PLASTICITY OF AXONAL GROWTH AND SPROUTING IN PHEOCHROMOCYTOMA-12 CELLS IN THE PRESENCE OF ACTIVATED FACTOR II (THROMBIN), A SERINE PROTEASE

A DISSERTATION SUBMITTED

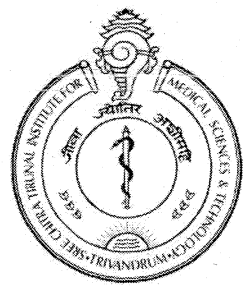
BY

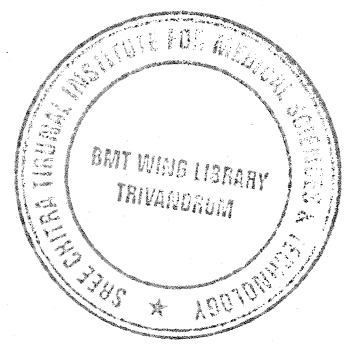
JESSY KURIAKOSE

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF PHILOSOPHY





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DECLARATION

I, **Jessy Kuriakose**, hereby declare that I had personally carried out the work depicted in the dissertation entitled "**Plasticity Of Axonal Growth And Sprouting In Pheochromocytoma-12 Cells In The Presence Of Activated Factor II (Thrombin), A Serine Protease**" under the direct supervision of **Dr. Anoopkumar Thekkuveettil, Scientist F, Molecular Medicine Laboratory,** Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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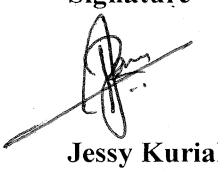

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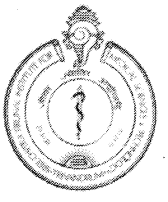

CERTIFICATE

This is to certify that the dissertation entitled "**Plasticity Of Axonal Growth And Sprouting In Pheochromocytoma-12 Cells In The Presence Of Activated Factor II (Thrombin), A Serine Protease**" submitted by **Jessy Kuriakose** in partial fulfilment for the **Degree of Master of Philosophy** in Biomedical Technology to be awarded by this Institute. The entire work was done by her under my supervision and guidance at Molecular Medicine Laboratory, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram-695011.

Thiruvananthapuram

Date: 21/8/2010

Signature

Dr. Anoopkumar Thekkuveettil

The Dissertation Entitled

PLASTICITY OF AXONAL GROWTH AND SPROUTING IN PHEOCHROMOCYTOMA-12 CELLS IN THE PRESENCE OF ACTIVATED FACTOR II (THROMBIN), A SERINE PROTEASE

Submitted

By

JESSY KURIAKOSE

For

Master of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY

THIRUVANANTHAPURAM – 695 011

Evaluated and approved

by

Signature 21/8/2010

Name of Supervisor

Dr. Anoopkumar Thekkeveettil.

Signature 21/8/2010

Examiner's name and Designation

Dr. A. Vijaya Nandkumar.

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Jessy Kuriakose

ABBREVIATIONS

°C	Degree Celsius
µL	microliter
µM	micromolar
AA	Arachidonic acid
ADP	Adenosine diphosphate
ATP	Adenosine diphosphate
BBB	Blood brain barrier
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CNS	Central nervous system
CO_2	Carbon dioxide
DAG	Diacylglycerol
DMEM:F12	Dulbecco's Modified eagle medium/Nutrient mixture F-12 Ham
dNTP	Deoxyribonucleotide triphosphate
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GPCRs	G protein coupled receptors

GTP	Guanosine triphosphate
ImageJ	Image Processing And Analysis in Java
iNOS	Nitric oxide synthase
IP3	Inositol (1,4,5) trisphosphate
IU	International Unit
MAPK	Mitogen-activated protein kinases
$MgCl_2$	Magnesium chloride
min	Minute
mL	milliliter
ng	Nanogram
NGF	Nerve growth factor
NO	Nitric oxide
PAR	Protease activating receptors
PBS	Phosphate buffer saline
PC12	Pheochromocytoma12
PKC	Protein kinase C
PLA2	Phospholipase A2
PLC	Phospholipase
PTX	Pertussis toxin
Pyk2	Proline-rich tyrosine kinase-2

RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SV	Synaptic vesicles
Syp	Synaptophysin
TAE	Tris-acetate-EDTA
VAMP	**Vesicle associated membrane proteins**
VAT-1	Vesicle amine transport protein 1
α-SNAP	Soluble NSF Attachment Protein α

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SYNOPSIS

SYNOPSIS

Neurological diseases as well as physical or biological insults can cause central nervous system injuries. Thrombin, a potent serine protease is known to play a critical role in some of the neurological disease such as Alzheimer's disease, Parkinson's disease etc as well as in intra cranial haemorrhages. Neuronal injuries lead to complex cellular and molecular interactions within the central nervous system in an attempt to recover from the insult. Since neuronal cells are post mitotic in nature, any insult to central nervous system will have a devastating effect. Currently there are only limited therapeutic interventions available to reduce extend of damage of this vital system. In this context it is essential to understand the biochemical pathways involved in neuronal damage so that appropriate therapeutic approaches can be developed. Thrombin, a serine protease involved in blood coagulation, has been reported to cause neurotoxicity in a concentration dependent manner. In this study we attempted to identify the role of thrombin in neuritic morphogenesis at a sublethal concentration. Our results suggest that thrombin has a significant role in the structural modulation of axonal and dendritic extensions.

In this study we have used Pheochromocytoma-12 (PC-12) cell line, which is the most widely used neuronal cell line. It was developed from a tumour of adrenal medulla by irradiation. PC12 differentiates into sympathetic like neurons in response to nerve growth factor (NGF). This neuronal culture system is an excellent model that resembles cells *in situ*. Neurons sense changes in the environment, communicate these changes to other neurons and command the body's responses to these sensations. The connectivity and functionality of neural networks is determined by the formation of synapses between the appropriate neurons. We used changes in the morphology of axons and dendrites as a marker for thrombin's effect. To measure the active zone changes, we used synaptophysin (Syp), an integral membrane glycoprotein selectively and permanently coupled with the membrane of SVs and have a critical role in active zone formation.

Polylysine coating of culture plate enhances PC12 cell attachment. PC12 cells were seeded onto polylysine coated tissue culture plate containing DMEM:F12 complete media. PC12 differentiates was then differentiated to sympathetic like neurons by the addition of nerve growth factor (NGF). Differentiated cells were exposed to thrombin at different sublethal concentrations (0.5 IU and 1 IU). The culture plates were incubated at 37˚C /5% CO_2 for 4 days without changing media or added components. Lengths of neuritic processes were quantified using Image Processing And Analysis in Java (ImageJ) software from the pictures taken on Day 1 to 4 of control and test samples. At the end of Day 4, control and test cells were checked for viability using Hoechst staining. RNA was isolated from control and thrombin treated PC12 cells. RT-PCR for β-actin (control gene) and synaptic vesicle protein, synaptophysin have been carried out to analyze the effect of thrombin at gene expression level. PCR products of the β-actin and synaptophysin were quantitated by densitometric analysis using UVIpro platinum software (UVIpro UK).

PC12 cells attached well onto polylysine coated plate and were differentiated into sympathetic like neurons in response to nerve growth factor (NGF). Differentiated PC12 cells on exposure to 0.5 IU and 1 IU thrombin showed variations in neuritic length at a concentration dependent manner. The data showed a 14 (P value = 0.02) and 20 (P value =0.001) percentile decrease in neuritic growth on day 4 in the presence of 0.5 and 1 IU thrombin, respectively. To get a better understanding on the rate of growth of neuritic sprouting, cell to cell follow up was carried out in all the three groups (control, 0.5 and 1 IU thrombin) and the data showed that the effect of thrombin in neuritic growth is highly significant and is concentration dependent. Presence of thrombin has shown differential effects on shorter and longer neurites. Shorter neurites showed an increase in length (4-5%) (P value =0.0001) (Figure 22) while longer neurites showed ~ 15% (P value =0.44) decrease in neurite length in the presence of 1 IU thrombin. Growth cone collapse and thus neurite retraction are caused by thrombin by activating thrombin receptor. One of the adaptative changes in neurons after an injury is to withdraw the injured axon and commit one of the dendrites as new axon. In this work we have not extended the period to see

such a morphological change, but the initial results suggest that such a possibility exist when neurons are exposed to thrombin. Presence of thrombin must be initiating signals almost similar to that of an axonal injury in neurons. Densitometric analysis revealed 18% reduction in synaptophysin gene level expression in thrombin treated cells with respect to control cells. Changes in the gene expression of integral membrane protein may have a role in the molecular basis of synaptic plasticity. Our preliminary result suggests that the variation of synaptophysin level could be due to variations in growth cone dynamics.

In this study we have shown that thrombin can influence changes in neurotic morphology and the dynamics of growth cone. Further studies will give a better insight on the role of thrombin in disease conditions like stroke, hypoglycemia, intra cranial haemorrhage, oxidative stress and during various neurological diseases like Parkinson's disease, Alzheimer's disease.

INTRODUCTION

CHAPTER I

INTRODUCTION

1.1 BACKGROUND

Central nervous system (CNS) injury is one of the major health problems in the world. Patients suffering from CNS injury (for example, stroke or spinal cord injury) endure irreversible disability and trauma. There are only limited therapeutic interventions available at present. It is essential to understand the molecular and cellular pathways activated during traumatic brain injuries.

Thromboembolic occlusion of arteries is one of the major causes of ischemic stroke. Thrombin, a serine protease, is essential in coagulation cascade. It is produced immediately after the occurrence of intracranial haemorrhage to stop bleeding. However it has been reported that direct infusion of thrombin results in brain edema and neuronal death [Lee *et al.*, 1997]. Action of thrombin in brain seems to be dose dependent. This study is an attempt to understand the physiological impact of thrombin at a low concentration in an *in vitro* neuronal model system.

1.1.1 NEURONS

Neurons sense changes in the environment, communicate these changes to other neurons and command the body's responses to these sensations. Neurons have the ability to transfer electrical signals throughout the brain and body. Size of the neuron ranges from 0.01 - 0.05 mm in diameter. Neurons have two distinguishable parts: soma or cell body, which is the central region containing nucleus; and neurites which are of two types: axon and dendrites. Figure 1 shows the structure of a neuron.

Soma consists of nucleus and cellular organelles. It's about 20 μm in diameter. Neuritis is the most remarkable structural feature of a neuron. Their elaborate branching is critical for information processing.

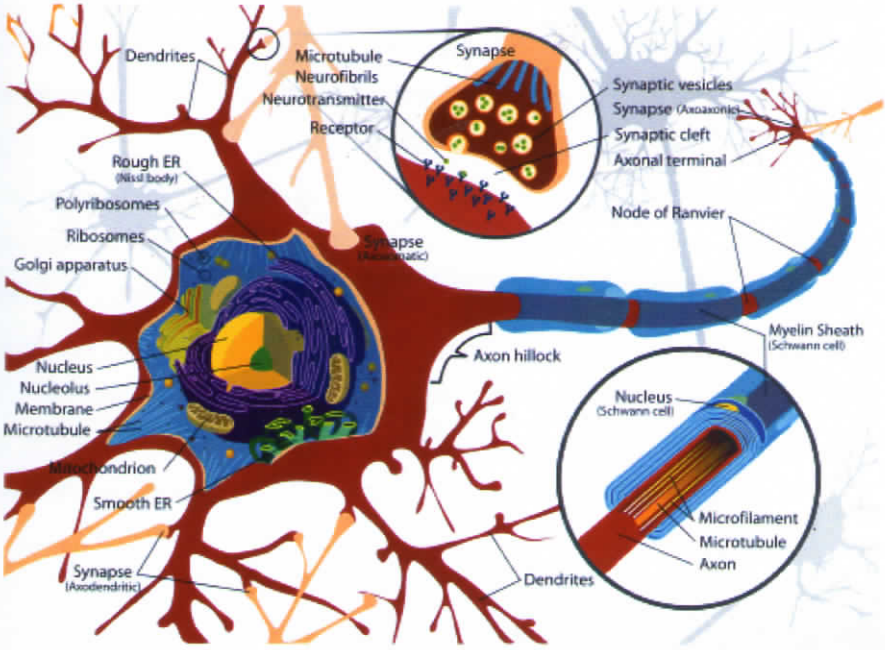

Figure 1: Structure of neuron

Classification of neurons:

Based on number of neurites	Based on dendritis	Based on dendritis
•Unipolar •Bipolar •Multipolar	•Stellate cells •Pyramidal cells	•Spiny •Aspinous

Based on connections	Based on axon length
•Sensory •Motor •Interneuron	•Golgi type I •Golgi type II

1.1.2 MODEL SYSTEMS TO UNDERSTAND NEURONS

Even though cell cultures have its own limitations, model biological systems helps us understand particular biological phenomenon, with the hope that it will provide insight to the real biological world. Many of our understandings of neurons come from the *in vitro* systems. Most culture systems are developed with the goal of obtaining neuronal cells that resemble cells *in situ*. Many neuronal cell cultures have been well established during the past decade most them being tumour cell lines. Pheochromocytoma line (PC12), C-1300 neuroblastoma, GT-1 cell line, P19 cell line, N-Tera 2 are some of the cell lines widely used for investigations in neurobiology. Among these PC12 cell lines are most widely used neuronal cell line. It was developed from a tumour of adrenal medulla by irradiation [Greene & Tischler, 1976].

1.1.3 THROMBIN AND NEURONS

Thrombin a serine protease is produced by the liver and is secreted to the blood. It plays a very important role in blood coagulation. Neurons on the other hand are protected from blood and blood borne factors by different mechanisms such as the blood brain barrier in the brain. However, prothrombin is found to be expressed in the brain of mammals. But what it does in the brain is not yet known. Thrombin has got neuroprotective and neurotoxic effects depending on its concentration and time of exposure to neurons. However, how this thrombin regulates plasticity of neuronal sprouting is under investigation. Thrombin is also found to play a critical role in some of the neurological disease such as Alzheimer's disease, Parkinson's disease etc. Nervous system is also exposed to thrombin during an injury. It has been found to regulate gene expression in muscle cells. But any gene regulation in neuronal cells needs to be investigated further.

1.2 REVIEW OF LITERATURE

1.2.1 PHEOCHROMOCYTOMA12 CELLS

The most commonly used neural clonal cell lines is the rat pheochromocytoma line PC12, which was derived from a tumour of the adrenal medulla following X-irradiation [Greene & Tischler, 1976]. PC12 differentiates into sympathetic like neurons in response to nerve growth factor (NGF). Under routine culture conditions, PC12 cells possess many characteristics of adrenal chromaffin cells, including round cell body and the ability to synthesize, store, and release catecholamine neurotransmitters [Greene & Tischler, 1976]. Differentiation of the cells is characterized by mitotic arrest, the elaboration of neuronal processes, and electrical excitability.

PC12 cells differ from most other transformed cell lines in their near diploid number of chromosomes and their relative slow growth. The extensive usage of the line is due to its relative stability, homogeneity, high degree of differentiation and of differentiative capacity, robust response to NGF and dramatic change in phenotype brought about by this factor, fidelity to many of the features of normal neuroblasts and neurons, potential for genetic manipulation and the accrual of a large number of studies regarding its characterization. Neurons derived from PC12 have the potential to form synapses with primary neurons [Zhou *et al.*, 2006]. Even though PC12 cells posses many advantages to be a neuronal model system, it has some limitation like: they are tumour derived cells and therefore differ from nontransformed cells in their behaviour, and hence can be used only as a starting point for investigations; they do undergo spontaneous mutations and there is consequent production of variants and finally the maintenance of neurites at all stages of treatment requires the continued presence of NGF. If the factor is withdrawn neurites will either retract or degenerate and the cells will resume proliferation.

1.2.2 NEURITE OUTGROWTH

Control of cell morphology is important for maintaining cellular structural design and normal functions. Cell processes like axons and dendrites maintain synaptic connections and signalling in neurons. Neuronal growth cones, located at the tip of the growing axon, are highly motile structures that respond to guidance cues by selectively altering the stability of the actin cytoskeleton and microtubules [Negishi *et al.*, 2005].

NGF-induced differentiation of PC12 cells to a neuronal phenotype involves transcription dependent phase (priming) and a transcription independent one (regeneration) [Levi *et al.*, 1988]. In the priming phase, activation of NGF receptor causes the expression of many genes such as protooncogenes (*c-fos, c-myc*), actin, ornithine decarboxylase. In the regeneration phase neurite outgrowth takes place. An alteration in gene expression occurs after NGF treatment and hence PC12 cells represent a model system to study neuronal differentiation [Kaplan & Stephens, 1994; Levi & Alema, 1991]. While comparing the gene expression of NGF treated and nontreated PC12 cells many mRNA was found equally expressed in both the group which have been designated as housekeeping enzymes which includes elongation factor 1-a, GAPDH, cyclophilin, secretogranin 1 and secretogranin II and the catecholamine-synthesizing enzyme tyrosine hydroxylase [Lee *et al.*, 1995]. Twelve differentially regulated genes were also identified which include ribosomal proteins L19 and L7, the G protein-coupled adenosine A2a and secretin receptors, cell trafficking proteins kinesin light-chain C and 13-COP, a synaptic glycoprotein SC2, synapsin 2, an insulin-induced growth response protein CL-6, superoxide dismutase and clusterin [Lee *et al.*, 1995].

1.2.3 SYNAPSE

The distinctive morphology of neuronal cells has exclusively developed to facilitate the rapid and reliable transmission of signals between neurons. Synapses are macromolecular structures that regulate intercellular communication in the nervous

system. The connectivity and functionality of neural networks is determined by the formation of synapses between the appropriate neurons. The specificity of synapse formation requires the clear-cut implementation of numerous developmental events, including cell fate specification, cell migration, axon guidance, dendritic growth, synaptic target selection, and synaptogenesis [Stoeckli & Zou, 2009]. Synapses are of electrical and chemical (Figure 2).

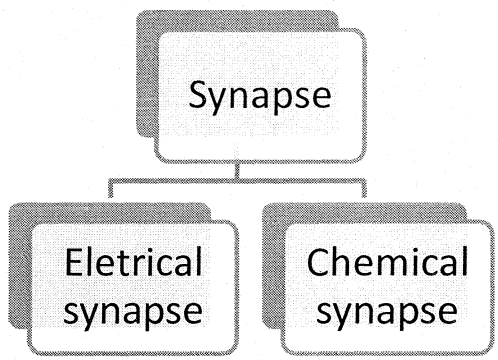

Figure 2: Types of synapses

Electrical synapses are gap junctions that allow bidirectional propagation of signals. They allow the direct transfer of ionic current from one cell to another. They have been characterized in synchronizing neural activity across networks [Hormuzdi *et al.*, 2004]. Chemical synapses links discontinuous neurons through extremely synchronized secretion of neurotransmitters. They are polarized junctions and permit the flow of information in a single direction [Eccles, 1982].

A synapse consists of three major components (Figure 3):

1. **The Presynapse:** It sends signals by releasing neurotransmitters stored in vesicles in response to depolarization.
2. **The Postsynapse:** It receives signals due to the opening of ligand-gated ion channels upon binding of neurotransmitter to its receptor.

3. **The Synaptic Cleft:** It is the extracellular space between the pre- and postsynaptic sites.

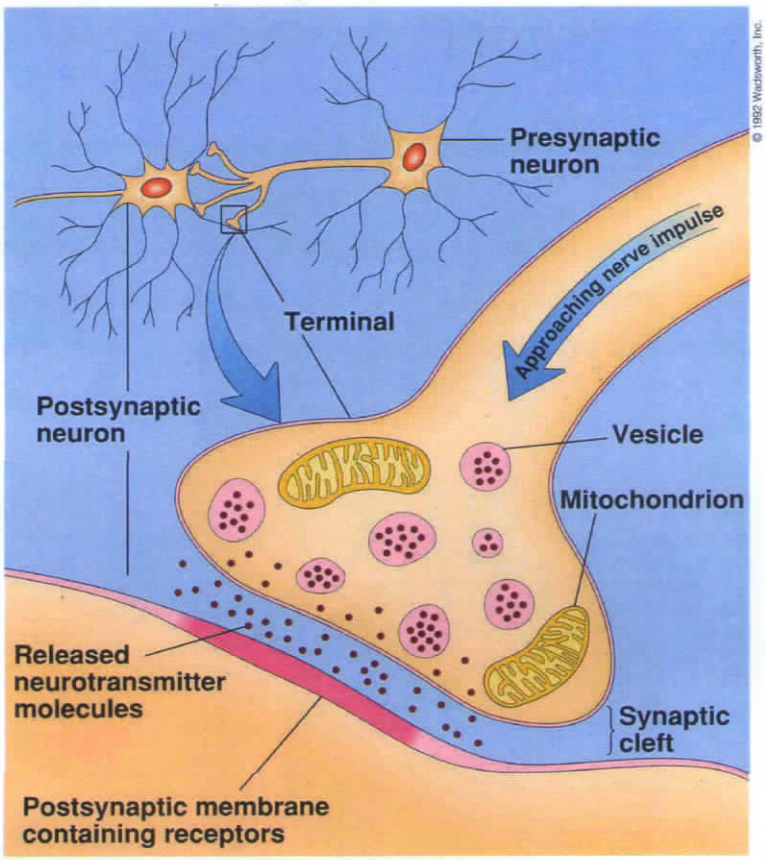

Figure 3: **The synapse** (www.unc.edu)

Synaptogenesis is the process of formation of synapses. It has two distinct developmental stages: synaptic specificity and synaptic assembly [Jin, 2005] (Figure 4).

Synaptic specificity describes the path where synapses form which includes the selection of the correct partner to the formation of synapses at the right subcellular compartment. Synaptic assembly involves the formation of synapses i.e. from the assembly of the macromolecular presynaptic structure to the formation of the postsynaptic specializations.

Proper synapse formation during childhood is essential for human perception, learning, memory, and cognition. Any improper formation or function of these synapses leads to neurodevelopmental disorders, including mental retardation and autism. The organized and reliable assembly, maintenance, and plasticity of synapses are significant to neuronal function. Numerous proteins play significant part in ensuring the reliability of synaptic transmission.

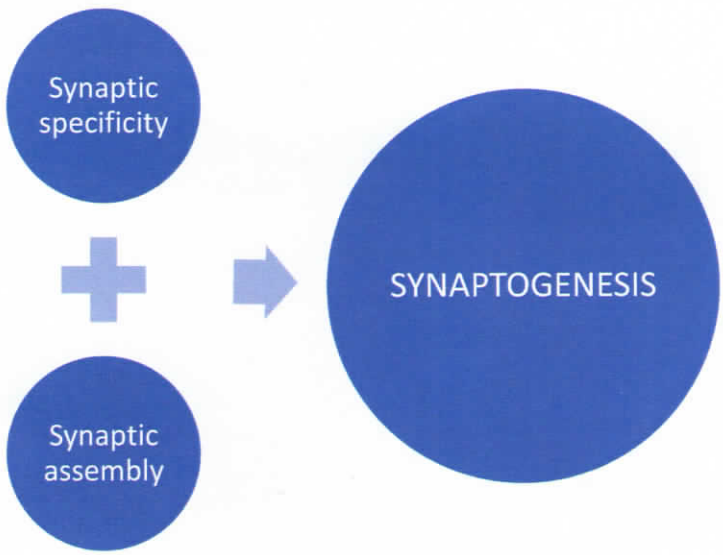

Figure 4: Synaptogenesis

1.2.4 SYNAPTIC VESICLE

The neuronal communication is mediated through the regulated transmission of neurotransmitters from a presynaptic nerve terminal upon the arrival of action potential. Synaptic vesicles (SV) are uniformly minute (~40 to 60 nm radius), abundant organelles which take up and release neurotransmitters. They are concentrated in clusters in defined subplasmalemmal areas of the nerve terminal. Synaptic vesicles undergo a trafficking cycle in the nerve terminal which is divided into [Valtorta & Benfenati, 1995] (Figure 5):

1. Transportation of neurotransmitters into synaptic vesicles.
2. Clustering of synaptic vesicles in front of the active zone.
3. Docking of synaptic vesicles at the active zone.
4. Priming of the vesicles.
5. Opening of the vesicle leading to the release of neurotransmitters.
6. Synaptic vesicles endocytosis and recycling.

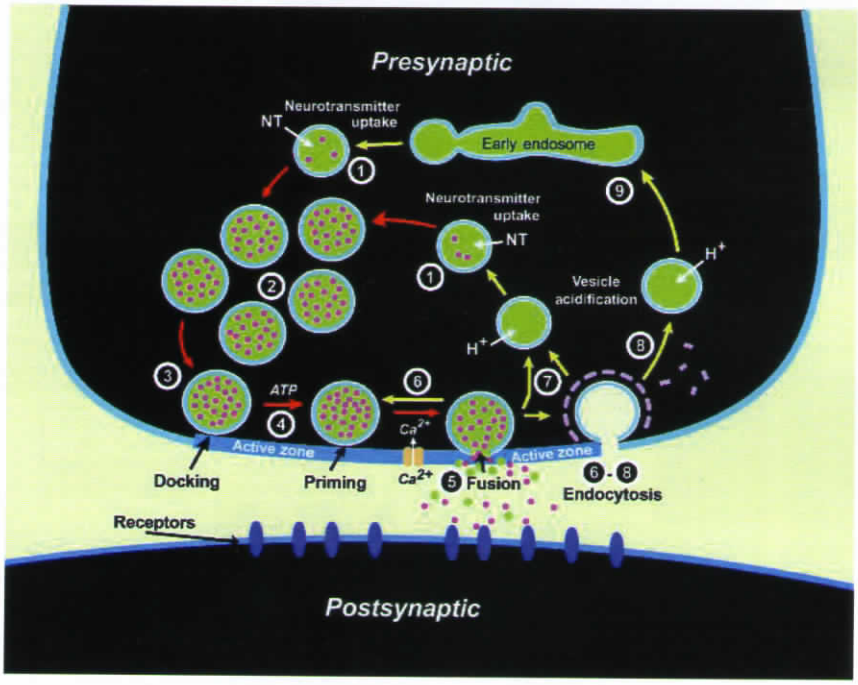

Figure 5: The synaptic vesicle cycle [Sudhof, 2004]

Purified vesicles have a protein:phospholipid ratio of 1:3 [Benfenati *et al.*, 1989]. Synaptic vesicles contain numerous proteins that are critical for their proper localization and calcium-dependent fusion [Sudhof, 2004] (Figure 6). SV proteins involved in synapse maintenance includes Bridging integrator, CSP, Dynamin, Hrs-2, Intersectin, NSF, NT transporter, Pallklin, Rab3, Rabphilin, RIMS binding protein, SCAMP, SV2, Synapsin, Synaptophysin, VAT-1, α-SNAP. SV proteins involved in exocytosis includes CAPS, CSP, Doc2, Rab3, Rabphilin, RIMS binding protein, SV2, synaptogyrin, synaptophysin, synaptotagmin, VAMP, α-SNAP. SV proteins involved in endocytosis include amphyphysin, dynamin, intersectin and synaptotagmin [Brachya *et al.*, 2006].

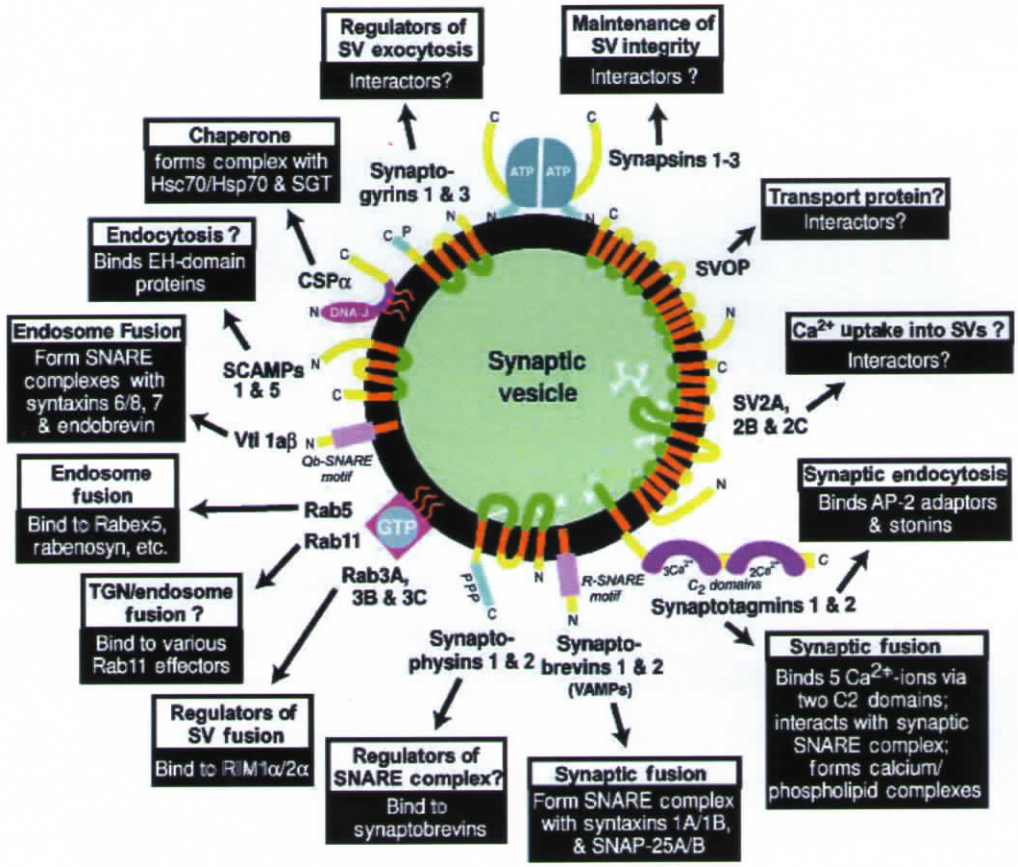

Figure 6: Structures, proposed interactions, and putative functions of synaptic vesicle trafficking proteins [Sudhof, 2004]

1.2.5 SYNAPTOPHYSIN

Synaptophysin (Syp) is an integral membrane glycoprotein selectively and permanently coupled with the membrane of SVs [Valtorta *et al.*, 2004]. Synaptophysin was the first synaptic vesicle protein to be cloned [Leube *et al.*, 1987]. It was discovered in 1985 by Wiedenmann B, et al., and Jahn R et al., independently [Jahn *et al.*, 1985; Wiedenmann & Franke, 1985]. Syp spans the SV membrane four times, with both the NH2- and the COOH-termini on the cytosolic side (Figure 7).

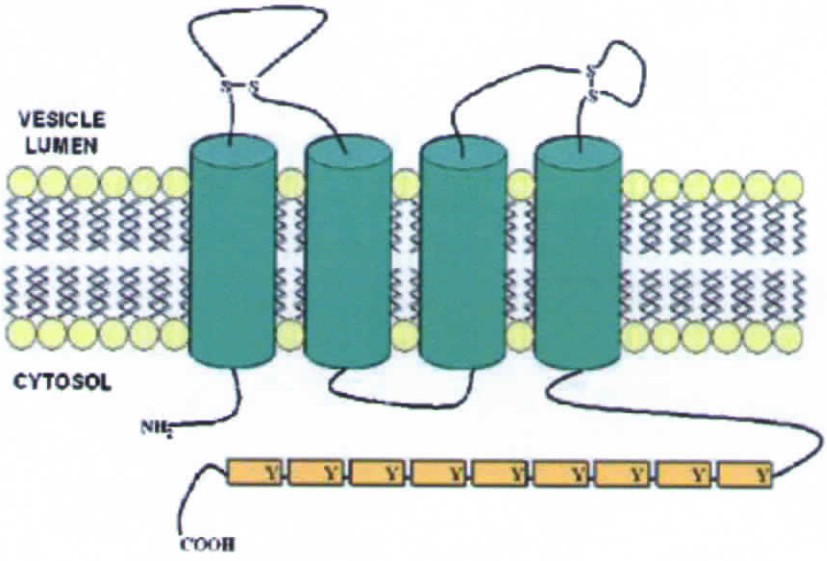

Figure 7: **The tertiary structure of synaptophysin**. [Valtorta *et al.*, 2004]

Synaptophysin is involved in synaptic vesicle exoendocytosis and SV biogenesis [Valtorta *et al.*, 2004] (Figure 8). Syp is said to play a major role in the assembly of specialized domains enriched in SV components in the Golgi membrane. During SV docking, Syp oligomers interact with an unknown plasmamembrane component allowing the release of neurotransmitters during the kiss-and-run mode of SV exocytosis. Syp along with dynamin participate in clathrin independent SV endocytosis [Daly *et al.*, 2000]. Syp has the ability to bind to cholesterol, [Thiele *et al.*, 2000] which is required

for both vesicle biogenesis and the formation of high curvature membrane domains [Hannah *et al.*, 1999].

Synaptophysin is widely used in neurobiology to trace the movements of SVs in living neurons or neuroendocrine cells [Li & Murthy, 2001; Pennuto *et al.*, 2002]; as a immunohistological marker for the determination of the density of synapses in brain disorders, to the diagnose and evaluate neoplasms of the central nervous system and of neuroendocrine or neuroectodermal tumours [Morrison & Prayson, 2000; Wick, 2000].

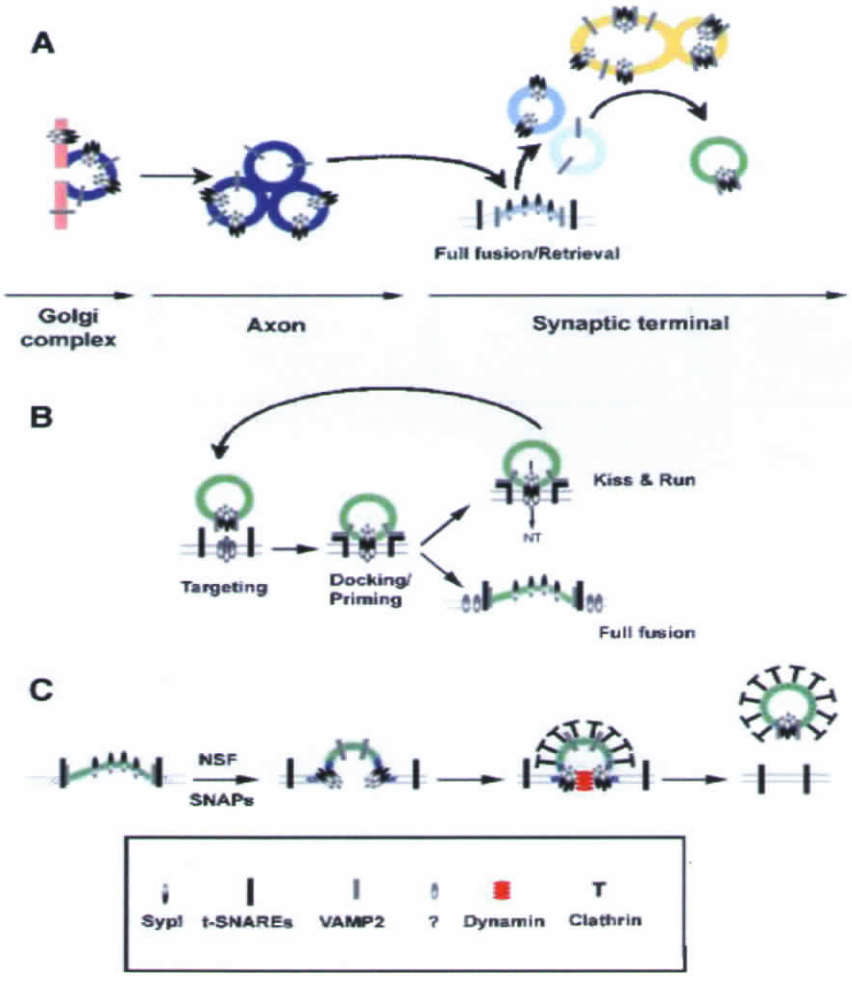

Figure 8 : **Role of synaptophysin in SV exoendocytosis and biogenesis** [Valtorta *et al.*, 2004]

1.2.6 PROTEASE

Among enzymes, protease forms the largest group. Owing to their highly specific mode of action, protease regulates wide variety of biological process including several pathological processes. They are hence called as 'signalling scissors'. Protease is classified based on the amino acid essentially involved in catalytic mechanism (Figure 9).

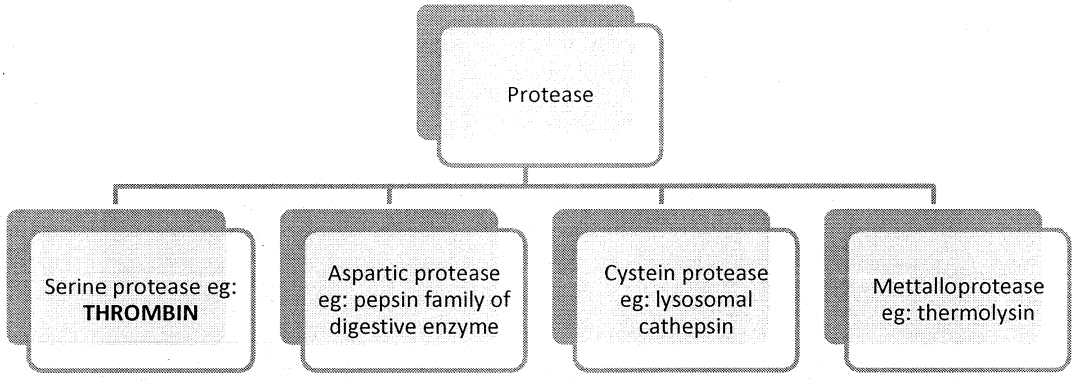

Figure 9: Classification of proteases

1.2.7 THROMBIN

Thrombin is produced in liver as prothrombin and is secreted. Concentration of prothrombin in plasma is about 1-5 μM level. Thrombin is rapidly produced from prothrombin at sites of tissue injury and catalyzes the final steps in blood coagulation. Thrombin converts circulating fibrinogen to fibrin monomer, triggers shape change in platelets and the release of the platelet activators ADP, serotonin, thromboxane A2, chemokines and growth factors, mobilizes the adhesion molecule P-selectin and the CD40 ligand to the platelet surface, activates the integrin αIIb/β3, triggers expression of

procoagulant activity on the platelet surface, regulate blood vessel diameter by endothelium-dependent vasodilation, in fibroblast or vascular smooth muscle cells, regulates cytokine production and is mitogenic, and triggers calcium signalling and other responses in T lymphocytes [Coughlin, 2000].

Thrombin or activated Factor II (IIa), a 39 kDa protein, is a serine protease generated by the cleavage of prothrombin by Factor Xa (Figure 10). It belongs to the peptidase family S1. The prothrombin gene is located on the eleventh chromosome (11p11-q12). The cleavage site of thrombin is Leu-Val-Pro-**Arg-Gly**-Ser. Thrombin cleaves fibrinogen to fibrin. Fibrin is insoluble and forms a mesh-like substance that forms the primary structure of blood clot.

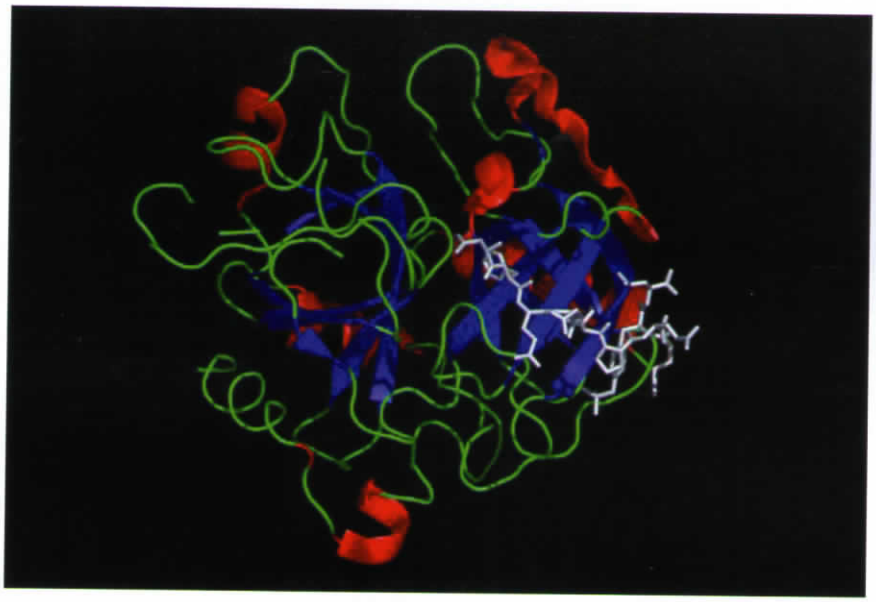

Figure 10: Structure of thrombin

The enzyme prothrombin is found to be produced in the human [Dihanich *et al.*, 1991], rat [Weinstein *et al.*, 1995] and mouse brain [Niclou *et al.*, 1994] especially in the olfactory bulb, cortex, cerebellum, hippocampus, and thalamus. The major factors

influencing the levels of thrombin in the brain are the levels of active thrombin in the blood, the integrity of the blood–brain barrier (BBB), and the levels of thrombin inhibitors in the brain. Physiological relevance of the proenzyme in the brain is not yet established. The ability of the CNS to convert prothrombin to thrombin has not been adequately addressed although Factor X was found to be expressed in the brain [Shikamoto & Morita, 1999]. However, high levels of protease nexin-1 and its mRNA synthesized by glia is found in the brain [Smith-Swintosky VL, 1995]. Sinnreich 2004 found that the formation of thrombin from prothrombin in the nervous system of a transgenic mouse seems required blood-derived factors [Sinnreich *et al.*, 2004]. Prothrombin expression is also seen in neuroblastoma as well as in glioblastoma cell lines [Sheehan & Tsirka, 2005].

Thrombin was the first neurite repellent identified [Hawkins & Seeds, 1989]. It is multifunctional and in the nervous system, depending on the concentration it can support growth or induce apoptosis, and can cause morphological changes. Hence it is a double edged sword. Thrombin activates various cell types including neurons, astrocytes and microglia [Wang & Tsirka, 2005]. Thrombin can induce neurite retraction and synapse reduction [Festoff *et al.*, 2001; Gill *et al.*, 1998], reverses astrocyte stellate morphology in culture and makes them flat and epithelial in shape [Cavanaugh *et al.*, 1990], enhances the synthesis and secretion of NGF in glial cells [Neveu *et al.*, 1993], induce astrocytic apoptosis that could lead to dysregulation of neurotransmitters, loss of structural support and collapse of the blood brain barrier [Sheehan & Tsirka, 2005] or enhance the survival of astrocytes after exposure to cellular insults [Striggow *et al.*, 2000], and retracts neurites on cultured neuroblastoma cells [Gurwitz & Cunningham, 1988] and human fetal neurons [Grand *et al.*, 1989]. Low concentrations of thrombin can induce neurogenesis [Yang *et al.*, 2008] and enhance the survival of hippocampal neurons and astrocytes after exposure to cellular insults including hypoglycemia, growth supplement deprivation, oxidative stress, and β-amyloid toxicity [Pike *et al.*, 1996; Vaughan *et al.*, 1995]. Thrombin also regulates gene expression [Okazaki *et al.*, 1992]. The central nervous

system is also exposed to thrombin upon breakdown of the blood-brain barrier. This occurs in acute trauma such as head injury or stroke and may also occur in chronic neurodegenerative diseases such as Alzheimer's disease [Akiyama *et al.*, 1992; Gingrich & Traynelis, 2000]. High concentration of thrombin within brain parenchyma causes the death of neurons and glia [Smirnova *et al.*, 1998], cause brain edema [Xi *et al.*, 1999], growth cone collapse and neurite retraction [Nurnberg *et al.*, 2008]. Thrombin seems to play an important role is neurological diseases (Table 1).

Table 1: List of neurological diseases where thrombin seems to play important roles.

Neurological diseases	References
Parkinson's disease	[Carreno-Muller *et al.*, 2003]
Alzheimer's disease	[Pike *et al.*, 1996]
HIV encephalitis brains	[Boven *et al.*, 2003]
Cerebral ischemia	[Rohatgi *et al.*, 2004]

Thrombin induces its effect by activating protease activating receptors (PARs) that is found in neuronal cell types. Cell culture studies demonstrating thrombin action on neurons and glia and changes in gene expression suggest a role for thrombin in CNS development, plasticity, and response to injury [Rohatgi T, 2004].

1.2.8 SIGNALING PATHWAY INVOLVED IN THROMBIN ACTION

Thrombin has a critical role in disease conditions like inflammation and injury in central nervous system. One of the cellular targets for thrombin is the protease activated receptors (PARs) [Lee *et al.*, 2000]. PARs maintain a delicate balance between neuroprotection and neurodegeration [Rohatgi *et al.*, 2004]. There are four subtypes of PARs: PAR-1, -2, -3 and -4 and thrombin can activate all of them except PAR-2 [Lee *et al.*, 2000]. Structurally PARs have seven transmembrane domains coupled to G protein

and are activated by proteolytic cleavage of the extracellular fraction of the receptor having an inhibitory site, which varies in amino acid sequences for different PAR subtypes [Vu *et al.*, 1991]. For example, amino acid sequence in the inhibitory site of PAR-4 is GGTQTPSVYDESGSTGGGDDSTPSILPAPR [Xu *et al.*, 1998].

PARs are distributed differentially in central nervous system (CNS). The major regions and cell types were PARs have been located in CNS is listed in the given Table 2.

Table 2: Major regions and cell types where PARs are found.

PAR	Found in	References
PAR-1	Neurons, Astrocyte, Oligodendroglial cells, Microglia, Hippocampus, Cortex, Thalamus, Hypothalamus, Striatum, Amygdale, Dorsal root ganglia, Near capillaries of CNS	[Junge *et al.*, 2004; Niclou *et al.*, 1998; Striggow *et al.*, 2001; Suo *et al.*, 2003; Wang *et al.*, 2004; Weinstein *et al.*, 1995]
PAR-2	Peripheral nervous system during embryogenesis, Primary spinal afferent neurons	[Jenkins *et al.*, 2000; Steinhoff *et al.*, 2000]
PAR-3 and PAR-4	Hippocampus, Cortex, Thalamus, Hypothalamus, Striatum, Amygdale	[D'Andrea *et al.*, 2003; Striggow *et al.*, 2001]

PAR activation has different effects on different types of cells in CNS and this include morphological, physiological, and biochemical variations. Major functional variations are listed in the Table 3.

All four PAR subtypes share high homology and are conserved across species. PAR-1, -2 and -3 genes are located on chromosome 5 while PAR-4 on chromosome 19. Gene structure includes two exons separated by an intron [Kahn *et al.*, 1998]. PAR possess a signal peptide, protease recognition site, protease cleavage site, tethered peptide ligand which binds to extracellular loop 2 for signal transduction, seven transmembrane domains and intracellular domain (Figure 11).

Table 3: Functional Consequences of Thrombin-Mediated PAR Activation in Different Cell Types of the Brain

Brain Cell Type	Consequences of PAR Activation	References
Neurons	Apoptosis; cytoprotection; cell process retraction; glutamate (*N*-methyl-D-aspartate) receptor potentiation	[Hua *et al.*, 2007; Yamazaki *et al.*, 2008]
Astrocytes	Apoptosis; cell proliferation; cytoprotection; endothelin-1 release; increase in cytokine induced nitric oxide (NO) release and iNOS (nitric oxide synthase) expression; loss of stellate morphology; reduced mRNA and protein levels of mGluR5	[Rohatgi *et al.*, 2004; Vaughan *et al.*, 1995; Wang *et al.*, 2002]
Microglia	NO production; potentiation of tumor necrosis factor-α production and release of other cytokines; cell proliferation	[Katsuki *et al.*, 2006; Nakanishi, 2003; Ryu *et al.*, 2000]

PARs possess an irreversible activation mechanism (Figure 12): protease cleavage at a specific site within the extracellular N- terminal region of the receptor allows the six amino acid residues within the N-terminal tethered ligand to bind with the receptor polypeptide chain with the amino acid sequence ITTCHDV that is conserved in PAR1,

PAR2, and PAR3, and sequence of CHD in PAR-4 which lies within the extracellular loop 2 of the receptor protein. This structural modification activates transmembrane signalling [Lee *et al.*, 2000]. In human PAR N-terminal the flanking residues of the cleavage sites contains protease recognition site defined as unprimed (P) and primed (P') sites. The sequence of the tethered ligand domains varies in all the PARs (Figure 13).

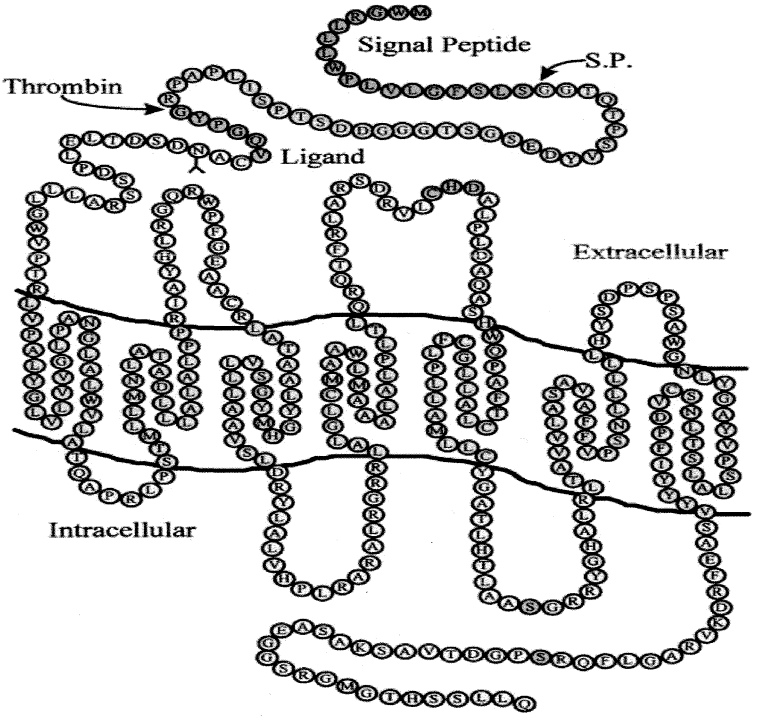

Figure 11: Seven transmembrane structure of PAR-4; green color - signal peptide; yellow - amino-terminal peptide cleaved by thrombin; blue - tethered peptide ligand; gold - seven transmembrane-domain regions; gray - remaining extracellular and intracellular regions; pink - CHD sequence in the second transmembrane loop present in the four known PAR proteins.[Xu *et al.*, 1998].

The intracellular signalling of PAR is coupled to G- protein activation, causing a catalytic exchange of GTP for GDP in the α-subunit of G protein and downstream activation of various ion channels [Rohatgi *et al.*, 2004]. PAR-1 activation has been found to result in inhibition of adenylyl cyclase and thus lowering the cAMP. But, most frequently, PAR activation results in Gq protein linked phospholipase (PLC) activation [Nurnberg *et al.*, 2008] and release of inositol (1,4,5) trisphosphate (IP3) and diacylglycerol (DAG).

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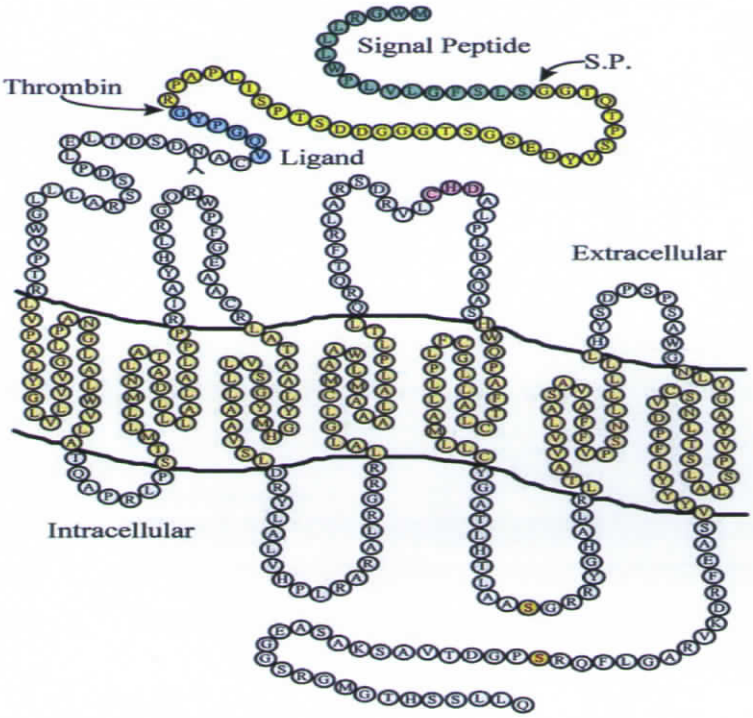

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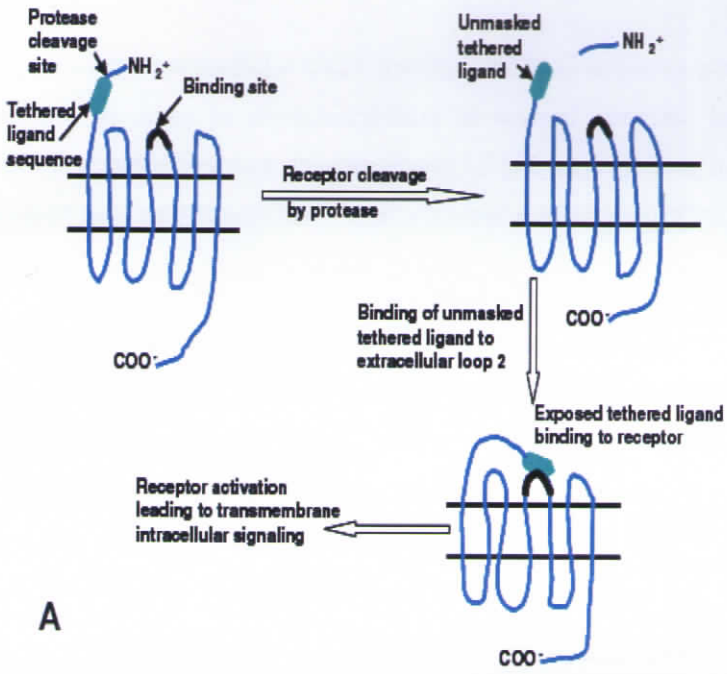


Figure 12: PAR structure and activation mechanism [Rohatgi *et al.*, 2004]

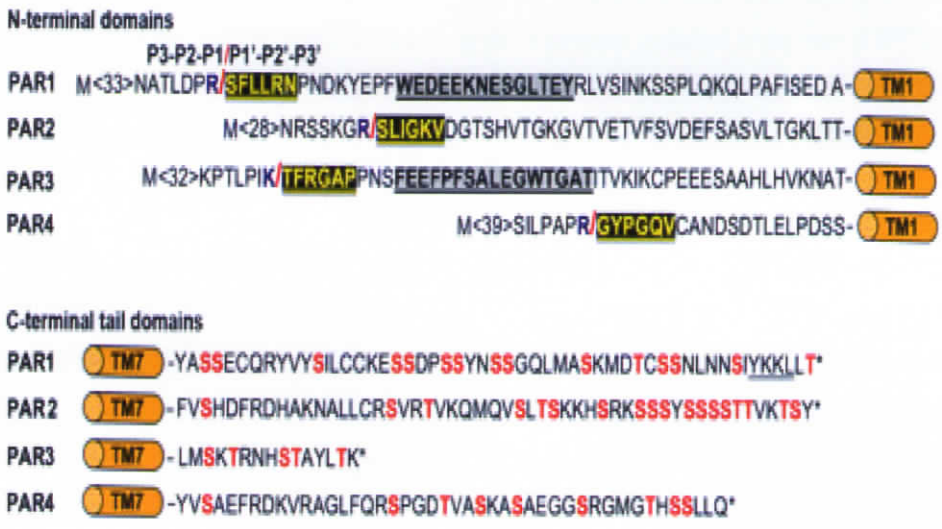


Figure 13: Human PAR N-terminal and C-terminal sequences. P3-P2-P1 – unprimed sequences; P1'-P2'-P3'- primed sequences; / - cleavage site; sequences in yellow - the tethered ligand domains; * - end of the PAR sequences [Arora *et al.*, 2007]

Generation of IP3 alters the intracellular Ca2+ levels, and DAG activates protein kinase C (PKC), an enzyme involved in phosphorylation of several proteins. In astrocytes thrombin stimulates calcium-dependent phospholipase A2 (PLA2) resulting in the release of the polyunsaturated fatty acid, arachidonic acid a second messenger in CNS [Strokin *et al.*, 2003].

PAR-1 signalling also results in activation of mitogen-activated protein kinases (MAPK). MAPKs have a central role in cellular growth and proliferation and are involved in phosphorylation of transcription factors, cytoskeletal proteins, and other protein kinases [Rohatgi T, 2004]. MAPK activation has been reported in astrocytes stimulated by thrombin and/or activated PAR-1 [Wang *et al.*, 2002]. PAR-1 downstream pathway can activate pertussis toxin (PTX)-sensitive Gi protein ($\beta\gamma$-subunits) which inturn activates phosphatidylinositol 3-kinase and ultimately results in MAPK phosphorylation [Wang *et al.*, 2002]. Besides, thrombin mediated PAR-1 activation in cultured rat astrocytes resulted in phosphorylation of proline-rich tyrosine kinase-2 (Pyk2) [Wang & Reiser, 2003]. Pyk2 interacts with G protein coupled receptors (GPCRs) and allows MAPK activation by recruiting several mediator proteins, such as Src, Shc, and Grb2 [Lev *et al.*, 1995]. PAR-1–mediated increase in intracellular calcium also occurs via PTX insensitive G protein [Wang *et al.*, 2002]

Cytosolic brain isoform of creatine kinase found to interacts with the C-tail of PAR-1 [Mahajan *et al.*, 2000]. Creatine kinase is an ATP-generating enzyme that regulates ATP homeostasis within subcellular compartments. Cytosolic creatine kinase provides energy in the form of ATP, required for PAR-1 signal transduction via $G\alpha 12/13$ and RhoA leading to localized protein phosphorylation, actin polymerization and actomyosin contraction [Mahajan *et al.*, 2000; Seasholtz *et al.*, 1999]. Activated RhoA activates Rho kinase which phosphorylates myosin light chain and regulatory subunit of myosin light chain phosphatase and thus inhibiting the enzyme [Kimura *et al.*, 1996]. Phosphorylated MLC in turn interacts with actin leading to actin rearrangement thus inducing neurite retraction in neuronal cell types. (Figure 14)

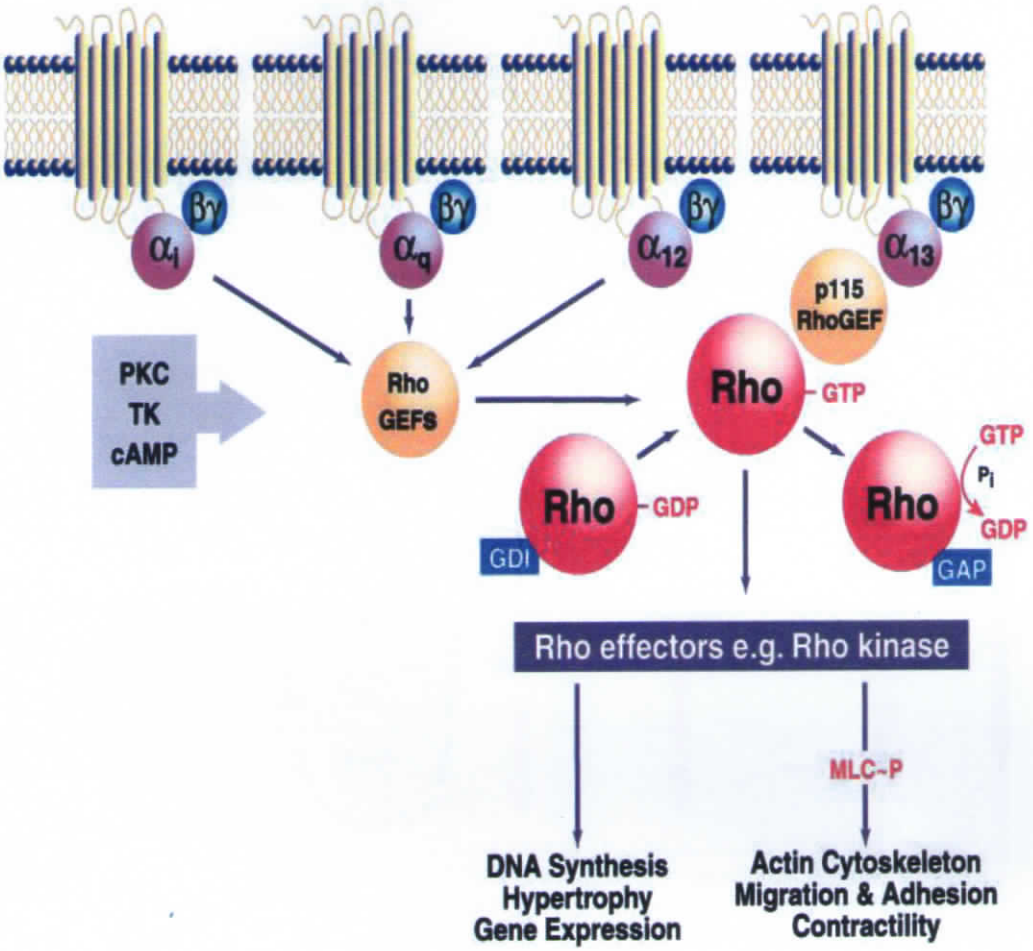


Figure 14: Signalling pathway of Rho activated by GPCR.[Seasholtz *et al.*, 1999]

Another protein interacts the C-tail of PAR-1 is Hsp90 [Pai *et al.*, 2001]. The change in astrocyte morphology and hence cytoskeletal rearrangement upstream of RhoA activation was revealed to be mediated via the PAR-1-Hsp90 complex. Figure 15 shows signaling pathways regulated through activation of protease-activated receptor (PAR)-1.

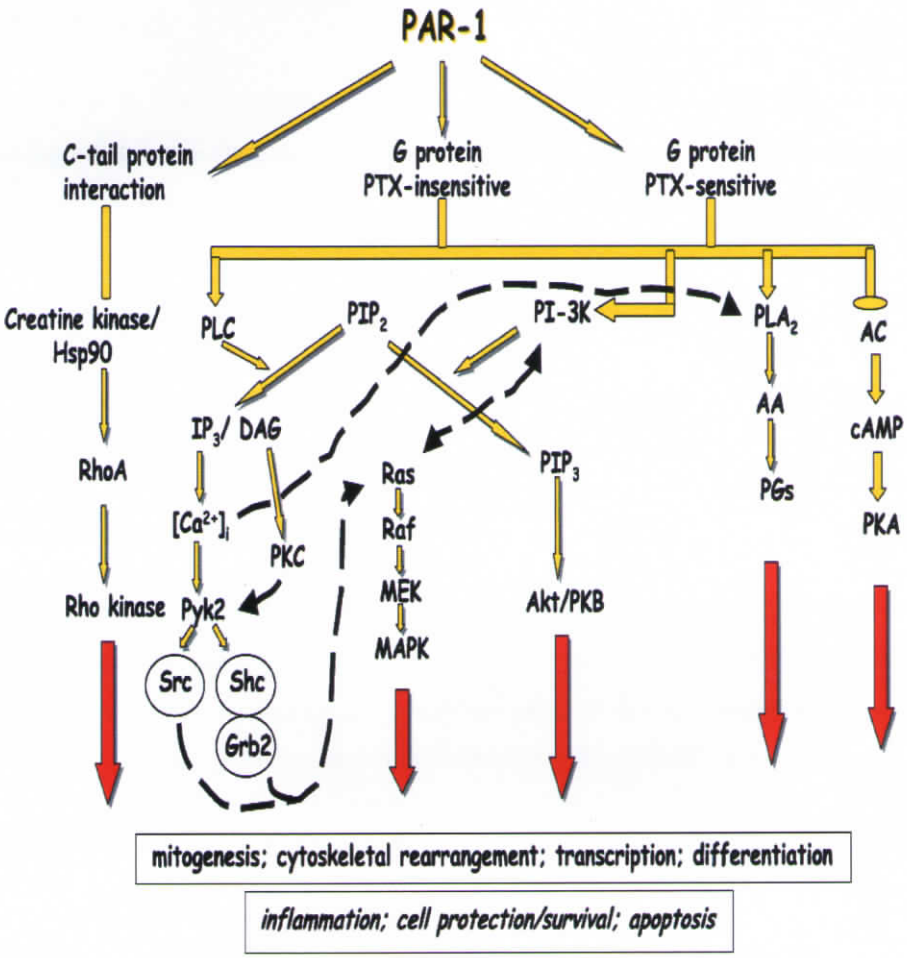


Figure 15: Signaling pathways regulated through activation of protease-activated receptor (PAR)-1 [Rohatgi *et al.*, 2004]

In the presence of thrombin, neuritic projections like axons and dendrites found to retract in its growth. Removal of thrombin found to results in regained growth of neural cells [Festoff *et al.*, 2001].

1.3 HYPOTHESIS

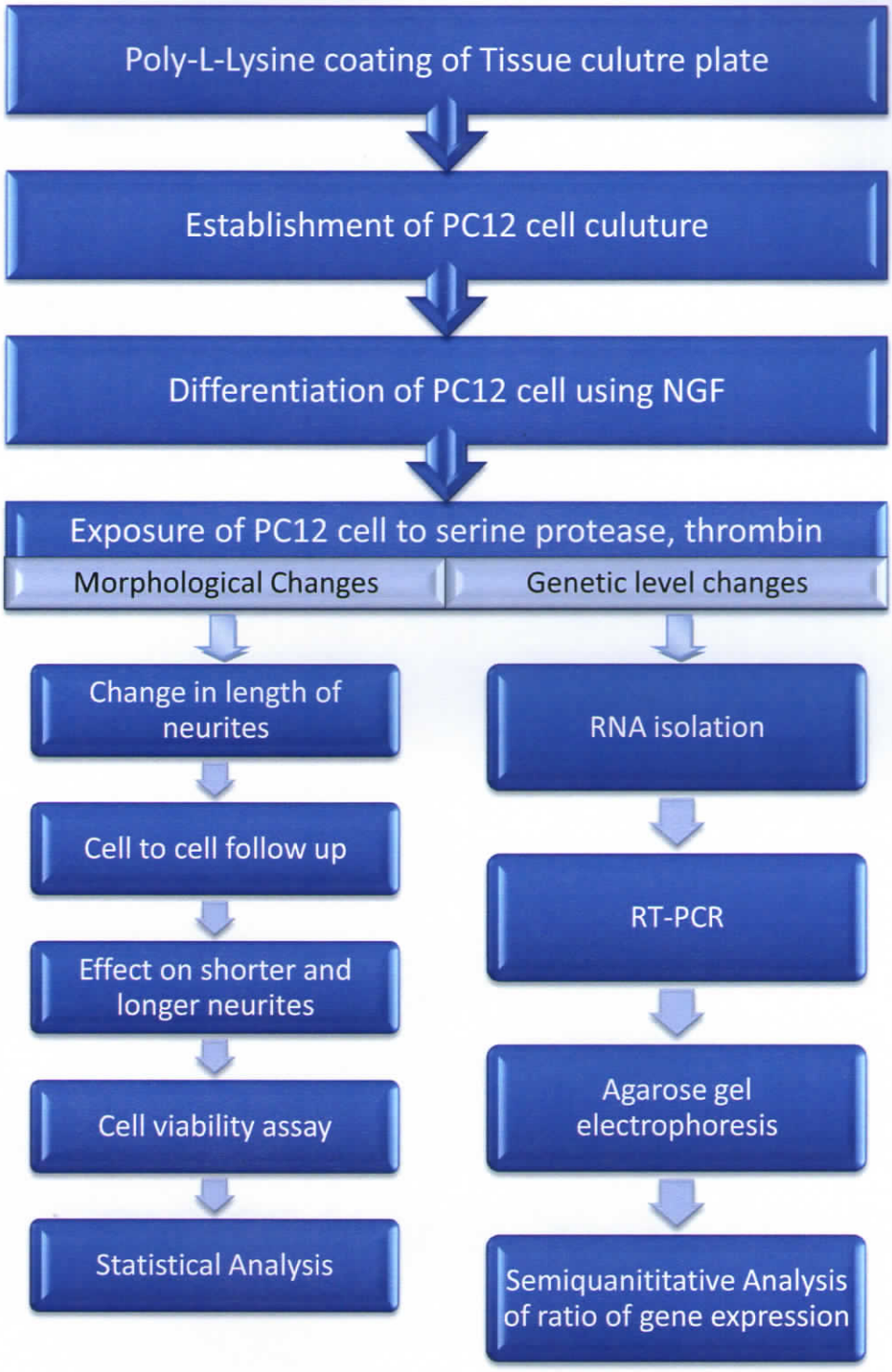
Thrombin at low concentration has neuroprotective effects [Striggow *et al.*, 2000]. We hypothesiszed that, thrombin at a low concentration might have an effect in neuromodulation in general and neuritic sprouting in specific. Such physiological effects can provide neurons a time-window to alter the network patterns in an injured site. This will help the CNS to recover to an extend from intracranial hemorrhages.

1.4 OBJECTIVES OF THE STUDY

Objectives of the study were:

1. Establish PC12 cell line as a *in vitro* model system for neuronal injury studies
2. Measurement of neuromodulative effects of thrombin at various concentrations
3. Gene expression variations of Synaptophysin, a critical presynaptic protein involved in the formation of functional synapse and neuronal networking.

Figure 16: FLOWCHART OF WORK PLAN



MATERIALS AND METHODS

CHAPTER II

MATERIALS AND METHODS

2.1 MATERIALS USED

Poly-L-Lysine hydrobromide (Sigma), Sterile tissue culture plate (Axygen), Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12 Ham (DMEM/F-12, 1:1 Mixture) (HIMEDIA), Fetal Bovine Serum (PAN), Fetal Horse Serum (PAN), Penicllin G sodium salt (Sigma), Streptomycin sulphate (Gibco), Forma Direct Heat CO_2 Incubator (Thermo Scientific), Nerve growth factor-7S (Sigma), Olympus IX51 inverted microscope with Qimaging Rolera-XR Fast 1394 camera, Hoechst 33342, trihydrochloride, trihydrate (Invitrogen), TRI Reagent (Sigma), 1.5 mL Microtubes (Axygen), Ultra-turrax Homogenizer (Ika Labritechnik), Chloroform GR (purity level 99.0-99.4%) (Merck), Centrifuge 5415R (Eppendorf), Isopropanol (purity level 99.5%) (Sisco Research laboratory Pvt Ltd.), Sodium chloride AR (HIMEDIA), Trisodium citrate AR (purity level 99%) (Sisco Research laboratory Pvt Ltd.), GeneRuler DNA Ladder Mix (10000 bp marker) (MBI Fermantas), Horizontal gel electrophoresis apparatus (Owl Pvt Ltd), UVIpro Platinum Gel documentation System (Uvitec), reagents for RT-PCR and PCR was from MBI Fermantas.

2.2 POLY-L-LYSINE COATING OF TISSUE CULTURE PLATE

Polylysine coating of culture plate enhances PC12 cell attachment. 1.5mL of 1X polylysine was added to sterile tissue culture plate. It was kept overnight at room temperature. Excess polylysine was removed and the well was rinsed with 1X PBS. 2 mL of DMEM:F12 complete media was added and the plate was kept at 37°C /5% CO_2 in the Forma Direct Heat CO_2 Incubator till the cells were seeded.

2.3 ESTABLISHMENT OF PC12 CELL CULTURE

PC12 cells a gift from the Neural Stem Biology Lab, RGCB were seeded onto polylysine coated tissue culture plate containing DMEM:F12 complete media, under aseptic condition. The culture plate was incubated at 37°C /5% CO_2.

2.4 DIFFERENTIATION OF PC12 CELLS

PC12 differentiates into sympathetic like neurons in response to nerve growth factor (NGF) [Greene & Tischler, 1976]. After 22-24 h of incubation of PC12 at 37°C /5% CO_2, the DMEM:F12 complete media was changed with 2 mL of DMEM:F12 PC12 differential media. NGF with a final concentration 100 ng/mL was added to the culture plate. Culture plate was incubated at 37°C /5% CO_2.

2.5 EXPOSURE OF DIFFERENTIATED PC12 CELLS TO THROMBIN

Thrombin, a gift from Thrombosis Research unit, SCTIMST, is a serine protease involved in the coagulation cascade of blood. After 22-24 h of incubation at 37°C /5% CO_2 of PC12 cells treated with NGF, cells were exposed to thrombin at different concentrations.

Cells	Thrombin (IU)
Control	0
Test 1	0.5
Test 2	1

The culture plate was incubated at 37°C /5% CO_2 for 4 days without changing media or added components. Images were taken using Olympus IX51 inverted microscope with Qimaging Rolera-XR Fast 1394 camera, under 20X objective with 22-24 h intermittence i.e.

Day 1: 22-24h after adding NGF in control and test but just before the addition of thrombin in test cells

Day 2: 46-48 h after adding NGF in test and control and 22-24 h after adding thrombin in test cells

Day 3: 70-72 h after adding NGF in test and control and 46-48 h after adding thrombin in test cells.

Day 4: 94-96 h after adding NGF in test and control and 70-72 h after adding thrombin in test cells.

2.6 QUANTIFICATION OF NEURITE OUTGROWTH

Length of neurite processes were quantified at pixel intensity using Image Processing And Analysis in Java (ImageJ) software from the pictures taken on day 1-4 of control and test using Olympus IX51 inverted microscope with Qimaging Rolera-XR Fast 1394 camera, under 20X objective.

2.7 CELL VIABILITY ASSAY USING HOECHST STAIN

The Hoechst dyes are cell permeable nucleic acid stains that are used for the viability measurements by monitoring the emission spectral shifts of the dye. At the end of Day 4, control and test cells were checked for viability using Hoechst stain. The media was removed and the cells were washed with 1X PBS. 700 µL of working solution of Hoechst stain was added and kept for 2 min. The cells were again washed two times with 1X PBS. Viability of the cells were viewed and images were taken using Olympus IX51

inverted microscope with Qimaging Rolera-XR Fast 1394 camera under 20X objective in violet filter (Excitation: 350nm and Emission: 461nm).

2.8 RIBONUCLEIC ACID (RNA) ISOLATION

RNA was isolated from control and thrombin treated PC12 cells [Sambrook & Russell, 2001] to study the gene level expression of synaptic vesicle protein. Both control and thrombin treated cells were washed with 1X PBS and 1 mL of TRI Reagent was added separately. It was kept for 10 min at room temperature and then transferred to sterile 1.5 mL Microtubes. The mix was subjected to homogenization for 15 s / 2 times using Ultra-turrax Homogenizer. The microtubes were kept at room temperature for 10min. 0.2 mL of Chloroform was then added and mixed well. The tubes were then centrifuged at 10,000 g/15 min/4˚C using Eppendorf Centrifuge 5415R. The aqueous layer was transferred to sterile 1.5 mL Microtubes. To that 250 µL of isopropanol and 250 µL of RNA precipitation solution was added and kept for 30 min at 4˚C. The solution was centrifuged at 10,000 g/10 min/4˚C. Supernantant was discarded and the pellet was washed with 500 µL of 75% ethanol. It was again centrifuged at 10,000 g/10 min/4˚C. Pellet was air dried and resuspended in sterile millipore water. The RNA was stored at -20˚C until use.

2.9 REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

RT-PCR is used to determine the abundance of specific different RNA molecules within a cell or tissue as a measure of gene expression. RT-PCR for β-actin (control gene) and synaptic vesicle protein, synaptophysin has been done to analyze the effect of thrombin on gene level expression.

Primer used:

Gene	Reverse primer	Tm	%GC
β-actin	5'AGCCACCAATCCACACAGAGTA'3	61.9°C	50
Synaptophysin	5'CTCGAGGACCAGATTACATCTGATTGG'3	67°C	41.1

Method:

In the reaction, 4 µL of the template RNA was denatured in the presence of 1 µL of reverse primer and immediately chilled on ice. After adding 4 µL of 5X reaction buffer for M-MuLV RT, 2mM of dNTP mix, 15 U of ribonuclease inhibitor, the reaction was continued at 37°C/ 5 min. 100 U of RevertAid M-MuLV reverse transcriptase enzyme was then added and the reaction mixture was incubated at 42°C/60 min. The reaction was stopped after keeping at 70°C/10 min.

cDNA synthesized was amplified by using the following primers

Primer used:

Gene	Primer	Tm	%GC	Product Size
β-actin	5'AGCCACCAATCCACACAGAGTA '3 (reverse primer) 5'GCCAACCGTGAAAAGATGAC'3 (forward primer)	61.9°C 59.6°C	50 47.4	691bp
Synaptophysin	5'CTCGAGGACCAGATTACATCTG ATTGG'3 (reverse primer) 5'CTCGAGATGGACGTGGTGAATC' 3 (forward primer)	67°C 64.2°C	48.1 54.5	941bp

The reaction mixture of 25 µL contains:

Sterile Millipore water	7.5 µL
Forward primer	1 µL
Reverse primer	1 µL
dNTP mix	2 µL
25mM $MgCl_2$	2 µL
10X PCR buffer	2.5 µL
Taq polymerase	2 µL

cDNA 8 µL

PCR condition:

β-actin		Synaptophysin	
94°C 4minutes		94°C 10minutes	
94°C 45seconds	35 cycles	94°C 30seconds	35 cycles
58°C 45seconds		60°C 1minute	
72°C 1minute		72°C 1minute	
72°C 5minutes		72°C 15minutes	

2.10 AGAROSE GEL ELECTROPHORESIS

PCR products of the β-actin and synaptophysin were run on agarose gel electrophoresis based on the standard protocol [Sambrook & Russell, 2001]. 1% agarose was prepared in 1X TAE buffer with final concentration of 0.5 µg/mL ethidium bromide. Sample DNA was mixed with 6X gel loading dye and was run at 70 V along with GeneRuler DNA Ladder Mix (10,000 bp marker) in horizontal gel electrophoresis apparatus. Gel was documented using UVIpro Platinum Gel documentation System.

2.11 SEMI QUANTITATIVE ANALYZES OF GENE EXPRESSION

The band intensities were calculated by densitometric analysis using UVIpro platinum software (UVIpro UK).

2.12 STATISTICAL ANALYSIS

Statistical analysis was carried out by unpaired t test using Graphpad software. $P < 0.05$ was considered as statistically significant value.

RESULTS AND DISCUSSION

CHAPTER III

RESULTS AND DISCUSSION

3.1 ESTABLISHMENT OF PC12 CELL CULTURE

PC12 cells seeded on polylysine coated plate has attached well (Figure:17.A). Surfaces treated with polylysine appear to promote cell adhesion by altering the surface charge on the substrate and this is essential for PC12 cell growth and proliferation [Letourneau, 1975]. In the absence of polylysine the cell-attachment was limited and excessive floating of the cell were noted (Data not shown). All the experiments were carried out in polylysine coated plates.

3.2 NGF INDUCES DIFFERENTIATION OF PC12 CELLS

PC12 cells were differentiated into sympathetic like neurons in response to nerve growth factor (NGF) (Figure: 17.B). The biological effects of NGF are usually observed at 5 to 100 ng/mL [Darling & Shooter, 1984]. The neuronal extensions were observed within 8 hours of NGF addition. The neuritic extensions were growing for upto Day 3 (Figure: 17.B-E). Mitotic division of the cells was also arrested in the presence of NGF [Greene & Tischler, 1976]. On Day 4 the neuritic extensions showed no additional growth and few cells even showed a retraction of neurites. This may be due to single treatment of NGF in all these cells; and the effect of single NGF treatment last for around 100 hours. It is known that PC12 cells retreat back from the neuronal morphology when NGF is withdrawn from the media [Greene & Tischler, 1976]. We confined to single NGF treatment mainly to avoid additional induction of neuronal sprouting due to the growth factor in the experimental conditions. Based on these results, we decided to conduct the thrombin treatment and follow-up for a maximum period of 3 to 4 days.

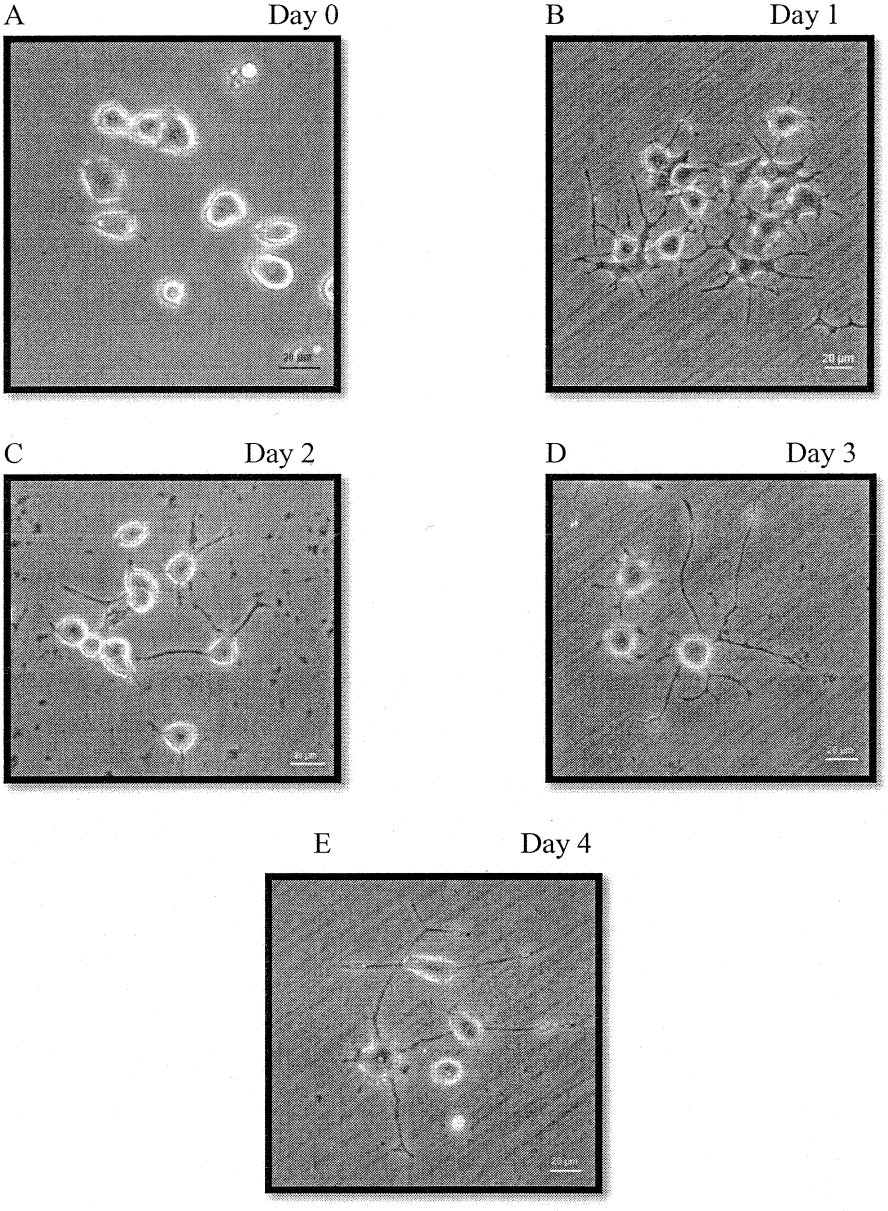


Figure 17: Establishment of PC12 cell culture and its differentiation into neuron like cells in the presence of NGF. Images were taken using Olympus 1X51inverted microscope with Qimaging Rolera-XR Fast 1394 camera under 20X phase contrast objective. Day 0: undifferentiated PC12 cells; Day 1: 22-24hr after adding NGF; Day 2: 46-48hr after adding NGF; Day 3: 70-72hr after adding NGF; Day 4: 94-96hr after adding NGF. NGF concentration: 100ng/mL

3.3 PLASTICITY OF NEURITIS AND CELL VIABILITY IN PRESENCE OF THROMBIN

Differentiated PC12 cells on exposure to 0.5 IU and 1 IU thrombin showed neuritic retraction on a concentration dependent manner. Figure 18 shows the overall length of neuritis in control and test group that was measured using ImageJ software. In the control group, there is a constant increase in number and length of sprouting. Compared to the control group, there is 14% (P = 0.02) and 20% (P =0.001) decrease in the rate of growth of neurites in the presence of 0.5 IU and 1 IU thrombin respectively (Figure 19).

The rate of axonal growth of an average of 600 neurons from control and thrombin treated were measured. The data showed a 14 and 20 percentile decrease in neuritic growth on day 4 in the presence of 0.5 and 1 IU thrombin, respectively. The data also suggested that 1 IU thrombin has a persistent effect on neurons compared to 0.5 IU; the later condition showed the neuronal growth can extend further with time. However in 1 IU thrombin condition had a significant effect in growth rate of neurites (Figure 19). To get a better understanding on the rate of growth of neuritic sprouting, cell to cell follow up was carried out in all the three groups (control, 0.5 and 1 IU thrombin) (Figure: 20). The data showed that the effect of thrombin in neuritic growth is highly significant and is concentration dependent (Figure: 21).

Neuron has two kind of neuritic extensions: axons and dendrites. Axons are longer projections from the cell body compared to the numerous dendrites which act as the post synaptic terminal for neuronal networking. Differentiated PC12 cells also produce neurites of different length to be destined as axons and dendrites (Figure: 22). As expected, number of shorter neurites always outnumbered the longer ones (Figure: 22). Presence of thrombin has shown differential effects on shorter and longer neurites. Shorter neurites showed a increase in length (4-5%) (P=0.0001) compare to control (Figure 23) while longer neurites showed ~ 15% (P=0.44) decrease in neurite length in the presence of 1 IU thrombin (Figure 24). This result suggests that there is a significant

morphogenic effect of thrombin on neurons, mainly in dendtritic extension. Often during neuronal injury axons are the most affected extensions. One of the adaptative changes in neurons after an injury is to withdraw the injured axon and commit one of the dendrites as new axon. Our results suggested that both axonal and dendritic extensions are affected in the presence of thrombin. Increase in dendritic length is intriguing observation as such changes occurs only when there is a extensive variation in neuronal networking and axonal retrieval. Though we have not found statistically significant decrease in axonal length, we hypothesized that axonal modifications might have been set-in in the presence of thrombin (see section 3.4).

Proteases have an impact on neuronal and glial differentiation and plasticity because of their capacity to amend intracellular biochemistry, regulate process outgrowth, and induce cell migration and proliferation [Monard, 1988]. The double edged sword, thrombin, if controlled, has the potential to increase plasticity of the CNS. Presence of prothrombin mRNA in the olfactory bulb, the cortex, the cerebellum, and other regions of the rat and human nervous system and in neural cell lines has been confirmed [Dihanich *et al.*, 1991]. Cellular architecture and normal functions can be maintained only by regulating the cell morphology. Axonal growth occurs by navigating complex cellular environments with the help of guidance receptors in growth cone. Growth cone collapse and thus neurite retraction are caused by thrombin by activating thrombin receptors [Nurnberg *et al.*, 2008]. This supports our observation that axonal sprouting is more affected than the dendrites because the growth cone density is mainly attributed to the growing axons. It is also known that PARs undergo densentization, internalization and resensitization [Hoxie *et al.*, 1993]. After activation these receptors undergo desentization and internalization. Resensitization occurs by the synthesis of new receptors. High concentration and continued presence of thrombin is required for its neurotoxic effects as a critical threshold signal should be reached or else it will only have protective effect [Donovan & Cunningham, 1998].

A

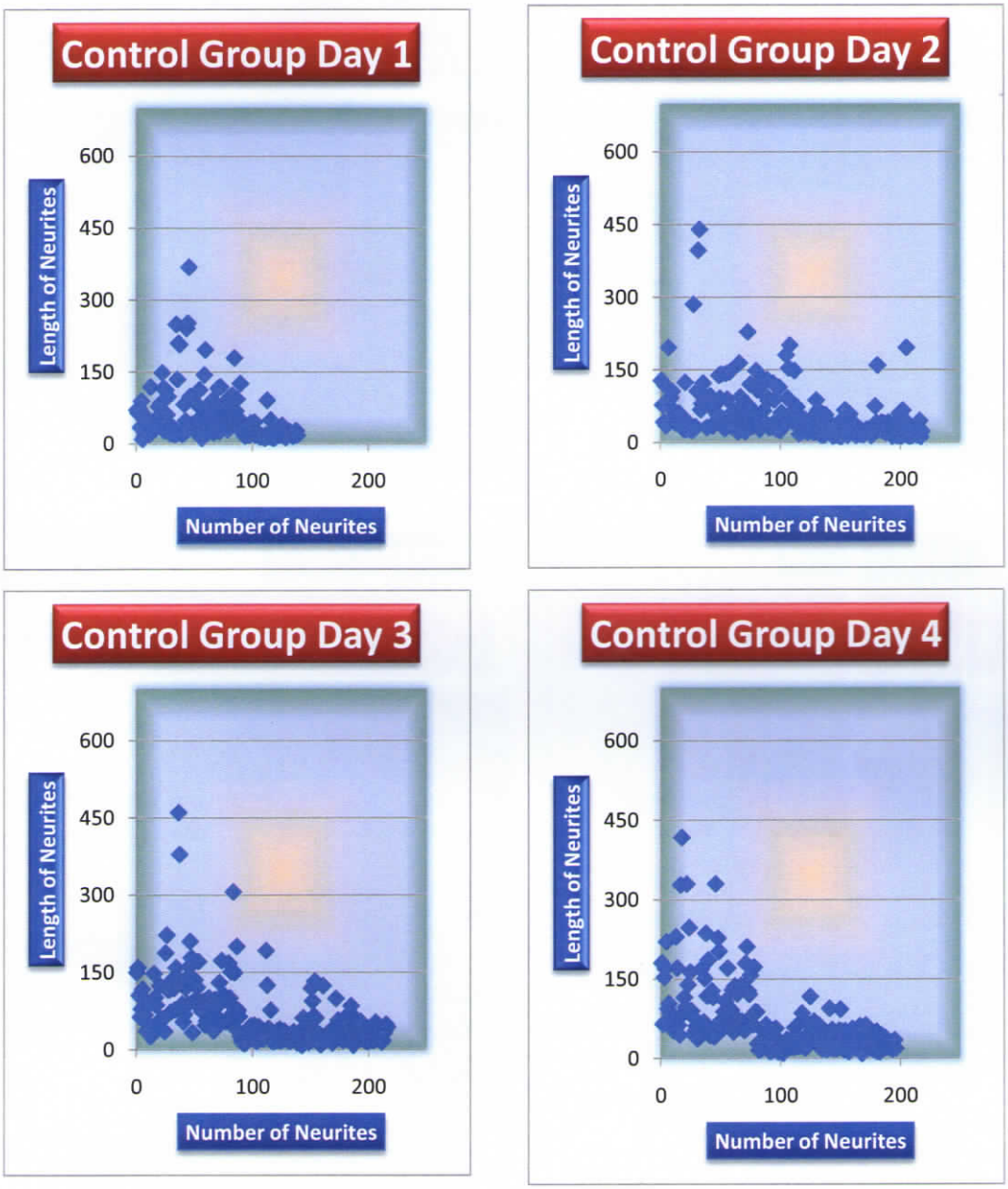


Figure 18: Scatter plot diagram of all the neurite length of control and test group measured using ImageJ software. Each graph shows the length of neurite measured on each day. **A:** Control group; **B**: Test group 1; **C**: Test group 2. Data from three independent experiments. Length is in arbitrary unit.

B

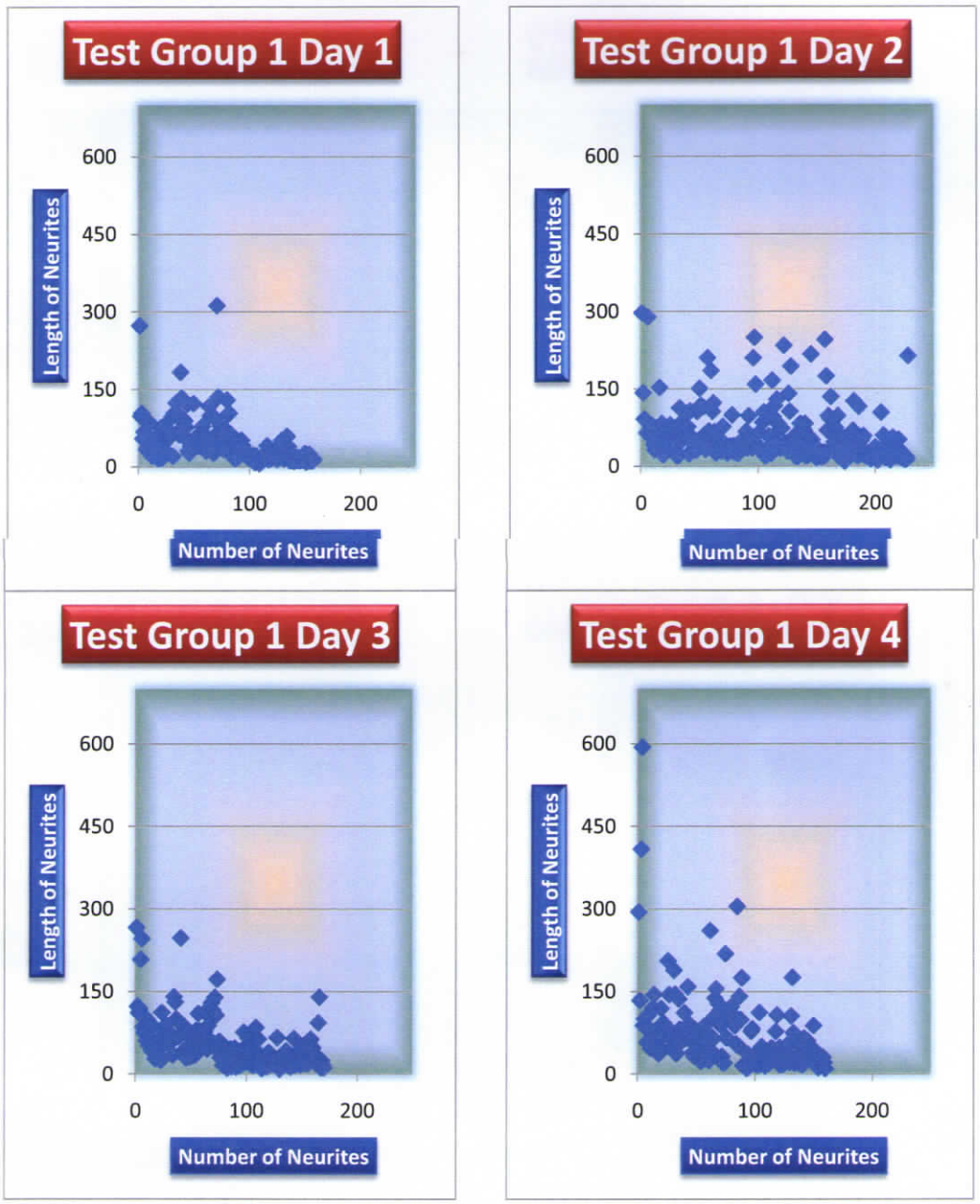


Figure 18: Scatter plot diagram of all the neurite length of control and test group measured using ImageJ software. Each graph shows the length of neurite measured on each day. **A:** Control group; **B**: Test group 1; **C**: Test group 2; Data from three independent experiments. Length is in arbitrary unit.

C

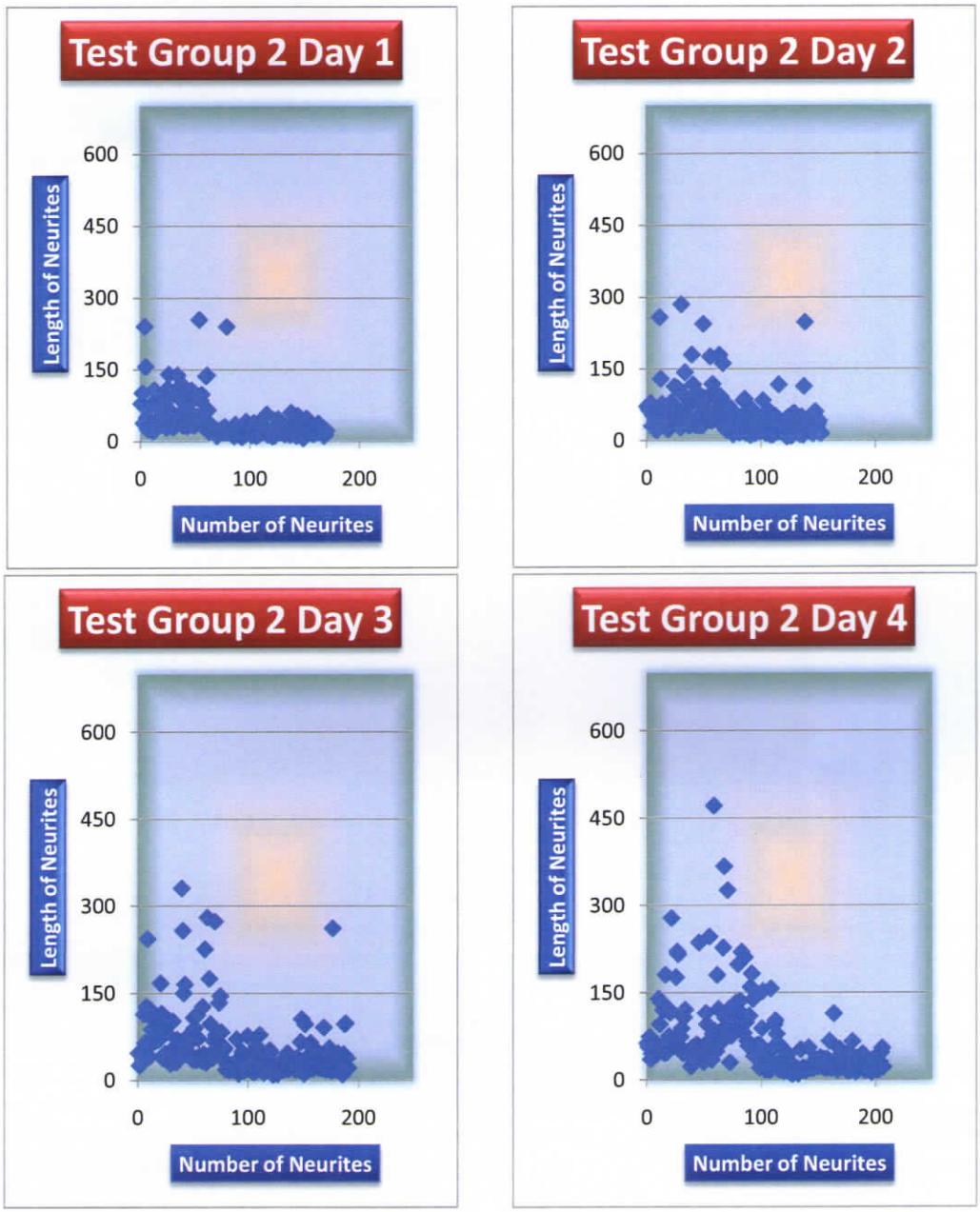


Figure 18: Scatter plot diagram of all the neurite length of control and test group measured using ImageJ software. Each graph shows the length of neurite measured on each day. **A:** Control group; **B**: Test group 1; **C**: Test group 2. Length is in arbitrary unit. Data from three independent experiments.

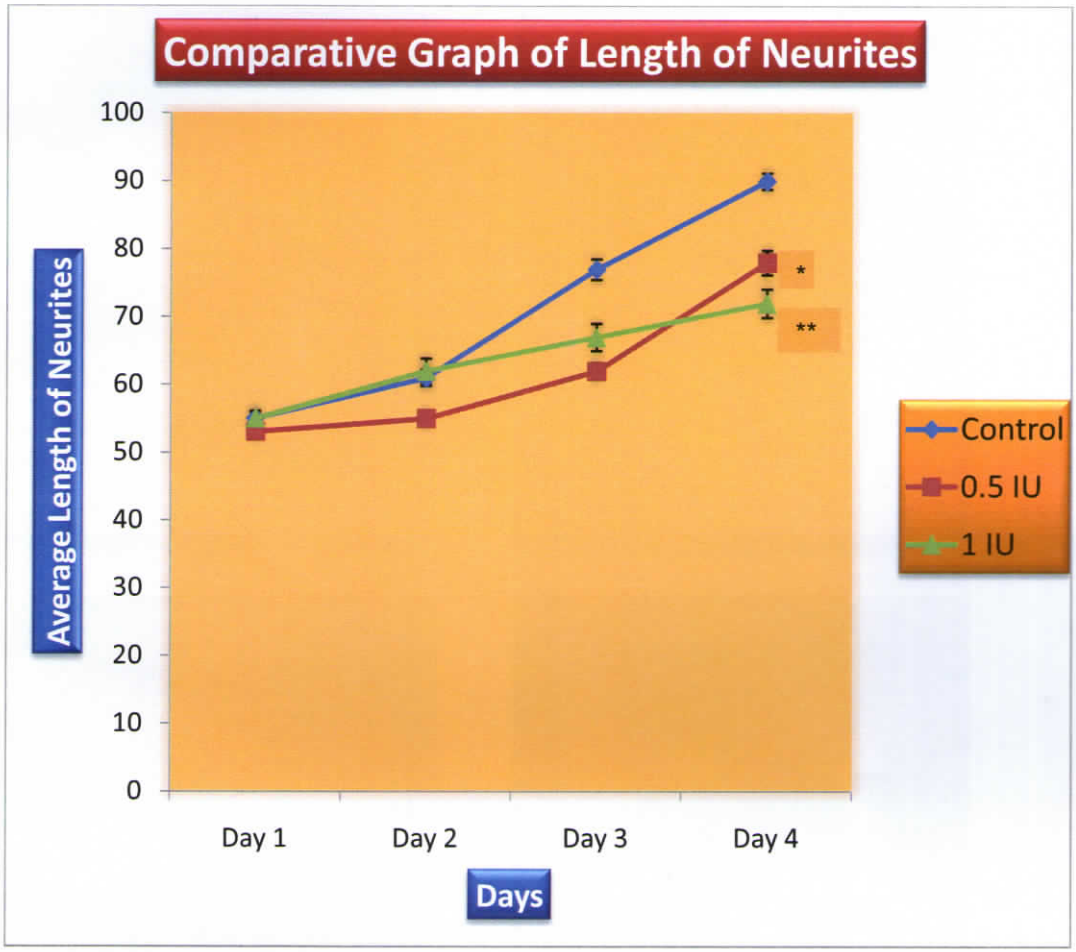


Figure 19: Comparative graph showing the average length of the sprouting of PC12 cells in all the groups. There is 14% and 20% decrease in the rate of growth of neurites in the presence of 0.5 IU and 1 IU thrombin respectively compared to control group on Day 4. Each data point is the mean of three independent experiments. Error bar: ±SEM; * P = 0.02; ** P =0.001 compared to day 4 control.

A: Control group

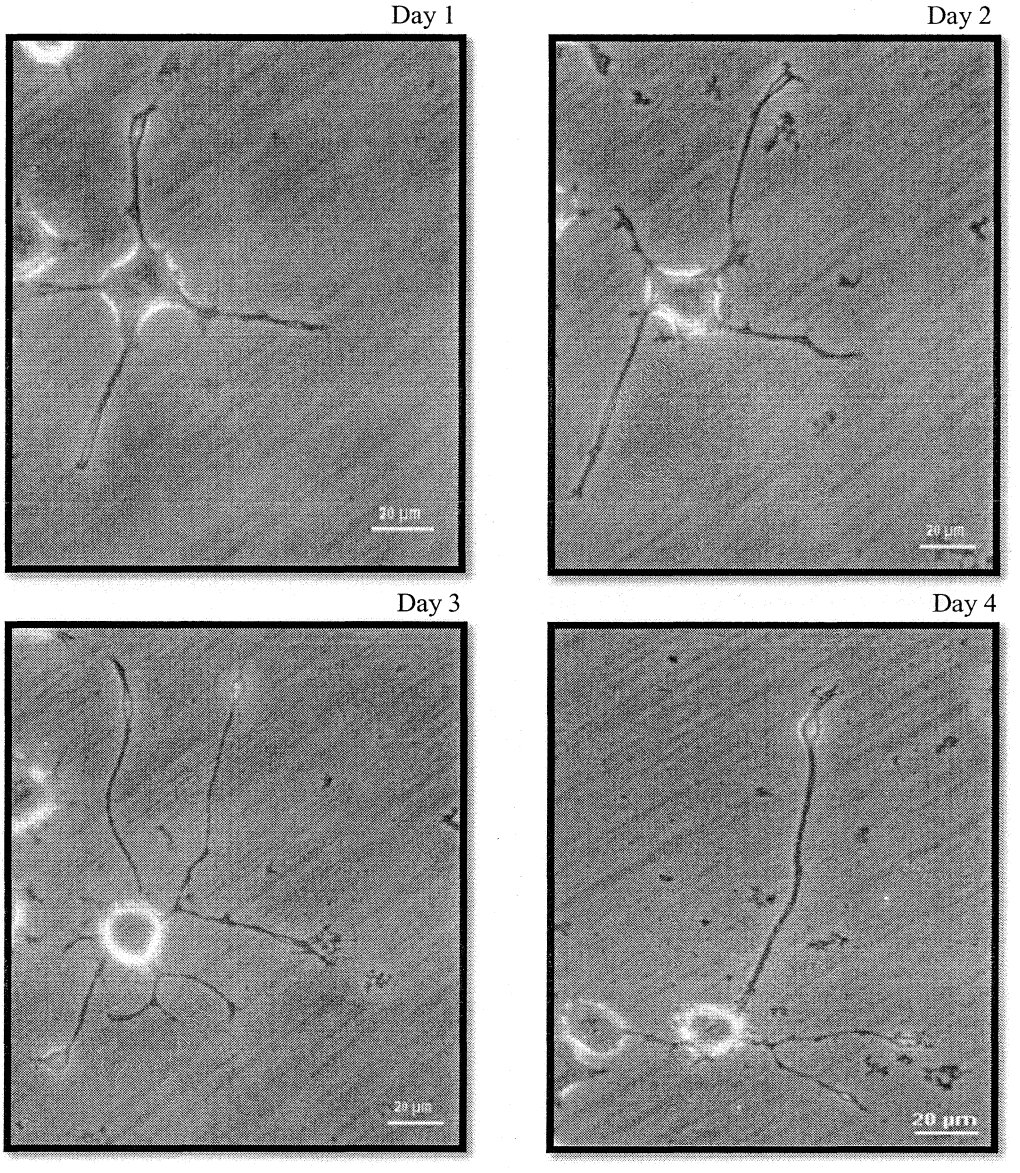


Figure 20: Cell to cell follow up images of control and test group. Images were taken using Olympus 1X51inverted microscope with Qimaging Rolera-XR Fast 1394 camera under 20X phase contrast objective. **A**: Control group; **B**: Test group 1 (0.5 IU Thrombin); **C**: Test group 2 (1 IU thrombin)

B: Test group 1

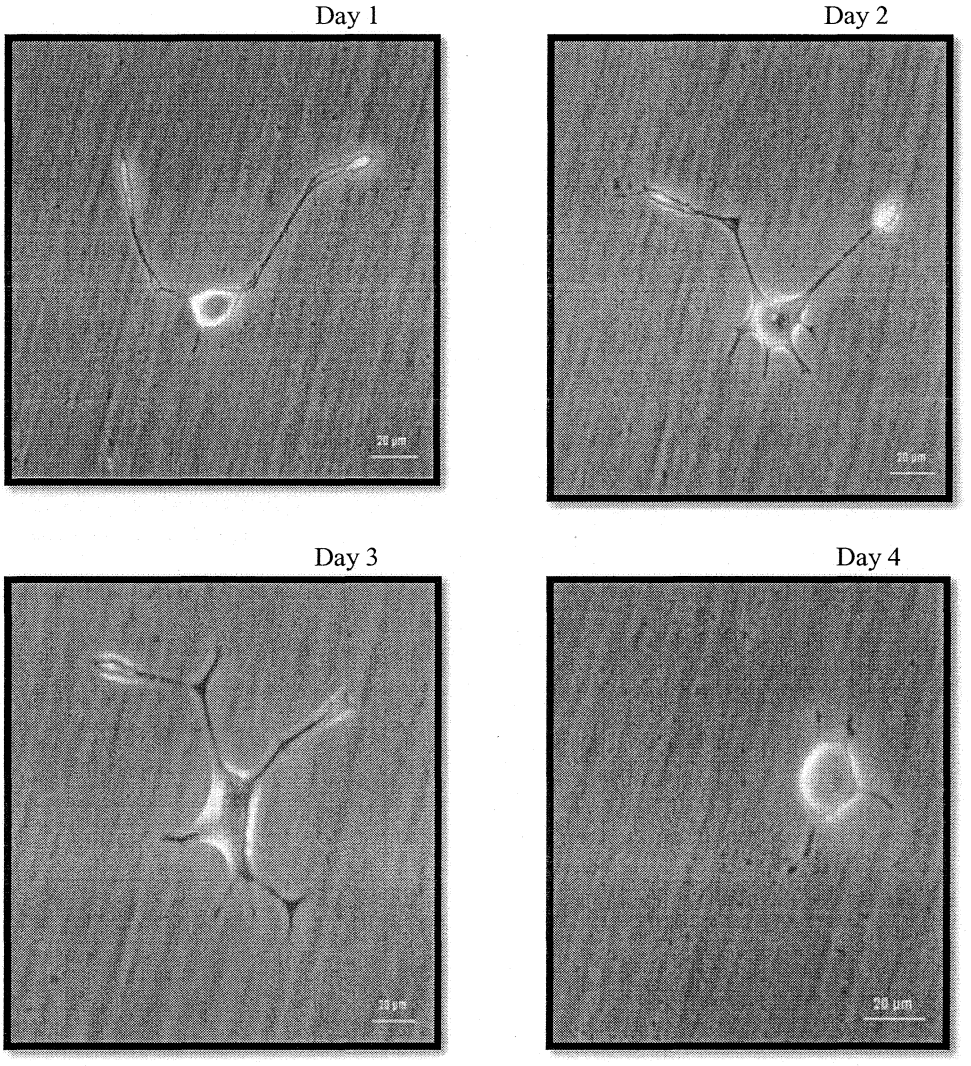


Figure 20: Cell to cell follow up images of control and test group. Images were taken using Olympus 1X51inverted microscope with Qimaging Rolera-XR Fast 1394 camera under 20X phase contrast objective. **A**: Control group; **B**: Test group 1 (0.5 IU Thrombin); **C**: Test group 2 (1 IU thrombin).

C: Test group 2

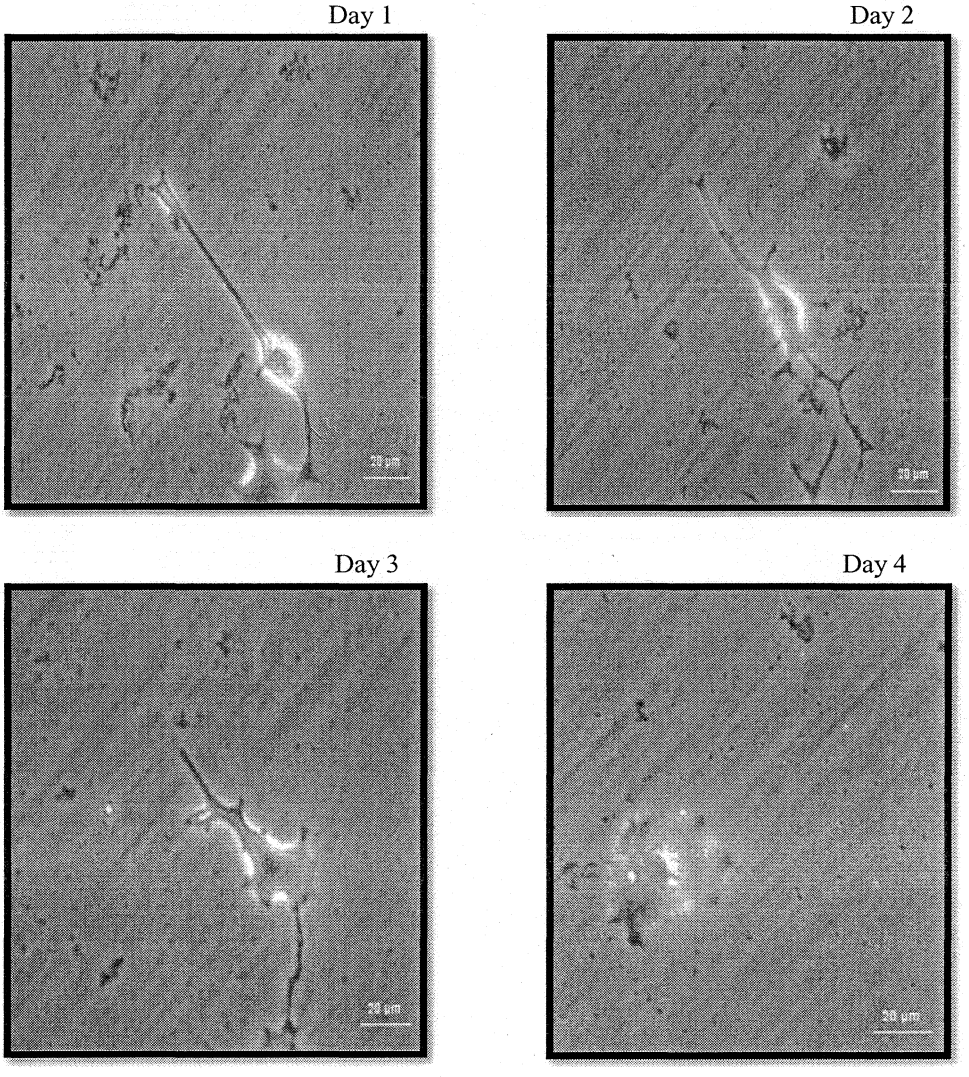


Figure 20: Cell to cell follow up images of control and test group. Images were taken using Olympus 1X51inverted microscope with Qimaging Rolera-XR Fast 1394 camera under 20X phase contrast objective. **A**: Control group; **B**: Test group 1 (0.5 IU Thrombin); **C**: Test group 2 (1 IU thrombin).

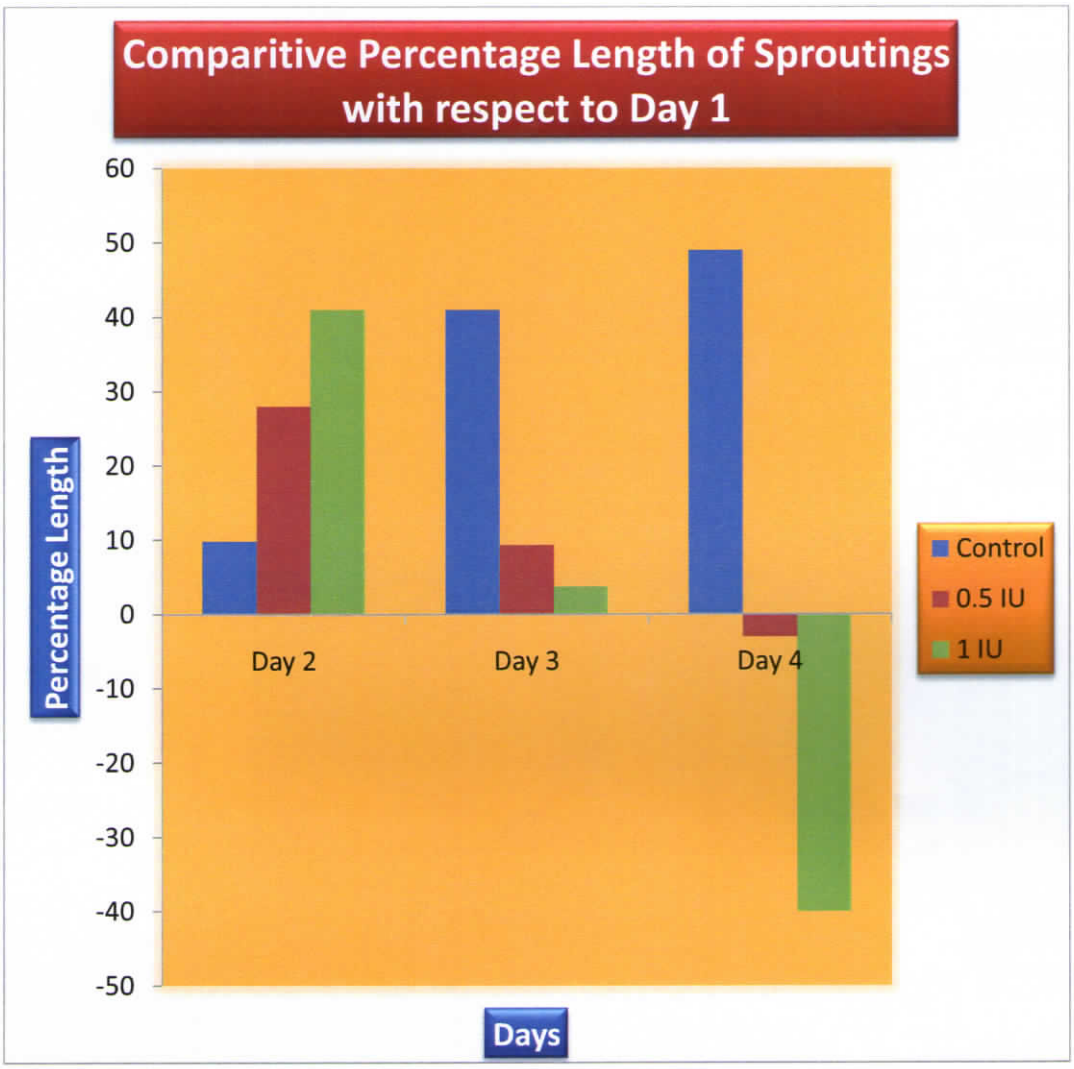


Figure 21: Percentage comparison of length of sprouting of the same PC12 cells with respect to day 1 followed up till Day 4 in control and test groups. Significant rate of reduction in growth of neurites was observed in thrombin treated cells with an increase in concentration of thrombin. Data points are mean value from two independent experiments.

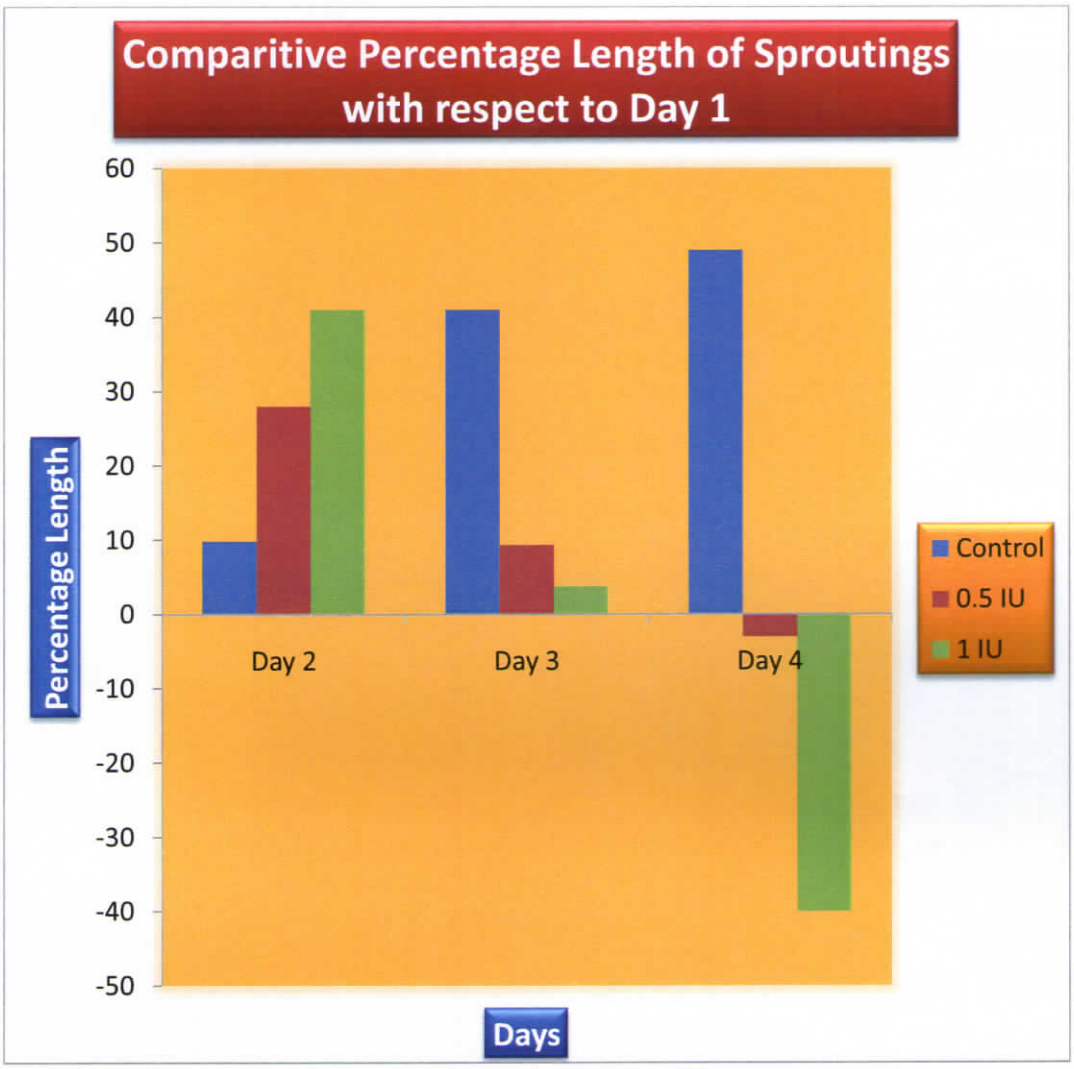


Figure 21: Percentage comparison of length of sprouting of the same PC12 cells with respect to day 1 followed up till Day 4 in control and test groups. Significant rate of reduction in growth of neurites was observed in thrombin treated cells with an increase in concentration of thrombin. Data points are mean value from two independent experiments.

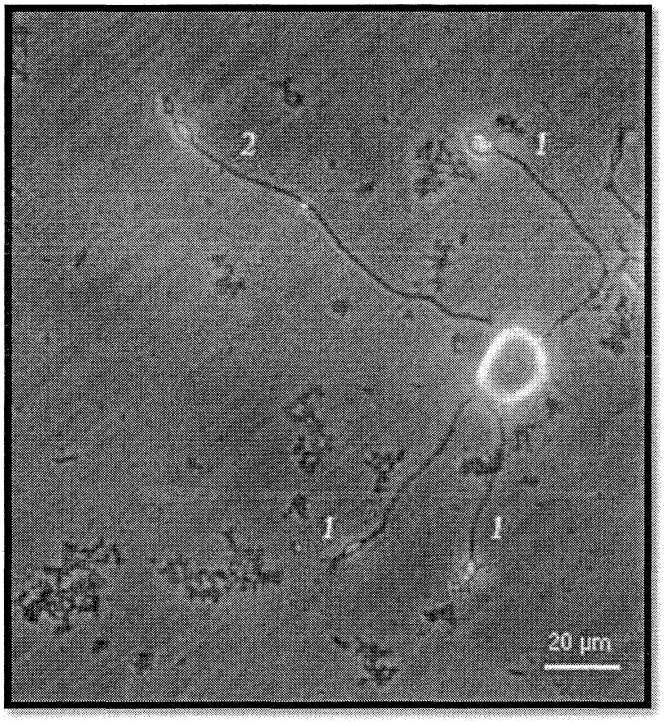


Figure 22: PC12 cells produce neurites of different length which can be broadly classified as shorter and longer neurites. Images were taken using Olympus 1X51inverted microscope with Qimaging Rolera-XR Fast 1394 camera under 20X phase contrast objective. 1: shorter neurites (dendrites); 2: longer neurite (axon).

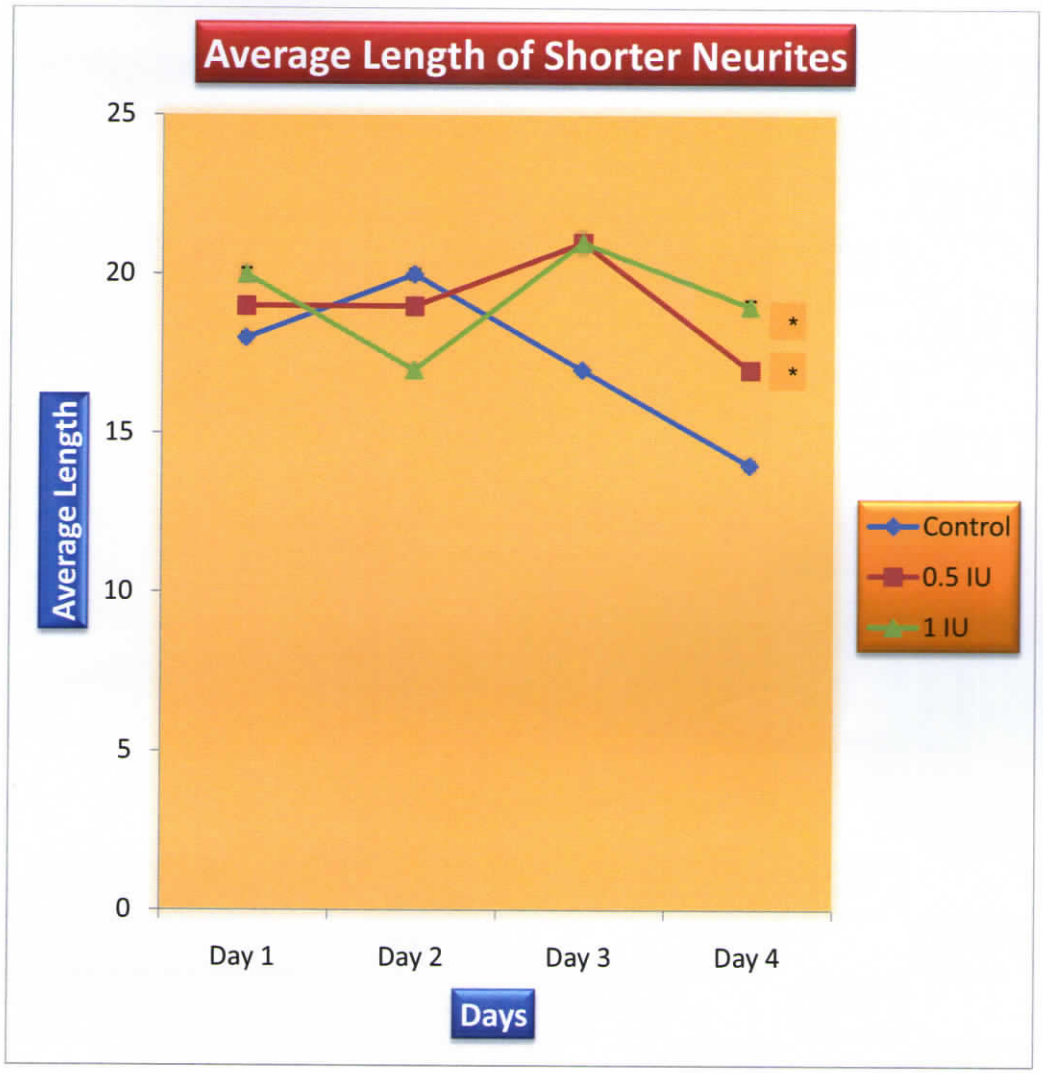


Figure 23: Average length of shorter neurites of all the groups. There is a marginal increase of 4-5% in neurite length in thrombin treated cells. *P=0.0001 compared to day 4 control. Error bar: SEM. Data points are mean valued from four independent experiments.

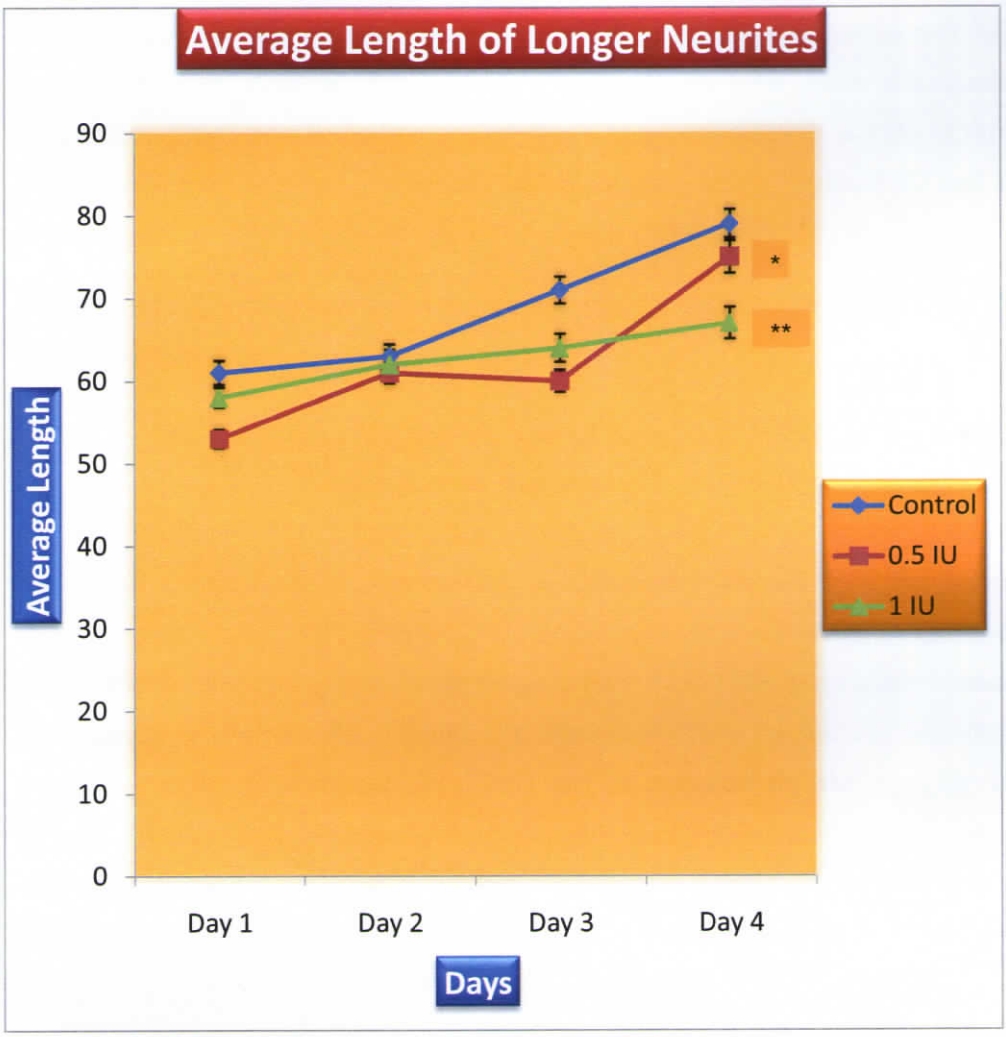


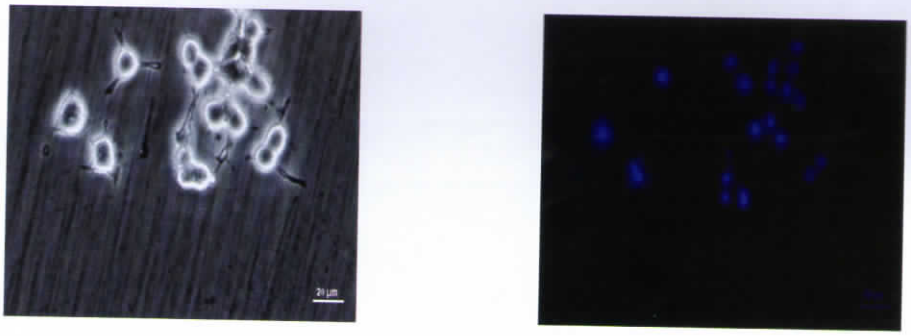
Figure 24: Average length of longer neurites of all the groups. There is a reduction of neurite length in thrombin treated cells, compared to control. *P=0.44;**P=0.43 compared to day 4control. Error bar: SEM. Data points are mean valued from four independent experiments.

Around 98–99% of PC12 cells in both control and test were found to be viable when stained with Hoechst dye (Figure: 25), indicating that the concentration of thrombin used in the experiment are not affecting survival of the cells. High concentration of thrombin is neurotoxic while low concentration is neuroprotective. 0.2 IU of thrombin increase astrocyte survival [Donovan & Cunningham, 1998], while 2 IU and higher concentration of thrombin are neurotoxic [Xue *et al.*, 2006].

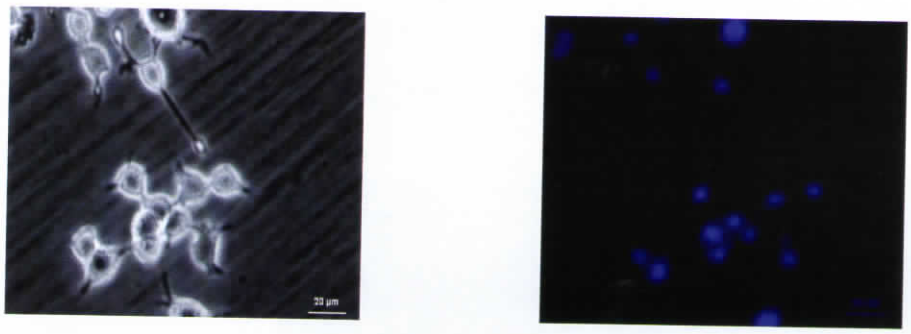
3.4 THROMBIN CAUSES DOWN REGULATION OF SYNAPTOPHYSIN GENE EXPRESSION

To identify whether the growth cone of axons are affected in the presence of thrombin, synaptophysin mRNA level was measured in control and thrombin treated PC12 cells using RT-PCR amplification of the gene. β-actin gene expression was used as the control. Amplified bands were verified on agarose gel (691 and 941bp for β-actin and synaptophysin respectively) (Figure: 26). Densitometric analysis using UVIpro software revealed 18% reduction in synaptophysin gene level expression in thrombin treated cells with respect to control cells (Figure: 27). Synaptophysin is exclusively present in the synaptic vesicles [Valtorta *et al.*, 2004] and is required for the synaptic vesicle exoendocytosis and biogenesis as well as growth cone formation. Changes in the gene expression of integral membrane protein may have a role in the molecular basis of synaptic plasticity [Thome *et al.*, 2001]. It is know that synptophysin is highly sensitive to the neuronal stress. For example acute and chronic immobilization stress can cause 30 to 50% reduction in synaptophysin expression level in hippocampus and cerebral cortex [Thome *et al.*, 2001]. Our preliminary result suggests that the variation of synaptophysin level could be due to stress as well as variations in growth cone dynamics. Further studies are needed how these effects alter the neuronal morphology and net working. This will give a better insight on the role of thrombin in disease conditions like stroke, hypoglycemia, ICH, oxidative stress and during various neurological diseases like Parkinson's disease, Alzheimer's disease [Carreno-Muller *et al.*, 2003; Pike *et al.*, 1996; Vaughan *et al.*, 1995].

A: Control group



B: Test group 1



C: Test group 2

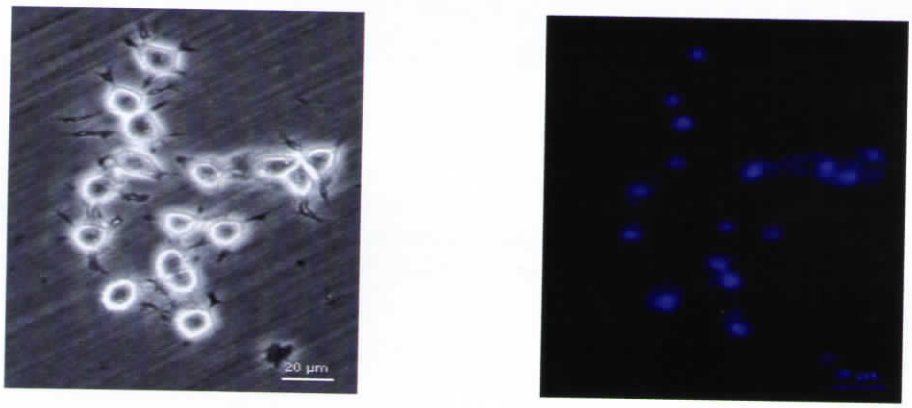
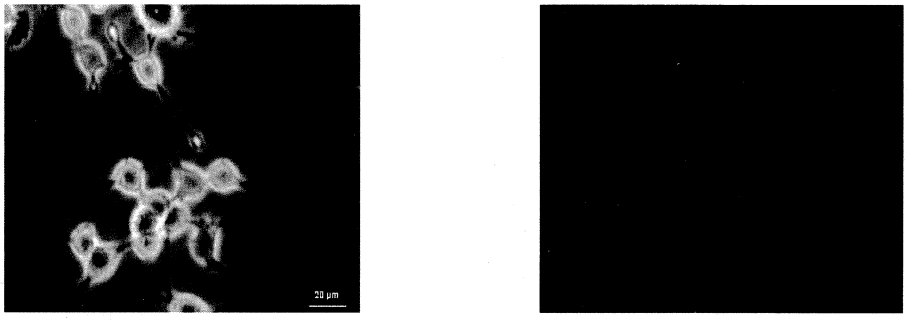


Figure 25: Cell viability assay using Heochst stain. Images were taken using Olympus 1X51inverted microscope with Qimaging Rolera-XR Fast 1394 camera under 20X objective. **A**: Control group; **B**: Test group 1 (0.5 IU thrombin); **C**: Test group 2 (1 IU thrombin). Column 1: phase contrast; Column 2: Heochst stained cells (Excitation: 350nm and Emission: 461nm)

A: Control group

B: Test group 1



C: Test group 2

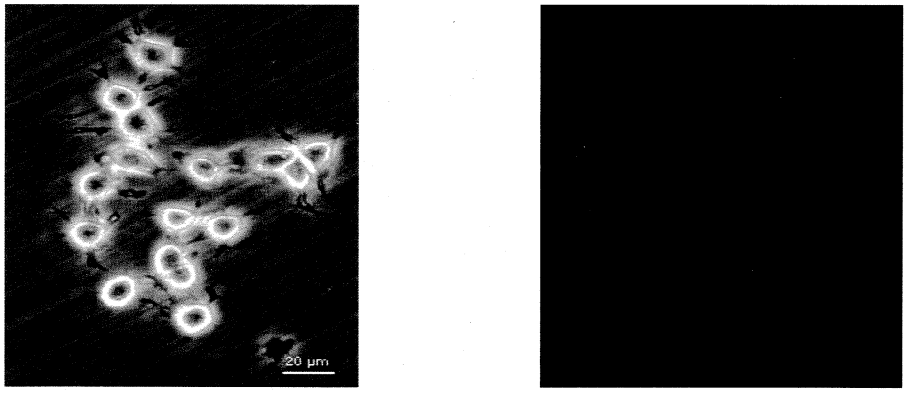


Figure 25: Cell viability assay using Heochst stain. Images were taken using Olympus 1X51inverted microscope with Qimaging Rolera-XR Fast 1394 camera under 20X objective. **A**: Control group; **B**: Test group 1 (0.5 IU thrombin); **C**: Test group 2 (1 IU thrombin). Column 1: phase contrast; Column 2: Heochst stained cells (Excitation: 350nm and Emission: 461nm)

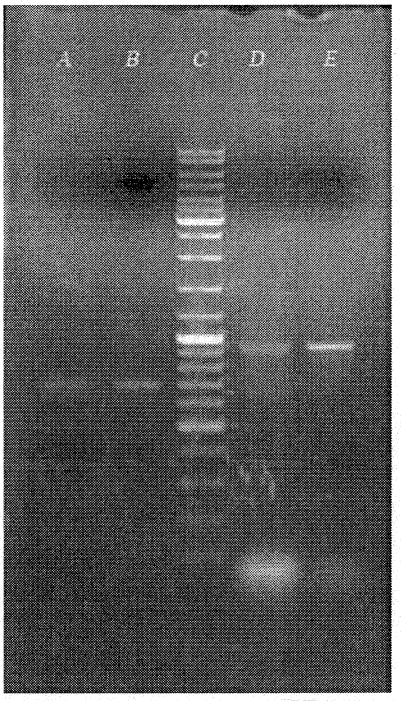


Figure 26: Agarose gel electrophoresis. Lane A: β-actin in control cells; Lane B: β-actin in 1 IU thrombin treated cells; Lane C: molecular weight marker; Lane D: synaptophysin in control cells; Lane E: synaptophysin in 1 IU thrombin treated cells.

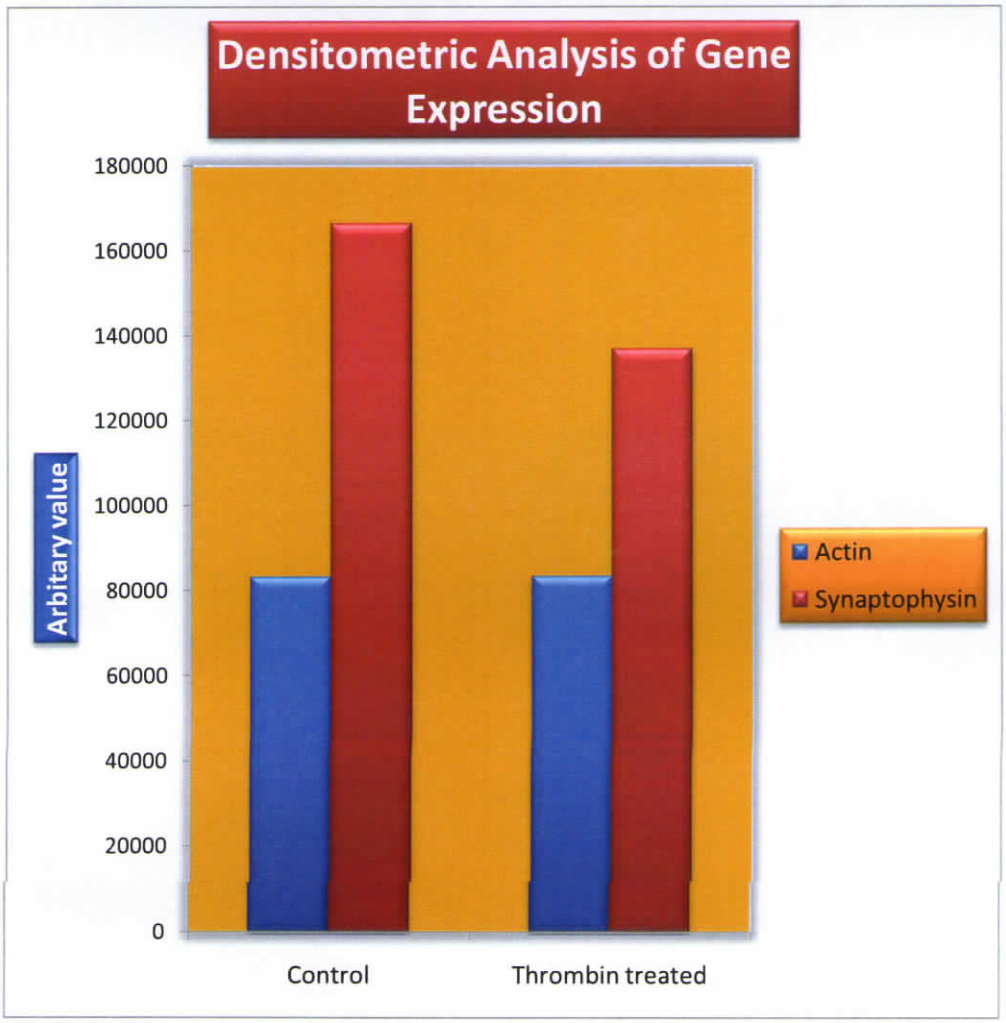


Figure 27: Densitometric analysis of gene expression in control and thrombin treated cells. ~18% reduction in synaptophysin gene expression is observed in thrombin treated cells.

SUMMARY AND CONCLUSION

CHAPTER IV

SUMMARY AND CONCLUSION

Central nervous system injury is major concern all over the world. Injuries to neurons are caused either by neurological disease or by some physical or biological insults. Thrombin is also found to play a critical role in some of the neurological disease such as Alzheimer's disease, Parkinson's disease etc. Nervous system is also exposed to thrombin during injury and intra cranial bleeding. Injuries to brain and spinal cord lead to complex cellular and molecular interactions within the central nervous system as a reaction to the insult as well as an attempt to repair the initial tissue damage. Thrombin has a complex functional role in central nervous system. It causes brain damage at high concentrations and induces neuroprotection at low concentrations. When a set of neurons dies, there is an immediate effect on neuronal network, because each neuron in central nervous system is a part of a highly interacting cellular network. On an intracranial bleeding, there will be a gradient effect of thrombin: a high concentration at the bleeding site resulting in extensive neuronal death and the periphery of the injured site where thrombin at low concentration, allowing the surviving neurons to reorganize. In this study we tried to understand the effect of thrombin at sublethal concentrations in neuronal cells. The main focus of the study was on the morphological changes of neurons, especially the axonal and dendtritic extensions. We have demonstrated that thrombin could cause significant changes in neurtic processes of the cells.

PC12 cells are one of the model systems widely used to study in neurobiology, especially to understand the behaviour of single neurons in culture. Our results suggested that 0.5 IU and 1 IU of thrombin are well tolerated by the PC12 cells causing minimal cell death. But these sublethal concentrations do cause statistically significant alterations in growth of neurites: 0.5 IU and 1 IU thrombin causing 14% and 20% reduction in overall neuritic extensions respectively. Cell to cell follow up for four days provided an excellent insight on the rate of growth of neuritic sprouting suggesting significant down

regulation in the growth with increase in thrombin concentration. Our study also demonstrates that thrombin has a negative regulation on the morphology of axons while an up regulation in the growth of dendrites. Thrombin is known to cause growth cone collapse which may be why the axon gets retracted. The up regulation of dendritic growth may be an adaptive change in morphology for the formation of new axon when an insult occurs. To understand whether low concentrations of thrombin affect the growth cone dynamics of axons, we analyzed the expression levels of synaptophysin, a critical protein in the active zone. The mRNA level of synaptophysin 1 was found to be reduced by 18% on 4^{th} day of 1U thrombin exposure. These results suggest that thrombin exposure in CNS could cause variations in neuronal architecture and will have significant effect in neuronal networking. This could be an adaptive feature for the system to recover from an intracranial damage due to bleeding. Conversely, the CNS might be using thrombin as a signalling molecule to alter the dynamics of neuronal networking and plasticity. Further studies are needed for a better insight on the role of thrombin in disease conditions like stroke, hypoglycemia, intracranial haemorrhage, oxidative stress and during various neurological diseases like Parkinson's disease, Alzheimer's disease.

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ANNEXURE

ANNEXURE

Reagents and buffers

DMEM:F12 1:1 complete media

For 50mL

DMEM:F12 1:1 solution	42.2mL
Fetal Bovine Serum	5mL
Fetal Horse Serum	2.5mL
Penicillin solution	50µL
Streptomycin solution	50µL

DMEM:F-12 1:1 mixture is made by dissolving 15.7g in 1L of sterile distilled water. Penicillin stock solution (1 lakhU/mL) is made by dissolving 30.165mg in 500µL of sterile distilled water. Streptomycin stock solution (1µg/mL) is made by dissolving 100mg in 1mL of sterile distilled water. Kept at 4˚C.

DMEM:F12 1:1 PC12 differential media

For 50mL

DMEM:F12 1:1 solution	49.4mL
Fetal Bovine Serum	500µL
Penicillin solution	50µL
Streptomycin solution	50µL

DMEM:F-12 1:1 mixture is made by dissolving 15.7g in 1L of sterile distilled water. Penicillin stock solution (1 lakhU/mL) is made by dissolving 30.165mg in 500µL of sterile distilled water. Streptomycin stock solution (1µg/mL) is made by dissolving 100mg in 1mL of sterile distilled water. Kept at 4˚C.

75% Ethanol

75 mL of absolute alcohol dilute to 100 mL with distilled water.

Ethidium Bromide

Dissolve 5 mg Ethidium Bromide and diluted to 1 mL with distilled water.

Hoechst 33342, trihydrochloride, trihydrate

Stock solution,

Hoechst dye	100 mg
Sterile distilled water	10 mL

Store at -20˚C.

Working solution is prepared by 1:500 dilution of the stock solution.

Nerve growth factor-7S stock solution

Nerve growth factor-7S	1mg
Sterile distilled water	1mL

Kept at -20˚C.

Phosphate buffer saline

NaCl	140mM
Phosphate buffer	15mM

To prepare 10X stock solution, dissolve NaCl (82g), sodium phosphate monobasic (NaH2PO42H2O, 4.3g) and sodium phosphate dibasic (Na2HPO4, 17.4g) in final volume of 1L. Kept at 4˚C.

10X poly-L-lysine solution

Poly-L-lysine hydrobromide	10mg
Sterile distilled water	10mL

Kept at 4˚C. Working solution concentration is 1X.

RNA precipitation solution

NaCl	1.2M
Trisodium citrate	0.8M

Store at 4˚C.

10X TAE

For 1 litre

Tris base	48.4 g
Glacial Acetic acid	10.9 g
EDTA	2.92 g

Dissolve to total volume of 1 litre in distilled water.

Thrombin

Stock solution contains 200U in 200µL of sterile distilled water. Kept at -20˚C.