual experiments. The black horizontal bars represent mean values for each experiment. All data were measured in terms of percentage change in gel density relative to loading controls. **A)** Neurons were treated with or without 200ng/mL NGF for times as indicated. **B) and C)** Quantification of data from 4 experiments similar to that illustrated in **A** to illustrate NGF activation of Erk and TrkA.

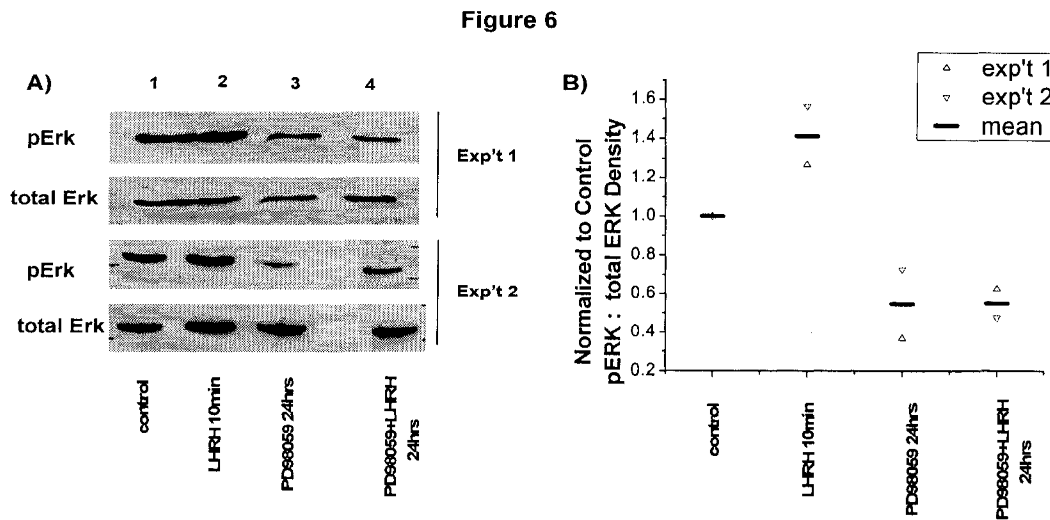


Figure 6. Lack of Erk activation by LHRH in the continued exposure to PD98059. The symbols, (triangles) indicate results from individual experiments. The black horizontal bars represent mean values for each experiment. All data were measured in terms of percentage change in gel density relative to loading controls. **A)** Untreated neurons, neurons treated with 100nM LHRH for 10 min, 50μM PD98059 24 hrs, or 50μM PD98059 plus 100nM LHRH for 24 hrs. **J)** Quantification of data from 2 experiments to illustrate the lack of Erk activation by LHRH in the continued exposure to PD98059.

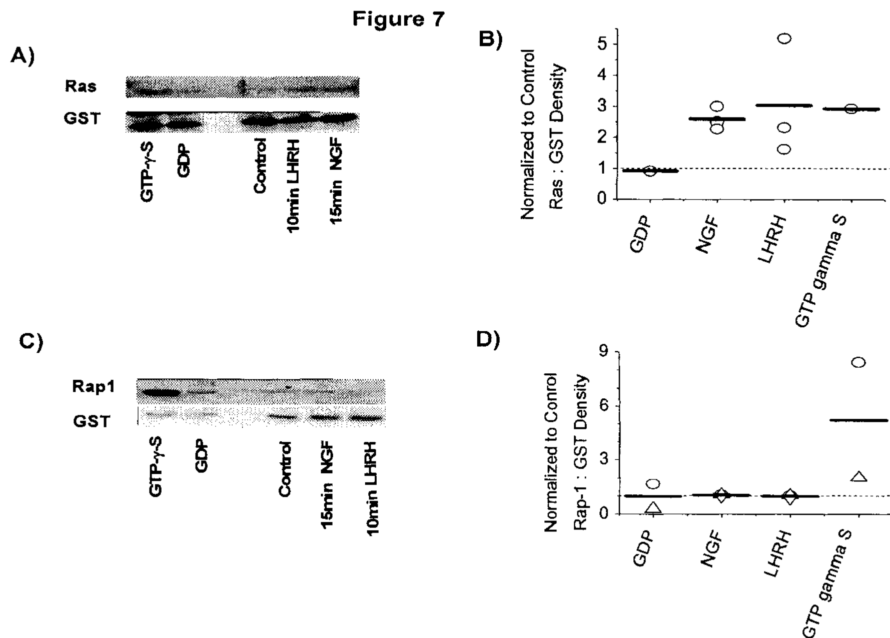


Figure 7. Activation of Ras but not Rap-1 by LHRH and NGF. A) Neurons were cultured for 5-6 days then were treated with 100 nM LHRH for 10min, 200ng/mL NGF for 15min, 10 mM GTPγS for 30 min, or 100 nM GDP for 30 min. The symbols, (diamonds, circles or triangles) indicate results from individual experiments. The black horizontal bars represent mean values for each experiment. **B)** Quantification of data such as that shown in **A** for three replicate experiments. **C)** Neurons were subjected to the same treatments as in **A**, then cell lysates were added to GST-RalGDS-RBD Swellgel[R] to pull down activated Rap-1 and the supernatants were loaded and separated by SDS-PAGE. GST immunoreactivity was used as a loading control. **D)** Quantification of data such as that shown in **C** for three replicate experiments.

Figure 8

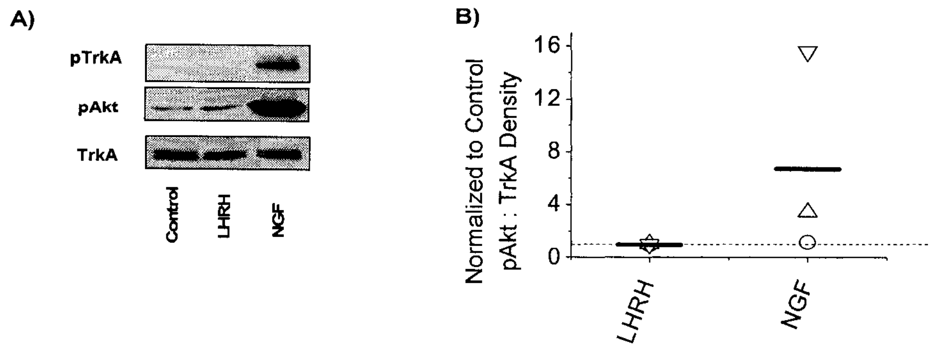


Figure 8. NGF activation of Akt and TrkA and lack of effect of LHRH on Akt and TrkA activation. The symbols, (circles or triangles) indicate results from individual experiments. The black horizontal bars represent mean values for each experiment. All data were measured in terms of percentage change in gel density relative to loading controls. **A)** Activation of Akt and TrkA by NGF and lack of effect of LHRH on Akt and TrkA activation. **B)** Quantification of data from 3 experiments similar to that illustrated in **A** to illustrate NGF activation of Akt and TrkA and lack of effect on Akt and TrkA by LHRH.

Figure 9

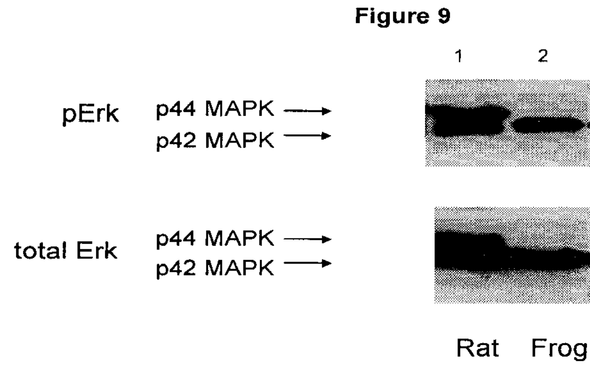


Figure 9. Erk expression in rat and frog neurons. Expression of Erk1 and Erk2 in rat superior cervical ganglia neurons (lane 1) versus expression of only Erk2 in BFSG neurons (lane 2).

Figure 10

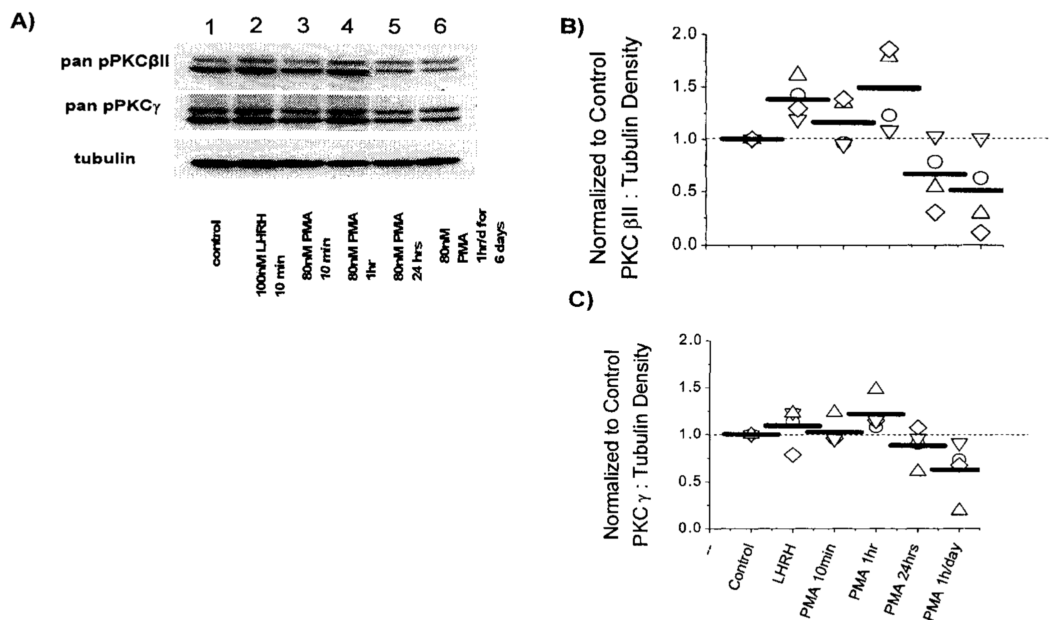


Figure 10. Activation of PKCβII and not PKCγ by LHRH and PMA. The symbols, (circles, diamonds or triangles) indicate results from individual experiments. The black horizontal bars represent mean values for each experiment. All data were measured in terms of percentage change in gel density relative to loading controls. **A)** Immunoblots to illustrate activation of PKCβII but not PKCγ by LHRH and PMA, Note maximal activation of PKCβII is seen after 1 h with PMA and activation is attenuated after 24 h exposure or during intermittent exposure as was done in electrophysiological experiments. **B) and C)** Quantitative representation of data from 3 replicate experiments similar to that presented in **A.**

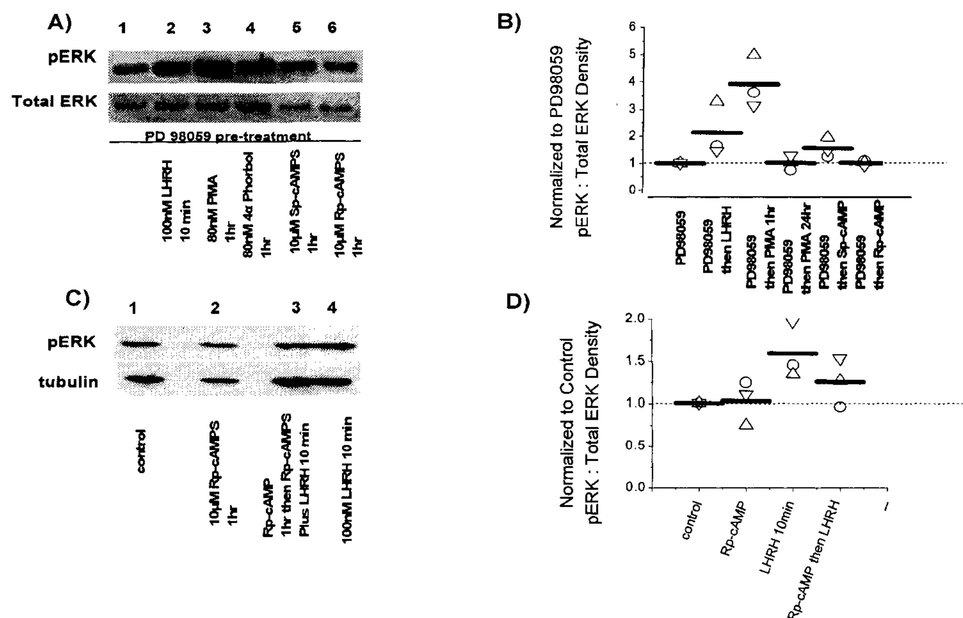
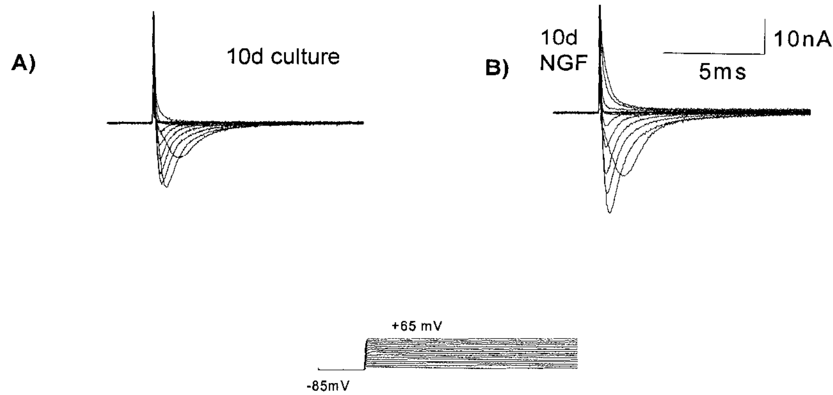


Figure 11. Activation of Erk by LHRH, PMA and Sp-cAMPS but not by Rp-cAMPS or 4α-phorbol and Inhibition of LHRH – induced Erk activation by Rp-cAMPS. The clear symbols, (circles, diamonds or triangles) indicate results from individual experiments. The black horizontal bars represent mean values for each experiment. All data were measured in terms of percentage change in gel density relative to loading controls. **A)** Activation of Erk by LHRH, PMA and Sp-cAMPS but not by Rp-cAMPS or 4α-phorbol. **C)** Inhibition of LHRH – induced Erk activation by Rp-cAMPS. **B) and D)** Quantitative representation of data from experiments similar to that presented in **A** and **C.**

Figure 12



Modified from Ford Dissertation 2003

Figure 12. Long-term regulation of I_{Na} by NGF. All recordings are families of I_{Na} produced in response to 10mV incremental steps from a holding potential of -85mV. **A)** Currents recorded after 10d in defined medium culture. **B)** Currents recorded after 10d in the presence of 200ng/ml NGF (note larger amplitude compared to **A**, lower trace is voltage recording from neuron illustrated in upper trace. Calibration bars in **B** refer to all current traces.

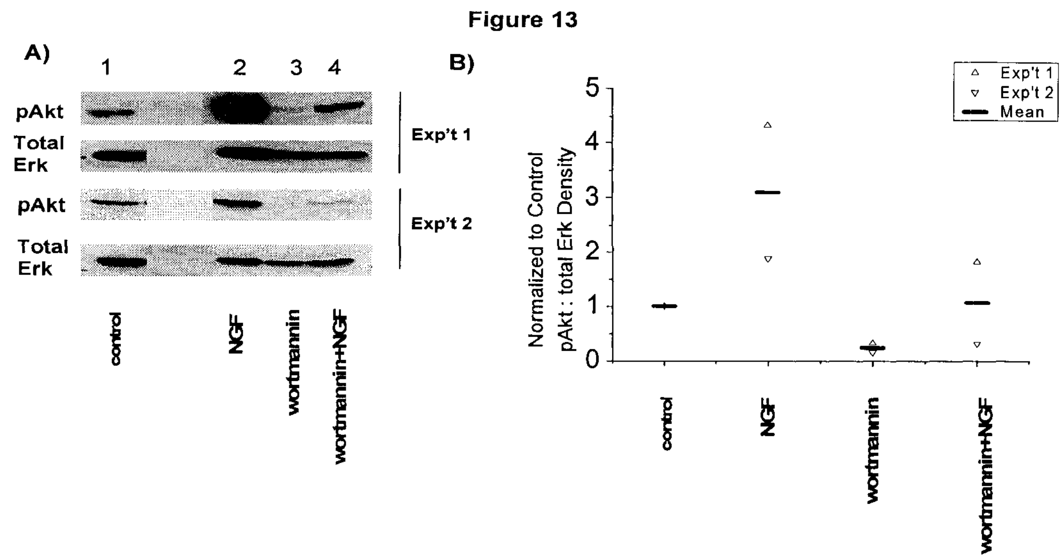


Figure 13. NGF activation of Akt and attenuation of Akt activation by wortmannin. The symbols, (triangles) indicate results from individual experiments. The black horizontal bars represent mean values for each experiment. All data were measured in terms of percentage change in gel density relative to loading controls. **A)** Phosphorylation level of Akt in untreated, NGF treated, wortmannin treated, NGF plus wortmannin treated neurons. **B)** Quantification of data illustrated in **A** from 2 replicate experiments illustrating the attenuation of NGF activation of Akt in the presence of wortmannin.

Figure 14. A

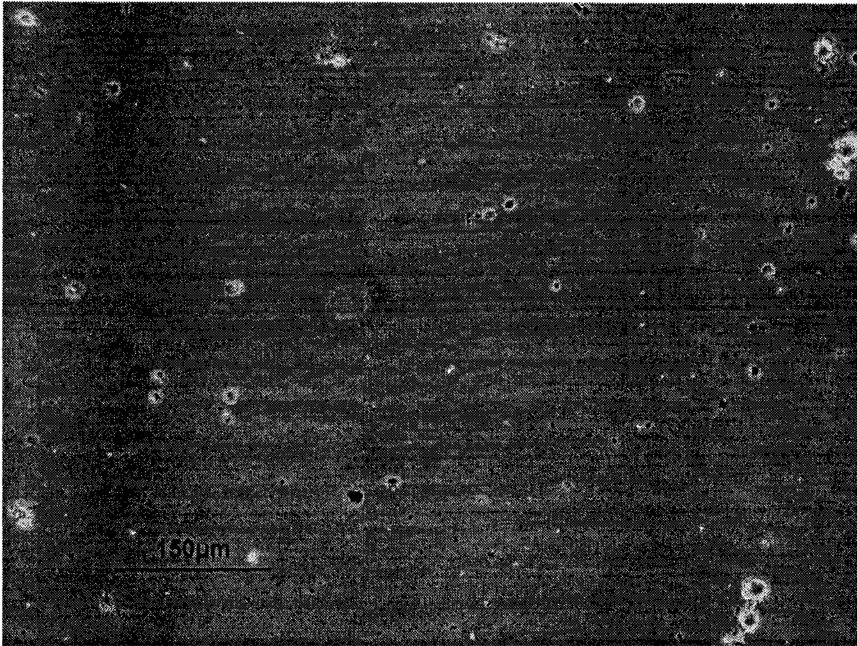


Figure 14. B

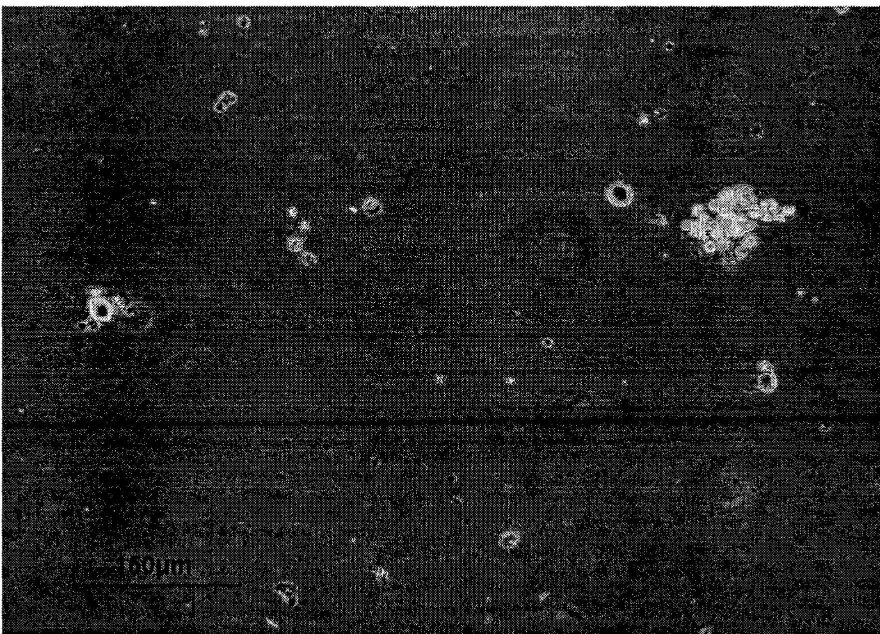


Figure 14. C

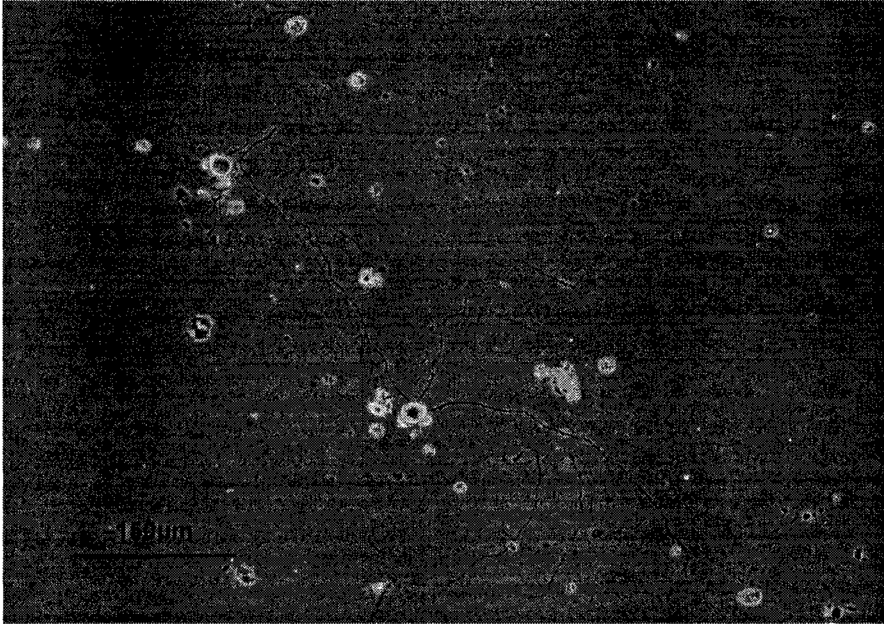


Figure 14. D

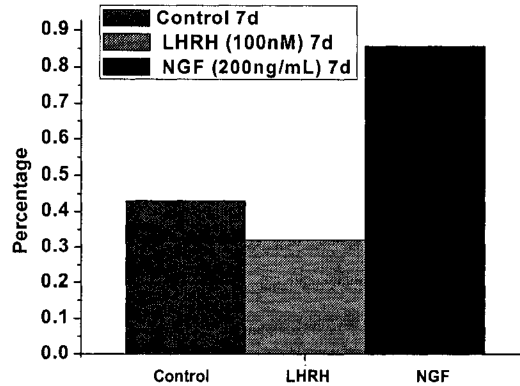


Figure 14. Cells from all three groups were capture captured at 100x magnification, counted, and grouped as having neurites or not having neurites. **A)** Representative picture of neurons in control group at day 7. **B)** Representative picture of neurons exposed continuously to 100nM LHRH at day 7. **C)** Representative picture of neurons exposed continuously to 200ng/mL NGF at day 7. **D)** Graph illustrating the percentage of neurons in each group having neurite outgrowth.

Chapter 4

Discussion and Future Directions

4.1 Discussion

All experiments in this thesis were performed on BFSG neurons following a pharmacological approach. This raises concerns about inhibitor concentration and specificity. Although molecular techniques such as RNA interference (Holen and Mobbs, 2004) or introduction of dominant negative or constitutively active Ras (Fitzgerald, 2000) may be considered, the lack of information regarding the bullfrog genome makes these approaches difficult. Since this study is a continuation of an extensive series of electrophysiological studies (Jassar et al., 1993; Lei et al., 1997; Lei et al., 1998; Lei et al., 2001; Petrov et al., 2001; Ford et al., 2003a) on BFSG neurons of *Rana Catesbeiana*, it is inappropriate to switch to a mammalian system which would make the molecular approaches more feasible (Black et al., 2003). However, as mentioned earlier, the BFSG system offers several important advantages as it has only two principal types of cells (B-neurons and C-neurons) that are easily identifiable on the basis on physical size and electrophysiological properties (Adams et al., 1986; Horn et al., 1988; Jassar et al., 1993; Smith, 1994; Ivanoff and Smith, 1995; Jobling and Horn, 1996; Thorne and Horn, 1997). Furthermore, adult BFSG neurons can be maintained in a serum-free, defined medium culture system for over 14 days (Lei et al., 1997). A serum-free, defined medium culturing system is free of complicating effects from exogenous neurotrophins that is critical for the study of trophic actions of neurotransmitters.

The main findings of this thesis confirm and extend electrophysiology findings from (Ford dissertation, 2003) and Lu et al., Society for Neuroscience 2002 abstract. They are 1) LHRH signals via the Ras-MAPK and not via Rap-1 pathway to

increase functional expression of N-type Ca^{2+} channel conductance in adult sympathetic neurons 2) LHRH signalling to Ras involves PKCβII 3) PKA is necessary but not sufficient to affect the transduction process 4) NGF signals via the PI3K pathway to increase Na^{+} currents 5) LHRH fails to affect functional expression of Na^{+} channel current. It is therefore suggested that downstream signalling from LHRH has selective access to MAPK and not to PI3K.

4.1.1 Role of Ras-MAPK in the LHRH-induced increase in I_{Ca}

Immunoblot analyses demonstrated that NGF phosphorylates and activates both Akt and Erk1/2 whereas; LHRH only induces phosphorylation of Erk1/2 and not Akt. These findings support the suggestion that the effect of LHRH is independent of the PI3K pathway (Koch et al., 1991; Lopez-Ilasaca et al., 1997). The effect of LHRH may involve PLC and Ras-MAPK is supported by the lack of effect of PPI and wortmannin and positive results with the inhibitors U73122 and PD98059 (Ford dissertation, 2003) (Table 1). In addition, immunoblot data indicates that LHRH failed to activate TrkA which gives further evidence against a role for transactivation of growth factor receptors by LHRH. However, this mechanism of action has been reported in other GPCR systems (Rajagopal et al., 2004). Even though Rap-1 has been shown to be activated by surrogate growth factor receptors in PC12 cells (Black et al., 2003), this phenomenon does not seem to occur with native TrkA receptors in sympathetic neurons. This is supported by immunoblot experiments showing the ability of LHRH and NGF to activate Ras but not Rap-1, which also suggests that Ras is involved with gonadotropin effect on Ca^{2+} current (Fig. 7A, 7B, 7C, and 7D).

Classically, PKC activation is shown by monitoring the translocation of the protein from the cytosol to the membranes (reviewed in Woodgett and Hunter, 1987; Shirai and Saito, 2002). Although numerous attempts were carried out in hope demonstrating PKC translocation using an established protocol in other systems by performing ultra-centrifugation (100000 x g) to partition the cytosolic and membrane fractions, recoveries of both the membrane and cytosolic fractions were poor and inconsistent in our samples. With the availability of antibodies against phosphorylated forms of certain PKCs, they were chosen as an approach to demonstrate PKC activation. Some studies have reported the significance of PKC phosphorylation with respect to the activation of the proteins such as: purified PKC could be inactivated following phosphatase treatments (Pears et al., 1992); phosphorylation in the T500 site within the activation loop is essential for PKCβ activation (Orr and Newton, 1994); and removal of phosphate from the three catalytic domains is crucial to desensitization of conventional PKCs (Hansra et al., 1996).

The basis of all the LHRH experiments is the neurotrophic effects are mediated by a heterotrimeric G-protein. Other mechanisms such as: internalization of peptide-bound receptors to induce transduction processes (Miller and Lefkowitz, 2001); or a G-protein-independent receptor, such as tyrosine kinase, may have been involved. These possibilities seem unlikely as all reported effects of LHRH in neurons, endocrine glands and cancer cells seem to progress through heterotrimer G-proteins (Grundker et al., 2001; Kakar et al., 2002; McArdle et al., 2002; Cheng and Leung, 2000; Naor et al., 1998a; Naor et al., 2000). In addition, it is commonly

accepted that in gonadotrophs, LHRH triggers the Ras-MAPK via a GPCR; hence, it is reasonable to believe that a similar pathway exists in neurons.

LHRH receptors have been shown to be capable of activating different heterotrimeric G-protein and signalling pathways in different cell types (Conn et al., 1979; Kuphal et al., 1994). In mammalian cells and cell lines, GnRH receptors have been reported to couple to $G_{q/11}$, G_S, and G_i (Hawes et al., 1993; Janovick and Conn, 1994; Stanislaus et al., 1997; Ulloa-Aguirre et al., 1998). Since LHRH activates PLC in BFSG neurons to increase IP_3 production and Ca^{2+} concentration (Pfaffinger et al., 1988), as well as, suppressing M-type K^+ current (Ford et al., 2003b; Ford et al., 2004), it is reasonable to suggest that BFSG LHRH receptors are also coupled to $G_{q/11}$ proteins. Interestingly, a novel mammalian receptor has been reported for the type II GnRH (Millar et al., 2001), which may correspond with the BFSG receptor. It is unclear if this is the only receptor used by LHRH signalling in BFSG or if $G_{q/11}$ is the principle and/or only Gα isoform used. However, a study by Boland and Bean reported that LHRH inhibited Ca^{2+} current in freshly dissociated BFSG neurons (Boland and Bean, 1993), which could imply that LHRH could act through $G_{\alpha i}$ in BFSG neurons.

In certain pituitary cell lines, GnRH effect on Ras-MAPK is tranduced through PKC (Sundaresan et al., 1996; Reiss et al., 1997). In addition, direct activation PKC with a phorbol ester has been shown to activate MAPK (Reiss et al., 1997). Electrophysiological findings with chelerythrine and U73122 indicate that LHRH receptor signals through PLC and PKC in BFSG B-cells (table 1) (Ford dissertation, 2003). As well, direct PMA stimulation of PKC is sufficient to increase

I_{Ba} (Fig. 2G) (Ford dissertation, 2003), which suggests the possibility of PKC being both "necessary and sufficient" to regulate BFSG Ca^{2+} channels. Furthermore, immunoblot studies using isoform specific antibodies suggest that PKCβII but not PKCγ is involved in the effect by LHRH. However, these studies do not exclude the possible role by other PKC isoforms. Since activation of PKC has been reported to influence inactivation of Na^{+} conductances in various neuronal systems (Franceschetti et al., 2000), it is reasonable to predict that LHRH may have a similar effect in BFSG neurons. This was not observed (Ford dissertation, 2003).

Although landmark studies have shown cAMP inhibition of ERKs through PKA in a Ras-dependent manner (Burgering et al., 1993; Cooks and McCormick, 1993; Graves et al., 1993; Wu et al., 1993), other studies have reported cAMP requiring PKA and Ras to activate Erk in selected neurons (Ambrosini et al., 2000; Iida et al., 2001). In certain cell types, cAMP can inhibit cell proliferation, but in other cell types cAMP can stimulate cell proliferation and differentiation via Erk activation (Young et al., 1994; Vossler et al., 1997). Known studies of cAMP-mediated cell proliferation is triggered by GPCR that are Gαs-coupled (Stork and Schmitt, 2002). In PC12 cells the PACAP receptor, Pac1, is capable of inducing the cAMP/PKA pathway and the PLC pathway to activate Rap1 and Ras and synergistically activating Erk (Bouschet et al., 2003). Although Rap1 activation was shown to be essential for Erk activation, Rap1 activation on its own was not sufficient for efficient stimulation of Erk activity (Bouschet et al., 2003). Perhaps a similar mechanism could exist for the LHRH receptor in BFSG. This would explain the observation that H-89 attenuated the electrophysiological effects of LHRH, but Sp-

cAMP (a cAMP analogue) failed to mimic this action (Ford dissertation, 2003) even though it was capable of inducing a weak activation of Erk (fig. 11A, lane 5, summarized in fig. 11B). Hence, PKA may be "necessary but not sufficient" in mediating the effects of LHRH. Another example of permissive role of Ras is shown by a wild type Ras ($p21^{Ras}$) study in PC12 cells that it is also "necessary but not sufficient" to mediate neurotrophin induction of Ca^{2+} channels (Pollock and Rane, 1996).

NGF and LHRH both have effects on Ca^{2+} channels; however, some differences do exist between their actions. The NGF effects as mentioned earlier involve an increase in $I_{Ca,L}$, decreased inactivation of total I_{Ca}, and an increase in $I_{Ca,N}$ (Lei et al., 1997). Whereas LHRH only increased $I_{Ca,N}$ but had no effect on activation or inactivation kinetics (Ford et al., 2003a). These findings may indicate that another pathway in addition to the Ras-MAPK pathway may be responsible for the $I_{Ca,L}$ and inactivation kinetics. Interestingly, the differences could perhaps relate to the transient Erk1/2 activation induced by LHRH (Fig. 4A, lanes 3 to 5, summarized in fig. 4C) compared to the ongoing activation triggered by NGF (fig. 5A, lanes 2 to 4, summarized in fig. 5C).

4.1.2 Role of PI3K in NGF-induced increase in I_{Na}

Electrophysiological data with LY294002 and wortmannin (Ford dissertation, 2003), and immunoblot experiments support the role of PI3K pathway in the action of NGF on Na^+ current. Since it has been reported that downstream signalling of PI3K often progresses via Akt (Sofroniew et al., 2001), NGF phosphorylation of Akt in our

BFSG system (fig. 8A, lane 3, summarized in fig. 8B) lends further support to the involvement of the PI3K in NGF-induced increase in I_{Na}.

Other studies have shown that in PC12 cells effects on Na^+ density is Ras-independent (Fanger et al., 1993; Fanger et al., 1997; Hilborn et al., 1998); however, the study by Fanger and coworkers in 1997 suggested that members of the Src non-receptor tyrosine kinase may be involved instead of PI3K. One possibility for this discrepancy is that our studies were performed on native TrkA on intact adult neurons whereas Fanger and co-worker in 1997 performed their experiments on PC12 cells expressing surrogate platelet-derived growth factor (PDGF) beta receptors with mutations that eliminate activation of specific signalling molecules. Another possibility is that our pharmacological approach which is reliant on the supposed selectivity of the inhibitors may be less reliable than molecular techniques.

4.1.3 LHRH effect is independent of neurite outgrowth

Erk mediated cellular effects are strongly dependent on the temporal pattern of Erk activation (Huang and Reichardt, 2003). Exposure of LHRH to BFSG neurons in our system resulted in transient activation of Erk (fig. 4A, lanes 3-5), whereas, exposure to NGF resulted in sustained Erk activation (fig. 5A, lanes 2-4). Morphological analysis showed 7 days LHRH treatment did not increase neurite outgrowth as only 25 out of 78 cells had neurite outgrowth (fig. 14A, 14C) compared to control cells which had 28 of 65 control cells with neurite outgrowth (fig. 14A, 14B). By contrast, NGF treatment increased neurite outgrowth with 48 out of 56 cells having outgrowth (fig. 14A, 14D). These results are not surprising since

neurotrophins are classically known to be responsible for growth, development, and repair of the nervous system. Furthermore, Huang and Reichardt have suggested that sustained activation of Erk is required for induction of axonal growth (Huang and Reichardt, 2003).

4.1.4 Lack of effect of LHRH on Na^+ currents

It appears that LHRH does not have an effect on I_{Na} (Ford dissertation, 2003), which could be explained by its lack of effect on the PI3K pathway that is implicated in NGF-triggered increases in I_{Na} (fig. 12A, and 12B). NGF appears to have access to both the PI3K pathway to regulate functional expression of Na^+ channels, and the Ras-MAPK pathway to regulate Ca^{2+} currents. LHRH, on the other hand, appears to only have access to the Ras-MAPK pathway, can only regulates Ca^{2+} currents.

As mentioned earlier, the physiological role of I_M may be to act as a voltage-clamp for cells to resist depolarizing stimulus influence (Adams et al., 1982a); therefore, suppression of I_M will give a powerful excitatory effect. Interestingly, LHRH has been shown to deplete PIP_2 causing M-channel suppression in BFSG neurons (Ford et al., 2003b; Ford et al., 2004) making the neurons more excitable. Since Ca^{2+} channels play an important role in the generation of action potential in BFSG cells (Pennefather et al., 1985; Smith, 1994), and our results indicate LHRH increases functional expression Ca^{2+} via the Ras-MAPK pathway suggest a pleiotrophic effect of LHRH in BFSG neurons.

In BFSG neurons functional up-regulation of Na^+ and Ca^{2+} channels by NGF is persistent provided that the target-derived neurotrophin is available. By contrast,

the neurotrophic effect by LHRH is more variable since its release is dependent on preganglionic nerve activity. These effects may be related to the transient effect of LHRH compared to the lasting effect of NGF on Erk1/2 phosphorylation. It has been shown that repetitive stimulation is more effective than burst stimulation at releasing LHRH in BFSG (Peng and Horn, 1991); therefore, LHRH regulation of Ca^{2+} channels may couple pre-ganglionic events to alterations in the electrical properties of post-ganglionic neurons. Since neurotransmitter release is dependent on Ca^{2+} influx, increased Ca^{2+} channel availability at sympathetic post-ganglionic terminals may govern sympathetic signals to target tissues. In comparison, Na^{+} channel regulation is dependent on the availability of target derived-NGF rather than ganglionic transmission. Hence, the differential regulation of channel types exists because of the ability of NGF to signal via both the MAPK and PI3K pathways compared to the selective activation of the Ras-MAPK pathway by LHRH (fig. 15). These results may provide a tool for the understanding of the cellular and biochemical mechanisms in disease states such as hypertension and congestive heart failure, by which sympathetic outflow to blood vessels, the heart, and other visceral organs is elevated.

4.2 Future Directions

The work performed in this thesis is a continuation of an extensive series of physiological studies (Jassar et al., 1993; Lei et al., 1997; Lei et al., 1998; Lei et al., 2001; Petrov et al., 2001; Ford et al., 2003a) on BFSG neurons of *Rana Catesbeiana*,

has provided biochemical verification for the differential regulation of Na^+ and Ca^{2+} channels by NGF and LHRH. Although molecular approaches such as introduction of dominant negative form of Ras (Fitzgerald, 2000), or using RNA interference (Holen and Mobbs, 2004) would provide more definitive evidence for the involvement of the proteins in question, these techniques are currently only available for mammals and are not feasible due to the lack of information on the *Rana Catesbeiana* genome, as well as time consuming to develop the protocols. Furthermore, the neurons may not survive after being subjected to such manipulations for the 6d duration of our experiments. In our system the neurons survived nicely as demonstrated by the morphology data. Since electrophysiological evidence suggest the upregulation of Na^+ currents by NGF and Ca^{2+} currents by NGF and LHRH, future experiments could be to investigate the expression of these channel proteins using pan-sodium channel and pan-calcium channel antibodies, which would provide direct evidence for channel upregulations. Even though the classical method of demonstrating PKC activation by looking at membrane translocation of PKC was unsuccessful, the phosphorylation of several serine/threonine sites in PKC has been shown to be essential for the full activation of the enzyme in response to different stimulations (Hofmann, 1997; Parekh et al., 2000; Newton, 2003). Our data suggest the involvement of PKC βII and not PKCγ, but this does not exclude the possible role of other PKC isoforms, hence future studies could examine phosphorylation levels of other PKC isoforms. Moreover, immunoblot assays could be performed using Chelerythrine to inhibit PKC and determine Erk activation could also support the role of PKC in the effect by LHRH. A possible role of Akt in NGF upregulation of Na^+ current has been

implicated without definitive evidence. Future biochemical experiments could examine the expression of Na^+ channel proteins in the presence of an Akt inhibitor such as FPA-124 (available from Echelon Biosciences Inc., Utah, USA), would support the role of Akt in the effect by NGF.

Interestingly, a study by Chieffi and colleagues on frog *Rana esculenta* testes showed that there are variations through out a year in the expression of Erk proteins and their states of activation do not always correlate (Chieffi et al., 2000). Hence another future study could analyze the months by which the experiments were conducted to note the expression levels of Erk as well as the level of Erk activation observed to see if, perhaps, there is any trend or correlation in this regard.

Figure 15

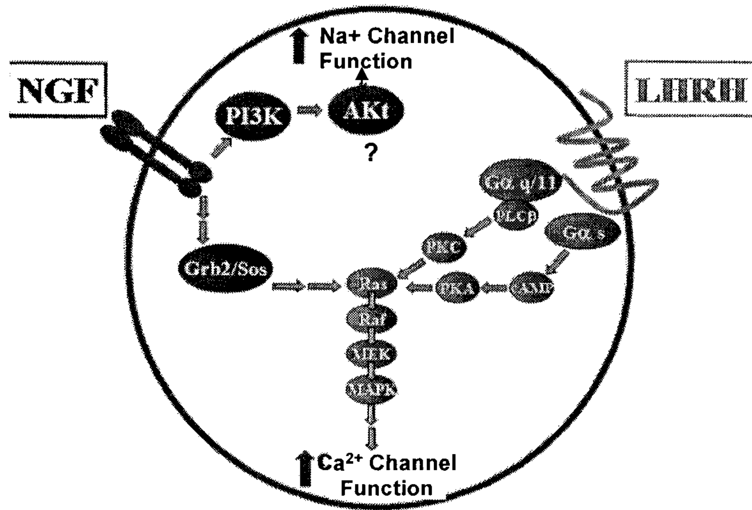


Figure 15. Scheme to illustrate proposed mechanisms for NGF and LHRH regulation of Na^{+} and Ca^{2+} Channels.

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