IN VTRO ANALYSIS OF ENRICHED SPINALCORD CULTURES AND THEIR SENSITIVITY TO THE BOTULINUM NEUROTOXIN A

by

ASWANI VUNNAVA

(Under the Direction of Julie A. Coffield)

ABSTRACT

Botulinum neurotoxin/A cleaves soluble N-ethyl Maleamide sensitive attachment receptor protein (SNAP-25) at the Neuro Muscular Junction, an important molecular protein in exocytosis and neurotransmitter release. This cleavage results in the blockade of the neurotransmission thus causing the flaccid muscle paralysis. Even though, heterogeneous spinal cord cultures provide the sensitive invitro model system to study the mechanism of action, the presence of the other population of cells in them, makes these mixed cultures not very pragmatic for the use of proteomics. The current study aims at addressing this issue of heterogeneity. In the present study motor neurons were enriched from the mixed spinal cord cell populations. Further, these enriched populations both quantitatively and qualitatively assessed for the motor neuron populations. Also, these enriched cultures were tested for the toxin sensitivity at different ages

INDEX WORDS: Botulinum neurotoxin/A, Neuromuscular junction, Motor neurons, SNAP-25.

INVTRO ANALYSIS OF ENRICHED SPINALCORD CULTURES AND THEIR SENSITIVITY TO THE BOTULINUM NEUROTOXIN-A

by

ASWANI VUNNAVA
M.Sc, University of Hyderabad, 1999
(India)

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment

of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2007

INVITRO ANALYSIS OF ENRICHED SPINALCORD CULTURES AND THEIR SENSITIVITY TO THE BOTULINUM NEUROTOXIN-A

by

ASWANI VUNNAVA

Major Professor:	Julie A. Coffield
Committee:	Gaylen L. Edwards
	Mary Alice. Smith

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2007

ACKNOWLEDGEMENTS

I express deep sense of gratitude towards my major professor Dr. Julie Coffield, who has given me a wonderful opportunity to work with the primary spinal cord cultures. The constant support and encouragement she has given me will always be remembered. I sincerely thank my committee members Dr.Edwards and Dr.Smith who were very helpful and guiding me throughout this project. I thank my lab members Paula Brooks, Dr. Xiaqing Li, Dr. Xiuzhen Yan and Kristen Kellock for making my stay very congenial. Also, I express my sincere thanks to Dr.Edwards lab members Kimberly Freeman and Reddy Gaddam who have generously shared their lab space with me. Also, I thank Dr. Greenspan and his lab members for allowing me to use their laboratory equipment during the early stages of the primary cell cultures.

TABLE OF CONTENTS

Page

CHAPTER-1

INTRODUCTION AND LITERATURE REVIEW

Initial understandings about Clostridium botulinum

Clostridium botulinum species are disreputably famous for the production of one of the most biologically potent substance Botulinum toxins known to mankind. This reputation can be well appreciated by the fact that the minimal lethal dose for the humans being 0.1-1µg/kg of body weight (Johnson et al., 2001). It is still amazing that even after 48 years of its (Neurotoxin/A) accredited role in causing the disease Botulism, we are still in search of the molecular partners involved in its mediation of entry into the neuron (Petro et al., 2006). Such lethality is due to its targeting to the important molecular components, SNARES which are involved in the neurotransmission thus, blocking the release of neurotransmitter, Acetylcholine (ACh) which mediates the nerve conduction resulting in the paralysis and respiratory failure. The authentic record about the awareness of this toxin to the mankind dates back to 18^{th} century (Ergberth, 2004). Botulism was mainly prevalent in those times due to the lack of proper understanding, for the importance of hygiene in the food processing and preservation. Initially, food poisoning was known as “sausage poisoning” due to the facts that sausage was the popular rural dish of those times and these blood sausages were prepared with and preserved with little or no hygienic standards. The understanding of the “food poisoning” has evolved gradually with the inception of scientific approaches. Justinus Kerner was the first person to officially publish the findings of the food poisoning and for the same reason this disease was also known as “kerner’s disease” until, 1895 when Emille Pierre Marie Van Ermengem, a microbiologist

successfully isolated the toxin producing bacteria from both the autopsies of the victims, and the infested ham. He coined the name for bacteria causing this “Food poisoning” as Clostridium botulinum after applying thoroughly the postulates by Robert Koch (Ergberth, 2004).

Even though, toxins produced by these clostridia species were deadly in nature to the human beings on one hand, on the other they have paved the way to open an entirely new subject in the field of Neurobiology, which later became a field unto itself. One is surprised at the vast diversity of the genus clostridium harboring 174 different strains. In this family, we find dangerous ones like botulinum and tetani, commercially important solvent producing C.acetobutylicum species, some species involved in the synthesis of Hydrolytic Enzymes of immense industrial importance (Johnson et al., 2001).

The literature review in this chapter gives general background information about the organism causing the disease, and the toxin it secretes which mediates the fatal botulism. Further, the information briefly describes the structural aspects of the toxin/A molecule, mechanism involving the neurotoxicity and the molecular targets involved as the substrates for the toxin.

Enriched spinal cord cultures were used as an in vitro model system to understand the toxin sensitivity. A brief introduction is given about the initial understandings, about the heterogeneous spinal cord cultures, and the rationale behind their utilization for the toxin studies.

Etiological agent

Clostridium botulinum is an anaerobic sporulative and gram-positive bacillus found ubiquitously in soil and in environments where anoxic conditions prevail. These bacilli of the

family clostridium occur in two forms based on the extent of proteolysis, damage to the food. Serotypes A, B and F are the proteolytic forms and C, D and E the non-proteolytic forms.

Botulinum Neurotoxin/A

Most of the studies (Zhang et al. 2003) on the neurotoxin/A come from the study of Botulinum neurotoxin type A hall strain. This strain has been widely exploited to understand the biochemical aspects, mechanism of action at the target site and most importantly for the purified toxin for the crystallographic studies.

The synthesis of neurotoxin is metabolically very expensive and hence is strictly regulated. The toxin is synthesized during the growth phase of the cell as progenitor complex of 900kDa and is either secreted or, released once the cell is lysed. This complex consists of highly activated neurotoxin, botR a transcription factor, number of heamagglutinin (HA) molecules, and a nontoxic non-heamagglutinin protein (NTNH). Media containing complex nutrients like meat infusions and corn steep liquor, calcium, glucose and individual amino acids enhance the synthesis of toxin. For the mass scale production of the toxin, the fermentation method using dialysis membranes submerged with bulk cultures are employed. In addition, the toxin production is remarkably affected by the nitrogen and CO_2 concentration. Dependence of toxin production on the nitrogen and glucose concentration implicates that the main controlling mechanism is through nitrogen levels and probably through catabolite repression.

The release or secretion of the toxin from the cells is not clearly understood, but is thought to occur during cell autolysis in the later stages of the growth phase. Reducing conditions prevail inside the cellular cytoplasm, nicking of disulphide linkage/oxidative linkage of the heavy and light chains, occurs extracellularly (Reviewed by Johnson et al., 2001).

Structural aspects of the toxin

Botulinum Neurotoxin/A belongs to the family of metalloproteases, and categorized into subfamily Zinicins due to its coordination with the metal zinc. Botulinum toxin A is a huge proteinaceous neurotoxin of 150-kDa molecular weight shares that structural and some functional overlap with tetanus neurotoxin. Hence, in ancient literature it was often confusing to describe the distinguishing symptoms of poisoning. The structural overlap approximates about 30% including similar secondary structure, and location of catalytic domain in the C-Terminal portion of the light chain (Singh et al., 1995; Li, Li et al., 2000).

The toxin is secreted into the cytosol of the bacteria under favorable conditions as a single polypeptide chain. This polypeptide undergoes posttranscriptional modification by either exo or endopepetidases. The mature polypeptide is comprised of a light chain of 50-kDa and a heavy chain of 100-kDa linked by a disulfide bond. Each of these chains is important for structural integrity, binding at the membrane and finally enzymatic cleavage of the substrate. The enzymatic function of the toxin resides in the light chain, which is classified as a metalloprotease due to the presence of the metal atom zinc in the catalytic domain. The light chain of the toxin contains a signature motif HEXXH that is the characteristic feature of the subfamily Zinicins.

It is widely known that various metabolic enzymes contain zinc in the catalytic domain. In general, the zinc atom coordinates with three types of motifs catalytic, co catalytic and structural. Various studies have been done to characterize the role of zinc in the botulinum toxin. Elimination of the zinc atom from the light chain using metal chelators resulted in the total loss of the enzymatic activity. Structural studies using far U.V and C.D (circular dichroism) indicated that this apoprotein did not show any change in the secondary structure difference from the native one. Contrastingly, the near U.V and fluorescence spectroscopic studies have indicated

that there is a perturbation in the tertiary structure and this change is irreversible. Metal replenishment could not retrieve the native tertiary structure. The zinc atom coordinates with the signature motif residues His 223, glu224, his227, glu262 and Tyr 366 and coordination with these amino acid residues is the essence of the catalytic activity. The coordination sphere creates a specific conformation, which is the reason behind the resistance to penetration at high temperatures. In addition, this interaction of metal atom with the amino acid residues creates a long distance influence stabilizing the structural integrity of the light chain (Bandyopadhyay et al., 1987).

Botulinum toxin along with associated proteins referred to as nontoxin associated proteins (NAP) is released/ secreted outside the bacterial cells. The whole of the complex i.e. the toxin and the NAP combined are known as progenitor toxin. These NAPS vary in the size and the number of units that are associated with the toxic component. In the case of botulinum neurotoxin A, strain synthesis occurs in either of three different forms. A 12S (300 kDa)complex that has one molecule of botulinum neurotoxin as the toxic component with one unit of non toxic non hem agglutinin (NTNHA) protein. This and does not have associated proteins for haemagglutinin activity, a 16S complex (500kDa) that contains all the components present in the 12Scomplex and, in addition has three heamagglutinin proteins (HA) HA17, HA33, HA70, and a 19S (900kDa) complex which a dimer of the 16S complex along with an extra HA33 component. These NAP's are thought to be involved in the recognition of the ganglioside and the glycolipids at the membrane allowing the toxin to gain access into the blood stream. It was also found that the presence of these NAP's increases the toxicity 100 fold than that of the nude toxin through the oral entry. So from these observations it is postulated that these NAP's are for the stability and protection of the toxin in the hostile environments. Of the three heamagglutinin

proteins, the major recognized and elucidated protein is HA 33(Reviewed by Raffestin et al. 2005).

Heavy chain:

The molecular weight of the heavy chain is 100-kDa and it is associated with the light chain through disulfide bond. Within the heavy chain, two functional domains have been identified (Lacy, D.B et al., 1998). A translocation domain is situated in the N terminal portion of the peptide and a binding domain in the C-Terminal domain. It is this binding domain, which initiates the interrelation between the toxin and the membrane receptors at the neuromuscular junction. It is proposed that there is a cross talk between a ganglioside and a protein at the membrane of presynaptic nerve terminal. This interaction that mediates serotype specific recognition and toxin internalization (Neale, E.A et al., 1995).

Binding domain:

The binding domain located at the C-terminal interacts with the plasma membrane and is involved in the internalization. The translocation domain is thought to initiate oligomerization and create pores through which the catalytic domain is released into the cytosol. Once the toxin is inside the vesicles, the presence of amphipathic residues between 548-685 senses the difference in pH and undergoes structural changes that lead to the formation of the pore in the endosomal membrane. The presence of the negative clusters around translocation domain increases the P^{Ka} of the residues thus allowing them to titrate against the endosomal pH (Singh et al. 2000). In addition, the presence of histidine residues along with these is thought to mediate the structural changes upon p^{H} change. Overall, there exists an inherent structural flexibility in this domain, which can proceed to formation of a pore followed by translocation of the catalytic domain (Lacy, D.B et al., 1999).

Catalytic domain

The light chain or catalytic domain consists of a 50-kDa length polypeptide with a conserved motif HEXXH. Only the zinc-binding domain shares the sequence homology among the family of clostridial toxins. Comparison with another metalloproteases, thermolysin indicates some structural similarities like the coordination sphere with the zinc atom involving the histidine residues His142, His146, a glutamate residue and water mediated glutamate residue. The translocation belt and the binding domain both block the active site in the 150-kDa holotoxin. There are seven strongly conserved residues H222, E223, H226, E260, E261, R362, Y365, which are found within the 6 Angstroms of the active site .The presence of these amino acids, is needed for zinc dependent endopeptidase activity (DeFilippis, V.et al.1995, Fu. et al.,. 1998 Singh et al.,2000 and Simpson et al.2001).

Mutational studies on the catalytic domain revealed key information regarding the importance of certain residues. Mutation of the G262A resulted in the reduction in the activity of the toxin. Y336A was found to retain some activity in this toxin whereas in the tetanus toxin resulted in a complete inactive mutant. This difference in activity for the same site mutation could be due to their different target substrates, which have different geometries. Another possible explanation for this disparity could be due to the lack of flexibility in the structure of tetanus toxin. (Reviewed by Acharaya 2002).

Journey of Neurotoxin from the site of its synthesis to its target

The uniqueness of the botulinum toxin can be appreciated by the way it reaches the final target the peripheral cholinergic nerve endings. Intoxication with the toxin can occur in many ways like ingestion of the spoiled food containing either the clostridium species or more resistant forms the spores. In the former situation, the bacterium synthesizes the toxin under the favorable conditions and the toxin is released by the autolysis of the cells, known as the primary intoxication. In the later situation of ingestion of the spores, these multiply in the gut releasing the toxin, known as primary infection followed by the secondary intoxication. In either case, toxin has to travel through very hostile environmental conditions, like the low p^H in the gut, strongly hydrophobic membrane barriers, but the toxin exploits these conditions to its advantage due to its inherent configuration and amino acid composition. During the voyage of the toxin to reach the neuromuscular junction of the peripheral cholinergic nerves, the toxin has to be endocytosed from the epithelial cells through the binding of the receptor on the cell surface reaching the general circulation. It is speculated that the binding domain for the receptor is present either on the Heamagglutinin (HA) component of the toxin or on the toxin itself. From here on, the path taken by the toxin to reach the target, the NMJ is only assumed. It is only later known that the toxin can block the release of the acetylcholine from all the cholinergic nerve endings, the initial understandings about the blockade of the toxin was exploited using the isolated neuromuscular junction preparations(Reviewed by Simpson., 2004). These preparations however provided major inputs to the understanding of both the functional mechanism targeted by the toxin using electrophysiology (Coffield et al., 2004). The presence of low abundant receptor protein, along with the muscle proteins in the preparations has created big impediments for the study of the proteomics.

Molecular Targets for the Toxin/A

It has been known that the toxin/A mediates its effect by blocking the release of the neurotransmitter from the presynaptic nerve terminal. Communication between the presynaptic side of the nerve terminal with the postsynaptic membrane of the participating neuron or the muscle cell in the synapse have a specialized molecular architecture needed for the process of the exocytosis i.e. release of the neurotransmitter. This communication occurs in highly dedicated sites known as active zones. In these zones, the plasma membrane of the presynaptic terminal comes in close contact with the postsynaptic membrane. At this site, the synaptic vesicles containing the neurotransmitter adhere and wait for the endocytotic event upon the opening of the calcium channels (Murthy et al., 2003). The entry of the calcium changes the conformational status of the SNARE complex from the cis orientation to the trans orientation. This conformational change, results in initiating the process of fusion and release of the neurotransmitter i.e. Acetylcholine into the synaptic cleft (Keller and Neale. 2000). The process of exocytosis is a highly regulated and is mediated by three set of proteins collectively known as Soluble N-Ethyl Maleimide Sensitive Factor Attachment protein receptors (SNARES) .These SNARES consists of, a 13 kDa vesicular protein Synaptobrevin also known as VAMP, a membrane associated syntaxin and membrane bound SNAP-25(synaptosomal associated protein) which is of 25 kDa molecular weight. Individually assembled recombinant SNARE proteins consisting of Synaptobrevin, Syntaxin, SNAP-25 into the lipid vesicles would be sufficient bring about the fusion of these vesicles to the plasma membrane and are thus, the minimal requirements for the exocytosis and the release of the transmitter (Mochida., 2000, Sollner et al., 1993).

Spinal cord cultures

Even though, it is well known that Botulinum neurotoxin/A acts within the central nervous system, the use of in vitro neuronal cell culture systems to demonstrate similar blockade of neurotransmitter release from the presynaptic terminals (Bigalke. 1985) has gradually replaced the use of in vivo systems to understand the mechanisms of action of this toxin. Interestingly, establishment of the spinal cord cultures dates back to early 1980's (Ransom and Neale et al., 1977), the use of these spinal cord cultures for the understanding and appreciation of the binding and identification of the binding partner the search is still ongoing (Ransom et al.,1977). The studies during these periods have used wide variety of approaches for culturing the spinal cord neurons. Most of these preparations involved simply enzymatic dissociation of the intact spinal cord and plating them on either feeder layer of muscle cells or collagen coated surfaces. Some of the difficulties posed during the development of these spinal cord cultures as the in vitro models were (i) developmental aspects of the neuronal cells in the developing spinal cord, (ii) heterogeneity of the cultures (iii) knowledge of the growth requirements for the growing neurons in vitro.

Development and differentiation of the motor neurons in the spinal cord

Understanding about the intricate and complex mechanisms involved in the development of the nervous system in general and the motor neurons residing in the spinal cord comes from studies using the approaches like molecular genetics and more surprisingly from the invertebrate systems like Drosophila, the fruit fly and the common nematode Ceanoraphbditis .elegans. Interestingly, use of such simple systems has given us broad understandings of the developing synaptic circuits and the importance of the local cell to cell signaling in the maturation of the neuronal population in the spinal cord. We are still far from the thorough

understandings of exactly the chronological and the spatial development from the induction of the neural plate to the completely organized spinal cord (Tanabe and Jessel., 1996).

Temporal positioning of the undifferentiated population of the cell types along the neural tube which in the future development is initiated through either contact mediated or cell-cell signaling processes, defines the fate of the individual population of the cell progenitors. From the experiments done on chick, it has been shown that motor neurons arise from the media neural plate, which is analogous to the ventral neural tube. Progenitors for motor neurons in specific are positioned in a longitudinal fashion on both sides of the neural tube dorsoventrally. Later on during the development expression of homeobox genes PAX3 and PAX7 from these progenitors is completely dominated by the neural plate expression of NKX2.2 and PAX6 whose, expression in a gradient fashion aids in the ventralization of these neural progenitors (Ellen., 1999). The differentiation of these future motor neurons is dependent on the expression of islet-1. From the targeted disruption of this specific islet-1 implicates its role in the differentiation of the motor neurons. Also, the studies from the same group have included one more player in the process of establishing the identification of motor neurons, MNR2. Along with the islet-1, MNR2 is involved in the identity of the subpopulation of the motor neurons. From then expression of neurotrophic receptor genes like tyrosine receptor kinase B (trkB), binding of the ligand brain derived neurotrophic factor (BDNF) ligands mediates the differentiation of the motor neuron progenitors (Yamada and Pfaf et al., 1993; Eisen., 1999).

References

Acharaya KR, Chaddock JA and Turton K etal (2002) Botulinum and tetanus Neurotoxins structure, function and Therapeutic utility. Trends in Biochemical Sciences **27** (11):552-558.

Bandyopadhyay S, Allen WC, Dasgupta BR and Sathyamoorthy V. (1987) Role of heavy and light chains of botulinum Neurotoxin in Neuromuscular paralysis. J Biol chem **262**(6):2660-2663.

Bigalke H, Dreyer F and Bergey G. (1985) Botulinum A Neurotoxin Inhibits Non-Cholinergic Synaptic Transmission in the Mouse Spinal cord Neurons in Culture. Brain Res **360**:318-324.

Coffield JA, Whelchel DD, Brehmer TM, Brooks PM and Darragh N. (2004) Molecular Targets of Botulinum Toxin at the Mammalian Neuromuscular Junction .Mov Disord.**19** (8):S7-S16.

DeFilippis V. (1995) Structural studies on the zinc endopeptidase light chain of Tetanus neurotoxin. Eur JourBiochem **229:** 61-69.

Ellen JS. (1999) Pattern motor neurons in the vertebrate nervous system. TINS. **22**(7):321-326.

Erbguth FJ. (2004) Historical Notes on the Botulism Clostridium botulinum, BotulinumToxin and the idea of the therapeutic use of the toxin. Mov Disord **19** (8):S2-S6.

Fu FN. et al. (1998) Role of Zinc in the structure and the toxic activity of botulinum neurotoxin. Biochemistry **37**: 5267-5278.

Johnson EA and Bradshaw M. (2001) Clostridium botulinum and its neurotoxins: a metabolic and cellular perspective. Toxicon **39**:1703-1722.

Keller JE , Neale EA, Oyler G, and Adler M. (1999) Persistence of botulinum neurotoxin action in the cultured spinal cord cells. FEBS letters **456**:137-142.

Lacy DB et al (1998) Crystal structure of Botulinum neurotoxin type A and implications for the toxicity. Nat struct Biol.**5**:898-902.

Lacy DB, Stevens RC. (1999) Sequence homology and structural analysis of clostridial neurotoxins. JMB **291:**1091-1104.

Li L, and Singh BR. (2000) Role of zinc binding in type a botulinum neurotoxin light chain's toxic structure. Biochemistry **39**:10581-10586.

Mochida S. (2000) Protein-Protein interactions in the neurotransmitter release. Neuro Res.**36**:175-182.

Murthy VN and Camilli PD. (2003) Cell biology of the Presynaptic Terminal. Annu.Rev.Neurosci.**326**:701-728.

Neale EA et al. (1995) Neurospecific binding, internalization and retrograde axonal transport. Curr top Micro immunol.**195**:21-241.

Petro KA, Dyer MA, Yowler BC and Schengrund CL et al. (2006) Disruption of lipid rafts enhances activity of botulinum neurotoxin serotype A .Toxicon **48**:1035-1045.

Raffestin S, Dupuy B, Marvuad JC and Popoff MR (2005) BotR/A and TetR are the alternative RNA polymerase sigma Factors controlling the expression of the neurotoxin and associated protein genes In Clostridium botulinum type A and Clostridium tetani. Mol Microbiol. **55**(1):235-241.

Ransom BR, Henkart P, Bullock PN, Nelson PG and Neale EA. (1977) Mouse spinal cord in cell culture. I. Morphology and intrinsic Neuronal Electrophysiological properties. Jour Neurophysiol **40**(5):1132-1150.

Simpson LL (2004) Identification of Major Steps in Botulinum Toxin Action. Ann Rev Pharm Toxicol. **44:** 167-193.

Singh BR, Li B and Read D. (1995) Botulinum versus Tetanus neurotoxin Why Botulinum is neurotoxin but not tetanus neurotoxin a food poison. Toxicon **33**(12):1541-1547.

Tanabe Y and Jessel TM. (1996) Diversity and pattern in the developing spinal cord. Science **274**:1115-1123.

Yamada T and Pfaf SL, Edlund T and Jessell T. (1993) Control of cell pattern in the neural tube: Motor neuron induction by the diffusible factors from the notochord and floor plate.Cell.**73**:673-686.

Zhang Z, Casey DM, Julien JP and Xu Z (2003) Normal Dendritic Arborizations in the Spinal Motorneurons Requires Neurofilament Subunit L.J Comp.Neurol.**450**:144-152

CHAPTER 2

SPINAL CORD CULTURES-OPTIMIZATION AND THEIR ENRICHNMENT FOR MOTOR NEURONS

Introduction

The development of the vertebrate nervous system, brain and spinal cord collectively called central nervous system (CNS), is marked by interplay of various intercellular and intracellular events. To begin with, thousands of undifferentiated cells proliferate to form millions of few distinct populations of neurons. The development and differentiation of the neurons in the spinal cord involves seemingly simple fine-tuning of the cell-cell interactions and require target induced polarized growth. The lack of complete characterization of the players involved in both the paracrine as well as autocrine signaling, the developmental and differentiation process of the vertebrate CNS in general and mouse in particular is still very complex and far from being completely understood (Schnaar and Schaffner, 1981; Jessel, 2002).

In order to appreciate and gain a thorough insight into the understanding of the factors involved in the development, a suitable in vitro system is definitely the first step towards that goal. Even though lower invertebrate models like Caenorhabditis elegans and Drosophila melanogaster would be a convenient choice initially, comparative and contrasting studies about the development and differentiation between invertebrate and higher mammalian models like the mouse will be very overwhelming. B.R Ransom and Neale et al. have laid the foundations and the initial understandings about the neuronal differentiation and the development, of the spinal cord neurons. Even though their goal was to study and understand the synapse formation, its function and the electrophysiological properties of the single neurons in cultures. It is very surprising that the methodology used by the above group for the isolation of the neurons is still

the accepted technique to date without any modifications in some of the research works (Sheridan et al., 2005). It was later in the early 90's that Camu and Henderson from France introduced a new strategy, for the isolation of motor neurons from both chicken and rat species in the early embryonic stages. This strategy known as 'immunopanning' involves the isolation of the motor neurons from the mixed populations of cells using either rat or chicken specific motor neuron markers. This strategy can well be extended to the mouse with substantial modifications with age of the embryo and the cultural conditions. The first attempt to use intact spinal cord neurons came in 1995 from the research group of Neale, who have used this system to demonstrate the sensitivity of these heterogeneous cultures, towards the clostridial neurotoxins. This group has employed the simple dissociation of the 13-day embryonic spinal cords and plated on to the collagen coated dishes. It is known that the embryonic spinal cord day 13, mostly contains post mitotic differentiated motor neurons. The presence of the other cell populations, both neuronal and nonneuronal cells (e.g. epithelial cells from the meninges), some them still in the proliferating stage, that could add to the complexity of the cultures, if this system is to be used for proteomic studies. Once the harvesting of the cells from the population is done, it is imperative to analyze the quality of the cultures for the enrichment of the motor neuron population. Surprisingly, not much of the research is focused on this quantitative assessment, because of the fact that early embryonic spinal cords consists predominantly of motor neurons. This even though convincing for the theoretical explanation, it does not extend to the practical approach. Since growth of motor neurons is target dependent, ChAT, Choline acetyl transferase enzyme as a marker, is the first choice, even though it is expressed at the later stages during development. Therefore, in search for the early markers for the motor neurons in 1993, Henderson group have used p^{75}NTR (P-75 Neurotropin receptor) for the immnuopanning

strategy in rats. According to these authors, this is the earliest marker selectively expressed in the motor neurons (CamuWN and Gallego et al 1993). Later on in 2000, Geula et al, have used SMI-32, a nonphosphorylated neurofilament antibody, in histochemical studies performed in the mammalian species rat, rhesus, marmoset and human spinal cord sections. These nonphosphorylated neurofilaments are unusually enriched in the motor neurons due to their physiological properties like fast conduction and increase in the axonal caliber (Geula et al. 2000).

In the present chapter, the growth of the spinal cord cultures across the three different ages was observed. Also, from the early 48 hours, mixed spinal cord cultures were estimated for the motor neuron expression marker P-75 Neurotrophin receptor. The cultures were grown with the growth factors and B-27 supplements from post 3 days hours onwards until 21 days. At the end of 21 days the cultures were stained for a neurofilament marker SMI-32 which intensely stains non phosphorylated neurofilaments in the motor neurons. Along with the SMI-32 the neurons were double stained with the ChAT, a cholinergic marker. The double immunostaining identifies both the motor neurons as well as their extensive arborizations.

Materials and Methods

Embryonic Spinal cord cultures

NIH Swiss 13 day timed pregnant mice were obtained from Harlan Laboratories .The mothers were euthanized using CO_2 chamber and the pups were aseptically removed from the womb. The pups were placed in ice cold solution of 1X sterile phosphate buffered saline (Sigma-Aldrich; St Louis, MO,) until their spinal cords were removed. The skin of each pup was gently removed from the dorsal side and the spinal cord was exposed. The underlying attachments like

the meninges and dorsal root ganglion were removed and the cords were cleaned. Cleaned cords were placed in the ice cold 1X Krebs Buffer (7.25% NaCl, 0.4% KCl, 0.14% NaH_2PO_4, 2.6% D-Glucose, 5.97%HEPES (acid form) and pH adjusted to 7.4). This 1X Krebs buffer was made into 5 different solutions I-V and used as indicated from processing of the cords to harvesting the cells. Solution I contained 10%of 10X Krebs buffer,0.8% of 3.82% $MgCl_2$, 0.3% Bovine Serum Albumin (BSA, Sigma-Aldrich; St Louis, MO), Approximately, 20-25 cords were collected from 2-3 mice and placed in the ice cold 1X Krebs buffer. Trypsinization was done in solution II that contained 0.025% Trypsin (Sigma-Aldrich, St Louis, MO) made in solution I for 25 minutes in a water bath preset at 37^0C. Once the trypsinization was complete, solution IV which was made by mixing 40% solution III and 60% solution-I was immediately added to stop any further process of dissociation. Trypsinized cords were collected after a brief centrifugation at 200 X g in the centrifuge and gently triturated with the1X Krebs Buffer containing 0.008% of DNAse, 0.0052% trypsin inhibitor, 1%$MgCl_2$ (solution III). The dissociated spinal cord cells were gently placed on a 4% BSA cushion made from the solution V which was made by adding 0.8% $MgCl_2$, 0.12% $CaCl_2$ to solution I. The gradient was spun at 350 g for 10 minutes and then the pellet was collected in approximately 2mL of solution V. This suspension was placed on the 6.7% Nycodenz (Sigma-Aldrich; St Louis, MO) made in solution-V and spun at 500 X g for approximately 15 minutes. The interphase enriched for the motor neurons was collected and then spun on 4% BSA for a second time at 300 X g for 10 minutes for collection. The cells were counted with an inverted Nikon microscope using haemocytometer and plated on 35mm Laminin and Poly-D-Lysine coated plates (BD Labware; Biocoat ® Cellware; Bedford, MA) in the L-15 Leibovitz Medium (Sigma-Aldrich; St Louis, MO,).

The L-15(Leibovitz-15) also known as plating medium contained the following composition. The stock concentrations are indicated in the bold. The concentrations used are mentioned as percentages. 10% of D-(+)-Glucose, 0.25% l-glutamine, 5% of horse serum, 7.5% of sodium bicarbonate, 1% of penicillin , 0.1% of streptomycin , 0.05% of insulin, 0.16% of putresciene, 1% of conalbumin, 0.1% of progesterone, 0.1% of sodium selenite, Embryonic 1% of fluid, at a density of 1.5 million cells per well. The cells were shifted from L-15 Leibovitz to Neurobasal (Gibco, U.S.A) with 2% of B-27 (Gibco, U.S.A). The growth factors (Biosource., CA and Sigma-Aldrich; St Louis, MO) 1ng/mL of Brain derived Neurotrophic factor BDNF, 100pg/mL of Glial derived Neurotrophic factor GDNF and 10ng/mL of Cilliary Neurotrophic factor CNTF. Also, along with the above mentioned growth supplements, 0.8% of chick embryonic extract (Sera Laboratories International Ltd) and 0.6% of embryonic fluid were added after 72 hours. From there on the Neurobasal medium was changed every 72 hours. The cells were either harvested or fixed after 1, 2 or 3 weeks depending on the nature of the experiment. For the immunofluroscence studies, the cells were grown on Poly-L-Lysine and Laminin coated coverslips (BD Lab ware; Biocoat ® Cellware; MA).

Estimation of the motor neuronal population in enriched cultures

The cells grown on 12 mm coated coverslips (BD Lab ware; Biocoat ® Cellware; MA) for 48 h were used for the immunofluorescence studies. The coverslips were washed with 1X PBS after the medium was completely removed. The cells were then fixed with 4% paraformaldehyde for 1 hr. Later on, the cells were washed with PBS for 30 minutes followed by permeabilization with 0.1% triton X-100 diluted in PBS for 10 minutes. The cells were again

washed 1X PBS for 10 minutes and blocked with 5% goat serum (Sigma-Aldrich, St Louis, MO), made in the antibody diluent 0.1% Triton X-100 0.1% BSA, 0.04% Sodium EDTA, 0.05% sodium azide (Sigma-Aldrich, St Louis, MO) made in 1X PBS for 1 h. The cells were then probed with P-75 Neurotropin receptor (1:200) the primary antibody diluted in antibody diluent overnight at 4^{o}C. The following day the cells were washed with 1X PBS. The cells were then incubated in the goat antirabbit secondary antibody tagged with the fluorescent dye 596 (GAR-596, Invitrogen, Molecular Probes; Eugene OR, 1:300) for 1 h in the dark. Nissl stain was used to distinguish neurons from non- neuronal cells (Invitrogen, Molecular Probes Eugene OR, 1:300). This Nissl stain was diluted in PBS, added to the coverslips and incubated for 10 minutes. The coverslips were then affixed to the slides using DAPI immunogold slow fade gold antifade (Molecular Probes; Invitrogen, Eugene OR). The cells were then visualized on an inverted Zeiss microscope with a 40X objective. Images were captured using a Spot Insight camera, and analyzed using Spot Advanced Software.

Enumeration of the motor neurons from 48 hour cultures

Under the 40X objective a total of 8 fields were chosen to count cells. First, neurons were differentiated from non-neuronal cells with the Nissl stain. Cells co-labeled for both P75NTR (Green) and Nissl (Red) were considered to be motor neurons. Motor neuron population is calculated from the total neurons and expressed as a percentage.

Qualitative staining for the identification of the motor neurons with SMI-32

The cells from 3 week old cultures were probed with a mouse monoclonal antibody to SMI-32 and were prepared as mentioned above. The cells were probed for colocalization of SMI-32 and the motor neuron marker Cholineacetyltarnsferase (ChAT) and incubated overnight

at 4^0C. From there onwards, the cells were processed for the secondary antibody as mentioned above.

RESULTS

The dissociated spinal cords were subjected first to a 4% BSA (Refer to the Diagram 1) cushion and then to the 6.8% Nycodenz density gradient where there appeared to be two prominent visible fractions. These included the motor neuron enriched interphase and the mixed population of neurons and non-neuronal cells in the pellet. This was further evident when the cells were processed for counting. In the enriched fraction, the population was uniform in cell size; whereas in the unenriched population that was not subjected to the Nycodenz gradient, the presence of various sizes of cells was observed suggestive of their heterogeneity during early development (Figures 2.1 A, B). Even though there are large cells morphologically similar to motor neurons in the pellet after the BSA cushion, their significant association quantitatively with the other non-neuronal fraction makes this mixed fraction difficult to understand or evaluate on the studies involving motor neurons. The neurons were allowed to mature for 3 different ages of 1week, 2 week and 3 weeks for the (Figure 2.2).

For the quantitative assessment for the motor neuron population (Figures 2.3-2.4) cells were stained with three sets of markers during the early development in 48 h, These were the nuclear stain DAPI which stains all nuclei (total cell population) and is used as a non-specific cell body marker, Nissl stain which identifies only neurons (total neurons) and the p-75NTR which is selectively expressed on the motor neurons. Motor neuron population is calculated by counting the neurons, which express both Nissl and p-75NTR (merge population) over the total neuronal population (Nissl positive). When these cultures were examined, approximately 74% of the population from the enriched fraction consisted of motor neurons.

In addition, cultures were also examined for colocalization of the motor neuron marker ChAT with the nonphosphorylated neurofilament marker SMI-32. These nonphosphorylated neurofilaments are selectively enriched in the motor neurons. In this experiment, it was observed that all the ChAT positive staining overlapped with the SMI-32 staining (Figure 2.4).

DISCUSSION

Primary spinal cord cultures

In vitro heterogeneous spinal cord cultures have been used to study basic functions of the CNS like electrophysiological properties of the neurons, intercellular signaling among the population of neurons, formation of the synapses, etc. The extensive use of these in vitro culture models is partly because of the easy of accessibility and amenability for various experimental conditions, otherwise complex in an in vivo situation. But, the amount of information that may be gained from these in vitro cultures needs to be weighed against very cautiously the fact that the system being established in vitro has totally dissociated the anatomical organization that existed in the intact tissue. Even though these spinal cord cultures served the purpose of understanding the basic science of the neuron, but use of this system in itself had posed several issues open to the researchers. The first and foremost being the age of the spinal cords that are most accessible as well as give the most efficient yield of neurons when dissociated. Several attempts towards this goal had mixed success depending on the total time the neurons are grown in vitro. Dissociation of the day 13 embryonic spinal cords in mice has shown better survivability than those from postnatal day1 mice. One of the reasons being complete differentiation of motor neurons occurs by this postnatal period and the neurons start to give out axonal processes. By the end of the gestation (22 days in total/postnatal day -1) neurites are extensively elongated to reach their peripheral targets, i.e. muscles. So, it is assumed that dissociation during the trypsinization

leads to the severing of these processes which cannot be regenerated, thus decreasing the survivability of the neurons. Much of the published research has utilized the embryonic spinal cords than the postnatal day -1 due to their cost effectiveness and involves much easier steps during the processing of the spinal cords.

Even though heterogeneous spinal cord cultures have served as a good in vitro system to understand the binding, uptake, and cleavage of Clostridial neurotoxin substrates (SNARE proteins), the important quantitative assessment of these cultures for SNARES is missing in these studies. As mentioned in chapter 1, axons from the spinal motor neurons compose the presynaptic component in the NMJ of the peripheral synapse.

Interestingly, the spinal cord culture system in itself has various cell populations in different stages of development even at the embryonic day 13, but the majority of the population is motor neurons. Until a decade ago, ChAT was the only known marker to identify the population of motor neurons. But, with the advancement of techniques both in the field of developmental biology and molecular biology, new players have been identified and characterized. The early motor neuron specific marker P^{75} NTR in the absence of neurotrophic factors mediates the apoptotic pathway in the early development of the CNS. This receptor begins to express selectively on the cell surface of the motor neurons from day 14 onwards and mediates the cell signaling pathway either towards apoptosis in the absence of the neurotrophic factors, or mediates survival and maturity in conjunction with the Trk receptors.

Neurofilament (NF) is the cytoskeleton component that provides the structural support and integrity to the large motor neurons. These neurofilaments are present in three different forms based on their molecular weight, lower MW of 68 kDa, medium MW of 145 kDa and heavy of 200 kDa. The later two forms are highly phosphorylated and occur mostly in the axonal

processes. The nonphosphorylated neurofilaments occur in the cell body and in the dendrites. SMI-32 detects both phosphorylated states and hence is seen in cell body as well as the neurites. Even though, neurofilaments form the network in the other neurons, its colocalization with ChAT further confirms the identification of the motor neurons.

Conclusion

As the cultures grow from the 1 week towards 3 weeks the neurons grow in size both at the cell body and the neurites. The early detection of the motor neuron population from the enriched cultures is advantageous in assessing the quality of the methodology for the enrichment procedure. In this regard, the motor neuronal markers P-75 Neurotropin receptor and the SMI-32 definitely served this purpose.

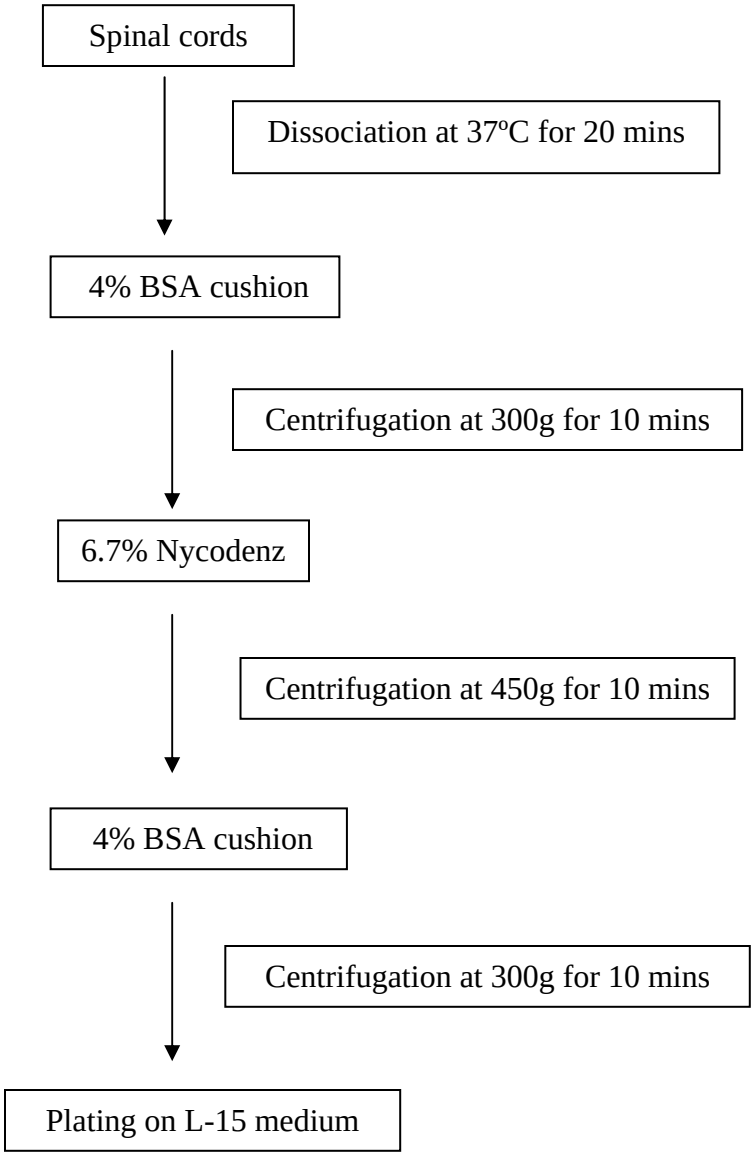


Diagram: 1 Representation of the steps involved in the enrichment of the motor neurons from the embryonic 13 day spinal cords.

Media and media supplements	**Source/Company**
L-15 Leibovitz Medium	Sigma-Aldrich; St Louis, MO
L-Glutamine,	Sigma-Aldrich; St Louis, MO
Horse serum, ,	Sigma-Aldrich; St Louis, MO
Penicillin and Streptomycin stabilized	Sigma-Aldrich; St Louis, MO
Conalbumin	Sigma-Aldrich; St Louis, MO
Progesterone	Sigma-Aldrich; St Louis, MO
Sodium selenite	Sigma-Aldrich; St Louis, MO
Embryonic fluid	Sigma-Aldrich; St Louis, MO
Neurobasal medium	Gibco; Grand island, NY
Chick embryonic extract	Sera laboratories international Ltd; West Sussex, UK
B-27 Supplement	Gibco; Grand island, NY
GDNF	Biosource ; Camarillo, CA
CTNF	Biosource ; Camarillo, CA
BDNF	Biosource ; Camarillo, CA
Poly-D-Lysine/Laminin Cellware	BD Biosciences; Bedford, MA

Table1: Source of the media and the media supplements used in the cell culture

Primary antibody	Source/Company
P-75 Neurotropin receptor	Sigma-Aldrich; St Louis, MO
SMI-32	Sternberger Monoclonals Incorporated ;MA
Cholineacetyltarnsferase (ChAT)	Novus Biologicals Inc, Littleton, CO

Table 2: Primary antibodies used and their sources

Figure 2.1: Phase contrast photomicrographs of spinal cord cultures grown for three weeks. A. Neurons in cultures that were processed for enrichment of motor neurons; B. neurons in cultures that were not processed for enrichment. Non-neuronal cells, most likely astrocytes, have completely covered the cell bodies and the neurites in the unenriched population. This is in contrast to the clearly visible neurons and their processes evident in the enriched population. Both images were taken under same magnification (40X).

A **B**

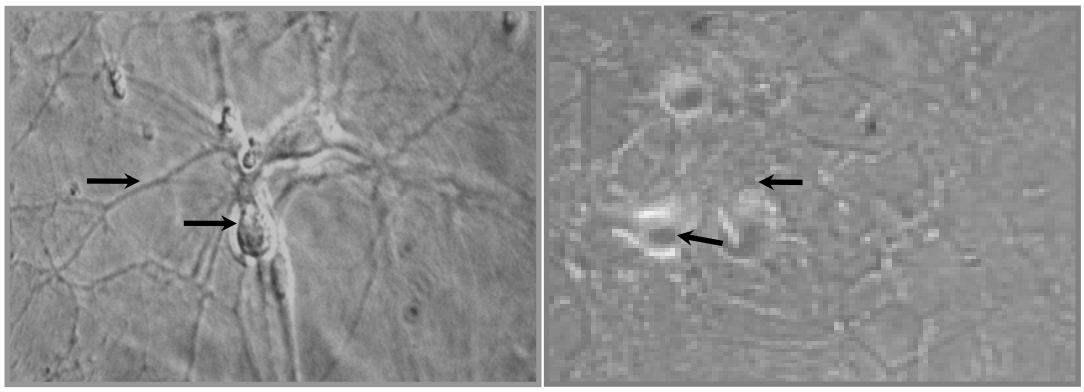


Figure 2.2: Phase contrast pictures of the spinal cord cultures enriched for motor neurons which are developing at different ages. A, B, C, D are at 48 hours,1week, 2week and 3weeks respectively. The increase in the size of the cell body that is initially flat grows three dimensionally. The neurites, which begin as small sprouts, extend to all directions as they grow and synapse on to the cell body of the other cells or, on to the other nerve endings. The increase in the strength of the neurites at the end of the third week (D) can be observed. All the images were taken under same magnification (40X).

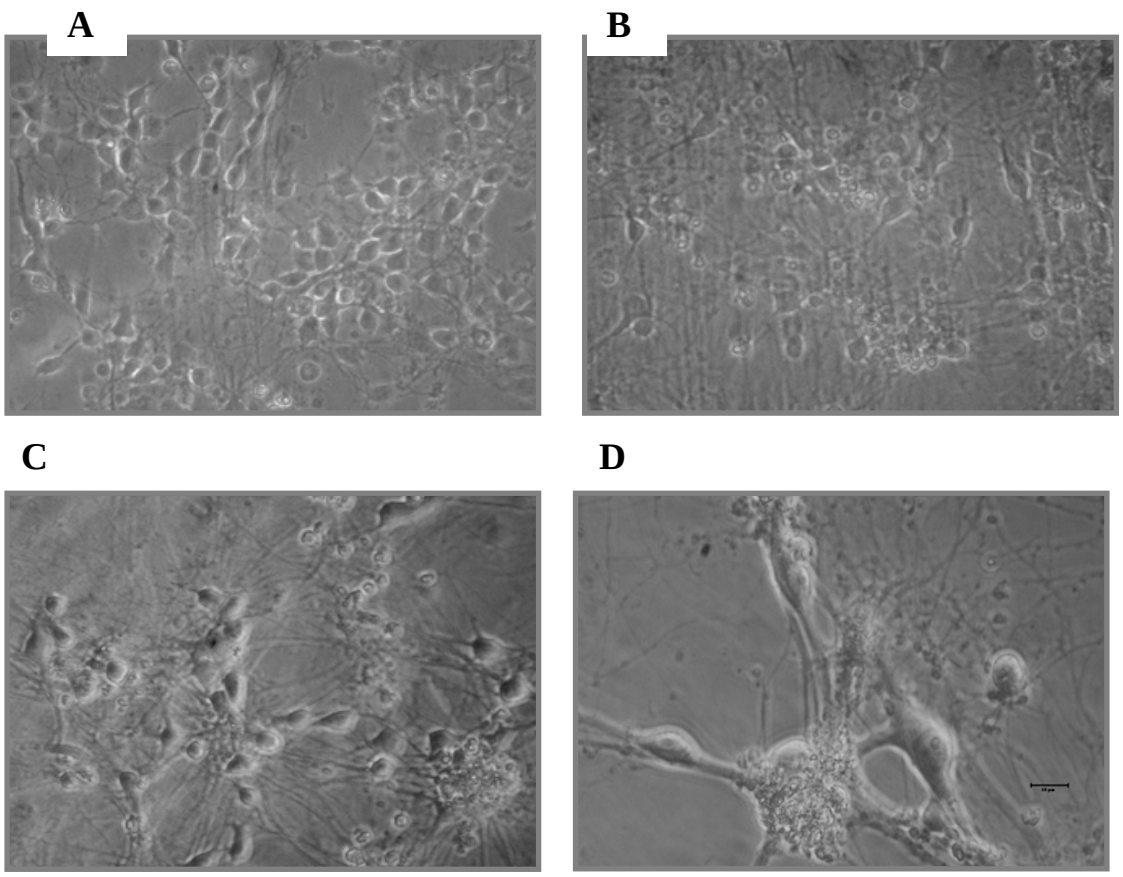
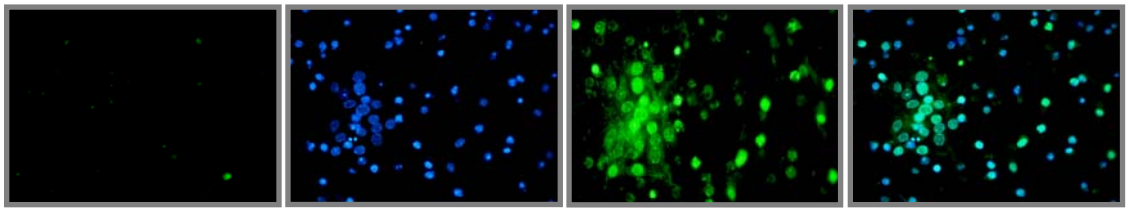

A
B
C
D

Figure 2.3: Immunofluorescent images illustrating enrichment for motor neurons. Cells were grown on the coated cover slips for 48 h and then stained for the early motor neuron specific marker. A. shows the control. B. neurons stained with DAPI identify the nuclei of both neurons and non neurons. C. neurons were stained with P^{75} NTR which is selectively expressed on the membrane of the motor neurons. D. Merge gives the total number of motor neurons. All the images were taken at 40X.

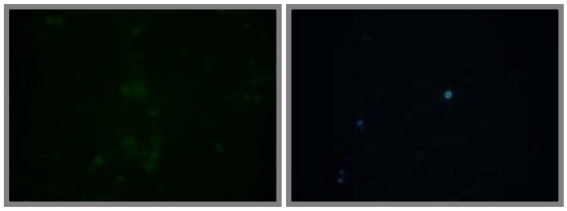
A **B** **C** **D**



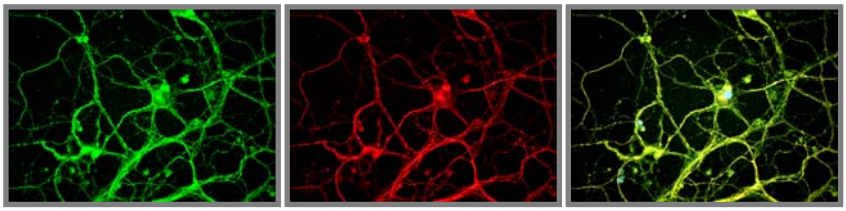
Field IDs	DAPI	Nissl	P75NTR	Merged
A1	15	13	10	10
A2	19	19	10	10
A3	36	26	29	26
A4	38	36	26	26
A5	19	19	16	16
A6	24	21	18	18
A7	12	10	7	8
A8	19	18	14	14
Total	**182**	**162**	**130**	**120**

Table 3. Estimation of number of motor neurons in the population from the enriched fractions. The motor neuron population is calculated by dividing the total number from the merged population with the number of neurons (Nissl positive).

Figure 2.4: Immunofluorescent images illustrating enrichment using SMI-32 in combination with ChAT. Cells were grown on coated coverslips for 3weeks and then probed for the late motor neuron specific marker SMI-32 which identifies Nonphosphorylated neurofilament of the motor neurons. DAPI is in blue. ChAT, a marker for cholinergic neurons is shown in green. Merged gives the total number of motor neurons. All the images were taken at the 40X.



CONTROL DAPI



ChAT SMI-32 MERGED

References

Camu W, Gallego BE and Henderson C E. (1993) Purification of spinal motor neurons from Chicken and Rat embryos by Immunopanning. Neuroprotocols **2**:191-199.

Schnaar RL and Schaffner AE.(1981) Separation of cell types from embryonic Chicken and rat spinal cord: Characterization of motor neuron enriched fractions. The J Neurosc **1** (2):204-217.

Nykjaer A, Willnow TE and Petersen CM.(2005) $p75^{NTR}$-live or let die. Curr Opin Neurobiol **15**:49-57.

Sheridan RE, Smith TJ and Adler M.(2005) Primary cell culture for evaluation of botulinum Neurotoxin Antagonists. Toxicon 45:377-382.

Tsang YM, Chiong F, Kuznetsov D, Kasarskis E and Geula C.(2000) Motor neurons are rich in Non-phosphorylated neurofilaments: cross species comparison and alteration in ALS. Brain Res. **861**:45-58

Wichterie H, Liberaman I, Poret JA and Jessel TM.(2002) Directed differentiation of Embryonic Stem cells into Motor Neurons. Cell **110**: 385-397.

Williamson LC, Halpern JL, Montecucco, Brown JE and Neale EA et al.(1996) Clostridial neurotoxins and substrate Proteolysis in the intact neurons. JBiol Chem. **271**(13) 7694- 7699.

CHAPTER 3

DEVELOPMENT OF SNARE PROTEINS IN CULTURES OF ENRICHED SPINAL CORD MOTOR NEURONS

Introduction

Spinal cord cultures are the most sensitive in vitro cell culture model to the clostridial neurotoxins. Even though this in vitro model is most demanding due to the need for optimization and establishment, more information can be obtained from these models than any other cell lines like Mouse N2a neuroblastoma (Sheridan et al., 2005, Dyer et al., 2006) From the initial studies on the spinal cord cultures it is notable that these cultures were allowed to mature for 3 to 4 weeks before they were used for the toxin studies. Studies utilizing these mature cultures have allowed the investigators to closely observe the path taken up by toxin on the surface of the neurons during its binding and internalization, etc.; something that could not be done in less accessible tissues. Few studies have focused on understanding and evaluating the molecular components involved in the actual developmental aspects of the motor neurons in culture. One of the facts implicated from these studies is that the embryonic day 13 spinal cord dissociated cultures have been allowed for the maturation and only after 21 days of in vitro these cultures were utilized for the toxin studies. The apparent reason might be the expression of the protein targets that are the substrates for toxin cleavage. Even though not many studies have been devoted to understand the role of the developmental expression of the substrates for neurotoxin/A in the cultures, similar studies on other counterparts like brain would be helpful full and can be extrapolated. This chapter aims at understanding the differential expression of the SNARES. In addition, acetylcholine the neurotransmitter which is released presynaptically at NMJ is also studied.

These studies performed in the present chapter aim at understanding the developmental aspects of motor neuronal cultures for the period of 21 days. The enriched populations were observed for the expression levels of the SNARE proteins across the three weeks.

Materials and Methods

Developmental Expression of SNARES

Immunofluorescence Studies

Cells grown on 12 mm coated coverslips (BD Lab ware; Biocoat ® Cellware; MA) were used for the immunofluorescence studies. The coverslips were washed with 0.1M PBS after the medium was completely removed. The cells were then fixed with 4% paraformaldehyde for 1 h. Later on the cells were washed with PBS for 30 minutes followed by permeabilization with 0.1% triton X-100 diluted in 0.1M PBS for 10 minutes. The cells were again washed 0.1M PBS for 10 minutes and blocked with 5% Goat serum, (Sigma-Aldrich, St Louis, MO) made in the Antibody diluent 0.1% TritonX-100, 0.1%BSA, 0.04% Sodium EDTA , 0.05%Sodium azide (Sigma-Aldrich, St Louis, MO) made in 1X PBS, for 1hour.The cells were then probed with the primary antibody made in the antibody diluent overnight . The following were the antibody sources and the dilutions used which were according to the manufacturers suggestions. Choline acetyl Transferase (Novus Biologicals Inc, Littleton, CO, 1:1000), SNAP-25(Sigma; 1:500), Synaptotagmin(Synaptic systems Germany; 1:500), Synaptobrevin (Wako Chemicals USA, Inc ;1:200), Syntaxin1A (Synaptic Systems; Gottingen, Germany;1:500), P75Neurotropin receptor(P^{75NTR})(Sigma St Louis, MO ;1:200), SMI-32(Sternberger Monoclonals Incorporated ;MA 1:300) ,VAChT(Sigma St Louis, MO ;1:500), Tubulin(Sigma St Louis, MO ;1:300) ,Cleaved SNAP-25(Research and Diagnostics; Concord, MA,1:500), Antitoxin/A antibody(Metabiologics Inc ; Madison, WI). The cells were then brought to the room

temperature and washed with the 1X PBS for 15 minutes and probed for the secondary antibody against primary host which is tagged to the fluorescent dye Alexa fluor 488 or 596 (Invitrogen, Molecular Probes; Eugene OR,1:300) for 1hour in dark.

Western Blots

Cells at 1, 2 and 3 weeks in culture were harvested using the M-PER (Pierce Co, Rockford, IL) protein extraction kit with addition of the protease cocktail (Sigma-Aldrich; St Louis, MO). The cell suspension was briefly sonicated and the cell lysate was centrifuged at 4°C for 15 minutes at 10,000 rpm. The supernatant was collected and the total protein content was measured with a spectrophotometer using the Bio-Rad DC protein reagent kit (modified Lowry method). The protein was solubilized in 4X sample buffer (50% glycerol, 8% SDS, 10 %(1M) Tris pH 6.8, 0.02% Coomasie stain, and freshly added 5.2%, ß-Mercaptoethanol, then boiled for 10 minutes. Equal protein amounts were loaded per gel lane. Samples of a synaptosomal preparation from the adult mouse brain were used as positive control. The protein was run on 4-20% precast gradient SDS criterion gels (Bio-Rad) at 200V for 90 min using 1X Tris glycine buffer. The protein was then transferred to methanol-treated PVDF membrane for 45 minutes at 100V in the Transfer buffer containing Tris-HCl (Bio-Rad) 0.48% glycine (Bio-Rad) 2.2%, 40% Methanol. The blot was then blocked in 3% nonfat milk diluted in 1X Tris TBS containing Tris-HCl 0.2%, NaCl 8% and pH adjusted to 7.6, then washed with 1X TBS for 30 minutes. The membranes were probed with primary antibodies at 4°C overnight, according to the manufacturers' recommendation. Their sources are indicated below in parentheses. Choline acetyl transferase (Advance targeting; San Diego CA), SNAP-25, synaptotagmin, synaptobrevin (Sigma-Aldrich; St Louis, MO), syntaxin (Synaptic Systems; Gottingen Germany). All were raised in rabbit except syntaxin, which is a mouse monoclonal antibody. On the following day

the blot was brought to the room temperature, washed for 30 minutes in 1X TBS. The blots were then incubated with the appropriate secondary antibody, either GAR-HRP or GAM-HRP (Bioscource; Camarillo, CA) for 1 h. Then the blots were washed for 2 h and then processed for chemiluminescent detection using ECLplus kit (GE Health Care). The blots were exposed to photographic film (Amersham Biosciences, Inc; UK) for the required amount of period and then developed. Images were captured using Quantity One image ready software.

Results

Differential expression of SNAREs

In the present study immunostaining for the expression pattern of the neuronal SNARES reveal that, the levels of cholinergic marker ChAT increase with the age of the cultures from 1 to 3 weeks(Figures 3.1(a) and 3.1(b)). This increase in the enzyme prepares the motor neurons to efficiently communicate with their target. Even though, protein levels for the VAChT were not performed, the conclusion from the ChAT can be extrapolated to the VAChT (Figure 3.2). Further, the protein levels for the neuronal SNARES consisting of VAMP (Figures 3.3(a) and 3.3(b)), SNAP-25 (Figures 3.5(a) and 3.5(b)) and syntaxin (Figures 3.6(a) and 3.6(b)) showed a gradual increase in the level of respective proteins. The expression levels of the calcium sensor protein, synaptotagmin (Figures 3.4(a) and 3.4(b)) showed an early constitutive expression pattern. The control used was the adult brain synaptosomal preparation, which is known to contain the high levels of SNARES. These simple experiments first of its kind collectively, revealed an interesting observations about the protein levels of the SNARES and the vesicular proteins involved in the neurotransmission.

Discussion

Protein up regulation occurs during the developmental or growth phase of any cell or tissue to meet its needs for both the survival and to carryout the physiological processes in which it is involved. Understanding of this expression pattern provides a basic understanding towards their functional relevance. In accordance, the neurons during the growth phase not only grow in size but also increase in the expression levels of machinery that is important for its function. These include, but are not limited to the SNARE proteins, vesicular proteins and vesicle associated proteins. Immunofluorescence staining methodology helps most importantly, to understand the localization, but quantitative interpretation cannot be made. In such cases, immunostaining (western blots) provide semi-quantitative information. These observations probably suggest vesicular proteins might also be involved in the functions other than exocytosis.

Conclusions

Neurons during the development involve the constitutive exocytosis, which helps in the recycling of the membrane associated proteins. Some of the proteins involved in this process are synaptobrevin and synaptotagmin. Hence, these proteins are constitutively expressed. But the proteins like syntaxin and SNAP-25 which involved in the exocytosis mediated neurotransmitter release, only start expressing at the later stages in the development.

Primary Antibody	Source
P-75 Neurotropin receptor	Sigma-Aldrich; St Louis, MO
SMI-32	Sternberger Monoclonals Incorporated ;MA
Cholineacetyltarnsferase (ChAT)	Novus Biologicals Inc, Littleton, CO
SNAP-25	Sigma-Aldrich; St Louis, MO
Synaptotagmin	Synaptic systems Germany
Synaptobrevin	Wako Chemicals Inc, USA
Syntaxin1A	Synaptic Systems; Gottingen, Germany
VAChT	Sigma St Louis, MO
Cleaved SNAP-25	Research and Diagnostics; Concord, MA
Antitoxin/A antibody	Metabiologics Inc ; Madison, WI

Table: 1 Sources of the primary antibodies used for both Immunofluroscence and Western blotting

Figure 3.1 (a): Developmental expression of the neurotransmitter ChAT. Enriched motor neuron cultures were grown on the coated coverslips for 1, 2, and 3 weeks. These coverslips were probed for ChAT, which is localized in the cytosol. All the images were taken at the 40X.

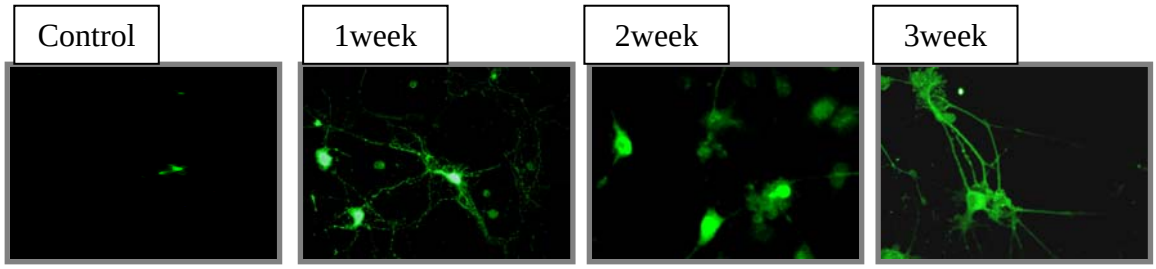

Control
1week
2week
3week

Figure 3.1(b): Immunodetection of ChAT in enriched motor neuron cultures grown on the 35mm wells for 1, 2, and 3 weeks. Equal amounts of protein (20µg) were loaded per lane, Adult brain synaptosomal preparation 5µg was used a control.

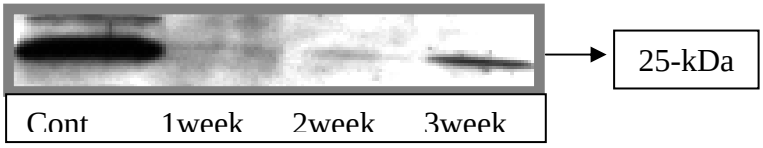

25-kDa
Cont
1week
2week
3week

Figure 3.2: Developmental expression of the neurotransmitter transporter VAChT. Enriched motor neuron cultures were grown on coated coverslips for 1, 2, and 3 weeks. Neurons were probed for VAChT. All the images were taken at the 40X.

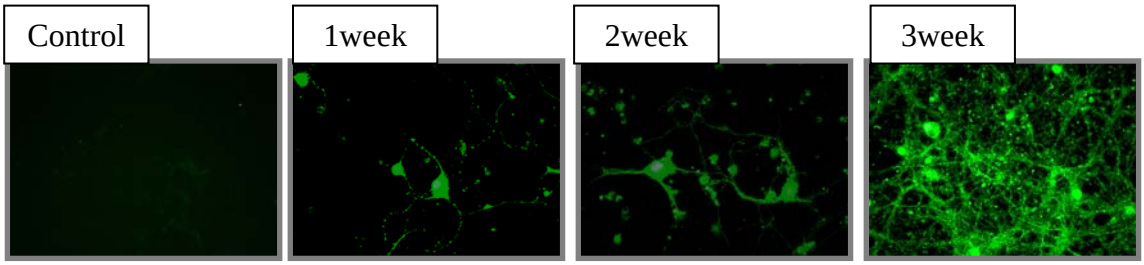

Control
1week
2week
3week

Figure 3.3(a): Developmental expression of synaptobrevin. Enriched motor neuron cultures were grown on coated coverslips for 1, 2, and 3 weeks. Cultured neurons were probed for the synaptobrevin. All the images were taken at the 40X

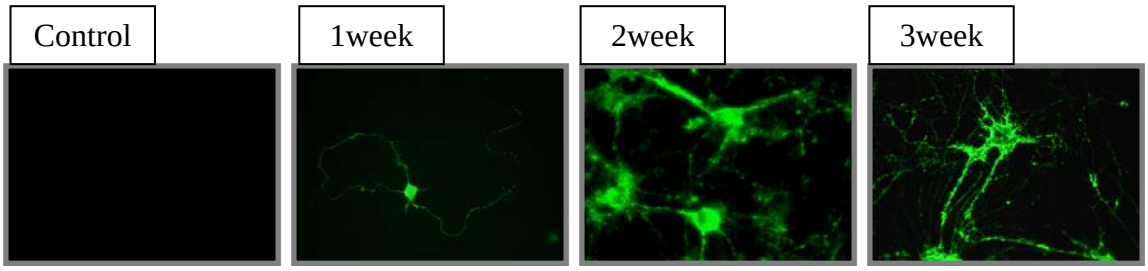

Control
1week
2week
3week

Figure 3.3(b): Immunodetection of synaptobrevin from enriched motor neuron cultures grown in 35 mm wells for 1, 2, and 3 weeks. Protein was harvested from all three ages and processed for the western blots. Equal amounts (5µg) of protein were loaded per lane. Samples of adult brain synaptosomal preparation (5µg) were used as a positive control.

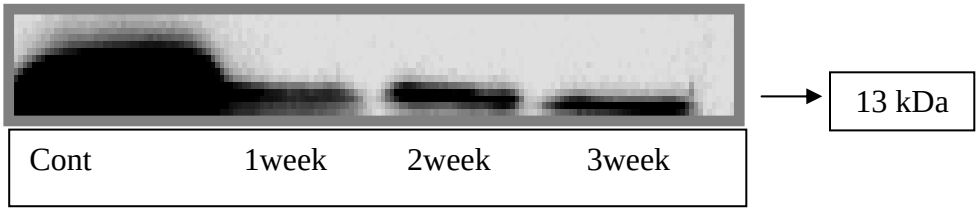

13 kDa
Cont
1week
2week
3week

Figure 3.4(a): Developmental expression of the calcium sensor synaptotagmin. Enriched motor neuron cultures were grown on coated coverslips for 1, 2, and 3 weeks. Cultured neurons were probed for synaptotagmin. All the images were taken at 40X.

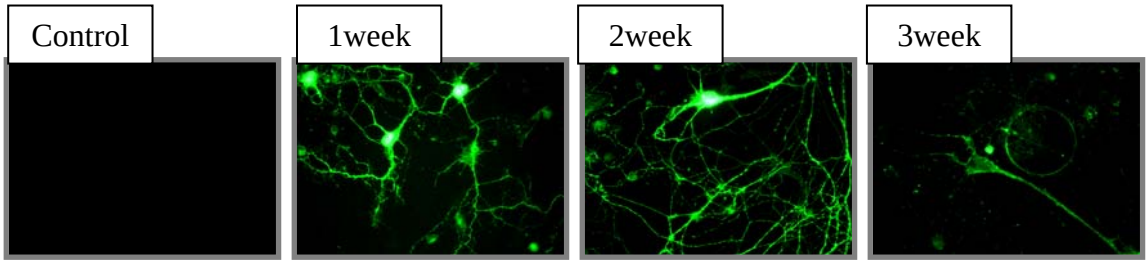

Control
1week
2week
3week

Figure 3.4(b): Immunodetection of synaptotagmin from enriched motor neuron cultures grown on 35 mm wells for 1, 2, and 3 weeks. Protein was harvested from all three ages and processed for the western blots. Equal amounts (20µg) of protein were loaded per lane. Samples from adult brain synaptosomal preparation 5µg were used as a positive control.

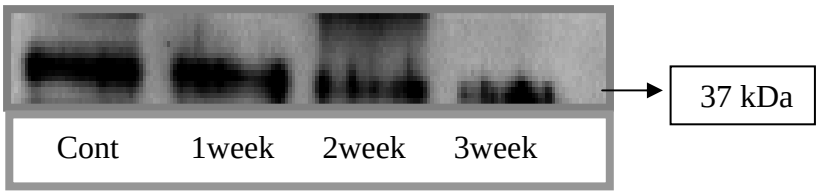

37 kDa
Cont
1week
2week
3week

Figure 3.5(a): Developmental expression of SNAP-25. Enriched motor neuron cultures were grown on coated coverslips for 1, 2, and 3 weeks. Cultured neurons were probed for SNAP-25. All the images were taken at the 40X.

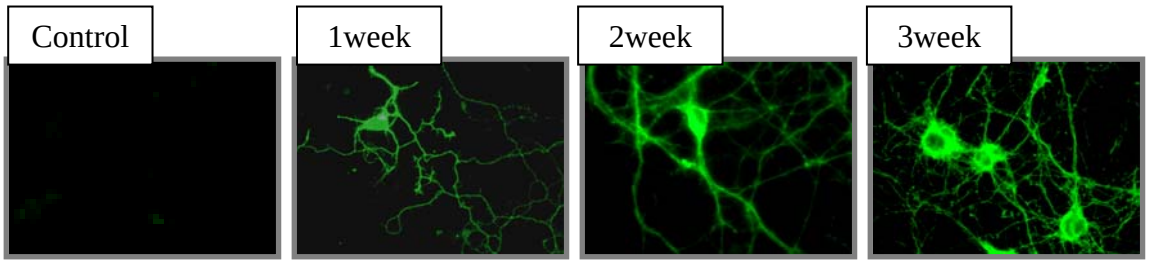

Control
1week
2week
3week

Figure 3.5(b): Immunodetection of SNAP-25 from enriched motor neuron cultures grown on 35mm wells for 1, 2, and 3 weeks. Protein was harvested from all three ages and processed for the western blots. Equal amounts of protein 1µg were loaded per lane. The blot was probed with a SNAP-25 antibody which identifies a 25 kDa band. Samples from adult brain synaptosomal preparation 5µg was used a positive control.

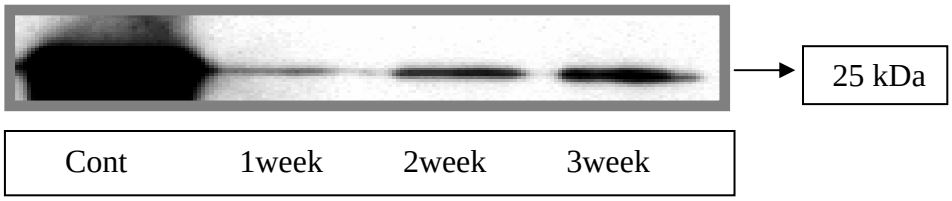

25 kDa
Cont
1week
2week
3week

Figure 3.6(a): Developmental expression of the syntaxin. Enriched motor neuron cultures were grown on coated coverslips for 1, 2, and 3 weeks. Cultured neurons were probed for the syntaxin. All the images were taken at the 40X

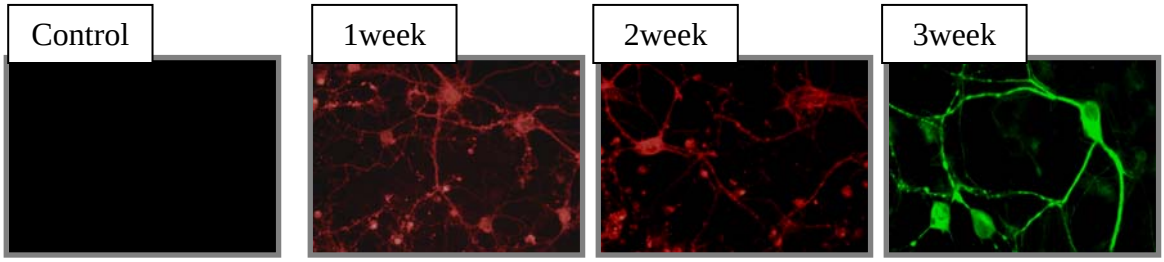
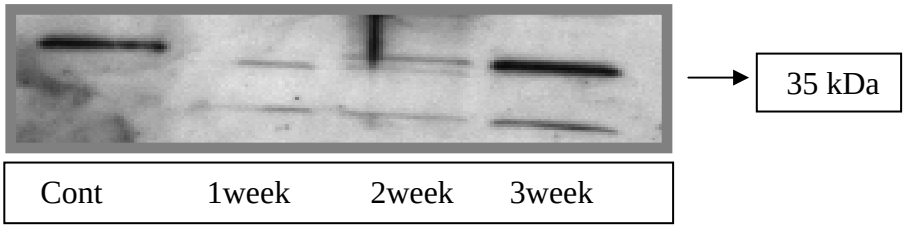

Control
1week
2week
3week

Figure 3.6(b): Immunodetection of syntaxin from enriched motor neuron cultures grown on 35mm wells for 1, 2, and 3 weeks. Protein was harvested from all three ages and processed for the western blots. Equal amounts of protein 20µg were loaded per lane. The blot was probed with a syntaxin specific antibody which identifies a 35 kDa specific antibody. Samples from adult brain synaptosomal preparation 5µg were used as a positive control.


35 kDa
Cont
1week
2week
3week

References

Arvindsson ULF, Riedl M, Elde R and Meister B.(1997) Vesicular Acetylcholine Transporter (VAChT)Protein: Novel and Unique Marker for Cholinergic Neurons in the Central and Peripheral Nervous Systems. J Comp Neurol. **378**:454-467.

Bark C, Bellinger FP, Kaushal A, Mathews JR, Partridge DL and Wilson MC.(2004) Developmentally regulated Switch in Alternatively spliced SNAP-25 Isoforms Alters Facilitation of Synaptic Transmission. J Neurosc **24**(40):8796-8805.

Dyer MA et al, Petro KA, Dyer MA, Yowler BC and Schengrund CL (2006) Disruption of lipid rafts enhances activity of Botulinum neurotoxin serotype A. Toxicon **48**:1035-1045.

Grosse G, Tapp R, Kuchinke J, Gorlston M, Fetter I, Zell BH, Gratzl M, Bergmann M and Grosse J.(1999)SNAP-25 Requirements for the dendritic growth of Hippocampal Neurons. J Neurosc Res **56**:539-546.

Hberman Y, Ziv I, Gorzalczany Y, Fukoda M, and Eisenberg RS. (2005) Classical protein kinase C(s) regulates Targeting of Synaptotagmin IX to the endocytotic recycling compartment. J Cell Sci. **118**:1641-1649.

Sendtner M, Pei G, Beck M, Schweizer and Weise S. (2000) Developmental motor neuron cell death and neurotrophic Factors. Cell Tiss Res **301**:71-84.

Sheridan RE, Smith TJ and Adler M. (2005) Primary cell culture for evaluation of botulinum neurotoxin Antagonists. Toxicon **45**:377-382.

Williamson LC, Halpern JL, Montecucco, Brown JE and Neale EA (1996) Clostridial neurotoxins and substrate Proteolysis in the intact neurons. JBiol Chem **271** (13):7694-7699.

Zurn AD, Winkel A, Menoud K, Djabali K and Aebischer P.(1996) Combined effects of GDNF, BDNF and CNTF On motor neuron differentiation invitro. J Neurosc Res. **44**:133-141.

CHAPTER 4

DEVELOPMENTAL SENSITIVITY OF ENRICHED MOTOR NEURON CULTURES TO THE BOTULINUM NEUROTOXIN SEROTYPE A

Introduction

Clostridial neurotoxins block neurotransmitter release by cleaving either one or two members of the SNARE proteins. The physiological significance of these toxins was only appreciated once it was understood that these toxins target the fundamental process of the exocytosis mechanism, in particular neurotransmission. Neurotransmission is the mechanism of release of the neurotransmitter from the presynaptic neuron into the synaptic cleft and this released neurotransmitter then binds to the receptors present on the postsynaptic cell. Even though these toxins target cells of secretory in nature, pancreatic cells it is the neurons which have grabbed the whole attention in the field of toxin research. The extreme sensitivity of the neurons to the toxin is due to the presence of the protein receptor on the surface of the neuron. Under experimental conditions other cell systems can be sensitized, but will not be suitable for the receptor and binding studies. The extreme sensitivity of the neuronal cultures on one hand offer researchers a valuable and appropriate in vitro model system for toxin-binding related research, on the other hand, it is one of the most demanding in vitro systems to maintain. This is evident by the fact that there is a very small amount of literature that has been published about the exploitation of this in vitro cell culture system. In the late 1990's Neale group were the first to initiate utilization of intact cultured spinal neurons to observe the proteolysis of the substrates for the clostridial toxins (Williamson and Neale., 1996). However, not much focus is given to studies utilizing these cultures; rather surprisingly, other cell line like pheocytochroma cell line (PC-12) is more widely used for the toxin studies. Neale's group has studied extensively the

proteolysis of the various substrates of the clostridial toxins and their different forms of expression. For the process of neuroexocytosis or the release of the neurotransmitter the minimum essential machinery are synaptobrevin, syntaxin and SNAP-25 collectively known as SNAREs. These three proteins interact with each other and form a thermodynamically stable complex known as the SNARE complex. This heterotrimeric ternary complex once formed is resistant to the action of the clostridial toxins and to detergent (SDS) denaturation. One alpha helical domain from each of the partners' syntaxin and synaptobrevin and two from the SNAP-25 form this complex (Montecucco and Schiavo., 2005).

Botulinum neurotoxin/A, which specifically targets SNAP-25 and cleaves between residues arginine and glutamic acid at the N-terminus before the complex formation results cleavage results in the loss of a 1 kDa fragment which in turn results in the inability of the formation of the SNARE complex.

From the studies performed on the developing hippocampal neurons which have been exposed to the clostridial neurotoxins in vitro, suggests the distribution of SNARE proteins SNAP-25, synaptobrevin and syntaxin distributed both on the axonal membranes as well as in the transport vesicles directed towards the growing axons and dendrites. Neurons actively carry out both constitutive and regulated exocytosis (Calcium dependent) during their development. The former mechanism supplies necessary molecular machinery required for the distribution of new dendrites and growing axons, where as the later process mediates the communication between the established synapses (Grosse et al., 1999).

The objective of the present study aims at analyzing the sensitivity of the different ages of cultures to a range of serotype A concentrations. Strategies like immunofluorescent localization as well as protein detection of the cleaved SNAP-25 were examined through

immunostaining. The preliminary observations from these studies performed would help to us to understand whether binding and internalization of the toxin requires mature 3 week cultures.

Materials and Methods

Immunofluroscent detection of SNAP-25 cleavage

Cells were grown for a period of 1, 2, or 3 weeks on 35,mm wells (BD Labware; Biocoat ® Cellware; Bedford, MA) for western blots or on 12 mm coated coverslips (BD Labware; Biocoat ® Cellware; Bedford, MA) for immunofluorescence. Cultures were washed with 1X PBS (Sigma-Aldrich; St Louis, MO) twice before adding the Botulinum Neurotoxin serotype A (Metabiologics Inc; Madison, WI). The toxin was diluted in stimulation buffer (Keller et al., 2004) containing 10mM HEPES pH 7.4, 56 mM KCl, 82 mM NaCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$. The toxin was serially diluted from 10^{-8}M to 10^{-12}M in pre-warmed stimulation buffer and then added to culture wells. The cells were incubated with the toxin for 30 minutes at 37^{o}C followed by a quick wash with PBS to remove unbound toxin. The cells were then allowed to rest at 37°C to allow for internalization and cleavage of SNAP-25 for 90 to 210 mins in Neurobasal medium. The cells were then washed with PBS, harvested and then processed for the western blots.

Immunofluorescent Detection of Neurotoxin/A Binding

Cells were grown for a period of 1, 2, or 3 weeks on 12mm coated coverslips (BD Labware; Biocoat ® Cellware; Bedford, MA) for immunofluorescence. The cells were washed with ice cold 1X PBS (Sigma-Aldrich; St Louis, MO) twice before addition of toxin serotype-A (Metabiologics Inc; Madison, WI). For these experiments, the toxin was diluted in ice cold PBS. The toxin was serially diluted from 10^{-8}M to 10^{-12}M in pre-chilled PBS and then added to culture wells. The cells were incubated with the toxin on ice for 30 minutes followed by a quick wash

with ice cold PBS to remove unbound toxin. The cells were then fixed with ice cold 4% paraformaldehyde for 1 h. Later on, the cells were washed with PBS for 30 minutes followed by permeabilization with 0.1% triton X-100 diluted in PBS for 10 minutes. The cells were again washed in 1X PBS for 10 minutes and blocked with 5% goat serum (Sigma-Aldrich; St Louis, MO) diluted in antibody diluent (0.1% TritonX-100, 0.1%BSA, 0.04% sodium EDTA, 0.05% sodium azide (Sigma-Aldrich; St Louis, MO) made in 1X PBS for 1 h. The cells were then probed with primary antibody specific for cleaved SNAP-25 (1:200) diluted in antibody diluent and incubated overnight. On the following day the cultures were brought to room temperature and washed with the 1X PBS for 15 minutes and probed with the secondary antibody which is tagged to the fluorescent dye Alexa fluor 488 and 596 (Invitrogen, Molecular Probes; OR, 1:300) for 1 hour in dark.

Western blots

Cells were harvested using the M-PER (Pierce Biochemical, IL) protein extraction kit with addition of the protease cocktail (Sigma-Aldrich; St Louis, MO). Then the cell suspension was briefly sonicated and the cell lysate was centrifuged at 4°C for 15 minutes at 10,000 rpm. The supernatant was collected and the total protein content was measured by a spectrophotometer (Beckman DU®-650 U.S.A) using the Bio-Rad DC protein reagent kit (Modified Lowry Method). The protein was solubilized in 4X sample buffer containing 50% glycerol, 8% SDS , 10% of 1M Tris pH (6.8), Coomasie stain 0.02 g and freshly added 5.2% ß-mercaptoethanol and then boiled for 10 minutes. The protein was separated on 12.5% SDS criterion gels (Bio-Rad) at 200V for 1 h in 1X Tris glycine buffer (Bio-Rad) and then transferred on PVDF membrane (Hybond-P; Amersham Biosciences Inc) for 45 minutes at 100V in transfer buffer containing 0.48% Tris-HCl, 2.2% glycine, and 40% methanol. The blot was then blocked

in 3% nonfat milk diluted in 1X TBS containing 0.2% Tris, 8% sodium chloride, pH adjusted to 7.6, then washed with 1X TBS for 30 minutes. The membranes were probed with SNAP-25 specific primary antibody raised in rabbit (Sigma-Aldrich, St Louis, MO) overnight. Then on the following day the blot was washed for 30 minutes in TBS. The blots were then incubated with the secondary antibody (GAR-HRP, Bioscource; CA) for 1 h. Then the blots were washed for 30 minutes followed by detection method using ECL kit (GE Health Care; UK).The blots were exposed to photographic film for required amount of period and developed. Images were captured using a Bio-Rad Imager equipped with Quantity One® software.

Results

In this study, immunofluorescence methodology was employed to detect the binding of serotype A at 3 different ages. In (Figure 4.1) binding of the toxin occurred and can be detected in all the ages of the cultures. The binding experiments were performed under cold conditions so that internalization was prevented. The cells were exposed to 10^{-8} M toxin for 30 minutes. Companion cultures treated under these same conditions were assayed for SNAP-25 cleavage. As can be seen in (Figure 4.2) cleavage was not evident in these cultures, indicating that bound toxin was not internalized but remained on the cell membrane.

Toxin-induced cleavage of SNAP-25 was investigated in neurons from cultures of 1, 2, and 3 week of age. Cultures were exposed to toxin concentrations ranging from 10^{-8}M to 10^{-12}M for 30 minutes and then harvested at several different time points post-toxin exposure. Dose and time-dependent SNAP-25 cleavage at different ages is illustrated in Figures 4.4 and 4.5. The intensity of the cleavage band for a given post exposure time point reflects the amount of SNAP-25. Also with the increase of the time allowed for the internalization, exposes more amount of its substrate SNAP-25 is available for the toxin to cleave. Immunofluorescent staining for the

SNAP-25 was also performed on the motor neurons the (Figure 4.3) for the cleavage of SNAP-25. The cultures were only exposed to 10^{-8}M toxin concentration for 30 minutes and rest 90 mins were allowed for the internalization.

Discussion:

In the present study, the dose responses for the toxin across different ages of the enriched cultures beginning from 1 to 3 weeks were analyzed. These cultures showed an increased sensitivity towards the toxin at lower doses as the neurons mature. The analysis of this increased sensitivity probably implicates the increase in the amount of the target SNAP-25 expressed as the age of the neuron increase. Even though it has already been shown that binding of the toxin under the suitable experimental conditions that occurs within 4 minutes of the toxin application, it has not been evaluated about the least amount of time required to bring about the cleavage of the target SNAP-25. Towards the contribution to that goal, these experiments demonstrate substantial sensitivity of 1 week and 2 week old cultures. Also, this experiment further corroborates that even though 3week old cultures are much more sensitive, the sensitivity does not arise at the end of third week. This observation most likely suggests that, once the motor neuron spread out at the end of 1week the number of receptors along with the increase in the intracytoplasmic membrane associated target SNAP-25, per given surface area. This might result in the sustained sensitivity for the toxin. Earlier experiments on the sustained action have shown that once internalized the toxin remains functional for very long periods of time (Keller and Neale., 2000). As the time allowed for the internalizations increases from 90 to 120 minutes the increase in the cleavage of the molecular target accumulates.

Conclusion

The present study aims at understanding the sensitivity of the early cultures to the neurotoxin/A. Even though, these early cultures require a higher dose of the toxin to produce the cleavage, suggests that the receptor protein involved in the binding of the toxin is present from the early stages itself. This preliminary observations would help to minimize the use of long term cultures for understanding the mechanism of binding of the toxin/A and further the action of serotype A on its target protein.

Figure 4.1: Immunofluorescent staining of toxin serotype A in 1, 2 and 3 week cultured motor neurons. All the images were taken at 40X.

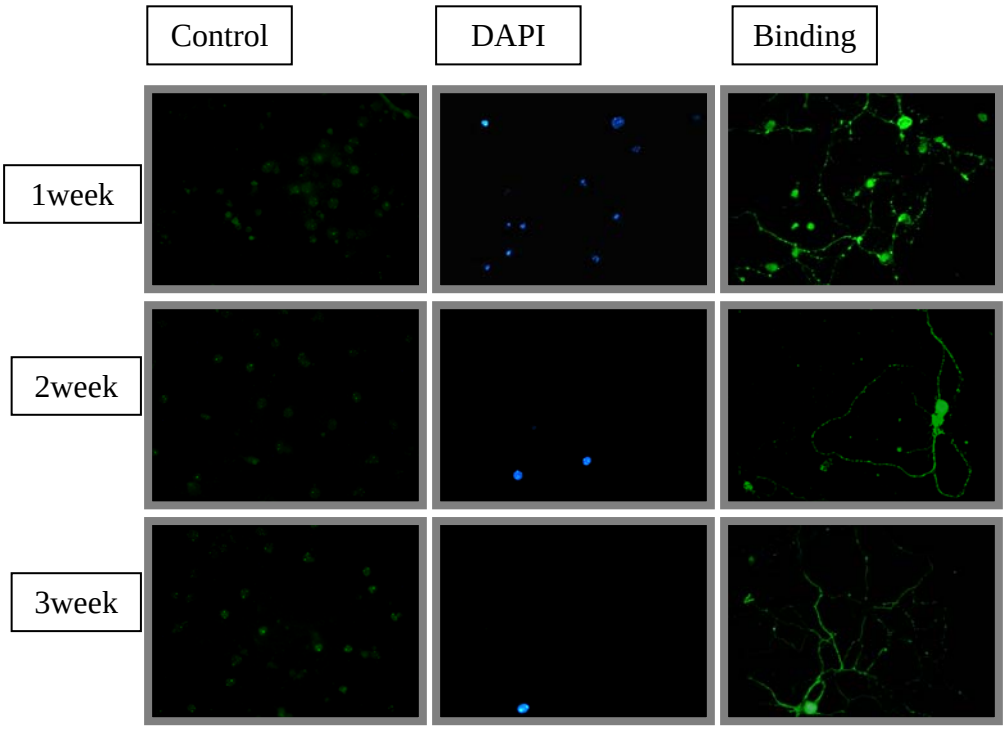

Control
DAPI
Binding
1week
2week
3week

Figure 4.2: Immunofluorescent imaging for SNAP-25 cleavage in 1, 2, and 3 week cultured motor neurons. All the images were taken at 40X.

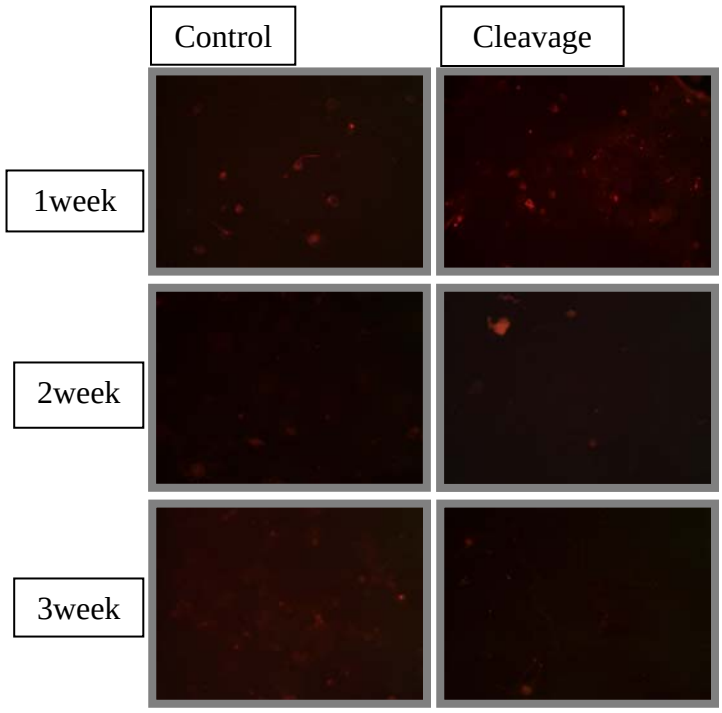

Control
Cleavage
1week
2week
3week

Figure 4.3: Immunofluorescent detection of cleaved SNAP-25 in motor neurons 1, 2, and 3 weeks in culture. All the images were taken at 40X.

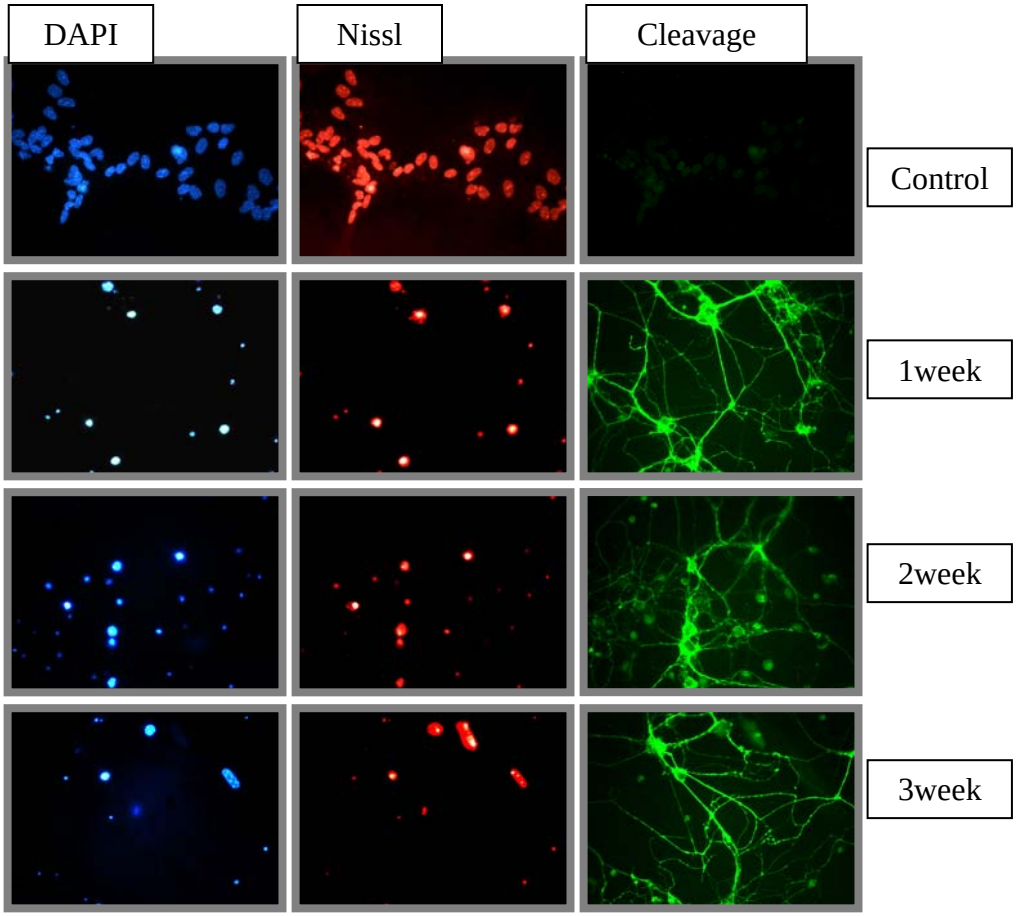

DAPI
Nissl
Cleavage
Control
1week
2week
3week

Figure 4.4: Immunoblot detection of SNAP-25 cleavage at 90 minutes post-toxin exposure. Neurons from 1, 2 and 3 week old cultures exposed for 30 minutes to serotype A at dilutions ranging from 10^{-8} to 10^{-12}. In this particular case the post-toxin exposure time was 90 min. The protein sample from these treated cultures along with the control were loaded onto the 12.5% SDS gels and transferred to PVDF membranes. The blots were probed for SNAP-25. Of the two bands visible in each blot the upper band represents the parent SNAP-25 of 25 kDa MW. The lower band represents the cleaved fragment of the SNAP-25 which is of 24 kDa MW.

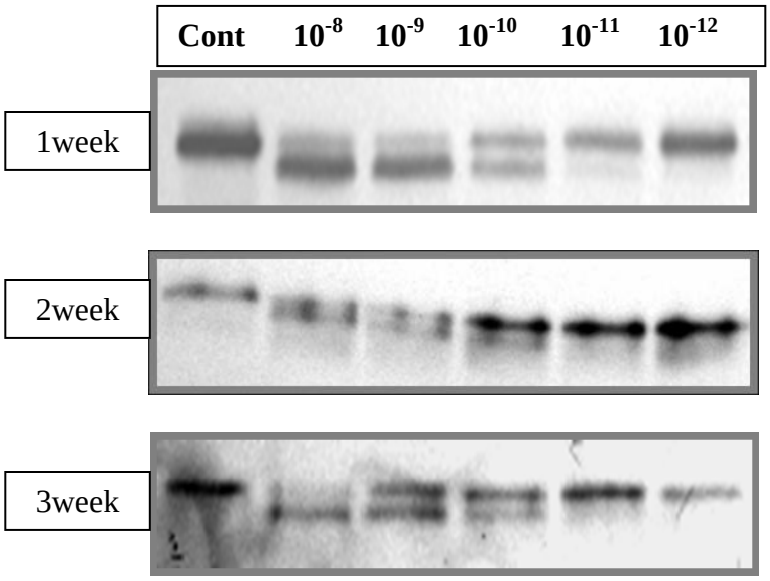
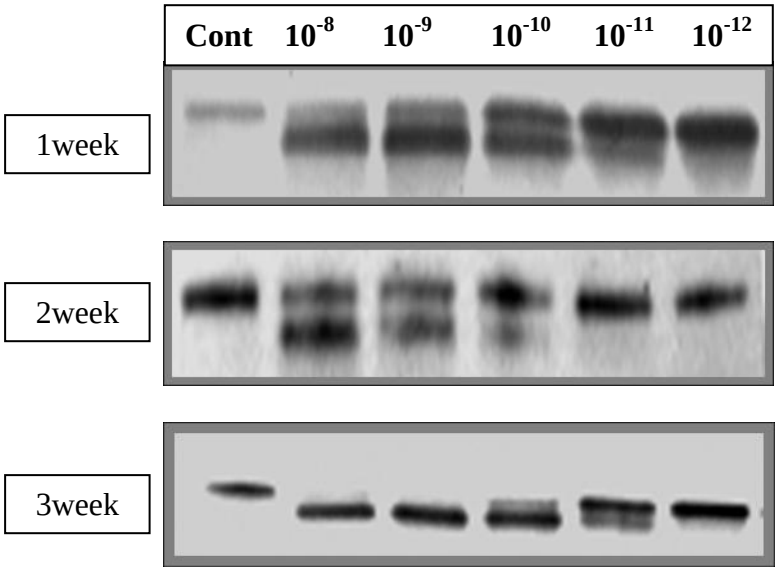

Cont
10-8
10-9
10-10
10-11
10-12
1week
2week
3week

Figure 4.5: Immunoblot detection of cleaved SNAP-25 at 210 min post-toxin exposure. Neurons from 1, 2 and 3 week old cultures were exposed to toxin for 30 minutes at dilutions ranging from 10^{-8}M to 10^{-12}M. In this case, the post toxin-exposure period was 210 min. The protein sample from these treated cultures along with the untreated control was loaded on to 12.5% SDS gels and transferred to PVDF membrane. The blots were probed for SNAP-25.


Cont
10-8
10-9
10-10
10-11
10-12
1week
2week
3week

References:

Grosse G, Tapp R, Kuchinke J, Gorlston M, Fetter I, Zell BH, Gratzl M, Bergmann M and Grosse J (1999) SNAP-25 Requirements for the dendritic growth of Hippocampal Neurons. J Neurosc Res **56**:539-546.

Keller JE and Neale EA.(2000) The Role of the Synaptic Protein SNAP-25 in the Potency of Botulinum Neurotoxin Type A. J Biol Chem **276**(16):13476-13482.

Keller JE and Adler M et al. (1999) Persistence of botulinum neurotoxin action in the cultured spinal cord cells. FEBS Lett **456**:137-142.

Keller JE, Cai F and Neale EA. (2004) Uptake of Botulinum Neurotoxin into Cultured Neurons. Biochem **43**:526-532.

Williamson LC, Halpern JL, Montecucco, Brown JE and Neale EA(1996) Clostridial neurotoxins and substrate Proteolysis in the intact neurons. JBiol Chem **271** (13) 7694-7699.

CHAPTER 5

CONCLUSIONS

Botulinum toxin serotype A is an extremely potent neurotoxin that targets the neuromuscular junction, inhibiting the release of acetylcholine, and bringing to halt neuromuscular transmission thus causing flaccid paralysis. Even though the mechanism of the action of this serotype has been well understood, the proteins that mediate the binding and internalization are still open to the speculation (Simpson. 2000). Research in the pursuit of finding these proteins on the native target has faced the challenge of using a rational in vitro model system.

Enrichment of spinal cord cultures

Earlier experiments attempting to optimize spinal cord cultures encountered some inherent drawbacks. Some of them were the presence of populations of other cell types such as dorsal root ganglion neurons (Ransom et al. 1997). Further, we have observed that horse serum, a common component of cell culture medium supports the growth and proliferation of glial cells, which eventually overrun the neuronal cultures. Although combinations of cell cycle inhibitors (e.g. mitotic inhibitors) like uridine and fluorodeoxyuridine were used to control early glial growth, their presence in the cultures was still notable. In an effort to overcome some of these inherent problems, the first aim of this thesis addressed the issue of population heterogeneity using Nycodenz, a density gradient. Utilization of this biochemical approach wherein only neurons of a larger size are selectively collected thus significantly reducing the heterogeneity of the spinal cord cultures. The optimized cultures were then quantitatively analyzed for the percentage of motor neurons in the whole population using the early motor neuron specific marker P^{75}NTR (Henderson, 1993). In our experimental system, approximately 74% of the

population was positive for the motor neuron marker. This protocol offers a very economical and quick means of assessing the motor neuron populations. Since the initial rate of motor neuron survival also depends on other factors such as reducing the amount of time from the dissociation step to the final step of plating the neurons, further addition of the immunopanning method to the existing density gradient method would hamper the survival and the productivity in the cultures. The immunopanning technique is quite time consuming and is not very cost effective. Hence use of the early motor neuron specific markers will not only assist in the early and quick method of identification of the motor neuron populations, but also assists for the long term survivability of the motor neuron populations.

Because supporting glial cells as well as the horse serum had been significantly reduced by the end of the first 72 h, optimum trophic support has to be provided to the motor neurons in some other form. This was done with B-27 supplement. The B-27 supplement mostly replaces the horse serum and along with it growth factors like GDNF, BDNF, and CNTF to facilitate sufficient nutritional requirements. This is important because it is known that motor neurons deprived of trophic support, and/or which fail to reach their target, undergo apoptotic cell death. In addition, as motor neurons start maturing and growing there is extensive metabolic stress resulting in oxidative stress-mediated cell injury, which may also culminate in cell death. Thus the presence of the added growth factors supports the neurons during such critical periods resulting in enhanced survivability.

Expression of SNARE proteins in cultured motor neurons

In general, botulinum neurotoxin serotype A targets/cleaves SNAP-25, one of the members of the SNARE machinery, by bringing to a halt the release of acetylcholine and inhibiting neurotransmission. The first step in the pursuit to evaluate the sensitivity of the

enriched motor neuron cultures to the toxin, it was first. necessary to check for the presence of the neuronal SNARES in the enriched populations along with the late motor neuron specific markers ChAT and VAChT which are entirely expressed by the motor neurons in the spinal cord. This was the second aim of this thesis. From these experiments, it was observed that as the cultures approached 3 week of age, the expression levels of ChAT and VAChT increased, suggesting that the motor neurons are approaching maturity. Interestingly, the levels of syntaxin, and SNAP-25 also increased considerably from the first week to the end of the third week but, further experiments such as measurement of transcripts levels for these proteins at different ages needs to be evaluated. Surprisingly, the vesicular proteins synaptobrevin and synaptotagmin did not show any variation in their levels of expression. These may be constitutively expressed and might be involved in other functions along with neuroexocytosis.

The final aim of this thesis was to examine the potential differential sensitivity of the cultures to the neurotoxin as they mature. It is already known that following uptake, serotype A remains functionally present in the cytosol for more than 5 days. So, it would be more exciting to see how quickly and how early in development, can the toxin bind and bring about cleavage of the substrate. In order to do so, three different ages of cultures (1 to 3weeks old) were selected and treated with the toxin. At the lower doses of the toxin 3 week cultures are found to be more sensitive and further, with the increase in the amount of time allowed for cleavage from 90 to 210 minutes the extent of SNAP-25 cleavage increased within a single age group. From the immunofluorescence studies examining the onset of cleavage of SNAP-25, it appeared that 30 minutes of toxin incubation given to cleave the target was not sufficient for the fluorescent detection of cleaved SNAP-25. This may be more reflective of the detection method than toxin sensitivity since this dose of toxin is known to inhibit transmission at the neuromuscular junction

within 30 minutes. Further experiments will be needed to address questions like 1) least amount of time required for the appearance of the cleavage products, 2) The rationale behind the early sensitivity of the 1 and 2 week old cultures to the toxin even though at lower dilutions is definitely an interesting area of research. More pertinently, if the later issue of the prior sensitivity of the motor neurons is addressed promptly, it will promptly facilitate the already belated research of finding the proteins involved in interaction with the Neurotoxin/A at the membrane surface. The findings from the present study demonstrate that motor neurons enriched from the heterogeneous population of spinal cord neurons are also sensitive to the neurotoxin A and offer a valuable in vitro model system for the toxin binding and internalization studies.

References

Camu W, Gallego BE and Henderson C E.(1993) Purification of spinal motor neurons from Chicken and Rat embryos by Immunopanning. Neuroprotocols **2**:191-199.

Ransom BR, Henkart P, Bullock PN, Nelson PG and Neale EA et al. (1977) Mouse spinal cord in cell culture. I. Morphology and intrinsic Neuronal Electrophysiological properties J Neurophysiol. **40**(5)1132-1150.

Simpson LL.(2004) Identification of Major Steps in Botulinum Toxin Action. Ann Rev Pharmacol Toxicol. **44**:167-193.

.