POTENTIAL ANALYSIS OF GENE EXPRESSION IN NERVE GROWTH FACTOR TREATED CELLS USING SEMI QUANTITATIVE PCR TECHNIQUE

(Spine title: NGF INDUCES CHANGES IN GENE EXPRESSION)
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by

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Abstract

Over sixty years ago, nerve growth factor (NGF) was originally discovered as a neurotrophic factor essential for the survival of sensory and sympathetic neurons during development. However, recent studies in patients with chronic pain indicate elevated levels of NGF in their sera and within injured and inflamed tissues. Abnormal neurotrophin action has been suggested to have an effect on acute and chronic pain, and in the development of degenerative diseases such as Alzheimer's, neuropathy, and cancer. NGF signals through two different types of receptors: TrkA and p75. These signals can be disrupted by the presence of small molecules inhibitors such as PD90780, ALE-0450, and PQC 083 by specifically binding to NGF. The binding by these molecules antagonizes the action of NGF from binding to its receptors, therefore blocking its signalling pathways. This could have a potential application in pain control and treatment of degenerative diseases. More recently, pain therapy has focused on selective NGF inhibition.

The aim of this study was to utilize gene expression analysis to investigate the intricate results of signalling in NGF treated cells compared to cells treated with both NGF and NGF inhibitors. A better understanding of genetic networks may contribute to the identification of novel drug targets, and more sensitive diagnostic strategies.

In this research, peak time expression for Egr-1 in NGF treated cells was evaluated. Also, Egr-1 gene expression was assessed in NGF treated cells using semi-quantitative RT-PCR. This may provide a better way to identify new drugs through the examination of cell signalling response. Moreover, these findings will be helpful as a model for *in vivo* applications.

Keywords: Nerve growth factor; TrkA, p75 receptors; PCR; gene expression.

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List of Abbreviations

CIPA	Congenital insensitivity to pain with anhidrosis
DNA	Deoxyribonucleic acid
Egr-1	Early growth response 1
ERK	Extracellular signal regulated kinases
HSAN	Hereditary sensory and autonomic neuropathies
NGF	Nerve growth factor
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
PI3K/AKT	Phosphinositide 3-kinase and protein kinase B
p75	p75 neurotrophin receptor
PCR	Polymerase chain reaction
PMA	Phorbol 12-myristate 13-acetate
ProNGF	Pronerve growth factor
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction

SEM	Standard error of the mean
SPR	Surface plasmon resonance

Chapter 1

Introduction

This chapter outlines pain definitions and mechanisms. The discovery of NGF, its structure, receptors, signalling pathway and role in chronic pain and inflammation will also be discussed. Moreover, therapeutic trials that targeted NGF receptors or signalling pathways, as an alternative method of pain management, will be presented. NGF inhibitory compounds and their mechanisms in NGF inhibition will be discussed and finally, different gene expression techniques will be outlined.

In this study, the main focus is to evaluate the capability of reported small molecule inhibitors to inhibit NGF signalling pathways in a PC12 cell line. It is a critical step towards the ultimate goal of finding a cure for chronic pain. Results of this research can determine the effectiveness of specific small molecule inhibitors and pursue further research *in vivo* models prior to introduction to clinical trials.

1.1 Pain

Understanding the mechanism of pain is crucial because it improves safe and efficient pain treatment and also it is a critical step in decreasing medical complications. Although no one has escaped the experience of pain, pain determination and management was ignored for many years. In 1968, the first definition for pain was introduced by McCaffery as "whatever the experiencing person says it is, existing whenever and wherever the person say it does" (McCaffery, 1972, p. 11). This definition was vague as

there are no standard tools to assess pain, and it depends mainly on the description provided by the patient in their own words.

The International Association for the Study of Pain (IASP) defines pain as an "unpleasant sensory and emotional experience associated with actual and potential tissue damage, or described in terms of such damage or both" (IASP, 2017, "Pain", para. 1). However, the relation between pain sensation and tissue damage is impractical to establish when guided by this definition (Price & Barrell, 2012).

In 1999, Price proposed a new definition for pain as a somatic perception containing a bodily sensation with qualities like those reported during tissue damaging stimulation of an experienced threat associated with this sensation, and a feeling of unpleasantness or other negative emotions based on experienced threat. This update canceled the demand to correlate between tissue damage and pain sensation (Price & Barrell, 2012).

In 2001, the Joint Commission on Accreditation of Healthcare Organizations (JCAHO) updated the definition of pain. This update included not only short-term hospitalized patients, but also included all different categories of long-term treated patients. Moreover, adverse physiological and psychological side effects related to unrelieved pain were added (Curtiss, 2001).

1.2 Pain signalling

It is fundamental to understand pain signalling mechanisms to understand some clues about the complex phenomenon of pain. The term “nociceptors” is derived from the Latin term "nocer", meaning injury or hurt; thus, receptors signalling noxious stimulus are called nociceptors and are present everywhere in the body.

Recent studies have classified nociceptors into four types: (1) thermal nociceptors activated by high or low temperatures, (2) mechanical nociceptors activated by extreme pressure to the skin, (3) polymodal nociceptors are enabled by chemical, mechanical and thermal stimuli, and (4) silent, or sleeping nociceptors which are activated by inflammatory mediators (Lemke, 2004).

Additionally, nociceptors have two major anatomical groups. The first group comprises of myelinated (Aδ) afferents, and is characterized by rapid onset, and mediate acute pain. The second group is the unmyelinated C fibers, which respond to both mechanical and thermal stimulus (polymodal), and demonstrate the slowest conduction (Willis, 1985). Additionally, neuroanatomical studies have shown that C fibers express the neurotrophin TrkA receptor which has high affinity to NGF (Fang *et al*., 2005).

Upon nociceptor stimulation with any noxious stimulus (e.g., chemical, thermal, mechanical), signals are identified and conveyed through the primary afferent nociceptors to the brain, where they are encoded (Basbaum *et al*., 2009). The most common conception of this process involves pain being elicited in a unidirectional pathway from the area of stimulus to the spinal cord. In fact, afferent fibers have been named pseudo-unipolar, as studies have shown that both central and peripheral terminals share the same axonal origin.

In other words, nociceptors can communicate from either end (central or peripheral) (Basbaum *et al*., 2009). This morphological finding is valuable as it opened the door to different treatment options; pain can be controlled through the peripheral pathway (as topical drug applications) or at the central pathway (as intrathecal injections; Alexander *et al.*, 2012; Nagano *et al.,* 2005). Figure 1.1 outlines central and peripheral signalling pathways in response to noxious stimuli.

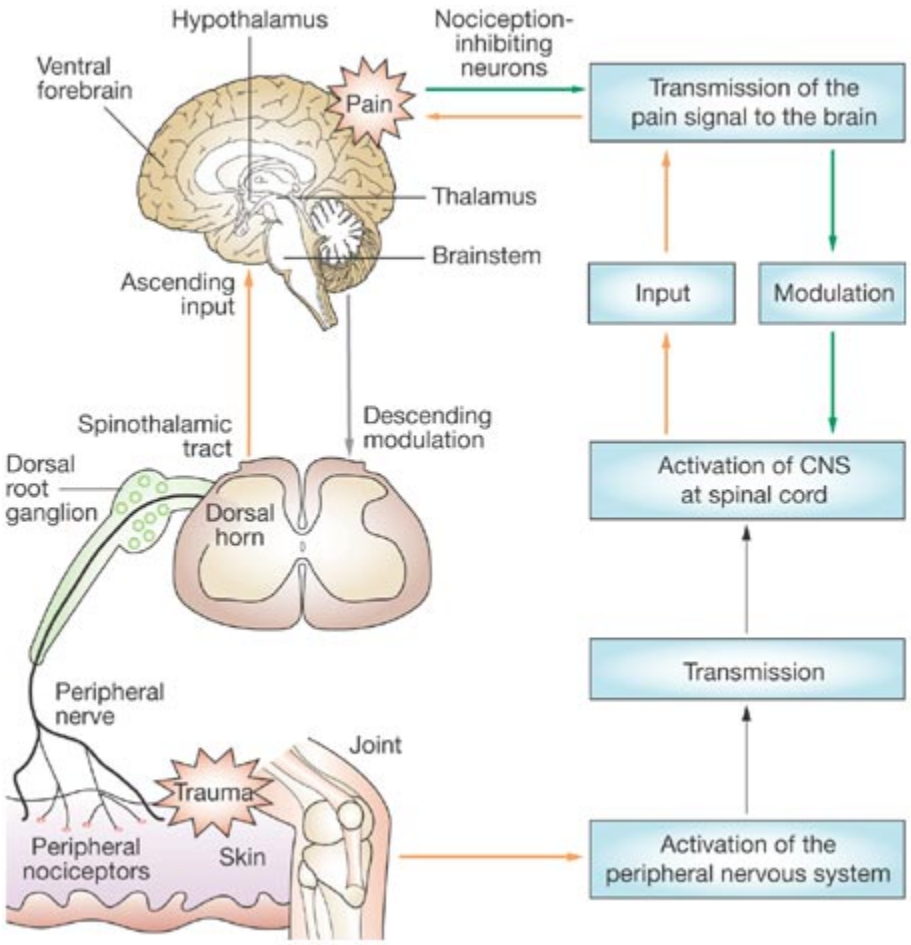


Figure 1.1: Central and peripheral system pain signalling pathways in response to noxious stimuli.
Upon nociceptor stimulation with any noxious stimulus signals are identified, and conveyed through the primary afferent nociceptors, where it is encoded. Image is originally published in Nature (Bingham *et al*., 2009).

1.3 Pain classification

Over many years, physicians, researchers, practitioners, and pharmacologists have been trying to agree upon an accurate pain classification system to improve pain management and treatment. Unfortunately, pain classification is still confusing and is not reliable. Various pain classifications were introduced based on anatomical classification (where pain is classified according to body locations), or etiological classification, based on the etiology of the disease, neglecting any other factors such as duration, location, and pain intensity (WHO, 2012).

The most commonly acceptable pain classification relies on pain duration, and is classified as follows:

Acute pain: Acute pain is characterized by a rapid onset, and acts as an alarm system that detects any noxious stimuli. It usually lasts for a few days to a maximum of two to three months. It emanates as a result of tissue injury, and is relieved after tissue healing (Jones, 2001).

Chronic pain: Chronic pain is defined as "aberrant somatosensory processing in the peripheral or central nervous system (CNS) that is sustained beyond the normally expected time course relative to the stimulus" (Greene, 2010, p.5). However, this definition does not include all the variables affecting chronic pain. In fact, chronic pain is a multi-directional complex phenomenon; the patient with chronic pain is not deregulated only by the pathological effects, but chronic pain also deregulates different systems including but not limited to behavior, cognition and emotional responses (Greene, 2010).

1.4 Pain and its impact

Pain is the most common medical complaint, but despite its prevalence, many individuals still suffer from chronic or untreated pain. According to a study done by Todd *et al.* (2007), pain is the most common reason for seeking healthcare, and it accounts for up to 78% of emergency hospital visits. However, many Canadians are left to suffer varying degrees of pain with little to no medical attention.

Despite having the knowledge and technology to help some of them, we still see many cases that cannot be treated by the means that we know of now. In 2008, the University of California conducted a survey to analyze the correlation between the impact of pain and quality of life of pain sufferers, and the different options of pain management (McCarberg *et al*., 2008). In addition, physicians were asked to identify the challenges faced when treating a patient's pain. The results of the survey showed that the chronic pain sufferers experienced multiple negative effects, which may severely impact their whole life. These effects included, but were not limited to: functional impairment and disability, anxiety, depression, sleep deprivation, and perhaps even their employment status and their ability to fulfill their basic duties (McCarberg *et al*., 2008).

The physician's survey indicated that pain management is a major health challenge, especially when taking into consideration the addiction problems that some patients face, the side effects associated with pain management medications, and the effects of dose withdrawal (Al-Hasani & Bruchas, 2011; McCarberg *et al.*, 2008). Studies also showed that people experiencing chronic pain are two times more likely to commit suicide compared to people without chronic pain (Tang & Crane, 2006). While a wide range of

therapeutic treatments are available, none of these are satisfactory.

1.5 Genetic defects in pain perception

According to the orthopaedic surgeon Paul Brand and authors (1993) in his book *The Gift Nobody Wants*, "pain is not the enemy, but the loyal scout announcing the enemy" (p. 20). Pulling your hand away when touching a hot surface is a protective mechanism because the hot surface causes you pain. However, individuals with hereditary sensory and autonomic neuropathies (HSANs) can have their hands burned, or can have broken legs without being aware, due to their inability to perceive pain. Despite the fact that hereditary sensory and autonomic neuropathies are rare, it is important to develop an understanding of them, as they can provide us with more information about the diversity and complexity of our nervous system.

HSAN is classified into five different types. Understanding molecular pathology for HSAN type IV is of significant advantage to explore the role of NGF-TrkA signalling.

HSAN type I: it is demonstrated between the second and fourth decade with ulcers at lower extremities, atypical pain and thermal perception, and anhidrosis (Hilz, 2002).

HSAN type II: it is recognised by impaired autonomic dysfunction and severe sensory impairment (Hilz, 2002).

HSAN type III: it is the most common type of HSAN's; it is known as familial dysautonomia. This disorder significantly alters the autonomic and sensory system (Hilz, 2002).

HSAN type IV: it is also known as "congenital insensitivity to pain and anhydrosis" (CIPA). CIPA is an autosomal recessive hereditary disorder, characterized by absence of pain perception to mechanical stimuli, anhydrosis, and recurrent episodes of fever as anhydrosis causes deregulation of homeostasis. Electrophysiological studies including nerve conduction and a set of experiments have been done in a trial to pinpoint the defects. The results indicate absence of small mylineated and unmyelinated fibers with the presence of the natural large myelinated fibers (Goebel *et al.*, 1980).

In 1986, a novel gene coding for the cell surface receptor tyrosine kinase family (Trk) was isolated from human colon carcinoma (Martin-Zanca *et al.*, 1989). Studies have since proven high affinity binding of NGF to TrkA, which is then phosphorylated upon binding and plays a fundamental role for intracellular signal transduction (di Mola *et al*., 2000).

TrkA knockout mutant mice have shown serious sensory and sympathetic neuropathies. Moreover, the mutant mice were born smaller in size and died within one month of birth (Crowley *et al*., 1994). Gathering all this information starting with the electrophysiological findings, and comparing CIPA with TrkA knockout mice, it has been shown that they both share severe impairment in sensory and sympathetic neurons, and insensitivity to any noxious stimulus. All this data suggest that TrkA gene mutation is the cause of CIPA (Indo, 2001).

Many of the TrkA signalling roles with NGF are still unstudied. Studying molecular pathology for CIPA is of significant advantage to explore the role of NGF-TrkA signalling.

HSAN type V: it is recognized within a few months after birth with selective loss of pain perception despite having a normal neurological response (Hilz, 2002).

1.6 Pharmacotherapeutics pain management

Chronic pain management is a challenging task. For many years, treatment was directed to the pathological cause with the assumption that the pain would be relieved. However, in many cases, even a combination of different medications would not help in the relief of pain.

As a step to improve pain treatment, in 2005, the Canadian Council on Health Services Accreditation added pain assessment and management in the Achieving Improving Measurement Standards (Accreditation Canada, 2013).

General pharmacotherapeutic strategies have been established to manage chronic pain. There are three major classes of medications used in chronic pain management: NSAIDs, acetaminophen and opioids. The mechanisms and side effects for these are discussed below:

1.6.1 Nonsteroidal anti-inflammatory analgesics (NSAIDs)

Following the pharmacotherapeutic principles, mild to moderate pain is treated with non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen and indomethacin. The anti-inflammatory and analgesic mechanism of action for NSAIDs is by the inhibition of cyclo-oxygenase (COX) enzyme, which enables prostaglandin release. Prostaglandins have a significant role in stimulating the inflammatory response, as shown by the major changes in prostaglandin production during inflammation (Ricciotti *et al.*, 2011).

Gastric and intestinal mucosal defense mechanisms and renal blood flow regulation are one of the physiological roles of the COX enzyme. Despite the effectiveness of NSAIDs in pain management, blocking COX enzyme causes a number of side effects, including gastric complications which can vary from gastric ulcer to gastric haemorrhage, renal dysfunction and increased risk of myocardial infarction (Marcum & Hanlon, 2010; Peskar, 2001).

1.6.2 Acetaminophen analgesics

The mechanism of action of acetaminophen is still unknown. Acetaminophen has an analgesic and antipyretic effects. While recognized as a safe drug, it may have a rare but mild side effect, including abdominal pain or diarrhea. Acetaminophen is contraindicated for patients with liver disease, as hepatic toxicity can be fatal (Lynch & Watson, 2006). Moreover, acetaminophen has a ceiling effect; so increasing the dose does not necessarily enhance its effectiveness (Hinz *et al*., 2008).

1.6.3 Opioid analgesics

Central and peripheral nervous systems produce natural endogenous opioids, which have a role in pain signalling. There are three groups of opioids receptors; μ, κ and δ. Most opioids antagonists have a considerably high affinity to opioid receptors through which they block their actions (Al-Hasani & Bruchas, 2011).

Opioid treatments have a broad range of side effects such as constipation, sedation, nausea and vomiting. Moreover, opioid treatment has the high risk of addiction and respiratory depression (Park & Moon, 2010). Unfortunately, despite all efforts, the quest for a safe and effective pain relief medication has not yet been achieved.

1.7 NGF discovery, and its role

With the discovery of NGF as a pain mediator, agents targeting NGF receptors or its signalling pathway can provide a new area to explore in therapeutic pain management with the potential of no adverse effects.

NGF was discovered by Rita Levi-Montalcini in the 1950's (Aloe *et al.*, 2004) as a "survival factor for sensory and sympathetic neurons in the developing nervous system" (Lötsch & Geisslinger, 2011, p. 453). Levi-Montalcini was interested in studying nerve development in chick embryo. She observed that if she cultured a tumor cell line anywhere in a developing chick embryo, the nerve fiber would grow more rapidly than the normal embryo (Aloe *et al.*, 2004).

In 1952, she speculated the presence of a biochemical agent that was capable of inducing the growth of nerve fiber. To study this agent further, she worked with Dr. Cohen to understand the workings of this biochemical agent. After a set of several experiments, they came to the discovery of the signalling protein NGF (Aloe *et al.*, 2004) .

Levi-Montalcini and Cohen were able to produce significant amounts of purified mouse salivary NGF as well as antibodies against NGF. Through sets of experiments, they were able to discover the function and significance of NGF in the development of sensory and sympathetic ganglia (Bocchini & Angeletti, 1969). In 1986, Levi-Montalcini was awarded the Nobel Prize in Physiology or Medicine for this discovery (Cowan, 2001). Figure 1.2 outlines different experimental sets to discover NGF.

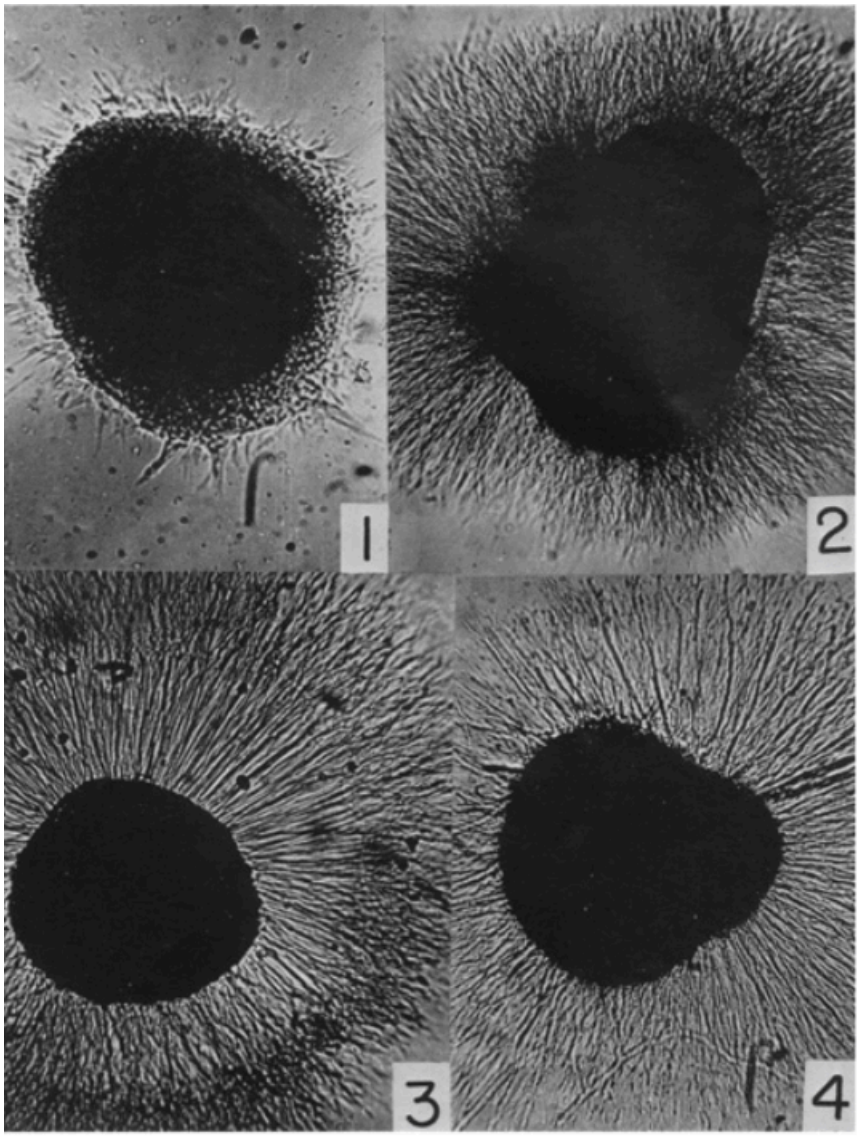


Figure 1.2: Experimental trials for NGF discovery.
Chicken embryo nerve tissue cultured with (1) control medium (Upper Left), (2), crude snake venom (Upper Right) (3), a partially purified protein fraction of snake venom (Lower Left), and (4) the protein fraction purified from sarcoma (Lower Right). Image is originally published in Proceedings of the National Academy of Sciences of the United States of America (PNAS) (Zeliadt, 2013).

Until recently, it was believed that NGF's sole role was to act as a survival factor for sensory and sympathetic neurons in the developing nervous system. Recent studies have shown that NGF's role is much more extensive and is crucial even beyond the nervous system (Petty *et al.*, 1994). One of their crucial findings was NGF's role in mediating inflammatory and immune response after tissue injury (McMahon, 1996). Furthermore, increased levels of proNGF (NGF precursor) has been found in neurodegenerative diseases such as Alzheimer and Parkinson's (Lorigados Pedre *et al.*, 2002; Williams *et al*., 2006). These findings can help in the development of new therapeutic strategies in the treatment of chronic pain and neurodegenerative diseases.

1.8 Biological development of the NGF in the nervous system

The development of synaptic connections with different cells begins during the early developmental stages. During this period, the axons extend from the neurons to their targets. As the development process is happening, it is expected that minimal cell death occurs. Unexpectedly, massive cell death occurs, and more than 50% of the neurons undergo cell death. The only accepted explanation that this programmed cell death provides more vast and more efficient nervous system (Frade & Barde, 1998).

Later developmental stages are characterized by target innervation. A unique feature for NGF, in comparison to other neurotrophins, is that it has two receptors with opposite actions. TrkA maintain neural survival, and p75 is an apoptotic inducer. During this stage, p75 enhances NGF binding to TrkA to maintain cell survival. During adulthood, all neurotrophins function to promote neural plasticity. During this period, NGF enhances

neurotransmitter expression during inflammation to perceive noxious stimuli (see Figure 1.3; Dekkers *et al.,* 2013).

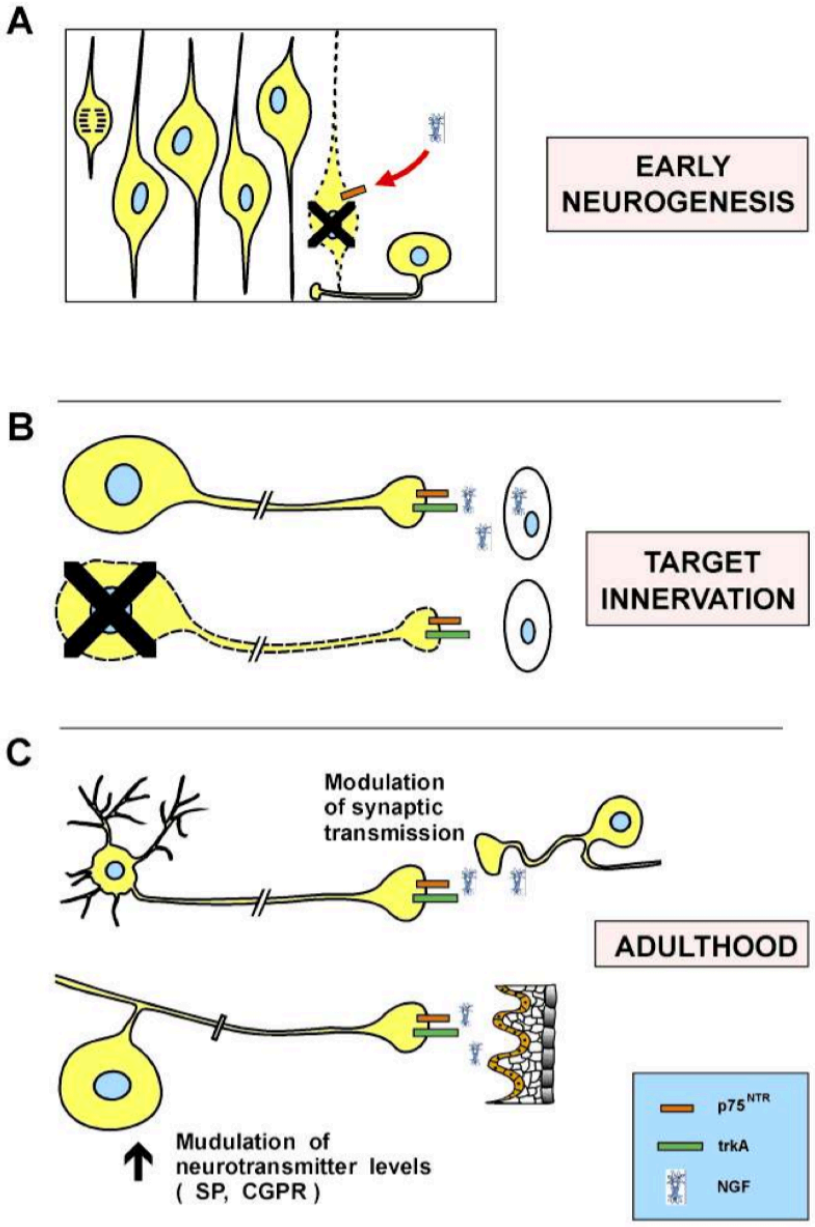


Figure 1.3: The Biological action of NGF in the developing and adult nervous system.
In newborn, NGF triggers the elimination of newly born neurons expressing p75. At later developmental stage, NGF and other neurotrophin express the appropriate TrkA receptors to maintain neuronal survival. In adulthood, NGF modulates neuronal plasticity and is involved in the regulation of hyperalgesia during inflammation. This picture is originally published in BioEssays (Frade & Barde, 1998).

1.9 NGF structure

It is crucial to understand the molecular structure of NGF and its receptors, so that we are able to design antagonists. This will pave the way to develop pharmacological and biological agents that will antagonize NGF mediated pathologies.

NGF is a member of the neurotrophin family. They are large protein molecules produced by target cells. They enable cell survival, differentiation, and neurite outgrowth (Korsching, 1993). Recent studies have shown that the neurotrophins have other fundamental functions including the promotion of synaptic plasticity, neuron protection and repair after tissue injury (Figure 1.3; Berry *et al*., 2012; Boyd & Gordon, 2003).

NGF structure was first discovered by X-ray crystallography, as a novel protein fold. NGF is composed of two 12.5 kDa monomers, and each monomer is composed of 118 amino acids (Angeletti & Bradshaw, 1971).

The three-dimensional structure of NGF was first identified in 1991 (McDonald *et al*., 1991). It is composed of antiparallel β strands folded around each other forming an elongated structure. β-hairpin structures are located at one end of the three-loop molecule, while the opposite end contains cysteine-knot. This arrangement stabilizes the fold and preserves the molecular structure (Bradshaw *et al.*, 1994). Studies have revealed a substantial variety in the loop region that differentiates NGF from the other neurotrophins (Wiesmann *et al*., 1999). Moreover, loop regions play a crucial role in receptor identification and binding. While Loop I, II, and IV are essential for TrkA binding, loop I and V are responsible for p75 binding (see Figure 1.4 ; Barker, 2007). These findings led

researchers and pharmacologists to target these specific loop regions in the NGF in attempts to inhibit NGF or its signalling pathways.

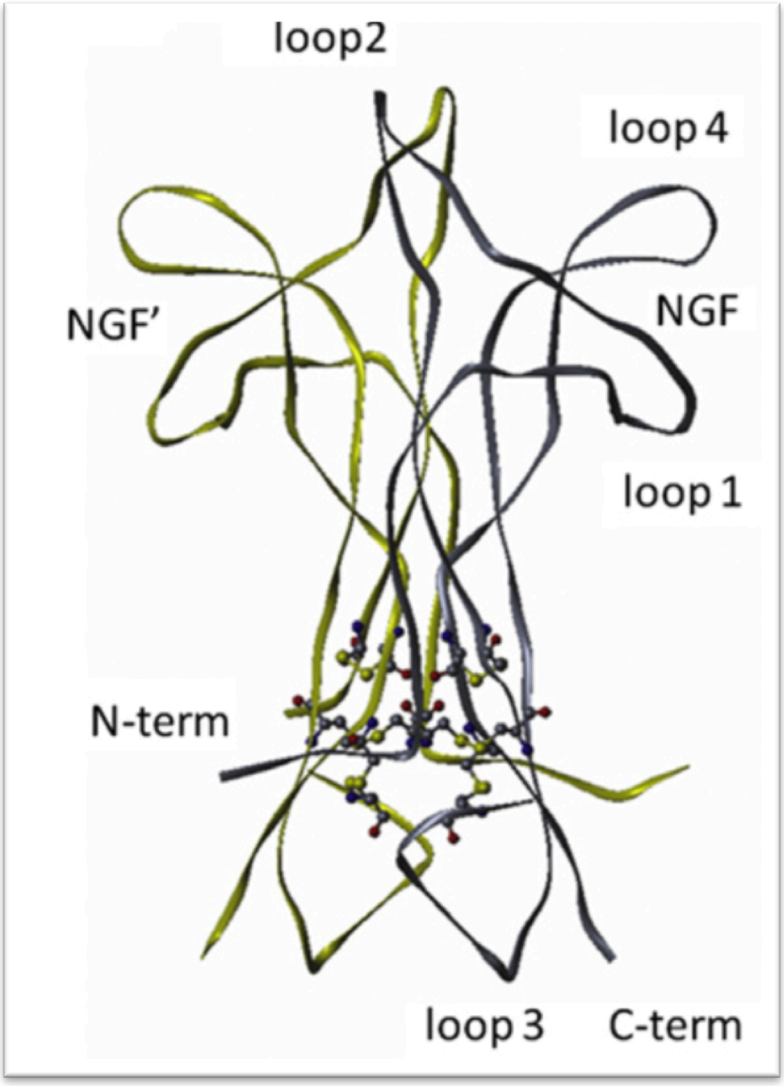


Figure 1.4: NGF structure.
The three-dimensional structure of NGF shows β-hairpin structures located at one end of the three-loop molecule. While the opposite end contains cysteine-knot. Moreover, it shows different loop regions suggested to be targeted by small molecule inhibitors. This picture is published in Neurochemistry International (Eibl *et al.,* 2012).

1.10 NGF receptors

For many years, p75 was believed to be the only receptor for NGF until the discovery of the TrkA receptor function. p75 is a transmembrane glycoprotein. It binds with almost the same equimolar affinity to all neurotrophin members. p75 is a member of the tumor necrosis receptor family (Bigda *et al*., 1994). Although p75 acts as an apoptosis inducer, studies have shown that it also has a role in neuronal cell survival and differentiation (Truzzi *et al*., 2011; Verdi *et al*., 1994). Upon stimulation of p75 expression, it leads to increase tyrosine phosphorylation of TrkA and potentiates cell survival and differentiation induced by NGF (Verdi *et al*., 1994).

TrkA is a single-path transmembrane glycoprotein. It is a member of tyrosine kinase growth factor receptors, and it plays a fundamental role in maintaining synaptic strength and plasticity of the nervous system (Kaplan & Stephens, 1994). TrkA binds to NGF with high affinity, and upon binding, Trk tyrosine kinases are activated and enable Ras stimulation. Ras, in turn, promotes activation of the mitogen-activated protein (MAP) kinases, and PI3K/Akt signalling pathways (Huang & Reichardt, 2001).

Although both receptors can function independently to bind to NGF, the decision between survival and death among NGF responsive neurons may be determined by the ratio of p75 to TrkA receptors (see Figure 1.5; Barrett & Bartlett, 1994).

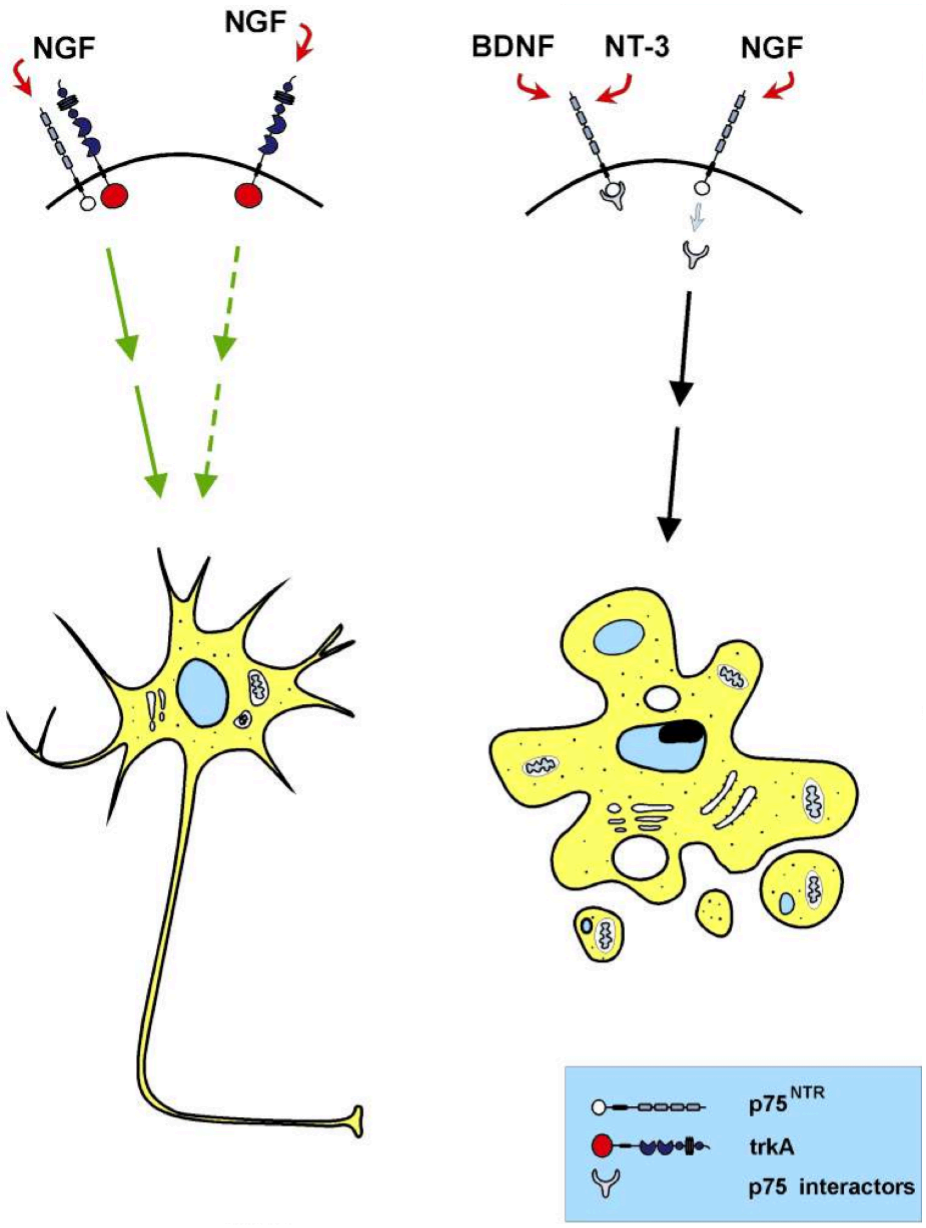


Figure 1.5: NGF receptors TrkA and p75.
TrkA activation leads to antiapoptotic signals that dominate over apoptotic signals of p75. The green arrow is survival, and the black arrow is programmed cell death through activation of p75. This figure is published in BioEssays (Frade & Barde, 1998).

1.11 NGF response to inflammation

Increased levels of NGF have been reported in a variety of acute and chronic diseases, including cystitis, rheumatoid arthritis, prostatitis and endometriosis (Barcena de Arellano *et al.*, 2011; Barthel *et al*., 2009; Chen *et al.*, 2016; Miller *et al*., 2002).

Following injury, the damaged tissue and the activated immune cells release inflammatory mediators. Mast cells, macrophages, and T cells all stimulate NGF release, which in turn binds to its receptors: TrkA and p75. The release of NGF results in the stimulation of many intracellular signalling pathways, leading to a variety of different cellular responses. Once binding occurs, the TrkA-NGF complex is transported to the cell body where it stimulates multiple gene expressions (see Figure 1.6; Nicol & Vasko, 2007).

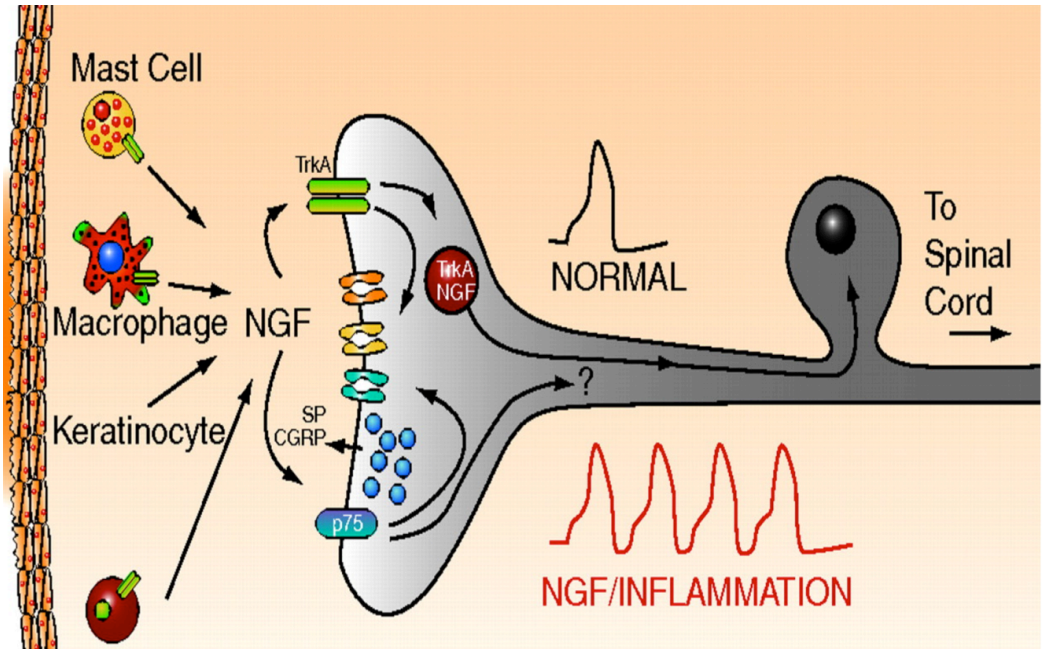


Figure 1.6: NGF response to inflammation, stimulating intracellular signalling and gene expression.
Following injury, the inflammatory mediators stimulate NGF release, causing activation of different cellular response. This figure was published in Molecular Interventions (Nicol & Vasko, 2007).

1.12 NGF and therapeutic interventions

NGF signalling has a very critical and valuable effect on neuronal protection and repair, which opens up the possibility for developing new therapeutic methods. However, due to the wide range of cells that respond to NGF signalling, many unwanted side effects may arise (Longo *et al*., 1999; Mantyh *et al*., 2011).

For example, in a study that was run for the safety of intravenous or subcutaneous NGF administration, patients suffered from sustained hyperalgesia (Andersen *et al.,* 2008). Dysregulation of NGF signalling also has been reported in diabetic patients (Ji *et al.,* 2002). Recombinant human NGF (rhNGF) has been tested in patients with diabetic neuropathy; it resulted in the same side effects such as hyperalgesia, and myalgia, and this clinical trial was terminated (Apfel *et al*., 2000).

One of the greatest challenges for NGF treatment is its inability to cross the blood brain barrier when administrated systemically. For this reason, another trial has been performed through the infusion of NGF into the cerebroventricular system of Alzheimer's patients. This also resulted in back pain after the infusion (Aloe *et al.*, 2012).

1.12.1 Human anti-NGF monoclonal antibodies

Due to these side effects, several approaches have been tried either by antagonizing NGF or blocking its binding to TrkA, which in turn can inhibit TrkA signalling. Several interventions have been undertaken for NGF inhibition *in vivo*. One of these trials used anti–NGF antibodies. This study involved testing analgesic and safety effects of human

monoclonal anti-NGF antibodies in adult osteoarthritic patients. These include Fulranumab, Tanezumab, and Fasinumab (Seidel *et al.*, 2013; Tiseo *et al*, 2014).

Although these drugs seemed promising in relieving pain in osteoarthritic patients, the trials were discontinued as patients were experiencing rapid joint destruction among other side effects (Seidel *et al*., 2012).

1.12.2 Mimetic peptides

Studies were directed to inhibit NGF-TrkA complex using NGF mimetic peptides. Many efforts have been devoted in an attempt to synthesize small molecules which mimic or antagonize NGF (Colangelo *et al*., 2008). Mimetic peptides are synthetic peptides that consist of short sequences of amino acids (5-10) resembling regions in NGF domains. Their mechanism relies on competing with NGF for its binding sites and blocking its signalling pathway (Beglova *et al.,* 2000). Many of these studies were promising, and it is hoped that they will have positive results in relation to second and third generation therapeutics with no side effect and more potency (Eibl *et al.*, 2012).

1.12.3 Small molecule inhibitors

The neurotrophins share many commonalities with dimeric proteins in terms of structure and chemical properties (Robinson *et al.,* 1995). Studies have reported a fundamental variety in the loop region that differentiates NGF from the other neurotrophin members (Wiesmann *et al.,* 1999). These findings lead researchers and pharmacologists to target this specific loop region in the NGF in attempts to inhibit NGF or its signalling pathway using small molecules inhibitors (see Figure 1.4). The small size of the molecule will

easily occupy NGF structure, altering NGF topology, thus inhibiting NGF signalling pathway (Niederhauser *et al.,* 2000).

Recent studies have reported small molecule inhibitors, which have shown inhibition of NGF signalling. These include, PD90780, ALE 0540, and Ro 08-2750 to name a few (Eibl *et al.*, 2010; Niederhauser *et al*., 2000; Owolabi *et al*., 1999; Spiegel *et al*., 1995). These small drug-like molecules have the ability to bind and modify NGF by inhibiting NGF/TrkA and NGF/p75. Their small size is of great value as they potentially could be administered orally, and may cross the blood brain barrier. This feature will provide a great advantage in treating central nervous system pathologies. Moreover, the ability of these compounds to act as inhibitors of NGF and proNGF has been tested using (SPR) surface plasma resonance spectroscopy (Sheffield *et al.*, 2015).

In this study, PD90780 was selected to assess its ability as a known NGF inhibitor in PC12 cell line. PD90780 is a non-peptide NGF inhibitor. It was first determined to inhibit binding of NGF to the p75 receptor by targeting loops I/IV cleft of NGF (see Figure 1.4; Spiegel *et al.,* 1995). A more recent study by Sheffield *et al.* (2016) used plasma resonance technology to demonstrate that PD90780 is the most effective inhibitor for TrkA and p75 receptors among the reported small molecules inhibitors. Moreover, PD90780 acts as an inhibitor on NGF and proNGF (Sheffield *et al.*, 2015).

1.13 NGF and regulation of gene expression

Advances in the fields of molecular and cell biology, and the massive data acquired from gene expression analysis have helped to gain a better understanding of cell signalling, function and response. Better understanding of genetic networks will contribute to identify novel drug targets, and more sensitive diagnostic strategies.

In 2000, studies were conducted by Angelastro *et al.* to explore how NGF regulates gene expression in neurons using serial analysis of gene expression (SAGE). The studies have identified hundreds of genes, which can be expressed or depressed after the exposure to NGF. Additionally, they focused on transcription factors that control gene function. In response to a range of external stimuli, a study by Sheng and Greenberg (1990) showed that early gene expression response was rapidly induced even in the absence of protein synthesis, such as Egr-1, c-fos, and c-jun. In this study, Egr-1 gene has been chosen to assess the signalling response of NGF and the effect of the inhibitory compounds in NGF treated cells.

1.13.1 Egr-1 discovery

Early growth response protein 1 (Egr-1) is a member of the immediate early gene family; it is rapidly induced within minutes after stimulation and it also rapidly decays (Mokin, 2005). When Egr-1 was first discovered in the mid-eighties, it was originally named nerve growth factor inducible A (NGFI-A). The name was given based on the fact that Egr-1 or NGFI-A is expressed in response to NGF stimulation of PC12 cells in the neural cell differentiation model (Milbrandt, 1987). Later on, it was reported in studies that were aimed at finding genes that contribute to growth, proliferation and differentiation of cells.

Following studies conducted on stimulated murine and human fibroblasts, the new name "Egr-1" was introduced by Sukhatme *et al.* (1987).

Recent studies have reported that Egr-1 is rapidly expressed in differing cellular responses such as: growth factors, stress, irradiation, injuries, neural plasticity and cytokines (Duclot & Kabbaj, 2017; Granet & Miossec, 2004; Lim *et al.*, 1998; Sukhatme *et al.*, 1988; Virolle *et al.*, 2001; Wu *et al*., 2009). Moreover, NGF is known to induce expression of Egr-1 (Kendall *et al.,* 1994).

1.13.2 Egr-1 gene downstream regulation

Egr-1 is a nuclear protein encoded by zinc finger transcription factor of Cys2-His2 class for 80 - 82 kDa (Thiel *et al.*,1994). The Egr-1 transcription factor is composed of independently operating domains: the activation domain and DNA binding domain. While the activation domain is located at the N terminal region, the DNA binding domain is located at C terminal region. DNA binding domain is composed of three-tandem zinc-finger, where it combines with their target molecules. Moreover, a repressor domain has been determined on the Egr-1 protein and it is positioned between the activation domain and the DNA binding domain (see Figure 1.7; Christy & Nathans, 1989). The repressor domain plays a crucial role in controlling gene overexpression by binding to NAB2 co-repressor leading to inhibition of its own downstream signalling and blocking its signalling pathway (Russo *et al.*, 1995).

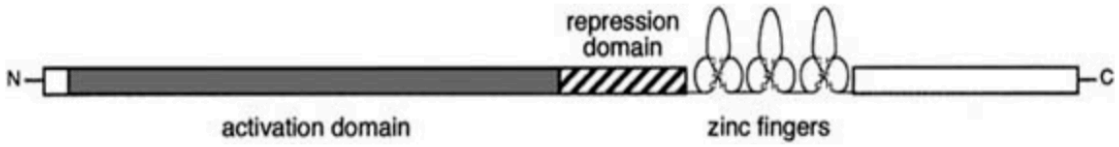


Figure 1.7: Functional domains in Egr-1 transcription factor.
The Egr-1 transcription factor structure is composed of an activation domain located on the N terminus and the three-tandem zinc finger located at the DNA binding domain. Moreover, an inhibitory domain in between the activation and the DNA binding domain. The figure was published in Journal of Cellular Physiology (Thiel & Cibelli, 2002).

1.13.3 Egr-1 and MAPK signalling

Egr-1 gene has a pivotal role in many biological functions, from regulating cell growth, to proliferation and cell survival (Nguyen *et al.*, 1993). The Egr-1 transcription regulator is characterized as a "master switch", as it acts as the connecting switch between the wide range of extracellular stimuli and the activation of the target gene (Yan *et al.*, 2000).

Studies have reported that Egr-1 is induced in a wide range of cell types involved in the mitogen process (Peng *et al.*, 1999). Moreover, mRNA for Egr-1 was synthesized in response to platelet derived growth factor, transforming growth factor and insulin like growth factor which are released on the activation of the mitogen activated protein kinase (MAPK) signalling cascades (Gineitis & Treisman, 2001).

The MAPK signalling pathway involves a series of proteins that act as convergent points. These convergent points work by transmitting signals on the cell surface from an extracellular stimulus until the signal translocates to the nucleus of the cell. The MAPK pathway structure is simply comprised of G proteins (RAS), and three consecutives

protein kinases (RAF, MEK and ERK). Kinases are enzymes that transfer phosphate groups to particular amino acids of a target protein.

The binding of extracellular stimuli, such as pathogens, stress or injury to the cell receptor, acts as a starting point that triggers all the signalling cascades. A ligand binding to its receptor causes dimerization of two-receptor tyrosine kinase subunits. This, in turn, stimulates phosphorylation and catalyzes the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP), changing the dormant RAS protein that binds to GDP to the active form of the RAS protein. Thus, active RAS protein phosphorylates, and activates the kinases RAF, MEK and ERK. Once ERK is activated, it translocates to the nucleus and activates several gene transcription factors such as Egr-1 transcription factor, and subsequent activation of genes expressions (see Figure 1.8; Zhang *et al.*, 2002).

Apparently, a wide range of extracellular stimulus can induce Egr-1 gene expression (Nguyen *et al.,* 1993). Knowing the fact that NGF is an Egr-1 gene inducer (Kendall *et al*., 1994), and that Egr-1 transcription factor acts as a "master switch" was of great value (Laity *et al.,* 2001). Taking this into consideration, it can be suggested that NGF signalling and the effects of NGF inhibitory molecules can be evaluated by examining this "master switch": Egr-1 gene expression. This means that the role of the Egr-1 gene in this study can be used as a marker to assess NGF signalling, to help us determine whether it is on or off.

Studying gene expression is a useful tool to help us gain a better understanding of the cell signalling response, which, in turn, can be useful in the discovery of novel drug targets.

Taking all of this information altogether, further research is needed to optimize the therapeutic potential of NGF inhibitors.

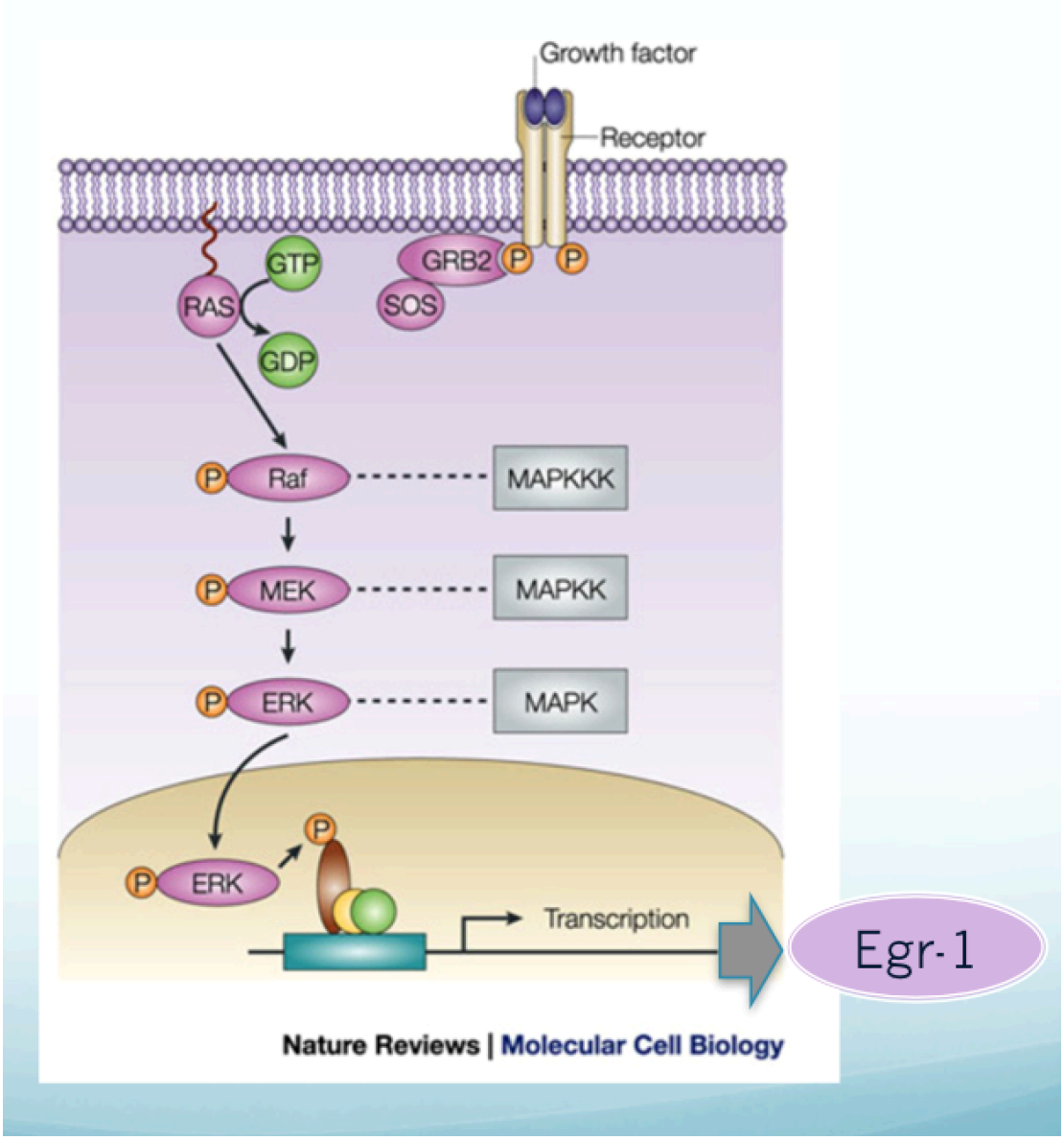


Figure 1.8: MAPK signalling pathway.
Binding of the extracellular stimuli to its ligand triggers the signalling cascades for MAPK pathway. Starting from RAF, REF, MEK to ERK. After ERK is translocated into the nucleus, transcription factor induces gene expressions. This figure was adapted from Molecular Cell Biology.

1.14 Neural cell line (PC12)

To have a better understanding of the NGF riddle, investigate NGF mechanism and explore different therapeutic interventions, a successful neural differentiation model should be applied. The most commonly used models in neural cell differentiation are either the primary cell culture or a neural cell line. The PC12 cell line is one of the mostly widely used models in neural cell differentiations. It was refined in 1976 by Greene and Tishler from adrenal medulla tumour of a rat.

PC12 cells share the same embryological origin with neuroblastic cells. Despite the fact that they share the same criteria of neurons, they are not considered as neuron. A distinguishable feature of PC12 cells is that they convert from chromaffin like cells to neuron like cells with dendritic extensions in response to NGF treatment (Eaton & Duplan, 2004).

The PC12 cell line was selected to investigate the effect of NGF inhibitory molecules. It is a straightforward and a useful model for in vitro studies. Its value relies on evaluating the results obtained from PC12 and deciding the following steps in investigating it in primary cell culture. Levkovitz *et al.* (2001) established that PC12 cells demonstrate a distinct expression of the Egr-1 gene when stimulated by NGF.

1.15 Phorbol 12-myristate 13-acetate (PMA)

Phorbol was first reported in 1934 as a product of the hydrolysis of cotton seeds. PMA is a diester of phorbol that has a controversial effect (Bueso *et al.,* 2016). While they are recognized by their ability to act as a tumour activator by inducing cell apoptosis, studies

have also reported its role in angiogenesis, and various effects in many cellular activities and different signalling pathways (Davis & Lipsky, 1985; Park *et al.,* 2001). Angiogenesis and cell proliferation are modulated by activating the Protein C kinase signalling pathway (Caino *et al.,* 2011).

Further studies have reported that PMA can stimulate the signalling pathway of MAPK, and is a potent mitogen that can induce different gene expressions (Chiu *et al.,* 1987). Moreover, induction of immediate early genes in response to PMA, such as c-fos, c-jun and Egr-1 gene has been also reported (Dunnmon *et al.,* 1990).

Cheng *et al*. (1994) found that, when treated with PMA, Egr-1 expression in PC12 cells is induced with peak expression 1 hour after treatment (Cheng *et al*., 1994). For this reason, PMA was chosen as a positive control for Egr-1 gene expression.

1.16 Gene analysis technique

For decades, biologists have used many tools, such as Northern or Southern blotting, to quantitate nucleic acids. In 1997, the Northern blot technique became a popular method for RNA quantification. The main idea for the Northern blot is to separate RNA according to their molecular size using a complementary hybridization probe (Alwine *et al*., 1977). However, it is a complex, and time-consuming procedure. Additionally, there is an increased risk of toxicity by the chemicals used for RNA denaturation, which makes this procedure unsafe to use (Goda & Minton, 1995). More attention was needed to generate more sensitive and specific quantification methods.

Nowadays, reverse transcriptase polymerase chain reaction (RT-PCR) is used as a standard tool for gene analysis. The invention of PCR by Kary Mullis in 1989 dramatically changed every scientific aspect (Mullis, 1990). This discovery made the impossible possible. PCR is a sensitive and a straightforward technique. The major strength of this technique is that small amount of RNA can be copied and amplified by PCR. Then, it can be quantified to analyze different gene expression.

Amplification of small amount of RNA has a tremendous impact in many scientific branches such as infectious disease detection, the discovery of different gene mutation, and forensic investigations. Moreover, PCR helped to solve the mystery of certain diseases in the past. For example, in 1967, the former vice president and presidential candidate Hubert. H. Humphrey had negative test results for bladder cancer, but passed away in 1978. When a urine sample was tested with PCR in 1990, surprisingly the results were positive for bladder cancer. The PCR technique is the invention for the past, present, and future. In 1993, Mullis won Nobel Prize in Chemistry for the PCR invention (Powledge, 2004).

In this study, we use gene expression analysis as functional readout, to assess the effect of the inhibitory compounds in NGF treated cells applied. Furthermore, Egr-1 expression time course is determined for Egr-1 in NGF treated PC12 cells. All the results were analyzed by semi quantitative RT-PCR.

1.17 Hypothesis and objectives

1.17.1 Hypothesis

Expression of genes downstream of NGF signalling can be used as an effective screening strategy for evaluating NGF inhibitors.

1.17.2 Objectives

i. Identify the peak time for Egr-1 expression in NGF treated cells.

ii. Determine the gene expression of Egr-1 in NGF treated cells in the presence of NGF inhibitors using semi quantitative RT-PCR.

1.17.3 Rationale

Chronic pain management is a challenging task. To date, many cases cannot be treated by the currently available techniques. Despite all the efforts done, the quest for safe and effective pain relief medications have not yet been achieved.

With the discovery of NGF as a pain mediator, studies were directed towards targeting NGF receptors or its signalling pathway. It is known that NGF release will induce different gene expression including Egr-1 gene. Exploring Egr-1 gene expression in NGF treated cells and comparing them to cells treated with NGF inhibitors may provide a better understanding of the NGF signalling riddle. Moreover, it may help to identify a novel drug target with the potential of no adverse effects.

1.18 Thesis organization

Chapter 2 focuses on the methodology and the materials used to run all the experiments. Chapter 3 presents the results obtained using semi quantitative PCR technique. Chapter 4 provides discussion and analysis of the results. Finally, Chapter 5 provides the conclusions derived from this thesis and highlight the major directions to extend this research in the future.

Chapter 2

Materials and Methodology

This chapter outlines the materials and the methodology used to run all the experiments. While the first step focuses on different treatments applied to cells, the second one deals with RNA extraction and DNAse treatment. Finally, PCR reactions will be analyzed.

2.1 Cell culture

PC12 cells were cultured in Dulbecco's Modified eagle Medium (DMEM), supplemented with 10% fetal calf serum (FCS) (Gibco; USA), 0.1 U/ml of penicillin and 0.1 U/ml of streptomycin. All cells were maintained at 37°C in a humidified incubator in 5% CO_2-95% air. The cells were grown to 80% to 90% confluence in T 75 cm^2 tissue treated flasks (Corning-USA). Using trypsin, (Trypsin-EDTA, Gibco; Canada), cells were dissociated. The cells were then counted and their viability was determined using a cell viability analyzer (Vi-cell XR, Beckman Coulter; Mississauga, Ontario) before being plated into 6 wells plates. Cells were seeded into 6 well plates at a density of 3x 10^6 in 2 ml media.

2.2 Cell treatments

2.2.1 First objective

Cells were treated with 0.5 nM of NGF obtained from Cedar Lane Labs (Burlington, ON, Canada), or 80 nM PMA (Sigma- Aldrich; Canada) for 30, 45, 60, 90 and 180 minutes.

2.2.2 Second objective

NGF treatment

Cells were treated with 0.5 nM of NGF and 100 μM of PD90780 obtained from Sussex Research (Ottawa, ON, Canada). NGF was pre-incubated with PD90780 for 60 minutes, and then the cells were incubated with the mixture for another 90 minutes.

PMA treatment

Cells were treated with 100 μM of PD90780, and 80 nM of PMA positive control. PMA was pre-incubated with PD90780 for 60 minutes, and the mix was incubated for another 60 minutes with the cells. All the samples were done in 6 replicates.

2.3 RNA extraction

Total RNA was extracted from the treated cells with TRIZOL reagent (Sigma-Aldrich), following the manufacture's protocol as follows. The media was removed; 500 μl of TRIZOL reagent was added to each well of the 6 well plates, for cell lysis. Cell lysates were transferred into an Eppendorf tube, followed by adding 100 μl of chloroform. The samples were then vortexed, and incubated at room temperature for 10 minutes. Samples

were centrifuged at 12,000 x g for 20 minutes at 4°C. The aqueous phase (top layer) was transferred into clean tube.

RNA was precipitated by adding 125 µl of isopropanol, then vortexed, and incubated at room temperature for 10 minutes. The samples were once again vortexed, before being centrifuged at 12,000 x g at 4°C for 8 minutes. RNA pellets were washed in 250 µl of 70% ethanol, centrifuged at 7,500 x g for 5 minutes at 4°C. Ethanol was then removed, and RNA pellets were air dried and dissolved in 17 µl of RNase free DEPC (diethyl pyrocarbonate-treated H_2O) at 37°C for 10 minutes.

2.4 Quantification of RNA concentrations

The quantification method was performed using a Nanodrop ND-1000 spectrophotometer at 260 nm. All measurements were carried out following the manufacturer's instructions, and by using a 1 µl sample. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of RNA. A ratio far from 1.8 - 2 gives an indication of contamination.

2.5 RNA integrity

RNA integrity was analyzed by using denaturing RNA electrophoresis. Agarose gel was prepared using 1% agarose and 1X Tris–borate electrophoresis buffer (TBE-Maniatis protocol). Ethidium bromide (0.5 µg/ml) was added to the liquid agarose mixture before being casted. After loading the samples, the gel was left to run for 60 minutes at 90 Volts.

RNA samples were prepared to a total volume of 12 µl by mixing 500 ng of the total RNA with 2 µl of 6X loading dye (Sigma- Aldrich) and DEPC water. 1 Kb plus DNA ladder (Sigma-Aldrich) was used as a molecular marker. RNA quantities and integrities were

analyzed using Nanodrop ND-1000 spectrophotometer (Biorad). After gel electrophoresis, RNA integrity bands were evaluated visually by comparing (28S to18S) bands intensity. The 28S band intensity should be twice as intense as the 18S bands 2:1 ratio (28S: 18S). Intact bands are an indication that the RNA is intact.

2.6 DNAse treatment

RNA samples were treated with DNAse before reverse transcriptase to remove all genomic DNA that could be present. The protocol was as follows: 2 μg of RNA was used as a template for reverse transcriptase in a total of 50 μl final volume. RNA samples were treated with the following; 2 μl of DNAse reaction buffer (20 mM Tris-HCl and 20 mM of $MgCl_2$) (Sigma –Aldrich), 2 μl of amplification Grade DNAse (Sigma –Aldrich), and DEPC-water to a final volume of 20 μl, followed by incubation at room temperature for 15 minutes. 2 μl of Stop solution (50 mM EDTA) was added to terminate the reaction, and then heated for 10 minutes at 70°C.

2.7 cDNA synthesis

First strand cDNA was synthesized by reverse transcriptase, to get single stranded complementary DNA. This cDNA in turn can be used directly as a template for further amplification using PCR technique. M-MuLV (Moloney Murine Leukemia Virus) reverse transcriptase was used following the manufacturer's instructions (Promega-Thermo Fisher Scientific Canada); as follows: random primers (1μl of 1 μg/μl) were added to the DNAse treated samples. The samples were heated for 5 minutes at 70°C, and then cooled on ice. Master mix was added to the samples, containing 2.5 μl of dNTPs (10 mM), 10 μl of 5X reaction buffer M-MuLV (Sigma-Aldrich), 12.5 μl of DEPC water and 2 μl of M-MuLV

RT to a total volume of 27 µl. The samples are then incubated for 60 minutes at 37°C using a thermo cycler (BioRad).

2.8 Polymerase chain reaction (PCR)

PCR was performed to amplify Egr-1 and β-actin genes using first strand cDNA as a template. The cDNA was synthesized from RNA isolated from PC12 cells as described in (Section 2.3). For PCR protocol, GoTaq flexi DNA polymerase chain reaction kit (Promega) was used following the manufacturer's instructions. The amplification reactions were performed using 2.5 µl of cDNA samples (0.05 µg/µl) that were added to a master mix prepared to a final volume of 25 µl. The master mix was prepared using the following; 0.5 µl of dNTPs (200 µM), 1.5 µl of $MgCl_2$ (1.5 mM), 5 µl of GoTaq flexi polymerase reaction buffer (Sigma-Aldrich) (1X), 0.5 µl of forward primer (100 ng/µl), 0.5 µl of reverse primer (100 ng/µl), 0.125 µl of GoTaq polymerase enzyme (5 U/µl) and DEPC water to a final volume of 25 µl. The PCR reactions were run in a thermo cycler machine (Biorad).

Amplification conditions for the PCR reaction were 1 cycle of 3 minutes at 94°C for denaturation; 20-35 cycles for 1 minute at 94°C, 1 minute at 57°C and 1 minute at 72°C; and a final extension cycle of 10 minutes at 72°C.

Table 2-1: PCR program set-up for Egr-1.
PCR program for Egr-1was as follow: total of 35 cycles, 34 cycles from step 2 to 4.

Steps	Temperature	Time
1	94°C	3minutes
2	94°C	1 minutes
3	58°C	1 minutes
4	72°C	1minute
5	72°C	10 minutes

Table 2-2: PCR program set-up for β-actin.
PCR program for β-actin was as follow: total 20 cycles, 19 cycles from step 2 to 4.

Steps	Temperature	Time
1	94°C	3 minutes
2	94°C	1minute
3	58°C	1 minute
4	72°C	1 minute
5	72°C	10 minutes

2.9 Primers

Egr-1 is the gene of interest and β-actin gene was used as a housekeeping gene for calibration; the primer sequence used was as follows:

Table 2-3: Primer specification for Egr-1 and β-actin.

Egr-1 (Reverse)	CTCAACAGGGCAAGCATACG
Egr-1 (Forward)	TTTCCACAACAACAGGGAGAC
β-actin (Forward)	GTATGGAATCCTGTGGCATCC
β-actin (Reverse)	GACTCATCGTACTCCTGCTTG

2.10 Gel electrophoresis for PCR

Agarose gel electrophoresis was used to analyze the PCR reaction. PCR samples were resolved on 2% agarose gel prepared from 1X TAE buffer; and ethiduim bromide (10 mg/ml) was added to the liquid agarose mixture before being casted. After loading the samples, the gel was left to run for 50 minutes at 90 volts. After electrophoresis, the gel was placed under Trans UV (Biorad) for imaging using Chemidoc XRS (Biorad).

2.11 Densitometry

For densitometry, images were analysed using Quantity One Software (Biorad). The density of each amplified band for both the housekeeping gene (β-actin) and the gene of interest Egr-1 were measured using 1-D Image software Data Analysis (see Figure 3.13). All data were analyzed by calculating the average ratio, between the gene of interest Egr-

1 and β-actin gene. Standard error was then provided for at least 6 samples of PCR for each of 3 biological experiments. Fold change was then calculated, using excel.

Statistical analysis was done using Graph Pad Prism 7 software. Unpaired two-tailed t-tests were used to validate the fold change significance within each treatment. A probability level of $p \leq 0.05$ was considered statistically significant.

Chapter 3

Results

This chapter presents the experimental design and the results obtained for identifying the best timing for Egr-1 gene expression in NGF treated cells. Moreover, it assesses the statistical significance and the coefficient of variation using semi quantitative RT-PCR.

3.1 Experimental design and analysis

The experiment was designed to evaluate cellular response of NGF, by studying early gene transcriptional expression profile. This study was applied to PC12 cells, which is a clonal cell line derived from rat phenochromocytoma. For many years, PC12 cells have been used as an effective model for neuronal study. They share identical properties with neurons, as they have embryological origin from neuroblastic cells (Greene & Tischler, 1976). The Egr-1 gene was selected because of its well-characterized response to NGF (Dijkmans *et al.,* 2009). Data analysis was carried out on the expression level for Egr-1 after treatment of NGF only (0.5 nM), or PMA only (80 nM), or a mixture of PD90780 (100 μM) and NGF; or PD90780 (100 μM) and PMA.

PD90780 is a non-peptide inhibitor that blocks NGF. It interacts with NGF and inhibit its binding to p75 (Spiegel *et al*., 1995). The expression profile for Egr-1 was normalized against the β-actin gene. PMA (Phorbol 12-myristate 13-acetate) has demonstrated the ability to induce the expression of Egr-1 in PC12 cells with peak expression after 1 hour before it decreases (Cheng *et al*., 1994).

PMA was chosen as a positive control to validate our results. The expression data were analyzed by semi quantitative polymerase chain reaction. Primers were designed as described in chapter 2. Agarose gel (2%) electrophoresis was performed to separate the PCR products.

3.2 Egr-1 time course expression in NGF treated cells

The effect of NGF on the expression of Egr-1 in PC12 cells was analyzed. PC12 cells were treated with NGF for 30, 45, 60, 90, and 180 minutes, and as a control. RNA was isolated from treated PC12 cells and the transcription level for Egr-1 and the β-actin genes were analyzed using RT- PCR.

Results from semi quantitative PCR performed on cDNA from NGF treated cells show no significant increase in the expression of Egr-1 after 30 minutes (0.9 fold), or 45 minutes (0.9 fold). At 60 minutes there was minimal fold increase (1.1 fold) (Figure 3.1). The highest level of expression was noticed at 90 minutes (2.7 fold; *p value $\leq$ 0.05) (Figure 3.2). Results from this study show that the expression for Egr-1 in NGF treated cells peaked at 90 minutes (2.7 fold) and declined at 180 minutes (1.0 fold) (Figure 3.2).

PMA treated cells show changed expression of Egr-1 at 30 minutes (2.2 fold), 45 minutes (0.5 fold), 60 minutes (2.9 fold; *p value $\leq$ 0.05) (Figure 3.1), and the fold is decreased at 90 minutes (2.6 fold) (Figure 3.2). Results from this study show that the expression of Egr-1 in PMA treated cells peaked at 60 minutes and Egr-1 expression declined at 90 minutes.

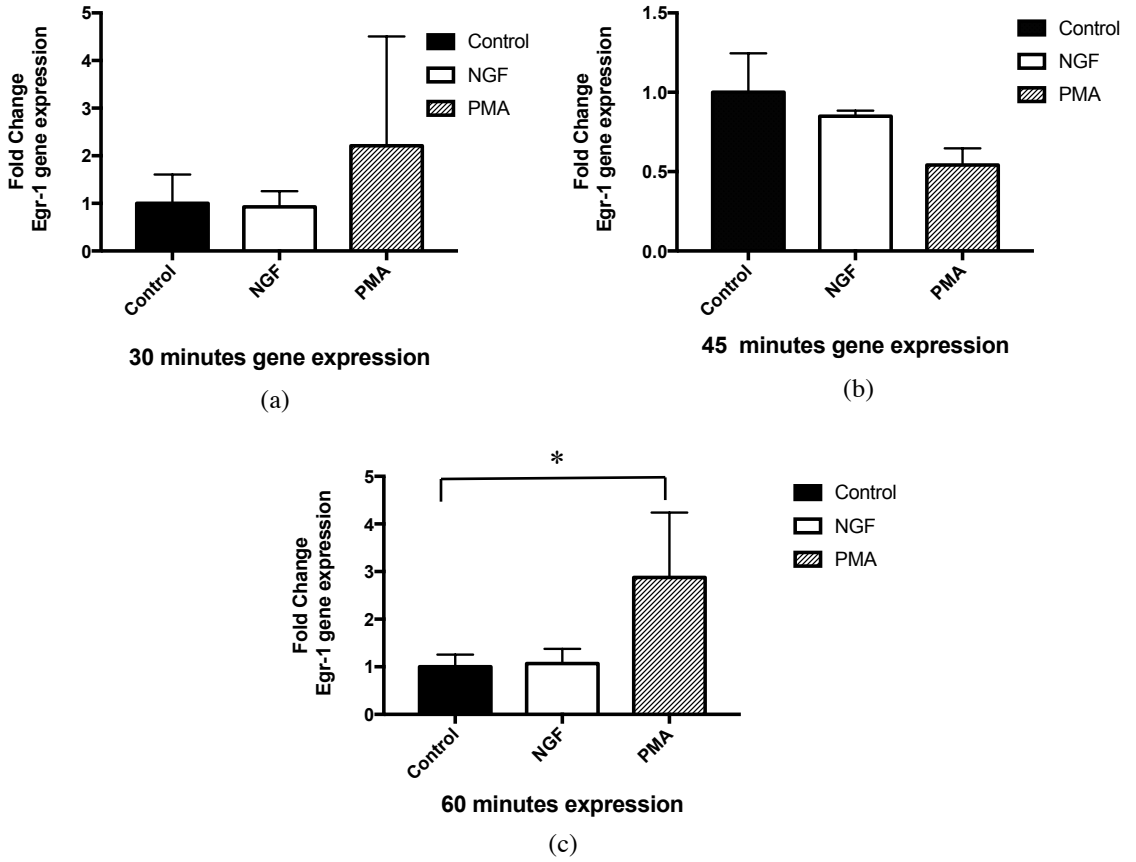


Figure 3.1: Fold change for gene expression in NGF and PMA treated cells at 30, 45, and 60 minutes.

(a) shows fold change for gene expression at 30 minutes for NGF treated cells (0.9 fold), and PMA treated cells (2.2 fold). (b) shows fold change for gene expression at 45 minutes for NGF treated cells (0.9 fold), and PMA treated cells (0.5 fold). (c) shows fold change for gene expression at 60 minutes for NGF treated cells (1.1 fold), and PMA treated cells (2.9 fold; *p value ≤ 0.05). The fold change for NGF or PMA treated cells relative to the control cells are represented in this Graph. Unpaired two-tailed t-tests determined statistical significance between the control and PMA by Graph Pad Prism. Error bar represent mean ± SEM, with n value =6.

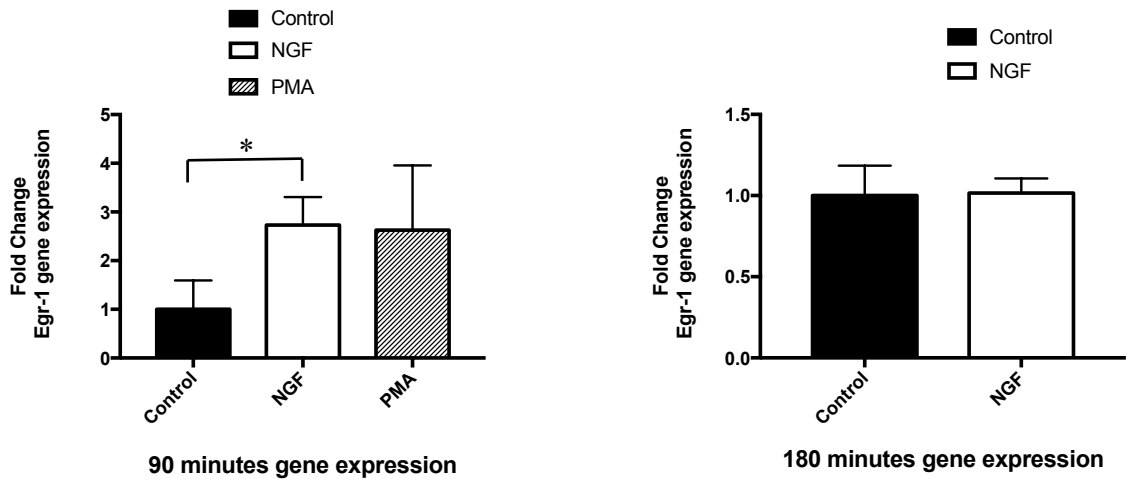


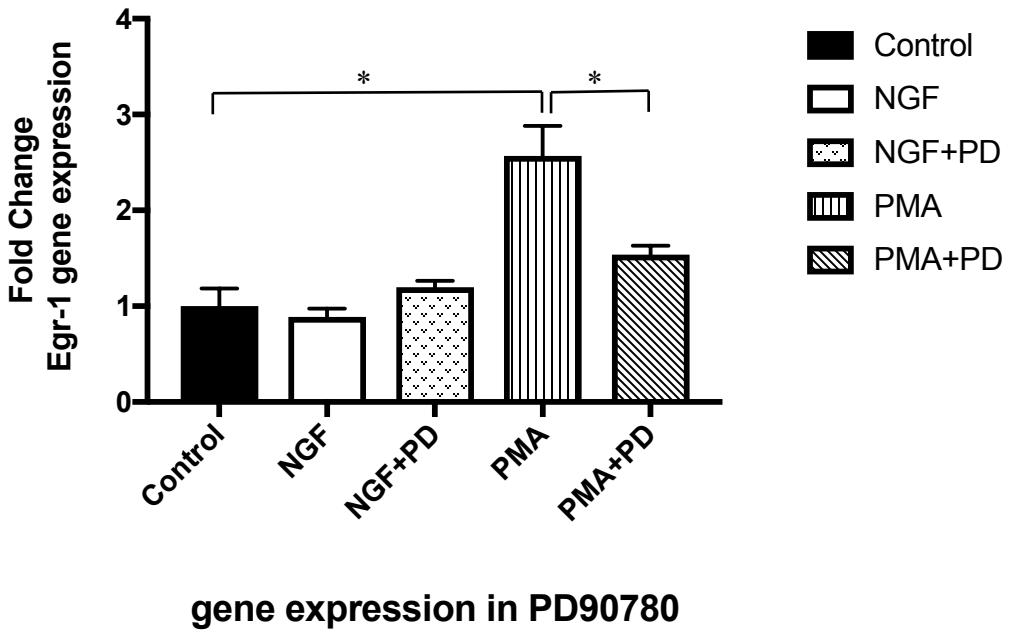
Figure 3.2: Fold change for gene expression in NGF and PMA treated cells at 90, and 180 minutes.

a) shows fold change for NGF treated cells at 90 minutes (2.7 fold; *p value ≤ 0.05) and, PMA treated cells (2.6 fold) at 90 minutes. (b) shows fold change for NGF treated cells (1.0 fold) at 180 minutes. The fold change for NGF or PMA treated cells relative to control cells are represented in this graph. Unpaired two-tail t-tests determined statistical significance between the control and NGF at 90 minutes by Graph Pad Prism 7. Error bar represent mean ± SEM, with n value =6.

3.3 The effect of PD90780 on the expression of Egr-1 in NGF treated cells.

Recent studies show that PD90780 is a potent NGF inhibitor (Sheffield *et al*., 2015). Based on these studies, PD90780 was chosen to analyze its effect on the expression of Egr-1 in NGF treated cells and validate its effect in cell signalling response.

The results show Egr-1 expression fold change in NGF treated cells (0.9 fold), combination of NGF and PD90780 (1.2 fold), PMA and PD90780 (1.5 fold) and PMA alone (2.6 fold; *p value $\leq$ 0.05). Statistical significance for the control and PMA only, was determined by using unpaired two-tailed t-test by Graph Pad Prism 7. Error bars represent mean ± SEM (n-6). Results were considered statistically significant with p value $\leq$ 0.05. The fold change results for Egr-1 expression from cells treated with NGF alone didn't show significant change through which the effect of the inhibitors can be assessed.



gene expression in PD90780

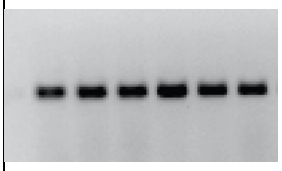
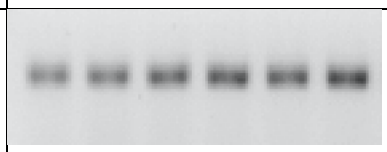
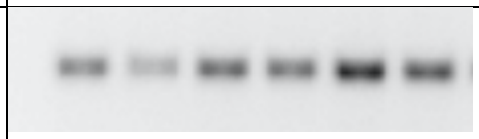
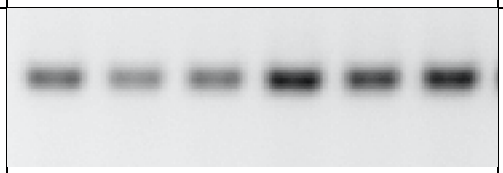
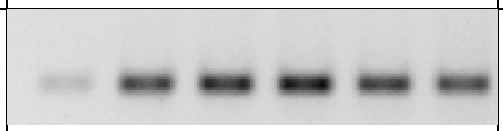
Figure 3.3: Fold change for gene expression in NGF, PMA, or a mixture of NGF and PD90780 or PMA and PD90780 treated cells.
Fold change of Egr-1 expression in NGF treated cells (0.9 fold),), or a mixture of NGF and PD (1.2 fold), or PMA treated cells (2.6 fold; *p value ≤ 0.05), or PMA and PD) treated cells (1.5 fold; p* value ≤ 0.05. Statistical significance for the control and PMA only, and PMA and PD was determined by using unpaired two-tailed tests by Graph Pad Prism 7. Error bar represent mean ± SEM, with n value=6.

3.4 Coefficient of variation

The coefficient of variation is a valuable statistical measure to compare different ranges of variability among various data. It is defined as the ratio of standard deviation to the mean value. The coefficient of variation for Egr-1 and β-actin gene expression between the six replicates for each treatment (PC12, NGF, PMA, and PD), at different timings (30, 45, 60, 90 and 180 minutes) was calculated to compare the outcome for each experiment with different treatments applied. Table 3-1 through Table 3-6 show the detailed numerical data for different treatments at different timing, while Figure 3.4 through Figure 3.9 present comparisons between the coefficient of variation for Egr-1 and β-actin gene in the different experimental sets. Table 3-7 and Table 3-8 present a summary of the data presented in Table 3-1 through Table 3-6 in normalized forms.

Table 3-1: PCR products for β-actin gene, and the coefficient of variation results for PC12 cells.

PCR products of β-actin amplified from 6 replicates of cDNA samples of PC12 cells for 30, 45, 60, 90, 180 minute and PD experiments respectively. PCR products are separated on 2% agarose gel, visualized with ethiduim bromide after electrophoresis. Coefficient of variation is calculated by the ratio of standard deviation to the mean.

Experiments	PCR products of β-actin in PC 12 cells	Mean	Standard deviation	Coefficient of variation
30 minutes		13772	8297	0.60
45 minutes		10933	4389	0.40
60 minutes		16826	10282	0.61
90 minutes		29108	15558	0.53
180 minutes		32865	9377	0.30
PD experiment		22755	9342	0.41

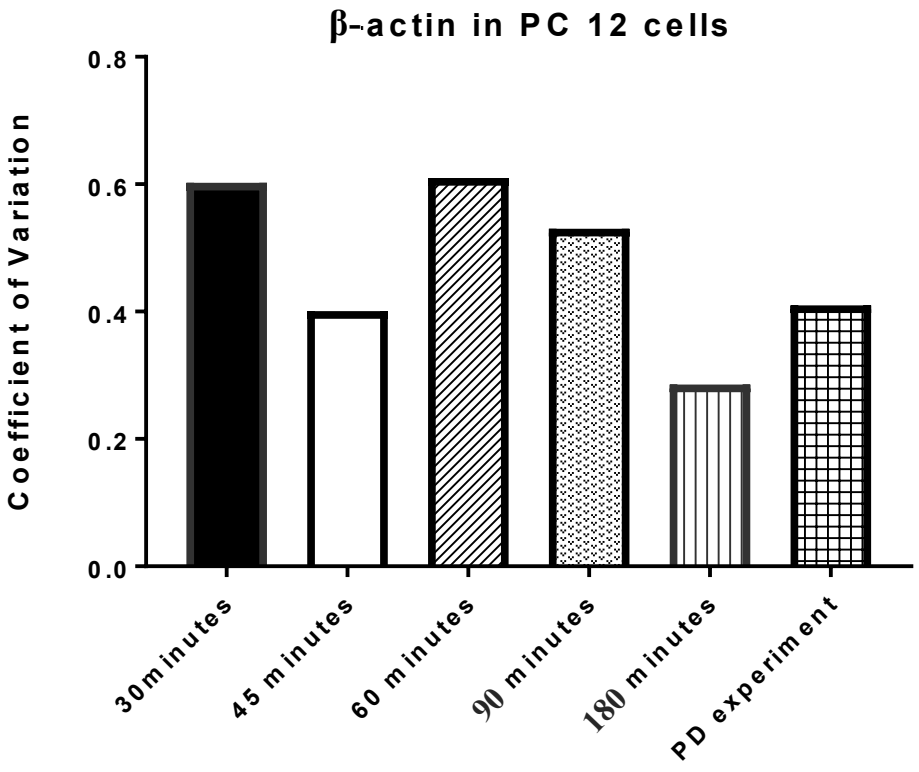
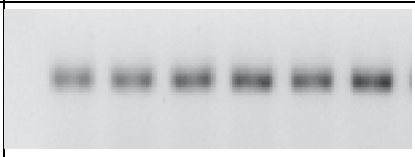


Figure 3.4: The coefficient of variation graph for β-actin gene for PC12 cells. The coefficient of variation for 6 replicates of PC 12 cells for β-actin. While, the 180 minute experiment shows the minimal coefficient of variation (0.30), the 60 minute experiment shows the highest one (0.61). There is no significant difference between the coefficient of variation for both the 30 minute and the 60 minute experiments.

Table 3-2: PCR products for Egr-1 gene, and the coefficient of variation results for PC12 cells.

PCR products of Egr-1 amplified from 6 replicates of cDNA samples of PC12 cells for 30, 45, 60, 90, 180 minute and PD experiments respectively. PCR products are separated on 2% agarose gel, visualized with ethiduim bromide after electrophoresis. Coefficient of variation is calculated by the ratio of standard deviation to the mean.

Experiments	PCR products of Egr-1 in PC12 cells.	Mean	Standard deviation	Coefficient of variation
30 minutes		28836	4745	0.16
45 minutes		14318	2658	0.18
60 minutes		14179	6857	0.48
90 minutes		21526	10624	0.49
180 minutes		53576	3864	0.07
PD experiment		10669	1932	0.18

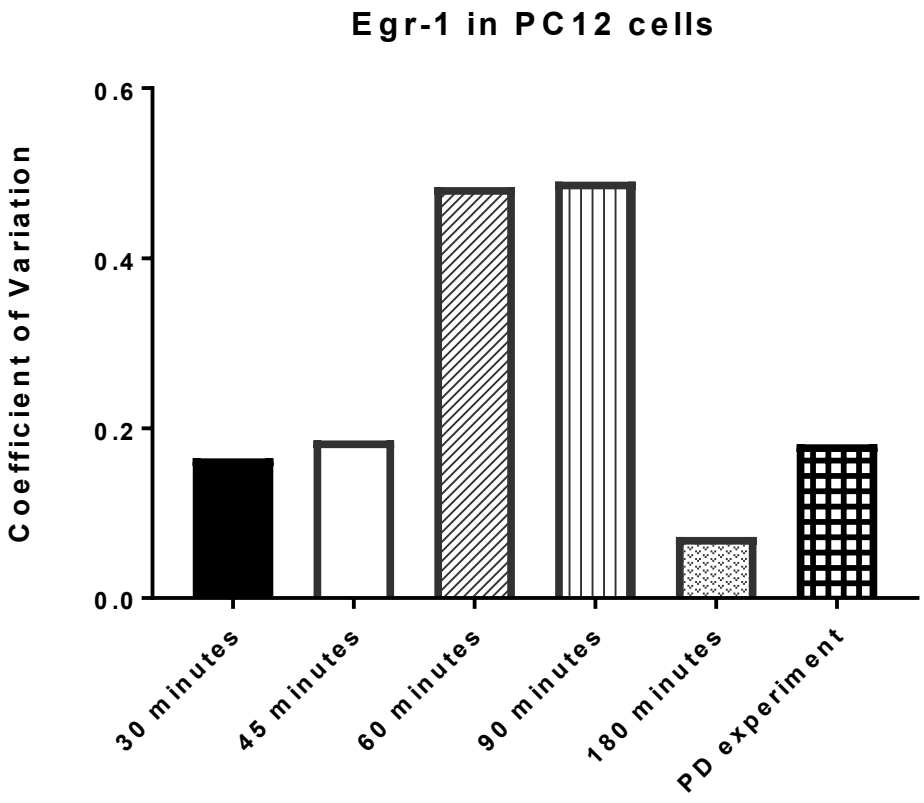
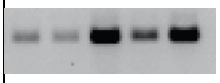
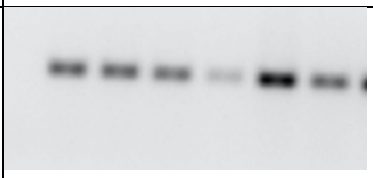
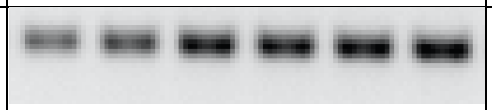
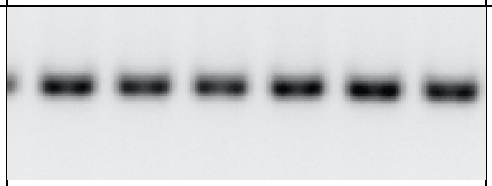


Figure 3.5: The coefficient of variation graph for Egr-1 gene for PC12 cells. The graph is representing the analysis for the coefficient of variation for 6 replicates of PC 12 cells for Egr-1 gene. The highest coefficient variation is noted at 60, and 90 minute experiments. Whereas the 180 minute experiment records the minimum coefficient of variation value (0.07).

Table 3-3: PCR products for Egr-1 gene, and the coefficient of variation results for NGF treated cells.

PCR products of Egr-1 amplified from 6 replicates of cDNA samples of PC12 cells for 30, 45, 60, 90, 180 minute and PD experiments respectively. PCR products are separated on 2% agarose gel, visualized with ethiduim bromide after electrophoresis. Coefficient of variation is calculated by the ratio of standard deviation to the mean. The 6th sample in 30 minutes was analysed in different gel.

Experiments	PCR products of Egr-1 in NGF treated cells	Mean	Standard deviation	Coefficient of variation
30 minutes		42333	8717	0.20
45 minutes		18610	1192	0.06
60 minutes		24957	9425	0.37
90 minutes		58203	8716	0.14
180 minutes		84747	4193	0.05
PD experiment		6287	1338	0.21

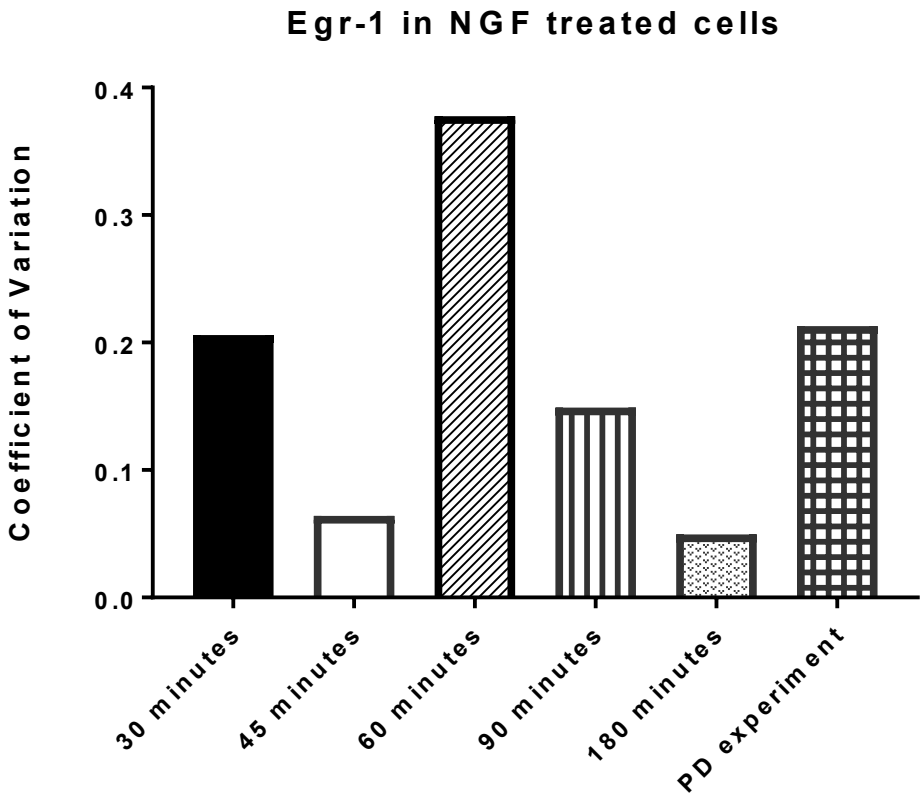
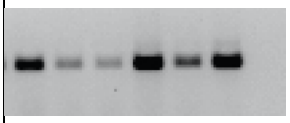
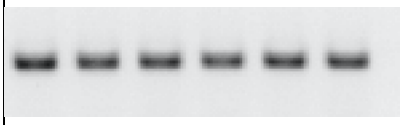
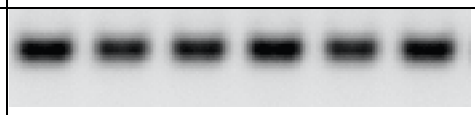


Figure 3.6: The coefficient of variation graph for Egr-1 gene in NGF treated cells. The graph represents the data for the coefficient of variation for 6 replicates of NGF treated cells for Egr-1 gene. While, the 180 minute experiment shows the minimal coefficient of variation (0.05), the 60 minute experiment shows the highest one (0.37).

Table 3-4: PCR products for β-actin gene, and the coefficient of variation results for NGF treated cells.

PCR products of β-actin amplified from 6 replicates of cDNA samples of NGF treated cells for 30, 45, 60, 90, 180 minute and PD experiments respectively. PCR products are separated on 2% agarose gel, visualized with ethiduim bromide after electrophoresis. Coefficient of variation is calculated by the ratio of standard deviation to the mean.

Experiments	PCR products of β-actin in NGF treated cells	Mean	Standard deviation	Coefficient of variations
30 minutes		18593	6080	0.32
45 minutes		14802	1046	0.07
60 minutes		24460	7259	0.29
90 minutes		16896	4033	0.23
180 minutes		48619	4713	0.09
PD experiment		12341	3514	0.28

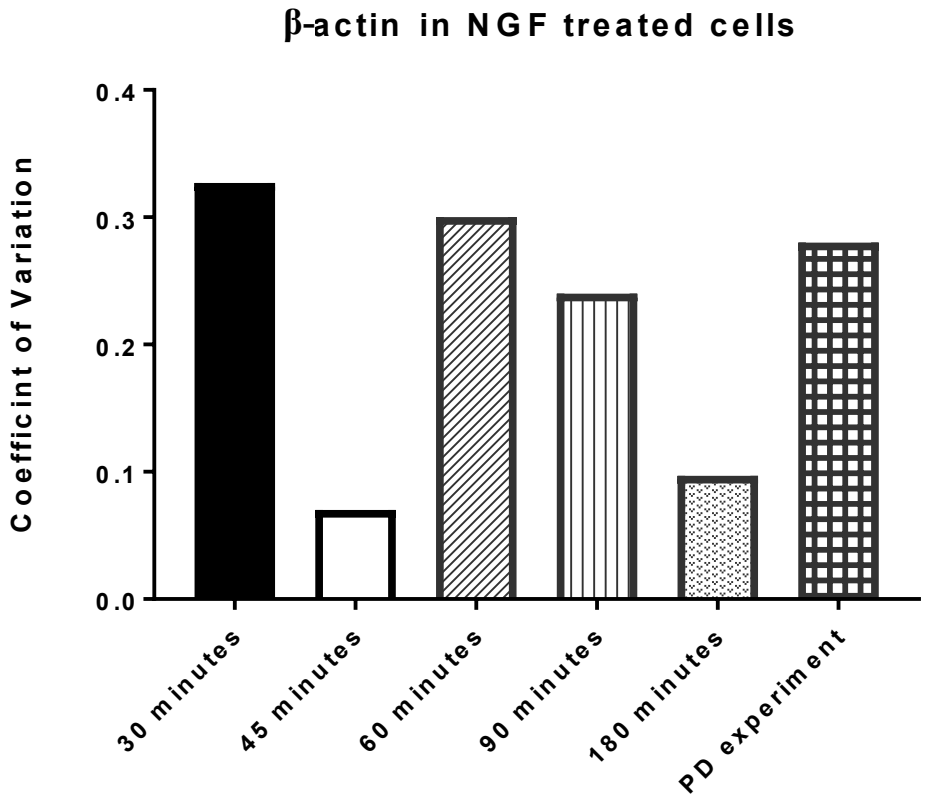
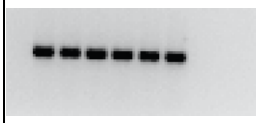
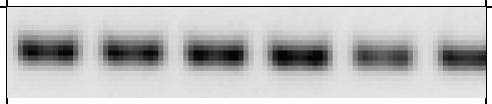


Figure 3.7: The coefficient of variation graph for β-actin gene in NGF treated cells. The coefficient of variation for 6 replicates of NGF treated cells for β-actin gene. The 45 minute experiment recorded the minimal coefficient of variation (0.07). The 30 minute experiment shows the highest one (0.32).

Table 3-5: PCR products for Egr-1 gene, and the coefficient of variation results for PMA treated cells.

PCR products of Egr-1 amplified from 6 replicates of cDNA samples of PMA treated cells for 30, 45, 60, 90, 180 minute and PD experiments respectively. PCR products are separated on 2% agarose gel, visualized with ethiduim bromide after electrophoresis. The coefficient of variation is calculated by the ratio of standard deviation to the mean.

Experiments	PCR products of Egr-1 in PMA treated cells.	Mean	Standard deviation	Coefficient of variation
30 minutes		34715	10214	0.30
45 minutes		16509	4241	0.25
60 minutes		22069	7415	0.33
90 minutes		63579	4074	0.06
PD experiment		17443	4065	0.21

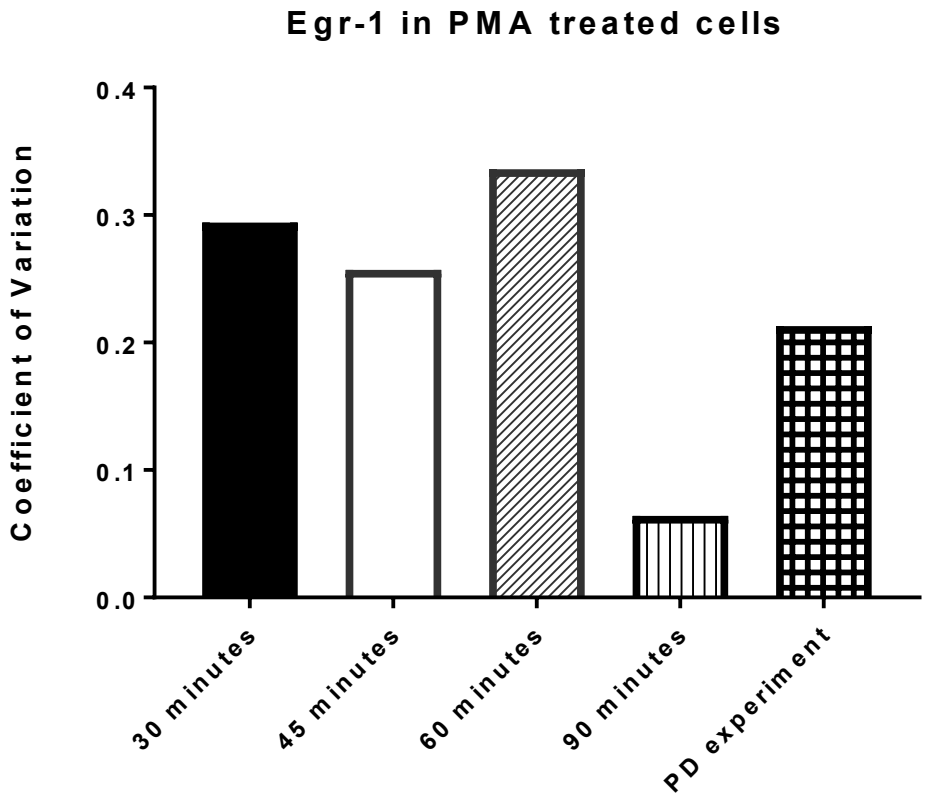
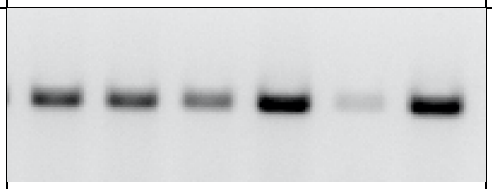
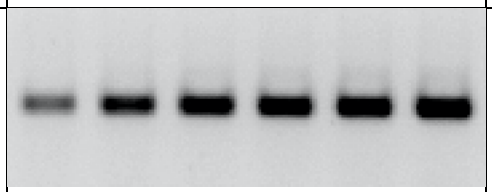


Figure 3.8: The coefficient of variation graph for Egr-1 gene in PMA treated cells. The coefficient of variation of 6 replicates of PMA treated cells for Egr-1 gene While, the 90 minute experiment shows the minimal coefficient of variation (0.06), the 60 minute experiment shows the highest one (0.33). The coefficients of variation for the 30 minute and the 45 minute experiments are (0.30) and (0.25) respectively.

Table 3-6: PCR products for β-actin gene, and the coefficient of variation results for PMA treated cells.
PCR products of β-actin amplified from 6 replicates of cDNA samples of PMA treated cells for 30, 45, 60, 90, 180 minute and PD experiments respectively. PCR products are separated on 2% agarose gel, visualized with ethiduim bromide after electrophoresis. Coefficient of variation is calculated by the ratio of standard deviation to the mean.

Experiments	PCR products of β-actin in PMA treated cells	Mean	Standard deviation	Coefficient of variations
30 minutes		9211	5413.	0.60
45 minutes		20921	1869	0.08
60 minutes		13075	7289	0.55
90 minutes		28034	15667	0.55
PD experiment		24852	8811	0.35

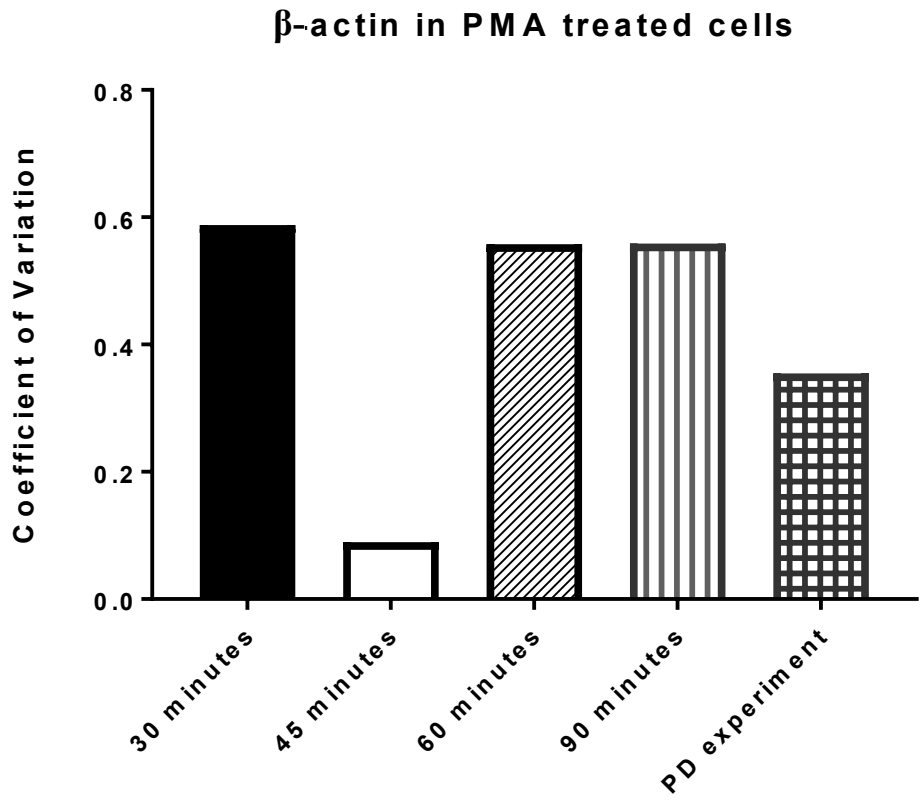


Figure 3.9: The coefficient of variation graph for β-actin gene in PMA treated cells. The coefficient of variations for 6 replicates of PMA treated cells for β-actin gene. The 45 minute experiment recorded the minimal coefficient of variation (0.08). The 30 minute experiment shows the highest one (0.6).

The results demonstrated that the coefficient of variation typically ranged from 0.4 to 0.6 for the β-actin gene expression and from 0.2 to 0.4 for Egr-1 gene expression. As the gene expression is the ratio of Egr-1 to β-actin (see section 3.5), the coefficient of variation is reported to be greater than 50% of the mean value.

Table 3-7: Normalized data for β-actin gene in PC12 cells, NGF, PMA and PD treated cells.

The table represents normalized data for β-actin gene for 6 replicates of PC12 cells, NGF, PMA and PD treated cells at 30, 45, 60, 90, 180 minute and PD experiments respectively.

		30 minutes	45 minutes	60 minutes	90 minutes	180 minutes	PD
PC12 cells	**Average**	13772	10933	16826	29108	32865	22755
	Standard deviation	8297	4389	10282	15558	9377	9342
	Coefficient of variation	0.6	0.4	0.61	0.53	0.3	0.41
NGF	**Average**	18593	14802	24460	16896	48619	12341
	Standard deviation	6080	1046	7259	4033	4713	3514
	Coefficient of variation	0.32	0.07	0.29	0.23	0.09	0.28
PMA	**Average**	9211	20921	13075	28034	N/A	24852
	Standard deviation	5413	1869	7289	15667	N/A	8811
	Coefficient of variation	0.6	0.08	0.55	0.55	N/A	0.35

Table 3-8: Normalized database for Egr-1 gene in pc12 cells, NGF and PMA, and PD treated cells.

The table represents normalized data for Egr-1gene for 6 replicates of PC12 cells, NGF, PMA and PD treated cells at 30, 45, 60, 90, 180 minute and PD experiments respectively.

		30 minutes	45 minutes	60 minutes	90 minutes	180 minutes	PD
PC12 cells	**Average**	28836	14318	14179	21526	53576	10669
	Standard deviation	4745	2658	6857	10624	3864	1932
	Coefficient of variation	0.16	0.18	0.48	0.49	0.07	0.18
NGF	**Average**	42333	18610	24957	58203	84747	6287
	Standard deviation	8717	1192	9425	8716	4193	1338
	Coefficient of variation	0.2	0.06	0.37	0.14	0.05	0.21
PMA	**Average**	34715	16509	22069	63579	N/A	17443
	Standard deviation	10214	4241	7415	4074	N/A	4065
	Coefficient of variation	0.3	0.25	0.33	0.06	N/A	0.21

3.5 Gene expression analysis with semi quantitative RT-PCR technique

Another goal in this thesis was to study cell-signalling response. Through this study, an inhibitory compound for NGF was tested by analyzing Egr-1 gene expression profile as a functional read out. Semi quantitative RT- PCR was applied to validate the results. Semi quantitative PCR technique is a standard tool in gene expression analysis. The great utility of this technique is a result of it being neither expensive nor difficult to perform. This method is semi quantitative as the amplification of the gene can be quantified to a certain degree. Differences in band intensity will also affect the interpretation of gene expression.

In this study, the experiments were repeated 3 times in 6 replicates. We noticed great variability in gene expression. For the first experiment, for NGF treated cells in the 90 minute incubation period, the fold change was 2.7 fold; (*p value ≤ 0.05), the second experiment was (1.5 fold) and the third experiment was (0.9 fold). P value was ≥ 0.05 for the second and third experiment (Figure 3.10).

For PMA treated cells in the 60 minute incubation period, the fold change for the first experiment was 2.9 fold; (*p value ≤ 0.05), the second experiment was (2.4 fold), and the third experiment was 2.7 fold; (*p value ≤ 0.05) (Figure 3.12). Statistical analysis was performed by using unpaired, two-tailed t-tests by Graph Pad Prism 7.

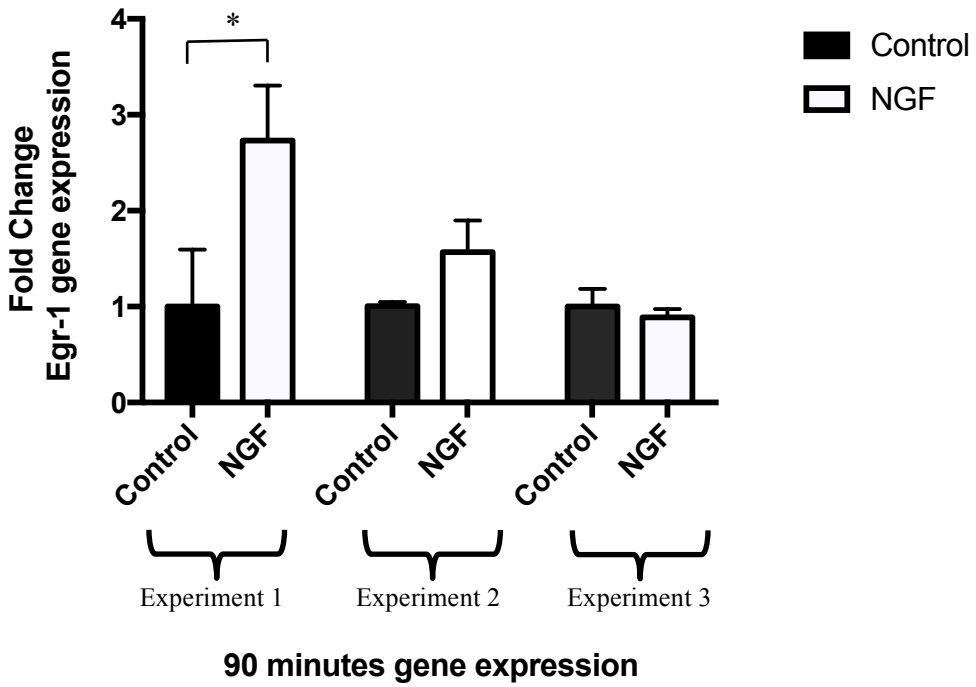


Figure 3.10: Three repeated experiments at 90 minutes for Egr-1 gene expression in NGF treated cells.
Experiment 1 (2.7 fold; *p ≤ 0.05), experiment 2 (1.7 fold), and experiment 3 (0.9 fold). Unpaired two-tailed t-tests determined statistical significance between the control and NGF for experiment 1 by Graph Pad Prism 7. Error bar represent mean ± SEM, with n value =6.

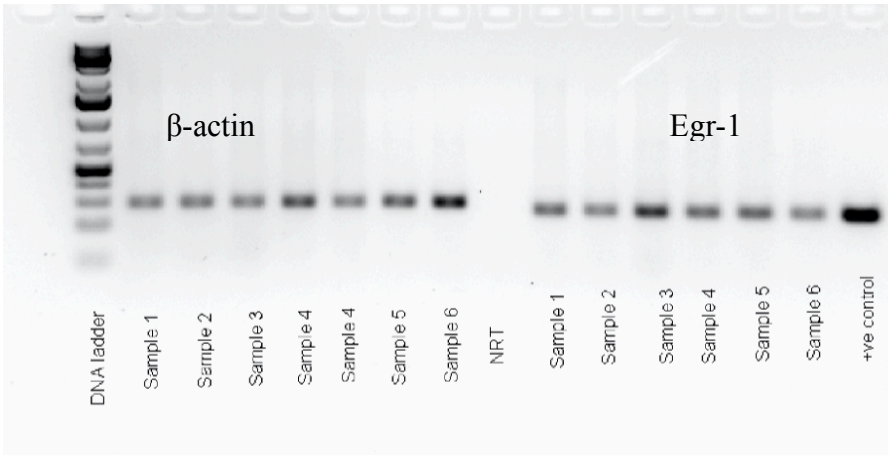


Figure 3.11: PCR bands for Egr-1 and β-actin gene.
PCR bands for Egr-1 and β-actin gene for 6 replicates of NGF treated cells, showing variable density between each sample.

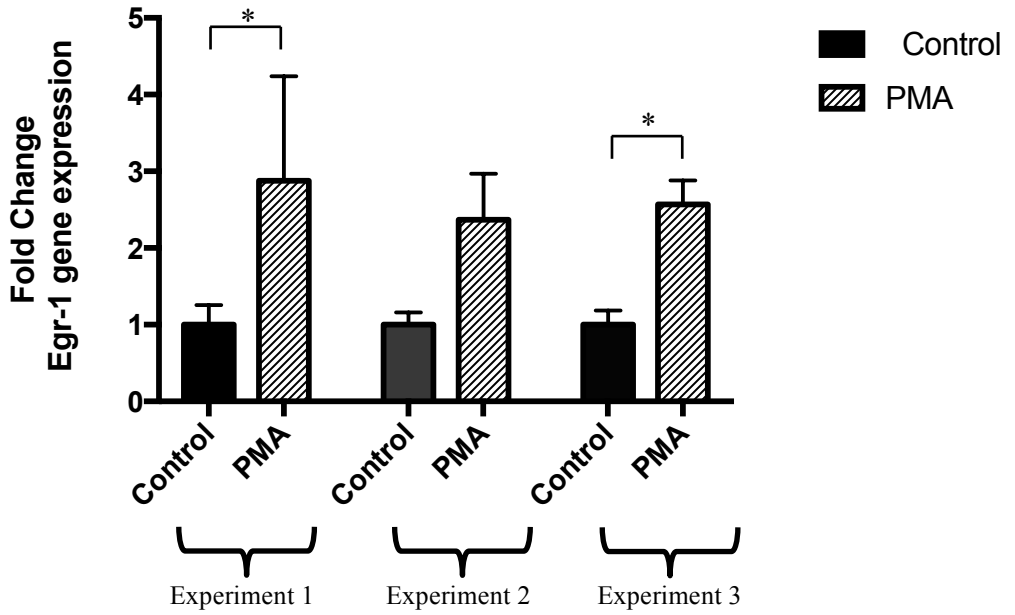


Figure 3.12: Three repeated experiments at 60 minutes for Egr-1 gene expression in PMA treated cells.

Experiment 1 (2.9 fold; *p ≤ 0.05), experiment 2 (2.4 fold), and experiment 3 (2.7 fold; *p value ≤ 0.05). Unpaired, two-tail t-tests determined statistical significance between the control and PMA for experiment 1 and experiment 3 by Graph Pad Prism 7. Error bar represent mean ± SEM, with n value=6.

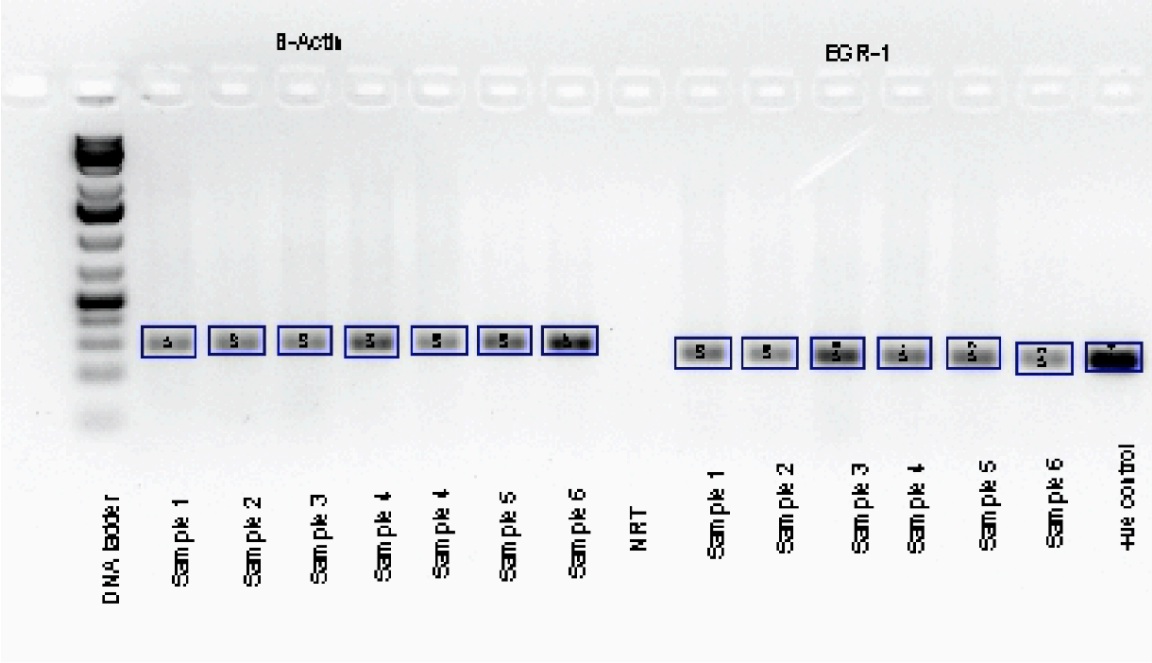


Figure 3.13: The band analysis for gene expression.
Band analysis for gene expression of 6 replicates for NGF treated cells, using densitometry analysed by 1-D Image Software Data Analysis.

3.6 Statistical analysis

Analysis of gene expression under different experimental conditions is important for informative functional analysis. In this study, semi quantitative RT-PCR was used for gene expression analysis. Statistical analyses were conducted using Graph Pad Prism 7. All experiments were repeated 3 times in 6 replicates, and statistical significance was determined using unpaired two-tail t-tests (p value ≤ 0.05).

Having a positive fold change suggests that there is a differential expression. To determine whether this expression is statistically significant, the p value was calculated using unpaired two-tailed t-tests.

Table 3-9: Statistical results for Egr-1 gene expression in NGF treated cells.

Egr-1 gene expression Time	Standard Error	P-Value
30 minutes	0.32	Ns
45 minutes	0.03	Ns
60 minutes	0.31	Ns
90 minutes	0.57	$P \leq 0.05$
180 minutes	0.03	Ns

Chapter 4

Discussion

This chapter discusses the results obtained from this study, and possible future work. The discussion begins by examining the importance of illustrating the time course expression level, and peak expression time for NGF treated cells. Moreover, it presents the advantages and disadvantages of common techniques to analyze gene expression profiles. Finally, it evaluates the application of the semi quantitative RT-PCR technique in using gene expression analysis as a functional read out.

4.1 Identifying peak time expression for Egr-1 in NGF treated cells

Several studies have been carried out to study the cellular response for NGF in PC12 cell line (Teng *et al.*, 2006). As previously discussed, NGF stimulates the expression of the Egr-1 gene in PC12 cells. This study was conducted on the assumption that PC12 cells have a constant response to NGF. Moreover, it is assumed that the cells' ability to express Egr-1 over time is constant.

These studies were not limited only to cellular phenotype, but also to transcriptional profiles (Kendall *et al.*,1994; Yan & Ziff, 1997). The studies of intracellular signalling pathways help determine the mechanisms by which all the neurotrophic factors carry out their biological response.

For many decades, pain management has been a challenging task, and yet many patients still suffer from chronic pain. Small molecule inhibitors have been reported to inhibit NGF such as PD90780, ALE-0540 and Ro 08-275 (Sheffield *el al.,* 2016). However, all

of these molecules have not been tested in primary cell line yet to evaluate their effectiveness before the attempt of clinical trials.

Many studies have shown that variability in NGF levels play a central role in chronic pain conditions such as arthritis and cystitis, some neuronal disorders such as Alzheimer's and multiple sclerosis, in addition to some metabolic disorders such as diabetes (Acosta *et al.*, 2013; Cattaneo & Calissano, 2012; Pittenger *et al*., 2003). We could pave the way for the development of new drugs for pain management and neuronal disorders by exploring the possibility of targeting NGF, and its signalling pathway, as well as by studying the effect of NGF inhibitors on the downstream signalling pathway.

Moreover, studying the effect of the small inhibitory molecules can be of great help in pain management and in minimizing different side effects. Therefore, it is crucial to gain a better understanding of different signalling pathways in response to different stimuli.

In this study, the aim was to determine gene expression of Egr-1 and to investigate the intricate results of signalling in NGF treated cells in comparison with cells treated with NGF together with NGF inhibitors. This study was based on the assumption that Egr-1 gene basal expression is constant in the absence of any treatment.

While signalling response and duration plays a fundamental role in assessing the results, it is crucial to identify the peak expression timing in NGF treated cells to analyze the response to these inhibitory molecules. Peak expression time for NGF treated cells using Egr-1 gene expression, as a functional read out analysis was studied.

Previous studies have analyzed gene expression at shorter time intervals, and have shown peak time expression for immediate early gene expression Egr-1 in NGF treated cells at 60 minutes (D'Onofrio *et al.*, 2011). In this study, the time course of the gene expression profile Egr-1 gene was extended to cover longer time intervals (30, 45, 60, 90, 180 minutes), using semi quantitative RT-PCR, and the results were analyzed and compared. The results indicated that the expression for Egr-1 in NGF treated cells is a 2.7-fold increase at 90 minutes, while it is a 1.1-fold increase at 60 minutes. Our results at 60 minutes are compatible with the outcomes from the previous studies found in literature (D'Onofrio *et al.*, 2011). The present study also revealed that there is no increase in gene expression at 180 minutes.

4.2 Strengths and limitations of semi quantitative technique RT-PCR in gene expression analysis in NGF treated cells

For many decades, eradicating or decreasing the side effects during drug development was considered to be a major challenge for pharmaceutical drug development. In 1961, the discovery of mRNA opened the door for new hopes to discover progression of different diseases, new medications and a better understanding of the physiological mechanisms (Cobb, 2015).

Over the past century, the evolution of molecular biology and advanced research in biochemistry has been used to identify the role of gene expression for different purposes. Gene expression analysis has three basic purposes: exploring different biological mechanisms, gene coding, and disease classification and subdivision. The study of the

disease subdivision is potent particularly in cancer research (Bittner *et al*., 2000; Golub *et al.*, 1999).

RNA is fundamental in studying protein regulation and gene expression profiles of multiple genes. Since the 1990's, new methods have been developed to study the expression levels and sequencing of genetic material. From Gene Arrays to Next Generation sequencing (NGS) and transcriptome analysis, research studies were focused on amplification and quantification of RNA (Fryer *et al*., 2002).

Biological pathway is the most reliable approach with regards to understanding the complexity of genetic networks, and their response to different stimuli (Cohen *et al*., 2000). Moreover, identifying different genes that can be expressed or inhibited can serve as a tool that can guide us to identify novel drug targets, as well as a sensitive diagnostic tools (Butte *et al*., 2000).

While mRNA is a rich source of data, the interpretation of these data to get valuable information remains a big challenge. Until now, the drug screening is a big challenge, as it may be influenced by various factors including cost, time, ease of application, efficacy, and reliability.

Despite having a wide variety of techniques, each one has its advantages and disadvantages. In general, the serial analysis of gene expression (SAGE) provides the best specificity with gene identity, but it is expensive and difficult to apply. With regards to the traditional Northern blot, it is considered to be the gold standard in quantifying RNA expression levels (Bhardwaj *et al.,* 2012). However, this technique is capable of analyzing only one gene at a time. This repetitive analysis needs several blots, and on a large scale,

this method is not only time consuming, but also extremely expensive. It uses extensive washing steps, and multiple film exposures to analyze the data. Furthermore, Northern blotting fails to detect low RNA abundance (Krumlauf, 1991).

Semi quantitative RT-PCR is an outstanding method for gene expression analyses, especially when compared to Northern blot. It is able to analyze hundreds of samples and multiple targets, even those with small amounts of RNA. As a result, RT-PCR is classified among the most sensitive gene expression analysis techniques (Freeman *et al*.,1999).

In this study, semi quantitative RT-PCR was applied to validate Egr-1 expression in NGF treated cells. The goal was to determine whether studying gene expression for NGF signalling is a valid and effective screening strategy to evaluate NGF inhibitors using semi quantitative RT-PCR. Another aim of this study was to test different NGF inhibitory molecules by gene expression analysis using RT-PCR technique.

Although RT-PCR is the most widely used technique for gene expression analysis, the reliability of the data produced from it can be affected by many factors, such as: primer optimization, normalization of gene expression, gel imaging resolution, and variability of the housekeeping gene. In order to standardize the PCR results, a housekeeping gene is usually employed to ensure consistent gene expression regardless of any treatment. In these experiments, a housekeeping gene is defined as a gene that does not vary in expression (i.e. has constant expression) regardless of all status, such as differentiation or manipulations. This study was based on six replicates for each treatment as a way to minimize the variation.

Statistical analyses were conducted for all the experiments to validate the data obtained. The standard error was calculated for different treatments at different time points. There were no significant differences between the treatment groups due to high variability within same groups of samples with different treatments (see Table 3-1,Table 3-2,Table 3-3,Table 3-4,Table 3-5, and Table 3-6).

To pinpoint the cause of variability, the coefficient of variation between the six replicates for each treatment at different timings were calculated. The coefficient of variation is a valuable statistical measure to compare different ranges of variability among various data. It is represented by the ratio of the standard deviation to the value of the mean, the higher the coefficient of variation the higher the dispersion.

The coefficient of variation for the PC12 cells for the β-actin gene (housekeeping gene), for the 30, 45, 60, 90, 180 minute and PD experiments, are 0.6, 0.4, 0.61, 0.53, 0.28, and 0.4 respectively (see Figure 3.4). The coefficient of variation for PC 12 cells in Egr-1 are 0.16, 0.18, 0.5, 0.5, 0.07 and 0.18 in 30, 45, 60, 90, 180 minute and PD experiments respectively (see Figure 3.5).

The coefficient of variation for Egr-1 data in NGF treated cells take the values 0.2, 0.06, 0.4, 0.14, 0.05 and 0.2 in the 30, 45, 60, 90, 180 minute and PD experiments, respectively (see Figure 3.6). The coefficient of variation for the NGF treated cells for the β-actin gene take the values 0.32, 0.32, 0.3, 0.24, 0.09, and 0.28 in the 30, 45, 60, 90, 180 minute and PD experiments, respectively (see Figure 3.7).

The coefficient of variation for PMA treated cells for Egr-1, it takes the values 0.3, 0.26, 0.33, 0.64 and 0.21 in the 0, 45, 60, 90 minute and PD experiments respectively, and for β-actin takes the values 0.5, 0.09, 0.55, 0.55 and 0.4, (see Figure 3.8 and Figure 3.9).

It is very important to consider the efficacy of amplification to ensure that the results collected are accurate and reliable. Moreover, studies have suggested that the most recommended housekeeping gene is the one that shows the lowest coefficient of variation in its expression. While the housekeeping gene role depends on keeping consistent gene expression regardless of any treatment, the coefficient of variation typically varied between 0.4 to 0.6 for the β-actin gene, and for Egr-1 it varied between 0.2 to 0.4.

To date, most studies determining β-actin signals as an internal control for gene expression analysis rely on the assumption that the expression level in the cells remains the same. However, there is evidence that shows that this is an unreliable assumption, with significant alterations in the expression for the β-actin gene in some studies (Waxman & Wurmbach, 2007). Unfortunately, a considerable reference gene for neural differentiation studies have not been proven yet.

Taking into consideration the fact that the housekeeping gene has to be constant, it was noted that the coefficient of variation fell within a wide range for the β-actin gene. Therefore, calculating the ratio of Egr-1 to β-actin to assess the fold change will not provide accurate results.

There are multiple advantages for using RT-PCR for gene expression analysis. It is simple to apply, inexpensive, and produces rapid results. Additionally, it is a sensitive technique that allows small amounts of RNA to be amplified to billions of copies,

(Templeton, 1992). However, it has several limitations (Smith & Osborn, 2009). One of these limitations includes the use of agarose gel for analysis. Using gels may not be able to resolve variability between samples, causing variability in bands intensities, and imprecise results (Figure 3.11). As the quantification method is based on evaluating the band intensity from gel electrophoresis, a difference in the intensity of one band may be interpreted differently leading to imprecise results.

Gene expression data obtained from PCR analysis is a complex procedure and several steps are required. Data collected from RT-PCR is at the end point of the reaction, which may not be precise. Moreover, these data are interpreted by band visualization in the gel cast under UV light. Agarose gel is used for the detection of the end point of PCR reactions (the plateau phase). The plateau phase detects gene expression results once the reaction is stopped. Moreover, the high variability for the coefficient of variation for the housekeeping gene has a significant role in the productivity of imprecise results.

Although the data showed a fold increase of 1.1 at 60 minutes for NGF treated cells, which is consistent with other published reports (D'Onofrio *et al*., 2011), due to the standard error variability, and the high coefficient of variation, these results obtained by semi quantitative PCR technique cannot be considered significant.

In this study, the results showed positive fold change throughout the time course, which suggests that the Egr-1 gene is expressed and that the signalling pathway is activated. Although, the data obtained contains plenty of valuable information, it is usually flawed with variability and consistency issues. Unfortunately, data analysis for gene expression

cannot depend only on fold change results, as there are many biological variables that can be significant and need to be studied.

By comparing the experimental results for NGF treated cells in 90 minutes (Figure 3.10), we noticed variability through each experiment, despite following the protocol very closely. In the first experiment, the fold change was 2.7 fold, the second experiment was 1.7 fold and the third experiment was 0.9 fold. Statistical analysis showed statistical significance for the first experiment and did not show significant difference from non-treated cells for the second and third experiment.

By comparing the experimental results in PMA treated cells at 60 minutes (Figure 3.12), it was also noticed variability in the fold change, experiment (1) 2.9 fold, experiment (2) 2.4 fold, and experiment (3) 2.6 fold. Experiments 1 and 3 are statistically significant. The statistical analysis did not show significant difference compared to non-treated cells for experiment 2.

Previous studies have shown peaked fold change for Egr-1 expression in NGF treated cells at 1.4 fold at 60 minutes (D'Onofrio *et al*., 2011). In this study, the Egr-1 expression increased to 2.7 fold and 1.7 fold at 90 minutes. However, due to the standard error variability, this data was also not significantly different from non-treated cells.

During this work, the effect of PD90780, a potent NGF inhibitor, was studied on cellular expression of Egr-1. This inhibitor has been chosen to test its effect on cellular response, which, in turn, will give a better understanding of its effect as an inhibitor (Spiegel *et al*., 1995). Statistical significance was shown between the control and PMA treated cells only.

Moreover, the results did not show the fold change in NGF treated cells by which PD90780 inhibitory effects can be validated.

In conclusion, and despite the variability of the results we obtained regarding the fold change in Egr-1 gene expression, a trend has been observed showing an increase in the expression levels following treatment with NGF. This is corroborated with previous publications showing similar results.

Whereas semi quantitative RT-PCR method can be used for initial studies, it has to be substantiated with other more accurate methods for gene expression analysis (e.g., real time quantitative PCR). Real time quantitative PCR monitors the DNA amplification during the exponential growth phase of PCR. Therefore, data can be collected at the exponential growth phase, which results in more sensitive and precise results. Although real time quantitative PCR technique allows for a dynamic monitoring of the reaction during the exponential phase, and thus analyzes the relative expression of different genes, real time PCR is an expensive technique to be used for drug screening.

While semi quantitative RT-PCR technique, as used in this study, is a simple and inexpensive method for gene expression analysis, it may not be the most suitable method for examining small differences in gene expression analysis due to variability and inaccuracy in the results.

Chapter 5

Concluding Remarks and Future Work

Gene expression analysis is a powerful technique to study the signalling pathway, and examine different responses. In this study, a semi quantitative technique has been used to validate the results. NGF expression was obtained throughout the time course and peak expression shown at 90 minutes. However, the results indicate a need for a more robust and reproducible method for gene expression analysis in NGF treated cells. It is suggested to apply the same study using real time quantitative PCR technique, as it detects amplification during the reaction precisely in the exponential phase (the optimal point for data analysis). This makes quantitation more accurate and more precise (Rutledge & Stewart, 2008).

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