Assessment of regenerative capability of NGF differentiated PC12 cells after neurite breakage

A DISSERTATION SUBMITTED

BY

PATTERSON CLEMENT C

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF PHILOSOPHY

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY

TRIVANDRUM – 695 011

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DECLARATION

I, **Patterson Clement C**, hereby declare that I had personally carried out the work depicted in the dissertation entitled "**Assessment of regenerative capability of NGF differentiated PC12 cells after neurite breakage"** under the direct supervision of **Dr. Anoop Kumar Thekkuveettil, SIC, Division of Molecular Medicine,** Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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CERTIFICATE

This is to certify that the dissertation entitled "**Assessment of regenerative capability of NGF differentiated PC12 cells after neurite breakage**" submitted by **Patterson Clement C,** in partial fulfillment for the Degree of Master of Philosophy in Biomedical Technology to be awarded by this Institute. The entire work was done by him under my supervision and guidance at **Division of Molecular Medicine**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram-695012.

Thiruvananthapuram

Signature

Date 30th July 2012

Name of Supervisor

The Dissertation

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Submitted

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For

Master of Philosophy

Of

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Acknowledgements

It gives me an immense pleasure to express my heart-felt gratitude and deep felt devotion to my venerable teacher and project supervisor **Dr. Anoop Kumar Thekkuveettil, Scientist in charge, Division of Molecular medicine**, BMT wing, Sree Chitra Tirunal Institute of Medical Science and Technology, Poojappura, Thiruvananthapuram, for his meticulous guidance, constant encouragement, inspiring discussions and valuable suggestions to do this work with confidence.

I express my sincere gratitude to **Dr. K. Radhakrishnan (Director), Dr. A.V George (Registrar), Dr. G.S Bhuvaneshwar (Head, BMT wing) and Dr. S. Sundar Jaya Singh (Deputy Registrar)**, Sree Chitra Tirunal Institute of Medical science and Technology, Poojappura, Thiruvananthapuram for granting me permission to work in this institute and to make use of the facilities that were needed for the work.

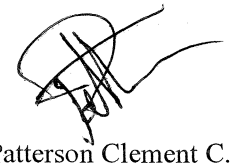
I thank **Dr. Lissy. K. Krishnan (SIC, Thrombosis Research Unit), Co-coordinator M.Phil Biomedical Technology** for organizing and managing the course

My sincere thanks to **Mr. Jose Jacob, Senior Technical assistant (instruments)**, molecular medicine, for his help and support.

I am thankful to my labmates **Sarish Sasidharan, Suraj L.S., Anupriya, Swapna Nandakumar and Bejoy Vijayan** for their comradie during my project. I also extend my thanks to my batch mates **Wulligundam Praveen, Mereena George, Vaishnavi, Shahana, Sree Lakshmi, Anusha, Surya Sree and Reshmi** and all other friend in BMT Wing and SCTIMST for their moral support

I thank all my teachers without whom I would have never been able to reach this far in my academic life.

Finally, I thank my beloved parents for their unfailing and constant support in every aspect throughout my life.



Patterson Clement C.

Abbreviations

°C	Degree Celsius
DMEM F12	Dulbecco's Modified Eagle Medium/ Nutrient Mixture F: 12 Ham
EDTA	Ethylenediamine Tetraacetic acid
g	gram
GFP	Green Fluorescence Protein
IU	International Units
L	Liter
M	Molar
mg	milligram
mL	milliliter
mM	Milimolar
mm	Millimeter
ng	Nanogram
PC12	Pheochromacytoma 12
rcf	Relative centrifugal force
rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulfate
UV	ultra violet rays
µg	Microgram
µl	microliter

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Synopsis

Neurons, which are the functional units of the nervous system, form extensive functional network for storing memory as well as part of learning process. These cells are post-mitotic in nature and hence very sensitive to injury. One way the neurons partially recover from injuries by its plasticity of regenerating neurites. Understanding of the regenerative mechanisms of neurites is necessary for devising strategies for therapies for injury and diseases which causes damage to the nervous system like head injuries, stroke, epilepsy, Alzheimer's, Parkinson's, etc.

Model systems like embryonic stem cells, different cells lines are often used to dissect the mechanisms of differentiation and regeneration. PC 12 cell is one such cell line isolated from pheochromocytoma; it neurites in the presence of nerve growth factor (NGF), and are extensively used for studying neurite growth and developments. The current models of neurite damage in PC12 cells like wound closure, individual neurite transection are not suitable for experiments that require studying damages associated with a network.

This study was aimed at the establishment of a PC12 model system for neuronal network for studying regenerative mechanisms in neurons by physical injury. In this model PC12 cells are differentiated for seven days under NGF and the differentiated PC12 cells are detached, neurites are broken by pipetting and replated in a new plate. The cells are then allowed to regenerate for 7 days.

The regeneration ability was assessed by quantifying morphological parameters, neurite sprouting percentage and neurite length. It was seen that regeneration of PC12 neurites was significantly faster after injury than sprouting of neurites during differentiation both in terms of increase in sprouting percentage and neurite length. This behavior partially resembles regeneration of peripheral neurons after injury.

The complexities of differentiated and regenerated neurites were analyzed using Sholl analysis, and dendritic maxima, critical value and Schoenen's ramification

index were calculated. It was observed that there is an increase in number and concentration of dendrites around the soma (Dendritic maxima), increase in the reach of the neurites around soma (Critical Value) and increase in branches formed (Schoenen's ramification index).

Synaptotagmin I GFP fusion protein was expressed in PC 12 cells by gene transfection to study the effect of synaptotagmin I in regeneration. Contrary to the earlier observation no significant difference in neurite length was observed between experimental and control cells. However there was good significance when the length regenerated neurites were compared with the one of differentiated neurite before neurite injury.

From the experiments performed it was found out that regenerating neurites of PC12 cells have the ability to form complex networks compared to neurites generated during differentiation.

The newly established model for regeneration of neurons in this study has advantages for investigating network of cells for neurite injury. The data can be gathered from a large statistically significant number of cells. Isolation of biomolecules for expression studies and epigenetic studies, assays which measures the levels of cellular components can be conveniently performed with this new model for neuronal injury.

Only the structural changes during regeneration were studied in this part of the work. Assessment of the functional efficiency of the regenerated neurites needs to be studied by verifying the ability to form synapse, electrical excitability and finally its ability to form functional connections.

The Nervous System is divided into

1. Central Nervous System

Central nervous System (CNS) includes brain and the spinal cord. Central nervous system has tight junctions around the capillaries known as the blood brain barrier, which allows small molecules and hormones to pass through it while restricting large molecules and majority of proteins. CNS contains supporting cells known as glial cells, which help in providing nutrients to the neuronal cells. The supporting cells in CNS are microglia, astrocytes and oligodentrocytes. Astrocytes functions as connective tissue, or skeletal tissue since it packages other CNS components, it surrounds them to protect the other cells from exitotoxic neurotransmitters like glutamate detoxifying it to glutamine. On Injury the astrocytes proliferate and accumulate glycogen and fill the injured portions. This injured part is known as glial scar. Microglia, the most mobile cell in the central nervous system takes part in inflammatory response, it also phagocytosize the debris formed during injury and wear and tear. Oligodendrocyte myleinates the neurons

2. Peripheral Nervous system

The Peripheral Nervous system (PNS) comprises of all other ganglia and nerves that is outside the central nervous system extended throughout the body. Unlike the central nervous system neurons PNS neurons do not have blood brain barrier. The supporting cells which surround the peripheral neurons are called Schwann cells that form the myelin sheath. Unlike an oligodendrocyte, a Schwann cell can only myelinate an axon or a part of it.(Siegel 2006)

Injury to the nervous system

CNS disorders are some of the gravest diseases that affect human beings. In addition to mortality, nervous system disorders also cause very serious disabilities, which drastically reduce the quality of life of an individual. Head injury and spinal injury are among the leading causes of death in human population. It is estimated that nearly 1.5 to 2 million persons are injured and 1 million

succumb to death every year in India (Gururaj 2002). Apart from death it causes severe impairments like memory disorders, locomotory disorders, emotional and intellectual disorders.

Since neurons are post mitotic cells, regenerative capacity of these cells in adult CNS are very limited. Diseases like Alzheimer's, Parkinson's, Huntington and Prion diseases contribute to extensive loss of neurons (Bertoli-Avella *et al* 2004). Metabolic disorders like phenyl ketonuria (Surtees *et al* 2000) diabetic ketoacidosis (Hoffman *et al* 2011) cause damage to the nervous system when not properly managed. Chronic starvation,(Auer 2004) intake of drugs environmental toxicants, (Rodier 1995) radiation, (Celikozlu *et al* 2012) assault, and pathogenic infections are some of the other causes for loss of neurons. Since neurons have extensively network and or interlinked, death of a single neuron causes information losses due to loss of connections. However neuronal plasticity helps in overcoming loss of neurons to an extent. Neuronal stem cells, which are present in low numbers in CNS, can proliferate and differentiate to replace lost neurons. (Siegel 2006)

Understanding the physiological and molecular mechanism of neurite plasticity and regeneration will help to devise strategies to accelerate the process pharmacologically so that minimum time is lost during recovery, increasing the chances of survival and quality of life.

The regenerative potential of a neuron can be studied by measuring the structural changes of axons after injury and its ability to form complex structures, to restore the original connections between the adjacent neurons.(Sholl 1953)

Neuronal injury can be overcome in two ways

1. Neurite Regeneration
2. Compensatory plasticity

Neurite regeneration is the regrowth of extensions from the damaged end after the neurites are severed. In compensatory plasticity, new neurites sprouts from

the soma and grow by working through or working around the injured tissue. Neonatal Brain shows extensive levels of compensatory plasticity. (Siegel 2006)

Regeneration in Peripheral Nervous System

In PNS after neurite injury, Schwann cell retracts myelin sheath, exposing the injured part of the neuron. Inflammatory cytokines such as TNF alpha and interleukin 1 alpha recruit macrophages to the injury site, and this leads to the removal of debris.

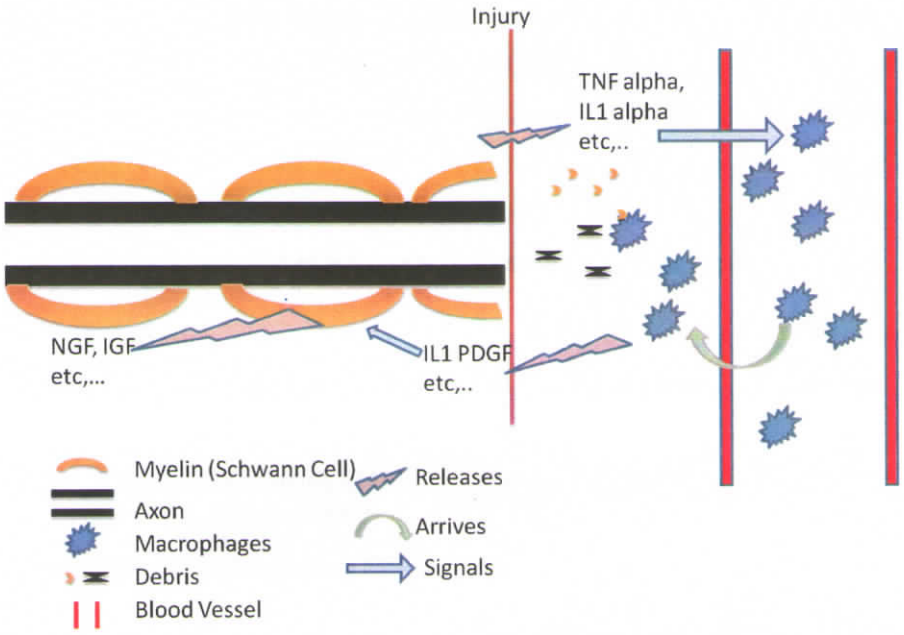


Figure 2. Injury Response in Peripheral Nervous system, upon injury the inflamatory cytokines such as TNF alpha, IL 1 alpha secretes macrophages which then act on injury debris and also secrets cytokines such as IL1, PDGF etc... This signals schwann cells to produce NGF, IGF etc...

Macrophages also secrete cytokines such as interleukin 1 and platelet derived growth factors, which signals the Schwann cells to dedifferentiate and proliferate at sites distal to the axonal injury (see Figure 2). Activated Schwann cells produce growth factors like nerve growth factor (NGF), insulin like growth factors I (IGF -1), and ciliary neurotrophic factors that promote axonal re-growth and helps in neuronal survival.(Siegel 2006)

General Signaling Pathways involved in Neurite Regeneration.

1. Injury of neurons causes the local activation of extracellular receptor kinase (ERK) and Janus kinases (JNK) (Cavalli *et al* 2005, Lindwall *et al* 2005). It has been shown that mitogen activated protein kinase (MAPK/ERK) and MAP kinase 2 (MEKK2) regulates the regrowth of neurites by balancing ERK1/2 and JNK2 pathways after injury (Waetzig *et al* 2005). The activation of JNK and ERK receptor pathways leads to their interaction with dyenin and dynactin retrograde motors, which transports cargo to the site of injury in axons (Cavalli 2005) (see Figure 3).

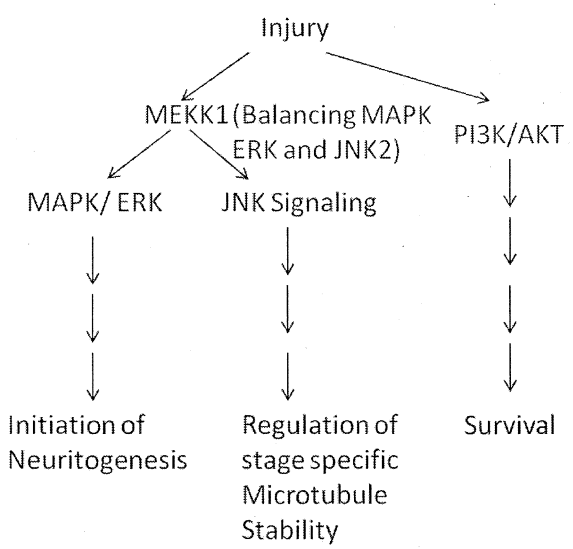


Figure 3, Outline of Activated Pathways Necessary for regeneration of Neurons after injury, on injury the MEKK1 is activated. It can further activate both JNK2 which regulates microtubule stability and MAPK/ERK which initiates pathway neuritogenesis, JNK2 supports differentiation by regulating microtubule stability at different stages of neurite outgrowth

2. JNK Pathway regulates elongation by regulating stage specific microtubule stability.(Hirai *et al* 2011)

3. Protein Kinase B Which is called AKT activation is needed for activation of cell survival to prevent the cell to undergo apoptosis(Read *et al* 2009).

Growth factors, NGF, BNGF, BDNG, NT3, NT4/5 are shown to enhance regeneration (Markus *et al* 2002)

Physiological Changes after injury

Plasmalemmal repair is required the formation regeneration of neurites after injury. This process takes place through vesicle mediated fusion (Detrait *et al* 2000). Axotomy also causes the membrane depolarization causing further calcium influx. The first step in the sealing of plasma membrane is the collapse of membrane thus reducing its diameter (see Figure 4 and 5). The axonal cytoskeleton has a central microtubule core surrounded by actin-spectrin layers. The collapse of membrane takes place because depolimerization of actin and microtubules and proteolytic cleavage of spectrin by calpain. Influx of calcium ions facilitates fusion of synaptic vesicles with the membrane and and forms a multivesicular sealing patch, thus preventing loss of cellular substances though the injured plasmalemma (Detrait 2000). The membrane structure that repairs plasmalemma includes lysosomes, endocytotic processes, myelin deaminations, and vesicles similar to synaptic vesicles.(Bradke *et al* 2012)

(EPAC) expanded as Exchange proteins directly activated by cAMP(Cazorla *et al* 2009), cytosolic oxidation and protein kinase A (PKA) pathways are involved in the sealing of plasmalemma (Spaeth *et al* 2011). The cytoskeleton reorganizes itself to form a new growth cone in the form of two dimensional actin rich lammelipodium

This growth cone development occurs because of differential polymerization of microtubules and actin. Microtubules repolymerize under low calcium concentration but actin fails to polymerize, giving it a characteristic retraction bulb. Micrutubule stabilization is necessary for growth cone formation. The destabilization of microtubules leads to the retraction of the active growth cone (Bradke 2012).

Once growth cone is formed, pro-regenerative proteins like Reg2, GAP43 are localized to the site of injury. GAP 43 is highly expressed protein in developing and regenerating axons and is involved in interaction between the growth cone and its microenvironment. Neurons in substantia nigra, hippocampus and olfactory bulb found to express GAP43 throughout its life (Siegel 2006). Reg – Regenerating protein, has many isoforms among which Reg2 is found mainly in mice and hamster; It has anti apoptotic effect and it is a schwann cell mitogen during motor neuron regeneration.(Parikh *et al* 2012)

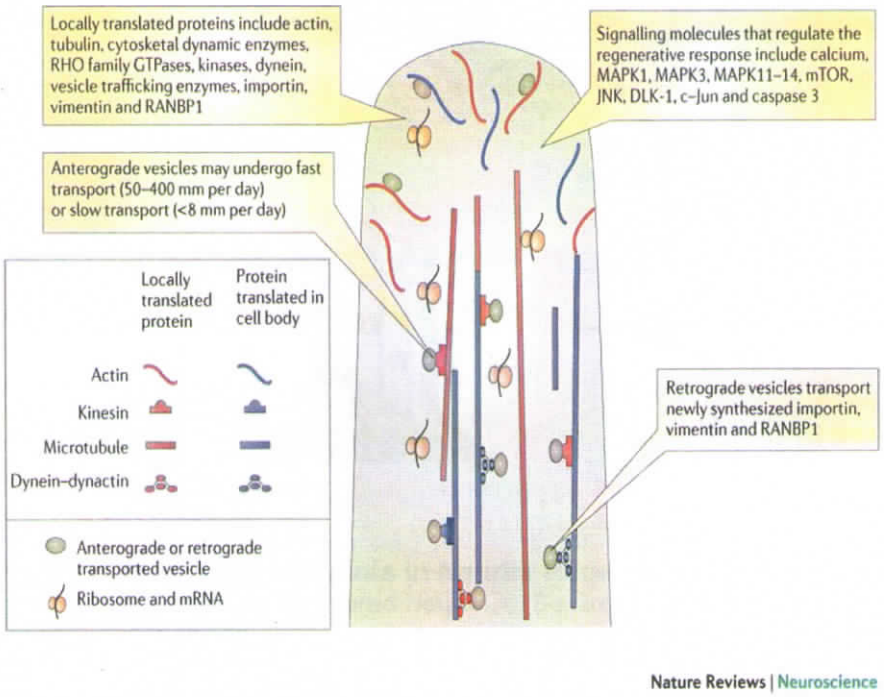


Figure 4 Molecular events in growth cone regeneration.(Bradke 2012)

Cell adhesion molecules such as N-cadherin, L1, and neuronal cell adhesion molecule (N-CAM) promotes the interaction between Schwann cells and axon and enhances myelination of the growing axon.(Siegel 2006)

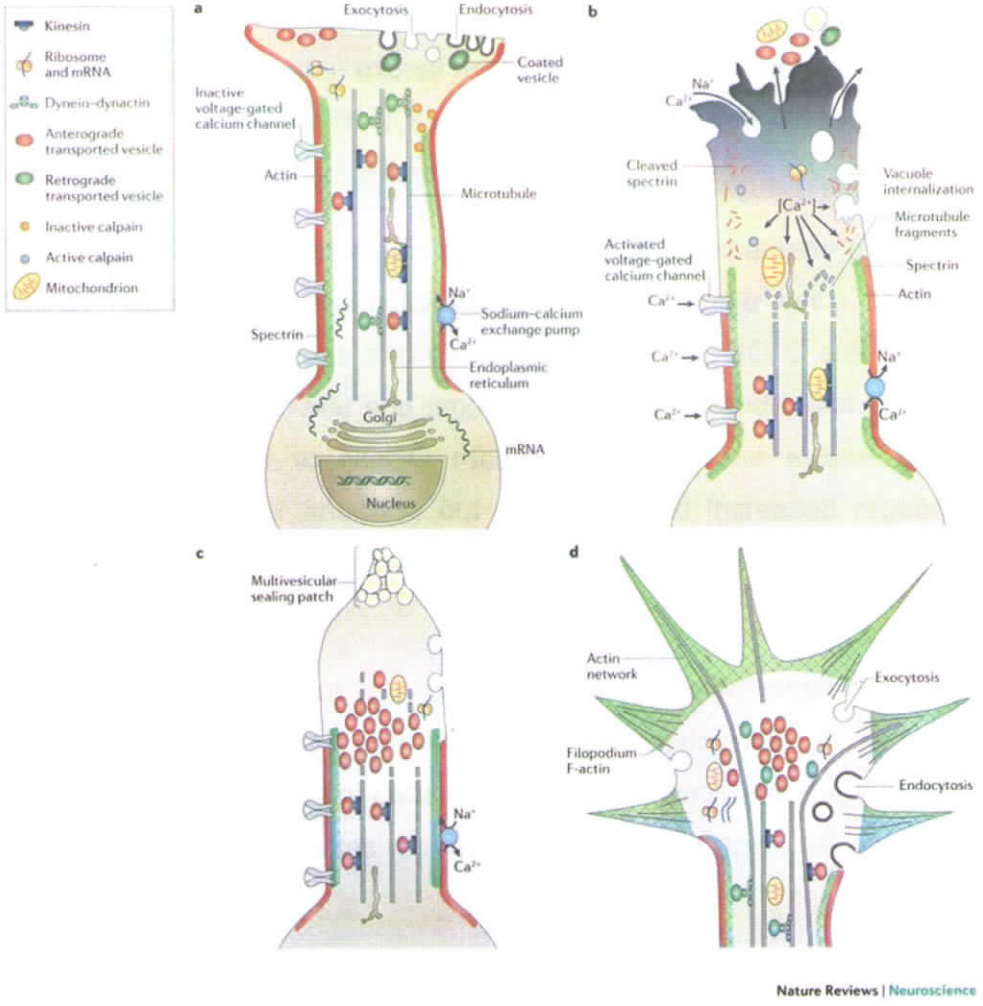


Figure 5. Over View of Events in Neurite Regeneration: a. components that take part in Regeneration, b. An Injured Neuron, c. Sealing Patch formation, d. A growth cone formed(Bradke 2012)

Regeneration in central Nervous System

CNS injuries are harder to regenerate. The principal difference in the regeneration of injured neurons in CNS versus PNS is that the macrophages that are acting in these injuries are fewer in number because of the blood brain barrier. Microglia are the first cells to reach the site of injury. Clearing of debris at the injury site takes much longer in CNS (see figure 6).

Inhibitory factors involved in axonal regeneration pathway include myelin proteins such as NOGO A, NOGO B, NOGO C, Mylein Associated Glycoproteins (MAG), and oligodendrocyte myelin glycoprotein.

NOGO A is mainly expressed in dorsal ganglion, sympathetic ganglion, motor neurons, hippocampal pyramidal is, cerebellar purkinje cells, NOGO B are expressed in adult neurons and oligodendrocytes. NOGO C are found outside CNS in cultured endothelial and smooth muscle cells and intact blood vessels. NOGO proteins bind to NOGO receptors (NgRs) and induce downstream pathways. Inhibiting or knocking out NgRs showed increased regeneration in neurons. Moreover, an increase in compensatory plasticity has been shown in these models.(Siegel 2006)

The action of NAGO can be inhibited with elevated cAMP concentrations conversely; NOGO A has been shown to be responsible for lesion induced neuronal plasticity. There are certain reports that neuronal NOGO A is involved in the regeneration of neurites as opposed to the actions of oligodendrocyte NOGO.(Siegel 2006)

MAG is another factor that is known to inhibit neurite regeneration. It belongs to Immunoglobulin super family.(Siegel 2006) It shares the NOGO receptor NgR along with oligodendrocyte myelin glycoprotein (OMpg). While NOGO A shows significant inhibition of regeneration in spinal cord axons and MAG and OMgp shows not very significant inhibition, their combined is significantly lesser than that of NOGO alone, indicating a synergic role of these three proteins in inhibition.(Cafferty *et al* 2010)

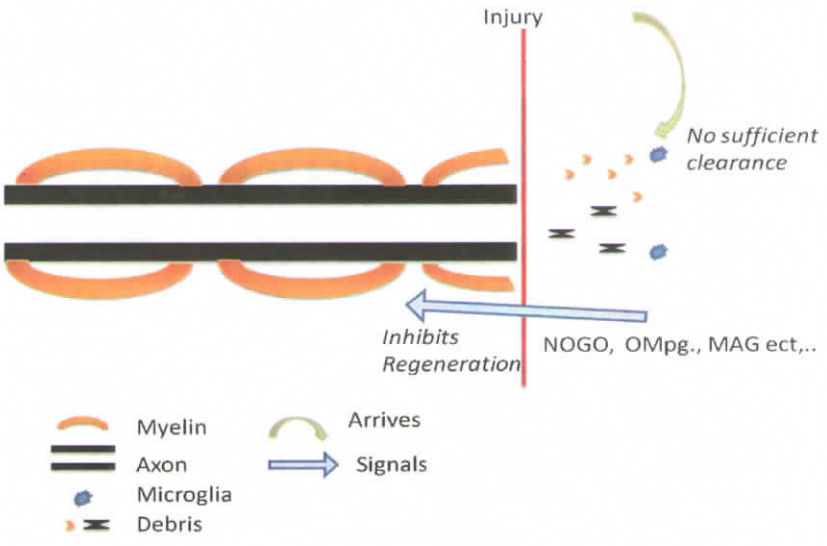


Figure 6. Injury Response in Central nervous System, only microglia clears debris in CNS injury. Hence unclear myelin debris NOGO-A , MAG, OMgp are not cleared inhibiting regeneration.

Astrocytes and meningial cells form a glial scar at the site of injury. Glial scars often inhibit the genesis of neurites. Extra cellular matrix (ECM) proteins such as chondriatin sulphate proteoglycans are mainly expressed in the glial scar. Common chondriatin sulphate proteoglycans are NG2, neurocan, versican, aggrecan and phosphorican. (Siegel 2006)

Pathways in PC12 Differentiation

PC12 cells are cells derived from pheochromocytoma, a rare type of adrenal gland cancer, which responds to a variety of growth factors by sprouting neurites. This property established PC12 as a model system to study structural, functional and molecular studies.(Vaudry *et al* 2002) PC12 cells have ability to form functional synaptic connection with neurons (Zhou *et al* 2006). They have even been shown to form functional connection inside brain(Freed *et al* 1986) and spinal cord (Zompa *et al* 1993).

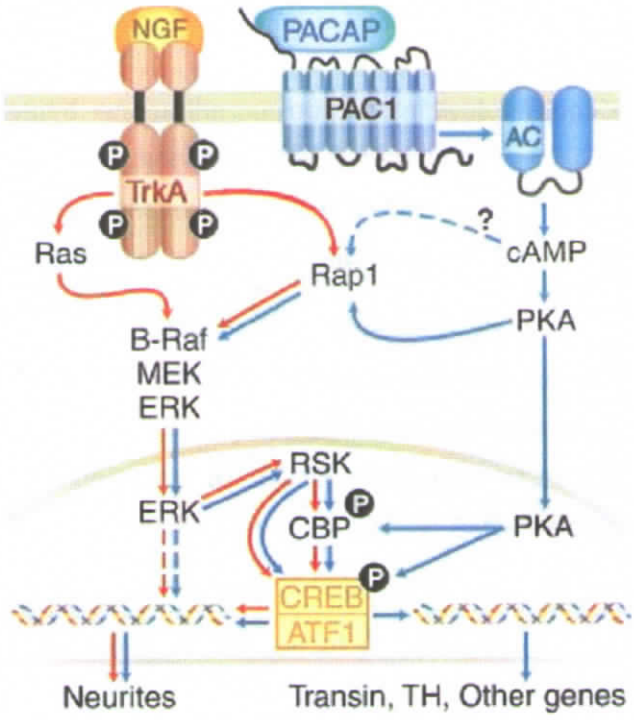


Figure 7. NGF response in PC12 cells (Vaudry 2002) Trk A is activated by NGF, Which activates Ras and Rap1, both Ras 1 and Rap 1 activate B-Raf, B-Raf activates MEK, MEK activates ERK, ERK enters nucleus and activates RSK, RSK activates CBP and CREB, CREB initiate transcription for differentiating genes. CBP coactivates CREB by binding with it. PACAP activates PAC1(GPCR) which recruits Adenylate Cylase (AC), AC produce cAMP, cAMP activates PKA, PKA activates RAP1 and ERK, leading to activation CREB.

There are host of growth factors that differentiates PC12 cells into neurons, including nerve growth gactor (NGF) transforming growth factor alpha (TGF Alpha), fibroblast growth factor (FGF), Interleukin 6 (IL6) (Nakafuku *et al* 1993), cerebrospinal fluid (Nabiuni *et al* 2012),. The pathway leading to sprouting of neurites in PC12 cells is initiated by the activation of cAMP Response Element Binding (CREB) Protein.

The main growth factor that has been studied to differentiate neurons is NGF. On activation with NGF the Trk A receptor either activates Ras Proximate -1(Rap1) or Ras. Both Ras and Rap1 inturn activate B Raf, Subsequently MAPK/ERK Kinase (MEK) and ERK are activated (Vaudry 2002). The ERK once it is

activated, enters the nucleus and activates Ribosomal S6 Protein Kinase (RSK) Which Phosphorylates CREB binding protein (CBP)(Xing *et al* 1996) which in turn is responsible for CREB to bind with the CRE. CREB once bound to CRE initiates transcription of a host of genes that are responsible for differentiation. (Vaudry 2002)

Trk A also recruits PI3K, which activates c-Jun Janus NH-terminal Kinase(JNK2) inturn activate c-Jun pathway which activates apoptosis in differentiated cells or differentiation in plain PC12 cells(Leppa *et al* 2001). Phosphoinositol 3 kinase (PI3K) survival signal is by activating Akt, which prevent the cell from apoptosis (Klesse *et al* 1999).

Differentiation of PC12 can also induced by cAMP signaling, mainly stimulated by the action of Pituitary adenylate cyclase activating peptide (PACAP). PACAP's exerts action through GPCR known as PAC1. On induction PC1 activates adenylate cyclase there by increasing cAMP levels, activating protein kinase A which inturn activates ERK. PACAP can also induce difference through Ras pathway through RAP1 which again results in the activation of ERK, ultimately leading to activation of CREB binding to CRE inducing differentiation.(Ravni *et al* 2006, Vaudry 2002)

Nerve Growth Factor in Adult Nervous system

NGF is a 14Kda protein, which interacts with TrK A with high affinity. It can stimulate outgrowth in sympathetic ganglia and is required for sympathetic neuron survival. NGF is present at a low concentration in CNS. Their presence is largely restricted to hippocampus and neocortex regions. NGF is also known to be secreted in response to pain.(Siegel 2006)

NGF belongs to neurotrophin family of growth factors. The other members include brain derived nerve growth factor (BDNF), neurotrophin 3 (NT3), neurotrophin 4/5 (NT4/5) and neurotrophin 6 (NT6). Usually the neurotrophins respond to specific receptors: Trk A for NGF, Trk B for BDNF, Trk C for NT3 Neurotrophins also respond to another receptor known as P75, which has lower

affinity towards neurotrophins than TrkA family. P75 can also bind to a precursor form of NGF and other immature pro-forms of neurotrophins with high affinity(Siegel 2006)

Delivery of neurotrophic factors to the nervous system as a pharmacological intervention for promoting regeneration during after neuronal injury has been considered (Thorne *et al* 2001). However, assessment of the regenerative potential of these factors is necessary to devise an optimal therapeutic strategy that can induce regeneration with minimal risks and side effects.

Hypodissertation

Neurons, being post-mitotic cells, most of them develop neuronal network early in the human life. Loss of neurons is mainly overcome by neurite regeneration and compensation. When there is neuronal injury and partial loss of neuritic connections, the injured neurons as well as the, adjacent neurons develop neuritic sprouting to partially repair the loss of network. This neuronal plasticity is critical for working of the nervous system as well as for the formation of complex connections accessing different type of neurons. Knowing the regenerative capability of individual neurons is essential in understanding the pathways involved in neuronal regeneration and its capacity to develop intricate network of functional connections. PC12 cells, having the ability to transform into a neuron like cell on treatment with the NGF, being used as a model system to study neuronal functions. This study tests the working hypothesis, whether neurotic processes developed after injury has any structural variations, especially in neurite length and number. The study also attempted to find the functional role of synaptotagmin, a critical function protein in neurons, in axonal growth.

Objectives

- To assess the differentiating potential of PC12 cells
- To compare the sprouting ability and changes in neurite length before and after breakage when treated with nerve growth factor.
- To compare the complexity of PC12 cells before and after neurite breakage on treatment with nerve growth factor using Sholl analysis.
- To assess the effect of synaptotagmin I expression on the sprouting ability and changes in the neurite length before and after the breakage of neurites on treatment with nerve growth factor.

Chapter II

Materials and Methods

DMEM F12 was purchased from Himedia Laboratories, India, horse serum and fetal bovine serums were purchased from Lonza, Germany. Poly L lysine hydrobromide, Penicillin G and nerve growth factor – 7s were purchased from Sigma Aldrich, Streptomycin Sulfate was purchased from GIBCO, Germany. Sodium Bi Carbonate was purchased from Sisco Research Laboratories, India. Nucleofector device was purchased from Amaxa, Lonza, Germany. All Optical Images were captured using Olympus IX51 microscope operated by the software application NIS Elements - Advanced Research supplied by NIKON. Polystyrene petriplates and T25 flasks were from Nunclon, Denmark.

Reagents and Buffers

DMEM F12 complete media

For 50 mL

DMEM: F12 1:1 Solution	42.2 mL
Fetal bovine serum	5.0 mL
Horse serum	2.5 mL
Penicillin solution	50 µL
Streptomycin Solution	50 µL

DMEM F12 differentiating media

For 50 mL

DMEM F12 1:1 solution	49.4 mL
Fetal bovine serum	0.5 mL
Penicillin solution	50 µL
Streptomycin Solution	50 µL

DMEM F12 1:1 mixture was made by dissolving 15.7 g in 1L of sterile distilled water. Penicillin stock solution (1lakh IU/mL) was made by dissolving 30.165 mg of penicillin in 500 µl of sterile distilled water and stored at 4°C streptomycin stock solution(100mg/ml) was prepared by dissolving 50 mg of streptomycin in 500 µl of Sterile distilled water and stored at 4°C.

Nerve Growth Factor - 7s stock

Nerve Growth Factor – 7s 1mg

DMEM F12 Differentiating media 1ml

Stored at -20°C in 10 µl aliquots to avoid repeated freeze thawing

100x poly L Lysine Stock Solution

Poly L Lysine hydrobromide	10 mg
Sterile Distilled water	10 ml

Stored at -20°C

10X TAE

For 1 Litre

Tris Base	48.4g
Glacial Acetic Acid	10.9mL
0.5 M EDTA	2 mL

Dissolved in 1 Liter of Sterile distilled water

6X gel loading dye

0.25% bromophenol Blue

0.25% Xylene cyanol FF

30% Glycerol in H_2O

Alkaline Lysis Solution I (GTE)

50 mM glucose

25mM Tris HCl

10mM EDTA

Alkaline lysis Solution II

0.2 N NaOH

1%(W/V) SDS

Alkaline Solution III

5M potassium Acetate	60 mL
Glacial Acetic Acid	11.5 mL
H_2O	28.5 mL

STE Buffer

10mM Tris-Cl (pH 7.8)

0.1M NaCl

1mM EDTA

Poly L Lysine Coating

One ml of 0.1mg/ml solution of Poly L Lysine hydro bromide solution was added to each 35mm polystyrene Petri dishes. The plates were then incubated for 5 minutes in a humidified atmosphere at 5% CO_2 concentration and 37° C in CO_2 incubator (Forma Direct heat, Thermo Scientific, USA). The solution was removed and the plates were allowed to dry at room temperature at least for 2 hours before seeding the cells.

Cell Culture Maintenance

PC12 cells were maintained in DMEM F12 Media supplemented with 10% Horse Serum and 5% Fetal Bovine Serum 100 IU/ml Penicillin and 100 µg/ml streptomycin, pH 7.4, under humidified atmosphere at 5% CO_2 concentration and 37° C in CO_2 incubator. Media was replenished in every third day.

Passaging of Cells

On attainment of 70 to 80% confluence, the cells were trypsinised by the following protocol. Media was removed from the T25 flask; the cells were washed with Phosphate Buffered Saline (PBS). PBS was removed and 800 µL Trypsin/EDTA mix was added. The flask was incubated at 37° C for 5 minutes. 800 µL of DMEM F12 complete media was added. The trypsinised cells were collected in a microfuge tube and centrifuged at 700 rcf for 5 minutes at room temperature. The supernatant was discarded and the pellet was resuspended in new DMEM F12 complete media and plated into new PLL coated T25 flask at a density of about $5x10^5$ cells per flask.

Cell Counting

10 µL of trypsinized cells was added to each side of the improved Newbauer counting chamber and number of cells in 5 large squares were counted manually in each side under the microscope. The total number of cells counted was multiplied by 10^3 to get the total number of cells per mL of cell suspension

Differentiating PC12 Cells

PC12 cells were seeded at a density of 60,000 cells per 35 mm Poly L Lysine coated polystyrene petriplate in DMEM F12 medium supplemented with 10% Horse Serum and 5% Fetal Bovine Serum, 100 IU/ml Penicillin and 100µg/ml streptomycin pH 7.4

After the cells were attached, new medium: DMEM F12 supplemented with 1% fetal bovine serum, 200ng/ml nerve growth factor-7s and 50mM potassium chloride, pH 7.4 was added.

Media was replenished every alternate day to ensure constant supply of the nerve growth factor and nutrients and the cells were allowed to differentiate for seven days. 5 images were taken with 10x objective each day and used for further analysis.

Neurite Breakage in PC12 cells

Differentiated PC12 cells were detached from the plates after seven days of NGF differentiation by pipetting forcefully and neurites are broken by triturating up and down using a micropipette. The media along with the cells were collected in a microfuge tube and spun down at 700g for 5 minutes in a swing bucket rotor centrifuge at room temperature. The pellet containing the cells were resuspended in one mL of DMEM F12 medium supplemented with 1% fetal bovine serum and 200ng/ml NGF and 50mM KCl and plated to a new poly L lysine coated 35 poly styrene petriplates in DMEM F12 medium containing 1% Fetal Bovine Serum, 200ng/ml Nerve growth factor and 50mM Potassium chloride. New media was added after the cells are attached to the surface of the plate. Media was

replenished every alternate day to maintain constant supply of nutrients and nerve growth factor. Cells were observed for 7 days for neurite regeneration.

Preparation of competent cells

Calcium Chloride method was used to prepare competent DH5 alpha bacteria. On day one DH5 alpha streaked in Luria Bertani (LB) agar plate (without Ampicilin) using an inoculation loop. The culture was allowed to grow overnight by incubating in bacterial incubator at 37° C. On day 2, 5 ml of LB Broth was inoculated with a single colony of DH5 alpha from the agar plate and incubated at at 37°C in a shaker incubator 300 rpm. Optical Density (OD) was monitored at 600 nm every 30 minutes until the value reaches 0.5.

The cells were then pelleted by centrifuging at 2500g for 5 minutes at 4°C. The supernatant was decanted and the pellet was resuspended in 5 ml of ice cold 200mM $CaCl_2$ till the suspension appears silky. The suspension was kept in ice for 10 minutes and 15 ml of 80mM $CaCl_2$ was added and kept in 4°C till use (This competent cells were used only for 7 days; fresh competent cells were prepared after 7 days) (Hindley 1983).

Transformation and plasmid isolation

The 50µl of competent cells were taken and around 1 microgram of plasmid DNA pEGFP C1 or pEGFP C1 Synaptotagmin I was added to it. The mixture was kept in ice for 15 minutes after which the tubes were transferred to 42°C (Thermomixure Comfort, Eppendorf) for 90 Seconds. The tubes were immediately chilled on Ice for 2 minutes. Then 950µl LB broth was added to the mixture. The tubes were kept at 37°C at 300 rpm for 60 minutes. 100 µl of the culture was spread in an LB agar medium plate (containing 50 µg/ml Ampicillin).

50 µl of competent cells were taken and treated in the same way without the addition of plasmid and used as control. These cells were spread in two different LB agar medium plates, one with 50µg/mL Ampicillin as negative control to rule out the possibility of contamination and one without ampicillin as positive control

to check the viability of the cells. Growths of bacterial colonies were recorded after 16-18 hours.

Alkaline Lysis Mini Preparation

Mini Preparation of pEGFP C1 and pEGFP C1 Synaptotagmin I from was performed according to the alkaline lysis method (Sambrook *et al* 2001)

1.5 ml of overnight culture of DH5 alpha cells transformed with pEGFP C1 and pEGFP C1 Synaptotagmin I, was transferred to a microcentrifuge tube. The tube was then centrifuged at 10,000 rpm for 5 minutes at 4°C to pellet bacterial cells. The supernatant was discarded and the pellet was resuspended 375µl of ice cold STE Buffer by vortexing. The tube was then centrifuged at 10,000 rpm for 5 minutes at 4°C. The supernatant was removed and the pellet was resuspended in 100 µl GTE (Alkaline Lysis solution I) by vortexing. The tube was then kept in room temperature for 5 minutes. After which 150 µl of freshly prepared alkaline solution II was added. The tubes were mixed by gently inverting and kept in ice for 5 minutes. Alkaline Solution III was added to the mixture and mixed by gently inverting the tube and kept in ice for 5 minutes and then centrifuged at 10,000 rpm for 5minutes at 4°C. Supernatant was transferred to a fresh microfuge tube and equal volume of tris-saturated phenol and chloroform isoamyl alcohol mixture was added, vortexed and then centrifuged at 10,000 rpm for 5 minutes at 4°C. Transfer the aqueous phase into a new microfuge tube and equal volume of chloroform iso- amyl alcohol was added, mixed by vortexing and centrifuged at 10,000 rpm for 5 minutes at 4°C. The aqueous layer was collected and equal volume of ice cold iso-propanol was added and kept at -20°C for 20 minutes. The mixture was then centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was removed and the pellet was washed with 70% ethanol and then centrifuged at 10,000 rpm for 5 minutes at 4°C. Supernatant was removed and the pellet was partially dried by keeping the tube open for 15 minutes. The pellet was then dissolved in 20µl sterile distilled water. The Presence of plasmid was confirmed by agarose gel electrophoresis for 1 hour at 70 Volts.

Restriction Digestion

pEGFP C1 and pEGFP C1 Synaptotagmin I plasmid were digested with PvuI restriction enzyme as follows:

Plasmid 3 µl

PvuI 0.5 µl (10 units)

10X PvuI buffer 2 µl

Water 17.5 µl

The reaction mixture was incubated for 3 hours. 10µl of digested sample was run on agarose gel electrophoresis at 80V.

Agarose Gel Electrophoresis

0.7 % agarose gel was prepared by dissolving 350 mg of agarose in 50 mL of in TAE buffer by heating in a microwave oven. 2 µl of 10 mg/mL Ethidium Bromide (EtBr) was added to agarose solution when the solution reaches the ear bearing temperature. Agarose solution was poured into gel casting tray, the comb was inserted and allowed to solidify.

The DNA samples were loaded with the gel loading dye and the gel was run at 80V in TAE buffer. DNA bands in the gel were visualized under UV light and documented using Uvipro platinum gel documentation system, Uvitec, UK.

Transfection

Transfection of PC12 cells were performed using Amaxa Nucleofector ii device, Lonza, and Amaxa nucleofector kit V as per the product protocol recommended by the manufacturer. Approximately 4 µg of DNA was used per transfection.

Cell counting and neurite Length Measurement

Differentiated cells are defined by the number of undifferentiated cells having length of the neurites at least half the diameter of the soma. The cells were counted using ImageJ plug-in Cell Counter (Rasband 1997-2007)

The length of the neurites was measured by tracing the each neurite using NIH Image J free hand selection tool, and measuring the length of the tracing using ROI manager. (Rasband 1997-2007) and P-Value was calculated by unpaired T-test (Wilson *et al* 1942).

Sholl Analysis

Tracing of neurites and sholl analysis was performed Fiji plugin Simple neurite Tracer (Longair *et al* 2011). Standard axis setting (Linear Method) (Sholl 1953) was used and Intersections were not normalized according to area enclosed by the circle. One representative cell was selected from each day for sholl analysis. One representative neuron was taken per day for analysis.

Parameters like dendritic maxima (dm), critical value (Cv) and maxmimum radius were obtained. Schoenen's ramification index (SRI) was calculated by the following formula.

SRI = dm/Cv

SRI – Schoenen's Ramification Index

Dm – Dendrite Maxima

N – Number primary neurites from soma.(Schoenen 1982)

Chapter III

Results and Discussion

PC12 cells were grown in DMEM F12 complete medium supplemented with 10% fetal bovine serum and 5 % horse serum, the cells were then trypsinized and replated and differentiated using DMEM F12 differentiating medium containing 1%fetal bovine serum with 200ng/ml NGF. Neuronal differentiation was monitored for seven days, with media change on every third day. The cells showed neurite sprouting on 2^{nd} day onwards (Figure 8C) but the total percentage of cells sprouting was varying from experiment to experiment. Hence the cell culture protocol was standardized by addition of potassium chloride. .

Effect of Potassium Chloride on differentiation PC12 cells

The effect of potassium chloride (KCl) on induction of neurite sprouting from PC12 cells in the presence of NGF was studied. PC12 cells were cultured in DMEM F12 differentiating medium containing 200ng/ml NGF with 50mM KCl on poly L lysine (PLL) coated 35mm polystyrene petriplate for seven days. PC12 cells cultured in the above medium without KCl served as the control.

The sprouting of PC12 cells were seen to be increased in plates which had depolarizing concentrations of KCl than that of the control (figure 1). This is in agreement with the earlier report (Saffell *et al* 1992). Hence forth in all the experiments 50mM KCl was used in the media for cellular differentiation.

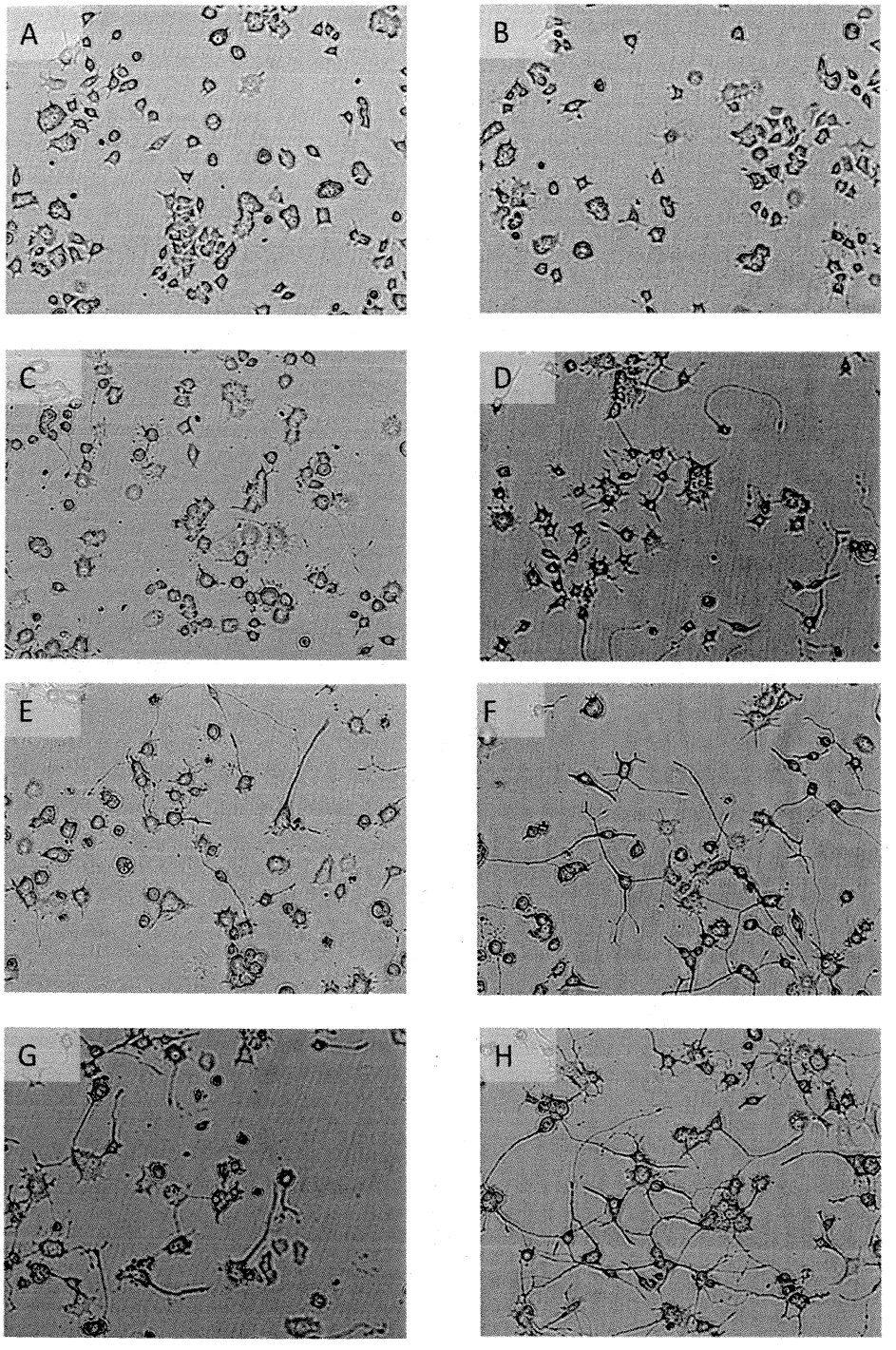
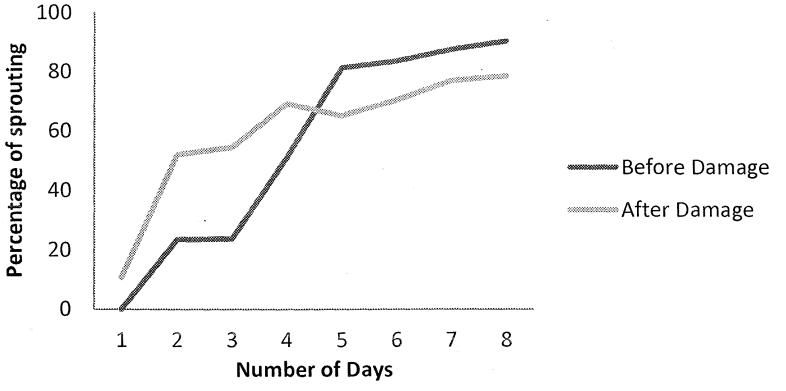


Figure 8: Neurite sprouting of PC12 cells cultured with DMEM F12 differentiating medium with and without KCl (A), (C), (E), (G) shows images Day 1, 3, 5, 7 respectively without KCl, (B), (D), (F) (H) shows images Day 1, 3 5, 7 respectively with KCl.

Quantification of differentiation of PC12 cells before and after damage.

To quantify the PC12 cell differentiation in its neurite growth, cells were differentiated and followed for seven days. On seventh day the neurites were damaged by detaching the cells from the plate by force pipetting the media and by triturating the cells (see Materials and Methods for details). These cells were replated and followed for another seven days to observe the recovery process. Parameters like sprouting percentage, number of surviving of cells, neurite length were measured both before damage and after damage cells. The data revealed that there was a progressive increase in the sprouting percentage, reaching almost 80-90% cells having sprouting on day 4 (Figure 9). When the spouting percentage plotted against the neurite length a sigmoid curve was obtained the percentage of sprouting started stabilizing around day 4 before damage. (Figure 9). Damaged cells showed a faster recovery with almost 50% cells sprouting on day 1 and stabilizing on 4th day with 70% of cells having neurite sprouting (Figure 2). Note that there were few cells (10%) without much breakage in their neurite extension after damage (Figure 9, Day 0 in after damage).



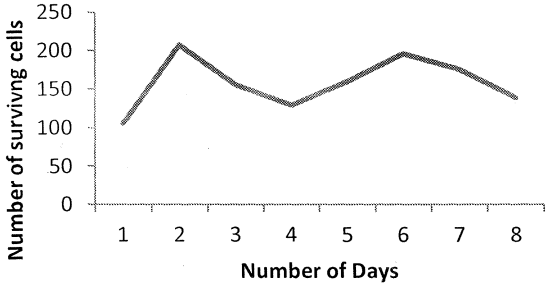
. **Figure 9,** PC12 sprouting percentage before and after damage

Mortality rate was around 50% after damage (see Figure 10a and 10b). However no further mortality was observed in the cells, which were followed for next seven days (Figure 10b). High mortality was expected in the experiment as the cells were forcefully detached from the plates. To counter the mortality rate initial

seeding rate was kept high (60,000 cells 35mm petriplate) so that sufficient cells will be available after damage.

PC12 cells on induction with NGF are committed to differentiation supported by the fact that the growth factor withdrawal causes apoptosis (Ferrari *et al* 1994). Since the cells were always maintained in NGF containing media, apoptosis of the cells after damage would have been prevented to a certain extent.

(a)



(b)

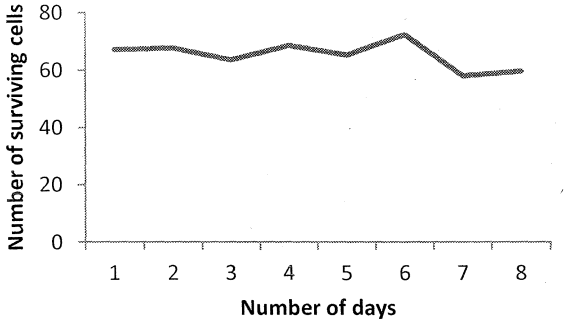


Figure 10, Day wise comparison of number of surviving cells a) before and b) after damage. The number of surviving cells reduced almost 50% after damage.

The average length of neurites was surprisingly much higher in cells which have undergone damage (Figure 11). The value was consistently high to a level of 50 to 60% for all the seven days. Both faster recovery (Figure 9) and increase in length of neurites suggests that the neuronal plasticity has been enhanced in these cells in the presence of growth factor. Even though axonal recovery has

been observed in a series of experiments conducted on single cell damage (Detrait 2000) studies, a group of cells undergoing consistent behavior is a first time observation to our knowledge. Extensive neurite sprouting requires enhanced exocytosis, and remodeling of actin- beta tubulin dynamics (de Forges *et al* 2012, Kapitein *et al* 2010). This model will greatly help in understanding those pathways. PC12 cells form a network of cells by 4th day in culture and damaging the network showed a spontaneous recovery of intrinsic network, though there was severe cellular mortality. This pattern of recovery mimics peripheral neuronal damage pathway (Geuna *et al* 2010), also to an extent to the changes involved in hippocampal sclerosis(Madden *et al* 2009, Thom *et al* 2009). Besides, this phenomenon of compensatory plasticity of neurons can be seen in the developing nervous where neurons near the injured portion in the nervous system extends neurites extensively to bridge the injured tissues (Siegel 2006)

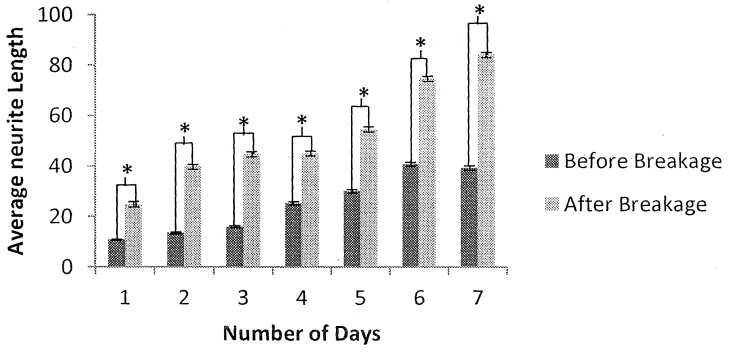


Figure 11, Day wise comparison of average neurite length of PC12 cells before and after damage. There is drastic increase in the length of neurites before and after damage. Error bars - standard estimated mean * p–values (unpaired student t-test) ranged from $1.6x10^{-21}$ to $1.9x10^{-10}$.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
N- Before Damage	272	173	190	223	221	179
N- after damage	166	224	245	259	266	259

Table 1, shows the number of neurites measured for neurite length measurement, N – Number

The complexity of the neurite extension was studied using sholl analysis. This analysis was done by drawing concentric circles having incremental increase in the radius by standard factor in length around the soma of the neuron. Number of intersections of neurites was counted for every concentric circle and a graph was drawn keeping the number of intersections in the Y axis and radius of the circle in X axis (Sholl 1953).

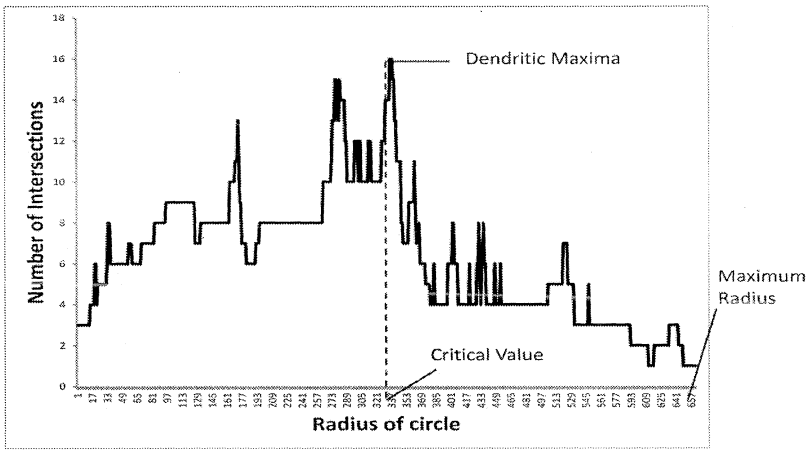


Figure 12, indicates the different parameters that are observed in typical sholl analysis graph. Highest peak is the dendritic Maxima; Corresponding reading in the radius is the critical value and maximum place where the neurites travel is given by maximum radius which is shown in the graph as maximum value present in the X axis.(Sholl 1953)

Values such as critical value (Cv), which is the radius of the concentric circle where there is a maximum number of intersections, Dendritic Maxima (Dm), which is the number at the intersections at critical value and maximum radius, which is the axonal length were calculated from Sholl analysis (see Figure 12) In addition, Schoenen's Ramification index (SRI) (Schoenen 1982) which is a measure of branching of neurites, obtained by the ratio of dendritic maxima and the number of neurites (N) arising from the soma. SRI gives number of sub branches in each dendrite.

SRI = Dm/N

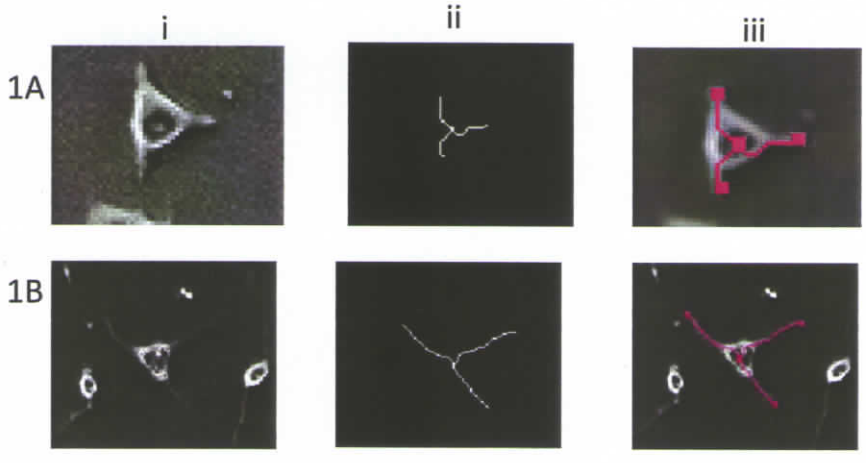
SRI – Schoenen's Ramification Index

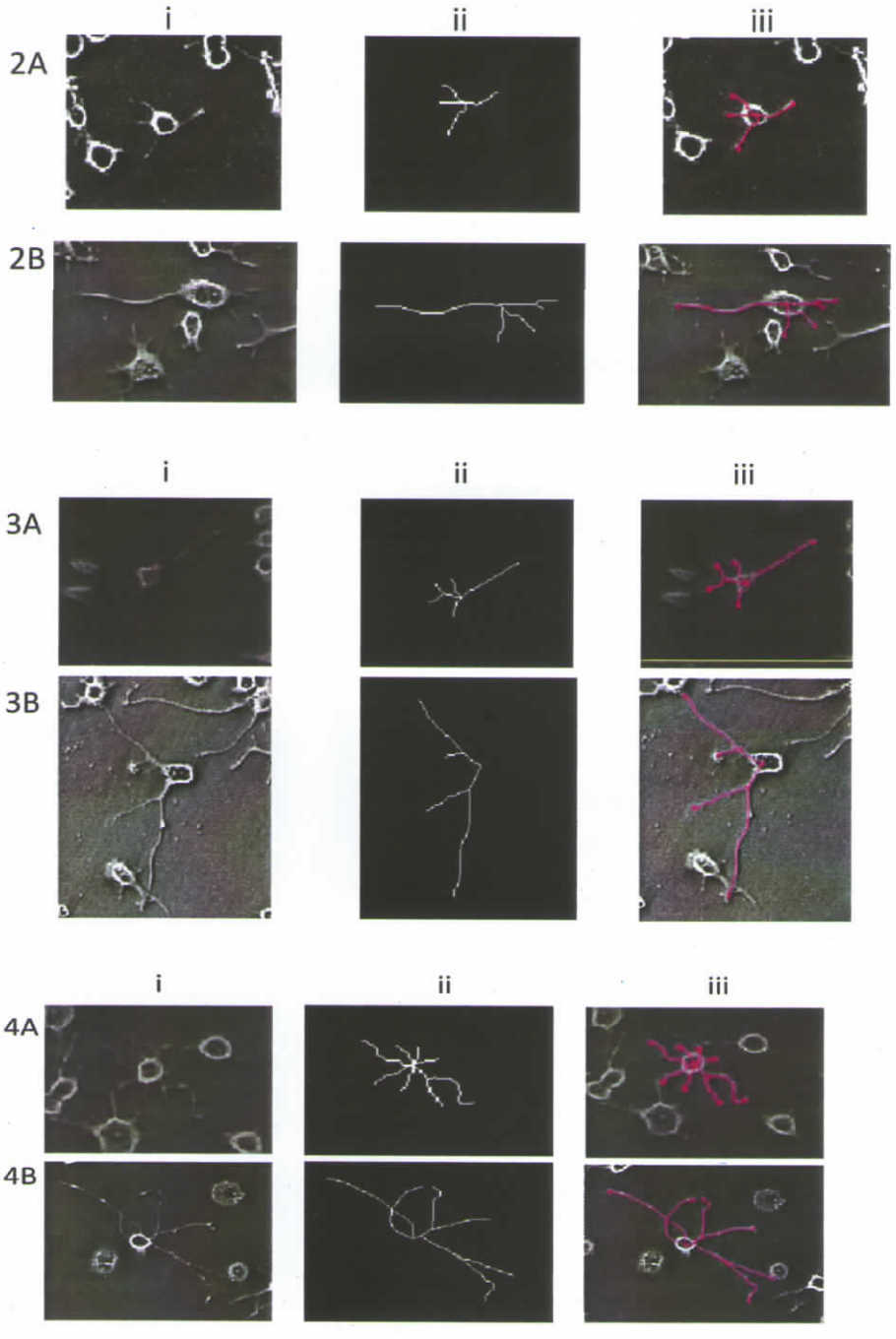
Dm – Dendrite Maxima

N – Number primary neurites from soma.

Sholl analysis gives the indirect measurement for dendrites and axons and their dynamics.

Representative neurons were taken from each day of experiment and the neurons were traced using Fiji plug-in: simple neurite tracer (Longair 2011). These traces were used for Sholl analysis (Fiji software). The cells along with their traces were shown in Figure 13




i
ii
iii
2A
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3A
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ii
iii
4A
4B

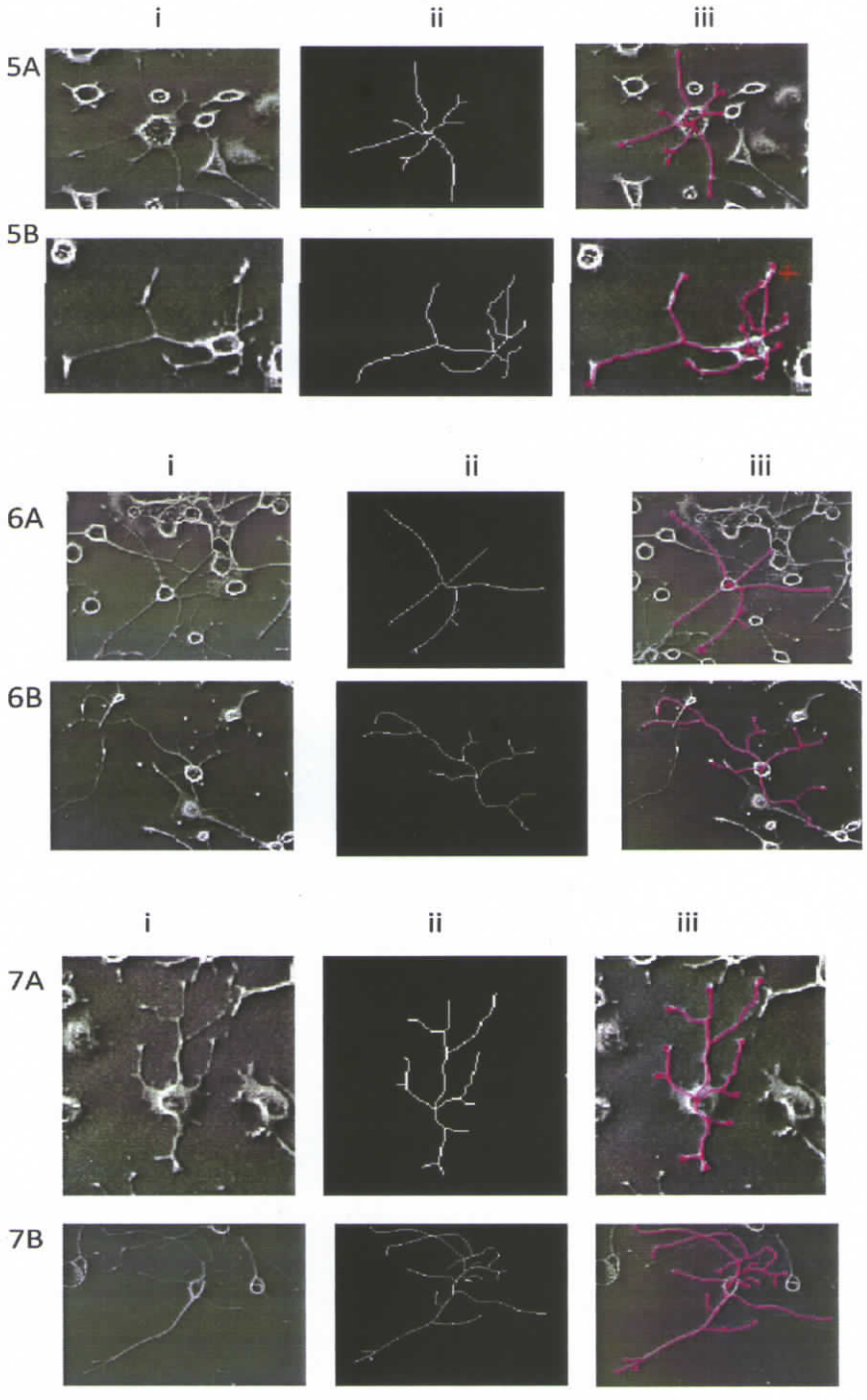
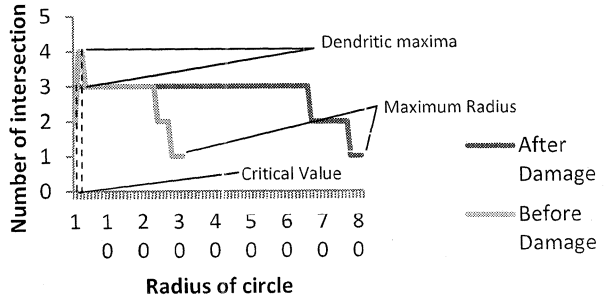


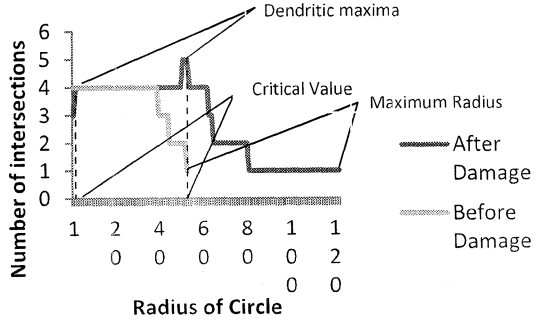
Figure 13, Differentiated PC12 cells selected for sholl analysis, 1,2,3,4,5,6,7 represents day 1, 2, 3, 4, 5, 6, 7 respectively, (A) Before damage (B) After damage, (i) Cells without tracing, (ii)Traced line stacks (iii) Merged Images

The Sholl graphs were drawn to compare the complexity of neurite extensions before and after damage (Figure 14). Critical value data though showed a variable data points because the cells traced on various days were not the same, there was a significant increase in the dendritic length in recovering cells after damage (Figure 15a). Dentritic maxima (Dm) showed substantial branching of dendrites after 4^{th} day of recovery, almost increasing exponentially on 7^{th} day (Figure 15b). SRI, which estimates sub-branches per dentrite, showed damaged dendrites undergo sub-branching at a higher rate compare to the control (Figure 15c). The data showed a strong correlation to enhancement of neuronal plasticity in the cells after damage, indicating an inborn mechanism within the cell to recover from neurite breakage. Increase in dendrite branching and length could be indicative of faster reestablishment of network. When the damage was induced, the cells were already formed a network. PC 12 cells are known to develop synaptic connections and form functional channels (Ogawa *et al* 1984, Zhou 2006). It is tempting to hypothesize that the cells, which are functionally connected, could recover faster by forming enhanced branching and establish functional connection.

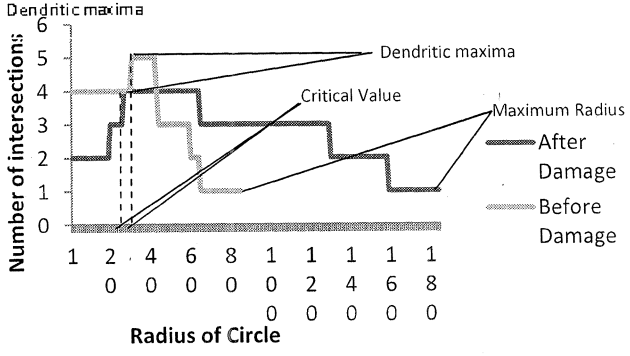
(A)


Number of intersection
Dendritic maxima
Maximum Radius
Critical Value
After Damage
Before Damage
Radius of circle

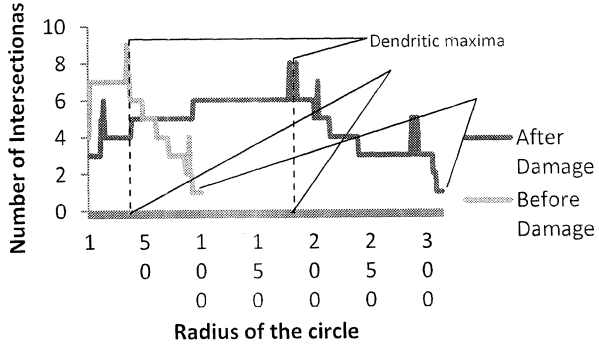
(B)


Number of intersections
Dendritic maxima
Critical Value
Maximum Radius
After Damage
Before Damage
Radius of Circle

(C)


Dendritic maxima
Number of intersections
Dendritic maxima
Critical Value
Maximum Radius
After Damage
Before Damage
Radius of Circle

(D)


Number of Intersectionas
Dendritic maxima
After Damage
Before Damage
Radius of the circle

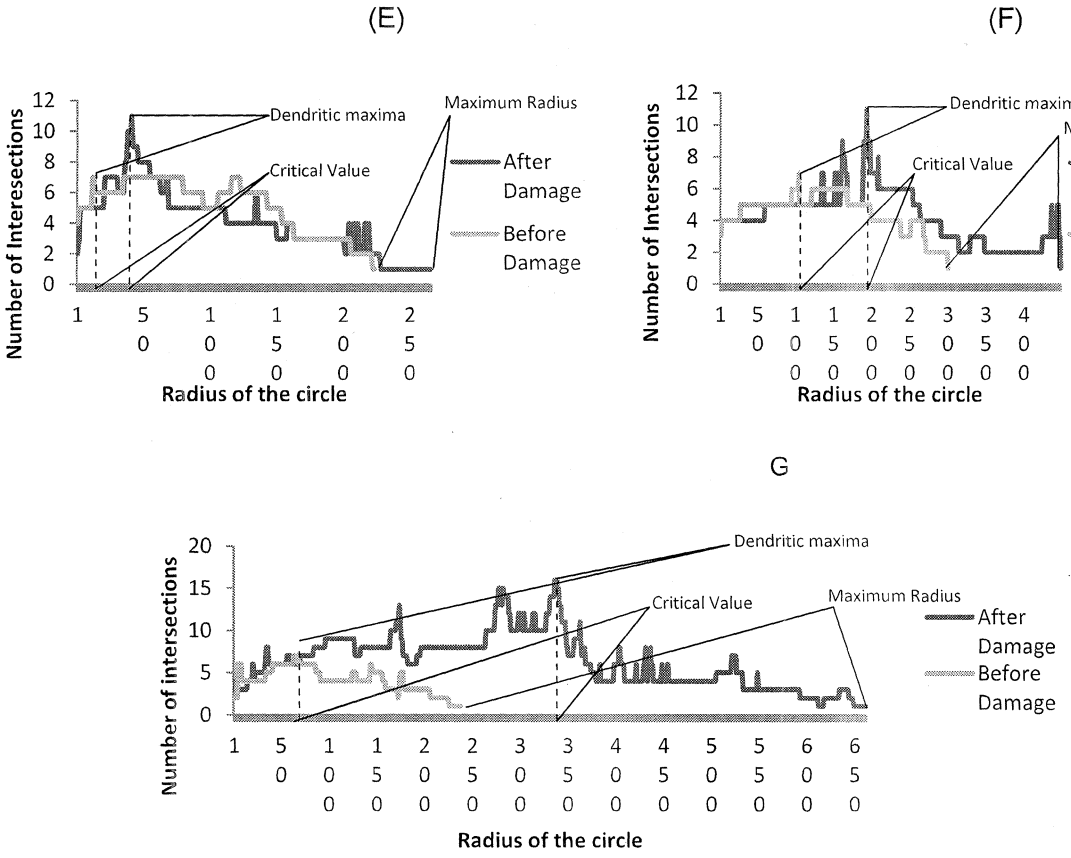


Figure 14, Comparison between sholl graph before and after damage (A) Day 1, (B) Day 2, (C) Day 3, (D) Day 4, (E) Day 5, (F) Day 6, G) Day 7

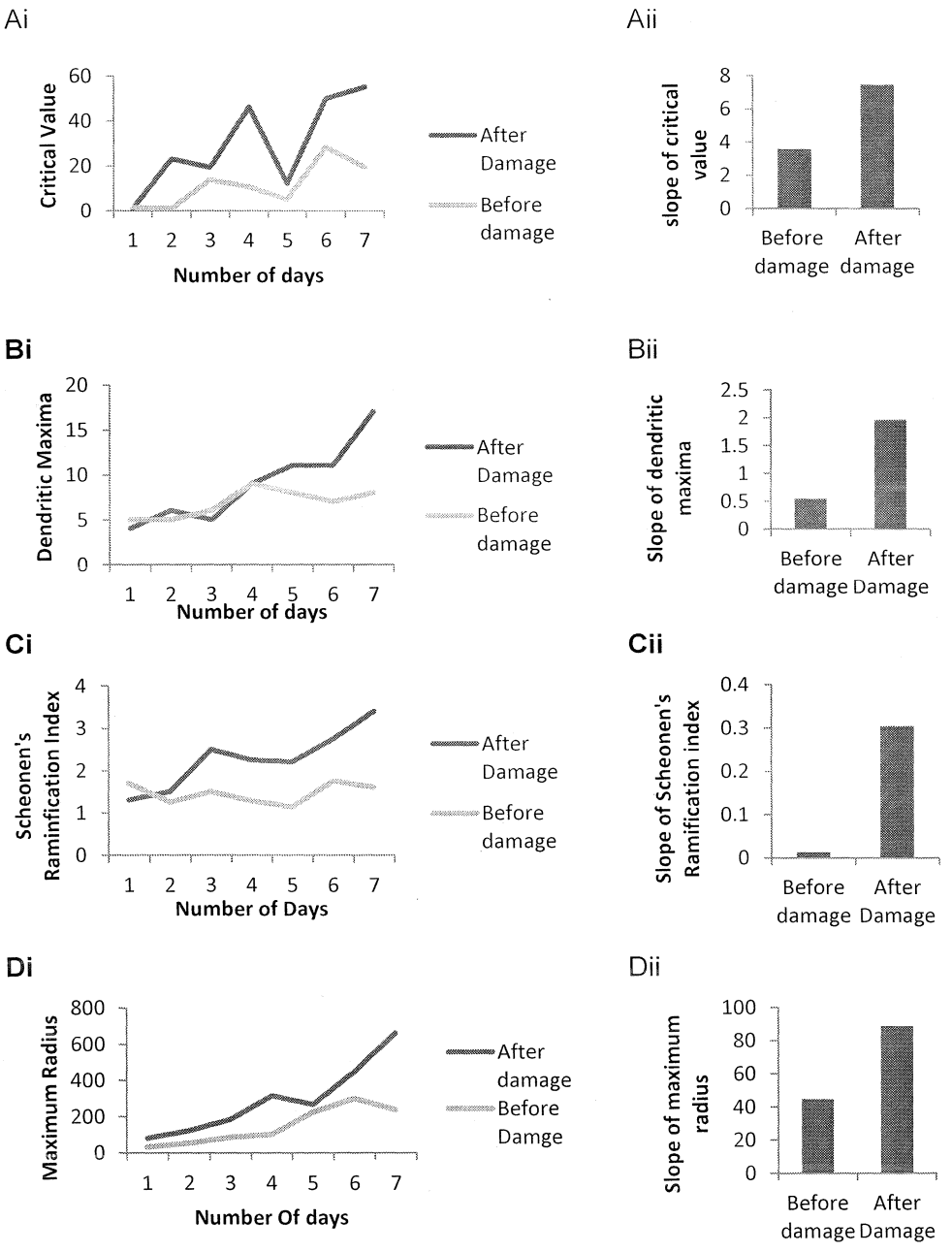


Figure 15, Comparison of sholl Parameters before and after neurite damage, (Ai) Critical Value (Aii) Slope of Critical Value (Bi) Dendritic Maxima (Bii) Slope of Dendritic Maxima (Ci) Schoenen's Ramification Index (Cii) Slope of Schoenen's Ramification Index (Di) Maximum Radius (Dii) Slope of Maximum Radius

	Day1	Day2	Day3	Day 4	Day 4	Day 7	Day 8
N per cell – Before damage	3	4	2	4	5	5	5
N per cell – After damage	3	4	7	5	5	4	6

Table 2, Number of neurites counted for calculating Schoenen's Ramification Index.

Studies on effect of NGF on PC12 cells have shown that a series of changes in protein expression occur at various stage of cell differentiation to neurons. In all the parameters calculated using sholl analysis, it can be seen that complexity increases with the progression of days. The slope of the values, dendritic maxima, critical value and Schoenen's ramification index of the regenerated PC12 cells is very high compared to PC12 cells undergoing normal differentiation (figure 15).

For example, proteins like GAP43 (Das *et al* 2004) caveolin 1, 2 (Galbiati *et al* 1998) are expressed in early phases of differentiation of PC12 cells. Proteins like Synapsin (Das 2004) are shown to have upregulated during later part. G proteins alpha and beta shown to increase during the early differentiation phase and attains maximum expression on day 4 while alpha i2 reaches the peak at day 4 (Zubiaur *et al* 1993) Synapsin and G proteins have critical function in development of functional networks (Valtorta *et al* 2011) and our results also suggested that the network of cells get established around day 4 (Figure 1F). When an established network gets damaged, the cells may undergo a complex biochemical change from that of the initial one. Understanding that pathways will give new insights on how the neurons behave after damage.

When network of functional neurons get established, cells communicate through neurotransmitters. PC12 cells have been found to develop functional connection with its partner cells (Ogawa 1984, Zhou 2006). One of the major proteins involved in neurotransmitter release is Synpatotagmin 1 and has critical role in

both exo and endocytosis (Rizo *et al* 2008). Hence preliminary experiments were carried out by over expressing synaptotagmin I in PC12 cells by gene transfection and compare how the cells behave after neurite damage.

Effect of synaptotagmin I on differentiation of PC12 cells before damage and after damage

Synaptotagmin I is a protein that is involved in the synaptic vesicles and it is involved in the exocytosis of synaptic vesicle in neurons. Since fusion of vesicles are important repair of injured neurites. Synaptotagmin I expression is necessary for regeneration of neurites. (Bradke 2012, Detrait 2000)

To study the effect of Synaptotagmin I on the regeneration of PC12 cells, PC12 cells were transfected with pEGFP C1 Synaptotagmin I, a Synaptotagmin I GFP fusion gene which was already cloned in the lab (Sunitha 2008). pEGFP C1 vector which expresses GFP is taken as control.

Both the vectors were verified for the inserts by restriction digestion (Figure 16)

The cells were transfected using Nucleofector kit V, devise ii and were cultured using DMEM F12 differentiating media containing 200ng/mL NGF and 50mM KCl in PLL coated 35mm polystyrene petriplates. Transfection efficiency was found to be at the range of 70% for GFP (Figure 17a, 17b). The GFP-Syt florescence was very weak and was difficult to get a clear estimate of transfection efficiency in these cells. However, we have seen cell started developing florescence as they differentiate into neurons (Figure 17).

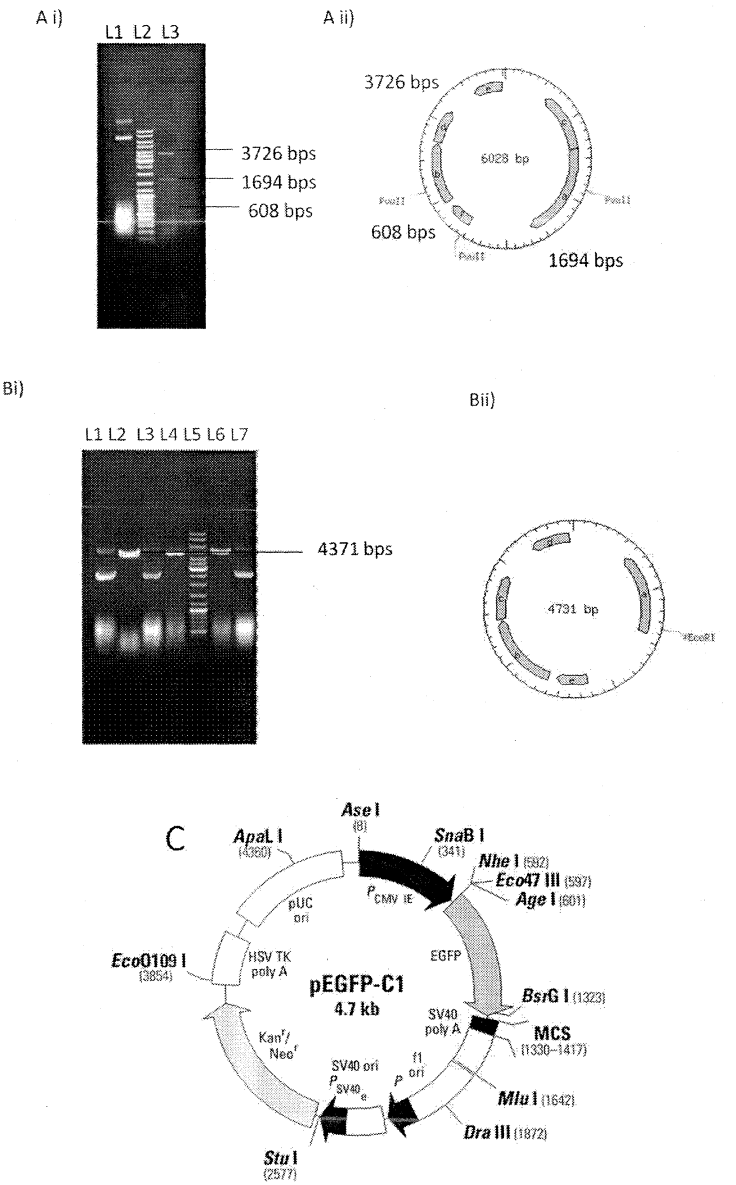


Figure 16, (A) agarose gel electrophoresis result for the confirmation of i) pEGFP C1 Synaptotagmin I plasmid by restriction digestion, lane 1 – undigested Plasmid, Lane 2 – Marker, Lane 3 – Pvu I digested plasmid. Ii) Pvu I restriction Sites in pEGFP 1 Synaptotagmin I (B) agarose gel electrophoresis result for the confirmation of i) pEGFP C1 Vector by restriction digestion, lane 1 – undigested Plasmid, Lane 2 – EcoR1 digested Vector, Lane 6 –.molecular Weight Marker ii) EcoR1 restriction Sites in pEGFP 1 C1, (C) Plasmid map of pEGFP C1

The effect of synaptotagmin I expression on regeneration of neurites after damage was studied by detaching the synaptotagmin I GFP expressing cells and GFP control cells by harshly pipetting, the neurites of the detached cells were broken and replated in a new petriplate containing DMEM F12 differentiating medium containing 200ng/ml NGF and 50mM KCl.

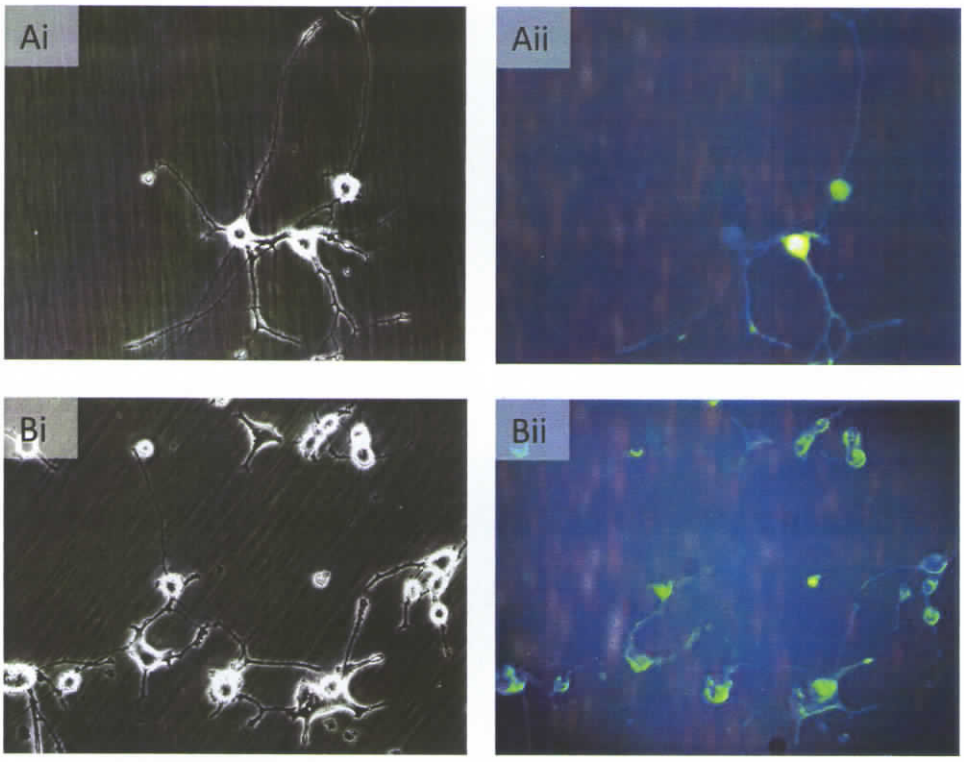
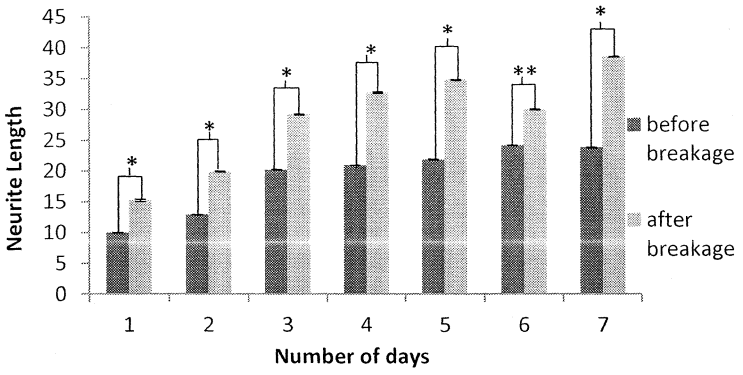


Figure 17, Transfected PC12 cells after differentiation, (Ai) and (Aii) shows phase contrast and fluorescent images of GFP transfected cells, (Bi) and (Bii) shows phase contrast and fluorescent images of Synaptotagmin I GFP transfected cells respectively

Synaptotagmin I expression does not affect the neurite length of PC12 cells

Neurite length of synaptotagmin I GFP and GFP alone transfected PC12 cells both before and after neurite damage were measured to study the effect The neurite length and pace of neurite growth of both GFP and Synaptotagmin I GFP expressing cells increased upon re-growth after damage (figure 18), as seen earlier.

A



B

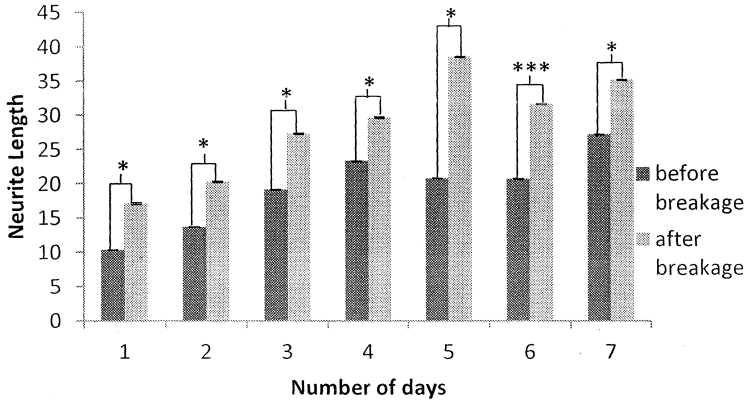


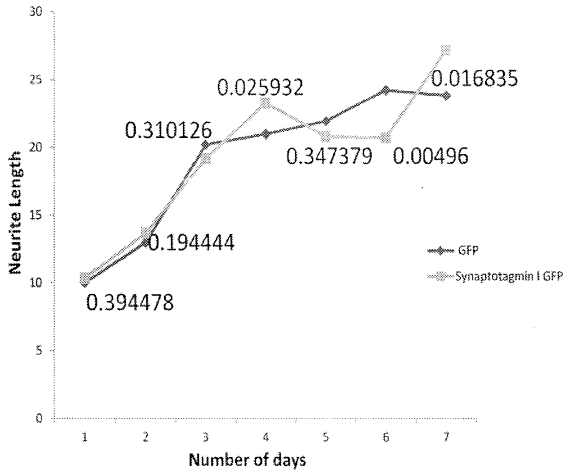
Figure 18, A comparison between neurite lengths of PC12 cells subjected to differentiation before and after damage: A) GFP expressing cells, B) Synaptotagmin I expressing cells. Error bars are standard estimated mean * p-values ranged from $2.1X10^{-6}$ to $1.9x\ 10^{-17}$; ** p-value 0.01 and *** p-value 0.008 from unpaired student t-test

Before Damage	Synaptotagmin I GFP	285	434	442	438	374	305	375
	GFP	124	387	362	322	280	296	298
After Damage	Synaptotagmin I GFP	48	134	138	167	220	198	278
	GFP	32	108	175	165	222	312	499

Table 3, shows the number of neurites measured for neurite length measurement, N – Number

When the neurite lengths of synaptotagmin I GFP Expressing cells are compared with GFP control cells no significant difference was observed either before or after damage (figure 12). This implicates that expression of synaptotagmin I do not promote neurite length in PC12 cells. The data is in contradiction with the observation that the expression of synaptotagmin I results in the increase in the neurite out growth(Fukuda *et al* 2000). However this variation could be due to low transfection efficiency of synaptotgamin I to the cells. The lab is working on a stable transfect so that this limitation can be avoided.

(A)



(B)

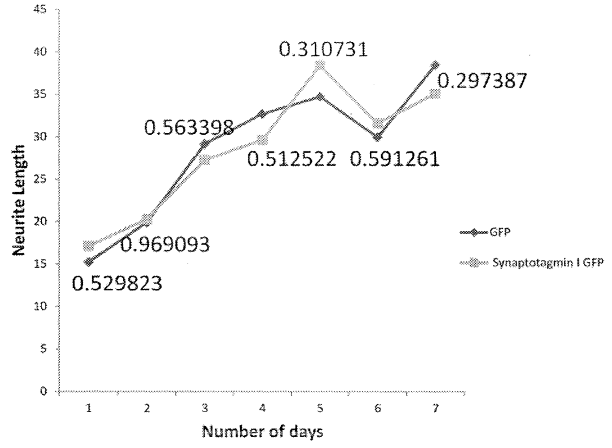


Figure 19, Comparison between the GFP Expressing and Synaptotagmin I Expressing cells A) Before damage B) After Damage. P-Value are represented near the respective points

PC12 cells which are replated after differentiation as a model system to study neurite regeneration in Neurons

The ability of differentiated PC12 cells after neuronal injury can be exploited for studying the mechanisms of regeneration of neurons. Neurite regeneration studies usually involve forming a mechanical wound in the culture and monitoring the closure of wound by regenerating neurites.(Detrait 2000, Nejatbakhsh *et al* 2011, Wu *et al* 2012). The main drawback of this technique is that number of neurites that are studied for regeneration is low. Studies like protein expression levels and mRNA expression are not possible due to the low availability of cells. Injured neurites are identified by the proximity to the wound, which is not a reliable indicator

These problems of the previous method can be overcome by replating after injuring neurites in a new plate, so that all the cells that survive the replating can be used for analysis to obtain higher statistical significance. Replating ensures that almost all the cells in the dish have undergone neurite injury, hence isolating proteins, mRNA and epigenetic modifications can be studied more easily.

Problems observed in performing the experiments are that there was a high degree of mortality when the neurites were damaged with mechanical force (Figure3), and around 10% cells did not lose their neurites after damage experiment. Though further standardization of this method is necessary for applying it as effective model system, the results suggests that the system is very promising to study a large network of cells and its recovery from damage. PC12 cells, though not perfect neuronal model cells, as demonstrated by the structural difference in its microtubule architecture (Zhou 2006) growth cones and varicosities (Mingorance-Le Meur *et al* 2009), they have the advantage to study the basic pathways involved in neuronal networking. This study has achieved standardization of a powerful model system to study neuronal networking pathways.

Summary and Conclusion

Understanding neuronal regeneration pathway is critical in developing efficient therapeutic strategies. One of the major impediment in studying neuronal growth and regeneration is lack of proper model systems. On majorities of the studies, single cells were used to study axonal damage with a serious limitation of cell selection and limit of number of cells one can induce damage. On this context in this study an attempt has been made to develop a model system with a large number of cells, which can reestablish cellular network after damage.

PC12 cells, a well-defined neuronal model cell, were used in this study for its ease in culture and maintenance. PC12 cells are used as a model system for studying mechanisms of functioning and structural composition of neurons. PC12 cells which are differentiated have been shown to communicate and form active connections with native neurons both in vivo and in vitro (Freed *et al* 1986, Zhou 2006) They have also shown to regenerate after neurite injury (Wu 2012). The vesicular protein Synaptotagmin I is necessary for regeneration of neurites after injury in PC12 cells (Detrait 2000).

The aim of this study was a) to establish a model system for the regeneration of neurons and b) to find the effect of Synaptotagmin I expression on the regenerative ability of these neurons. As it was reported earlier that depolarization of PC12 cells using KCl leads to the improvement in neuronal sprouting (Khan *et al* 1996, Rosen *et al* 1994), we adapted that protocol and established a predictable neuronal phenotype for PC12 cells. The damage experiment was a new protocol developed in the laboratory and has high reproducibility. Though there was high mortality rate, the surviving cells could regrow significantly faster after neurite damage. This could be due to enhanced exocytosis and part of the recovery process. Besides the cells are already transformed into neurons before the damage was initiated, and this could have enhanced the faster regrowth of neurites. The most surprising result was the larger extensions the neurons produced after damage. We have found that cells grown for 14 days without damage were not producing longer neurites (data not

shown), hence suggesting that the neurite extensions are somehow modified by damage caused. The complexity of neurite growth was also increased after damage, shown by parameters like Critical Value, and Dendritic maxima, which were calculated using Sholl analysis. Schoenen's ramification index showed increased branching capability of the neurites after damage hence forming complex connections. These results suggest that the regenerated PC12 neurites have more complex structural and functional connections than the ones which have not undergone breakage. One of the possibilities was the role synaptic vesicle proteins like synaptotagmin, which could have positive roles in these pathways. As a preliminary experiment the cells were transformed with synaptotagmin gene and verified for variations in neurite extensions, but to no avail. Possible reasons could be low efficiency of transfection and its limitation in expression level in cells. One way to overcome these limitations is to establish stable cell lines with the gene of interest. This work has been currently standardized in the laboratory.

This study could develop an interesting model system without the major draw backs faced in the previous model systems like transecting single neurites, (Detrait 2000) or wound closure (de Forges 2012). This model helps in studying a neural network having statistically significant number of neurites and also has sufficient cells to perform RNA and protein isolation experiment.

Further studies

The ability of the regenerated PC12 neurites to form functional connections like differentiated PC12 cells has not been studied yet. Fluorescent molecular dyes and electrophysiological techniques can be employed to study the function efficiency of regenerated PC12 cells. The functional efficiency can be studied in differentiated PC12 cells and PC12 cells co-cultured with primary neurons.

The regenerative capability of neurons has been assessed only *in vitro. In vivo* cells face a different environment with complex stimuli and interaction. This could make it more difficult to study the events clearly because of various new parameters involved. Regeneration response injury of fluorescently labeled PC12

neurites can be studied in vivo after transplantation into a suitable host nervous system. Chromaffin cells have been shown to be transplanted into humans to treat Parkinson's disease as a trial study. (Drucker-Colin *et al* 1999) PC12 has its origin from the same tissue studying the regenerative potential in a mammalian system will provide insights into the in vivo mechanisms of regeneration of neurites and its ability to form functional network.

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