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CICLO XXVI

STUDY OF THE MECHANISM OF ACTION OF SCORPION NEUROTOXINS

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BEV	*Buthus eupeus* venom
BSA	Bovine serum albumin
CGNs	Cerebellar granular neurons
DBP	Disulfide bridged peptides (DBPs
GST	Glutathione *S*-transferase GST
HCV	Hepatitis C virus
MNs	Motor neurons
NDBP	Non-disulfide bridged peptides
OSV	*Orthochirus scrobiculosus* venom
SA	Sinoatrial node
SNAP 25	Synaptosomal associated protein of 25 kDa
SNARE	Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
SVMP	Snake venom metalloprotease
TSV	*Tityus serrulatus* venom
VAMP	Vesicle associated protein

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RIASSUNTO

Gli Scorpioni sono importanti rappresentanti del phylum Artropodi. Essi si sono ben adattati a condizioni ambientali estreme e giocano un ruolo fondamentale in diversi ecosistemi. Allo stesso tempo gli scorpioni sono responsabili di più di 1.2 milioni di punture per anno con quasi 3000 morti in tutto il mondo. Sono due le famiglie di scorpioni pericolose per l'uomo: Buthida e Hemiscorpiidae. Le punture di scorpione possono causare da effetti lievi come rossore, e dolore a effetti gravi che causano danni a diversi organi ed eventualmente la morte del soggetto colpito.

Il veleno di scorpione è costituito da diversi componenti come peptidi a basso peso molecolare, lipidi ed enzimi. Gli effetti patologici della puntura di scorpione sono causati dalla presenza di diverse tossine (neurotossina, cardiotossina, nefrotossina, tossina emolitica) e diversi enzimi (fosfodiesterasi, fosfolipasi, ialuronidasi) nel veleno. Dato che gli scorpioni hanno una lunga storia evolutiva, durante questo lungo periodo hanno sviluppato un serie di peptidi e proteine che possiedono diverse funzioni biologiche.

Grazie alla loro abbondanza e quindi alla facilità d'isolamento, i componenti del veleno più studiati sono le tossine che esplicano la loro azione sui canali ionici i quali sono stati molto studiati e descritti in dettaglio in letteratura. Recentemente è stata descritta una nuova classe di metalloproteasi di scorpione capace di proteolizzare le proteine SNARE che è stata denominata Antarease. Fino ad ora le proteine SNARE che hanno un ruolo chiave nel processo di neuroesocitosi, sono state descritte essere il bersaglio molecolare solo di neurotossine batteriche quali le tossine del tetano e del botulismo. Per studiare questa nuova classe di metalloproteasi, abbiamo analizzato l'azione di diversi veleni di scorpioni sia su proteine SNARE ricombinanti sia su modelli di neuroni primari in coltura.

Questo studio ha dimostrato la presenza di metalloproteasi simili all'Antarease in specie di scorpioni *Buthus eupeus* e *Orthochirus scrobiculosus* e che questi enzimi sono in grado di proteolizzare in maniera specifica le proteine SNARE.

Summary

Scorpions are important representative of arthropods. They have been well adapted to the extremes of environmental conditions. Scorpions also play a vital role in ecological systems by maintaining balance between different populations in an ecosystem. Apart from their positive role scorpions are responsible for more than 1.2 million stings per year with almost 3000 deaths worldwide per year and thus posing serious threat to public health. Two families of scorpions i.e. Buthida and Hemiscorpiidae are dangerous for humans. Scorpion sting can result in mild effects of redness and pain to very severe and lethal effects which can result failure of multiple organs eventually leading to death.

Scorpion venom is composed of many different components such as low molecular weight peptides, nucleotides, lipids and certain enzymes. The diverse detrimental effects of scorpion sting result due to presence of many different types of toxins (neurotoxin, cardiotoxin, nephrotoxin, hemolytic toxin) and different enzymes (phosphodiesterases, phospholipases and hyaluronidases) in their venom. Since scorpions have a long evolutionary history during this long time period scorpions have developed a series of venom peptides that display a diverse range of biological functions.

The most widely studied components of scorpion venom are the ion channel-modulating toxins which have been studied and described in detail in literature. Antarease a new class of unique scorpion metalloproteases capable of cleaving SNARE proteins has been described only recently with no previous evidence on presence of such enzymes in scorpions. Up till now SNARE proteins have only been shown to be targets of clostridial neurotoxins. To investigate such a class of metalloproteases we have analyzed the action of different scorpion venoms both on recombinant SNARE proteins as well as in neuronal cell models.

Conclusion: This study shows for the first time the presence of antarease like proteins in scorpion species: *Buthus eupeus* and *Orthochirus scrobiculosus.* Both of the scorpion species contain active components i.e. metalloproteases that are able to cleave SNARE proteins in a mechanism similar to antarease.

CHAPTER 1

INTRODUCTION

CHAPTER I

INTRODUCTION

1.1 Scorpion Biology

Scorpions are known to be one of the oldest animals living on the planet and their existence dates back to more than 400 million years ago (Jeyaprakash and Hoy 2009). Scorpions are important representative of arthropods. They have been well adapted to the extremes of environmental conditions and have maintained the primary characteristics of Paleozoic scorpions such as pectin, book lung and venom apparatus. For these features scorpions have also been termed as living fossils (Polis 1990). Up till now almost 2000 different species of scorpions have been reported worldwide. Scorpions are found in vast geographical ranges occupying almost all continents except Antarctica. Scorpions venom system evolved long ago as their main weapon against the preys as well as against their predators. The venom gland has been reported from a scorpion fossil dating back to Silurian period, which is approximately 418 million years ago (Dunlop et al 2008 and Cao Z. et al, 2013).

Scorpions also play a vital role in ecological systems by maintaining balance between different populations in an ecosystem. Scorpions are nocturnal animals, which primarily feed on insects, small vertebrates and gastropods (Zwicky 1968 and Rao 1973). Another interesting characteristic of scorpions is the release of fluorescence when irradiated by ultraviolet light. This feature of scorpions has been widely used in the field to locate the scorpions (Gaban and Farley 2002).

A recent epidemiological survey estimates 1.2 million scorpion stings in year leading to more than 3000 deaths (Chippaux and Goyffon, 2008). Two families of scorpions i.e. Buthida and Hemiscorpiidae are dangerous for humans. The species of family buthidae that cause significant hazards to human health include Mesobuthus and Hottentotta species in Asia, Leiurus species in the Middle East, Androctonus and Buthus species in North Africa, Tityus

species in South America, Centruroides species in North America and Parabuthus species in South Africa (Murthy, 2000 and Yugandhar et al, 1999).

The scorpion venom apparatus is composed of a venom vesicle that contains a pair of venom glands in the last segment of post-abdoment called as telson (Figure 1.1). The venom glands are located on the tail lateral to the tip of the stinger and contain two different types of tall columnar cells. One type is responsible for the production of the toxins, while the other one produces mucus. There is a layer of striated muscle around the venom vesicle that regulates the ejection of the venom (Chippaux 2012).

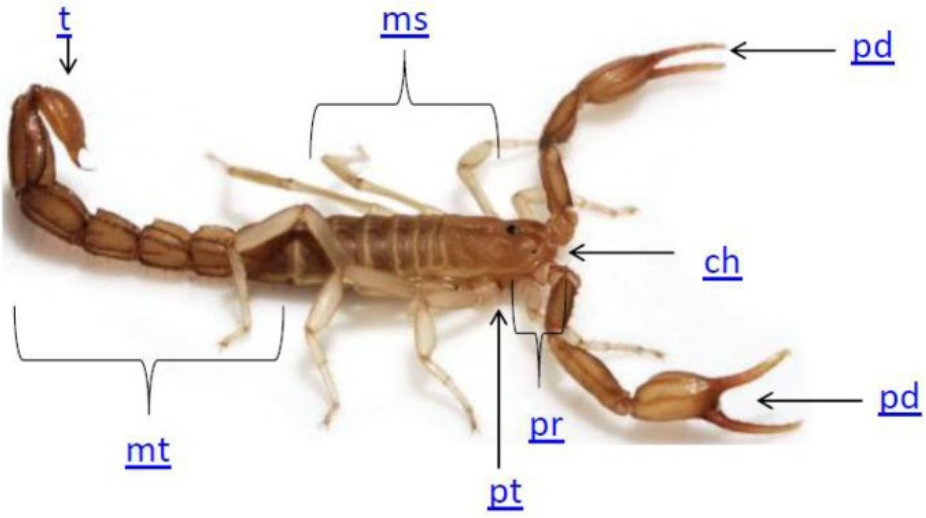
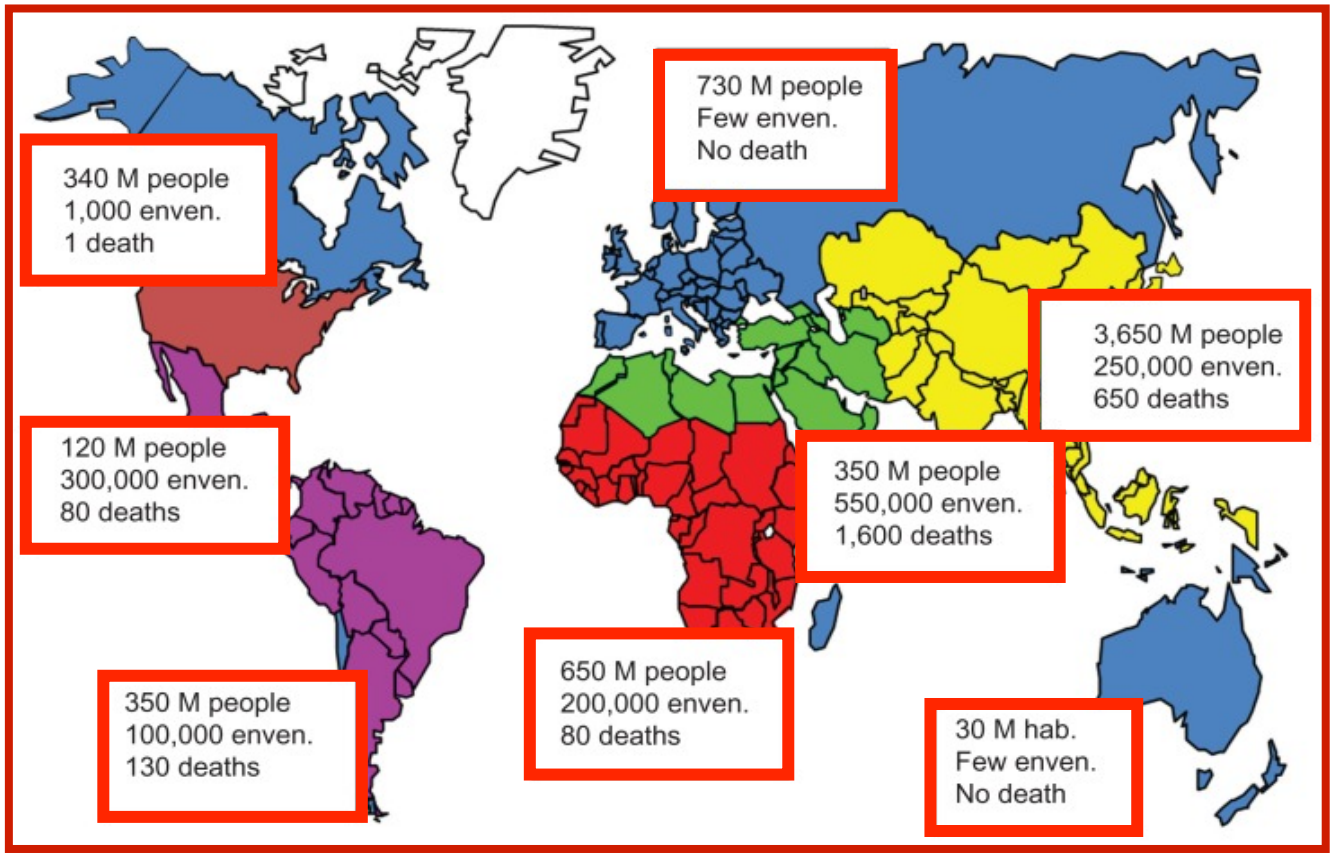


Figure 1.1: Basic morphology of a scorpion. The body is divided into three major sections, the tail or metasome (mt**); the abdomen or mesosoma (**ms**); and the head region or prosoma (**pr**); Distinct structures are also highlighted including pincers or pedipalp (**pd**); mandibles or chelicerae (**ch**); contact chemosensors (pectines-pt), and the venom apparatus or telson (**t**).** (Bergeron and Bingham, 2012, Adapted from Weber et al, 2012).

Scorpions grasp their prey with the help of pincers; soon after which they arch their tails and drive stingers close to the prey followed by injecting their prey with the venom. The quantity of venom to be injected with each sting can be voluntarily regulated by the scorpion. The

striated muscles in the stinger allow regulation of the amount of venom ejected, which is usually 0.1-0.6 mg. It normally takes several days for the production of venom once the whole supply of venom has been used from the venom glands. Depending on the scorpion and amount of venom it produces for example in the case of *Parabuthus* species with large venom sacs, are capable of even squirting their venom (Cheng et al., 2013).



(Chippaux J. P 2012). Abbreviations enven: envenoming

Figure 1.2: World incidence and mortality following scorpion stings. Abbreviations enven: envenoming and M: million (Chippaux J. P 2012).

1.2 Symptoms and pathophysiology of scorpion stings

Scorpionism incidences have been reported to occur more frequently in urban areas (approximately 70% of reported scorpionism) to increase in summers because of higher temperatures and high level of scorpion activity (Figure 1.2). The extent and severity of scorpion envenomation depends on many different factors, for example the species of scorpion, amount of venom, age and health status of victims etc. Almost all age group population faces scorpion accidents but most of the lethal cases are reported to occur in individuals younger than 14 years. Scorpion envenoming more or less produces similar symptoms in both humans as well as experimental animals regardless of the species of scorpion. A lot of information on pathology of scorpion sting has been made available through animal models. Scorpion envenoming causes a lot of different symptoms with most common ones including swelling, redness and pain at the site of sting (Bucaretchi et al, 1995 and Ministério da Saúde, 2001).

Symptoms of scorpion envenoming can be grouped into three different stages depending upon the severity of poisoning.

- The first stage, which is seen in most of the cases of scorpion envenoming is characterized by intense pain, fever, sweating and blood pressure fluctuations.
- In severe cases, a second stage occurs with symptoms including sweating, vomiting, cramps, diarrhea, hypotension, bradycardia, and dyspnoea.
- The most severe cases result in last stage, the third stage, that can be lethal with severe symptoms including respiratory complications, myocardial ischemia and cardiac arrhythmia (Chippaux and Goyffon, 2008).

Severe envenoming can result in failure of multiple organs eventually leading to death (Figure 1.3). The major effects caused by scorpion venom results due to toxins that interfere with the ion channels (Gwee et al, 2002).

In the case of heart the scorpion venom affects specially the sinoatrial node (SA) that is the heart's natural pacemaker. The specific effects include alterations in junctional rhythm, sinus arrest and atrial standstill. The interference caused by the venom on the heart electric activity

regulation results in the imbalance of ions between intra and extra-cellular spaces (Tilley, 1992).

The exact mechanism by which scorpion venom toxins induce severe cardiac dysfunction is still unknown. The cardiac damage seems to occur in different ways, a direct action of the toxins in the venom and at the same time an indirect mechanism by the autonomic alterations. It has also been hypothesized that the release of catecholamines and cytokines resulting from scorpion stings leads to myocarditis and congestive heart failure (Bahloul et al., 2010).

Recently direct effect of scorpion venom has also been identified on pancreas basically lead by the observation of severe pancreatitis resulting in patients of scorpion stings. It has been demonstrated that the toxins from the scorpions of the *Tityus* genus possess a powerful secretagogue effect on the pancreas. Furthermore it was also shown that scorpion venom was able to stimulate the release of amylase in the pancreas lobes of guinea pigs (Possani et al., 1991; Jiménez-Ferrer et al., 2005). Acute pancreatitis has been reported in the stung by *Leiurus quinquestriatus* (Sofer et al., 1991) and in the case of *Mesobuthus tamulus* whose venom has been shown to act directly on exocrine pancreas to cause acute pancreatitis in experimental models of dogs and rabbits (Murthy et al., 1989; Ortiz et al., 2013).

Scorpion envenoming also causes massive loss of water and electrolytes resulting from the systemic effects of venom toxins and ultimately leading to electrolytic imbalances in respiratory and gastrointestinal systems (Ismail 1995). As a result several electrolyte disorders occur including hyperglycemia, hypermagnesemia, hyperkalemia, hypocalcemia and hyponatremia (Andrade et al, 2004; Murthy et al., 1986; Angsanakul and Sitprija 2013).

Just like all major organs kidneys are also adversely affected due to scorpion envenoming which causes renal toxicity as indicated by presence of hemoglobinuria, proteinuria and hematuria ultimately leading to renal failure, which can result in more severe cases. At the same time blood toxicity, abnormalities in neurotransmitter releases and inflammatory responses further complicate the pathology and effects of scorpion envenoming (Pipelzadeh et al., 2007).

It has also been observed that the lethal fractions acquired from venoms of different scorpion species have very similar effects on autonomic nervous system. In one study carried out on the victims of scorpion bites indicates prevalence of hypertension in over 80% of the victims. Other more commonly observed symptoms include pulmonary edema (a major cause of deaths resulting due to scorpion stings), increased release of glucagon, liver enzymes, cortisol and serum amylase. The treatment for the scorpion envenomation involves symptomatic relief and support of vital functions along with serum therapy (Amitai, 1998).

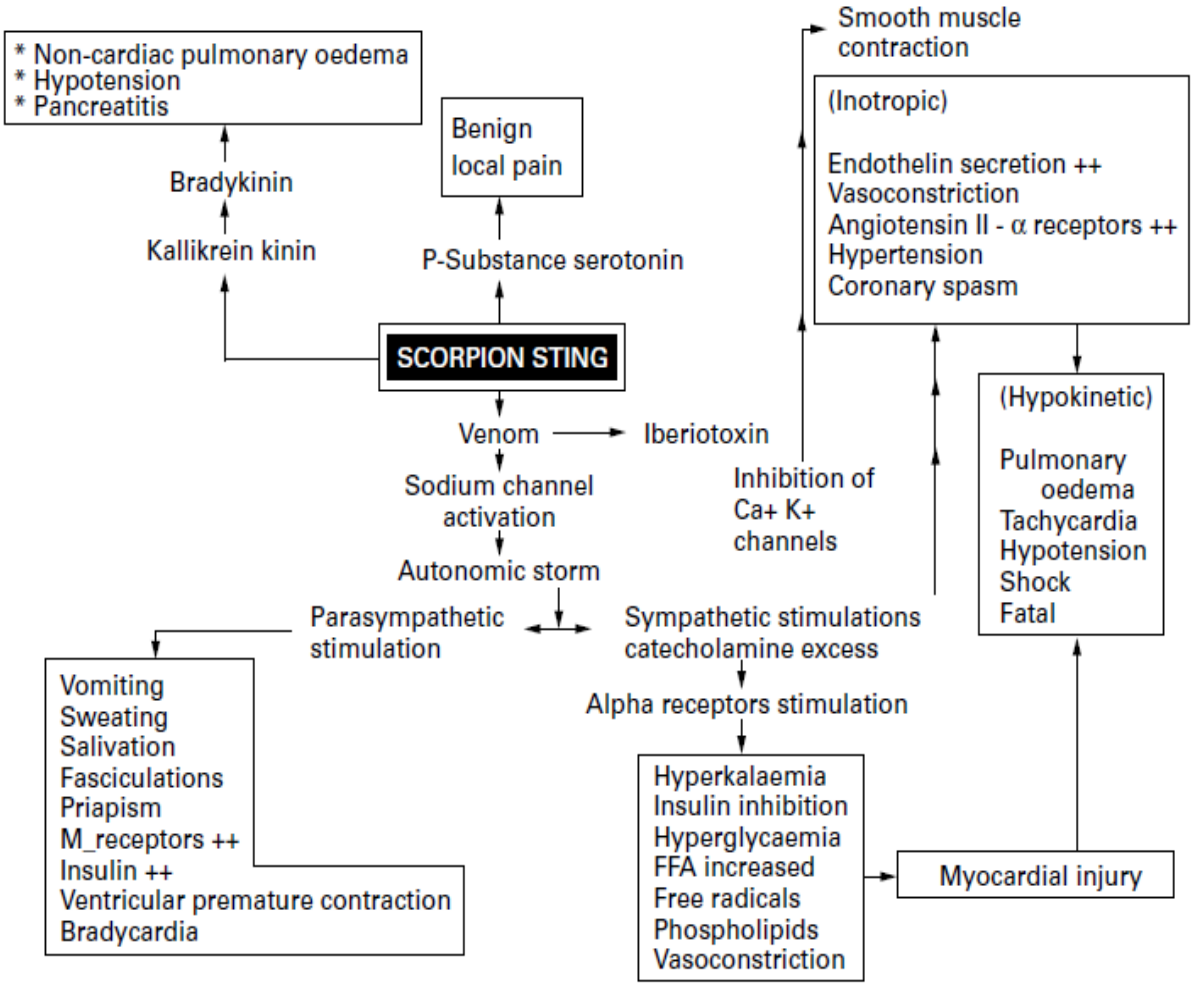


Figure 1.3: A flowchart showing the pathophysiology as a result of scorpion envenoming (Deshpande and Alex 2000).

1.3 Management of scorpion stings

Proper management of scorpion stings can lead to reduced mortalities and fast recovery rates in the victims. Stings from scorpion generally follow different stages depending on the severity of the stings which in turn depends on many factors for example species of scorpion, age of victim and health status of victim. Generally analgesics are used immediately to relieve the intense pain, which is very common with scorpion stings. In certain cases these are administered along with anti-inflammatory drugs. Out of the total stings it is estimated that almost 15-20% would actually progress to more severe state. Similarly depending upon the symptoms different management measures are taken for example specific drugs, which aim at cardiac and pulmonary treatment (Ismail 1994). Certain vasodilators for example Prazosin is recommended for scorpion stings in particular in India (Bawaskar et al, 1996).

Passive immunotherapy, which is administration of antibodies that have been produced in animals by injecting similar venoms, is also recommended when scorpion species is same or very similar to one that has been used to produce antibodies (Boyer et al, 2009).

1.4 Scorpion venom peptides

Scorpion venom is composed of many different components for example low molecular weight peptides, nucleotides, lipids and certain enzymes whose main functions for scorpion are: capturing prey and protecting scorpion against predators and microorganisms. The venom from scorpion species is not always considered to be homogenous particularly in case of some scorpions which release pre-venom followed by release of more lethal and peptide rich venom, in a situation where scorpion is faced with powerful preys. Scorpion venom may contain multiple toxins for example neurotoxin, cardiotoxin, nephrotoxin, hemolytic toxin and many different types of enzymes for example phosphodiesterases, phospholipases, hyaluronidases, glycosaminoglycans, histamine, serotonin, tryptophan, and cytokine releasers (Inceoglu et al, 2003).

Scorpions have a long evolutionary history and during this long time period scorpions have developed a series of venom peptides that display a diverse range of both biological as well as pharmacological function (Rodriguez de la Vega et al, 2010).

The peptides from scorpion venom are broadly classified into two classes:

- Disulfide bridged peptides (DBPs)
- Non-disulfide bridged peptides (NDBPs)

The DBP are generally small ion channel modulator peptides with three to four disulfide bridges. These peptides are further divided into different subclasses depending on the type of ion channel affected i.e. Na^+-channel toxins, K^+-channel toxins, Cl^--channel toxins, and Ca^{2+}-channel toxins. The NDBPs are a smaller group of peptides with a lot of different and diverse functions (Chen et al, 2003 and Zeng et al, 2000).

1.4.1 Disulfide bridged peptides

A lot of different types of ion channel modulators have been identified and isolated from scorpion venoms. Some of them are detailed below:

Sodium channel toxins

Na^+-channel scorpion toxins are long chain scorpion toxins with approximately 60-76 amino acids. Generally this class of toxins contains four disulfide bridges. Sometimes these toxins are also classified as anti-mammal and anti-insect neurotoxins. The ones, which affect mammals are further divided into two subtypes depending upon the mechanism of action. The α type toxins slow down the quick inactivation of Na^+-channels and prolong the action potential of Na^+-channels. The β type toxins affect the activation of Na^+-channels. Insect specific neurotoxins are generally further classified as excitatory or depressant (Ji et al, 1994, and Xiong et al, 1997).

Potassium channel toxins

Almost 140 different K^+-channel toxins have been reported along with their physiological properties. These toxins are composed of 20-70 amino acids. Maximum toxins have been reported to be less than 40 amino acids with much larger sequence diversity. These toxins contain three disulfide bridges. These toxins are generally classified into three further subtypes α-KTx, β-KTx, γ-KTx. α-KTx contains short chain toxins and is further subdivided into 19 subfamilies. β-KTx contains almost 60-65 amino acids containing three disulfide

bridges and further classified into four subcategories. In addition to these types there are also some other toxins composing of short amino acid sequences that have been designated to kappa-KTx family because of their distinct bi-helical scaffold (Tytgat et al, 1999, Wang et a., 2005 and Chagot et al, 2005).

Chloride channel toxins

These are generally low molecular weight proteins which contain from 35 to 38 amino acids. Generally these are also categorized by presence of four disulfide bridges. The properties of these toxins are very different from other disulfide bridged toxins, for example potassium channel modulators with four disulfide bridges. These toxins share considerable sequence similarity (50-74%) among them. One of the most important member of this group is Chlorotoxin. Chlorotoxin (CTX) is composed of 36 amino acids and four disulfide bridges. It has been isolated from scorpion *Leiurus quinquestriatus*. This toxin inhibits low conductance chloride channels in epithelial cells. It has also been reported to contain binding affinity to the glioma- specific chloride channels and this property has been exploited in many different studies to investigate the effects of chlorotoxins on brain tumors (Tytgat et al., 1998 and Kasai et al, 2012).

Calcium channel toxins

The final category of disulfide-bridged proteins is the toxins responsible for modulation of calcium channels. In this context IpTxA and Mca isolated from scorpion *Pandinus imperator* and *Scorpio maurus* palmatus respectively are quite important. Both of these are single chain polypeptides that contain a high number of basic amino acids. These toxins contain 33 amino acids and are cross linked by three disulfide bridges. Another toxin named as Kurtoxin has been isolated from *Parabuthus transvaalicus* venom. It binds to T type calcium channel with high affinity and carries out inhibition of the channel by altering voltage-dependent gating (Chuang et al, 1998, Fajloun et al, 2000).

1.4.2 Non-disulfide bridged peptides

This class of peptides contains huge variety in their activities and is active against a wide range of biological targets. The huge functional diversity of these peptides can be explained

by different genetic and molecular events for example gene duplication, polymorphisms and trans-splicing events. Some of the functions from these peptides are as follows:

Anti-bacterial peptides:

These peptides are active against bacteria, viruses, protozoa yeast and fungi. One antimicrobial peptide named as Hadrurin has been isolated from scorpion *Hadrurus aztecus*. It is a 41 amino acid long peptide which is reported to inhibit growth of both Gram positive as well as Gram-negative bacteria along with some other cytolytic and hemolytic functions (Torres-Larios et al, 2000). Similarly Meucin- 13 and Meucin 18 containing 13 and 18 amino acids respectively have been identified by cDNA library generation from the genome of *Mesobuthus eupeus.* These peptides have cytolytic effects on bacteria, fungi, yeast as well as rabbit erythrocytes (Gao et al 2009). Other peptides with similar functions include Im-1 a 56 amino acid long peptide isolated from *Isometrus maculates*, Mucroporin a 17 amino acid peptide isolated from *Lychas mucronatus* and Pantinin from *Panidnus imperator* (Zeng et al, 2005). It is also worth mentioning that there have been reports on some antimicrobials isolated from scorpions, which are quite effective against antibiotic resistant strains like penicillin resistant pneumococci, penicillin resistant *E. faecalis* and pencillin resistant *S. Aureus* (Yuan et al, 2010).

Anti-malarial peptides

Malaria is one of the most hazardous disease that has caused millions of deaths worldwide. In this context scorpion venoms can be a rich source of acquiring anti-malarial peptides. The first evidence for the role of venom proteins to target *P. falciparum* comes from the identification of Meucin-24 and Meucin-25, which are both 24 and 25 amino acid long peptides identified through cDNA library of *M. eupeus.* These peptides have been shown to specifically target intraerythrocytic *P. falciparum*. Apart from this property these peptides were not toxic to other microbes and had no adverse effect viability of mammalian cells (Gao et al, 2010).

Anti-cancer peptides

Recently scorpion venoms have also been shown to contain certain peptides, which specifically target the cancer cells apparently by a mechanism involving differential cell membrane morphology of the cancer cells in comparison to the normal cells. In this context two anti-cancer peptides- TsAP-1 and TsAP-2 have been identified from *Tityus serrulatus* venom through cDNA library. Both of these peptides are linear alpha helical peptides with primary structural similarity to anti-microbial peptides from amphibian skins and to phylloseptins (Guo et al, 2013). Mauriporin another peptide, which is 48 amino acid long with anti-cancer activity has been identified in *Androctonus mauritanicus*. Mauriporin was found to be cytotoxic against three different cancer cell lines. When checked for non-cancerous cells mauriporin showed almost negligible cytotoxic effect (Almaaytah et al, 2013).

Immune modulatory peptides

Many different peptides from scorpion venoms have been studied for potential immune system modulatory effects including Opsitoporin 1, Parabutoporin and BmKbpp. Similarly, two different peptides with a similar amino acid sequence isolated from South African scorpion have been shown to activate granulocytes at submicromolar concentrations by stimulating chemotaxis and degranulation. These have shown to act as pore formers at higher concentrations (Willems et al, 2002).

Anti-HCV activity

Hepatitis C virus (HCV) is the major cause of chronic hepatitis affecting more than 200 million people worldwide posing a major public health problem. Along with identification of certain antimicrobial peptides in scorpion venoms anti-HCV peptide has also been reported from scorpion *H. petersii.* It is a α-helical peptide cloned and characterized from the natural venom and named as Hp1090. The functional assay showed that this peptide is virocidal for HCV in vitro by directly interacting with the viral particles and destabilizing their phospholipid membranes. The discovery of this peptide has provided a new approach to target viruses through the use of natural antimicrobials (Yan et al, 2011).

Bradykinin potentiating peptides

Bradykinin plays important role in the regulation of blood pressure by increasing vasodilation and decreasing the blood pressure. The peptides responsible for the inactivation of bradykinin are known as bradykinin potentiating peptides which are also used for the treatment of human hypertension. Different bradykinin potentiating peptides have also been isolated from scorpion venoms including:

Peptide T, a 13 amino acid long peptide, isolated from venom of scorpion *T. serrulatus.*

Peptide K12, a 21 amino acid long peptide isolated from the venom of the scorpion *B. occitanus.*

Peptide BmKbpp, a 47 amino acid peptide identified from the venom of the Chinese scorpion *Mesobuthus martensii* Karsch (Ferreira et al, 1993, Meki et al, 1995 and Zeng et al 2012).

1.5 Scorpion species used in this study

Three different scorpion species have been used in this study. These species are as follows.

Tityus serrulatus

Buthus eupeus

Orthochirus scrobiculosus

1.5.1 Tityus serrulatus

Out of the total species of scorpion the genus Tityus comprises the highest number of scorpion species with containing more than 60% of all scorpions which are found in tropical and subtropical regions of the world (Figure 1.4). The most important species of this genus include *Tityus serrulatus* also known as yellow scorpion, *Tityus bahiensis*, and *Tityus stigmurus.* All of these species are considered to be dangerous species because envenoming by these species can be lethal in humans. *Tityus serrulatus* is endemic in Brazil and it is widely distributed in the whole country reaching all states (São Paulo, Minas Gerais, Bahia, Espírito Santo, Goiás, Paraná and Rio de Janeiro). It is responsible for most of the serious scorpion accidents in Brazil, with approximately 1% mortality rate among children

(Ministério da Saúde, 2001). *Tityus serrulatus* is quite unique among scorpions because of its parthenogenetic mode of reproduction (Lourenco et al 2008).

Figure 1.4: An image of the Brazilian scorpion *Tityus serrulatus* Copyright © Dr. Wolfgang Wüster

1.5.2 Buthus eupeus

Buthus eupeus also known as lesser Asian scorpion or the mottled scorpion is a species of family buthidae (Figure 1.5). It is one of the most widespread species of scorpions in Asia inhabiting wide range of geographical areas (Shi et al, 2007). The adult scorpion can reach almost 2 inches in length. *B. eupeus* is a polymorphic species and it generally feeds on small insects. The stings from *B. eupeus* are not as dangerous as other members of the family Buthidae but usually symptoms include intense pain, swelling and hyperemia (Sun and Sun 2011).

Figure 1.5: An image of the Asian scorpion *Buthus eupeus*. Buthus paris (C. L. Koch, 1839) - Female - Locality: Meknes (Morocco) - Copyright: Frantisek Kovarik.

1.5.3 Orthochirus scrobiculosus

Orthochirus scrobiculosus belongs to family Buthidae and is found in almost all of the countries of Asia (Figure 1.6). It is a small sized scorpion ranging in size from approximately 1-2 inches long. It can be found in various colors ranging in shades of Olive to brown. It is often recognized easily because of its distinct tail, which appears as short and thick. Generally this species of scorpions is very quiet and envenoming is not lethal in most of the cases. Stings from *O. scrobiculosus* cause a severe pain a common symptom of scorpion stings. Generally the itching symptoms appear soon after sting that usually disappear after 10 to 15 hours without any need of medical care (Dehghani and Fathi 2012).

Figure 1.6: An image of the Asian scorpion of *O. scrobiculosus* (Dehghani R and Fathi B 2012).

1.6 Proteases

Proteases are a group of enzymes that catalyse hydrolysis (breakdown) of peptide bonds of proteins. They are also known as proteolytic enzymes or proteinases. Proteases are different in their ability to hydrolyze different peptide bonds. Each type of protease has a specific kind of peptide bonds it breaks. Proteolytic enzymes are found in many venoms (snake, spiders, sea animals, lizards, insects and toads), but there are few studies showing the presence of proteases in scorpions The presence of proteolytic enzymes in scorpion venoms was demonstrated by Nget-Hong and Gnanajothy (1992) that published comparative study of the enzymes present in different scorpion genera.

1.7 Antarease- a SNARE cleaving metalloprotease from Scorpion species

The effects of different components of scorpion venoms have not been investigated in detail apart from certain abundantly present enzymes for example hyaluronidase that facilitates tissue penetration and distribution of different venom components and vastly studied ion channel modulator peptides (Feng et al 2008). In fact most of the effects of scorpion envenoming and management of scorpion stings have been described and related to ion channel modulators ignoring the other possible important components adding to pathologies of scorpion stings.

Up till now two different classes of proteases have been described from the scorpion venom. These include **serine proteases** and **metalloproteases**. In 2010 Fletcher et al., reported for the first time evidence of presence of a new class of scorpion venom metalloproteases that are responsible for the cleavage of SNARE proteins. The active metalloprotease has been identified in the Brazilian scorpion *Tityus serrulatus* and has been named as Antarease. Antarease has been shown to cleave VAMP protein at specific site ultimately changing the secretory mechanisms that can lead to varied pathologies including acute pancreatitis, which is often associated with scorpion envenoming. Currently only the clostridial toxins, tetanus and botulinum neurotoxins have been reported to target and cleave the different SNARE proteins.

1.8 SNARE Proteins

1.8.1 Structure and function of SNARE proteins

SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) are responsible for the fusion of transport vesicles (v-SNAREs) with their target membranes (t-SNAREs). This is a large family of proteins, which are characterized by the presence of a characteristic amino acid motif known as SNARE motif (Figure 1.7). The interaction of t-SNAREs and v-SNAREs leads to formation of trans-SNARE complex that brings the opposing membranes together eventually leading to their fusion. After the fusion event the SNARE complex undergoes disassembly and the SNARE components are recycled. SNARE complex formation is vital for membrane fusion in both neuronal and non-neuronal cells. A lot of available information on SNARE complex actually comes from studies of these complexes in neuronal cells. Different isoforms of SNARE proteins have been identified in different types of exosecretory systems.

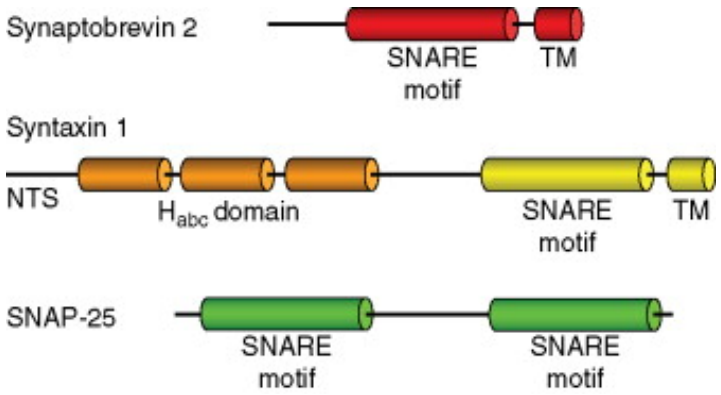


Figure 1.7: Domain structures of the neuronal SNAREs. The SNARE motifs, TM regions, and the helices of the H_{abc} domain are represented by cylinders; other sequences are represented by black lines. NTS, N-terminal sequence; SNAP-25, synaptosomal associated protein of 25 kDa; SNARE, soluble *N*-ethylmaleimide-sensitive factor attached protein receptor; TM, transmembrane (Rizo 2009).

The best characterized SNARE proteins are the ones, which are responsible for the chemical synaptic transmission during synaptic vesicle exocytosis (Figure 1.8), (Jahn and Fasshauer

2012; Jurado et al., 2013). These neuronal SNARE proteins that are responsible for the neurotransmitter release include:

Vesicle associated protein 2 VAMP2 (or synaptobrevin 2)

Plasma membrane associated protein Syntaxin 1

Synaptosomal associated protein of 25 kDa also known as SNAP 25.

Other important components that play critical role in fusion of synaptic vesicles include synaptotagmin I and complexins that are involved in tight regulation of vesicle fusion (Chen and Scheller 2001, Rizo 2009).

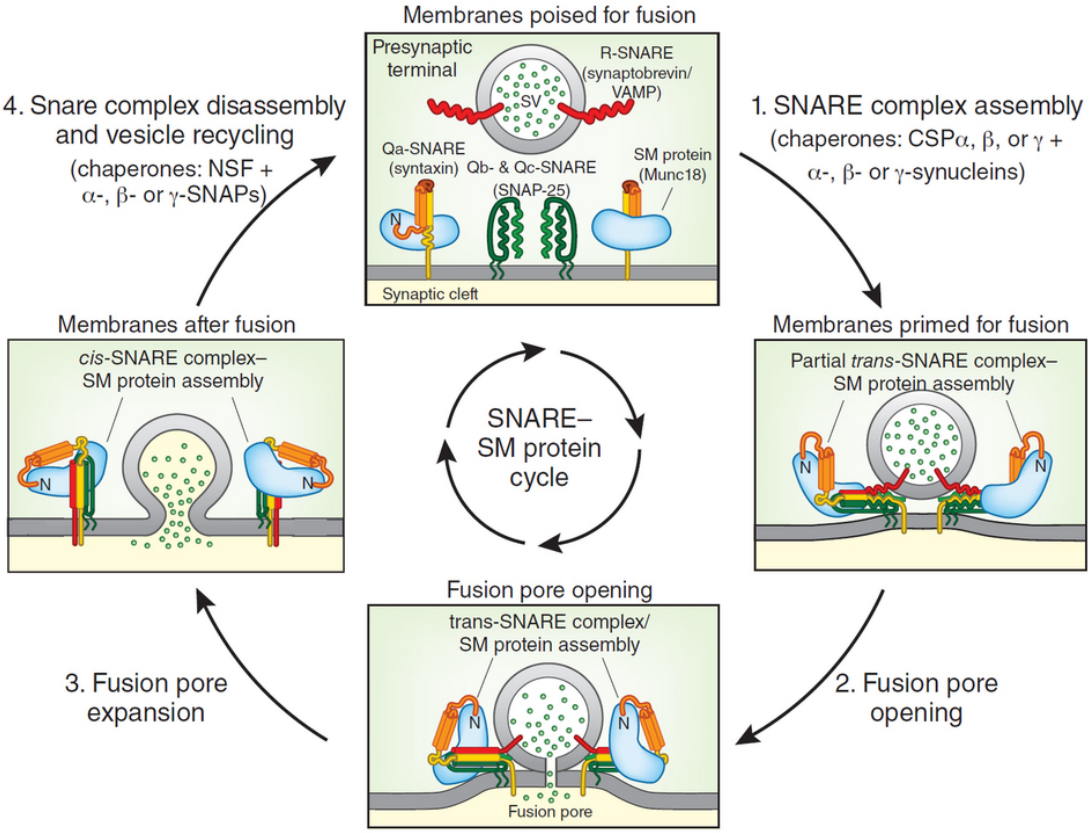


Figure 1.8: Model of the SNARE protein cycle during synaptic vesicle fusion. The four different steps followed during SNARE protein cycling include 1) SNARE complex assembly; 2) fusion and opening of pore; 3) expansion of fusion pore; 4) SNARE complex disassemble and vesicle recycling (Südhof 2013).

1.9 SNAREs discussed in this study

1.9.1 Syntaxin

Syntaxin is approximately 35 KDa protein mainly found on the neuronal plasmalemma. Syntaxin plays important role in neuronal development and plasticity. Syntaxin is a family of t-SNAREs with 17 different gene products in animal cells and are responsible for regulation of different membrane trafficking pathways (Advani et al., 1998). Several isoforms of this protein undergo alternative splicing mechanism to form variable forms of this protein (Quiñones et al., 1999). Syntaxin interacts with other SNARE proteins i.e. SNAP 25 and VAMP 2 to generate synaptic SNARE complex that forms the core of neuroexocytosis apparatus. The amino terminal portion of syntaxin is exposed to cytosol and takes part in protein-protein interaction and formation of four-helix bundle structure (Chen et al., 1999, Schiavo et al., 2000).

1.9.2 SNAP 25

SNAP 25 is one of the major proteins in the central nervous system. It is one of the highly conserved proteins in yeast as well as humans and shows similarity in both length of the protein and in amino acid sequence. SNAP 25 forms a complex with synaptotagmin and plays important role in calcium dependent neurotransmitter release. Like syntaxin, SNAP 25 is also involved in neuronal development and plasticity. Two different forms of SNAP 25 i.e. A and B are developmentally regulated and show different expression levels during development. SNAP 23 is another isoform of SNAP 25 that is expressed in non-neuronal systems and play similar role to SNAP 25 i.e. in regulated secretory pathways (Ravichandran et al., 1996).

1.9.3 VAMP

VAMP 1 and 2 (also called synaptobrevin) are approximately 13 KDa proteins that are localized to the synaptic vesicles. The protein is anchored to the synaptic vesicle membrane via a single transmembrane domain, which is followed by a poorly conserved intravesicular tail of variable length in different species. Ten different isoforms of VAMP have been reported and are found in all tissues of vertebrates. VAMP 1 and 2 associate with

synaptophysin, a major component of synaptic vesicle membrane and with subunits of the V-ATPase (Schiavo et al., 2000).

1.10 SNARE proteins and neurotransmission

The fusion between the transport vesicle and its target membrane is one of the most fundamental process essential for all eukaryotic cells. Several different conserved protein families are involved in this process. One of the major group of these conserved proteins include the SNARE proteins which are responsible for docking and fusion of transport vesicle with its target membrane.

The functions of nervous system depend on regulated neurotransmitter release. The synaptic vesicles are small homogenous secretory organelles that are used for signaling between the nerve cells. These are approximately 50 nm in size and contain neurotransmitters such as γ-aminobutyric acid (GABA) and acetylcholine. Synaptic vesicles occurring in the resting nerve terminals exocytose at slow rates than the ones, which are depolarized. At the nerve terminals these tiny vesicles cycle through many different steps (Mundigl and De Camilli 1994). The events in the synaptic cycle include docking of the neurotransmitter-filled synaptic vesicles at the presynaptic active zones. The second step is the maturation and priming of synaptic vesicles to fuse with the membrane (Klenchin and Martin 2000) (Figure 1.9). Finally the primed vesicles undergo fusion to release neurotransmitters under the influence of action potential induced calcium influx. The final step includes the retrieval of the protein constituents from the plasma membrane by endocytosis and the synaptic vesicles are ready for another round of exocytosis (Li and Chin 2003).

The SNARE proteins form the core of the membrane fusion machinery and these proteins are localized to the different compartments of the endocytic trafficking pathways and lead to specific membrane fusion process intracellularly. In case of neuronal cells the synaptic vesicle exocytosis depends on the three different SNARE proteins i.e. vesicle associated membrane protein (VAMP 2), syntaxin, and synaptosomal–associated protein (SNAP 25) (Pelham 2001; Chen and Scheller 2001).

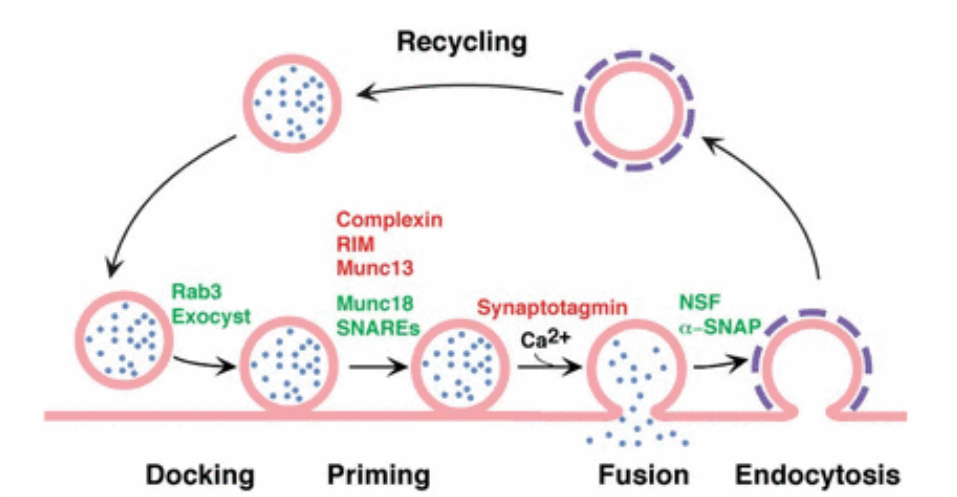


Figure 1.9: The synaptic vesicle cycle at the nerve terminal. The different steps in synaptic vesicle recycling are indicated i.e docking, priming, fusion and endocytosis. The protein components that are conserved in this process are indicated in green color and the ones that have unique function are indicated in red color (Li and Chin 2003).

1.11 Identification of Antarease

Antarease has been identified through purification procedures carried out on the dried whole venom from *tityus serrulatus* (Fletcher et al., 2010). The metalloprotease was purified as single peak following gel filtration and further by Supelco wide pore C_{18} reverse phase chromatography. The N-terminal sequencing initially had been carried out through automated Edman degradation for the first 29 residues followed by chemical (CNBr) and proteolytic enzyme cleavages. The overlapping amino acid sequences has been used to assemble the peptide map with a single protein sequence.

The amino acid sequence of Antarease indicates that it belongs to **reprolysin** family of peptidases. Antarease belongs to **snake venom metalloproteinase or venom metalloproteinase (M12B)** subfamily of reprolysins as indicated by the presence of a conserved peptidase M12B domain present in many venom metalloproteases. The term reprolysin was first coined because of the fact that some members of the subfamily belong to reptiles (*rep*) whereas other members were discovered as a group of proteinases identified in mammalian reproductive tissues (*repro lysin*). However, it is now recognized that there are representatives of reprolysins in a wide variety of organisms and tissues (White 2003).

Reprolysins are a class of peptides mostly found in snake venoms, which interfere with the blood coagulation cascades in their preys. Another interesting feature of reprolysins found in some snakes is their homologous similarity with factors responsible for blood coagulation which are series of trypsin-like serine peptidases. The ADAM (a disintegrin-like and metalloproteinase)/MDC (metalloproteinase,disintegrin, cysteine-rich) proteins are the homologs of reprolysins which are found in mammals and birds. Despite being homologs of reprolysins, ADAM proteins are functionally different from the snake venom peptidases.

Reprolysins have been reported in a wide variety of invertebrates for example in certain annelids, drosophila, honey bee, beetle, human louse, tick *Ixodes ricinus* and wasp. Among all these species their respective reprolysins differ in domain composition for example reprolysin protein from Drosophila Kuzbanian has reprolysin as well as disintegrin domains. Whereas the reprolysin from wasp *Pimpla hypochondriaca* lack the disintegrin domain. None of these reprolysins have been biochemically characterized (Parkinson et al, 2002 and Barrett et al 2004).

The Snake venom metalloproteinases (SVMPs) are further divided into 4 subcategories depending on their domain organization P-I, P-II, P-III and P-IV (Figure 1.10). Out of these four groups the groups P-I and P-IV are abundantly found in viper snakes. The general domain organization classifies SVMPs in four major groups (as shown in Figure. 1.10):

a) P-I SVMPs being the smallest representatives of SVMPs with an independent metalloproteinase domain. b) P-II SVMPs contain a metalloproteinase plus a disintegrin domain, but frequently found in venoms as a processed form with only the disintegrin domain c) P-III SVMPs, with the catalytic domain, disintegrin-like and cysteine-rich domains. P-III SVMPs mostly occur in venoms as monomeric or dimeric catalytic forms (metalloproteinase, disintegrin-like and cysteine-rich domains) or the enzymatically inactive forms that retains only disintegrin-like and cysteine-rich do- mains. d) P-IV SVMPs consist of a subunit having a P-III domain organization plus a lectin-like domains linked by disulfide bonds (Moura-da-Silva et al 2007).

These venom metalloproteases have diverse effects on biological systems for example cellular receptors, plasma proteins and extracellular matrices. Different properties of these metalloproteases have been studied in detail in particular their interactions with platelet

glycoprotein (GP) Ib, GP VI, integrin α2β1, neutrophil PSGL-1, endothelial adherens junction, plasma vWF, fibrinogen and other extracellular matrix, e.g. collagen, in causing antiplatelet, antiinflammation, and endothelial apoptosis (Hsu and Huang 2011).

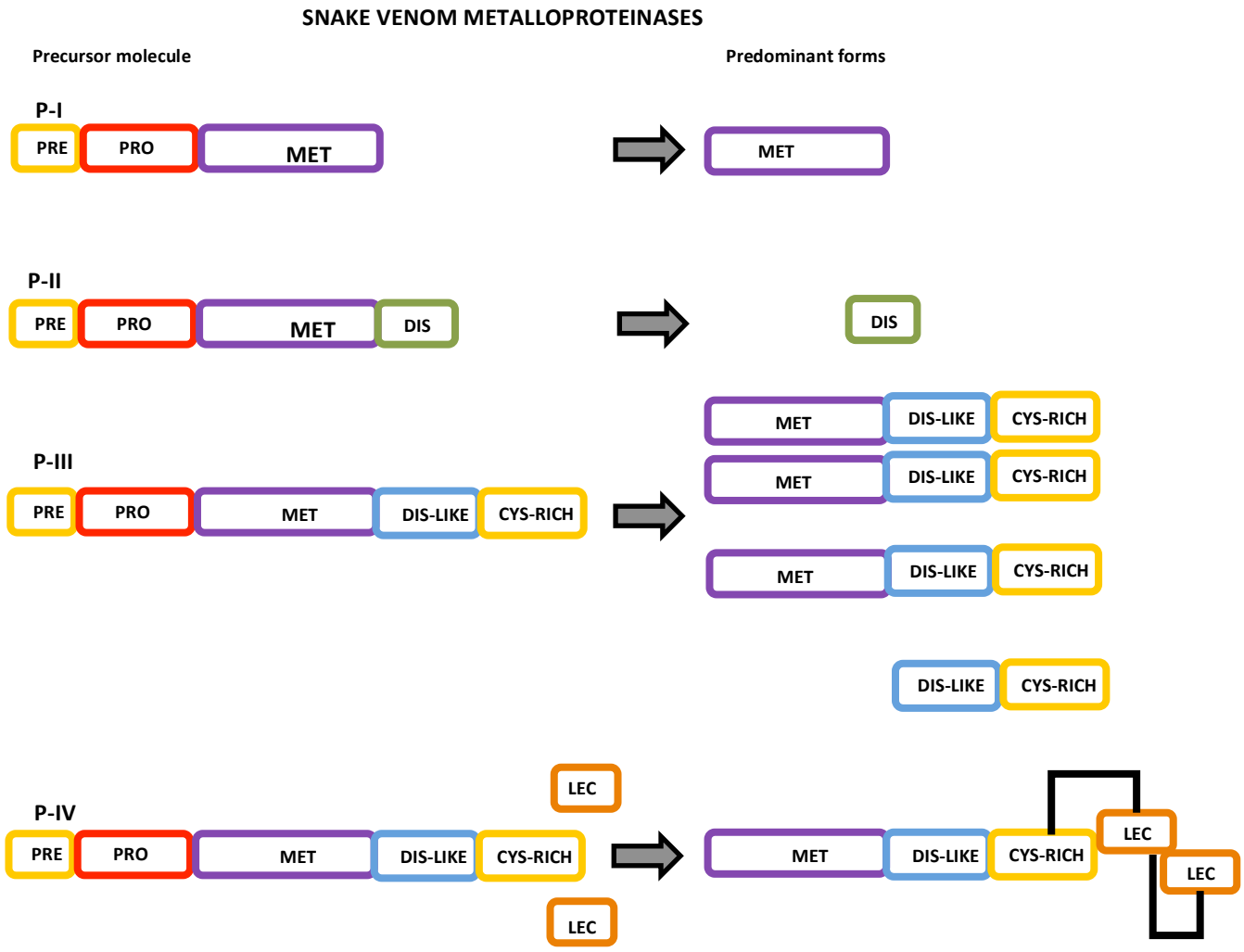


Figure 1.10: Diagrammatic depiction of predominant forms of SVMPs groups. SVMPs are synthesized as multidomain precursors, which further undergo different processing fates (Moura-da-Silva et al., 2007).

1.12 Mechanism of action of Antarease

The Antarease has been shown to cleave at specific sites in VAMP 2 and VAMP8 (Fletcher et al., 2010). The VAMP2 cleavage site is situated between the SNARE motif and the transmembrane domain of VAMP2 (Fig. 1.11). Both of these SNARE proteins are found in the zymogen granule membranes of the pancreatic acinar cells suggesting a potential link between cleavage of these proteins and development of pancreatitis in the case *Tityus serrulatus* stings in humans. The action of Antarease on the substrates has been shown to be inhibited by 10mM EDTA. The sequence analysis of the purified Antarease from the whole venom revealed presence of sequence **(HESVHLLGSPHD).** This sequence has been identified as a conserved zinc binding motif also found in other types of metalloproteases.

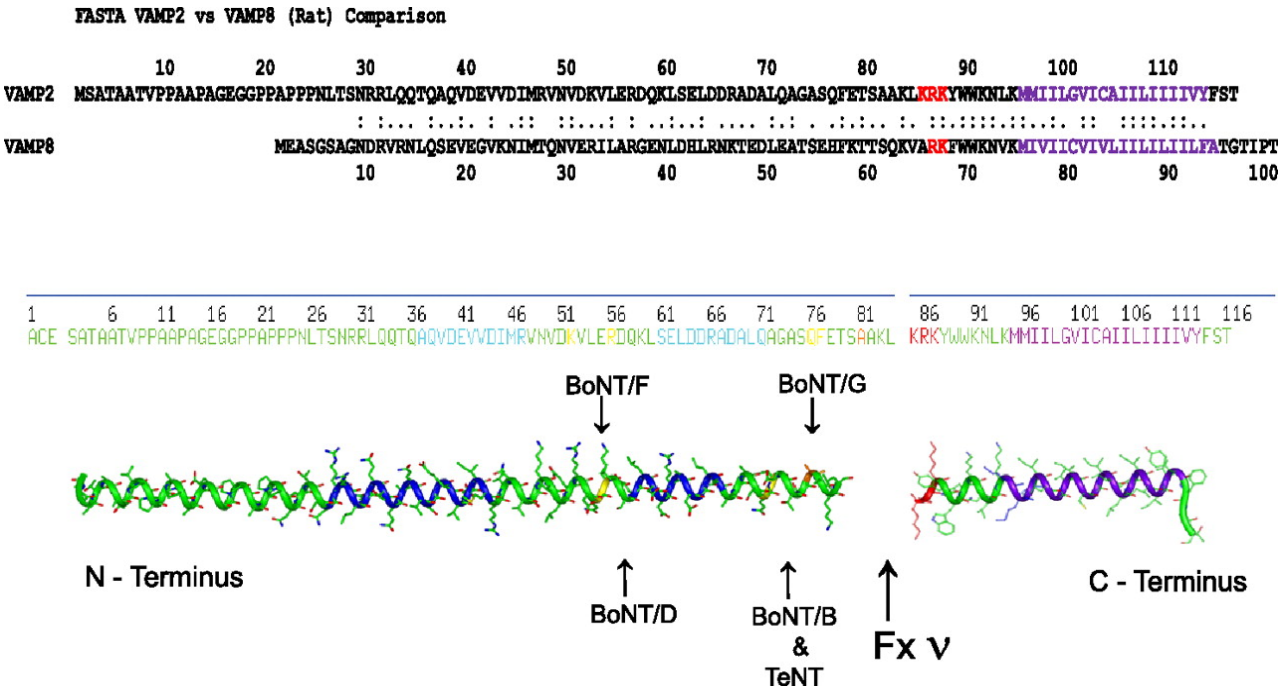


Figure 1.11: VAMP2 and its cleavage sites by Antarease and by clostridial toxins. Top, VAMP2 and VAMP8 FASTA homology alignment with cleavage sites by Antarease indicated in red and transmembrane segments indicated in violet. Bottom, VAMP 2 amino acid sequence coordinated with a molecular model, where the cleavage site by a venom fraction-containing Antarease (Fx ν) is indicated in red, cleavage sites by clostridial metalloproteases botulinum (BoNT) and tetanus toxin (TeNT) are indicated in yellow and

orange, and SNARE motifs are indicated in blue. The intervening sequence ribbon is green (Fletcher et al. 2010).

1.13 Evidence of other metalloproteases from scorpion venom

Antarease like proteins have also recently been identified in other scorpion species. Some of the examples are listed below (www.uniprot.org; Ortiz et al., 2013).

Sequence similarity to venom metalloprotease from *Mesobuthus eupeus*

Sequence similarity to M13 metalloprotease from the *Hottentotta Judaicus* scorpion.

Sequence similarity to Antarease from *Tityus stigmurus*.

Sequence similarity to Antaresae-Lyc from Lychas buchari

1.14 Other SNARE cleaving metalloproteases

Before the discovery of the metalloproteolytic activity of Antarease, the only enzymes known to cleave SNARE proteins were the clostridial neurotoxins. These bacterial toxins contain a typical **His-Glu-Xaa-Xaa-His** signature sequence in the enzymatically active light chain. The use of metal chelators such as ortho-phenanthrolin results in inactivation of the active neurotoxins by removing the bound zinc without making any changes in the secondary structure of the light chain. Similarly the activity of the neurotoxins can be gained back in presence of zinc (Schiavo et al., 1992).

Clostridial neurotoxins (BoNTs and TeNT) are expressed in the cytosol of bacteria without any peptide leader sequence and soon after bacterial cell lysis these toxins are released into the culture medium (Das Gupta 1994). Botulinum toxins are encoded by bont genes which are approximately 3880 bp long. Eight antigenically different serotypes of botulinum (BoNT A–BoNT H) are described in detail in literature (Rossetto et al., 2013; Barash and Arnon, 2014). Apart from main serotypes many subtypes have also been reported (Binz and Rummel 2009, Lacy and Stevens 1999). The eight serotypes of botulinum toxins exhibit 34-97% sequence similarity among them. Both botulinum and tetanus toxins are generated as single chain pro-toxins with 150 KDa molecular weight (Poulain, 2008 and Arndt et al., 2006) which is either cleaved by host or clostridial proteases to generate 50 KDa light chain and

100KDa heavy chain which are linked together by disulfide bridge and another belt segment (Rossetto and Montecucco 2008). The light chain contains the active zinc–protease activity. The heavy chain contains the N- terminal domain responsible for translocation of light chain and a C-terminal domain that is responsible for binding to the receptors (Montal 2010; Tighe and Schiavo 2012), (Figure 1.12).

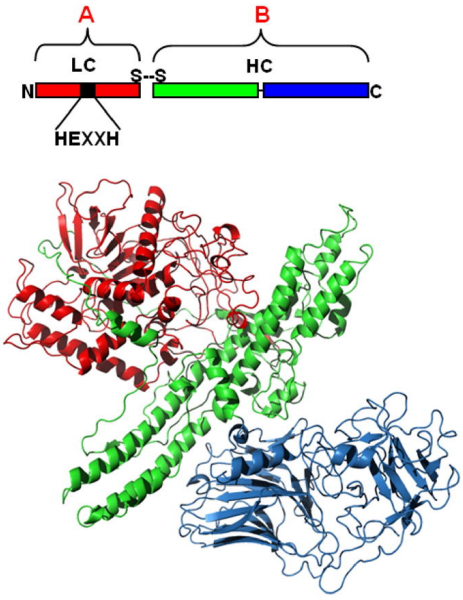


Figure 1.12: Structural and functional organization of botulinum neurotoxins. Upper panel is showing main domain organization of botulinum toxins. Botulinum toxin containing two major domains a light chain containing the catalytic activity and a heavy chain that is divided further into two domains i.e. a translocation domain and a receptor binding domain.Lower panel showing the crystal structure of Botulinum A with three functional domains light chain indicated by red color, heavy chain translocation domain indicated by green color and heavy chain receptor binding domain is indicated by blue color (Lacy et al 1998; Kroken et al, 2011).

1.14.1 Mechanism of action of clostridial neurotoxins

The neuron intoxication by the clostridial neurotoxins is generally divided into four steps, which are binding of the neurotoxins, internalization of the neurotoxins, translocation of the light chain in the neuronal cytosol and finally the cleavage of the substrates (Montecucco et al., 1994), (Figure 1.13).

(1) The neurotoxins bind to their respective receptors on the surface of neuronal cells. The receptors are polysialogangliosides and specific synaptic vesicle proteins depending upon the serotypes. These receptors generally cluster in the lipid microdomains (yellow segment). (2) The second step include internalization and sorting of these neurotoxins to specific intracellular pathways, TeNT following non-acidified fast retrograde pathway whereas the BoNTs following the endocytic pathway (3) the translocation of the catalytically active light chain takes place in the presence of acidic lumen of endosomes. The acidic pH triggers a conformational change in the toxin molecule leading to insertion of heavy chain N-terminal domain into the endocytic membrane and formation of a trans-membrane channel that will allow translocation of light chain into the cytosol. (4) Once in the cytosol the clostridial neurotoxins cleave their respective SNARE proteins depending on the specific serotype (Lalli et al., 2003).

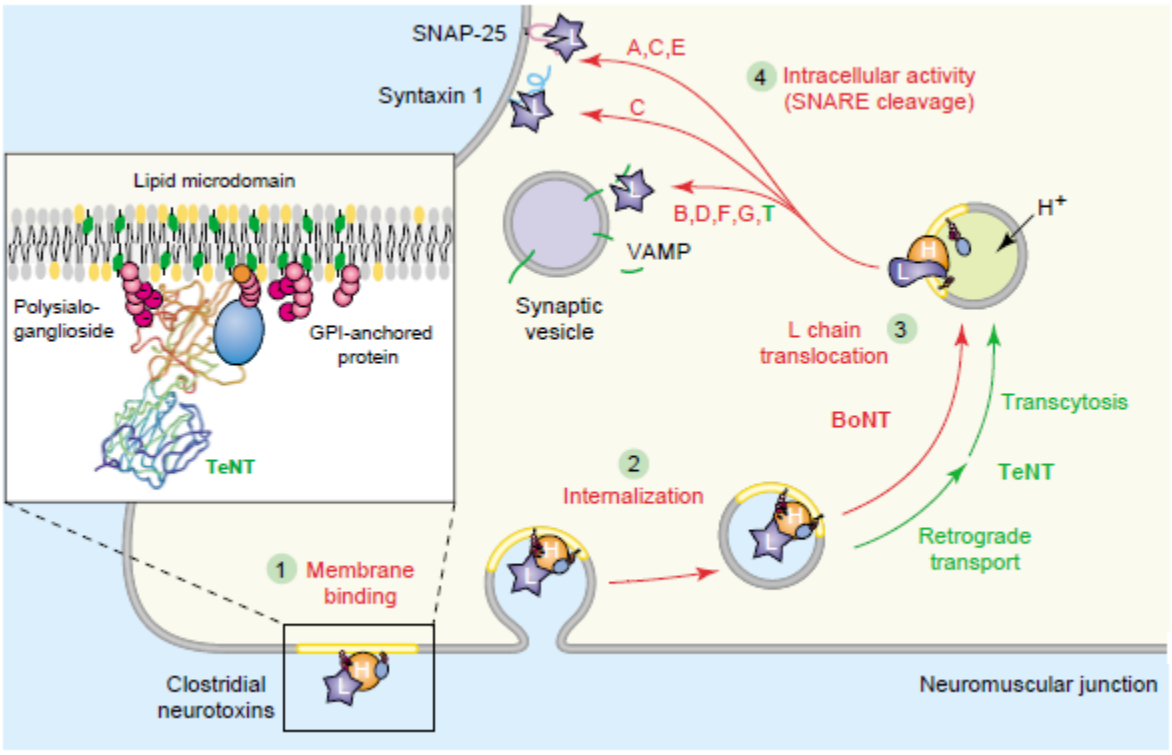


Figure 1.13: The mechanism of action of the clostridial neurotoxins. Tetanus and botulinum neurotoxins follow a four-step mechanism of action i.e. binding (1), internalization (2), translocation of light chain (3) and cleavage of substrate (4) (Lalli et al., 2003).

1.15 Cell Models used in this study

1.15.1 Cerebellar granular neurons

Granule cells of the cerebellum contain the largest homogenous neuronal population in the mammalian brain. Occurring in cerebellum these cells are generally characterized as small cells with few dendrites (Contestabile 2002). Primary culture of cerebellar granule neurons are important tools to study neurological and molecular aspects of the neuron survival and death. In particular this type of primary neuronal culture has been extensively used to study signaling pathways related to neuronal survival, migration and differentiation. A lot of different neuroprotective factors have been identified as a result of studies that have been conducted on the cultures of cerebellar neurons. These factors include insulin-like growth

factor 1, cyclic adenosine monophosphate AKT, and myocyte enhancer factor (Dudek et al. 1997 and Li et al. 2001). Cerebellar granular neurons also provide extra benefits of having readily distinguishable axons and dendrites, ease of culturing and maintaining. Granule cells are excitatory and use glutamate as their neurotransmitter (Contestabile 2002).

1.15.2 Motor neurons

Motor neurons originate from the neural tube. These are known to be the first type of cells that are born in the spinal cord. Motor neurons play very important role in voluntary movement by releasing acetylcholine at the neuromuscular junction of the target skeletal muscles. In turn each of the muscle fiber contains a single axon terminal. The number of synapses that a single motor neuron forms with muscle fibers can be highly variable ranging from as few as ten to several hundred. The firing rates of the motor neurons and the resultant muscle fiber contractions depend on the inhibitory and excitatory signals that are carried out by the motor neurons (Altman and Bayer 1984; Carp and Wolpaw 2001). In terms of morphology the soma of the motor neurons is larger than the other types of neurons for example cerebellar granular neurons. Motor neurons are also generally characterized based on their long axons for example a motor neuron that originates from the lumbar spinal cord targets the muscles of foot and can be in length up to 1 meter (Carp and Wolpaw 2001). The primary motor neuronal culture can shed important information on several important cellular and molecular aspects of neurobiology as well as in understanding several neurobiological diseases. Cultures used can be studied as nearly pure or mixed with other cell types depending on the protocol followed (Ullian et al. 2004). Primary motor neuronal cultures have been used mainly in studies such as glial-neuronal interactions, axonal transport, synaptogenesis as well as in the studies of degeneration, regeneration, apoptosis of neurons (Hanson et al. 1998).

1.15.3 Cortical neurons

The cortical neuronal culture are traditionally prepared from the embryonic animals as at this time neurons have not developed extensive axons and dendrites. Furthermore the optimum developmental age for preparation of cortical neurons can vary depending upon the type of experiments required and the number and type of cells required (Sciarretta and Minichiello

2010). From the structural and functional point of view the cortical neurons contain soma (cell body) that is responsible for integration of incoming signals to produce variation in the membrane potential. The signaling between different neurons takes place with the help of synapse. Cortical neurons possess an extensive network of dendrites that function as connection points of synapses. All of the excitatory connections are present of dendrites. The neocortex region contains many diverse types of neurons and the circuits that they make can in turn perform wide variety of functions (Wells 2005).

1.16 Aims and significance of this study

This study was initiated because of a report of the presence of a novel zinc-metalloprotease, named antarease, in the venom of *T. serrulatus.* This enzyme, which was not described before appeared to penetrate the intact tissue and carry out cleavage of *soluble N-ethylmaleimide*-sensitive factor attachment protein receptors (SNAREs) that are involved in the pancreatic secretion ultimately leading to disruption of normal vesicular traffic. SNAREs till now had been demonstrated to be the targets only of the clostridial neurotoxins tetanus and botulinum neurotoxins, which have been subject of study of our laboratory for many years.

Since reports on antarease or antarease-like sequences have been based on cDNA library construction and bioinformatics tool, we aimed to use practical or experimental tools to provide evidence to characterize these enzymes, which has not been achieved yet apart from the work done by Fletcher et al 2010.

For this purpose we aimed to investigate following facts:

- To understand mechanism of action of antarease and to identify and screen for antarease- like metalloproteases from other scorpion venoms and to understand mechanism of action of these unique metalloproteases from different venoms.
- To study the effects of these venoms on SNARE proteins in different neuronal cell culture model since these are ideal models in which SNARE proteins have been vastly studied. We also aimed to get some information on mechanism of entry of these metalloproteases in neuronal cells and their effects on viability of the cells.

- To clone by recombinant DNA technology, and express these metalloproteases, or to purify the active metalloprotease from the whole venom of *Tityus serrulatus*.
- To get information on cleavage of recombinantly expressed SNAP 25 by these active metalloproteases.

Scorpion envenoming and resulting pathology is often a neglected topic particularly in the developing countries where a lot of mortalities take place due to scorpion stings. The significance of this study lies in the fact that this work is based on investigation of a new class of metalloproteinases in the scorpions, which was not previously described before. This study can be helpful to provide better understanding on the pathology of scorpion stings as well as to develop better ways to overcome pathological effects of scorpion envenoming. Furthermore it proposes to investigate this unique class of metalloproteinase as a potential target for anti-venom therapeutics that may lead to improvement of current anti-venom therapies. Finally this new class of metalloproteases could be used as new tools in cell biology to affect SNAREs-regulated cellular traffic.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

Materials and Methods

2.1 Chemicals and materials used

All the chemicals used in this project were of analytical grade. All the chemical solutions, buffers and growth media were prepared in MilliQ water (MilliQ plus system, Millipore). The media for the growth of bacteria were routinely sterilized by autoclaving at 121°C and 15 p.s.i for 15-20 min. Heat-sensitive compounds and antibiotics were filter-sterilized. For the preparation of growth media, tryptone, yeast extract and agar were purchased from Melford Laboratories. DNA manipulating enzymes (restriction endonucleases, T_4 DNA ligase, Antarctic phosphatase) were purchased from New England Biolabs. DNA and protein ladders were obtained from Fermentas and Bio-Rad. SDS-PAGE gel system used was from Novex. Nitrocellulose membrane were from Biorad. Hoechst dye to stain nuclei was bought from Sigma, Alexa fluor (555 and 488) secondary antibodies against mouse and rabbit were purchased from life technologies. Anti-VAMP2 Mab, anti-Syntaxin Mab were from Synaptic System. Polyclonal anti-SNAP25 was produced by injecting in rabbit the C-terminal peptide ANQRATKMLGSG of SNAP25 conjugated to keyhole lymphet haemocyanin. Polyclonal anti-syntaxin was prepared by injecting recombinantly-expressed syntaxin in rabbit. Scorpion venoms were provided by our collaborators Maria Elena de Lima (Universidade Federal de Minas Gerais) and Eugene Grishin (Biological Department of Moscow State University).

2.2 Cell cultures

2.2.1 Primary culture of cerebellar granular neurons (CGNs)

Cerebellar granular neurons were prepared according to the protocol described by Bilimoria and Bonni 2008 with some modifications. CGNs were prepared from three P5-P7 Wistar rats. The rats were decapitated and the skin was removed from the head. The portion of the brain containing cerebellum was removed and immediately placed in ice cold solution I (120 mM NaCl, 5.3 mM KCl, 14 mM glucose, 30 μM phenol red, 1 mM NaH_2PO_4, 20 mM HEPES,

0.5 mM $MgSO_4$, 40 µM BSA in Milli Q, pH 7.4). Cerebellum was separated from rest of the brain parts and meninges and blood vessels were removed. Cerebella were placed in an empty dish thoroughly minced with lancet and resuspended in 10 ml of solution II (Solution I containing 30 µM Trypsin). The suspension was transferred in a sterile tube and left at 37 ºC for 15 minutes with constant agitation. After 15 minutes 10 ml of solution III(16% solution I containing 3 µM DNase I, 30 µM trypsin inhibitor and 1.6 mM $MgSO_4$ and 84% solution I) was added and suspension was centrifuged at ~200*g* for 2 minutes. The supernatant was removed and the cell pellet was resuspended in 3 ml of solution IV (solution I containing 3 µM DNase I, 30 µM trypsin inhibitor and 1.6 mM $MgSO_4$). The cell pellet was thoroughly re-suspended by repeated aspiration and expiration. The suspension was left to let settle all un-dissociated tissues. The supernatant was transferred into another sterile tube and equal volume of solution IV (solution I containing 1.2 mM $MgSO_4$ and 0.1 mM $CaCl_2$). After this cells were centrifuged at ~200*g* for 8 minutes at room temperature. The supernatant was removed and cells were resuspended in 10 ml of Complete medium (BME containing 0.1% of 50 mg/ml gentamicin, 2mM L-glutamine, 20mM KCl and 10% FBS). The cells were counted and plated in poly-lysine coated plates with $0.3x10^6$ cells per well. Arabinofuranosyl Cytidine (Ara C) 10 µM was added to cells after 24 hours.

2.2.2 Primary culture of spinal cord neurons

The primary culture of motor neuron was prepared from E15 Wistar rats as described by Graber and Harris 2013, with some modifications. In summary 5 spinal cords were dissected from the embryos. Meninges and extra tissue was removed and spinal cords were thoroughly minced with help of lancet. The minced spines were transferred in sterile tube containing 0.025% trypsin and incubated at 37º C for 15 minutes with agitation. After the incubation the trypsinized tissue was transferred to sterile tube containing 80% L15, 10 % of BSA (4%) 10 % of DNAase (1 mg/ml) and agitated vigorously until the tissue fragments were disaggregated. The suspension was left to settle for 2 minutes and the supernatant was transferred to sterile tube containing 90% L15, 10 % of BSA (4%) and 2% of DNAase (1 mg/ml). The suspension was further triturated and the supernatant was transferred in sterile tube. To the pooled supernatant 2 ml of 4% BSA was added at the bottom and the suspension was centrifuged at 245 *g* at room temperature for 5 minutes. Finally cell pellet was

resuspended in complete medium (Neurobasal supplemented with 1% B27, 2% horse serum, 0.5 mM L-glutamine, 25 µM mercaptoethanol, 25 µM glutamic acid). The cells were counted and seeded on polyornithine (overnight coated) and laminin (two hours) coated plates. The cells were allowed to grow for a week before setting up the experiment.

2.2.3 Primary culture of cortical neurons

Primary cortical neurons were prepared as previously described by Banker and Goslin 1998; Hilgenberg and Smith 2007, with some minor modifications. Briefly, forebrains from newborn 1 to 2 day old rat pups were dissected and the cortex region of the brain was placed in ice-cold solution I (120 mM NaCl, 5.3 mM KCl, 14 mM glucose, 30 µM phenol red, 1 mM NaH_2PO_4, 20 mM HEPES, 0.5 mM $MgSO_4$, 40 µM BSA in Milli Q, pH 7.4). The cells were then mechanically dissociated and digested with trypsin (30 µM Trypsin) for 15 min. After 15 minutes 10 ml of solution III (6% solution I containing 3 µM DNase I, 30 µM trypsin inhibitor and 1.6 mM $MgSO_4$ and 84% solution I) was added and suspension was centrifuged at ~200*g* for 2 minutes. The supernatant was removed and the cell pellet was resuspended in 3 ml of solution IV (solution I containing 3 µM DNase I, 30 µM trypsin inhibitor and 1.6 mM $MgSO_4$) followed by centrifugation at ~200*g* for 8 minutes at room temperature. The cells were finally re-suspended in complete medium (Neurobasal A supplemented with 2% of 50X B-27, 2mM L-glutamine, 0.1% of 50 mg/ml Gentamycin solution in deionized water). The cells were kept at 37°C in a humidified 5% CO2/95% air atmosphere for a week before setting up the experiment.

2.3 Immunoflourescence in rat primary neuronal cultures

Depending upon the experiment the primary neuronal cultures (CGNs, CNs, or MNs) were prepared as described previously. The cells were counted and seeded on the sterile coverslips (previously coated with polylysine for CGNs and CNs and polyornithine-laminin coated for MNs) in 24 well plates. For CGNs 250000 cells per well, for CNs 150000 cells per well and for MNs 10000 to 30000 cells per well. The cells were allowed to grow for a week. For immunofluorescence cells were washed twice with PBS (phosphate buffered saline pH 7.2 to 7.4) to remove all media. The cells were fixed with 4% PFA in BSA at room temperature for

20 minutes on shaker incubator.. After this cells were thoroughly washed four times with 500 µl of sterile PBS. The cells were then permeabilized with 0.1 % triton X100 in PBS for 15 minutes. This step was followed by five times washing of cells with 500 µl of sterile PBS for 5 minutes for each washing. The cells were then saturated with 0.5% BSA in PBS for 30 minutes at room temperature on shaker incubator. The cells were then incubated with primary antibodies against syntaxin and VAMP (1µl/200µl of 0.5% BSA in PBS), and SNAP 25 (1µl/300µl of 0.5% BSA in PBS) at 37ºC for 1.5 hours in a humidified incubator. The cells were washed 5 times with 500 µl of sterile PBS for five minutes before incubating with the secondary antibodies. The secondary antibodies used were anti-rabbit Alexa flour 555 and anti-mouse Alexa flour 488 with a dilution 1:200 µl of 0.5% BSA in PBS for 45 minutes at 37ºC in a humidified incubator. The cells were then washed 5 times with 500 µl of sterile PBS for five minutes each. After washing cells were incubated with the Hoechst dye to stain cell nuclei. The Hoechst was used at final concentration 1µg/ml in PBS for 1 minute at room temperature. This step was followed by washing the cells with PBS twice for 5 minutes each with 500 µl of sterile PBS. Finally the cover slips containing the cells were mounted on glass slides in presence of small volume of mounting solution and sides of coverslips were properly sealed before viewing under oil immersion by using Leica immunoflourescence microscope. The cells were visualized for alexa fluor 488 (absorption 495 nm and emission 519 nm) alexa fluor 555 (absorption 555nm and emission 565nm), and hoechst (absorption 346 nm and emission 460 nm).

2.4 Immunoflourescence in drosophila larvae

To carry out investigation on the cleavage of SNARE proteins under the action of scorpion venom in drosophila third instar fly larvae were used. In short flies were raised on a standard yeast–glucose–agar medium and were maintained at 23°C, 70% relative humidity, in a 12 hour light: 12 hour dark cycle. Experiments were carried out on F1 third instar larvae. And the protocol for immunofluorescence was followed as described in detail in previous section.

2.5 *In vitro* cell viability assay (MTT assay)

The effect of scorpion venoms on viability of cells was determined by using MTT assay as described by Mosmann, 1983. The viability of the treated and control cells was measured

with the help of MTS (3-4,5-dimethylthiazol-2-yl-5-(3-carboxymethoxyphenyl-2-(4-sulfonphenyl)-2H-tetrazolium)- based assay (CellTiter 96 aqueous one solution cell proliferation assay by Promega). It is a colorimetric method to determine the viability of the cells in the proliferation or cytotoxicity assays. The principle of this assay is based on the conversion of a tetrazolium salt into a formazan product. This conversion is accomplished by the NADPH or NADH produced bydehydrogenase enzymes in metabolically active cells.

Briefly CGNs were grown in 96 well plates with total number of cells between 50000 to 60000 cells per well. Cells were incubated overnight with 30ug/ml of whole venom or venom fractions in a humidified atmosphere of 5% (v/v) CO_2 at 37^{o}C. After overnight incubation, 20µl of CellTiter 96One Solution Reagent was added to each well containing 100µl of media. The plate was incubated further for 3 to 4 hours in a humidified atmosphere of 5% (v/v) CO_2 at 37 ^{o}C. For the positive control 1% triton X was used. The absorbance was measured at 490 nm with a microplate reader. Absorbance from untreated cells was considered as 100% of growth and used for percentage viability calculation. Finally the effect of scorpion venom or venom fractions on the viability of cells was expressed as the percentage viability.

2.6 Protein techniques

2.6.1 Total protein quantification

For the quantification of total protein content Bicinchoninic acid assay or BCA assay was followed according to the protocol described by Smith et al., 1985. The protocol is based on two processes, the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium followed by the colorimetric detection of the Cu^{1+} by the bicinchoninic acid. The dark purple color product results due to chelation of BCA with the cuprous ion. This assay is influenced mainly by the presence of cysteine or cystine, tyrosine, and tryptophan in the proteins under study.
Bovine serum albumin controls ranging in concentration 0 µg/ml for the blank to 2000 µg/ml were prepared in deionized distilled water. Similarly different dilutions were prepared for the samples under analysis. The microplate protein quantification protocol was then carried out by pipetting 25 ul of each protein samples into microplate well. After this 200 µl or working solution (containing 50 parts of reagent A [sodium carbonate, sodium bicarbonate,

bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide] and 1 part reagent B [4% cupric sulfate] was added to each well containing the samples and the blank. The microplate was thouroughly shaked on a shaker and kept in dark at 37º C for 30 minutes. The microplate was equilibrated at room temperature for five minutes and the absorbance was measured at 562 nm. Standard curve for the control was plotted after blank subtraction and the total protein concentration in each sample was determined using the standard curve.

2.6.2 Toxin reduction

Tetanus (TeNT) and botulinum toxins (BoNT) were used as a positive control for the cleavage of recombinantly expressed SNARE proteins. In *in vitro* cleavage assay, the disulfide-bridge which keeps together the light and the heavy chain of clostridial neurotoxins, which needs to be reduced so that the toxins are active. For this purpose, TeNT and BoNT/A were incubated with a reducing buffer (150 mM NaCl, 10 mM NaH_2PO_4, 15 mM DTT pH 7.4) for 30 min at 37°C. The reduction of the disulfide bond of the toxins was assessed by carrying out SDS-PAGE of reduced and non-reduced toxins.

2.6.3 SNARE cleavage assay

Cleavage assay was carried out on the recombinantly expressed SNAP 25,VAMP and GST protein under the action of whole venoms from different scorpion species. For this purpose the substrate proteins were incubated with the whole venoms of *Tityus serrulatus*, *Buthus eupeus* and *Orthochirus scrobiculosus* for 3 hours at 37 ºC in a humified chamber. The proteolytic action of metalloproteases in the venoms was also accessed in presence or absence of metal chelating agent phenanthrolin. For positive controls of SNAP 25 and VAMP cleavage, botulinum A and tetanus toxins respectively were used.

2.6.4 Sample preparation

Before preparing total cell lysate for western blotting the cells were washed twice with PBS to remove all media. Appropriate volumes of lysis buffer (100mM Tris-HCl, 1% SDS along with the protease inhibitor cocktail [complete Mini EDTA-free, Roche]) were added to the wells (40 µl for 24 well plate and 80µl for 12 well plate). The cells were scraped carefully from the bottom of plates using cell scraper and keeping the plate on ice during whole time.

The lysate was collected in sterile eppendorf. The BCA assay was carried out to estimate the total protein concentration. Bromophenol blue was added to the samples and the SDS-PAGE was carried out as described before.

2.6.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis or also commonly known as SDS-PAGE is a method to separate different proteins according to their molecular weights. In this work the method followed for carrying out SDS-PAGE is based on the protocol described by Laemmli 1970 that relies on the use of tris-glycine gels with two main components stacking and resolving gels (Sambrook et al 1989). Novex® Tris-Glycine precast mini gradient gels with 4-12% polyacrylamide concentrations have been used to carry out protein gel electrophoresis. The protein samples were prepared as described before. Before loading the samples the wells of the gels were thoroughly washed with 1 ml syringe with 1X Novex tris-glycine SDS running buffer (prepared by adding 100 ml of 10X Novex tris-glycine SDS running buffer in 900 ml of deionized water). The protein samples were loaded into each well along with protein ladders. The gels were run in running buffer (Invitrogen) in the NuPage® Novex® *Gel* apparatus at 125 V constant for 90 minutes.

For staining and visualization of protein gel SimplyBlue™ SafeStain a ready-to-use, fast, sensitive, and safe Coomassie® G-250 stain was used. The instructions from the manufacturer were followed. After the electrophoresis the gels were rinsed thoroughly three times for 5 minutes with 100 ml of deionized water to remove the salts, SDS etc. after that the gel was stained with sufficient volume of SimplyBlue™ SafeStain for 1 hour on shaker at room temperature. After incubation the stain was discarded and gel was thoroughly washed with 100 ml of deionized water on shaker at room temperature. Finally gel was analysed using Biorad gel doc system.

2.6.6 Western blotting

Total protein concentration was determined by BCA method (Pierce BCA protein assay, Thermo Scientific) and equal amounts of total proteins were loaded into each well and separated by electrophoresis according to the SDS-PAGE protocol. The gel was carefully removed from the gel apparatus the stacking gel was removed with a gel cutter.

Nitrocellulose and whatman filter papers were already cut according to the dimensions of the gel. The gel, membranes and filter papers were soaked in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). A layer of filter paper followed by gel, nitrocellulose and finally filter papers was assembled and placed in the transfer apparatus (Biorad) the apparatus was assembled and finally the buffer tank was completely filled with transfer buffer. The transfer was carried out at maximum voltage (300V) and maximum amperage (400 amp) for 1.5 hours.

After the transfer, the nitrocellulose membranes were blocked in 5% BSA in PBS-Tween (1 ml Tween 20 in 1000 ml PBS Buffer [137mM NaCl, 2.7mM KCl,10mM Na_2HPO_4, 1.8mMKH_2PO_4, pH adjusted to 7.4]) for 1 hour followed by incubation with primary antibodies (against syntaxin, VAMP or SNAP 25) overnight at 4ºC. the antibodies dilution was prepared in 5% BSA in PBST according to the manufacturer's instructions. The membranes were washed thrice with PBS-Tween 5 minutes each. The membrane was then incubated with HRP conjugated secondary anti-mouse and anti-rabbit antibody for an additional 1 hour, washed with PBS-Tween three times and visualized with ECL Plus Western Blotting Detection System (GE Healthcare).

2.6.7 Venom extraction

All venom extraction was done by our collaborators in Brazil and Russia as described by Rates et al., 2008. In short venom was extracted from several individuals by electrical stimulation directly on the hindmost abdominal segment. The venom was transferred to chilled acidified water (0.1% aqueous trifluoroacetic acid) and centrifuged (15,000 rpm for 5 min) to remove cellular debris and a mucous pellet. The supernatant fractions were lyophilized and stored at −20 °C until required. To be used in experiments each vial containing lyophilized venom or venom fractions were equilibrated at room temperature for 60 minutes followed by dissolving in buffer (20 mM hepes-Tris-HCl, 150 mM NaCl, pH 7.4)

2.6.8 Liquid chromatography

T. serrulatus venom (250 mg) was applied to a Sephadex G-50 (Superfine) column and eluted with 0.15 M ammonium formiate buffer, pH 6.5 using a 0.7 mL/min flow rate.

Obtained fractions were pooled according to the chromatographic profile and then lyophilized (Rates et al., 2008).

2.6.9 Mass spectrometry (MS) analysis

The analysis of gel fragments by MS was carried out as described by Arrigoni et al., 2008. Samples of recombinant SNAREs (venom digested) and different fractions of TSV were separated by conventional SDS−PAGE, the desired bands of proteins in gel lanes were horizontally cut in slices and the gel pieces were subjected to reduction/alkylation and trypsin digestion. In brief crushed gel pieces were washed with acetonitrile, dried under vacuum followed by reduction with 10 mM DTT for 1 h at 56 °C. The samples were then alkylated with 55 mM iodoacetamide for 45 min at room temperature while kept away from light in the darkness. After which the samples were extensively washed with 100 mM ammonium bicarbonate and acetonitrile followed by the dehydration with acetonitrile. The samples were further dried under vacuum, and finally treated with 12.5 ng/μL sequencing grade trypsin at 37 °C overnight. After digestion, the peptides were extracted by three changes of 50% acetonitrile/0.1% formic acid (20 min between changes). This step was followed by drying samples under vacuum, and resuspending with 10 μL of 0.1% formic acid, and finally analyzed by LC−MS/MS.

2.6.10 Recombinant protein expression and purification

Recombinant proteins for example GST tagged SNAP 25, VAMP and Antarease were expressed in *E. coli* BL21 DE3 as GST fusion proteins and isolated as previously described (Tonello et al., 1999). In short expression was induced for 3 hours at 37°C with 1 mM IPTG (isopropyl-β-D-thiogalactoside). Cells were then lysed by two passages through a precooled French pressure cell (1300 pounds/inch22). The lysate was centrifuged (10,000*g,* 10 min), and the supernatant was incubated for 1.5 h with GSH–Sepharose resin and proteins were isolated on a glutathione–Sepharose 4B affinity column (Amersham Biosciences) according to the manufacturer's instructions. Resin-bound GST tagged proteins were eluted in a buffer containing 20 mM reduced glutathione and dialysed overnight in 10 mM Hepes buffer pH 7.4 with 150 mM NaCl. The recombinant proteins fused to a N-terminal His_6 tag and purified by FPLC with a Cu-charged Hitrap chelating column (Amersham Biosciences) according to

the protocol described in the Recombinant Protein Handbook (Amersham Biosciences). Protein expression levels and purity was checked by SDS–PAGE and western blotting.

2.7 Bioinformatic analysis

For the bioinformatics antalysis of antarease the sequence of antarease was retrieved from UniProtKB database (www.uniprot.org) under the accession number P86392. To identify similar sequences blast was carried out on retrieved sequence of antarease. Sequences with high similarity were aligned and further analysed.

2.8 Bacterial strains used in this study

E. coli strain OmniMAX^{TM} 2 (Invitrogen)

The genotype of this strain is as follows**:** F' [*pro*AB *lac*I^{q} *lac*ZΔM15 *Tn*10(Tet^{R}) Δ(*ccd*AB)] *mcr*A Δ(*mrr hsd*RMS-*mcr*BC) φ80(*lac*Z) ΔM15 Δ(*lac*ZYA-*arg*F)U169 *end*A1 *rec*A1 *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A *pan*D. Some of the characteristics associated with this strain include resistance to phage T1 and T5 infections and efficient transformation of highly methylated DNA.

E. coli strain BL21-Star (DE3) (Invitrogen)

The genotype of this strain is as follows**:** *E. coli* B F^{-} *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm rne131(DE3)*. Use of this strain sometimes enhances protein expression since it contains a truncated gene for RNase E, thus increasing mRNA stability.

E. coli strain C43 (DE3)

The genotype of this strain is as follows**:** F^{-} *ompT hsdS*$_B$(r_B^- m_B^-) *dcm lon λ(DE3).* Use of this strain can sometimes enhance the expression of toxic membrane proteins (Miroux and Walker, 1996).

2.9 Preparation of competent cells

For preparation of competent cells a fresh LB agar plate containing an appropriate antibiotic was streaked with cells of the desired *E. coli* strain, such as Omnimax^{TM} 2, and incubated

overnight at 37°C. Next morning 2 ml of LB broth with appropriate antibiotic in a sterile Falcon tube was inoculated with a single colony from the overnight plate. The tube was incubated in an orbital incubator at 37°C and 200 rpm overnight. The following morning 500 µl of the resultant pre-culture was used to inoculate 50 ml of LB medium in 250 ml flask. The flask was then incubated in an orbital incubator at 37°C and 200 rpm until the D_{600nm} of the culture was 0.4-0.6. Once the desired D_{600nm} value was reached the flask was chilled on ice. The cells were then harvested in pre-chilled Falcon tubes by centrifugation at 1000 *x g* at 4°C for 10 min. The cell pellet was resuspended in ice-chilled Tfb-I buffer (30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM manganese chloride and 15% (v/v) glycerol, the pH adjusted to 5.8 with 0.2 M acetic acid) and left on ice for 5 min. The cells were again harvested by centrifugation at 1000 *x g* at 4°C for 10 min. The supernatant was removed carefully. The cell pellet was next resuspended in Tfb-II buffer (10 mM MOPS [3 [(N-Morpholino)]-propanesulfonic acid], 10 mM rubidium chloride, 75 mM calcium chloride and 15% (v/v) glycerol, with the pH adjusted to 6.5 with KOH). The cells were incubated on ice for 15 min and finally dispensed as 50 µl aliquots into sterile tubes and stored at -80°C.

2.10 Transformation

For the transformation 50 µl of the appropriate competent cells were thawed on ice. After the cells were thawed approximately 10-30 ng of plasmid DNA or a ligation mixture was added. The cells were gently mixed and left on ice for 30 min, then heat shocked at 42°C for 30 sec, and finally incubated once for 2 min. LB medium (100 µl)was next added and the cells were further incubated at 37°C for 1 h at 200 rpm. They were then spread on a LB agar plate supplemented with appropriate antibiotics and incubated at 37°C overnight. The next day colonies were selected for plasmid isolation for further analysis or were used for the expression experiments.

2.11 Media used for bacterial growth

Luria-Bertani medium

Luria-Bertani (LB) medium was prepared by dissolving 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl in about 950 ml of MilliQ. The pH was adjusted to 7.0 with 1 N NaOH and the final volume

was made up to 1 litre with MilliQ water. For making LB agar 1.5 g of agar was dissolved in 100 ml of LB medium.

2.12 DNA techniques

2.12.1 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out according to the method of Sambrook *et al.* (1989) for the analysis of DNA from PCR, small-scale plasmid preparations ('minipreps'), restriction digestion, etc. Agarose gels (0.8% to 1% agarose) were prepared in TAE buffer (0.04 M Tris, 0.7 mM EDTA, 20 mM acetic acid, pH 8.0) by melting the agarose (SeaKem ® LE Agarose) in a microwave for 1 to 2 min. After the agarose was completely melted SYBR Safe™ dye (Invitrogen; 1/10,000 dilution) was added. The gel was poured carefully and kept at room temperature for 30 to 45 min to set before loading the samples. The DNA samples were prepared by adding DNA loading dye (0.25% bromophenol blue and 30% glycerol). Appropriate DNA markers (Mass ruler™ DNA ladder mix, Fermentas) were also run alongside of the samples. Electrophoresis was performed in a horizontal gel tank (Flowgen Bioscience) at constant voltage of 90 to 100 V for 30 - 45 min. The DNA bands in the agarose gel were analysed using blue light (GeneGenius, Syngene).

2.12.2 Quantification of DNA samples

The quantification of DNA was carried out in one of two ways. The amount of DNA in samples was routinely estimated by comparing the intensity of sample bands on SYBR Safe™-stained agarose gels with those for bands of known DNA content in the DNA ladder used as a marker. Alternatively, DNA was quantified spectrophotometrically. For this method, the concentration of DNA was calculated on the basis that an A_{260nm} value of 1 corresponds to a DNA concentration of 50 µg/ml.

2.12.3 Purification of DNA fragments

DNA samples were resolved by agarose gel electrophoresis, as described in section 2.8.1. The desired DNA fragments were next excised using a scalpel and placed in pre-weighed, sterile Eppendorf tubes. DNA was then isolated using a QIAGEN QIAquick gel extraction kit, according to the instructions given by the manufacturer.

2.12.4 Dephosphorylation of vector DNA

Dephosphorylation of DNA fragments was carried out using the enzyme Antarctic phosphatase (New England BioLabs) which removes the 5' phosphate groups from DNA, thus decreasing the self-ligation of vectors cut with a single restriction enzyme. The dephosphorylation was carried out according to the manufacturer's instructions. In a typical reaction 8 µl of miniprep DNA was mixed with 1 µl of Antarctic phosphatase (5000 U/ml), and 1 µl of 10 X Buffer (500 mM Bis Tris-Propane, 10 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 6.0). The final volume was made to 10 µl with autoclaved MilliQ water if required. The reaction mixture was incubated at 37°C for 1 h. After which the enzyme was deactivated by heating for 5 min at 65°C. Thedephosphorylated vector could then be used for ligation reaction with the gene of interest.

2.12.5 Ligation reactions

Purified DNA fragments were used to set up ligation reactions with the appropriate vectors. T4 DNA ligase carries out phosphodiester bond formation between the 5' phosphate and 3' hydroxyl groups in blunt- or cohesive-ended DNA molecules. Optimization of such ligation reactions may be required in certain cases, but as a starting point, in the present study ligation reactions were typically carried out using an insert:vector molar ratio of - 3:1. The appropriate amount of the insert to be ligated into the vector was calculated using the following formula:

$$\text{ng of insert} = \text{ng of vector} \times \frac{\text{size of insert}}{\text{size of vector}} \times \text{desired molar ratio of insert:vector}$$

The ligation reaction was set up by mixing together the appropriate amounts of insert and vector DNA, 1 µl T4 DNA ligase (400000 U/ml) and 1 µl of 10X T4 DNA ligase buffer (500 mM Tris-HCl, pH 7.5, containing100 mM $MgCl_2$, 100 mM dithiothreitol and 10 mM ATP)

and the final volume made up to 10 µl. The reaction mixture was kept at 16°C overnight before carrying out transformation as detailed in section 2.7.

2.12.6 Isolation of plasmid DNA

Plasmid DNA was isolated by culturing the *E. coli* cells carrying the plasmid in 3 ml of LB medium containing an appropriate antibiotic overnight at 37°C, continuously shaking at 200 rpm. The cells were then pelleted down in benchtop centrifuge (Sanyo 15/O5) at 10000 *x g* and 4°C. A Sigma Gene-elute plasmid mini-prep kit was then used according to the manufacturer's instructions to isolate the plasmid DNA. After elution, the plasmid DNA was stored at -20°C.

2.12.7 Restriction enzyme analysis

The restriction enzymes and the suitable buffers for obtaining the maximum activity of the enzymes used were provided by New England Biolabs. For a typical reaction mixture 100-500 ng of plasmid DNA was digested for 3 h at 37°C with a suitable amount of restriction endonuclease, typically 1 µl (4000 U/ml) in the optimal buffer recommended by the manufacturer, in a final volume of 10-40 µl. The products were then analyzed by agarose gel electrophoresis (section 2.8.1).

2.12.8 Polymerase chain reaction

The polymerase chain reaction was carried out for the amplification of DNA fragments, for example that encoding green fluorescent protein (GFP), using KOD hot start DNA polymerase (Novagen) according to the manufacturer's instructions. The following components were added in a typical reaction mixture: 5 µl of 10 X PCR buffer, 1.5 µl of each of the forward and reverse primers (each at 10 uM) 3 µl of 25 mM $MgSO_4$ (1.5 mM final concentration), 5 µl of 2 mM dNTPs (0.2 mM final concentration), 1 µl (50 ng) of template DNA, 1 µl of KOD polymerase (1 U/µl) and 32 µl of autoclaved milli Q H_2O. The primers used were as follows:

Forward primer 5'-AGGAATTCGCCATATGGACG-3'

Reverse primer 5'-CGCTCGAGGGATCCTTAATC-3'

2.12.9 DNA sequencing

The DNA sequences of plasmids were determined by Direct Sanger performed using the ABI Prism Big Dye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, CA) on an ABI Prism 3700 DNA analyzer (Applied Biosystems, CA).

CHAPTER 3

RESULTS

CHAPTER 3

RESULTS

3.1 Evidence of zinc metalloproteases in different scorpion species

With the first evidence of a unique metalloprotease antarease from *Tityus serrulatus*, we investigated if such sequences are conserved among different species of scorpions. For this purpose the antarease sequence was blasted on uniprot database to find any known similar sequences. As shown in **Figure 3.1** antarease shows similarity with different metalloproteases present in different scorpion species in particular with the *Buthus eupeus* species. This similarity basically lead us to investigate the role of metalloproteases from different scorpion species that are spread in different geographical areas.

3.2 *In vitro* effects of venoms on recombinant SNARES

The *in vitro* effects of three different venoms were studied first of all on recombinantly expressed SNARE proteins VAMP and SNAP 25. It is evident from the **Figure 3.2 (a)** and **(b)** and **Figure 3.3** that venoms from all three scorpion species i.e. *Tityus serrulatus* venom (TSV), *Buthus eupeus* venom (BEV) and *Orthochirus scrobiculosus* venom (OSV) are able to cleave the recombinantly expressed SNARE proteins SNAP-25 and VAMP. For the positive control of SNAP 25 cleavage, a known metalloprotease specifically active against SNAP 25, botulinum toxin type A was used, whereas for the positive control of VAMP protein cleavage, tetanus toxin that is specific for VAMP was used. Among all these venoms the activity of the TSV seems to be highest when used at same concentration as other venoms. Apart from the SNARE proteins the activity of all three venoms was also tested on Glutathione *S*-transferase (GST) and Bovine serum albumin (BSA) proteins. The three venoms are not effective against GST and BSA as indicated in **Figure 3.2 (c)** and **(d).**

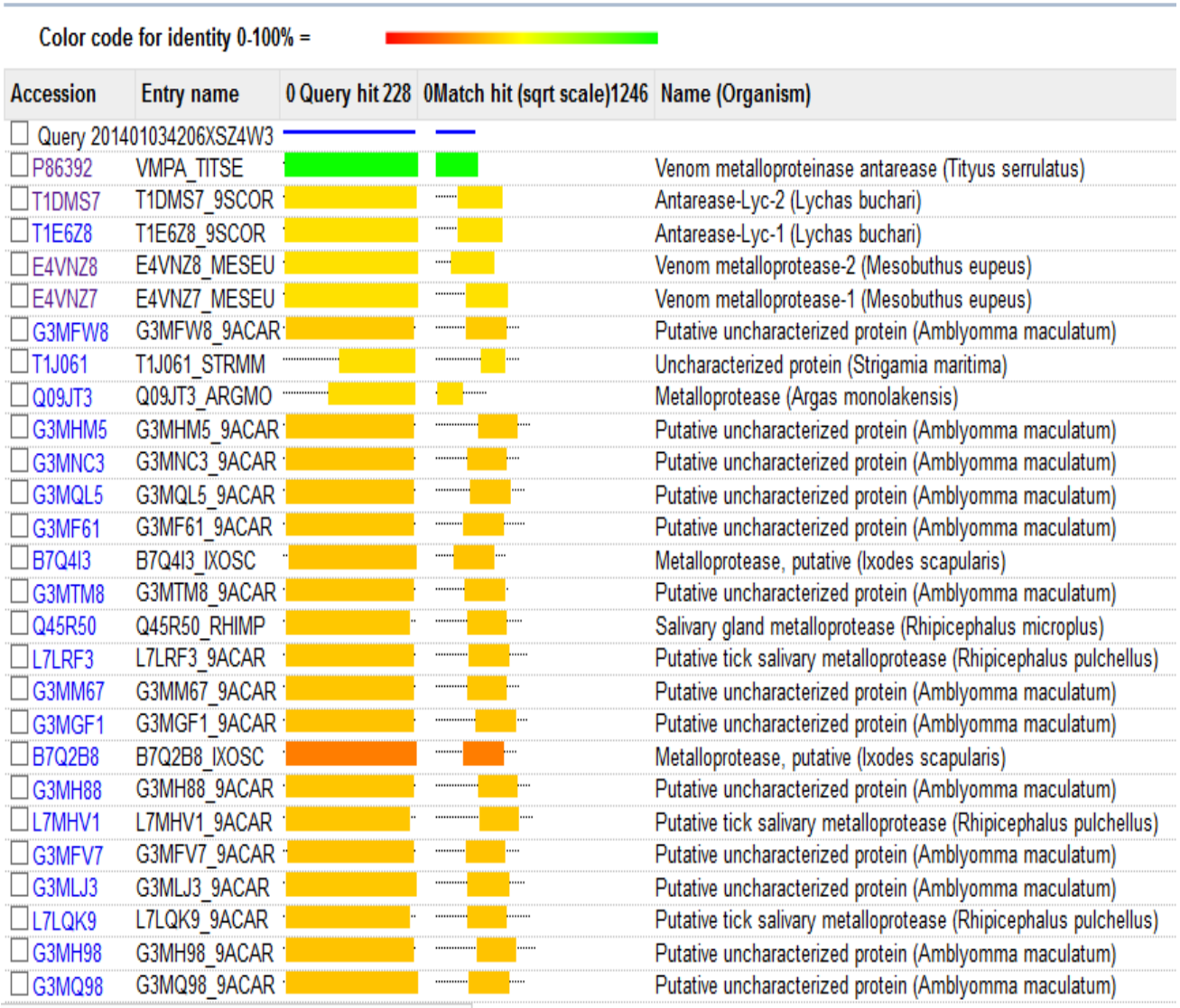


Figure 3.1: Graphical representation of blast first 26 results on uniprot database carried out with antarease amino acid sequence to identify similar sequences in other species. The similarity in sequences is represented by a color code described in the figure with green showing 100% similarity and red showing 0% similarity.

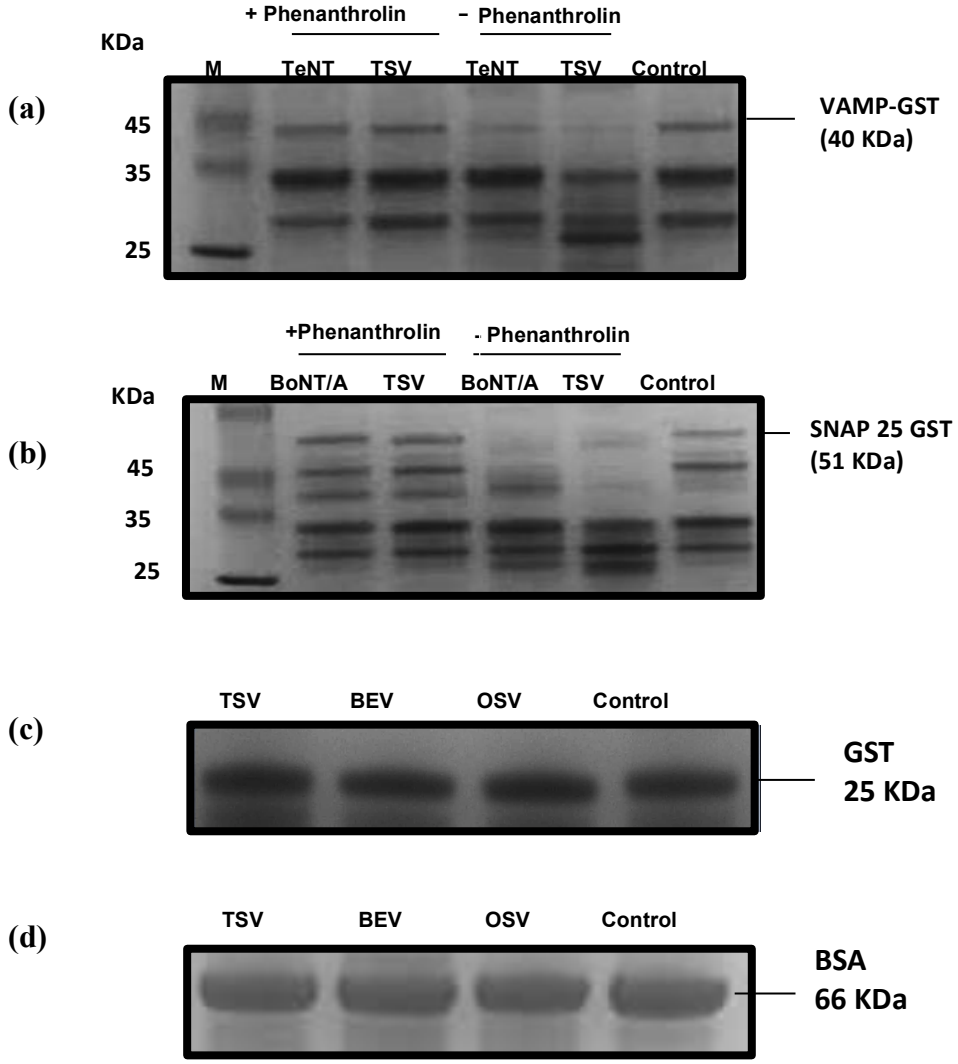


Figure 3.2: Cleavage assay of recombinant proteins under the action of three venoms. **(a)** SDS-PAGE showing the effect of TSV (6 ug of total venom) on recombinant VAMP-GST (6ug) and **(b)** SNAP 25-GST (6ug) after incubation at 37 ºC for 3 hours in presence or absence of 2mM phenanthrolin. TeNT is used as a positive control for VAMP cleavage while BoNT/A is used as positive control for SNAP 25 cleavage. TSV is *Tityus serrulatus* venom, OSV is *Orthochirus scrobiculosu s* venom and BEV is *Buthus eupeus* venom. **(c)** and **(d)** Effect of different venoms on recombinantly expressed GST (Glutathione *S*-transferase and BSA (Bovine serum albumin) when incubated at 37 ºC for 3 hours.

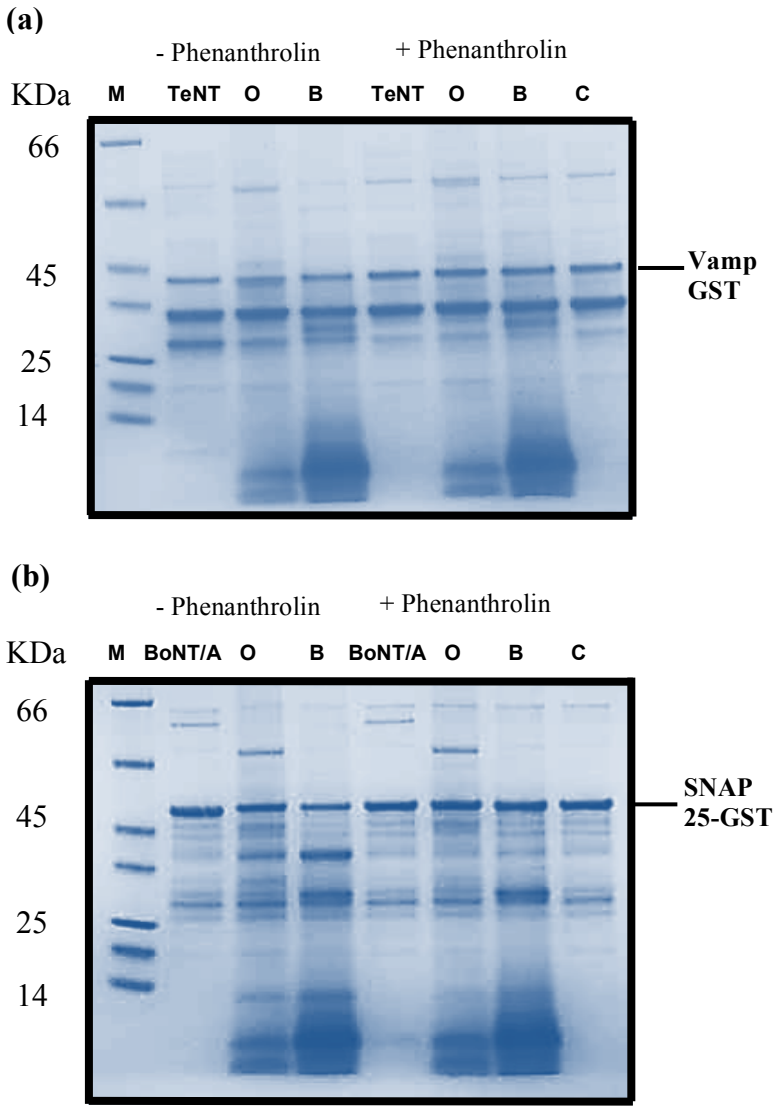


Figure 3.3: Cleavage assay of SNAREs under the action of venoms. **(a)**SDS-PAGE showing the effect of different venoms (6 ug of total venom) on recombinant VAMP-GST (6ug) and **(b)** SNAP 25-GST (6ug) after incubation at 37 ºC for 3 hours in presence or absence of 2mM orthophenanthrolin. Tetanus toxin (TeNT) is used as a positive control for VAMP cleavage while botulinum A (BoNT/A) toxin is used as positive control for SNAP 25 cleavage. In sample incubated with BoNT/A without orthophenathrolin, the SNAP25-GST band is slightly lower than in the control (C) since it cleaves only 9 residues at the C terminal of SNAP25. O is *Orthochirus scrobiculosus* venom, B is *Buthus eupeus* venom, – and + represent absence and presence of orthophenanthrolin.

Since the antarease has been described as a metalloprotease active on VAMP and SNAP 25, and with the finding that BEV and OSC venoms were also active on the recombinant SNARE proteins we aimed to investigate if these two venoms also contain active metalloproteases specific for the SNARE proteins. For this purpose we used the metal chelator orthophenanthrolin and tested its effects on the activity of the venoms on the recombinant SNARE proteins. Orthophenanthrolin is a hetrocyclic compound that forms strong complexes with most of the metal ions. **Figure 3.2** (a and b) and **Figure 3.3** (a and b) show the inability of the venom to cleave SNAP 25 and VAMP protein in the presence of orthophenanthrolin, further pointing to the idea that probably the active component in the scorpion venoms specific on the SNARE proteins are the metalloproteinases similar to the known zinc metalloproteinase antarease.

3.3 Effects of venoms on SNARE proteins in motor neurons

The effect of three venoms on SNAP 25 and VAMP was assessed in different neuronal cell models. In particular the effects of these venoms have been tested in motor neurons and cerebellar granular neurons both of which provide a good model systems to study SNAREs.

Figure 3.4 is the representative image of an immunoblot and the quantification of the cleavage of SNAP 25 and VAMP in the motor neurons. Neurons were incubated with 30μg/ml of whole venoms from the three scorpion species. The cells were lysed as usual in lysis buffer and western blot was carried out according to standard procedure. SNAREs were visualized with antibodies against syntaxin, SNAP 25 and VAMP. The immunoblots shown in **Figure 3.4** indicates substantial cleavage of SNAP 25 and VAMP in the treated samples as compared to the negative control. The extent of cleavage is expressed as percentage cleavage against negative control and syntaxin is used as an internal standard. In the case of all three venoms maximum cleavage has been obtained by TSV, followed by BEV and finally OSV.

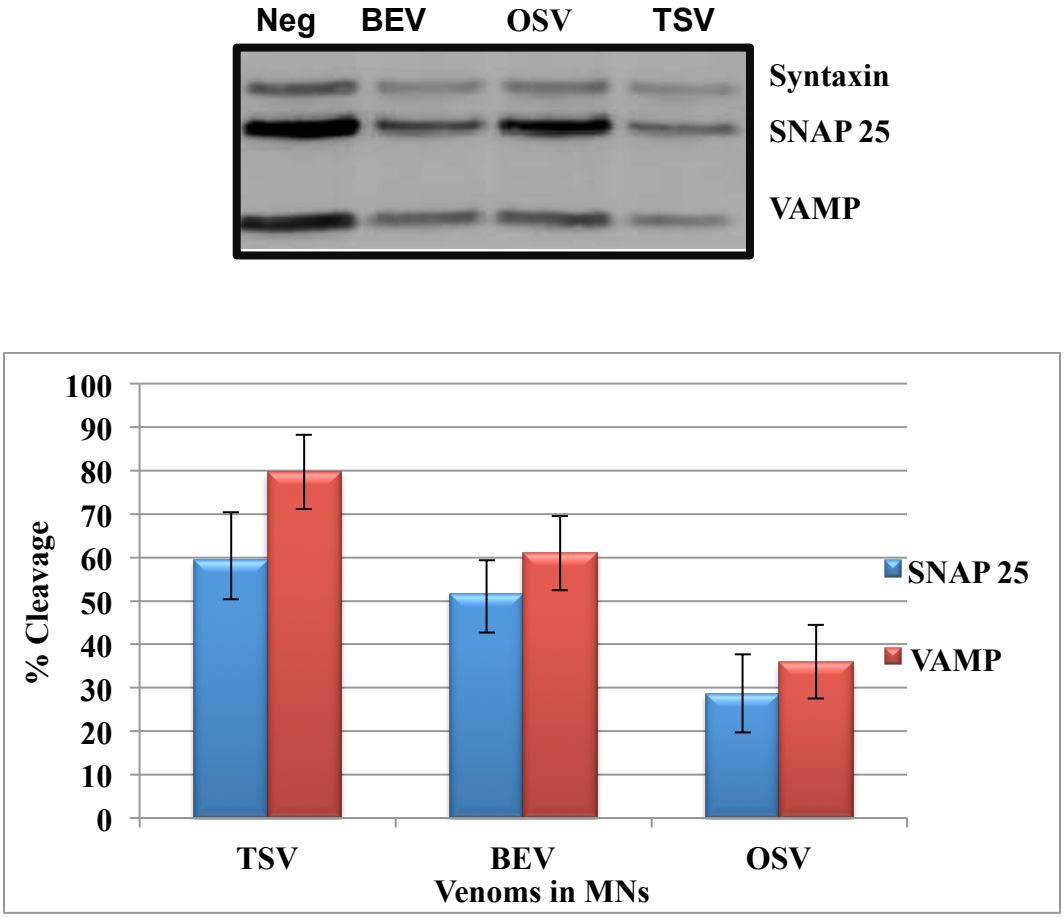


Figure 3.4: Top panel is the western blot showing effects of three venoms, *Tityus serrulatus* (TSV), *Buthus eupeus* (BEV) and *Orthochirus scrobiculosus* (OSV) on motor neuron (MNs) when incubated at concentration 30µg/ml overnight. Lower panel is the quantification of the western blot represented as percentage cleavage of SNAP 25 and VAMP against negative control. Syntaxin, which is not affected by the venoms, is taken as an internal standard.

3.4 Immunofluorescence in Motor neurons

To further assess the effect of the three venoms on the SNARE proteins, immunofluorescence analysis was also carried out in different neuronal cell models. Since motor neurons provide an ideal system to study SNAREs as well as we have already conducted immunoblot analysis of motor neurons under action of different venoms. We further investigated the effects of different venoms on motor neurons by immunofluorescence. The effects of the three venoms on VAMP in motor neurons are shown in **Figure 3.5** to **Figure 3.7**. Similarly the effects of TSV, BEV and OSV on SNAP 25 in motor neurons are shown in **Figure 3.8** to **Figure 3.10**. In short the cells were incubated with 30 µg/ml of whole venom from each scorpion species. The cells were fixed and SNAREs were visualized with antibodies against syntaxin, SNAP 25 and VAMP. In the case of VAMP the treated samples show significant decrease in VAMP staining as compared to the negative control or the untreated samples. TSV and BEV cause a more significant decrease in staining of VAMP as compared to OSV. Similar effects can be seen on the disappearance of SNAP 25 under the action of three venoms in motor neurons. As can be seen in Figure 8 to 10 the loss of staining is higher for TSV and BEV as compared to the OSV. This is in consistence with the previous data showing more drastic effects of TSV in comparison to the OSV when used at same final concentration.

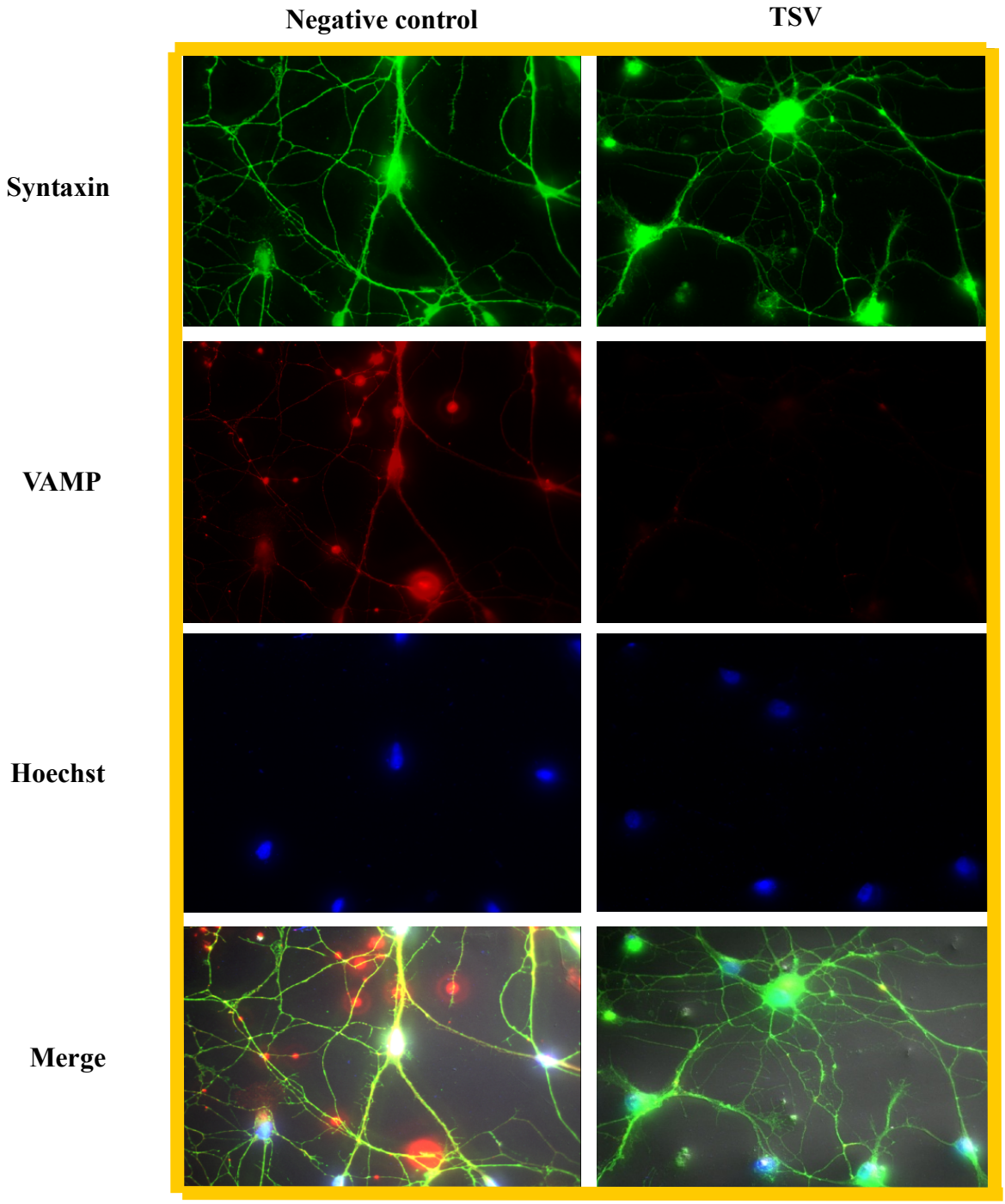


Figure 3.5: Immunofluorescent images showing the effect of TSV (30 µg/ml) on VAMP in the rat motor neurons. The cells were incubated overnight with the indicated amount of venom. Syntaxin (green) was visualized with polyclonal antibody whereas VAMP (red) was visualized with monoclonal antibody. Cell nuclei (blue) were stained with Hoechst.

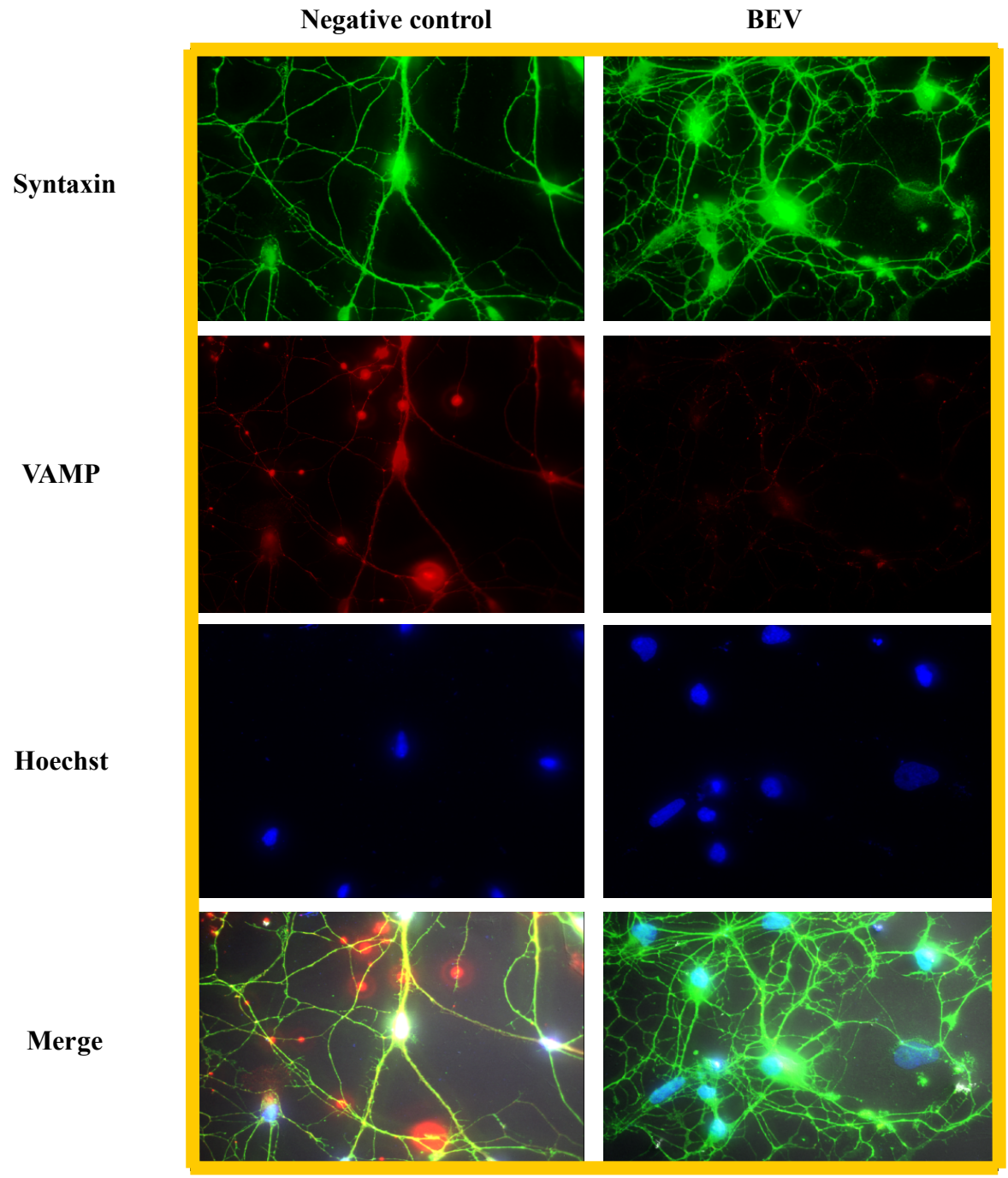


Figure 3.6: Immunofluorescent images showing the effect of BEV (30 µg/ml) on VAMP in the rat motor neurons. The cells were incubated overnight with the indicated amount of venom. Syntaxin (green) was visualized with polyclonal antibody whereas VAMP (red) was visualized with monoclonal antibody. Cell nuclei (blue) were stained with Hoechst.

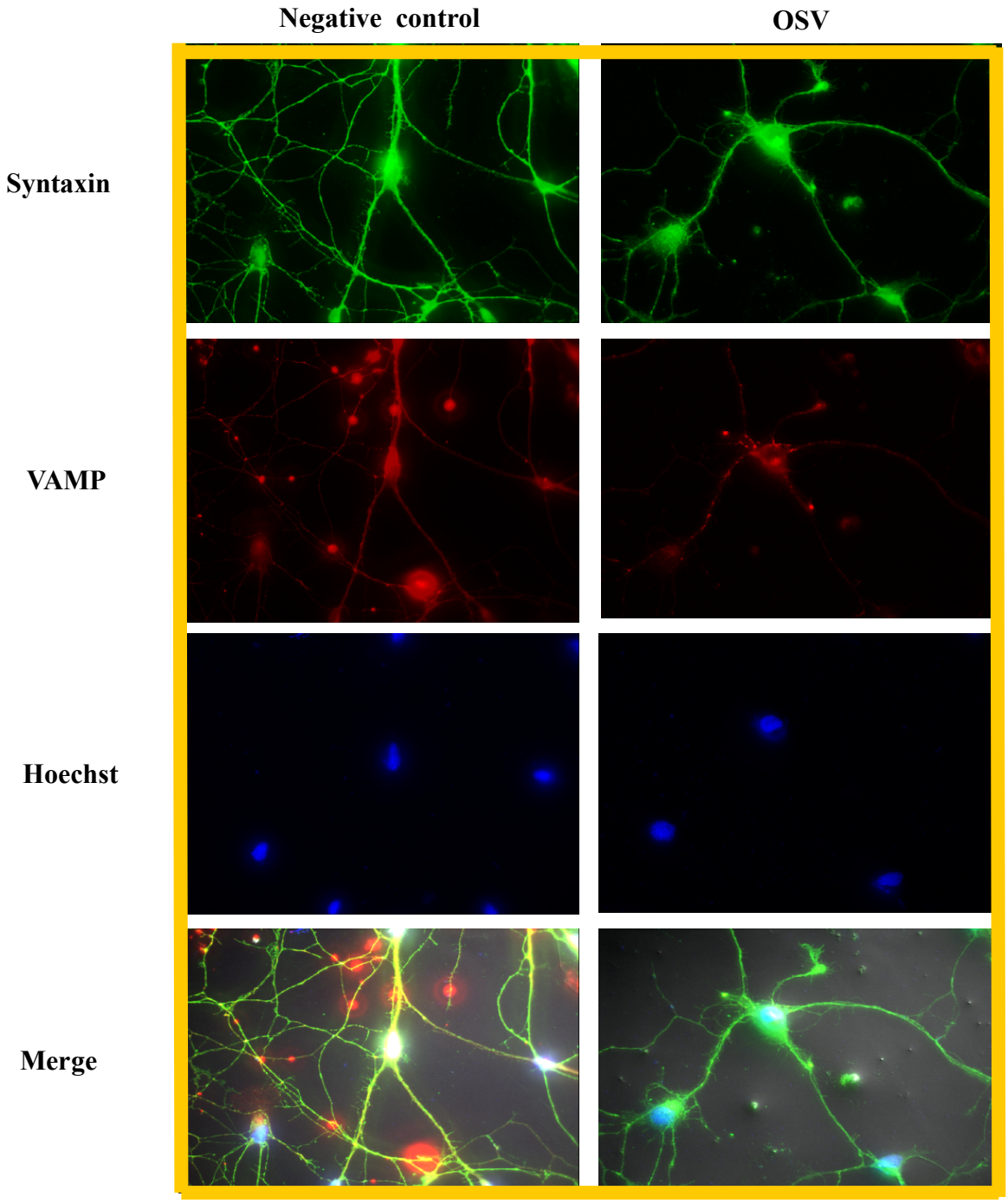


Figure 3.7: Immunofluorescent images showing the effect of OSV (30 µg/ml) on VAMP in the rat motor neurons. The cells were incubated overnight with the indicated amount of venom. Syntaxin (green) was visualized with polyclonal antibody whereas VAMP (red) was visualized with monoclonal antibody. Cell nuclei (blue) were stained with Hoechst.

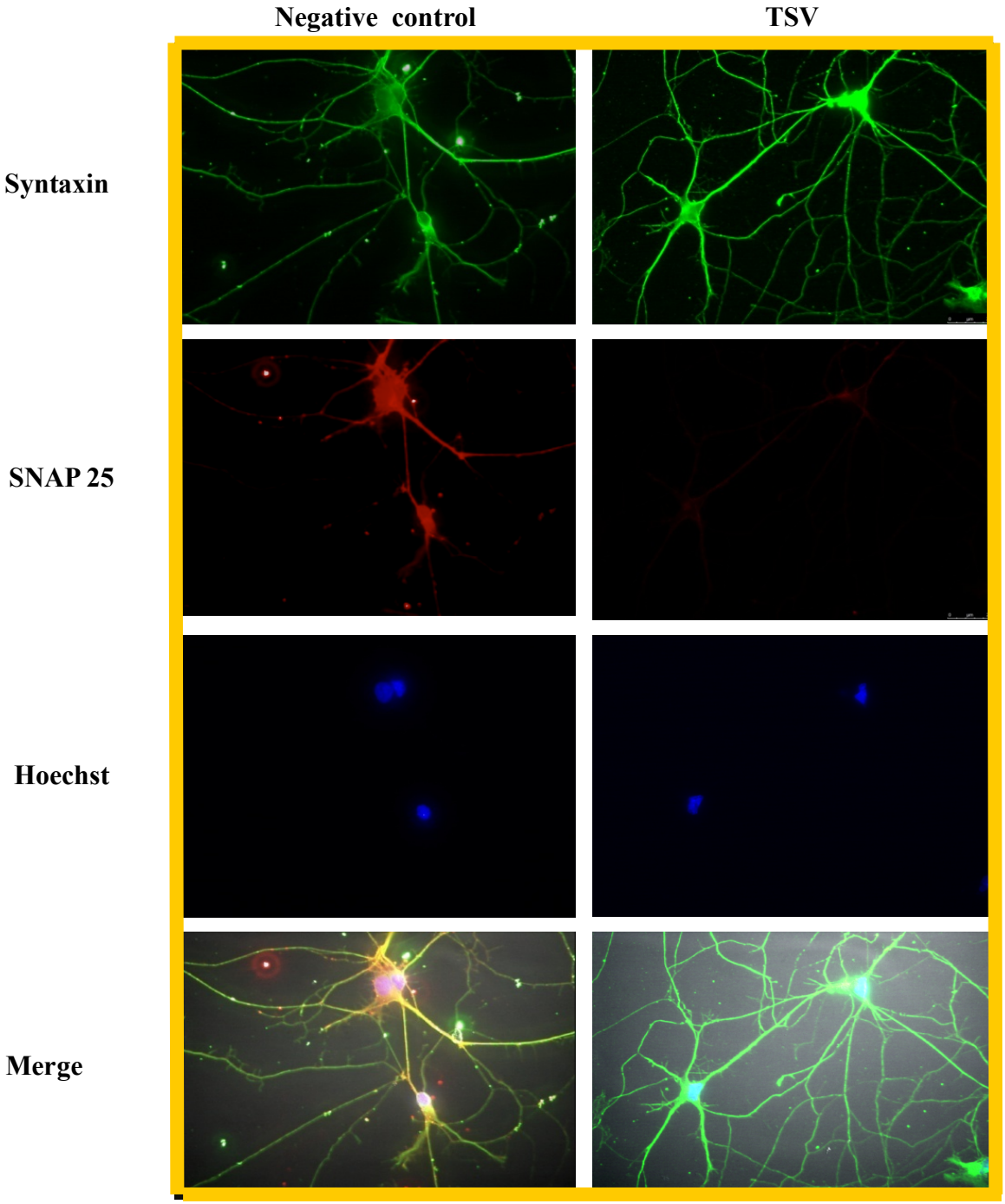


Figure 3.8: Immunofluorescent images showing the effect of TSV (30 µg/ml) on SNAP 25 in the rat motor neurons. The cells were incubated overnight with the indicated amount of venom. Syntaxin (green) was visualized with monoclonal antibody whereas SNAP 25 (red) was visualized with polyclonal antibody. Cell nuclei (blue) were stained with Hoechst.

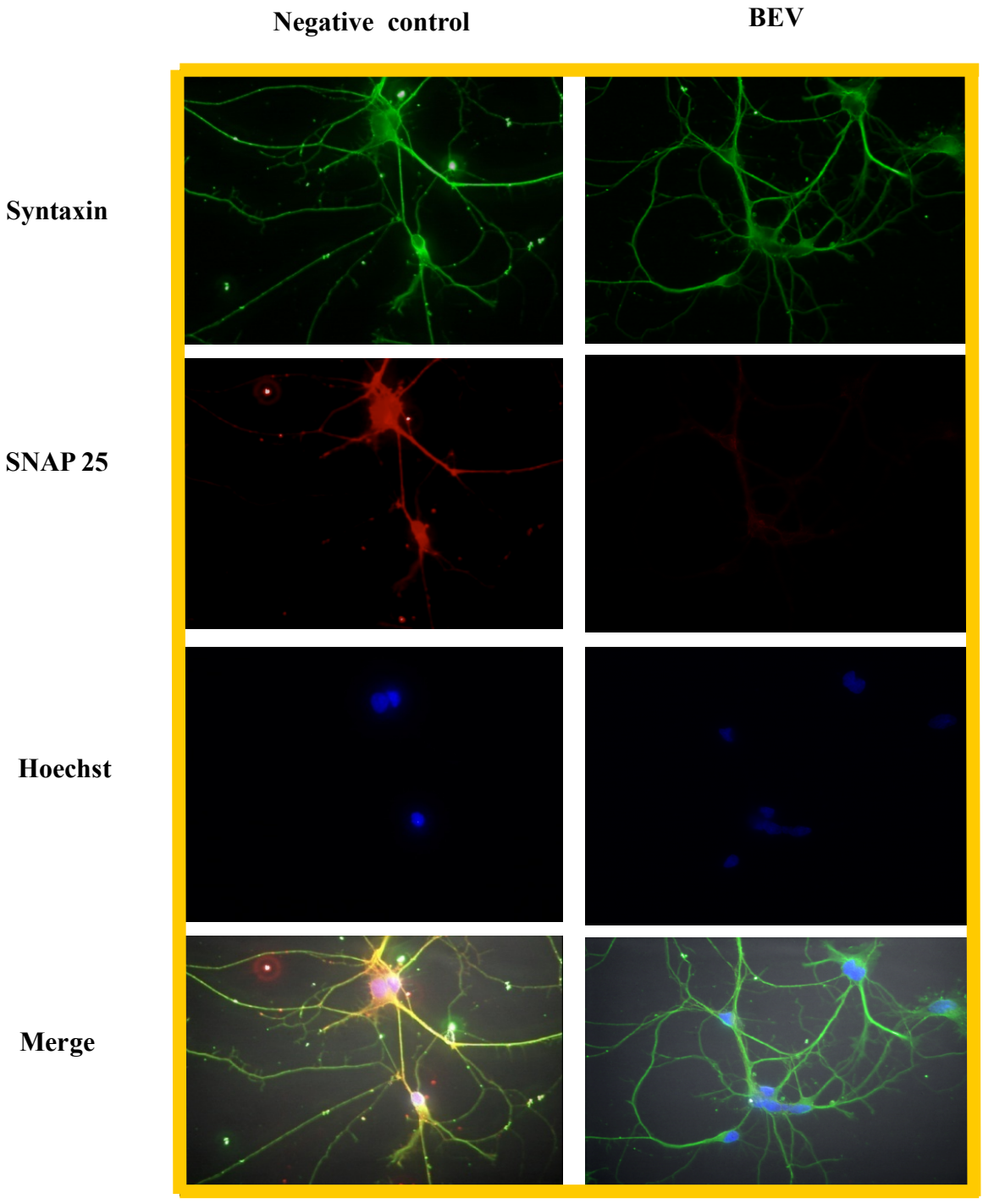


Figure 3.9: Immunofluorescent images showing the effect of BEV (30 µg/ml) on SNAP 25 in the rat motor neurons. The cells were incubated overnight with the indicated amount of venom. Syntaxin (green) was visualized with monoclonal antibody whereas SNAP 25 (red) was visualized with polyclonal antibody. Cell nuclei (blue) were stained with Hoechst.

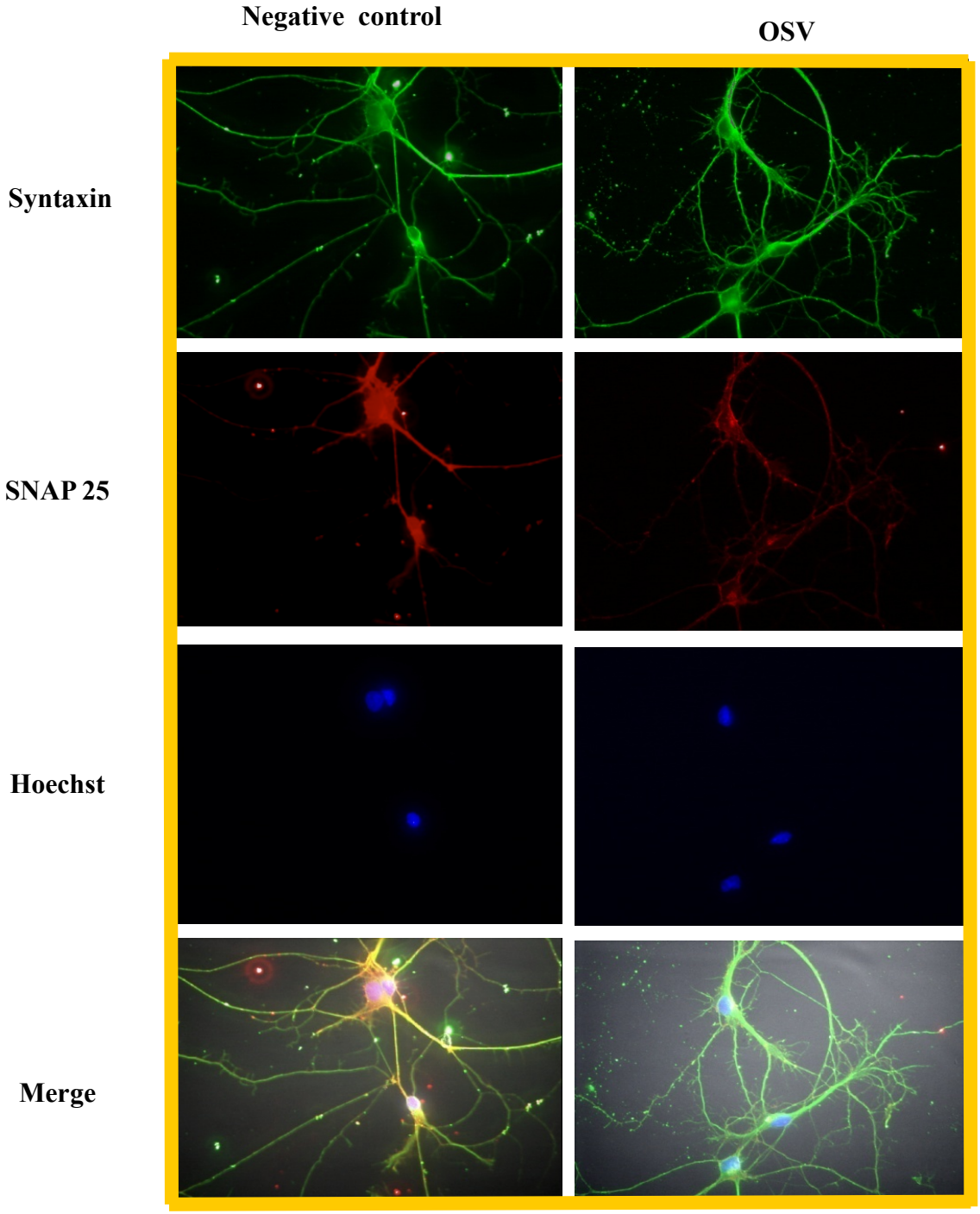


Figure 3.10: Immunoflourescent images showing the effect of OSV (30 µg/ml) on SNAP 25 in the rat motor neurons. The cells were incubated overnight with the indicated amount of venom. Syntaxin (green) was visualized with monoclonal antibody whereas SNAP 25 (red) was visualized with polyclonal antibody. Cell nuclei (blue) were stained with Hoechst.

3.5 Effects of venoms on SNARE proteins in cerebellar granular neurons

The effects of whole venoms form the three scorpion species were also tested in the cerebellar granular neurons. The cells were incubated overnight with 30µg/ml of whole venom. The western blot was carried out according to standard protocol. The immunoblots from the cerebellar granular neurons incubated with venom overnight showed clearly loss of SNAP 25 and VAMP as compared to the negative control. TSV showed the highest cleavage of SNAP 25 and VAMP followed by BEV and OSV as shown in **Figure 3.11 (a)** and **(b)**, confirming what we observed in motor neurons. The viability of the cells was also assessed under the same venom concentration as used for immunoblot analysis i.e. 30 µg/ml. The viability of cells was almost comparable to negative control as shown in **Figure 3.11 (c)**, indicating the fact that this concentration of venom was not lethal in the cells and that the venom metalloproteinases probably entered the neuronal cells to cleave the SNARE proteins without disrupting cells overall structure and morphology.

3.6 Immunoflourescence in cerebellar granular neurons and cortical neurons

The effects of three venoms were also investigated in cerebellar granular neurons and cortical neurons by immunofluorescence. In the case of cerebellar granular neurons the overnight incubation of the neurons with these three different venoms resulted in substantial decrease in both, VAMP staining as shown in **Figure 3.12**, and SNAP 25 staining as shown in **Figure 3.13**, when compared with their respective negative controls.

Since cortical neurons also represent important neuronal cell model system we also tested effects of TSV in this cell model. As expected in the light of previous experiments, the overnight incubation of cortical neurons with TSV also resulted in reduction of VAMP and SNAP 25 staining as compared to their respective negative controls as shown in **Figure 3.14**.

3.7 Immunoflourescence in drosophila

Since in nature, scorpions are known to prey on small insects we tested the effects of TSV on SNAREs in drosophila larvae. For this reason the effects of TSV on the SNAP 25 in drosophila third instar larvae were studied. As indicated in the **Figure 3.15** the SNAP 25 is partially reduced in the larvae treated with TSV for two hours. Furthermore this effect was observed to be concentration dependent as shown in **Figure 3.15 (a)** and **(b)**, with more lesser staining of SNAP 25 with a higher concentration of TSV. Unfortunately because of the unavailability of antibody against drosophila VAMP proteins the experiments could not be carried out to study the effects of TSV on VAMP protein in drosophila.

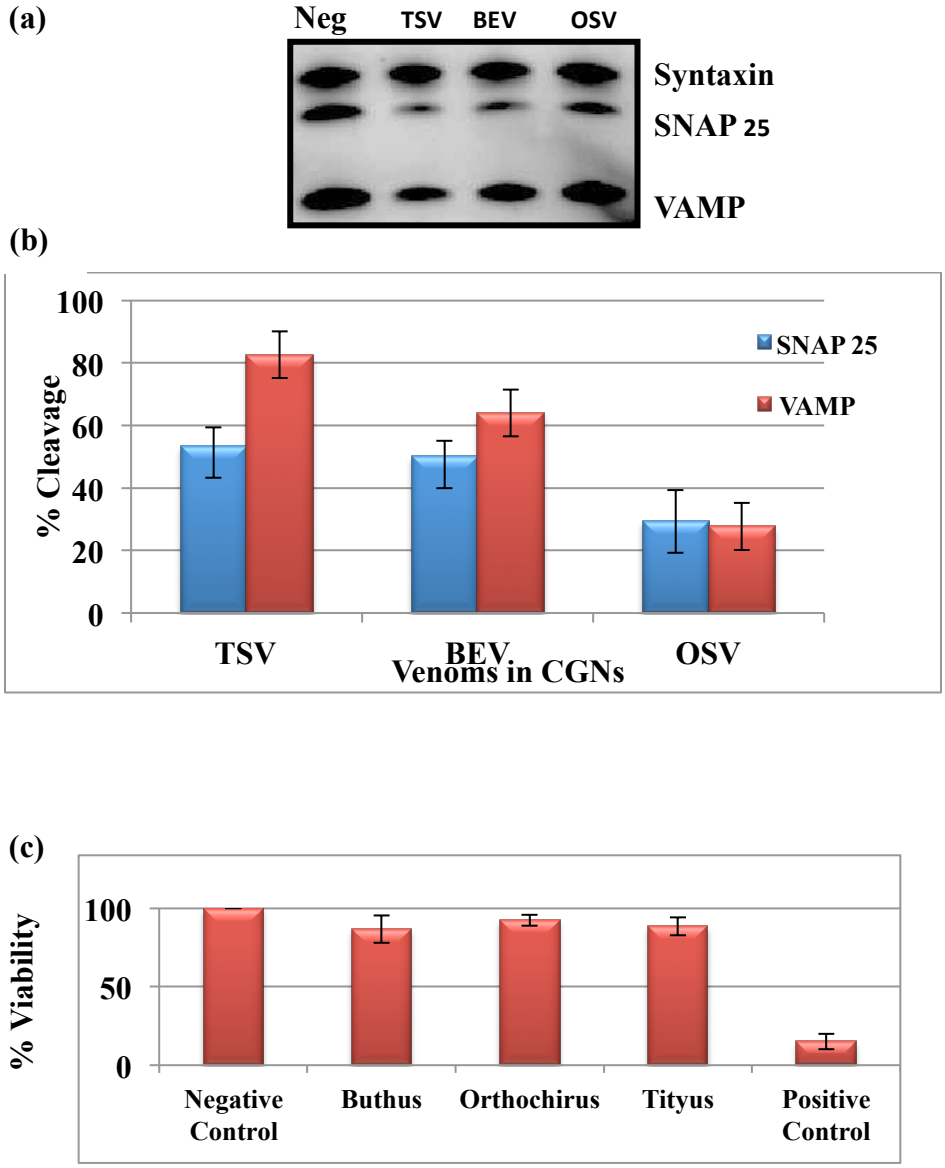


Figure 3.11: Effects of different veneoms on cerebellar granular neurons (CGNs). **(a)** Western blot showing effects of three venoms (TSV, BEV and OSV) on CGNs when incubated at concentration 30µg/ml. **(b)** Quantification of western blot represented as percentage cleavage of SNAP 25 and Vamp against negative control. **(c)** MTT or viability assay of CGNs after overnight incubation with 30µg/ml of each *Tityus serrulatus* (TSV), *Buthus eupeus* (BEV) and *Orthochirus scrobiculosus* (OSV) venoms.

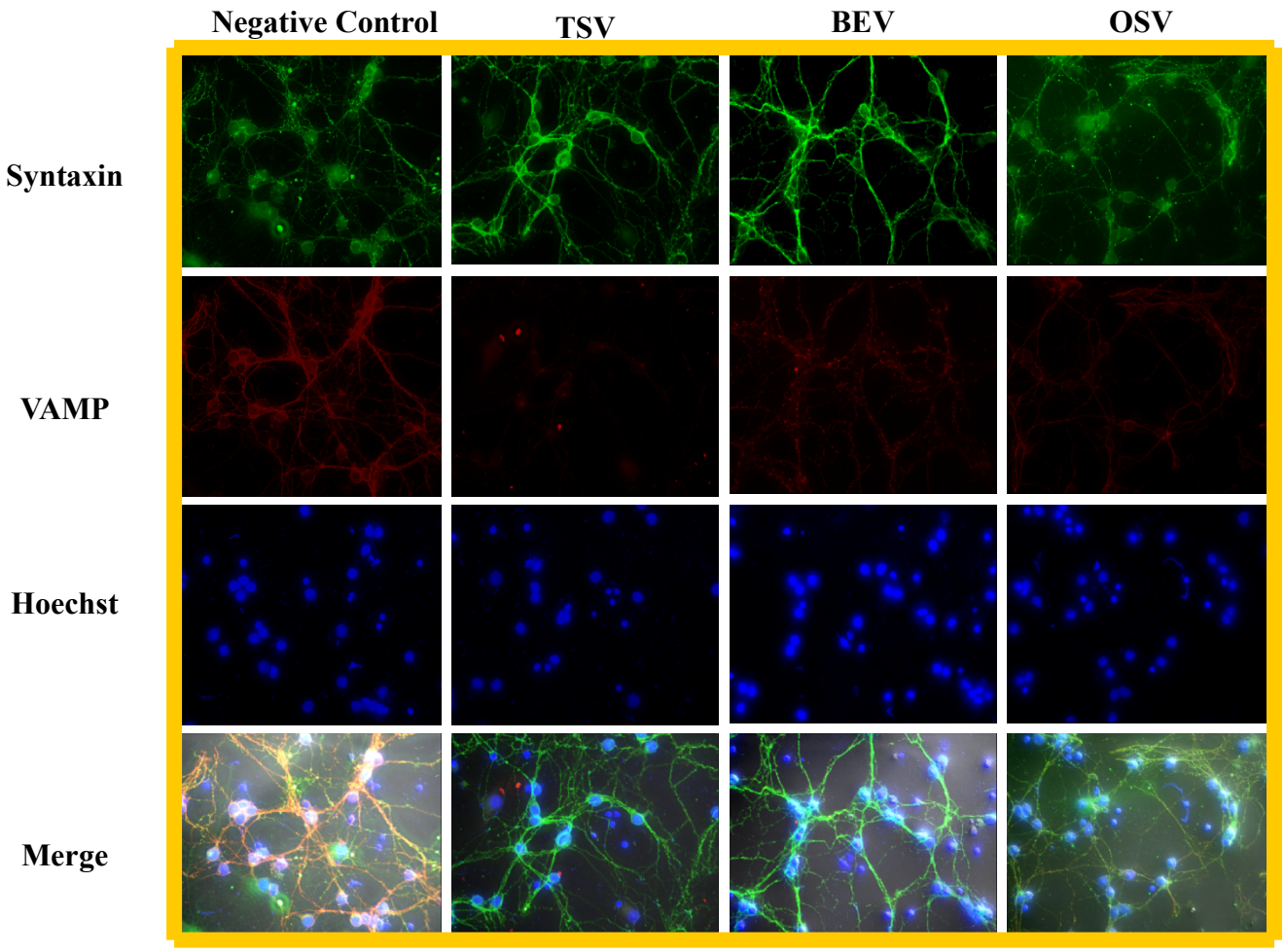


Figure 3.12: Immunofluorescent images showing the effect of TSV, BEV and OSV (30 µg/ml) on VAMP in the cerebellar granular neurons. The cells were incubated overnight with the indicated amount of venom. Syntaxin (green) was visualized with polyclonal antibody whereas VAMP (red) is visualized with monoclonal antibody. Cell nuclei (blue) were stained with Hoechst.

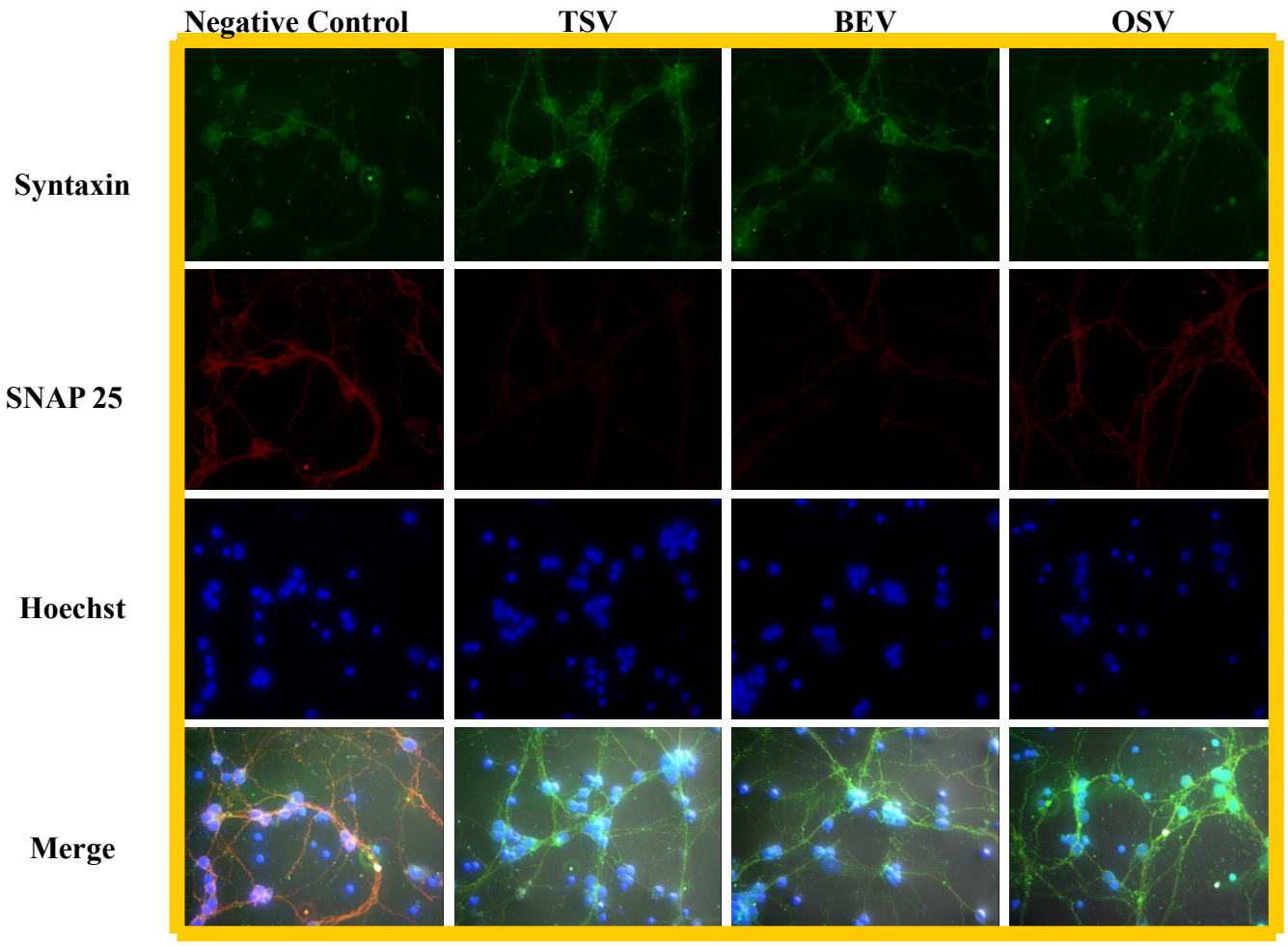


Figure 3.13: Immunofluorescent images showing the effect of TSV, BEV and OSV (30 µg/ml) on SNAP 25 in the cerebellar granular neurons. The cells were incubated overnight with the indicated amount of venom. Syntaxin (green) was visualized with monoclonal antibody whereas SNAP 25 (red) is visualized with polyclonal antibody. Cell nuclei (blue) were stained with Hoechst.

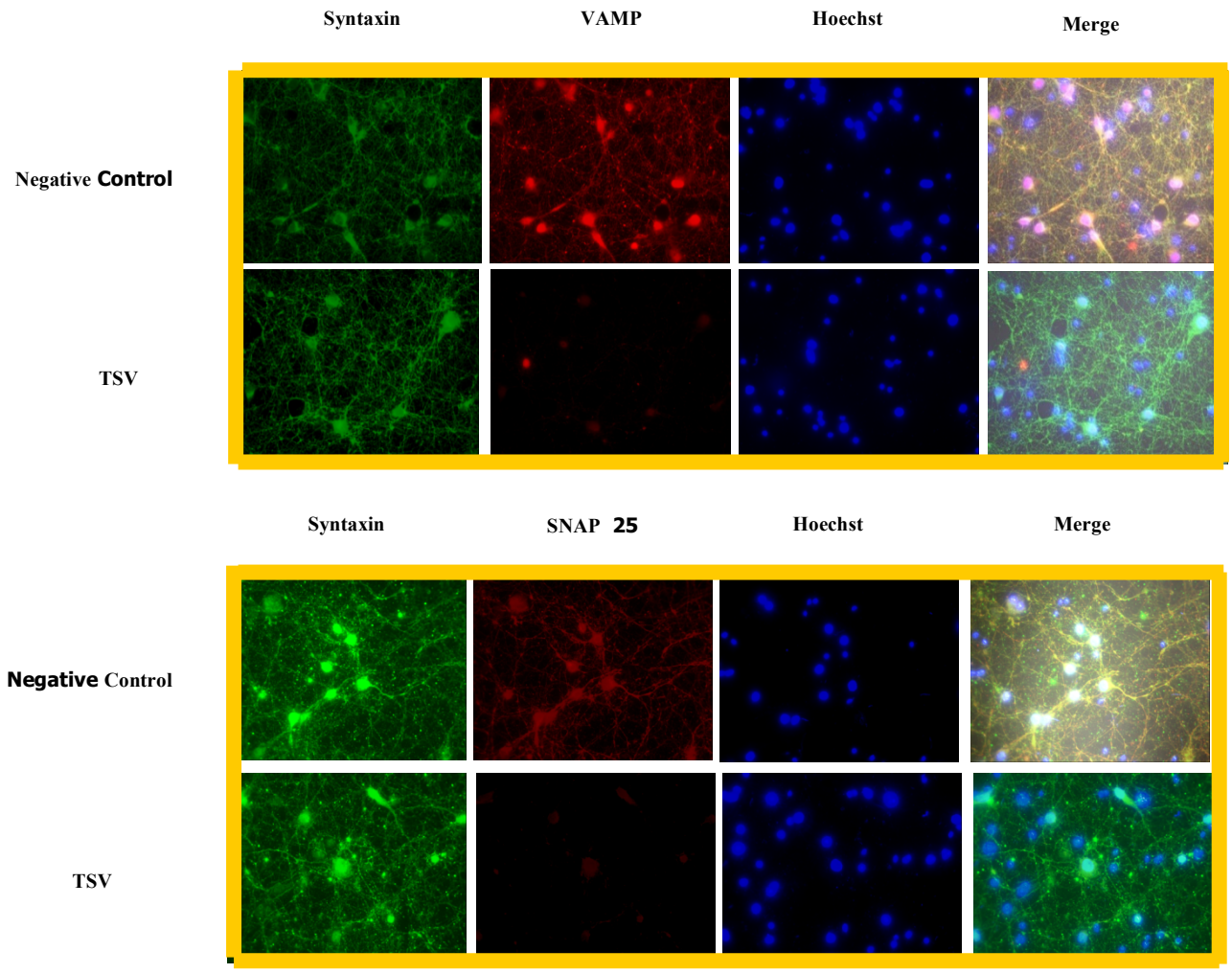


Figure 3.14: Effect of TSV (30µg/ml) on the SNARE proteins (VAMP shown in upper panel and SNAP 25 shown in lower panel) in cortical neurons. The cells were incubated overnight with the indicated amount of venom. In the upper panel syntaxin (green) was visualized with polyclonal antibody against VAMP (red) monoclonal antibody In lower panel syntaxin (green) was visualized with monoclonal antibody against SNAP 25 (red) polyclonal antibody. Whereas cell nuclei (blue) were stained with Hoechst.

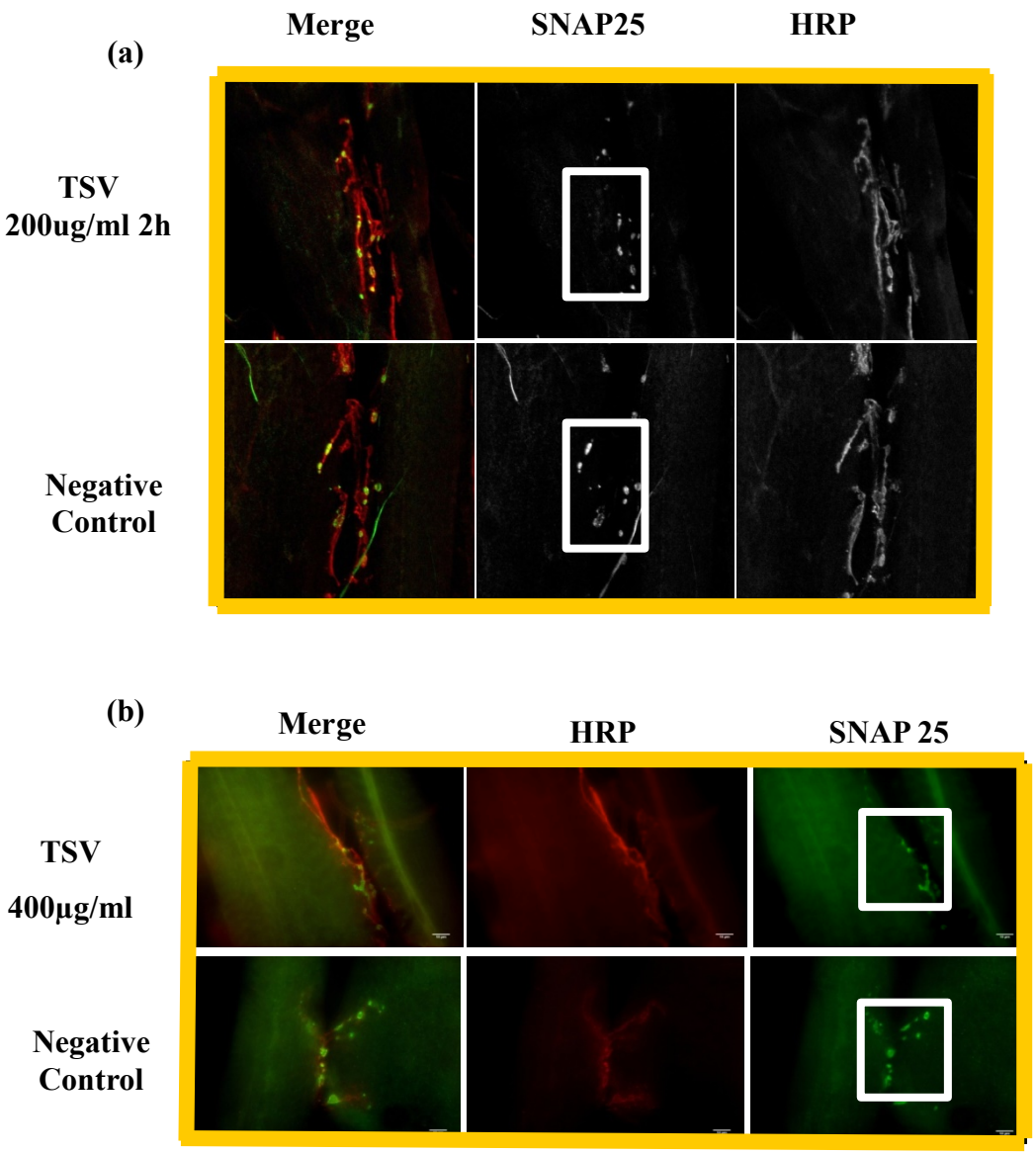


Figure 3.15: Effect of different concentrations of TSV on SNAP 25 in Drosophila third instar larva envenomed at 28°C for 2 hours, where **(a)** 200ug/ml of TSV and **(b)** 400ug/ml of TSV. HRP is the control antibody against the neuronal membrane.

3.8 Entry mechanism of Antarease

The mechanism of entry of antarease was investigated in cerebellar granular neurons under the depolarization condition at high potassium chloride concentration or in presence of bafilomycin which inhibits acidification of synaptic vesicles and which is known to block the entry of the clostridial neurotoxic metalloproteases. As shown in **Figure 3.16** the presence or absence of bafilomycin had no effect on the entry of antarease and thus cleavage of SNAP 25 and VAMP remains unaffected as compared with the control tetanus toxin which is known to enter central neurons through synaptic vesicle recycling and to utilize acidification of synaptic vesicles to translocate into the cytosol eventually cleaving the SNARE protein VAMP. Similarly, effect of TSV on SNAP 25 and VAMP in presence or absence of high stimulation media remains almost same as indicated by the similar percentages of cleavages of SNAP 25 and VAMP in **Figure 3.17**.

3.9 Expression of recombinant antarease

The sequence of antarease was retrieved from uniprot database (accession number P86392) and submitted to Blue heron to be gene synthesized in entry vector pUC. The antarease gene was then cloned into pET 19b vector (data not shown). After which expression of his-tagged antarease was assessed in different expression strains of *E.coli.* Since the expression level of recombinant His-tagged antarease was very low and difficult to observe in SDS-PAGE, a more specific western blotting approach was utilized to assess expression levels of different strains by anti-His antibody as shown in **Figure 3.18 (a)**. The strain with maximum expression level i.e. DE 3 was further subjected to bulk expression and purification. The expression levels were assessed by western blotting against His-tag **Figure 3.18 (b).** Since the expression levels were very low and could only be visualized with western blotting a PCR based cloning strategy was followed to clone antarease sequence into pGEX 4T vector to be expressed as GST tagged protein. The summary of the strategy followed is shown in **Figure 3.18 (c).** The PCR product approximately 740 bp as shown in **Figure 3.19 (a)** was purified and cloned into pGEX 4T vector. Expression trial was carried out and bulk purification from induced DE 3 cultures showed optimum levels of expression of GST tagged antarease as can be seen in **Figure 3.19 (b) and (c).**

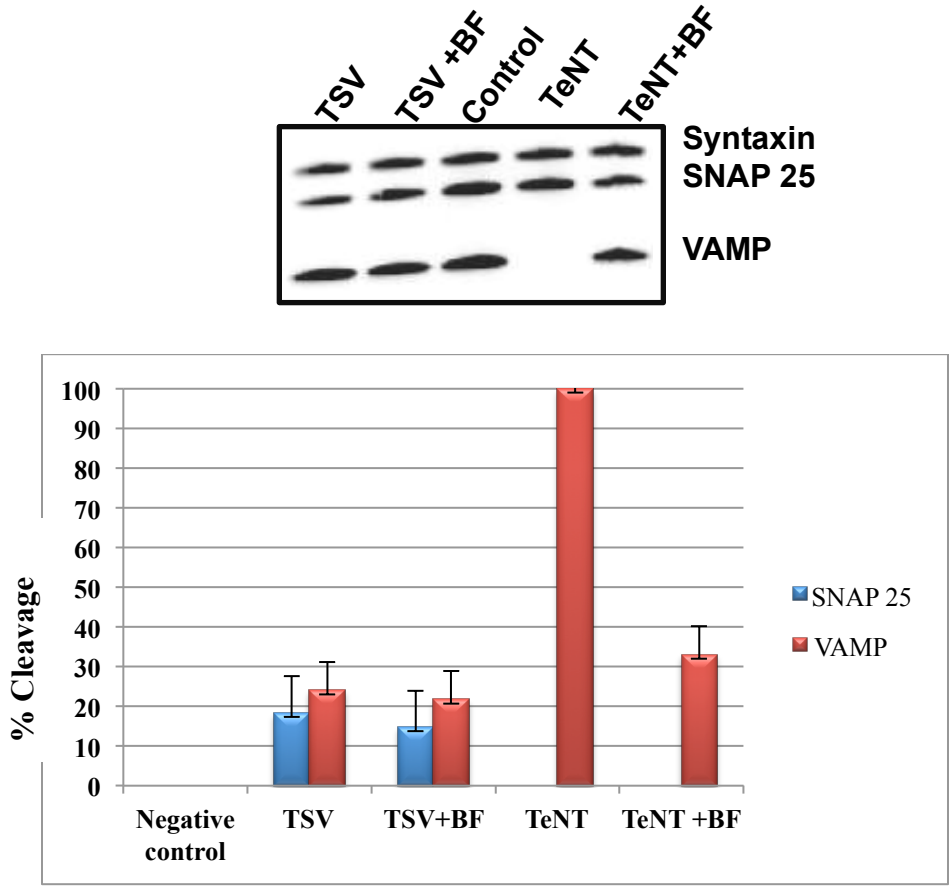


Figure 3.16: A representative blot and its quantification showing the effect of Bafilomycin (100nM) on the cerebellar granular neuron (CGNs) incubated with the TSV (30 ug/ml) when incubated for 5 hours at 37 ºC. Abbreviation include TeNT for tetanus toxin used as positive control, BF for bafilomycin and TSV for *Tityus serrulatus* venom. The incubation time with whole venom was reduced to five hours to reduce toxic effects of BF on the cells.

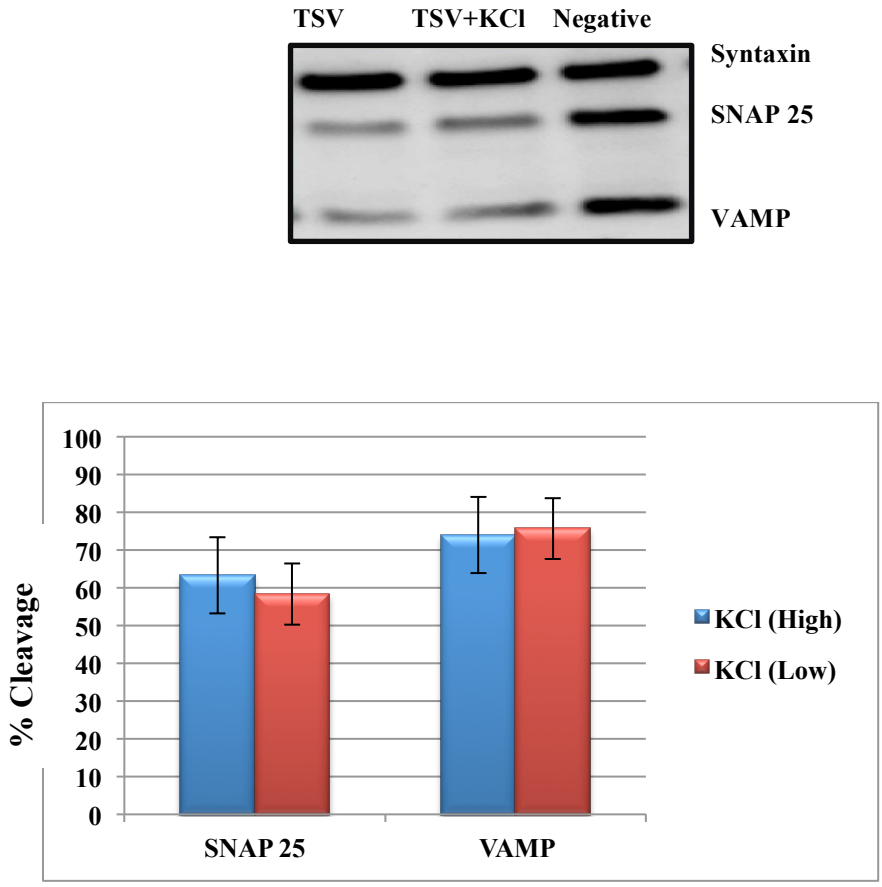
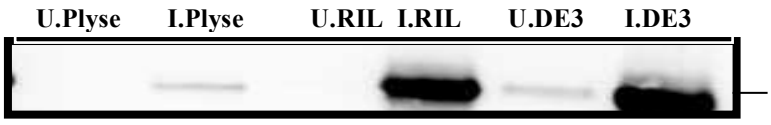


Figure 3.17: A representative blot and its quantification showing the effect of different concentration of KCl upon cleavage of SNAP 25 and VAMP in the cerebellar granular neuron (CGNs) incubated with the venom (30 ug/ml) overnight at 37ºC. Where high KCl = 60mM KCl and low KCl represents 25mM KCl.

(a)



(b)

(c)

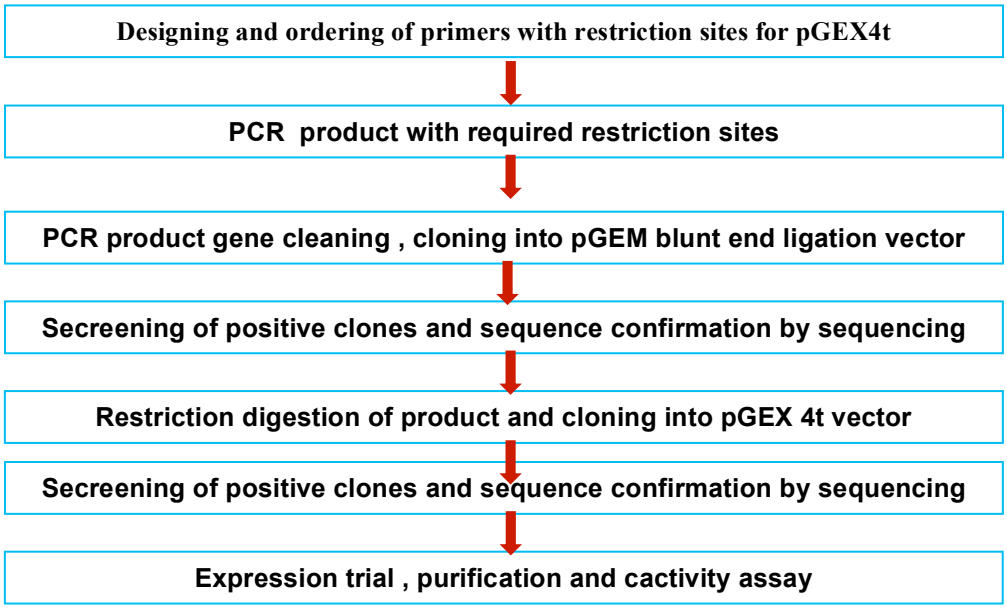


Figure 3.18: Recombinant expression of antarease. **(a)** western blot showing the expression of His tagged antarease in different expression strains when induced with 1mM IPTG for 3 hours at 37 ºC. Letters U represent uninduced, I represent Induced and Plyse, RIL and DE3 are different *E.coli* expression strains **(b)** Western blot showing the purified His-tagged antarease with gradient imidazole (20mM-500mM) from 2 liters of induced DE 3 cultures harbouring pET-antarease vector.**(c)** Cloning strategy followed to clone antarease into pGEX 4t vector to introduce GST tag in antarease.

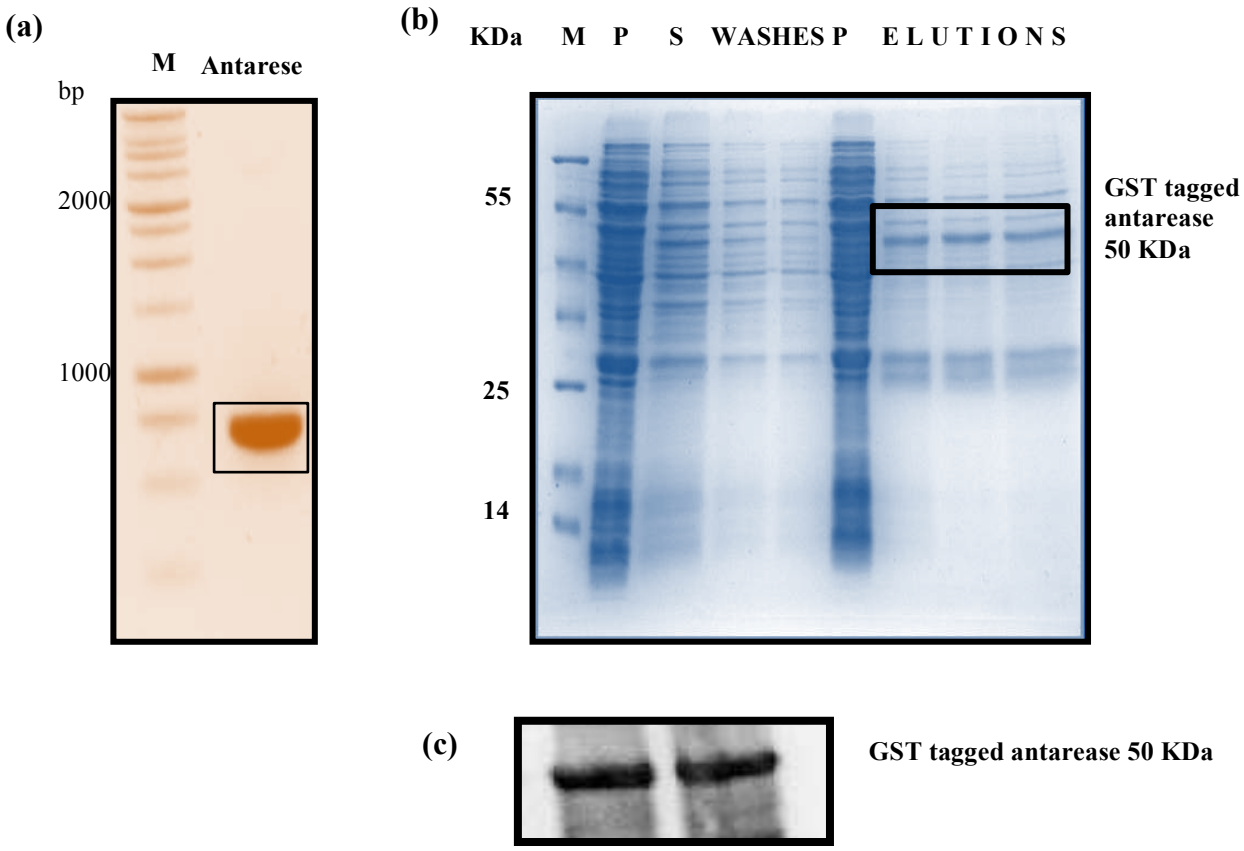


Figure 3.19: Recombinant expression of antarease in pGEX vector. **(a)** PCR product (740 bp) of antarease amplified to insert restriction sites to clone in pGEX 4T vector. **(b)** and **(c)** SDS-PAGE showing the purified antarease expressed with a GST tag. **(c)** A western blot of the purified antarease expressed with a GST tag and visualized with antibody against GST tag. P is Pellet and S is Supernatant.

3.10 Activity assay of recombinant Antarease

The activity of GST-tagged antarease was assessed by overnight incubation of recombinant antarease with the recombinant GST tagged substrates i.e. GST-SNAP 25 and GST-VAMP. Unfortunately the recombinant antarease remained inactive against SNAP 25 and VAMP proteins as can be seen by SDS PAGE in **Figure 3.20 (a)** and **(b)**. At the same time western blotting was also carried out using antibody against SNAP 25 as well as antibody against VAMP protein to access the activity of recombinant antarease as shown in **Figure 3.20 (c)** further confirming the inactivity of recombinant antarease. At the same time TSV, which is used as positive control can be seen to cleave and thus reduce the staining of VAMP as well as SNAP 25 (**Figure 3.20)**.

3.11 Purification of Antarease

To obtain further information on mechanism of action of venom zinc metalloprotease, and since the recombinant antarease was not catalytically active, antarease was purified by size exclusion chromatography from 250 mg of whole TSV. Different fractions were obtained as shown in **Figure 3.21**. SDS-PAGE was carried out on first three fractions obtained as shown in **Figure 3.22**. To gain more information on the domain structure of antarease the fractions were run under different conditions i.e. in presence and absence of beta-mercaptoethanol. Different bands were cut ranging in molecular weights 20-35 KDa and submitted for Mass spectrophotometry analysis to identify antarease. The mass spectrometry analysis (Data not shown) showed presence of antarease at 25 KDa position in presence and absence of beta-mercaptoethanol indicating high probability of antarease to be expressed as a single subunit protein of 25kDa.

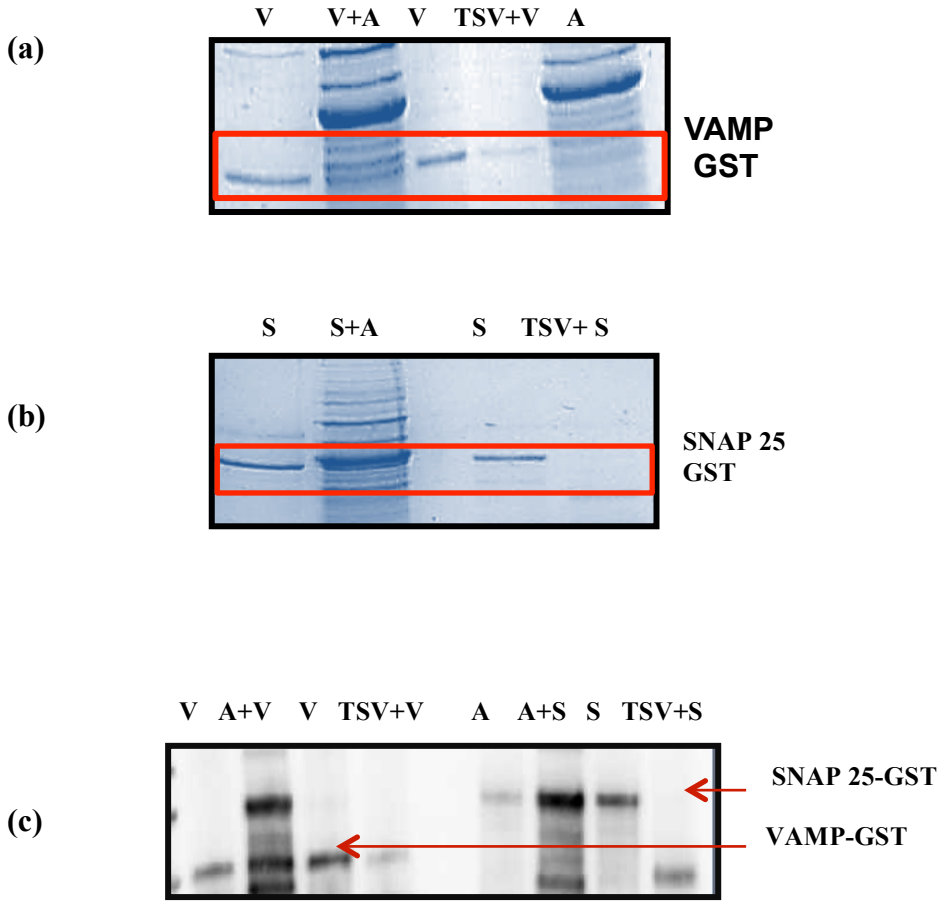


Figure 3.20: Activity assay of recombinant antarease. **(a)** SDS-PAGE showing overnight VAMP (1.7µg) digestion with antarease-GST (50µg). **(b)** SDS-PAGE showing overnight SNAP 25 (1.7µg) digestion with antarease-GST (50µg). **(c)** Immunoblot of overnight SNARE digestion as in **(a)** and **(b).** Abbreviations used are V, VAMP-GST; A, Antarease-GST; TSV, *Tityus serrlulatus* venom; S, SNAP 25-GST.

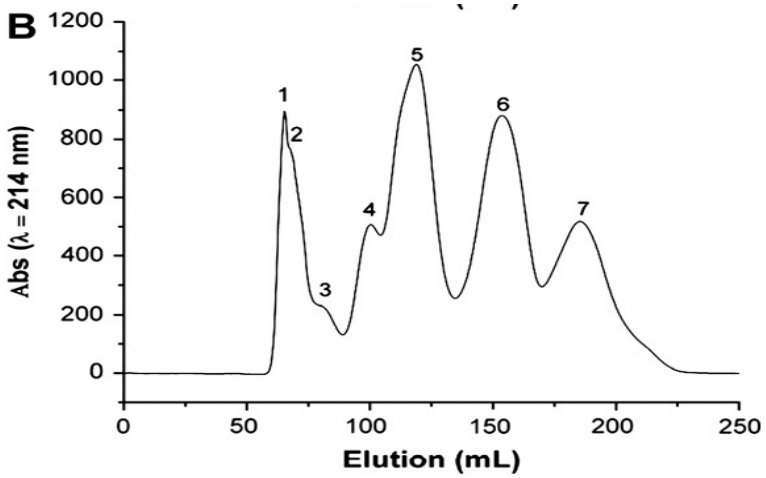


Figure 3.21: Partial purification of antarease. Venom (250 mg) was applied to a Sephadex G-50 (Superfine) column and eluted with 0.15 M ammonium formiate buffer, pH 6.5 (0.7 mL/min flow rate). Elution was monitored by absorbance readings at 214 nm.

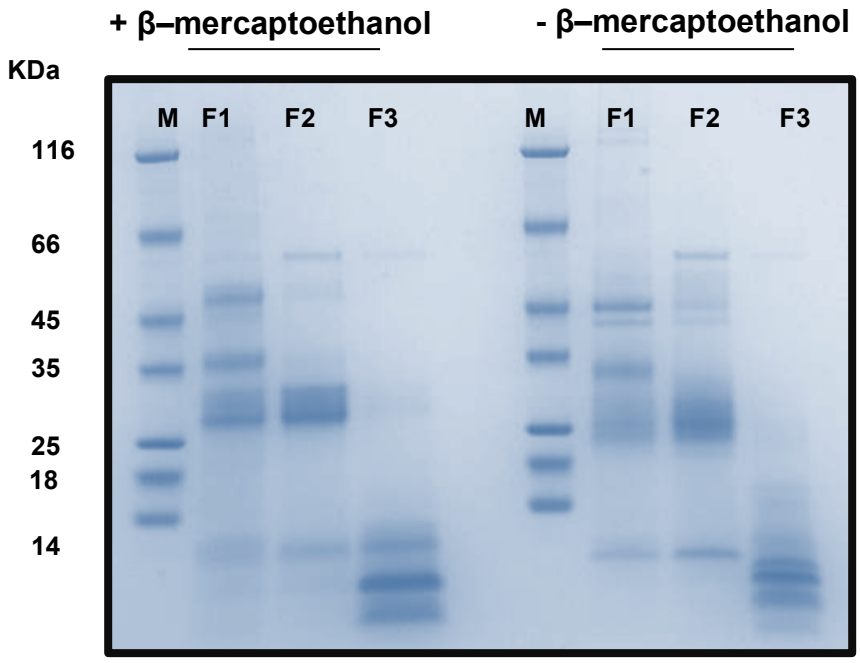


Figure 3.22: SDS-PAGE of different fractions of TSV (*Tityus serrulatus* venom) purified from Sephadex G-50 (Superfine) from 250 mg of whole venom. F1, F2, and F3 correspond to the different fractions obtained whereas M represents protein marker of known molecular weights.

3.12 *In vitro* effects of TSV fractions

Different fractions obtained as a result of TSV fractionation were subjected to SNARE cleavage assay. Out of the three fractions only fraction 1 and 2 were active against the SNAP 25 and VAMP (data not shown). The activity of fraction 1 and 2 were further assessed under presence and absence of phenanthrolin. Both fraction 1 and 2 retain much higher SNARE cleaving ability as can be seen **in Figure 3.23 (a)** and **(b).** Furthermore this activity of the fractions was inhibited in presence of metal chelating agent orthophenanthrolin indicating presence of functional metalloproteainase antarease in the first two fractions.

3.13 Effects of TSV fractions in cells

The western blot from the cells intoxicated with fraction 1 and 2 indicate considerable reduction in both SNAP 25 as well as VAMP whereas the inactive fraction 3 had no effects on SNAP 25 and VAMP and the staining of the two proteins seemed comparable to the negative control as can be seen in **Figure 3.24 (a)** and **(b)**. At the same time all three fractions had no considerable effect on the viability of the cerebellar granular neuron after overnight incubation **(Figure 3.24 c).**

The effects of the TSV fractions were further investigated by immunofluorescence in cortical neurons as well as in cerebellar granular neurons. The data from both neuronal cultures suggests that fraction 1 and fraction 2 contain the active metalloproteinase that is able to enter cells and cleave the VAMP and SNAP 25 proteins as shown in **Figure 3.25** to **Figure 3.27.**

In both cell models the two active fractions Fraction 1 and Fraction 2 of TSV showed significance decrease in VAMP as well as SNAP 25 staining as compared to the negative control. At the same time fraction 3 had no effect on the staining of both SNAP 25 and VAMP. In fact the SNARE protein staining in the negative control and in cells incubated with fraction 3 was quite similar indicating absence of active metalloproteases in this fraction. The effects of active fractions on the SNARE proteins are more drastic as compared to whole venom even when the fractions are used at much less concentration than the whole

venom further emphasizing the specificity of these active metalloproteinases against the SNARE proteins without disrupting overall cellular morphology **Figure 3.24** to **Figure 3.27.**

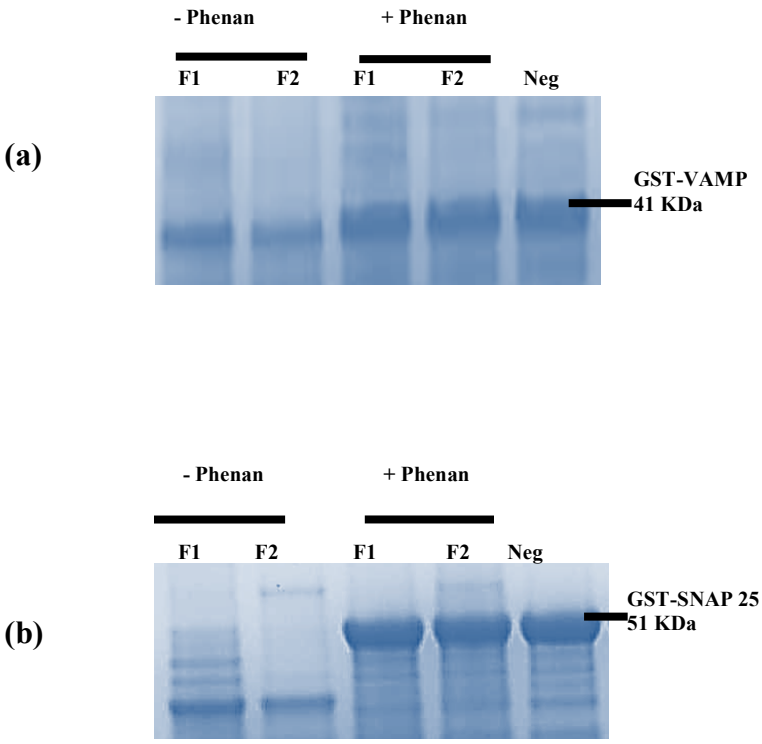


Figure 3.23: *In vitro* effects of different fractions of TSV on recombinant SNAREs. **(a)** and **(b)** In vitro effect of different fraction F1(I), F2(II) and F3(III) on GST tagged recombinant VAMP and SNAP 25. Each fraction is used at concentration of 0.5µg per 6µg of respective SNARE protein.

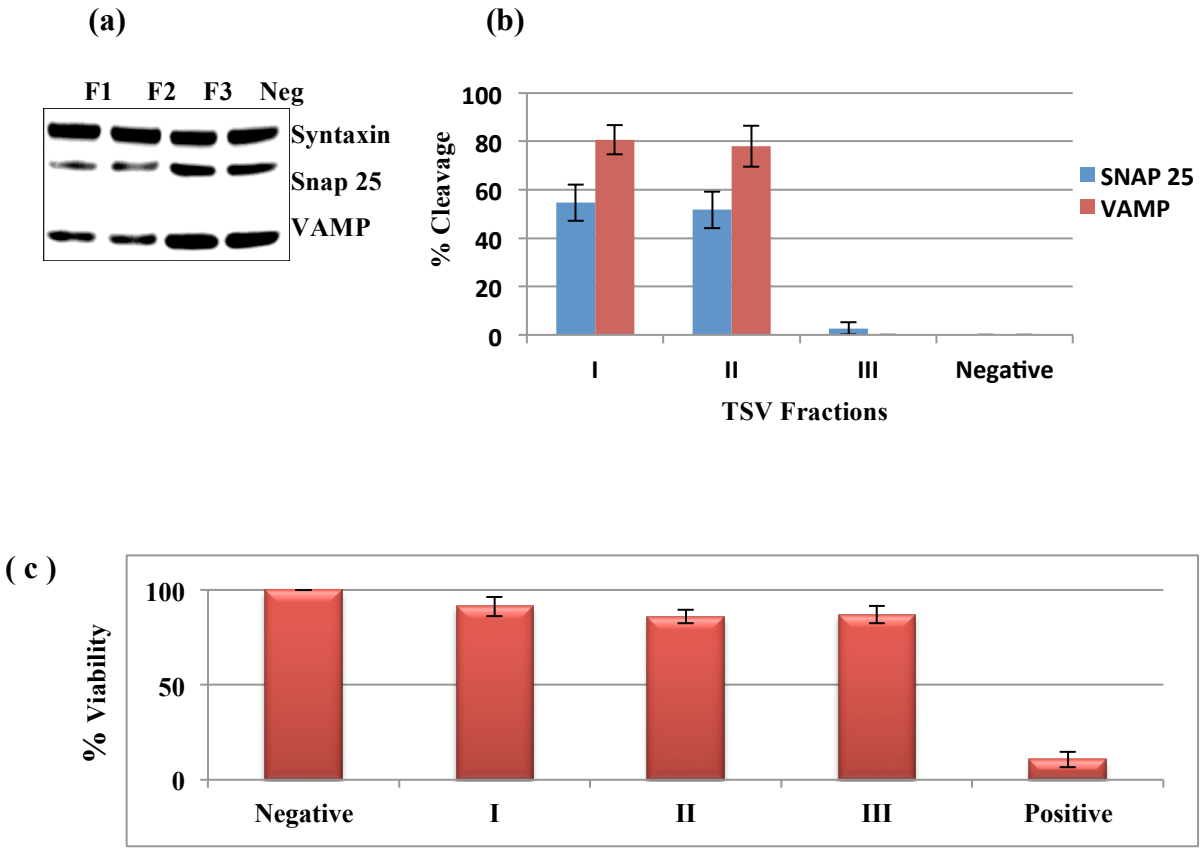


Figure 3.24: Effects of different fractions of TSV on CGNs. **(a)** Western blot showing the effect of TSV fractions 15 ug/ml (overnight) on SNARE proteins in CGNs as **(b)** quantified by western blotting. Percentage cleavage of SNAP 25 and VAMP represented as percentage against negative control. **(c)** Graph showing the effects of different fractions of TSV (15ug/ml) on viability of CGNS after overnight incubation.

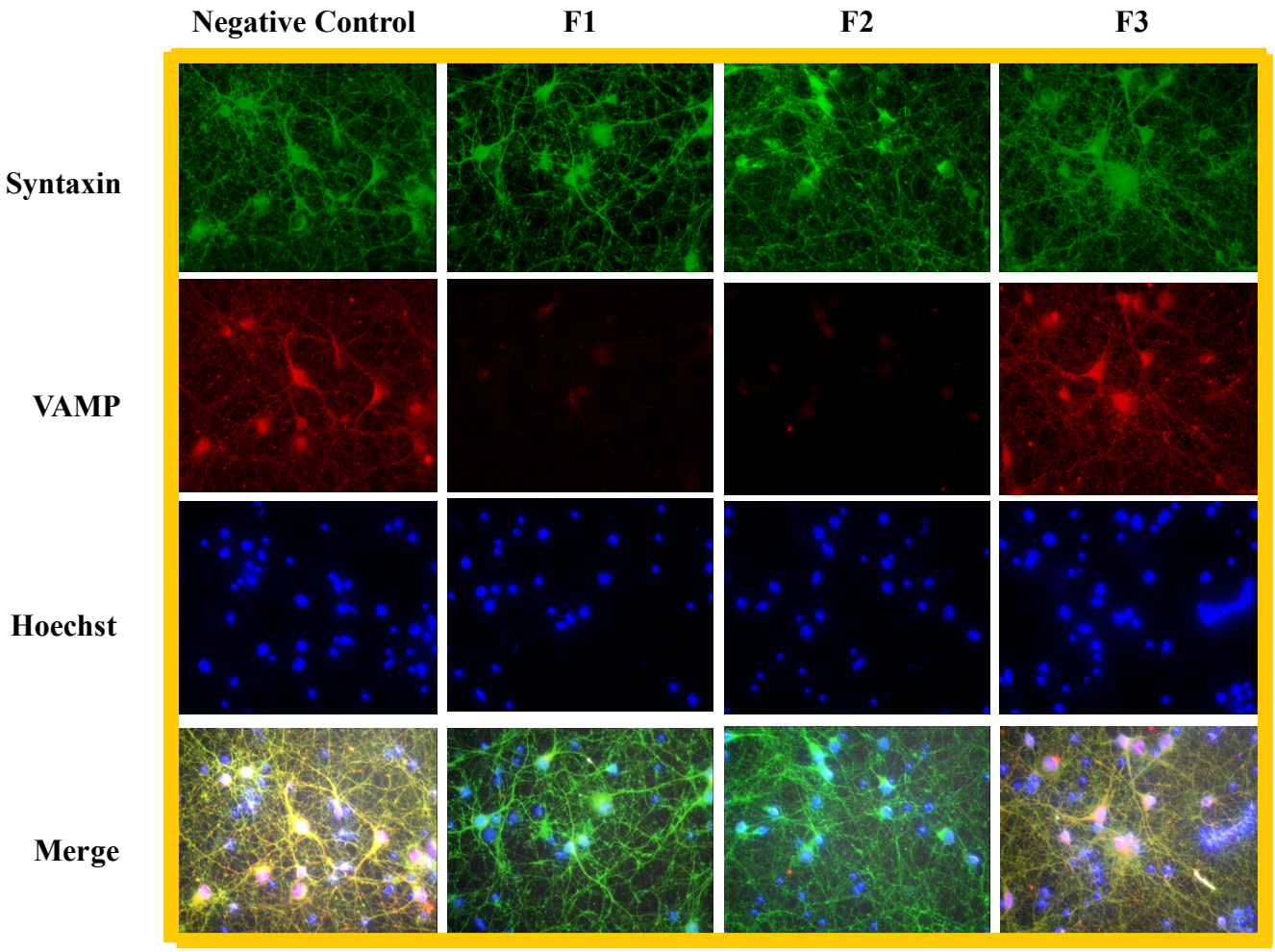


Figure 3.25: Effect of different fractions (fraction 1, fraction 2 and fraction 3 at concentration 15µg/ml) of TSV on VAMP in cortical neurons after overnight incubation. Syntaxin (green) was visualized with polyclonal antibody against VAMP (red) monoclonal antibody Whereas cell nuclei (blue) were stained with Hoechst.

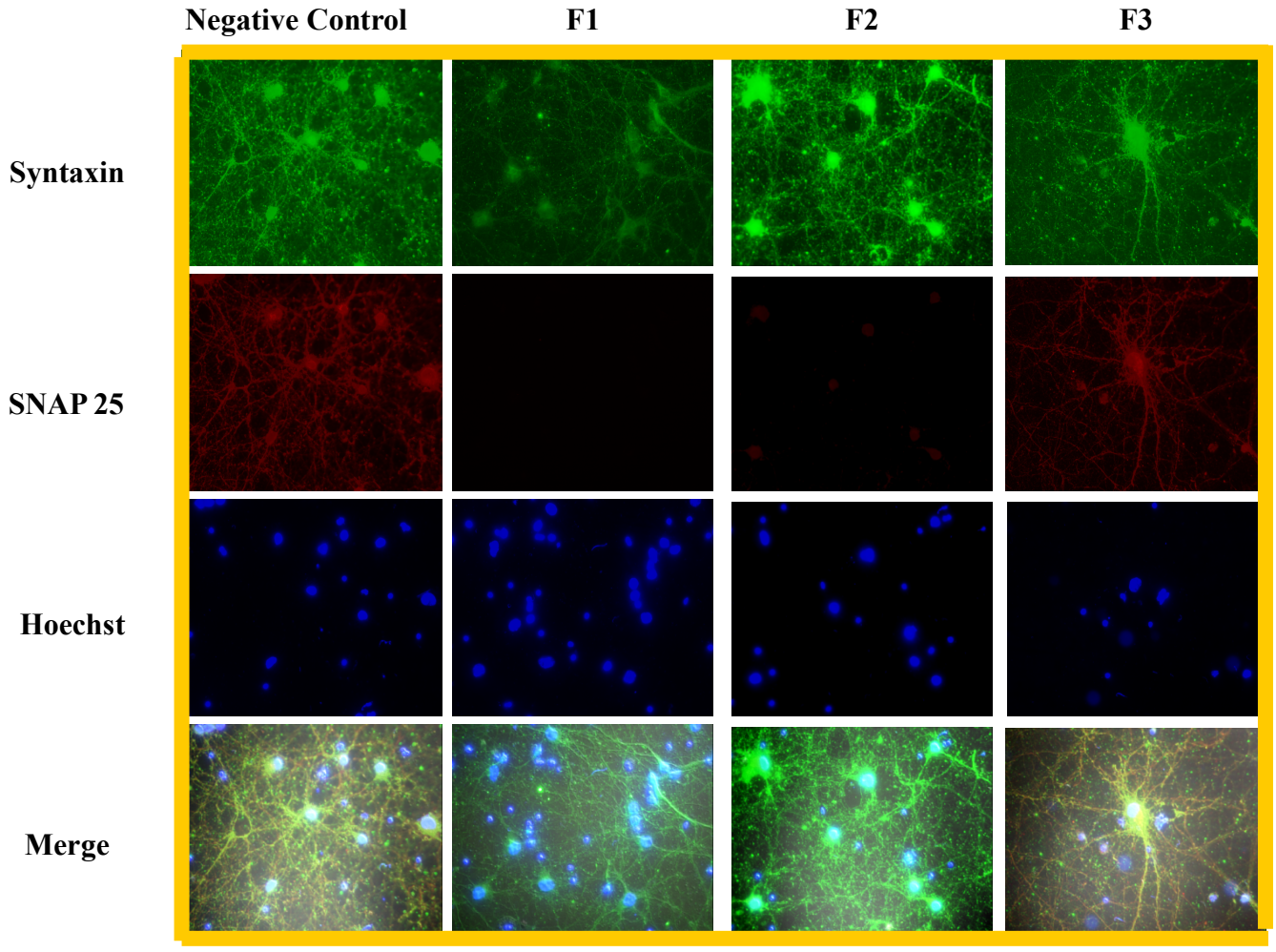


Figure 3.26: Effect of different fractions (fraction 1, fraction 2 and fraction 3 at concentration 15µg/ml) of TSV on SNAP 25 in cortical neurons after overnight incubation. Syntaxin (green) was visualized with monoclonal antibody against SNAP 25 (red) polyclonal antibody. Whereas cell nuclei (blue) were stained with Hoechst.

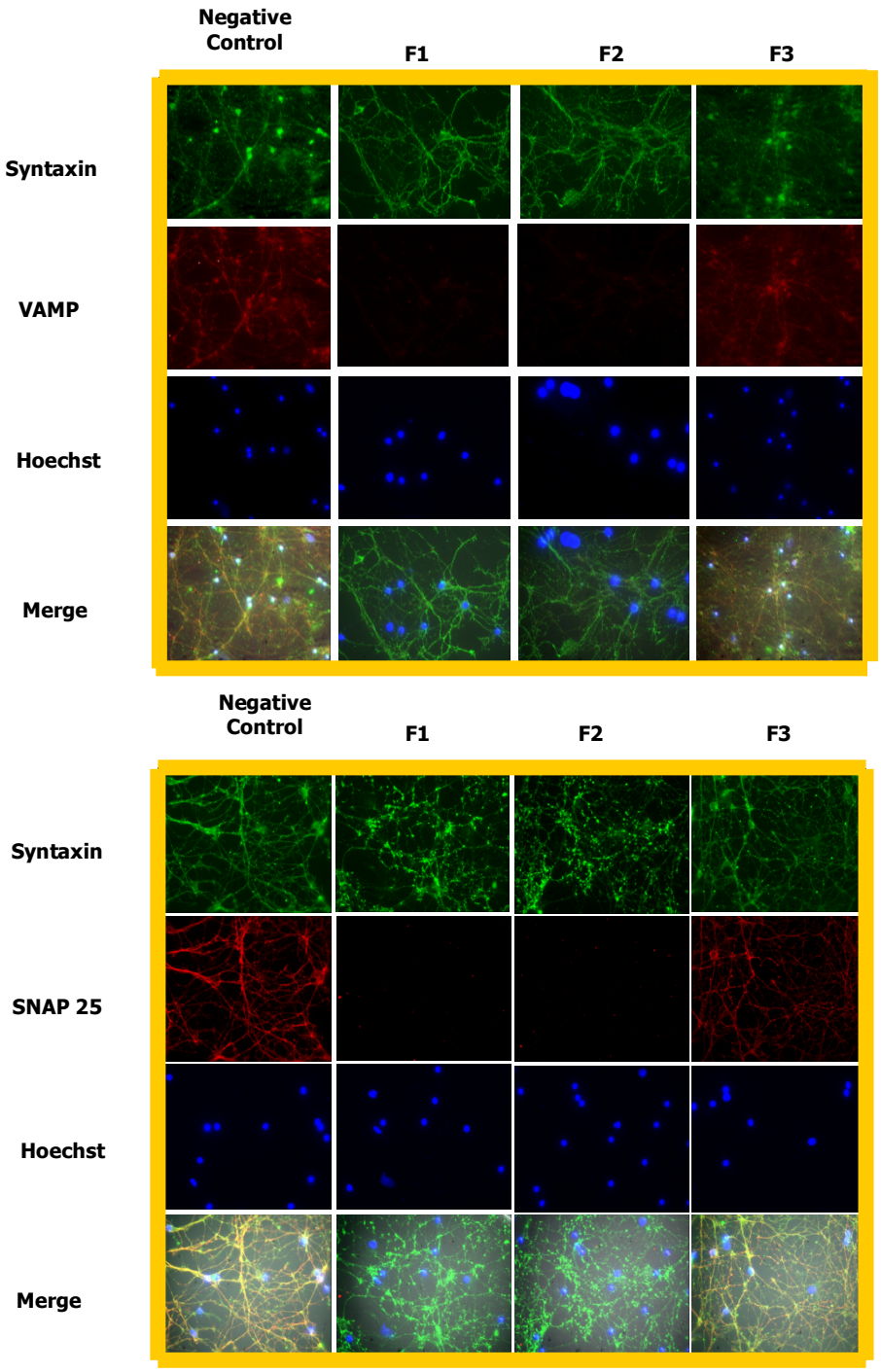


Figure 3.27: Effect of different fraction 1, fraction 2 and fraction 3 (15µg/ml) of TSV on the SNARE proteins (SNAP 25 and VAMP) in cerebellar granular neurons after overnight incubation In the upper panel syntaxin (green) was visualized with polyclonal antibody against VAMP (red) monoclonal antibody. In lower panel syntaxin (green) was visualized with monoclonal antibody against SNAP 25 (red) polyclonal antibody. Whereas cell nuclei (blue) were stained with Hoechst.

CHAPTER 4

DISCUSSION

CHAPTER 4

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4.1 Phylogeny and metalloproteinases

Scorpions are one of the oldest venomous living organisms with family Buthidae being the most largest and medically significant family of scorpions. During their 400 million years of evolution scorpion venoms have evolved and diversified to exert toxic effects on wide range of biological preys by evolving unique venom toxins. This toxin diversity can also be attributed to their long geological history and slow migration rates. The most appreciated scientific hypothesis on diversification and evolution of the venoms in venomous organism is generally based on variations among these organisms in their prey choices or in their predatory habits (Kordiš and Gubenšek 2000; Brust et al 2013; Sunagar et al., 2013). This fact has also been proven by a recent study conducted on Na^{+}-channel modulators in the scorpion species from different areas of Amazon Basin, which revealed the diversity and variations in toxin components among the species of same genus but occupying different ecogeographical habitats (Guerrero-Vargas et al., 2012).

Up till now antarease-like metalloproteinases have only been identified in the scorpions of the Buthidae family, among which these metalloproteases are ubiquitous to a broad range of scorpion species (Ortiz et al., 2013). Here I present evidence of the presence of such metalloprotease in the scorpions of Buthidae family as indicated by the sequence similarity analysis.

It can thus be hypothesized that all Buthidae must be possessing this class of metalloproteinases. In terms of feeding habits Buthidae spp. are characterized by comparatively smaller pincers as compared to other families of scorpions. But at the same time their venom is highly potent compensating for the need of larger pincers to be used for preying (Sunagar et al., 2013). It can be possible that the evolutionary driven acquisition of such metalloproteinases that contribute to paralysis by cleaving the SNARE proteins also

improve prey capture. In this study we analysed the venom of three different scorpions of Buthidae family that are spread in different geographical areas and found in all of them the presence of such kind of metalloproteolytic activity.

In general it would be interesting to study this class of unique enzymes in different families of scorpions which will also be helpful to track phylogeny among different groups of scorpions based on their geographical habitats and feeding habits.

4.2 Structure and function of antarease

From structure point of view antarease sequence possesses a highly conserved ADAM metalloprotease domain. Many of the proteins belonging to this category are known to have a zinc protease domain and a disintegrin domain. In general, among the ADAM metalloproteinase the ones secreted by arthropods contain a consensus hydrophobic sequence region (HExxHxxGxxHx) that coordinates zinc and contains a catalytic Glu and a distally located methionine (Rawlings and Barrett 1993). Because of these two conserved characteristics (Met and the longer zinc-binding motif), this metalloprotease family is also known as the metzincins. The sequence of antarease and other such venom metalloproteinases from scorpion species contain this type of conserved zinc binding motif (Ortiz et al., 2013). Consistent with this, the action of antarease was also shown to be inhibited by the metal chelating agent EDTA. In the present the effects of orthophenanthroline, another metal chelator, were studied *in vitro* on recombinant SNARE proteins incubated with different scorpion venoms. As expected the active components in the venom of different scorpions responsible for the cleavage of SNARE proteins could not retain their activity in the presence of metal chelating agent o-phenanthroline. This points out the fact that these metalloproteinases specific for SNARE proteins, need metal ions to be active. The exact mechanism of action of antarease or other antarease-like metalloproteinases is not known and further research needs to be carried out before conclusive results are obtained. Attempts are also in progress to determine the exact cleavage site in recombinant SNAP 25 after SNAP 25 has been cleaved under the action of three venoms (data not shown). The information on the site of cleavage of SNAP 25 (which has not yet been reported) can further highlight the mechanism of action of these metalloproteases.

4.3 Pathogenic mechanism of action of antarease-like metalloproteinase

Pathology of scorpion sting has been attributed to the major presence in scorpion venom of ion channel mediator activities (Ismail, 1995). These neurotoxins are modulators of voltage-gated sodium and potassium channels, chloride channels, and calcium channels and cause toxic effects on the central and peripheral nervous systems, the cardiovascular system and other detrimental effects on metabolism (Freire-Maia et al., 1994; Ismail 1995). At the same time, information on the mechanism of action of this newly reported class of scorpion venom metalloproteinases is lacking. Until now studies have been carried out on the effects of such enzymes on the pancreatic exocrine secretory discharge, which under normal condition is regulated by cholinergic as well as peptidergic control (Fletcher et al., 1992). Similarly, the acute pancreatitis, which follows scorpion stings has been linked to the presence of such metalloproteinases in the scorpion venoms (Possani et al., 1991). Due to its activity, antarease might be one of the components responsible for the onset of acute pancreatitis observed after *T. serrulatus* intoxication (Fletcher et al., 2010). If this proves to be the case, this protease should be considered as a therapeutic target for the generation of antivenoms able to neutralize the toxic effect of the whole venom, an effort that has until now focused on the ion-channel modifying toxins (Amaro et al., 2011). This study has highlighted the fact that apart from the direct effects of these venom metalloproteinases on the pancreatic systems these enzymes possess capability to impart direct effects on neuronal system. Furthermore, the present work has shown that the metalloprotease contained in the scorpion venoms tested is able to enter intact neuronal cells by a mechanism independent from synaptic vesicle recycling and to cleave VAMP2 and SNAP25 proteins without affecting cell vitality, though the mode of entry remains unknown. The antarease-like metalloproteases share some similarities with the clostridial neurotoxins in being zinc-endopeptidases that target the SNARE proteins. This fact renders them unique and important to study. However, they do not enter neurons in the same way the botulinum neurotoxins do as bafilomycin and increased synaptic vesicle recycling did not have any significant effect on the cleavage of SNAP 25 and VAMP in the cerebellar granular neurons.

As the preys of scorpions are insects and other invertebrates, the effect of antarease was also assessed in dissected third instar larva of *Drosophila melanogaster*. The results showed entry

of antarease into the dissected tissues of the larvae and partial cleavage of SNAP 25 after just two hours of incubation. This result has an important significance since insects are typical preys of scorpions and it is consistent with a role of toxins with SNARE-specific metalloproteolytic activity in predation and/or feeding of scorpions.

4.4 Expression, purification and activity of antarease

The study of venom components from venomous animals is a difficult and strong effort requiring in particular for the venomous arthropods for example scorpion or ticks, which release a very small volume of venom. This can further be aggravated by the amount of expression of the specific toxin under study. The biggest challenges in such a study requiring arthropod venom are to overcome the issue of supply of venom or such components under study. The amount of antarease to be estimated in one recent publication is almost 0.5% of the total venom components (Ortiz et al., 2013). With these problems it was ideal to clone and express antarease recombinantly and therefore I used this strategy on the basis of the antarease sequence reported in Fletcher et al., 2010. Unfortunately the recombinantly expressed antarease was not proteolytically active against SNARE protein. Recently Ortiz et al., 2013 reported the translation of the cDNA sequence obtained for *T. serrulatus* completing the originally reported partially sequenced antarease in which a region of 8 aminoacids (between Val 30 and Leu38) and Glu183 were missing. A possible explanation for the lack of activity of our recombinant antarease could be the crucial role of these residues in the catalysis.

After this attempt, I turned to the traditional approach of venom collection and fractionation in collaboration with prof. M.E. de Lima (Universidade Federal de Minas Gerais, Brazil). With the limited amount of venom provided, I attained a partial purification of antarease. Since fraction 1 and fraction 2 retained much better activity as compared to the whole venom when used at same concentration (data not shown) we used fractions at half of the concentration of TSV. The fractions contained active metalloproteinases that was active on recombinant SNARE as well as was effective against SNAREs in the neuronal cell models.

4.5 Conclusion

The present study has evidentiated the presence of a unique class of metalloproteinase which is recently identified, in different scorpion species of the genus *Buthidae*. The study of these metalloproteinases can provide many valuable informations. In particular it can help to overcome pathological condition such as pancreatitis resulting from the scorpion envenoming. The identification of such components in venom can not only help in better management but also can play important role in development of specific antivenoms. Furthermore these specific SNARE cleaving enzymes just like botulinum toxins can prove to be a useful tool for cell biology, biotechnological and medical applications. At the same time analysis of these unique proteases can provide deeper insights in to biology of scorpions in particular preying habits, geographical habitats and phylogeny of scorpion.

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