CHANGES IN THE EXPRESSION LEVELS OF SEMAPHORIN 3A (SEMA3A) IN AN *IN VIVO* MODEL OF ACUTE EXCITOTOXICITY

Dissertation submitted to
Department of Biotechnology

Cochin University of Science and Technology, Cochin

In partial fulfillment for the degree of

Master of Science in Biotechnology

By

ARCHANA R

Reg. No: 30318003

Department of Biotechnology,

Cochin University of Science and Technology, Cochin,
Kerala

Under the guidance of

Dr. R. V. Omkumar
Scientist F
Molecular Neurobiology Division
Rajiv Gandhi Centre for Biotechnology
Thiruvananthapuram

RGCB
DISCOVERIES FOR A BETTER TOMORROW

Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram 695014, Kerala State, India.
An Autonomous National Institute for Discovery, Innovation & Translation in Biotechnology and Disease Biology,
Government of India, Ministry of Science & Technology, Department of Biotechnology.

राजीव गाँधी जैव प्रौद्योगिकी केन्द्र, तिरुवनन्तपुरम 695 014, केरल, भारत.
जैवप्रौद्योगिकी और रोग जीवविज्ञान में आविष्कार, नवीनता एवं अनुवाद
की स्वायत्त राष्ट्रीय संस्थान,
भारत सरकार विज्ञान एवं प्रौद्योगिकी मंत्रालय, जैवप्रौद्योगिकी विभाग.

CERTIFICATE

This is to certify that the project titled **'Changes in the expression levels of semaphorin 3A (SEMA3A) in an *in vivo* model of acute excitotoxicity'** is a bonafide work done by **Ms. Archana R (Reg. No: 30318003)**, M.Sc. Biotechnology student, Department of Biotechnology, Cochin University of Science and Technology, who carried out the work under my supervision at Molecular Neurobiology Division, Rajiv Gandhi Centre for Biotechnology, Thycaud P.O., Thiruvananthapuram - 695014, Kerala, India.

Place: Thiruvananthapuram
Date: 22-05-2020

Dr. R. V. Omkumar
Scientist F
Molecular Neurobiology Division

तैक्काट पी.ओ., पूजप्पुरा, तिरुवनन्तपुरम - 695 014, केरल, भारत. फोण: 0471-2529400 (30 लाईन्स), 2347975, 2348104, 2348753, 2345899
फैक्स: 0471-2348096, ई-मेल: director@rgcb.res.in, info@rgcb.res.in, वेब: www.rgcb.res.in
Thycaud P.O., Poojappura, Thiruvananthapuram - 695 014, Kerala, India. Phone: 0471-2529400 (30 Lines), 2347975, 2348104, 2348753, 2345899
Fax: 0471-2348096, E-mail: director@rgcb.res.in, info@rgcb.res.in, Web: www.rgcb.res.in

DEPARTMENT OF BIOTECHNOLOGY

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

COCHIN - 682 022, INDIA Phone: 0484-2576267

E-mail: biotech@cusat.ac.in Fax: 91-484-2576267, 2577595

Ref. No. DBT

Date : 15.06.2020

CERTIFICATE

This is to certify that the research work presented in the project entitled "**Changes in the expression levels of semaphorin 3A (SEMA3A) in an *in vivo* model of acute exitotoxicity**" is an authentic record of work done by **Ms. Archana R. (Reg. No. 30318003)**, under the guidance of **Dr. R. V. Omkumar,** Scientist F, Molecular Neurobiology Division, Rajiv Gandhi Centre for Biotechnology, Trivandrum, in partial fulfilment of the requirements for the award of the Degree of Master of Science in Biotechnology from Cochin University of Science and Technology, Cochin-682022 and that no part of this work has been presented for the award of any other degree or diploma to any other Institute or University.

Dr. SARITA G. BHAT

Professor and Head

EXAMINERS:

Sl No	Name of Examiner	Signature
1	Dr. P M Sherief	
2	Dr. Ajith Vengellur	
3	Dr. Baby Chakrapani P S	
4	Dr. Mohanan Valiyaveettil	
5	Dr. Anusha Ashokan	
6	Dr.Deepa Narayanan	
7	Dr. Sreeja Narayanan	
8	Dr. Manjusha S	
9	Dr. Bindiya E. S.	

Department of Biotechnology

Cochin University of Science and Technology, Cochin

CERTIFICATE

This is to certify the research work presented in the project entitled "'**Changes in the expression levels of semaphorin 3A (SEMA3A) in an *in vivo* model of acute excitotoxicity**'" is an authentic record of work done by **Ms. Archana R** (Reg. no. **30318003)** under the guidance of, Dr. R.V. Omkumar, Scientist F, Molecular Neurobiology Division, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, in partial fulfilment of the requirements for the award of the degree of Master of Science in Microbiology from Cochin University of Science and Technology, Cochin, and that no part of this work has been presented for the award of any other degree or diploma to any other Institute or University.

Dr. Sarita G Bhat

Head of the Department

Examiner 1: **Examiner 2:**

Examiner 3: **Examiner 4:**

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my external guide, Dr. R. V. Omkumar (Principal investigator, Division of Neurobiology, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram) who gave me an opportunity to do my dissertation under his guidance. I would like to extend my gratefulness towards Ms. Reena Sarah Jacob (Ph.D. student) who was my co-guide, without whom I would never be able to navigate the facilities offered by RGCB. She has guided me through every step of this work. I would also like to mention Ms. Sowmya Gunasekaran (Ph.D. student) and Ms. Sruthi Sankari (JRF) who helped me a lot by ensuring a safe and friendly lab atmosphere and also by giving necessary guidance and support throughout my project work. I would like to thank the group of project trainees who were in the lab during my time there, Ms. Kavya K.G., Ms. Sukanya Dudeja and Ms. Anupriya and extend my gratitude towards all the staff members of my lab and RGCB who ensured a clean functional lab.

In addition, I would like to thank my HoD. Dr. Sarita G. Bhat who made sure that the students of the Department of Biotechnology worked hard to our maximum capacity to ensure a bright future for all of us. I would also like to thank Dr. Ajith Vengallur and Dr. Baby Chakrapani P. S. who were there with me along my journey. Both of them have been a constant support and care during my time in the university as well as during summer training and dissertation work. They both have played a major role in shaping the aspiring scientist in me. I am sure that the light they gave me will stay throughout my career in future. I owe a deep gratitude to all the faculty and non-teaching staff at the Department of Biotechnology, CUSAT and the administrative staff at CUSAT. Finally, I would like to express my profound gratitude to my classmates, friends and family - these amazing humans have helped me look into myself and grow to the person I am today.

I would like to thank my amazing husband Amarnath Harichandran, without whom I would never be able to do what I do on a daily basis. I would also like to thank my parents and in-laws who also helped me get to where I am today. Getting to know that my formal education is ending here makes my heart drop, but thanks to God Almighty, who took care of me all these years. Once again, I am thankful to have crossed paths with all these amazing individuals in this wonderful journey.

Archana R (30318003)

DECLARATION

I hereby declare that the work embodied in the dissertation entitled **'Changes in the expression levels of semaphorin 3A (SEMA3A) in an *in vivo* model of acute excitotoxicity'** submitted to Cochin University of Science and Technology, in partial fulfillment for the award of Master of Science in Biotechnology is a record of bonafide dissertation done by me under the supervision of Dr. R. V. Omkumar, Scientist F, Molecular Neurobiology Division, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram and that no part of this work has been submitted for the award of any degree or diploma to any candidate of any university.

Cochin

25.05.2020 Ms. Archana R (30318003)

DISCLAIMER

The data generated by Ms. Archana R, as a part of her M.Sc. dissertation entitled **'Changes in the expression levels of semaphorin 3A (SEMA3A) in an *in vivo* model of acute excitotoxicity'**, is proprietary to Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram. The principal investigator, Dr. R.V. Omkumar, Scientist F, Molecular Neurobiology Division, RGCB, reserves the rights to communicate this work in any form whatsoever.

Ms. Archana R (30318003)
25.05.2020

Dr. R.V. Omkumar,
Scientist F,
Molecular NeurobiologyDivision,
Rajiv Gandhi Centre for
Biotechnology,Thiruvananthapuram

vi

ABSTRACT

The nervous system is undoubtedly one of the most important and complex tissue systems that exist in the animal body. It is involved in controlling and coordinating the body at multitudinous levels. At the cellular level, the neurons along with neuroglia function in a choreographed manner to transmit information across synapses using neurotransmitters. Glutamate is one of the major excitatory neurotransmitters which is involved in synaptic plasticity and excitotoxicity. During excitotoxicity, over stimulation of the glutamate receptor, N-methyl-D-aspartate receptor (NMDAR) results in influx of lethal doses of Ca^{2+}. Semaphorins (SEMA) are a class of transmembrane proteins that are secreted upon processing and are involved in the development of nervous system and axonal guidance. SEMA and associated proteins function by inducing cell death and has been found to be associated with various neurodegenerative diseases in the adult brain (Quintremil *et al*., 2018).In this study, we have analyzed the changes in the expression level of SEMA3A in cortical tissue samples obtained from an *in vivo* acute excitotoxicity model, created by injecting saline and NMDA (40 µg/Kg body weight) via intracerebroventricular (ICV) route into the left and right lateral ventricles respectively (Kumar *et al*.,2019). No significant change in the expression ofSEMA3A protein was observed30 mins after NMDA treatment. This study indicates that the level ofSEMA3A may not be affected during an acute excitotoxic event.

Keywords: NMDAR, Excitotoxicity, SEMA3A

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMPAR	α-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid Receptor
AD	Alzheimer's disease
AP	Action potential
ATD	Amino or N-terminal domain
BBB	Blood brain barrier
BSA	Bovine serum albumin
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTD	C-terminal domain
ECL	Enhanced chemiluminescence
GABA	γ- Aminobutyric acid
Glu	Glutamate
GPCRs	G-protein coupled receptors
GPI	Glycosylphosphatidyl inositol
HD	Huntington's disease
ICV	Intracerebroventricular
IHC	Immunohistochemistry
iGluRs	Ionotropic glutamate receptors

LBD	Ligand or agonist binding domain
LTP	Long term potentiation
mGluRs	Metabotropic glutamate receptors
NMDA	N-Methyl-D-Aspartate
NMDAR	N-Methyl-D-Aspartate Receptor
NPN	Neuropilin
PD	Parkinson's disease
PKC	Protein kinase C
PNS	Peripheral nervous system
PSD	Post synaptic density
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
SAP	Synapse associated proteins
SDS- PAGE	Sodium Dodecyl sulphate-polyacrylamide gel electrophoresis.
SEMA	Semaphorin
TBI	Traumatic brain injury
TMD	Transmembrane or pore domain
VGCCs	Voltage gated calcium channels

CHAPTER: 01

INTRODUCTION

The nervous system plays an important role in the animal body by coordinating its behaviour and signal transmission across different body parts. In vertebrates, it consists of two parts, the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS consists of the brain and spinal cord which acts as the central processing and controlling unit. The PNS consists of nerves that are composed of long fibres that connect the CNS to every part of the body. It includes the peripheral ganglia, sympathetic and parasympathetic ganglia and the enteric nervous system.

The human brain controls all functions of the body, interprets information which is received through the sense organs and also embodies the essence of mind and soul, apart from being the seat of intelligence, consciousness, creativity, memory and sense of self. Protected within the skull and meninges, the brain is composed of the cerebrum, cerebellum and brain stem which extend through the foramen magnum into the spinal cord. The cerebrum can be further divided into two hemispheres and four lobes. The brain also consists of deep structures and the cerebrospinal fluid (CSF) filled ventricles that help in its buoyancy.

At the cellular level, the entire nervous system is composed of neurons and glial cells. Neuron or the nerve cell is a special type of cell, that allows electrochemical signal transmission rapidly and precisely between each other. They possess an axon, a protoplasmic protrusion which forms thin fibers that extend to distant parts of the body and make thousands of synaptic contacts which release chemicals, called neurotransmitters. These help in communication between nerve cells and also with other cell types to function in a coordinated manner. These innumerous connections in the system create complex patterns of dynamic activities. Along with neurons, there are glial cells in the system which gives the structural and metabolic support the neurons require, alongside playing a crucial role in forming the neuronal circuitry (Allen & Barres, 2009).

The neurotransmitters are essentially body's chemical messengers which help in communication between two neurons by changing the electrical impulse into a chemical response and transmitting it across the synapses. Their actions can be broadly classified into three categories: excitatory, inhibitory or modulatory.

There are six classes of neurotransmitters involved in carrying out these responses:

1. amino acid neurotransmitters like γ-amino-butyric acid (GABA) and glutamate
2. peptide neurotransmitters including oxytocin and endorphins
3. monoamines like epinephrine, nor epinephrine, histamine, dopamine and serotonin
4. purines like adenosine and adenosine triphosphate
5. gas transmitters like nitric oxide and carbon monoxide
6. acetylcholine

Together these chemicals help in defining who we are.

Memory is one of the most complex functions among the different brain processes. It includes three phases: encoding, storing and recalling. A sensory input increases the release of neurotransmitters and sets up a series of reactions in the hippocampus to store the long-term memory before transferring them to cortex. The transmitter used for long-term potentiation is the amino acid glutamate, which binds to receptors in the postsynaptic cell (Kandel, 2009). This allows calcium (Ca^{2+}) to flow in and activate the associated kinases. Working memory mostly functions with the action of dopamine associated cells.

Glutamate is one of the most abundant amino acid that functions in multiple metabolic pathways. It has a significant excitatory effect on the nerve cells; wherein in excess amounts, it can excite the cell to its death, which is referred to as 'excitotoxicity', caused by over activation of the glutamate receptors present on the cell surface (Zhou & Danbolt, 2014). Glutamate receptors are complex and are classified into ionotropic and metabotropic receptors. Ionotropic receptors are nonselective cation channels that allow the passage of Na^+ and K^+ ions and small amounts of Ca^{2+} ions.

The NMDA subfamily (N-methyl-D-aspartate receptors) of glutamate receptors are ionotropic receptors that display a rather interesting property of allowing the entry ofCa^{2+}in addition to other monovalent cations when activated by glutamate and glycine. They play an important role in the process of learning and memory. As the brain age, the receptors become hypofunctional leading to decrease in cognitive abilities (Kumar, 2015).

When neurons are exposed to high levels of glutamate, it causes persistent activation of NMDARs and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors(AMPARs), resulting in influx of lethal doses of Ca^{2+}.This results in the cell death mechanism known as

excitotoxicity which can be associated with several neurodegenerative conditions like Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), traumatic brain injury (TBI), stroke and seizures.

Excitotoxicity can be induced in a rat model by injecting NMDA into the brain. Previous experiments from the Molecular Neurobiology laboratory at Rajiv Gandhi Centre for Biotechnology (Kumar *et al*., 2019) have shown that injection of NMDA into the ventricles of the cerebral hemispheres causes biochemical changes associated with cell death indicating excitotoxicity. Tissue samples from this acute model of excitotoxicity were further analyzed for the expression levels of semaphorins and associated proteins. Semaphorins are a class of secreted proteins which are involved in extracellular signaling. They play a significant role in the development of many organs and tissues.

CHAPTER: 02

REVIEW OF LITERATURE

Neuroscience research can be considered to be an umbrella term, as the area that encompasses diverse fields including neuroanatomy and physiology to molecular genetics to neuroinformatics and neuropsychology. The common factor between all these diverse fields is the unstoppable urge of human race to conquer and understand the complexities of human nervous system that continues to marvel generations of scientists. As enigmatic and awe inspiring it is to the scientific community, researchers are trying to find a way to tap into the unlimited potential one's brain can possess and to expand their horizons.

1. THE HUMAN NERVOUS SYSTEM

The nervous system is essentially a complex network of cells that run through the body, which regulates and coordinates multiple body activities. The specialised cells of the nervous system known as nerve cell or neurons use electrical and chemical signal to communicate between each other. By doing so, they transmit signals between different parts of the body. Structurally, the nervous system has two components:

- Central nervous system (CNS) that comprises of the brain and spinal cord and
- Peripheral nervous system (PNS) that consists of the neural elements

In addition to the brain and spinal cord, the nervous system also comprises of the sensory organs (eyes, ears, nose and tongue) and sensory receptors (skin, gut, muscles) located in various regions of the body. They are part of the PNS which gathers information and relays it to the CNS.

• Central Nervous System (CNS)

The CNS is comprised of the brain and spinal cord. The brain is one of the largest and most complex organs in the human body which is made up of 100 billion neurons that communicate in trillions of connections across different parts, which makes us humans. The brain is divided into forebrain, midbrain and hind brain. The brain is not just involved in controlling and

coordinating intelligence, emotions, sense of self and consciousness and motor coordination, but also plays an equal role in regulating various body functions by working hand in hand with the endocrine system. The bony skull encapsulates the brain and the vertebral column protects the spinal cord. In addition to this, a three-layered protection is ensured by the meninges and also bathed in the cerebrospinal fluid (CSF) which helps in the buoyancy of the brain. Blood vessels that enter the CNS are surrounded by a blood-brain barrier (BBB), which protects the cells in the CNS from any potential toxic chemicals.

- **Peripheral Nervous System (PNS)**

The nervous tissue that lies outside the CNS forms the PNS.A majority of these cells have their cell bodies in the brain or spinal cord, while their axons projects out of the CNS. They connect to the brain and spinal cord to ensure coordinated functioning of the body. The PNS can be further divided into two:

- the autonomic nervous system which comprise of the sympathetic and parasympathetic nervous systems and
- the somatic nervous system

The vertebrate nervous system can also be divided into two areas based on anatomy. The grey matter (pink or light brown colour in living tissue) which contains the cell bodies that are not myelinated and the white matter which is composed of the myelin coated axons. The grey matter can be found as clusters in brain and spinal cord. The cluster of cell bodies outside the brain is known as ‘nucleus’ or ‘ganglion’ based on its location. The white matter consists of body’s nerves and the interior of the brain.

2. CELLS OF THE NERVOUS SYSTEM

Like any other tissue, the tissues of the nervous system are also made of the basic building block, cell. The nervous system has specialised cells, called neurons, which can send and receive electrical and chemical impulses across different body parts. Networks formed by these interconnected groups of cells are capable of a wide variety of functions. Some of them are feature detection, pattern generation and timing (Dayan & Abbott, 2005). There is another set of specialised cells known as glial cells that nourish the neurons.

- **Neurons**

Neurons can be distinguished from other cells as they are anatomically distinct cells. The most fundamental difference between neurons and other cells is that they communicate between themselves via synapses, which are the junctions at the nerve endings that contain the molecular machinery to allow rapid transmission of chemical or electrical signals. Structurally, they are very distinct and most of the neurons possess a cell body, dendrons and axon. Axons from the cells of CNS extend outside the spinal cord forming the part of PNS. There are three different types of neurons, classified based on their function:

- Sensory neuron,
- Motor neuron and
- Interneuron

These are distributed in extensive networks throughout the body and through their collective work; the body is able to make decisions in a matter of seconds.

- **Glial cells**

The glial cells are non-neuronal cells, present in the nervous system, that provide support and nutrition, maintain homeostasis, forms myelin that insulate neurons electrically, destroys any pathogens and removes dead neurons. Studies have revealed that they can also participate in signal transmission in the nervous system (Allen & Barres, 2009) and provide guidance cues directing the axons to their targets. There are different types of glial cells according to their structure and specific function:

- astrocytes,
- oligodendrocytes,
- ependymal cells,
- Schwann cells,
- microglia

These cells are equally important as the nerve cell. Although the proportions vary in different brain regions, the total number of glial cells is roughly equal to the number of neurons (Azevedo *et al*., 2009).

- **Neurons and synapses**

Neurons communicate with each other by sending signals via axons. The propagation of signals across the axon is carried out by a mechanism known as action potential (AP). Upon reaching the nerve endings, cell-to-cell signals are relayed, through point of contacts called synapses, with the help of neurotransmitters. Synapses can be electrical or chemical. Electrical synapses are faster and they pass ions directly between neurons (Hormuzdi *et al*., 2004). Chemical synapses are more common and diverse in function. At a chemical synapse, the neuron which sends the signal is the pre-synaptic neuron, the one which receives the signal is the post-synaptic neuron and the space between them is called the synaptic cleft.

The nerve terminals of the pre-synaptic neuron contain spherical vesicles encapsulating neurotransmitters that aid to convey signals. When calcium enters the pre-synaptic terminal through voltage-gated calcium channels as a result of an incoming stimulation, an array of calcium-sensitive proteins embedded in the vesicle membrane is activated and causes the movement of vesicles to the surface of the membrane and its subsequent rupture results in the release of neurotransmitters into the narrow synaptic cleft. These chemical messengers bind to specific receptors present on the post-synaptic membrane resulting in its activation. Depending on the type of receptor, the effect on the postsynaptic cell may be excitatory, inhibitory, or modulatory in more complex ways. According to Dale's principle, a neuron releases the same neurotransmitter at all of its synapses (Strata & Harvey, 1999). So, the same neurotransmitter is able to elicit different responses depending on the type of receptor it activates. There are more than a hundred neurotransmitters known and most synapses use more than one neurotransmitter to function efficiently.

The post-synaptic receptors can be generally divided into two broad categories: ligand-gated ion channels and G-protein coupled receptors (GPCRs). A ligand-gated ion channel when activated opens a channel that allows the flow of ions across the membrane. A GPCR, on the other hand, rely on second messenger signaling. When it is activated, a cascade of molecular interactions is triggered in the target cell that produces a wide variety of complex effects.

3. NEUROTRANSMITTERS

When an AP arrives at the synapses via the axon of the pre-synaptic neuron, chemical

messenger molecules called neurotransmitters are released from the axon terminal, which bind to receptors present on the membrane of the post-synaptic cell (Kandel *et al.*, 2000). By using a fast acting small-molecule neurotransmitter such as glutamate or GABA along with a peptide neurotransmitter which is slower-acting helps in achieving higher efficiency (Greengard, 2001). Most of the neurotransmitters are small amines, amino acids or neuro-peptides that can be classified into following six classes:

1. Amino acid neurotransmitters like γ-amino-butyric acid (GABA) and glutamate
2. Peptide neurotransmitters including oxytocin and endorphins
3. Monoamines like epinephrine, nor epinephrine, histamine, dopamine and serotonin
4. Purines like adenosine and adenosine triphosphate
5. Gasotransmitters like nitric oxide and carbon monoxide
6. Acetylcholine

Two of the major neurotransmitters are glutamate and GABA. While glutamate has several widely occurring excitatory or modulatory receptors, GABA has inhibitory receptors. There are few exceptions to this when GABA exhibits excitatory effects during early development (Marty & Llano, 2005). The glutamatergic neurons are commonly termed as 'excitatory' neurons and GABAergic neurons as 'inhibitory' neurons.

4. GLUTAMATE

The amino acid glutamate (Glu) is the anion of glutamic acid which plays a central role in both the normal and abnormal functioning of the CNS. Glutamate is the main excitatory neurotransmitter in the CNS. At the same time, it is also an excitotoxin that can destroy neurons by excessive activation of excitatory receptors on cell surfaces (Newcomer *et al.*, 2000). Glutamate is also the metabolic precursor of GABA. Being one of the abundant neurotransmitters, glutamate plays a key role in the neural circuitry involved in synaptic plasticity, which helps the brain to accommodate new information and form memories without actually growing new neurons. They are the central elements in memory formation and retrieval as they have a key role in the cellular mechanism of memory and learning called long-term potentiation (LTP) (Mayadevi *et al*., 2012). Glutamate signalling plays an important role

in cognitive functions and is widely expressed throughout the CNS – both in neurons and glial cells.

Glutamate is stored in vesicles in the pre-synaptic neuron until a nerve impulse triggers its release. Post-synaptic neurons with glutamate receptors respond by initiating a receptor-mediated signalling process (Zhou & Danbolt, 2014). There are two general types of glutamate receptors – ionotropic glutamate receptors (iGluRs), that allows the entry of ions, and G-protein coupled receptors or metabotropic glutamate receptors (mGluRs). The mGluRs modulate synaptic transmission by regulating the activity of a wide variety of ion channels including iGluRs as well as receptors of other neurotransmitters (Gerhard, 2013; Meldrum, 2000).

5. IONOTROPIC GLUTAMATE RECEPTORS (iGluRs)

Ionotropic receptors are membrane-bound receptor proteins that respond to ligand binding by opening an ion channel to allow influx of ions. They contain two functional domains: an extracellular domain that can bind the neurotransmitter and a membrane spanning domain that forms the ion channel. The neurotransmitter binds to the receptor that causes a conformational change which is passed along to the closely associated ion channel, resulting in passage of ions through the channel. There are three classes of iGluRs:

- α-amino-3-hydroxyl-5-methyl-isoxazole propionate receptors(AMPARs)
- kainate receptors and
- N-methyl-D-aspartate receptors (NMDARs)

• N-Methyl-D-Aspartate Receptors (NMDARs)

NMDARs are the major ion channel receptors involved in excitatory synaptic transmission, synaptic plasticity, development of neurons and excitotoxicity in the nervous system. Apart from glutamate, these receptors also have a specific chemical agonist, N-methyl-D-aspartate (NMDA), which gives the receptor its name. They are mostly located at post-synaptic sites and some are located on the pre-synaptic site as well (Loftis & Janowsky, 2003).

NMDAR is a both ligand-dependent (responds to binding of the agonist glutamate and co-agonist glycine (D-serine) to its subunits) and voltage-dependent ion channel (responds to changes in membrane potential). It is selectively permeable to monovalent cations like Na^+ and divalent Ca^{2+}ions (Vyklicky *et al*., 2014). The voltage-dependent activation of NMDARs is due to the presence of a Mg^{2+} ion block at the opening of the channel. Glutamate binding to neurons stimulates two processes simultaneously, resulting in the receptor to detect synaptic coincidence (simultaneous activation of pre-synaptic and post-synaptic neuron) and to have associative properties (able to associate two events together at the synaptic level) (Wigström & Gustafsson, 1985; Cooke & Bliss, 2006).

The receptor possesses unusually slow activation and deactivation kinetics (Cull-Candy & Leszkiewicz, 2004). The NMDARs are unique channels as their opening requires two events viz., pre-synaptic glutamate release and strong membrane depolarization resulting in removal of the Mg^{2+} block (Mayer *et al*., 1984). Phosphorylation enhances NMDA receptor function. NMDA receptors can be phosphorylated by serine/threonine kinases such as protein kinase C (PKC), protein kinase A (PKA) and calcium/calmodulin-dependent protein kinase II (CaMKII), as well as by tyrosine kinases (Salter *et al*., 2009).

- **NMDAR subunits**

The NMDA receptor is composed of three subunits–GluN1, GluN2 and GluN3 (formerly known as NR1, NR2 and NR3) – which is further subdivided into seven subtypes. There is one GluN1 subunit, four subunit types of GluN2 (A-D) and two subunits of GluN3 (A and B) (Paoletti, 2011). GluN1 and GluN3 subunits contain the glycine binding site and GluN2 contains the glutamate binding site. NMDARs assemble as a heterotetramer. They have two obligatory GluN1 subunits and either two GluN2 (GluN2A–GluN2D) and/or GluN3 (GluN3A–GluN3B) subunits which results in a heterogeneous population of NMDARs that exhibit diverse pharmacological properties, kinetics, and downstream signaling pathways (Cull-Candy *et al*., 2001; Köhr, 2006; Paoletti *et al*., 2013; Paoletti & Neyton, 2007; Sanz-Clemente *et al*., 2013). Most of the NMDARs in the CNS exist as diheteromers of GluN1 / GluN2A or GluN1 / GluN2B or as a triheteromer of GluN1 / GluN2A /GluN2B (Szydlowska & Traynelis, 2010).

The four subunit subtypes of GluN2 (A-D) arise from four distinct genes and subunit

expression exhibit developmental variability. GluN2B and GluN2D are expressed mainly in prenatal stages and decrease postnatally, during which the expression of GluN2A and GluN2C increases (Monyer *et al*., 1994). In the adult brain, GluN2A is expressed throughout the brain, while GluN2B is limited to forebrain, GluN2C to cerebellum and GluN2D is expressed in only some selected regions of the brain (Monyer *et al*., 1992). The partition of the subunits is not absolute; GluN2B can be found at synaptic sites (Harris &Pettit, 2007; Liu *et al*., 2004; Petralia *et al*., 2010; Thomas *et al*., 2006). GluN2A at synaptic and GluN2B at extrasynaptic regions (Groc *et al*., 2006; Martel *et al*., 2009; Sanz-Clemente *et al*., 2013; Tovar & Westbrook, 1999) is the most widely observed pattern of distribution. Simultaneously, the association of GluN2A-type NMDARs with cell survivaland GluN2B-type NMDARs with cell death signaling is also being studied (Lai *et al*., 2011).

The GluN1, GluN2A and GluN2B combination is the major receptor subtype found in mature neurons of the forebrain (Luo *et al*., 1999), Various membrane associated proteins like synapse associated proteins (SAP) and post synaptic density (PSD) proteins anchor to NMDARs at the synaptic region which helps in downstream signaling (Kim *et al*., 2005). Splicing of the GluN1 subunit transcript yields eight functional splice forms. The splice variants arise by addition and deletion of three exons in the N-terminal (N1) and C-terminal (C1, C2) domain of the subunit (Hollmann *et al*., 1993; Loftis & Janowsky, 2003). Each splice variant of GluN1 affects NMDAR channel properties. The presence of exon5 splice variant at the amino terminal end interferes with the pH sensitivity of NMDARs. At normal pH, receptors with GluN1 splice variant containing exon 5 are highly active while its absence can block its activation (Traynelis *et al*., 1995). GluN2 and GluN3 subunits are transcribed from six genes. GluN2 subunit is responsible for impulse conduction through channel, sensitization to Zn^{2+} ions and polyamines and in the activation/deactivation of the channel (Cull-Candy *et al*., 2001).

Although both GluN2A-NMDARs and GluN2B-NMDARs share some common binding partners such as postsynaptic density protein95 (PSD95) (Sheng & Kim, 2011), they bind differentially to some other proteins. NMDAR is phosphorylated at the Ser^{1303} position of GluN2B by CaMKII (Omkumar *et al*., 1996). NMDAR opening results in CaMKII activation, which translocates to PSD and binds to GluN2B subunit of NMDAR. CaMKII binds to the C- terminal end of GluN2B subunit with high affinity (Gardoni *et al*., 1998).

- **Structure of NMDAR**

NMDA receptors are heterotetramers composed of two obligate GluN1 and typically two GluN2 subunits (Salussolia *et al*., 2011). The structure of NMDAR has four different domains namely amino or N-terminal domain (ATD), ligand or agonist binding domain (LBD), transmembrane or pore domain (TMD) and C-terminal domain (CTD). The ATD end controls the channel opening, the LBD binds to agonists and contributes to control of ion channel open probability, deactivation speed and contains binding sites for subtype-specific allosteric modulator compounds, the TMD forms the heterotetrameric ion channel having voltage sensing capacity and the CTD interacts with PSD proteins and facilitates in neuroplasticity (Karakas & Furukama, 2014).

- **Role of NMDAR in Nervous system**

NMDA receptor channel acts as an associative switch for the induction of LTP - turning on when postsynaptic depolarization is paired temporally with the synaptic release of glutamate. Studies also suggest that the receptor plays a key role in divert forms of synaptic plasticity including LTD (Bliss & Collingridge, 1993; Malenka & Nicoll, 1993). It is also involved in experience-dependent synaptic plasticity during development (McDonald & Johnston, 1990; Mori & Mishina, 1995).

Apart from the role of the receptor in synaptic plasticity, it is also involved in neuronal cell death. Excess glutamate release can cause receptor hyperactivation leading to high concentration of Ca^{2+}ions in the cytosol, resulting in neuronal death. This condition is called excitotoxicity and has been implicated in neurodegenerative disorders like seizures, ischemic stroke, AD, HD and PD (Fan & Raymond, 2007). On the other hand, as the brain ages, the NMDA receptor can progressively become hypofunctional as well and this has been associated with memory and learning impairments, psychosis and ultimately with neurotoxic brain injury (Newcomer *et al*., 2000).

6. EXCITOTOXICITY

Excitotoxicity is a term coined by John Olney (Olney, 1969). It is the condition in which the level of glutamate increases with subsequent over excitation of glutamate receptors, majorly the NMDARs, leading to neuronal death. Excitatory amino acids play a crucial role in mediating pathological neuronal injuries (Choi, 1988). Excess glutamate binds to over activate iGluRs and mGluRs resulting in increase in cytosolic free Ca^{2+}. The increase in Ca^{2+} inflow as a result of this over activation can trigger the cells to undergo apoptosis (Choi, 1987). This condition is considered to be a major cause of cell death in neurodegeneration diseases (Dong *et al*., 2009). The requirement of Ca^{2+} is described by calcium hypothesis of neurotoxicity which states that neuronal Ca^{2+} overload leads to subsequent neuronal damage (Sattler *et al*., 1998).

The route of Ca^{2+} entry through NMDARs, but not voltage-gated calcium channels (VGCCs), is the main determinant of excitotoxicity (Szydlowska & Tymianski, 2010). Lethal Ca^{2+} signals are also determined by the molecules that interact with NMDARs such as PSD95. Another event that contributes to excitotoxicity is high glutamate release and fewer uptakes in the synaptic cleft, which results in excessive activation of receptors (Obrenovitch *et al*., 2000).

Neurotoxicity is mainly mediated by GluN1 and GluN2B containing NMDA receptors in primary cortical neurons. The cells are highly sensitive to glutamate treatment from DIV8 (days *in vitro*) to DIV11, since they expressGluN1 and GluN2B subunits but not GluN2A subunit (Mizuta *et al*., 1998). Antagonists specific to GluN2B subunit like ifenprodil perturbs induction of excitotoxicity in cultured cells (Chenard & Menniti, 1999). Few promising results have been reported for uncompetitive NMDAR antagonists in the treatment of several forms of dementia (Olivares *et al*., 2012).

The toxic change induced by glutamate has two components - (a) swelling of neurons and (b) slowly evolving neurodegeneration (Choi, 1988). Intracellular Ca^{2+} overload also activates neuronal nitric oxide (NO) synthase, resulting in excess production of NO and formation of reactive oxygen species (ROS) and lipid peroxidation of membrane phospholipids (Görlach *et al*., 2015; Yermolaieva *et al*., 2000).

Neuronal cell death follows either necrotic or apoptotic pathway during excitotoxicity. Apoptosis induced by glutamate, even at a small concentration, can lead to necrosis (Cheng

et al., 2008). Cell death is induced by the death-associated receptors present on the cell surface, as well as by mitochondrial pathways (Thornberry & Lazebnik, 1998). Deregulation and loss of mitochondrial membrane potential is another event that occurs during excitotoxicity. Impaired intracellular Ca^{2+} homeostasis leads to the activation of cytoplasmic cysteine proteases like calpains. Calpain cleaves cytosolic enzymes involved in homeostatic maintenance and proapoptotic proteins to initiate cell death (Szydlowska & Tymianski, 2010).

Excitotoxity is the major underlying cause of several neurodegenerative disorders like epilepsy, HD, AD, ischemia, TBI and stroke (Lynch & Guttmann, 2002). Glutamate neurotoxicity may participate in pathogenesis of four types of acute injury in the nervous system leading to prolonged seizures, compromised blood supply, glucose deprivation and mechanical trauma or traumatic brain injury (Choi, 1988; Parsons& Raymond, 2014).

The NMDA receptor is one of the most important and widely studied topics. Researchers are striving to develop clinical NMDA receptor antagonists to block the process of excitotoxity without interfering with normal functions of signal transmission and synaptic plasticity.

7. SEMAPHORINS

Semaphorins (SEMA) are a large family of transmembrane, or glycosyl phosphatidyl inositol (GPI)-anchored proteins that are secreted upon processing and bind to plexin/neuropilin or integrin receptors, providing axonal guidance cues towards a proper target (Terman, 2017), thereby aiding in the development of functional neuronal networks (Hu& Zhu, 2018). Soon after their discovery, their role in CNS was unveiled. Emerging evidence suggests that beyond their role in guidance, they also function in a broad spectrum of pathophysiological conditions, including various neurodegenerative diseases apart from various signaling mechanisms. Semaphorin family members can induce death in different types of cells including neurons and one of the best characterized cases is SEMA3A-induced death of embryonic sensory neurons that mimic functions similar to apoptosis (Ben-Zvi *et al*., 2008).

- **Structure and functions:**

The major defining feature of the SEMA protein family is a conserved sema domain, which is an approximately a 500 amino acid extracellular domain (Kolodkin *et al*., 1993). The overall protein organization within the family is quite different, apart from this conserved sema domain (Kolodkin *et al*., 1993). There are more than 20 members in this family, with diverse structures, which are either secreted or transmembrane or cell surfaceattached proteins. They are characterized by a single cysteine rich extracellular sema domain (Alto & Terman, 2017), also known as the plexin-semaphorin-integrin (PSI) domain, because of its homologous nature to the beta chain of integrins and its presence in plexin family members (Siebold & Jones, 2013). The sema domain exists as a single copy at the N-terminus of the SEMA proteins and is essential for signalling. It is also found in the plexin family of proteins and in several receptor tyrosine kinases (Siebold & Jones, 2013). These proteins are also included in the SEMA super family (Siebold & Jones, 2013). Semaphorin structure has revealed that the sema domain undergoes homophilic dimerization and this dimerization is important for its functioning (Klostermann *et al*., 1998; Koppel & Raper, 1998).

Semaphorins are majorly grouped into eight major classes based on phylogenetic tree analysis and their structure (Bamberg *et al.,* 1999). The first seven are ordered by number and the eighth group is class V for virus. Classes 1 and 2 are found in invertebrates and classes 3, 4, 5, 6 and 7 are found in vertebrates. Members belonging to classes 1, 4, 5, and 6 are transmembrane proteins, classes 2, 3 and V members are secreted proteins, and class 7 members are GPI-linked (Alto & Terman, 2017).

- Class 1- SEMA1A, SEMA1B
- Class 2-SEMA2A
- Class 3- SEMA3A, SEMA3B, SEMA3C, SEMA3D, SEMA3E, SEMA3F
- Class 4- SEMA4A, SEMA4B, SEMA4C, SEMA4D, SEMA4E, SEMA4F, SEMA4G
- Class 5- SEMA5A, SEMA5B
- Class 6- SEMA6A, SEMA6B, SEMA6C
- Class 7- SEMA7A
- Class V- SEMAVA, SEMAVB

In humans a variety of genes code for semaphorins. These include:

- *SEMA3A, SEMA3B, SEMA3C, SEMA3D, SEMA3E, SEMA3F, SEMA3G*
- *SEMA4A, SEMA4B, SEMA4C ("SEMAF"), SEMA4D, SEMA4F, SEMA4G*
- *SEMA5A, SEMA5B*
- *SEMA6A, SEMA6B, SEMA6C, SEMA6D*
- *SEMA7A*

The major function of SEMA protein is during embryonic development, where they are essential for the maintenance of many organs and tissues (Alto & Terman, 2017). They are also expressed in the adult brain. Recent studies have shown that they have an active role in refining synaptogenesis, dendritic and axonal exuberance, remodelling of the synaptic network and may also play a role in neuronal response to ROS during neuronal apoptosis (Pasterkamp & Giger, 2009; Okuno *et al*., 2011; Mecollari *et al*., 2014; Van Battum *et al*., 2015; Goshima *et al*., 2016).They function as a key regulator of morphology and motility in tissue systems like cardiovascular, immune, endocrine, hepatic, renal, reproductive, respiratory and musculoskeletal systems and sometimes can be found associated with cancer cells too (Alto & Terman, 2017).

Even though different protein families are known to directly bind to SEMA proteins and function as receptors, most of the signalling is mediated through plexin receptors. Members from all classes of SEMA proteins have been found to interact with plexin receptors (Perälä *et al*., 2012).

Neuropilin (NPN) is also another class of well characterized SEMA receptor that is best known for its association with class 3 SEMA proteins. NPN receptors containshort intracellular domains that work with various signal transduction receptors, including plexins and cell adhesion molecules. SEMA also binds to other receptors including CD72, TIM-2, integrins and proteoglycans (Alto & Terman, 2017).

• Expression of semaphorins in the body

There is no particular expression pattern for different classes of semaphorins. They are widely and dynamically expressed in particular regions during different stages of development and

this diverse expression pattern suggests that they are important in a variety of functions during development and throughout adulthood. They are widely associated with cellular processes such as adhesion, aggregation, fusion, migration, patterning, process formation, proliferation, viability and cytoskeletal organization (Yazdani & Terman, 2006).

- **SEMA3A**

There are seven members belonging to the secreted class 3 of semaphorins, named SEMA3A through SEMA3G, which are expressed in the nervous system (Hashimoto *et al.*, 2004; Hou *et al.*, 2008) and function through the NPN and plexin receptors, forming complexes in which NPNs serve as the ligand-binding moiety and plexins act as the signal transduction component (Sharma *et al.*, 2012).

SEMA3A, first designated as collapsin-1, was found to regulate stimulation-induced growth cone slump (Luo *et al.*, 1993) and repulsion of neuron axon growth in the nervous system (Kolodkin *et al.*, 1997; Tamagnone & Comoglio, 2000; Dent *et al.*, 2004). They contain immunoglobulin like domain, one PSI domain and one semaphorin domain. SEMA3A can function as both a chemo attractive agent and chemo repulsive agent (Messersmith *et al.*, 1995).

Previous studies suggest that SEMA3A has both membrane-associated and free forms. Proteolytic cleavage might mediate initial activation and release of the protein and further proteolysis may partially attenuate its activity (Adams *et al.*, 1997). At least three predicted proteolytic cleavage sites, designated PCS1 (residues 555–556), PCS2 (residues 735–736) and PCS3 (residues 761–762), have been identified in mammalian forms of SEMA3A (771 residues) (Good *et al.*, 2004). Western blot analysis of SEMA reveals differential distribution of three bands migrating with apparent molecular masses of approximately 160, 120–125 and 95–100 kDa (Good *et al.*, 2004).

8. PLEXINS

Plexins are large transmembrane receptors similar to SEMA. They contain an extracellular sema domain, a PSI domain and an Ig-like, plexins, transcription factors (IPT) domain (Hu& Zhu, 2018). The plexin family includes four subfamilies based of their structure. Two classes

are present in invertebrates (A and B) and four classes in vertebrates (A–D) (Perälä *et al*., 2012; Alto & Terman, 2017).

- Class A
- Class B
- Class C
- Class D

- **Structure and functions:**

Plexins are large transmembrane receptors with ten domains in the extracellular region containing an extracellular sema domain, which mediates it's interaction with SEMA proteins, several other known extracellular protein domains including, two or three Met-related sequences (MRS; also called 'domains found in plexins, semaphorins and integrins, PSI), glycine-proline-rich immunoglobulin (Ig)-domains shared by plexins and transcription factors (IPT), all of which can also be found in Met family tyrosine kinases (Perälä *et al*., 2012), and a highly conserved intracellular domain containing a GTPase activating protein (GAP) homology domain with a Rho GTPase-binding domain insert (Kong *et al*., 2016). They also have a PSI domain, and an Ig-like, plexins, IPT domain. The cytoplasmic domain contains two GTPase activating protein domains which include the GTPase-binding domain and PDZ domain (Hu & Zhu, 2018).

Plexin expression is regulated by nine genes which is categorized into four subclasses based on homology and structure (Kong *et al*., 2016). They are:

- Class A: *PLXNA1*, *PLXNA2*, *PLXNA3*, *PLXNA4*
- Class B: *PLXNB1*, *PLXNB2*, *PLXNB3*
- Class C: *PLXNC1*
- Class D: *PLXND1*

Each plexin receptor class interacts with a particular SEMA class or classes to mediate signaling. They are majorly involved in the expression of SEMA on the surface of axon growth cones band involvement in signal transduction to steer axon growth away from the source of

Semaphorin (Winberg *et al*., 1998) as they are mediators of repulsive signals (Mauti *et al*., 2006). They also have a role in many other aspects of development of the nervous system including axon guidance, fasciculation, branching and synapse formation (Kruger *et al*., 2005; Tran *et al*., 2007). Their functioning in association with class 3 semaphorins, as a co-receptor, is the most widely studied one (Mauti *et al*., 2006). Plexin is inactive in the absence of SEMA. Binding of SEMA to the extracellular region activates the intracellular signaling pathway of plexin (Pascoe *et al*., 2015). Some plexin family members also have a role in controlling tissue homeostasis and regeneration after a trauma or injury to the nervous system (Shim *et al*., 2012).

- **Expression of plexin subclasses in the body**

The expression and distribution of classA plexins has been studied in more detail than any other plexin subfamilies as they are often associated with the functioning of class3 SEMA. Plexin-As also have an NPN-independent expression. The expression of class-B plexins has not been associated with any specific function and they are expressed in neurons and transiently in glial cells. PlexinC1 expression is associated with late stages of neural development and PlexinD1is expressed transiently in motor neurons during certain stages of development (Mauti *et al*., 2006).

- **PLEXIN A3**

Plexin A3 is a 220kDa cell surface receptor protein. It acts as a co-receptor for SEMA3A and SEMA3F, and might play a significant role in semaphorin-mediated cell death in the developing nervous system. They are involved in axon guidance and regulation of migration of sympathetic neurons, but not of neural crest precursors (Kong *et al*., 2016). It is also essential for dorsal turning of the corticospinal tract axons, development of hippocampus and maintaining the normal dendrite spinal morphology of pyramidal neurons (Ben-Zvi *et al*., 2008; Cheng *et al*., 2001).

- **Role of semaphorin and associated proteins in the human nervous system**

Proteins of SEMA family are best known for their roles in nervous system development, majorly in axon guidance (Kruger *et al*., 2005). An important way by which they guide growing axons is by either repelling them or preventing them from entering certain regions. Studies with normal and mutant SEMA proteins, both *in vivo* and *in vitro*, have revealed that at least some semaphorins form molecular boundaries to prevent axons and cells from entering inappropriate areas (Yazdani & Terman, 2006). They also play important roles in physiological and pathological processes during adulthood. In the nervous system, altered semaphorin function has been linked to epilepsy, retinal degeneration, AD, motor neuron degeneration, schizophrenia and PD (Rice *et al*., 2004; Sahay *et al*., 2005; Pasterkamp & Kolodkin, 2003; Eastwood *et al*., 2003). Semaphorins may also limit the ability of axons to re-grow after injury and prevent abnormal sprouting of axons involved in pain or autonomic function (de Wit & Verhaagen, 2003; Moreau-Fauvarque *et al*., 2003; Goldberg *et al*., 2004; Tang *et al*., 2004).

CHAPTER: 03

AIM AND OBJECTIVES

AIM

To analyze changes in the expression level of the protein semaphorin 3A (SEMA3A) in an *in vivo* model of acute excitotoxicity.

OBJECTIVES

1. To estimate the protein concentrations of brain tissue samples treated with saline and NMDA by intracerebroventricular injection.

2. To determine the expression level of SEMA3A using western blotting and to quantitate changes in its expression level upon NMDA treatment, in comparison to saline treatment.

3. To determine the expression level of Plexin A3 using western blotting and to quantitate changes in its expression level upon NMDA treatment, in comparison to saline treatment.

CHAPTER: 04

MATERIALS AND METHODS

I. Materials

1. Rat brain tissue homogenates

The cortical regions of brain tissues from six Wistar rats subjected to intracerebroventricular (ICV) injection of saline (left cortex) and NMDA at a dose of 40µg/Kg body weight (right cortex) (Kumar *et al*.,2019) were provided.

The samples were labelled as mentioned below:

Table 4 1: Samples of rat brain tissue homogenates

Animal No.	Left cortex (Saline)	Right cortex (NMDA)
1	ALC1	ARC1
2	ALC2	ARC2
3	ALC3	ARC3
4	A2LC3	A2RC3
5	LC1	RC1
6	LC2	RC2

2. Protein estimation using BCA method

2.1. Chemicals Required:

2.1.1. BSA working solutions (10 mg/mL – 50 mg/mL): The required quantity of BSA was dissolved in 1 mL of distilled water to prepare the desired working solutions.

Table 4.2: BSA working standard solutions

Working Concentration (mg/mL)	BSA (mg)
10	10
20	20
30	30
40	40
50	50

2.1.2. BCA reagent: For a 50 mL reaction mixture, add the following:

- Reagent A – 49 mL
- Reagent B – 1mL

3. Western Blotting

3.1. Chemicals Required:

1. Trizma® base
2. 30% Acrylamide (acrylamide/bisacrylamide) solution
3. Ammonium Persulphate (APS)
4. Tetra methyl ethylene diamene (TEMED)
5. Glycine
6. Methanol
7. Sodium chloride (NaCl)
8. Tween-20
9. Bovine Serum Albumin (BSA)
10. Sodium Dodecyl Sulphate (SDS)
11. Ponceau stain
12. β-mercapto ethanol
13. Bromophenol blue
14. Glycerol
15. Tris-HCl
16. Hydrochloric acid

17. Anti-SEMA3A antibody (Santa Cruz)
18. Anti-β-actin antibody (Sigma)
19. Anti-Rabbit antibody conjugated to HRP (Sigma)
20. Anti-Mouse antibody conjugated to HRP (Sigma)
21. Clarity western ECL Kit (Bio-Rad)

3.2. Composition:

1) **10% APS (10mL):**

   1g APS was dissolved in 10mL of distilled water.

2) **30% Acrylamide solution:**

   - Acrylamide -29.22g
   - N,N'-Methylene bis(acrylamide) -0.78g

   Dissolve the above in 100mL of distilled water and filter the solution through Whatman filter paper.

3) **Tris Buffer(4X) (100mL) (pH 8.8)**

   - Trizma® base – 18.17 g
   - 20% SDS - 2mL

   Adjust the pH to 8.8 using HCl

4) **TrisBuffer (4X) (100mL) (pH 6.8)**

   - Trizma® base – 6.06 g
   - 20% SDS - 2mL

   Adjust the pH to 6.8 using HCl

5) **SDS-loading dye (5X) (100 mL)**

   - β-Mercapto ethanol - 5 mL
   - Bromophenol blue - 0.02 g

- Glycerol - 30 mL
- SDS - 10g
- Tris-HCl (pH 6.8) - 3.94 g

The volume was made up to 100 mL with distilled water.

6) Tris-Glycine buffer (10X) (1000 mL)

- Trizma® base -30 g
- Glycine – 144g

The pH was adjusted to 8.3 and volume was made up to 1000 mL with distilled water.

Tris-Glycine buffer (1X) (500 mL)

50 mL of 10X Tris-Glycine buffer was diluted with 450 mL of distilled water.2.5 mL of 20% SDS stock solution was added prior to use.

7) Towbin's buffer (1000 mL)

- Trizma® base - 3 g
- Glycine - 14.4 g
- Methanol - 200mL

The volume was made up to 1000mLwith distilled water.

8) TBS buffer (10X) (500 mL)

- Trizma® base – 6.057 g
- NaCl - 58.4 g

The pH was adjusted to 7.5 using 1N HCl and the volume was made up to 500 mL with distilled water.

TBS buffer (1X) (1000 mL)

100 mL of 10X TBS buffer was diluted with 900 mL of distilled water.

9) TTBS buffer (1000 mL)

- TBS buffer (1X) - 980 mL
- 10% Tween-20 - 20 mL

10) Blocking solution (50 mL)

2.5 g BSA was dissolved in 50 mL of TTBS buffer.

11) Antibody dilution buffer (50 mL)

1 g BSA was dissolved in 50 mL of TTBS buffer.

12) ECL Substrate

Enhanced luminol reagent and the oxidizing reagent were mixed in a 1:1 ratio in dark.

10% SDS-PAGE gel:

Table 4.3: 10% SDS-PAGE gel composition

Solution components	Resolving gel(mL)	Stacking gel(mL)
Distilled water	2.79	2.86
30%acrylamide solution	2.3	0.80
Trisbuffer	1.73 (pH 8.8)	1.25 (pH 6.8)
10% APS	0.17	0.10
TEMED	0.01	0.01

4. Equipments Used

- -80ºCfreezer
- -20°C freezer

- 4ºC cold room/refrigerator
- Vortex
- Micropipettes
- Eppendorf tubes
- Rotospin
- Magnetic stirrer
- pH meter
- Orbital Shaker
- 96-well plate
- 37°C Incubator
- TECAN microplate reader
- Blotting apparatus
- Molecular Imager® VersaDoc™ MP 4000 system

5. Softwares used

1. iControl Software, provided with TECAN microplate reader, was used for protein estimation by BCA assay.
2. Quantity One Software, provided with Molecular Imager® VersaDoc™ MP 4000 system, was used for blot development by enhanced chemiluminescence method.
3. ImageJ software, downloaded as a free tool from http://image.nih.gov/ij/download.html (National Institutes of Health, 1997), was used for the densitometry analysis of blot.

II. Methodology

1) Protein estimation using BCA method

1. In a 96-well plate, the protein samples were added in triplicates in a 1:10 dilution (1µL protein in 9µLdistilled water).
2. A blank (10µLdistilled water) and five BSA standards (10mg/mL, 20mg/mL, 30mg/mL, 40mg/mL and 50mg/mL) were also added in triplicates.
3. 190µL of BCA reagent mixture was added to each well and mixed well.
4. The plate was incubated at 37ºC for 30 minutes.
5. The absorbance values at 526nm were measured using TECAN microplate reader.
6. The average value of the triplicates used for each sample was calculated and normalized to the blank.
7. A standard graph was plotted with absorbance values (y-axis) vs. BSA standard concentrations (x-axis).
8. Using the straight-line equation y = mx+c, with the intercept value c set at zero, the concentrations of the unknown protein samples were determined.

2) Sample preparation for SDS-PAGE

1. Protein samples at a concentration of 60-120µgwere used for western blotting.
2. 5X SDS loading dye at a final concentration of 1X were added to all the samples.
3. The volume of the samples was made up to 25 µL with distilled water.
4. The samples were boiled at 100ºC for 15 minutes.

3) SDS-PAGE

1. The acrylamide gel was placed in the electrophoresis apparatus and filled with 1X Tris-glycine buffer.
2. The protein samples were loaded into each well along with a protein marker.
3. The gel tank was connected to the power supply.

4. Electrophoresis was carried out with a voltage setting as follows:
    - Stacking gel -75V
    - Resolving gel -100V
5. The power supply was stopped when the dye front reached the bottom of the gel

4) Western Blotting

1. The resolving gel was removed from the plate using a scoop and kept in fresh Towbin's buffer for 5-10 minutes.
2. Polyvinylidene fluoride (PVDF) membrane (9 cm x 6 cm) was activated with methanol for one minute and soaked in fresh Towbin's buffer for 10-15 minutes.
3. Similarly, the sponges and Whatman filter paper (9 cm × 6 cm) were also soaked in Towbin's buffer for 10-15 minutes.
4. The blot cassette was arranged by placing the gel and membrane between the filter paper and sponges. The gel was placed near the cathode (-ve) and membrane was placed near the anode (+ve). Prior to closing the cassette, the membrane roller was rolled over the membrane to eliminate air bubbles.
5. The blot cassette was placed in the apparatus and cold Towbin's buffer was added to it.
6. The apparatus was connected to the power supply.
7. Blotting was carried out for 1.5 hours at 100V.
8. After transfer, the PVDF membrane was removed and stained with Ponceau to check for protein transfer.
9. The blot was cut into two halves – upper half (>75 kDa) and lower half (<75 kDa).
10. The membranes were washed with TTBS for 10 minutes, thrice.
11. The membranes were blocked with 5% BSA for 1hour at room temperature.
12. The membranes were washed with TTBS for 10 minutes, thrice.
13. Primary antibody at the required dilutions (SEMA3A-1:200, β Actin-1:3000) were added to the membrane and incubated at 4°C overnight.
14. The membranes were washed with TTBS for 10 minutes, thrice.
15. Secondary antibody at the required dilutions (Anti-Rabbit-HRP-1:3000, Anti-mouse-HRP-1:5000) were added to the membrane and incubated at room temperature for 1 hour.
16. The membranes were washed with TTBS for 10 minutes, thrice.

17. The membrane was washed with 1X TBS for 10 minutes, twice and stored in 1X TBS at 4°C till development

5) Blot development using ECL method

1. In a 1.5 mL tube, equal volumes of enhanced luminol reagent and the oxidizing reagent were mixed in dark.
2. The membrane was placed in a tray and positioned in Molecular Imager® VersaDoc™ MP 4000 system.
3. The ECL reagent mixture was layered onto the membrane in dark (~ 5-10 times).
4. The blot images were captured at different exposure settings, optimized according to the protein of interest.

6) Blot quantitation using ImageJ

1. The captured blot images were exported in .tif image format and opened in ImageJ software.
2. The desired area from the blot image was cropped and the cropped image was converted to 8-bit format.
3. The noise present in the image was removed and the blot was aligned to a straight line, if skewed.
4. Using the rectangular tool, the desired bands were selected and the peaks for each lane were plotted.
5. By using the straight-line tool, the desired peak for each lane was demarcated.
6. The areas inside the selected peaks were obtained using the wand tool.
7. The values obtained were copied to an Excel file and labelled accordingly.
8. The densitometric value of SEMA3A (125 kDa and 95 kDa) was normalized with that of the loading control (β-actin).
9. The relative value of SEMA3A (125 kDa and 95 kDa) level upon NMDA treatment was calculated taking its corresponding saline control as 1.
10. Bar graphs were plotted with the calculated relative values.

CHAPTER: 05

RESULTS AND DISCUSSION

1. RESULTS

5.1 Description of Samples

Samples used in this study were previously generated and were reported (Kumar *et al*., 2019). In this *in vivo* model, saline and NMDA (at a dose of 40 µg/Kg body weight) were injected into the left and right lateral ventricles respectively using a stereotactic apparatus and the animals were sacrificed 30 mins later (Kumar *et al*.,2019). The cortical samples isolated from these animals that were available in the host laboratory were provided for this study.

5.2 Protein estimation by BCA method

The amount of protein in 1µL samples of homogenized cortical tissue were estimated using BCA method. Five different BSA standard concentrations, ranging from 10-50 mg/mL were used to construct the standard graph which was then used to calculate the unknown protein concentrations of the homogenate samples. A graph was plotted with absorbance value vs. protein concentration (Figure 5.1).

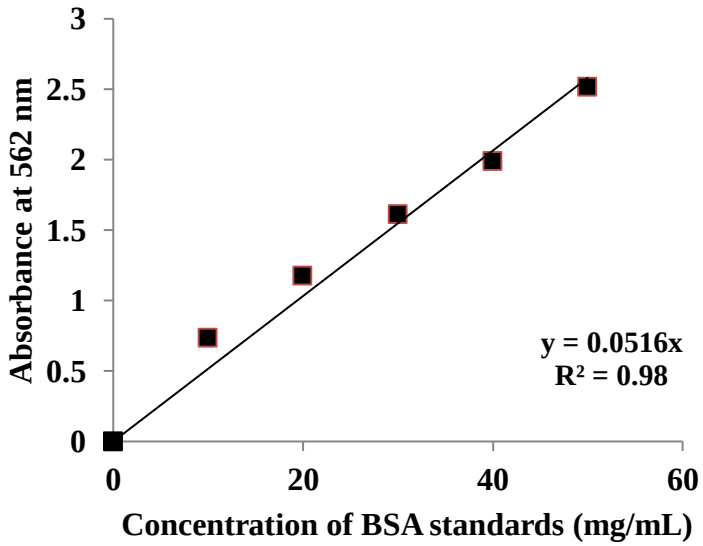

Figure 5.1 Graph depicting absorbance value vs. standard protein concentration

The unknown protein concentrations were calculated (Table 5.1) using the line equation y = mx + c, with the intercept c set at zero (Figure5.1).

Table 5.1: Protein concentration of saline and NMDA treated rat brain homogenate

Saline treated samples	Concentration (µg/µL)	NMDA treated samples	Concentration (µg/µL)
ALC1	28.29	**ARC1**	31.73
ALC2	34.03	**ARC2**	30.28
ALC3	27.12	**ARC3**	30.50
A2LC3	29.5	**A2RC3**	25.23
LC1	37.74	**RC1**	43.90
LC2	36.69	**RC2**	37.46

120 µg protein was used for western blot analysis.

5.3 Western Blotting

Western blotting was carried out with 120µg of each protein sample. The proteins were separated by SDS-PAGE. After separation, they were transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was probed against SEMA3A (1:200 dilution of primary antibody) and β-actin (1:5000dilution of primary antibody) (Figure 5.2).

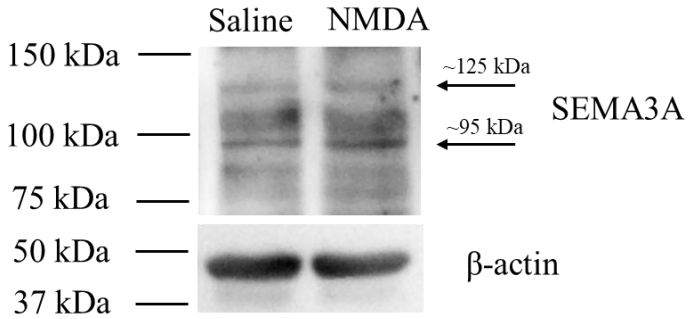

Figure 5.2 Representative immunoblots showing the expression level of SEMA3A (~ 125 and95 kDa) with its corresponding β-actin (~42 kDa) in saline and NMDA treated cortical tissue samples

Two prominent bands of SEMA3A at ~125 kDa and ~95 kDa were observed upon analysis of the developed blots. While 125 kDa form of SEMA3A represents the membrane-associated, full-length protein, the 95 kDa form represents the active protein generated by proteolysis by furin (Adams *et al.*, 1997). The blots of SEMA3Aand β-actin were quantified using ImageJ software. The values obtained were normalized against their corresponding β-actin. The relative value of SEMA3A level upon NMDA treatment for each sample was calculated taking its corresponding saline control as 1. The average and standard deviation of the relative values were calculated and plotted in bar graphs (Figure 5.3 and Figure 5.4). Student's t test was used to calculate the p value.

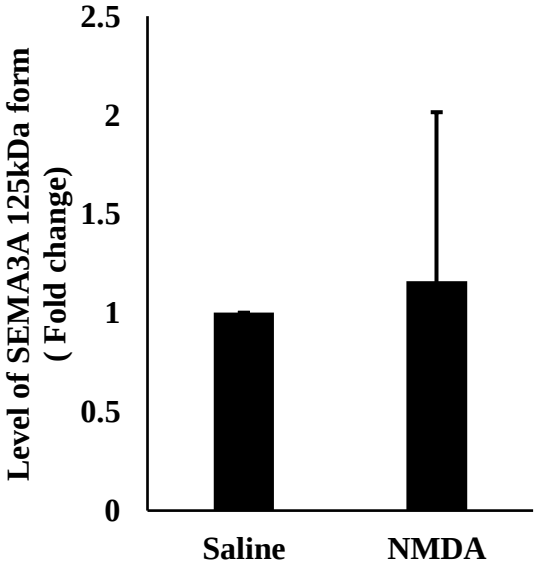

Figure 5.3: Bar graph representing the level of 125 kDa form of SEMA3Ain NMDA treated sample compared to saline treated sample. Band intensity of SEMA3A was normalised to corresponding β-actin. The values are expressed as fold change with respect to saline treated sample (n=6, p=0.656)

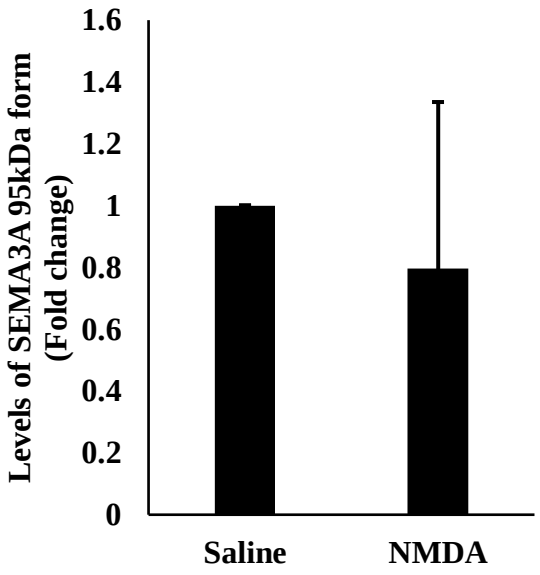

Figure 5.4: Bar graph representing the level of 95 kDa form of SEMA3A in NMDA treated sample compared to saline treated sample. Band intensity of SEMA3A was normalised to corresponding β-actin. The values are expressed as fold change with respect to saline treated sample (n=6, p=0.376)

No significant change in the expression levels of both 125 kDa and95 kDa forms of SEMA3A was observed between the saline and NMDA treated samples.

2. DISCUSSION

Proteolysis of SEMA precursor proteins, by furin, is essential for the initial activation and release of the mature protein (Adams *et al.*, 1997). Differential post-translational processing of full-length SEMA3A results in forms of varying molecular weights. The 125 kDa form of SEMA3A that represents the full-length protein (Adams *et al.*, 1997) is majorly observed in hippocampus and thalamus (Good *et al.*, 2004). The 125 kDa band observed in this study (Figure 5.2) may represent the membrane-associated SEMA3A protein. In human thalamic samples, this form presents a punctate surface staining pattern that represents its membrane-associated existence (Good *et al.*, 2004).

Proteolytic cleavage at site 3 (PCS3) releases the 95 kDa SEMA3A fragment (Figure 5.3) and renders SEMA3A fully active (Adams *et al.*, 1997). In AD, it has been previously

reported that the cleaved, active form of SEMA3A accumulates in a granular pattern in the cytoplasm of neurons in the CA1 field of the hippocampal formation and in the subiculum (Good *et al.*, 2004). Aberrant regulation of SEMA3A signaling is proposed to contribute to the progressive neurodegenerative changes observed during AD (Good *et al.*, 2004). Also, SEMA3A can induce death of embryonic sensory neurons (Ben-Zvi*et al*., 2008).

The absence of a significant change in the expression levels of both the 125 kDa and 95 kDa forms of SEMA3A upon NMDA treatment in this study might be due to the short exposure (30 mins) of brain tissue to an excitotoxic insult, in contrast to the previously reported changes in progressive forms of AD (Good *et al.*, 2004). Further experiments with a larger sample size (n>6) along with immunohistochemistry (IHC) and real-time PCR are essential to draw definite conclusions on the expression of SEMA during excitotoxicity. As semaphorin class3 proteins function in association with Plexin A3 receptors, a better understanding on how plexin A3 levels are altered during NMDAR-mediated excitotoxicity, along with other semaphorin associated proteins like neuropilins, will help in deciphering the role of SEMA proteins during neuronal cell death.

Note:

The initial proposal of the project work also included analyzing the expression levels of SEMA3A associated protein, plexin A3. However, due to the current COVID-19 pandemic situation, I was unable to complete the experiments with plexinA3.

SUMMARY AND CONCLUSION

SUMMARY

i. Protein estimation of homogenized brain tissue samples were performed using BCA method.

ii. The tissue samples were used for western blotting to check for the expression of Semaphorin 3A (SEMA3A).

iii. No significant change in the expression levels of 125 kDa and 95 kDa forms of SEMA3A were observed with NMDA treatment compared to saline treatment.

CONCLUSION

Aberrant regulation of SEMA3A signaling mechanism has been reported to contribute to progressive neurodegeneration observed during AD (Good *et al.*, 2004). In this study, no significant change in the protein expression levels of 125 kDa and 95 kDa forms of SEMA3A were observed in an *in vivo* model of acute excitotoxicity. Further experiments with a larger sample size along with immunohistochemistry (IHC) and real-time PCR are essential to draw definite conclusions on the expression of SEMA during excitotoxicity.

REFERENCES

- Adams, R. H., Lohrum, M., Klostermann, A., Betz, H., &Püschel, A. W. (1997). The chemorepulsive activity of secreted semaphorins is regulated by furin-dependent proteolytic processing. *The EMBO journal*, *16*(20), 6077-6086.
- Allen, N. J., &Barres, B. A. (2009). Neuroscience: Glia - more than just brain glue. *Nature*, 457 (7230): 675–7.
- Alto, L. T., &Terman, J. R. (2017). Semaphorins and their Signaling Mechanisms. *Methods in molecular biology (Clifton, N.J.)*, *1493*, 1–25.
- Azevedo, F. A., Carvalho, L. R., Grinberg, L. T., Farfel, J. M., Ferretti, R. E., Leite, R. E.,JacobFilho, W., Lent, R.,&Herculano-Houzel, S. (2009). Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. *Journal of Comparative Neurology*, *513*(5), 532-541.
- Bamberg, J. A., Baumgartner, S., Betz, H., Bolz, J., Chedotal, A., Christensen, C. R., P. M. Comoglio, J. G. Culotti, P. Doherty, H. Drabkin, A. Ensser, M. C. Fishman, B. Fleckenstein, G. J. Freeman, H. Fujisawa, A. Ghosh, D. D. Ginty, C. S. Goodman, S. Guthrie, S. Inagake, R. Keynes, T. Kimura, M. Klagsbrun, A. L. Kolodkin, J. Y. Kuwada, Y. Luo, J. D. Minna, S. L. Naylor, T. P. O'Connor, D. D.M. O'Leary, A. Pini, M. M. Poo, A. W. Poschel, J. A. Raper, J. Roche, C. J. Shatz, W. D. Snider, E. Soriano, M. K. Spriggs, S. M. Stritmatter, S. Sullivan, L. Tamagnone, M. Tessier-Lavigne, T. Tohyama, J. Verhaagen, F. S. Walsh, & T. Yagi (1999). Unified nomenclature for the semaphorins/collapsins [1]. Cell, 97(5), 551-552.
- Ben-Zvi, A., Manor, O., Schachner, M., Yaron, A., Tessier-Lavigne, M., & Behar, O. (2008). The Semaphorin receptor PlexinA3 mediates neuronal apoptosis during dorsal root ganglia development. *Journal of Neuroscience*, *28*(47), 12427-12432.
- Bliss, T. V., &Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature*, *361*(6407), 31-39.
- Chenard,B.L.&Menniti,F.S.(1999).AntagonistsselectiveforNMDAreceptorscontaining the NR2B subunit. *Curr pharm Des*,5,381-404.
- Cheng, H. J., Bagri, A., Yaron, A., Stein, E., Pleasure, S. J., & Tessier-Lavigne, M. (2001). Plexin-A3 mediates semaphorin signaling and regulates the development of hippocampal axonal projections. *Neuron*, *32*(2), 249-263.

- Cheng,H.Y.,Hsieh,M.T.,Wu,C.R.,Tsai,F.H.,Lu,T.C.,Hsiech,C.C.,Li,W.C.,Lin, Y. T.& Peng, W. H. (2008). Schizandrin protects primary cultures of rat cortical cells from glutamate-induced excitotoxicity. *J PharmacolSci,* 107(1), 21-31.
- Choi, D. W. (1987). Ionic dependence of glutamate neurotoxicity. *Journal of Neuroscience, 7*(2), 369-379.
- Choi, D. W. (1988). Glutamate neurotoxicity and diseases of the nervous system. *Neuron*, *1*(8), 623-634.
- Cooke, S. F., & Bliss, T. V. P. (2006). Plasticity in the human central nervous system. *Brain*, *129*(7), 1659-1673.
- Cull-Candy, S. G., &Leszkiewicz, D. N. (2004). Role of distinct NMDA receptor subtypes at central synapses. *Sci. STKE*, *2004*(255), re16-re16.
- Cull-Candy, S., Brickley, S., & Farrant, M. (2001). NMDA receptor subunits: diversity, development and disease. *Current opinion in neurobiology*, *11*(3), 327-335.
- Dayan, P., &Abbott, L. F. (2005). *Theoretical Neuroscience: Computational and Mathematical Modeling of Neural Systems*. MIT Press.
- de Wit, J., &Verhaagen, J. (2003). Role of semaphorins in the adult nervous system. *Progress in neurobiology*, *71*(2-3), 249-267.
- Dent, E. W., Barnes, A. M., Tang, F., and Kalil, K. (2004). Netrin-1 and semaphorin 3A promote or inhibit cortical axon branching, respectively, by reorganization of the cytoskeleton. *J. Neurosci.* 24, 3002–3012.
- Dong, X. X., Wang, Y., & Qin, Z. H. (2009). Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. *ActaPharmacologicaSinica*, *30*(4), 379-387.
- Eastwood, S. L., Law, A. J., Everall, I. P., & Harrison, P. J. (2003). The axonal chemorepellantsemaphorin 3A is increased in the cerebellum in schizophrenia and may contribute to its synaptic pathology. *Molecular psychiatry*, *8*(2), 148-155.
- Fan, M. M. & Raymond, L. A.(2007). N-Methyl-D-Aspartate (NMDA) receptor function and excitotoxicity in Huntington's disease. *Progress in neurobiology*, 81(5-6),272- 293.
- Gardoni, F., Caputi, A., Cimino, M., Pastorino, L., Cattabeni, F., & Di Luca, M. (1998). Calcium/calmodulin-dependent protein kinase II is associated with NR2A/B subunits of NMDA receptor in postsynaptic densities. *Journal of neurochemistry*, *71*(4), 1733-

1741.

- Gerhard D. (2013). Neuroscience. 5th Edition. *The Yale Journal of Biology and Medicine*, *86*(1), 113–114.
- Goldberg, J. L., Vargas, M. E., Wang, J. T., Mandemakers, W., Oster, S. F., Sretavan, D. W., & Barres, B. A. (2004). An oligodendrocyte lineage-specific semaphorin, Sema5A, inhibits axon growth by retinal ganglion cells. *Journal of Neuroscience*, *24*(21), 4989-4999.
- Good, P. F., Daisy Alapat, Amy Hsu, Caryn Chu, Daniel Perl, Xiaping Wen, David E. Burstein and D. Stave Kohtz (2004). A role for semaphorin 3A signaling in the degeneration of hippocampal neurons during Alzheimer's disease. *Journal of neurochemistry*, *91*(3), 716-736.
- Görlach, A., Bertram, K., Hudecova, S., &Krizanova, O. (2015). Calcium and ROS: A mutual interplay. *Redox biology*, *6*, 260–271. https://doi.org/10.1016/j.redox.2015.08.010
- Goshima, Y., Yamashita, N., Nakamura, F., & Sasaki, Y. (2016). Regulation of dendritic development by semaphorin 3A through novel intracellular remote signaling. *Cell adhesion & migration*, *10*(6), 627-640.
- Greengard, P. (2001). The neurobiology of slow synaptic transmission. *Science*, *294*(5544), 1024-1030.
- Groc, L., Heine, M., Cousins, S. L., Stephenson, F. A., Lounis, B., Cognet, L., &Choquet, D. (2006). NMDA receptor surface mobility depends on NR2A-2B subunits. *Proceedings of the National Academy of Sciences*, *103*(49), 18769-18774.
- Harris, A. Z., & Pettit, D. L. (2007). Extrasynaptic and synaptic NMDA receptors form stable and uniform pools in rat hippocampal slices. *The Journal of physiology*, *584*(2), 509-519.
- Hashimoto, M., Ino, H., Koda, M., Murakami, M., Yoshinaga, K., Yamazaki, M., & Moriya, H. (2004). Regulation of semaphorin 3A expression in neurons of the rat spinal cord and cerebral cortex after transection injury. *ActaNeuropathol.* 107, 250–256.
- Hollmann, M., Boulter, J., Maron, C., Beasley, L., Sullivan, J., Pecht,G.& Heinemann, S.(1993).Zincpotentiatesagonist-inducedcurrentsatcertainsplicevariantsoftheNMDA receptor. *Neuron*,10(5),943-954.

- Hormuzdi, S. G., Filippov, M. A., Mitropoulou, G., Monyer, H., &Bruzzone, R. (2004). Electrical synapses: a dynamic signaling system that shapes the activity of neuronal networks. *BiochimicaetBiophysicaActa (BBA)-Biomembranes*, *1662*(1-2), 113-137.
- Hou, S. T., Keklikian, A., Slinn, J., O'Hare, M., Jiang, S. X., and Aylsworth, A. (2008). Sustained up-regulation of semaphorin 3A, Neuropilin1, and doublecortin expression in ischemic mouse brain during long-term recovery. *Biochem. Biophys. Res. Commun.* 367, 109–115.
- Hu, S., & Zhu, L. (2018). Semaphorins and their receptors: From axonal guidance to atherosclerosis. *Frontiers in physiology*, *9*, 1236.
- Kandel, E. R. (2009). The biology of memory: a forty-year perspective. *Journal of Neuroscience*, *29*(41), 12748-12756.
- Kandel,E.R., Schwartz,J.H.&Jessell,T.M.(2000).Principlesofneuralscience.*McGraw-Hill*, NewYork.
- Karakas, E., & Furukawa, H. (2014). Crystal structure of a heterotetrameric NMDA receptor ion channel. *Science*, *344*(6187), 992-997.
- Kim, M. J., Dunah, A. W., Wang, Y. T.& Sheng, M.(2005). Differential roles of NR2A and NR2B containing NMDAR in Ras-ERK signalling and AMPA receptor trafficking. *Neuron*, 46(5),745-760.
- Klostermann, A., Lohrum, M., Adams, R. H., &Püschel, A. W. (1998). The chemorepulsive activity of the axonal guidance signal semaphorin D requires dimerization. *Journal of Biological Chemistry*, *273*(13), 7326-7331.
- Köhr, G. (2006). NMDA receptor function: subunit composition versus spatial distribution. *Cell and tissue research*, *326*(2), 439-446.
- Kolodkin, A. L., Levengood, D. V., Rowe, E. G., Tai, Y. T., Giger, R. J., and Ginty, D. D. (1997). Neuropilin is a semaphorin III receptor. *Cell* 90, 753–762.
- Kolodkin, A. L., Matthes, D. J., & Goodman, C. S. (1993). The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. *Cell*, *75*(7), 1389-1399.
- Kong, Y., Janssen, B. J., Malinauskas, T., Vangoor, V. R., Coles, C. H., Kaufmann, R., Ni, T., Gilbert, J.C. R., Parra, S.P., Paterkamp,J. & Jones, E. Y. (2016). Structural basis for plexin activation and regulation. *Neuron*, *91*(3), 548-560.

- Koppel, A. M., &Raper, J. A. (1998). Collapsin-1 covalently dimerizes, and dimerization is necessary for collapsing activity. *Journal of Biological Chemistry*, *273*(25), 15708-15713.
- Kruger, R. P., Aurandt, J., & Guan, K. L. (2005). Semaphorins command cells to move. *Nature reviews Molecular cell biology*, *6*(10), 789-800.
- Kumar, A. (2015). NMDA receptor function during senescence: implication on cognitive performance. *Frontiers in neuroscience*, *9*, 473.
- Kumar, M., John, M., Madhavan, M., James, J., &Omkumar, R. V. (2019). Alteration in the phosphorylation status of NMDA receptor GluN2B subunit by activation of both NMDA receptor and L-type voltage gated calcium channel. *Neuroscience Letters, 134343*.
- Lai, T. W., Shyu, W. C., & Wang, Y. T. (2011). Stroke intervention pathways: NMDA receptors and beyond. *Trends in molecular medicine*, *17*(5), 266-275.
- Liu, D. D., Yang, Q., & Li, S. T. (2013). Activation of extrasynaptic NMDA receptors induces LTD in rat hippocampal CA1 neurons. *Brain research bulletin*, *93*, 10-16.
- Liu, L., Wong, T. P., Pozza, M. F., Lingenhoehl, K., Wang, Y., Sheng, M., Yves, P.A., & Wang, Y. T. (2004). Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. *Science*, *304*(5673), 1021-1024.
- Loftis, J. M.&Janowsky,A.(2003). The N-methyl-D-aspartate receptor subunit NR2B: localisation, functional properties, regulation and clinical implications. *Pharmacology & Therapeutics,* 97(1),55-85.
- Luo, Y., Raible, D., and Raper, J. A. (1993). Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell* 75, 217–227.
- Luo,T., Chen, N.&Raymond,L. A.(1999). Subtype-dependence of NMDA receptor channel open probability.*J.Neurosci,* 19(16),6844-6854.
- Lynch, D. R., & Guttmann, R. P. (2002). Excitotoxicity: perspectives based onN-methyl-D-aspartate receptor subtypes. *Journal of Pharmacology and Experimental Therapeutics*, *300*(3), 717-723.
- Malenka, R. C., & Nicoll, R. A. (1993). NMDA-receptor-dependent synaptic plasticity: multiple forms and mechanisms. *Trends in neurosciences*, *16*(12), 521-527.
- Martel, M. A., Wyllie, D. J., &Hardingham, G. E. (2009). In developing hippocampal neurons, NR2B-containing N-methyl-D-aspartate receptors (NMDARs) can mediate

signaling to neuronal survival and synaptic potentiation, as well as neuronal death. *Neuroscience*, *158*(1), 334-343.

- Marty, A., &Llano, I. (2005). Excitatory effects of GABA in established brain networks. *Trends Neurosci.* 28 (6): 284–9.
- Mauti, O., Sadhu, R., Gemayel, J., Gesemann, M., &Stoeckli, E. T. (2006). Expression patterns of plexins and neuropilins are consistent with cooperative and separate functions during neural development. *BMC developmental biology*, *6*, 32.
- Mayadevi, M.,Archana,G.M., Prabhu, R.R., &Omkumar, R. V. (2012).Molecularmechanisms in synaptic plasticity. *Neuroscience-Dealing with Frontiers*.InTech.
- Mayer, M. L., Westbrook, G. L., & Guthrie, P. B. (1984). Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones. *Nature*, *309*(5965), 261-263.
- McDonald, J. W., & Johnston, M. V. (1990). Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. *Brain Research Reviews*, *15*(1), 41-70.
- Mecollari, V., Nieuwenhuis, B., &Verhaagen, J. (2014). A perspective on the role of class III semaphorin signaling in central nervous system trauma. *Frontiers in cellular neuroscience*, *8*, 328.
- Meldrum, B. S. (2000). Glutamate as a neurotransmitter in the brain: review of physiology and pathology. *The Journal of nutrition*, *130*(4), 1007S-1015S.
- Messersmith, E. K., Leonardo, E. D., Shatz, C. J., Tessier-Lavigne, M., Goodman, C. S., &Kolodkin, A. L. (1995). Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. *Neuron*, *14*(5), 949-959.
- Mizuta,L., Katayama, M., Watanabe, M. & Ishii, K.(1998).Development expression of NMDA receptor subunits and the emergence of glutamate neurotoxicity in primary culture of murine cerebral cortical neurons. *Cell. Mol. Life*,(54)721-725.
- Monyer, H., Burnashev, N., Laurie, D. J., Sakmann, B., &Seeburg, P. H. (1994). Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron*, *12*(3), 529-540.
- Monyer, H., Sprengel, R., Schoepfer, R., Herb, A., Higuchi, M., Lomeli, H., Burnashev, N., Sakmann, B.,&Seeburg, P. H. (1992). Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science*, *256*(5060), 1217-1221.

- Moreau-Fauvarque, C., Kumanogoh, A., Camand, E., Jaillard, C., Barbin, G., Boquet, I., , Love C, Jones EY, Kikutani H, LubetzkiC,Dusart, I. (2003). The transmembrane semaphorin Sema4D/CD100, an inhibitor of axonal growth, is expressed on oligodendrocytes and upregulated after CNS lesion. *Journal of Neuroscience*, *23*(27), 9229-9239.
- Mori, H., &Mishina, M. (1995). Structure and function of the NMDA receptor channel. *Neuropharmacology*, *34*(10), 1219–1237.
- National Institute of Health, 1997. Wayne Rasband, Image J,1.51w, NIH, USA.
- Newcomer, J. W., Farber, N. B., & Olney, J. W. (2000). NMDA receptor function, memory, and brain aging. *Dialogues in clinical neuroscience*, *2*(3), 219–232.
- Obrenovitch,T.P., Urenjak, J.,Zilkha,E.& Jay, T.M.(2000). Excitotoxicity in neurological disorders-the glutamate paradox. *Int.J Dev Neurosci*., 18 (2-3),281-287.
- Okuno, T., Nakatsuji, Y., &Kumanogoh, A. (2011). The role of immune semaphorins in multiple sclerosis. *FEBS letters*, *585*(23), 3829-3835.
- Olivares, D., Deshpande, V. K., Shi, Y., Lahiri, D. K., Greig, N. H., Rogers, J. T., & Huang, X. (2012). N-methyl D-aspartate (NMDA) receptor antagonists and memantine treatment for Alzheimer's disease, vascular dementia and Parkinson's disease. *Current Alzheimer research*, *9*(6), 746–758. https://doi.org/10.2174/156720512801322564
- Olney, J. W. (1969). Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamate. *Science*, *164*(3880), 719-721.
- Omkumar, R.V., Kiely, M.J., Rosenstein, A.J., Min, K. T.&Kennedy,M.B. (1996). Identificationofaphosphorylationsiteforcalcium/calmodulindependentproteinkinaseII in theNR2B subunit of N-Methyl-D-Aspartate receptor. *Journal of Biological Chemistry*, 271(49),31670-31678.
- Paoletti, P. (2011). Molecular basis of NMDA receptor functional diversity. *European Journal of Neuroscience*, *33*(8), 1351-1365.
- Paoletti, P., &Neyton, J. (2007). NMDA receptor subunits: function and pharmacology. *Current opinion in pharmacology*, *7*(1), 39-47.
- Paoletti, P., Bellone, C., & Zhou, Q. (2013). NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nature Reviews Neuroscience*, *14*(6), 383-400.
- Parsons, M. P., & Raymond, L. A. (2014). Extrasynaptic NMDA receptor involvement in central nervous system disorders. *Neuron*, *82*(2), 279-293.

- Pascoe, H. G., Wang, Y., & Zhang, X. (2015). Structural mechanisms of plexin signaling. *Progress in biophysics and molecular biology*, *118*(3), 161–168.
- Pasterkamp, R. J., & Giger, R. J. (2009). Semaphorin function in neural plasticity and disease. *Current opinion in neurobiology*, *19*(3), 263-274.
- Pasterkamp, R. J., &Kolodkin, A. L. (2003). Semaphorin junction: making tracks toward neural connectivity. *Current opinion in neurobiology*, *13*(1), 79-89.
- Perälä, N., Sariola, H., &Immonen, T. (2012). More than nervous: the emerging roles of plexins. *Differentiation*, *83*(1), 77-91.
- Petralia, R. S., Wang, Y. X., Hua, F., Yi, Z., Zhou, A., Ge, L., Stephenson, F. A.,&Wenthold, R. J. (2010). Organization of NMDA receptors at extrasynaptic locations. *Neuroscience*, *167*(1), 68-87.
- Quintremil, S., Ferrer, F. M., Puente, J., Pando, M. E., & Valenzuela, M. A. (2018). Roles of Semaphorins in Neurodegenerative Diseases. In *Neurons-Dendrites and Axons*. IntechOpen.
- Rice, D. S., Huang, W., Jones, H. A., Hansen, G., Ye, G. L., Xu, N., Wilson, E.A., Troughton, K., Vaddi, K., Newton, R.C.&Zambrowicz, B. P. (2004). Severe retinal degeneration associated with disruption of semaphorin 4A. *Investigative ophthalmology & visual science*, *45*(8), 2767-2777.
- Sahay, A., Kim, C. H., Sepkuty, J. P., Cho, E., Huganir, R. L., Ginty, D. D., &Kolodkin, A. L. (2005). Secreted semaphorins modulate synaptic transmission in the adult hippocampus. *Journal of Neuroscience*, *25*(14), 3613-3620.
- Salter, M. W., Dong, Y., Kalia, L. V., Liu, X. J., & Pitcher, G. (2009). Regulation of NMDA receptors by kinases and phosphatases. *Biology of the NMDA Receptor*, *7*, 123-48.
- Salussolia, C. L., Prodromou, M. L., Borker, P., &Wollmuth, L. P. (2011). Arrangement of subunits in functional NMDA receptors. *The Journal of neuroscience: the official journal of the Society for Neuroscience*, *31*(31), 11295–11304.
- Sanz-Clemente, A., Nicoll, R. A., & Roche, K. W. (2013). Diversity in NMDA receptor composition: many regulators, many consequences. *The Neuroscientist*, *19*(1), 62-75.
- Sattler, R., Charlton, M. P., Hafner, M., &Tymianski, M. (1998). Distinct influx pathways, not calcium load, determine neuronal vulnerability to calcium neurotoxicity. *Journal of neurochemistry*, *71*(6), 2349-2364.

- Sharma, A., Verhaagen, J., and Harvey, A. R. (2012). Receptor complexes for each of the class 3 semaphorins. *Front. Cell Neurosci.* 6:28.
- Sheng, M., & Kim, E. (2011). The postsynaptic organization of synapses. *Cold Spring Harbor perspectives in biology*, *3*(12), a005678.
- Shim, S. O., Cafferty, W. B., Schmidt, E. C., Kim, B. G., Fujisawa, H., &Strittmatter, S. M. (2012). PlexinA2 limits recovery from corticospinal axotomy by mediating oligodendrocyte-derived Sema6A growth inhibition. *Molecular and Cellular Neuroscience*, *50*(2), 193-200.
- Siebold, C., & Jones, E. Y. (2013). Structural insights into semaphorins and their receptors. In *Seminars in cell & developmental biology* (Vol. 24, No. 3, pp. 139-145). Academic Press.
- Strata, P., &Harvey, R. (1999). Dale's principle. *Brain Res. Bull.* 50 (5–6): 349–50.
- Szydlowska, K and Tymianski, M.(2010). Calcium, ischemia and excitotoxicity. *Cell calcium*, (47)122-129.
- Tamagnone, L., and Comoglio, P. M. (2000). Signalling by semaphorin receptors: cell guidance and beyond. *Trends Cell Biol.* 10, 377–383.
- Tang, X. Q., Tanelian, D. L., & Smith, G. M. (2004). Semaphorin3A inhibits nerve growth factor-induced sprouting of nociceptive afferents in adult rat spinal cord. *Journal of Neuroscience*, *24*(4), 819-827.
- Terman, J. R.(2017). *Semaphorin Signaling: Methods and Protocols*. 1st Edition, Humana Press.
- Thomas, C. G., Miller, A. J., & Westbrook, G. L. (2006). Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons. *Journal of neurophysiology*, *95*(3), 1727-1734.
- Thornberry, N.A.&Lazebnik, Y. (1998).Caspases: enemies within. *Science*, 281(5381), 1312-1316.
- Tovar, K. R., & Westbrook, G. L. (1999). The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. *Journal of Neuroscience*, *19*(10), 4180-4188.
- Tran, T. S., Kolodkin, A. L., &Bharadwaj, R. (2007). Semaphorin regulation of cellular morphology. *Annu. Rev. Cell Dev. Biol.*, *23*, 263-292.
- Traynelis,S.F.,Hartley,M.&Heimann,S.F.(1995).ControlofprotonsensitivityofNMDA receptorby RNA splicing and polyamines. *Science*,268(5212),873-876.

- Van Battum, E. Y., Brignani, S., &Pasterkamp, R. J. (2015). Axon guidance proteins in neurological disorders. *The Lancet Neurology*, *14*(5), 532-546.
- Vyklicky, V., Korinek, M., Smejkalova, T., Balik, A., Krausova, B., Kaniakova, M.,Lichnerova, K., Cerny, J., Krusek, J., Dittert, I., Horak, M.& Vyklicky L.(2014). Structure, function, and pharmacology of NMDA receptor channels. *Physiological research*, *63*,Suppl 1:S191-203.
- Wigström, H., &Gustafsson, B. (1985). On long-lasting potentiation in the hippocampus. a proposed mechanism for its dependence on coincident pre-and postsynaptic activity. *Actaphysiologicascandinavica*, *123*(4), 519-522.
- Winberg, M. L., Noordermeer, J. N., Tamagnone, L., Comoglio, P. M., Spriggs, M. K., Tessier-Lavigne, M., & Goodman, C. S. (1998). Plexin A is a neuronal semaphorin receptor that controls axon guidance. *Cell*, *95*(7), 903-916.
- Yazdani, U., &Terman, J. R. (2006). The semaphorins. *Genome biology*, *7*(3), 211.
- Yermolaieva, O., Brot, N., Weissbach, H., Heinemann, S. H., & Hoshi, T. (2000). Reactive oxygen species and nitric oxide mediate plasticity of neuronal calcium signaling. *Proceedings of the National Academy of Sciences*, *97*(1), 448-453.
- Zhou, Y., &Danbolt, N. C. (2014). Glutamate as a neurotransmitter in the healthy brain. *Journal of neural transmission*, *121*(8), 799-817.