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Zinc-Mediated Regulatory Function of PSD-95 on NMDAR at Post-Synapses

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ZINC-MEDIATED REGULATORY FUNCTION OF PSD-95 ON NMDAR AT POST-SYNPASES

A Thesis

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ABSTRACT

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Postsynaptic density-95 (PSD-95) is a membrane-associated guanylate kinase that mediates localization of receptors in the excitatory postsynaptic density. It has been reported that PSD-95 mediates postsynaptic localization of NMDA receptors and anchors postsynaptic AMPA receptors mainly through its postsynaptic membrane targeting by its N-terminal palmitoylation. Recent studies have shown that Ca^{2+}/calmodulin blocks palmitoylation of PSD-95 by binding at the N-terminus of PSD-95, which promotes dissociation of PSD-95 from the postsynaptic membrane and causes loss of surface AMPARs in cultured neurons. In addition to calcium, zinc is another metal ion found in various areas of the brain. As an endogenous neuromodulator, zinc plays a role in synaptic transmission and is important in the maintenance of postsynaptic density stability. However, whether or not Zn^{2+} regulates PSD-95 palmitoylation and changes NMDA receptors localization remain unknown. This study was carried out in human embryonic kidney 293 (HEK-293) cells expressing PSD-95. The palmitoylation of PSD-95 was assessed using acyl-biotinyl exchange (ABE) method. Our data showed that zinc stimulation decreased PSD-95 palmitoylation by 40%. To identify the

effects of zinc on surface NMDA receptor expression via the regulation of PSD-95, HEK-293 cells were transfected with PSD-95 and NMDAR subunits. Cells were treated with 0.1 mM ZnCl2 for 5 minutes and 10 minutes and the surface expression of NMDA receptors was assessed using a surface expression assay method. The data demonstrated that zinc stimulation did not change the surface expression of NMDA receptors at corresponding incubation periods (5 and 10 minutes). The potential effect of the Zn2+-induced depalmitoylation of PSD-95 will be further studied, such as PSD-95 postsynaptic stability and NMDA receptor postsynaptic localization in response to longer zinc stimulation.

DEDICATION

The completion of my masters' studies would not have been possible without the immense support from my family and friends. I especially want to dedicate this to Maria Isabelle Sanchez and her mother who passed away last year and never stopped believing in me. I also want to thank my parents who always made me put school first and always supported me even when I thought it was not possible. Thank you for always pushing me and never letting me give up on my ambitions.

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CHAPTER I

INTRODUCTION

Chemical Synaptic Transmission

The human brain is a part of the central nervous system (CNS) and is structurally composed of three major segments: the cerebrum, cerebellum, and brainstem. Although this nervous organ tissue has been studied intensively and has been recorded that its main purpose is to form memories, its profound branched structure has yet to be entirely explained (McGilchrist, 2010). There are two forms of cells that can be located in the brain, which are neurons and glia cells. Neuronal cells are found in the CNS (includes the brain and spinal cord) and in the autonomic ganglia; these cells contribute to the transmission of messages throughout different regions of the brain, and among the brain and the rest of the nervous system by the use of chemical and electrical signals (Maldonado *et al.,* 2022). There are various types of neurons that contain their own unique and respective function. In general, there are three universal classes of neuronal cells: sensory, motor and interneurons. Sensory neuronal cells have the ability to segregate between the diverse types of sensation, recognized by sensory organs through an input, from the environment to the brain (Lallemend & Ernfors*,* 2012). As for the motor neurons, they govern voluntary muscle activity and carry information

from nerve cells in the brain to the skeletal or smooth muscles of the body. Interneurons are the most abundant neurons in the body and aid in processing information by integration between sensory and motor neurons or with each other, which in return forms a complex circuit of transmission of information in the brain.

These nerve cells can also be categorized depending on their structural distinctions, such as unipolar, pseudo-unipolar, bipolar, and multipolar. Despite of the structural differences, the morphology of all neuronal types contains the same common parts that is the cell body, the dendrites, the axon, and the axon terminals (Maldonado *et al.,* 2022).

Continually, the brain contains a variety of functions that are mediated through a process known as chemical synaptic transmission. Communication between nerve cells happens through the combination of electrical and chemical signals. The rapid transmission of communication between neurons occurs at synapses, which are gaps between two neuronal cells regions known as the pre-synapse and post-synapse that will allow for chemical transmission (Lovinger, 2008). These small gaps contain an array of properties, such as the neurotransmitter kind, release probability, post-synaptic receptor configuration, and the presence of neuromodulatory receptors (Südhof, 2021).

Neuronal communication

The communicational process involves the suprathreshold excitation of a neuronal cell near the soma that produces an action potential, that will propagate into the axonal arbor allowing the release of chemical messengers, known as neurotransmitters, that relay information from the presynaptic neuron to the postsynaptic cell membrane (containing neurotransmitter receptors)

from axon terminals (Cox & Beatty, 2017). The pre-synaptic portion of the neuron is specifically designed for Ca^{2+} neurotransmitter discharge, and on the other hand, post-synapses are committedly utilized as the neurotransmitter reception of the neuronal cell (Südhof, 2021). The inflowing of calcium after the action potential will generate the exocytotic fusion of synaptic vesicles containing neurotransmitters that will then be released into the synapse and produce a post-synaptic signal (Katz, 1969; Südhof, 2013). A key difference between the two regions of the synapse specializations is that the release machinery principle of the pre-synapse is the same, whereas the post-synapse detects distinct neurotransmitters via diverse specific receptor machineries and postsynaptic densities (PSD)
(Südhof, 2021). The molecular composition of the pre-synapse is autonomous of the type of neurotransmitter exhibited in the post-synapse, with the use of similar proteins and quick Ca^{2+} dependent fusion of synaptic vesicles, in contrast, the post-synapse contains diverse molecular components (Südhof, 2013; Südhof, 2021). The different types of neurotransmitter receptors that are associated within this post-synaptic region (from most to least common) include: tetrameric glutamate receptors (NMDARs, AMPARs, and kainate receptors), pentameric cys-loop receptors (GABAA, glycine, nicotinic acetylcholine and ionotropic serotonin receptors), trimeric P2X (ATP receptors), and metabotropic G protein- coupled receptors (GPCRs) (Südhof, 2021).

Various components work alongside this transmissional mechanism to work effectively, for instance neurotransmitters (glutamate, GABA, glycine, dopamine), synaptic receptors (AMPA & NMDA-glutamate receptors), scaffolding proteins (PSD-95, PSD-94, SAP-102), and the protein-receptor interaction and its association with the postsynaptic membrane at synapses (Zhang *et al*. 2021).

Glutamate Neurotransmitter

Glutamic acid (glutamate) is a non-essential amino acid that is one of the most abundant in nature and is a major excitatory neurotransmitter in the mammalian central nervous system (Moldovan *et al.,* 2021). As a neurotransmitter, glutamate, plays an essential role in physiological and pathological processes. Glutamate is the most recurrent neurotransmitter found in the brain and it is heavily regulated upon its consumption through various mechanisms to prevent excessive or insufficient amounts. One of the major roles of Glu in the CNS is synaptic plasticity, which underlies memory, both (verbal expression, declarative memory) and also implicit (execution of procedures).

It has been recorded that glutamate contains large concentrations in the synapse (~ 100 mM) via synaptic vesicles that are released into the synaptic cleft through a process known as exocytosis, thus playing a role in synaptic plasticity (Meldrum, 2000; Zhou et al. 2014). This particular neurotransmitter can be received by two major types of glutamate receptors (GluRs) that are classified as ionotropic receptors and metabotropic receptors (Moldovan *et al.,* 2021). Ionotropic receptors (iGluRs) are voltage sensitive and are usually coupled with ionic channels, which include N-methyl-D-aspartate (NMDA) receptors, a-amino-3-hydroxy-5-methyl-4-isoxasolepropionic acid (AMPA) receptors, and kainate receptors, these types of receptors are able to facilitate fast excitatory neurotransmission (Dingledine *et al*., 1999; Crupi *et al.*, 2019). On the other hand, metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors (GPCRs) and ligand sensitive that modulate the effects of glutamate neurotransmission at the CNS level (Moldovan *et al*., 2021). These mGluRs contain three major groups of metabotropic receptors that are categorized based on their signal transduction pathways and pharmacological profiles: Group I (mGluR1 and mGluR5), II (mGluR2 and mGluR3) and III (mGluR4 and

mGluR6-8) (Crupi *et al.*, 2019). For this research study, it will focus on the iGluRs specifically the NMDAR molecular composition and its protein-receptor interaction.

Problem Statement

The balance of neurotransmitters and the regulation of the chemical transmission process is crucial for the prevention of neurological disorders. For instance, the excess of glutamate is knowingly associated with excitotoxicity by excitation of cells to their death through an excessive presence of glutamate receptors, bearing a role in the pathogenesis of Alzheimer disease and Parkinoson disease (Lau & Tymianski, 2010; Sheffler et al. 2022). There are many glutamate receptor proteins that were identified to interact with amino acid glutamate neurotransmitter, that is N-methyl-D-aspartate (NMDA) receptors (Gonda 2012; Bonaccorso et al. 2011; Santangelo et al. 2012), a-amino-3-hydroxy-5-methyl-4-isoxasolepropionic acid (AMPA) receptors (Rogawski 2013), kainate receptors (Lerma and Marques 2013) and metabotropic receptors (Gregory et al. 2013; Zhou et al. 2014).

The dynamics of the neuronal network transmissional process is required to be tremendously regulated and must preserve a balance of neurotransmitters for proper function. In the presence of an imbalance in neurotransmitters it can lead to various neurological disorders, such as epilepsy, Alzheimer disease, Parkinson disease and transmission impairments. Even so, the molecular mechanism is not quite understood.

CHAPTER II

REVIEW OF LITERATURE

Protein-Receptor Interactions

NMDA Receptor

N-methyl-D-aspartate receptors (NMDARs) are ionotropic glutamate receptors (iGluR) that play a main role in excitatory neurotransmission of the mammalian central nervous system (CNS). NMDARs moderates the influx of Ca^{2+} which in turn increases the postsynaptic strength causing long-term potentiation (LTP), which has been attributed to the basis of learning and memory (Morris, 2013; Izquierdo, 1994; Rockstroh *et al*., 1996; Scatton *et al*., 1991). NMDARs have been demonstrated to be involved in the development of Alzheimer's disease, Parkinson's disease, schizophrenia, and epilepsy. This tetrameric ionotropic receptor can be found mainly in the post-synaptic membrane but can also be expressed in the presynaptic neuronal membrane. Its overall functional composition entails two necessary GluN1 (GluNR1) subunits with either two GluN2 subunits or a combination of GluN2 (GluNR2) and GluN3 (GluNR2) subunits and require either amino acid, glycine or D-serine, to serve as a co- agonist for expression (Balu, 2016; Paoletti, 2011). Quinolinic acid and kynurenic acid are also known to be an endogenous agonist and antagonist, respectively, of NMDA receptors, and receptor activity is modulated by protons and Zinc. These subunits exist within the receptor in diverse subtypes formed by the variation of seven subunits (GluN1, GluN2A-D and GluN3A-B) into its tetrameric complex (Hansen *et al*, 2018). The NMDA

receptor pore is blocked by Mg^{2+}, which can be released by the depolarization of the membrane potential that accompanies the activation of a-amino-3-hydroxy-5-methyl-4-isoxasolepropionic acid (AMPA) receptors. Hence, the current moderated by the NMDAR is dependent on both the membrane potential and the frequency of synaptic release (Hansen *et al*, 2018). The binding of glutamate to GluN2 subunit and glycine to GluN1 subunit allows the formation of a central cation-permeable channel pore.

Postsynaptic density protein – 95 (PSD-95)

Postsynaptic density protein 95 (PSD-95) is a membrane-associated guanylate kinase (MAGUK) that acts as a scaffolding protein to stabilize synaptic expression of NMDARs and serves an essential role in synaptic plasticity. PSD-MAGUKs are the most abundant scaffolding proteins at central excitatory synapses, including PSD-95, PSD-93 and SAP102. This protein family plays a key role in regulating synaptic structure and plasticity. Each member contains similar structures such as the PDZ domains, one SH3 domain and one GK domain. Each specific protein of this family has unique properties and distinct expression patterns, but all serve similar roles. For instance, in terms of AMPA synaptic trafficking, all PSD-MAGUKs demonstrate to have overlapping roles (Levy *et al.,* 2015).

PSD-95 plays an important role in scaling down, which is a response towards an elevation of neuronal activity expressed by down-regulation of synaptic ionotropic receptor abundance. This protein structure involves three PDZ domains, SRC Homology 3 Domain (SH3), and Guanylate Kinase domain (GK). In the tetrameric structure of NMDA receptors, the GluN2A/GluN2B C-terminal carry a ESDV motif that exhibits a high affinity towards PSD-95 PDZ domains one and two (Patriarchi *et al*. 2018). PSD-95 is palmitoylated at two cysteine residues (C3 and C5) at the N-terminus, which is required for its postsynaptic localization.

Palmitoylation refers to a universal and important lipid modification, it is involved in various cellular processes, such as regulating membrane trafficking, protein stability and protein aggregation (Hu *et al*. 2011). It has also been discovered that protein palmitoylation plays an essential role in neuronal development and synaptic plasticity (Fukata *et al*. 2010).

Research studies have shown that palmitoylation of PSD-95 is blocked by the binding of calmodulin (CaM) to PSD-95, which prompts an increase in Ca^{2+} influx (Zhang *et al*, 2014). The determination of the role of PSD-95-CaM binding in controlling PSD-95 synaptic abundance impacting AMPAR downregulation in chemical long-term depression (cLTD) was recorded to have a significant reduction in surface levels of GluA1 after NMDA treatment (Chowdhury *et al*. 2019.) The results revealed that PSD-95-Ca^{2+}/CaM interaction reinforced the removal of PSD-95 and AMPARs from synapses by the reduction of PSD-95 palmitoylation (Chowdhury *et al*., 2019). However, the mechanism of PSD-95 for postsynaptic NMDARs differential targeting, localization and functions remains unclear.

Zinc Regulatory effects

Zinc is an essential micronutrient that accumulates in the brain and is required for normal development and function (Takeda, 2001). Zn^{2+} has been reported to directly inhibit NMDA receptor by two separate mechanisms: high affinity binding to the N-terminal domains of GluN2A subunits reducing the channel opening probability and low affinity voltage dependent binding to pore lining residues that blocks the channel (Amico-Ruvio *et al*. 2011). The regulatory function of PSD-95 with the effect of Zn^{2+} has yet to be explored. We hypothesize that zinc changes PSD-95 palmitoylation level which regulates surface NMDA receptors expression. This can be achieved by addressing the following two specific aims:

Aim I: Evaluate the effects of Zn^{2+} on the regulation of PSD-95 palmitoylation.

Aim II: Determine the involvement of Zn^{2+} in the NMDA receptor surface expression.

CHAPTER III

METHODOLOGY AND FINDINGS

Methodology

Cell culture

The use of human embryonic kidney 293 (HEK 293) cells was implemented in this project. The HEK 293 cell line has been used to overexpress the target protein particularly PSD-95 and NMDA receptors. The cell culture medium (complete medium) used is Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). These cells were cultured in 100 mm Falcon™ Standard Tissue Culture Dishes.

Cell Transfection

Cells are plated in 60 mm tissue cell culture dishes 24 hrs prior to cell density reaching ~90% confluency. The cells were transfected using LipoD293 (SigaGen) as described in the manufacture's instruction. Wild type PSD-95 plasmid is a gift of Dr. J.W. Hell, (University of California, Davis) and plasmids of NMDAR subunits are gifts of Dr. X-M Yu (Florida State University). LipoD293 mixture is composed of 250 ul of DMEM and 15 ul of LipoD293 reagent. The DNA mixture is composed of 250 ul of DMEM and 5 ug of DNA. The LipoD293 mixture will be decanted onto the DNA mixture, not in reverse order. This two will be vortexed and spun down briefly. Allow the mixture to rest for only 15-20 min at room temperature (RT) to allow

DNA-LipoD293 reagent complexes to form. Add 500 ul of DNA-LipoD293 reagent to each 60 mm HEK293 subculture dishes. Incubating for 5 hrs, a change of cell medium to fresh complete medium will be necessary. After the change is made the dishes will be incubated for 24-48 hrs in 37ºC, 5% CO_2 incubator.

Palmitoylation Assay

PSD-95 palmitoylation is assessed using the acyl-biotinyl exchange (ABE) method as described previously (Chowdhury *et al*. 2019). After the incubation period of transfection is complete, cells are introduced with 0.1 uM- 0.5 uM of $ZnCl_2$ and collected at different time-points, such as 5 min, 10 min and 15 min; the control is added with PBS. The cells are rinsed with PBS and lysed in 0.1 ml of lysis buffer (LB), composed of 150 mM of NaCl, 50 mM pH 7.5 Tris-HCl and 5 mM of EDTA, containing 2% SDS and N-ethyl-maleimide (NEM) (that will block free thiols). Sonication agitation is performed briefly. It will be kept at 37ºC for 15 min, after the extraction at 37ºC, the samples are then diluted 10-fold with LB that contained 2% Triton X-100 and 25 mM of NEM and incubated to nutate the cell lysate for 1 hr at 4ºC in rotator. Lysates are centrifuged at 13,000 rcf for 10 min, and the supernatant is transferred to a 15 ml centrifugation tube. chloroform-methanol (CM) precipitation is performed, and the protein pellets are then dissolved in 0.2 ml of 50 mM pH 7.5 Tris-HCl, 5 mM EDTA, 4% SDS, and water (4SB) at 37ºC for 10 min. Protein is then diluted five-fold with LB contain 0.2% of Triton X-100 and 1 mM NEM and incubated at 4ºC overnight in rotator. Three sequential CM precipitation is followed to remove any excess NEM, then 0.24 ml of 4SB is added. This will cleave thioester bonds and it will allow for synthesis of a biotin moiety. Each of the samples is diluted five-fold in 0.7 M hydroxylamine (HA), pH 7.4 0.2% Triton X-100, 1.5 uM pepstatin A, 2.1 uM leupeptin, 0.3 uM aprotinin, 0.2 mM PMSF (HB) containing the 1 mM HPDP-biotin.

Without hydroxylamine, palmitate groups are not removed to prevent biotinylation. The mixture in sample are incubated at RT for 1 hr in rotator and followed by CM precipitation. The protein is precipitated and solubilized in 4SB buffer and diluted five-fold with LB containing 0.2% Triton X-100 and 0.2 mM HPDP-biotin. Incubation for 1 hr at RT in rotator is exhibited. The unreactive biotin is removed by the two sequential CM precipitation, the pellets are then solubilized in 0.1 ml of PBS, pH 7.2, 2% SDS, 5 mM EDTA (2SB). Once the protein pellets are solubilized in 2SB, two-fold dilution in LB composed of 0.2% Triton X-100 10% of each sampled is recorded as input to assess the total expression. Further dilution is used by five-fold in LB (0.2% Triton X-100) and incubated for 30 min at RT in rotator. Brief centrifugation is concluded, and the supernatant is incubated with 40 ul of agarose beads for 90 min at RT in rotator to isolate the biotinylated proteins. Agarose beads are washed with LB (0.2% Triton X-100 and 0.2% SDS), the bound proteins were eluted with 50 ul of SDS-PAGE sample buffer at 95ºC for 5 min. The samples that were eluates and inputs are ready for SDS-PAGE and analyzed by Wester blotting.

Surface Expression Assay

Surface NMDAR expression assay is performed as described previously (Fang, et al, 2015) In the assay, the cell surface proteins were biotinylated with cell impenetrable non-cleavable EZ-Link Sulfo-NHSLC-LC-Biotin (Pierce, Appleton, WI). The cultured cells without treatment or treated with Zinc are incubated with cold PBS consisting of 1mg/ml bioting for 1 hr at 4ºC. Three sequential cold PBS washes removed the unbound biotin. The cells were then harvested in 1.5 ml of cold PBS with a rubber policeman and lysed in 400 µl of cold cell LB containing 50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1 % Igepal CA-630, 0.5 % sodium deoxycholate, 0.1 % SDS, 2 mM EDTA, 1 mM sodium orthovanadate, and the protease

inhibitors Pepstatin A (20 μg/ml), Leupeptin (20 μg/ml), Aprotinin (20 μg/ml) and Phenylmethylsulfonyl fluoride (1 mM). The homogenates were centrifuged at 20,000× g for 5 min at 4 °C. The supernatant (300 μl) was then incubated with NeutrAvidin agarose (Thermo Scientific, Rockford, lL) to precipitate biotinylated protein. The NMDA receptor proteins expressed on cell surface were determined through detection of NMDAR proteins in pull downed proteins with NeutrAvidin agarose.

Western Blot Analysis

The gel used for SDS-PAGE and Western blot analysis are 4-20% Mini-PROTEAN® TGX™ Precast Protein Gels (BioRad). The filters are probed by corresponding primary antibodies and horseradish peroxidase conjugated secondary antibodies. The primary antibodies that are utilized in this experiment are: rabbit anti-PSD-95 (Cell Signaling Technology), anti-GluN2A (BD Pharmingen), anti-GluN2A C-terminal (BD Pharmingen), anti-GluN2A N-terminal (Santa Cruz Biotechnology, Inc.), and mouse anti-actin (Santa Cruz Biotechnology, Inc.) antibodies. Chemiluminescence signals are visualized by Pierce ECL Western Blotting Substrate (ThermoFisher Scientific) and captured by iBright CL1500 (ThermoFisher Scientific). Immunoreative bands are quantitated using NIH-imageJ software (NIH, Bethesda, ML).

Statistical analysis

SPSS software Amos was used for statistical analysis and all data were presented as the mean ± SEM (≥ four replicates). Student's t-test was used. $P<0.05$ was considered to indicate a statistically significant difference.

Findings

Zinc negatively regulates PSD-95 palmitoylation

48-hour post-transfection of PSD-95 plasmid, HEK-293 cells were treated with 0.1 mM $ZnCl_2$ for 5 minutes. Cells were then harvested and palmitoylation of PSD-95 was assessed using the acyl-biotinyl exchange (ABE) method and Western blot analysis. The Western blot analysis demonstrated a decrease in PSD-95 palmitoylation level in response to 0.1 mM $ZnCl_2$ stimulation for 5 minutes than the control group (Figure 1). PBS was used in the control group in place of the ZnCl2. Hydroxylamine (NH_2OH) served to detect palmitoylation of PSD-95. This chemical releases the thioester-linked palmitoyl moieties and unmasks of free palmitoylated thiol groups (-SH) that can be labeled by biotion and separated by streptavidin pulldown. Tris was used in the negative control, and it could not break the thioester bond and release free thiol groups. Therefore, PSD-95 palmitoylation could not be detected in negative control group. This observation of decrease of palmitoylation level in PSD-95 was then quantified and compared with control group using the palmitoylation/total protein expression ratio. It was determined that there was a significant reduction in palmitoylation levels of PSD-95 of about 40% after treatment of zinc for 5 minutes when compared to the control (Figure 2).

H28A PSD-95 MT demonstrated no change in PSD-95 palmitoylation induced by zinc stimulation

Our previous analysis (Figure 3) has shown that PSD-95 N-terminal domain contains two conserved cysteines (Cys^3 and Cys^5) and two conserved histidines (His^{24} and His^{28}). The four residues form a Cys2His2-type zinc figure motif and are essential for zinc to bind with PSD-95 N-terminal. In this study, HEK-293 cells were transfected with PSD-95 and PSD-95 H28A mutant plasmids. We are interest if this mutation could affect PSD-95 palmitoylation at

its adjacent palmitoylation sites (Cys^3 and Cys^5). Cells were treated with 0.1 mM $ZnCl_2$ for 5 min and PBS was used in the control group. Following the treatment, cells were harvested and the palmitoylation of PSD-95 was assessed using ABE method. Our findings demonstrated a slight decrease in the palmitoylation level of PSD-95 H28A MT after zinc treatment compared to PSD-95 wild type (Figure 4). Quantification of Western blot analysis demonstrated that the zinc stimulation did not change palmitoylation level of PSD-95 H28A mutant that of control (Figure 5). Therefore, the palmitoylation presence in the PSD-95 WT after zinc stimulation, showed a greater significant change (decrease palmitoylation levels) than that of PSD-95 H28A MT (Figure 2). This suggests that PSD-95 WT provided more zinc binding than PSD-95 H28A MT.

Surface NMDA receptor expression did not show significant change after short-term zinc treatment

PSD-95 interacts with NMDA receptors at its C-terminal and maintains NMDA receptors surface localization. Since PSD-95 surface anchoring controlled by N-terminal palmitoylation can be regulated by zinc, we next continued to study if zinc simulation can change NMDA receptor surface expression via PSD-95 depalmitoylation. In this study, HEK-293 cells were transfected with PSD-95 and NMDA (GluN1 and GluN2A) subunit plasmids. Tissue culture dishes were coated with Poly-D-Lysine (PDL) prior to seeding to facilitate better cell attachment. 48-hour post-transfection, the cells were stimulated with 0.1 mM $ZnCl_2$ for 5 minutes and 10 minutes. Following the stimulation, cells were harvested and surface expression of NMDA receptors was assessed using the surface expression assay and Western blot analysis. PBS was used in the control group in place of the $ZnCl_2$. The samples were probed with GluN1 for western blot analysis and clear sharp bands were detected for the pulldown samples but not

for the input samples (Figure 6). It was also seen that pulldown samples of GluN1 did not show a significant change between different treatments. To understand the unclear signals from the input samples, we tested different antibodies against NMDA receptors, including anti- GluN1 antibody (BD Pharmingen), anti-GluN2A C-terminal antibody (BD Pharmingen), and anti-GluN2A N-terminal antibody (Santa Cruz Biotechnology, Inc.). GluN1 subunit molecular weight is about 120 kDa and GluN2A subunit molecular weight is about 170 kDa. Samples used in the troubleshooting were cells transfected with GluN1 subunit, GluN2A subunit, and PSD-95 plasmids in cell culture dishes with or without Poly-D-Lysine (PDL) coating. As shown in Figure 7, GluN1 and GluN2A subunits were detected in the three blots Figure 7A-C. Figure 7A showed GluN1 subunit and Figure 7B and 7C showed GluN2A subunit. Among the three antibodies, anti-GluN2A C-terminal antibody provided the best signals and were used in the later NMDA receptor surface assay study. Also, samples that did not have PDL coating prior to cell seeding showed weaker protein expression than those with PDL coating samples. This might be caused by more cell death due to poor cell attachment. Based on the data collected from the Western blot analysis, it was determined that anti-GluN2A C-terminal antibody was used to assess the surface NMDA receptor expression. The samples were probed with anti-GluN2A C-terminal antibody and bands were detected in both input and pulldown samples (Figure 8). A quantitative analysis was then done based on the pulldown (surface)/input (total) ratio and it was determined that there were no significant changes of NMDA receptor surface expression following zinc stimulation for 5 minutes and 10 minutes (Figure 9).

CHAPTER IV

DISCUSSION

Postsynaptic density-95 (PSD-95) is a membrane-associated guanylate kinase that mediates localization of receptors in the excitatory postsynaptic density. PSD-95 mediates postsynaptic localization and stability of NMDARs through direct interactions with the receptors and N-terminal palmitoylation-regulated membrane association. Zinc is an important trace metal and plays a key role in synaptic plasticity. Pre-dominantly free zinc can be found in synaptic vesicles within presynaptic terminals. Zinc can be released from pre-synapses and interact with proteins located at post-synapses. In our study we investigated if zinc could regulate PSD-95 postsynaptic localization. Our data showed that zinc stimulation significantly decreased PSD-95 palmitoylation by 40% when compared to control. Continually, the histidine mutations at PSD-95 N-terminal did not change the palmitoylation modification reduction upon stimulation of $ZnCl_2$. PSD-95 N-terminus contains two palmitoylation sites (Cys^3 and Cys^5) that form a putative zinc finger (Cys2His2-type) motif with two adjacent histidines. These two histidines (H24, H28) on PSD-95 are critical residues for Zn^{2+}-binding and were chosen to create mutations that could aid in further determining the effects of zinc on PSD-95 palmitoylation. The results demonstrated that the Zn^{2+}- regulation of PSD-95 palmitoylation levels was demonstrated in wildtype, which in histidine mutants (H28A) provided additional evidence for this Zn^{2+}

regulatory effect on PSD-95 when compared to wildtype. We presume that the reason for the regulation of Zn^{2+} is due to the
higher binding affinity that PSD-95 WT had towards zinc than H28A MT. Suggesting that zinc binding to PSD-95 shifts towards its depalmitoylation state.

NMDA receptors are glutamate excitatory receptors and are localized to the postsynapses. PSD-95 regulates NMDARs at postsynaptic membranes via direct interaction of its PDZ domains with NMDARs C-terminal tSXV motifs. In this study, the effect of zinc on NMDA receptor localization mediated by PSD-95 interaction was further studied. Our data showed that treatment of 0.1 mM of $ZnCl_2$ for 5 minutes and 10 minutes did not produce significant change in NMDA receptor surface expression. This might be due to longer and sufficient time is required for the NMDA receptors to respond to the zinc stimulation induced PSD-95 depalmitoylation. In the future, this experiment will be carried out by increasing zinc stimulation time and NMDA receptor surface expression will be analyzed following extended zinc stimulation. We are expecting our study will ultimately provide a better understanding of zinc inhibitory effects on surface NMDA receptor expression mediated by PSD-95 depalmitoylation.

This study provides a molecular mechanism for understanding PSD-95 postsynaptic targeting and regulatory function of NMDARs controlled by the PSD-95 N-terminal interaction with Zn^{2+} and reveals the potential molecular targets for therapy and prevention of neurological diseases. In the future, the potential effect of the Zn^{2+}-induced

depalmitoylation of PSD-95 will be further studied, such as PSD-95 postsynaptic stability and PSD-95 postsynaptic localization. More work needs to be done to unveil the mechanism underlying the impact of the depalmitoylation of PSD- 95 on the postsynaptic localization of NMDARs and AMPARs in response to zinc stimulation.

REFERENCES

Amico-Ruvio, S. A., Murthy, S. E., Smith, T. P., & Popescu, G. K. (2011). Zinc effects on NMDA receptor gating kinetics. *Biophysical journal*, *100*(8), 1910-1918.

Andersen, J. V., Markussen, K. H., Jakobsen, E., Schousboe, A., Waagepetersen, H. S., Rosenberg, P. A., & Aldana, B. I. (2021). *Glutamate metabolism and recycling at the excitatory synapse in health and neurodegeneration. Neuropharmacology, 196, 108719.*

Balu, D. T. (2016). The NMDA receptor and schizophrenia: from pathophysiology to treatment. In *Advances in Pharmacology* (Vol. 76, pp. 351-382). Academic Press.

Chowdhury, D., & Hell, J. W. (2019). Ca2+/calmodulin binding to PSD-95 downregulates its palmitoylation and AMPARs in long-term depression. *Frontiers in synaptic neuroscience*, *11*, 6.

COX, C. L.; BEATTY, J. A. (2017). *The multifaceted role of inhibitory interneurons in the dorsal lateral geniculate nucleus. Visual Neuroscience, 34(), E017.*

Crupi, R.; Impellizzeri, D.; Cuzzocrea, S. (2019). *Role of Metabotropic Glutamate Receptors in Neurological Disorders. Frontiers in Molecular Neuroscience, 12(), 20–.*

Dingledine, R., Borges, K., Bowie, D., Traynelis S.F. (1999). The glutamate receptor ion channels. Pharmacol Rev. ;51(1):7-61.

Francois Lallemend; Patrik Ernfors (2012). *Molecular interactions underlying the specification of sensory neurons. 35(6).*

Fukata, Y., & Fukata, M. (2010). Protein palmitoylation in neuronal development and synaptic plasticity. *Nature Reviews Neuroscience*, *11*(3), 161-175.

Hansen, K. B., Yi, F., Perszyk, R. E., Furukawa, H., Wollmuth, L. P., Gibb, A. J., & Traynelis, S. F. (2018). Structure, function, and allosteric modulation of NMDA receptors. *Journal of General Physiology*, *150*(8), 1081-1105.

Hu, L. L., Wan, S. B., Niu, S., Shi, X. H., Li, H. P., Cai, Y. D., & Chou, K. C. (2011). Prediction and analysis of protein palmitoylation sites. *Biochimie*, *93*(3), 489-496.

Katz, B. (1969). *The Release of Neural Transmitter Substances.* Liverpool Univ Press; Liverpool.

Krall, R. F.; Moutal, A.; Phillips, M. B.; Asraf, H.; Johnson, J. W.; Khanna, R.; Hershfinkel, M.; Aizenman, E.; Tzounopoulos, T. (2020). *Synaptic zinc inhibition of NMDA receptors depends on the association of GluN2A with the zinc transporter ZnT1. Science Advances, 6(27).*

Lau A, Tymianski M. (2010). Glutamate receptors, neurotoxicity and neurodegeneration. Pflugers Arch. ;460(2):525-42.

Levy, J. M., Chen, X.; Reese, T. S., Nicoll, R. A. (2015). *Synaptic Consolidation Normalizes AMPAR Quantal Size following MAGUK Loss. Neuron, 87(3), 534–548.*

Lovinger, D. M. (2008). "Communication networks in the brain: neurons, receptors, neurotransmitters, and alcohol." *Alcohol research & health: the journal of the National Institute on Alcohol Abuse and Alcoholism* vol. 31,3; 196-214.

Maldonado KA, Alsayouri K. Physiology, Brain. (2021). In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing.

McGilchrist I. Reciprocal organization of the cerebral hemispheres. Dialogues Clin Neurosci. 2010;12(4):503-15.

Meldrum, B. S. (2000). "Glutamate as a neurotransmitter in the brain: review of physiology and pathology," *Journal of Nutrition*, vol. 130, no. 4, pp. 1007S–1015S.

Moldovan, O.-L., Rusu, A., Tanase, C., & Vari, C.-E. (2021). *Glutamate - A multifaceted molecule: Endogenous neurotransmitter, controversial food additive, design compound for anti-cancer drugs. A critical appraisal. Food and Chemical Toxicology, 153, 112290.*

Morris, R. G. (2013). NMDA receptors and memory encoding. *Neuropharmacology*, *74*, 32-40.

Patriarchi, T., Buonarati, O. R., & Hell, J. W. (2018). Postsynaptic localization and regulation of AMPA receptors and Cav1. 2 by β2 adrenergic receptor/PKA and Ca2+/CaMKII signaling. *The EMBO journal*, *37*(20), e99771.

Pierre, P. (2011). *Molecular basis of NMDA receptor functional diversity. , 33(8), 1351–1365.*

Rockstroh S, Emre M, Tarral A, Pokorny R. (1996). Effects of the novel NMDA-receptor antagonist SDZ EAA 494 on memory and attention in humans. Psychopharmacology. 124:261–266.

Scatton B, Carter C, Benavides J. (1991). NMDA receptor antagonists: treatment of brain schemia. Drug News Persp. 4:89–102.

Sheffler ZM, Reddy V, Pillarisetty LS. Physiology, Neurotransmitters. (2022). In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing.

Südhof, T. C. (2021). "The cell biology of synapse formation." *The Journal of cell biology* vol. 220,7: e202103052.

Südhof, T. C. (2013). "Neurotransmitter release: the last millisecond in the life of a synaptic vesicle." *Neuron* vol. 80,3: 675-90.

Takeda, A. (2001). Zinc homeostasis and functions of zinc in the brain. *Biometals*, *14*(3-4), 343-351.

Victor, A., Li-Huei, T., Jürgen G., (2016). "Glutamate Receptors in Alzheimer's Disease: Mechanisms and Therapies", Neural Plasticity, vol. 2016, Article ID 8256196, 2 pages.

Zhang, Y., Matt, L., Patriarchi, T., Malik, Z. A., Chowdhury, D., Park, D. K., ... & Hell, J. W. (2014). Capping of the N-terminus of PSD-95 by calmodulin triggers its postsynaptic release. *The EMBO journal*, *33*(12), 1341-1353.

Zhang Y, Fang X, Ascota L, Li L, Guerra L, Vega A, et al. (2021). Zinc- chelating postsynaptic density-95 N-terminus impairs its palmitoyl modification. *Protein Science*, 1–12.

Zhou Y, Danbolt NC. (2014). Glutamate as a neurotransmitter in the healthy brain. *J Neural Transmitter (Vienna)*. 121(8):799

Izquierdo I.(1994). Pharmacological evidence for a role of long-term potentiation in memory. FASEB J. 8:1139–1145

Griesius, S., O'Donnell, C., Waldron, S., Thomas, K. L., Dwyer, D. M., Wilkinson, L. S., Hall, J., Robinson, E. S. J., Mellor, J. R., (2022). Reduced expression of the psychiatric risk gene DLG2 (PSD93) impairs hippocampal synaptic integration and plasticity, Neuropsychopharmacology, 10.1038/s41386-022-01277-6

APPENDIX

APPENDIX

FIGURES

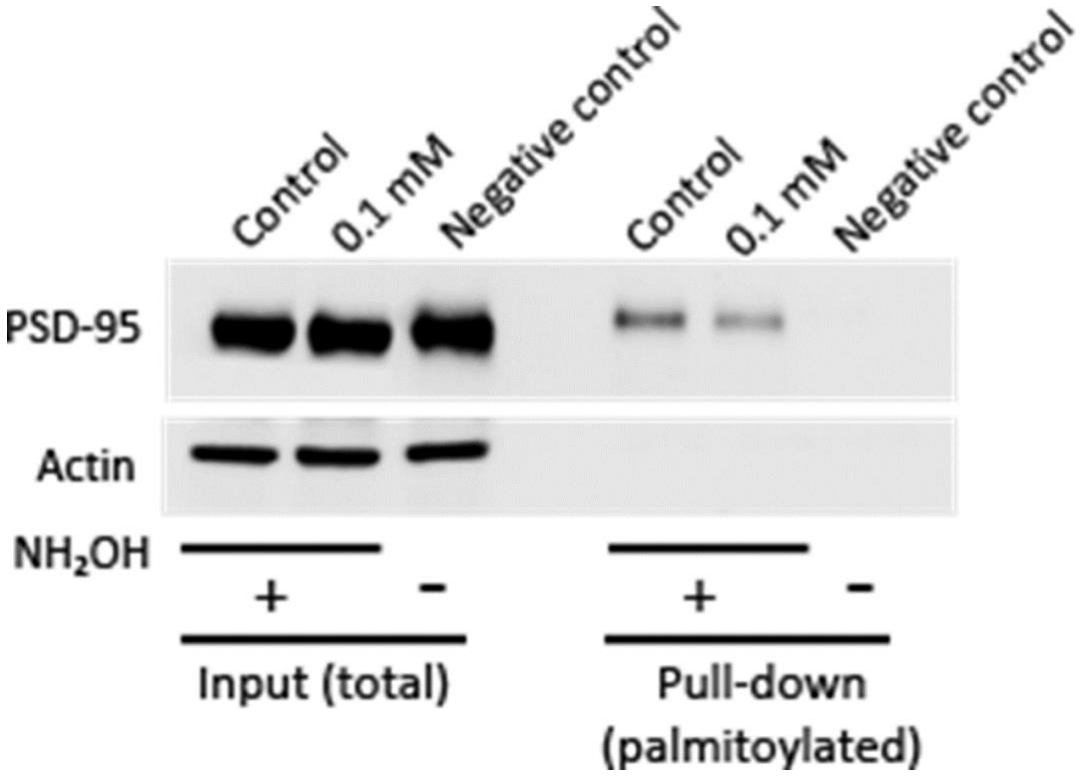

Figure 1. Western Blot analysis of PSD-95 palmitoylation with zinc treatment. Cells were transfected with PSD-95 plasmids. After incubation for 48 hours, the cells were stimulated with 0.1 mM $ZnCl_2$ for 5 min. And then the cells were harvested and the palmitoylation of PSD-95 was assessed using acyl-biotinyl exchange (ABE) method and Western blot. PBS was used in the control group in place of the $ZnCl_2$. NH_2OH serves to release thioester-linked palmitoyl moieties and restores the modified cysteine to thiols.

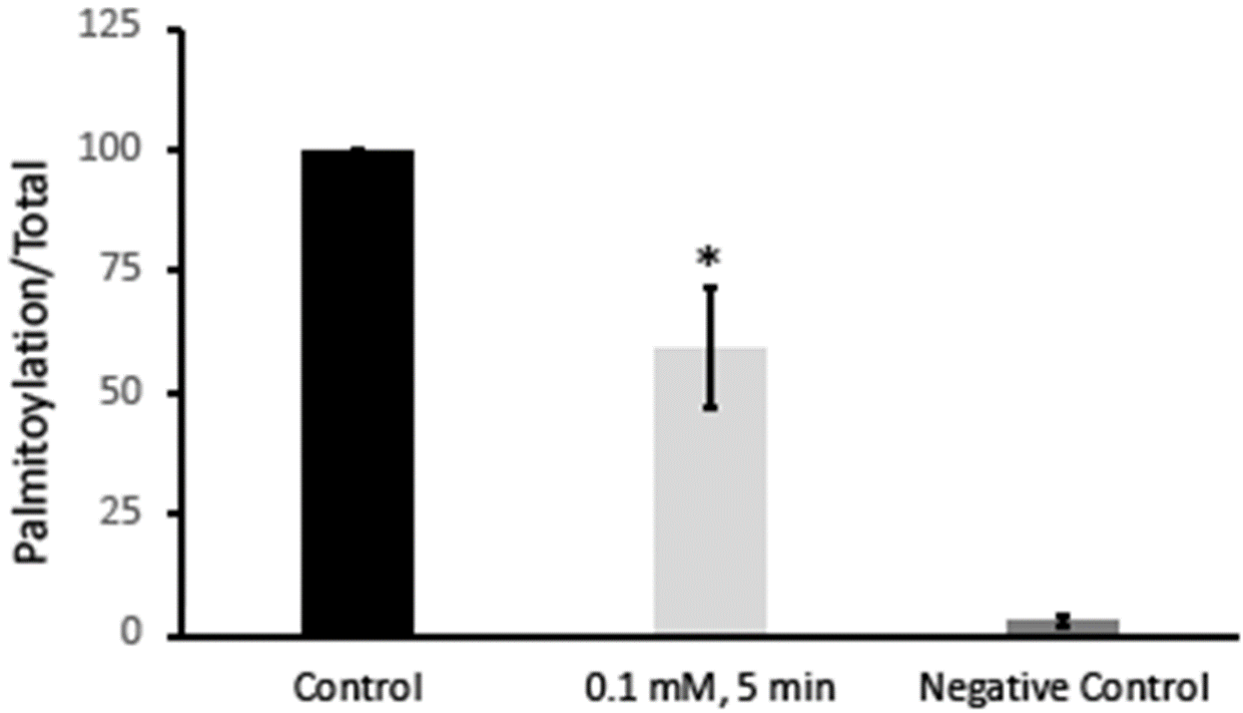

Figure 2. Quantitative analysis of Western blot PSD-95 palmitoylation with zinc treatment. Cells were transfected with PSD-95 plasmids. After incubation for 48 hours, the cells were stimulated with 0.1 mM $ZnCl_2$ for 5 min. And then the cells were harvested and the palmitoylation of PSD-95 was assessed using acyl-biotinyl exchange (ABE) method and Western blot. PBS was used in the control group in place of the $ZnCl_2$. NH_2OH serves to release thioester-linked palmitoyl moieties and restores the modified cysteine to thiols. Data represent the mean ± S.E.M of three replicates. * $p < 0.05$.

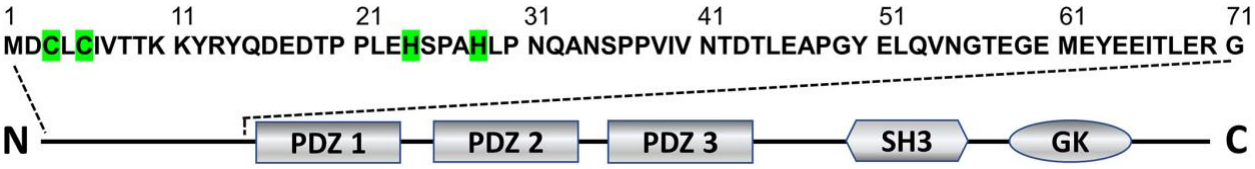

Figure 3. Schematic diagram of PSD-95 and its N-terminal amino acid sequence (1–71). Zhang et al., 2021.

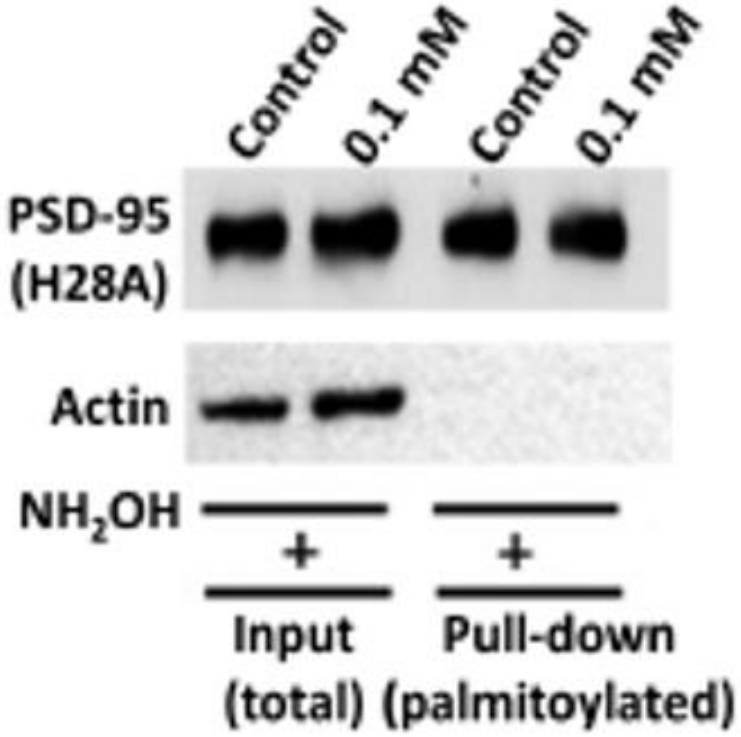

Figure 4. Western Blot analysis of PSD-95 palmitoylation with zinc treatment including MTs. HEK293 cells were transfected with PSD-95 mutant H28A plasmids. After incubation for 48 hours, the cells were stimulated with 0.1 mM $ZnCl_2$ for 5 min and extracted. PBS was used in the control group in place of the $ZnCl_2$. Cells were then harvested and the palmitoylation of PSD-95 was assessed using acyl-biotinyl exchange (ABE) method.

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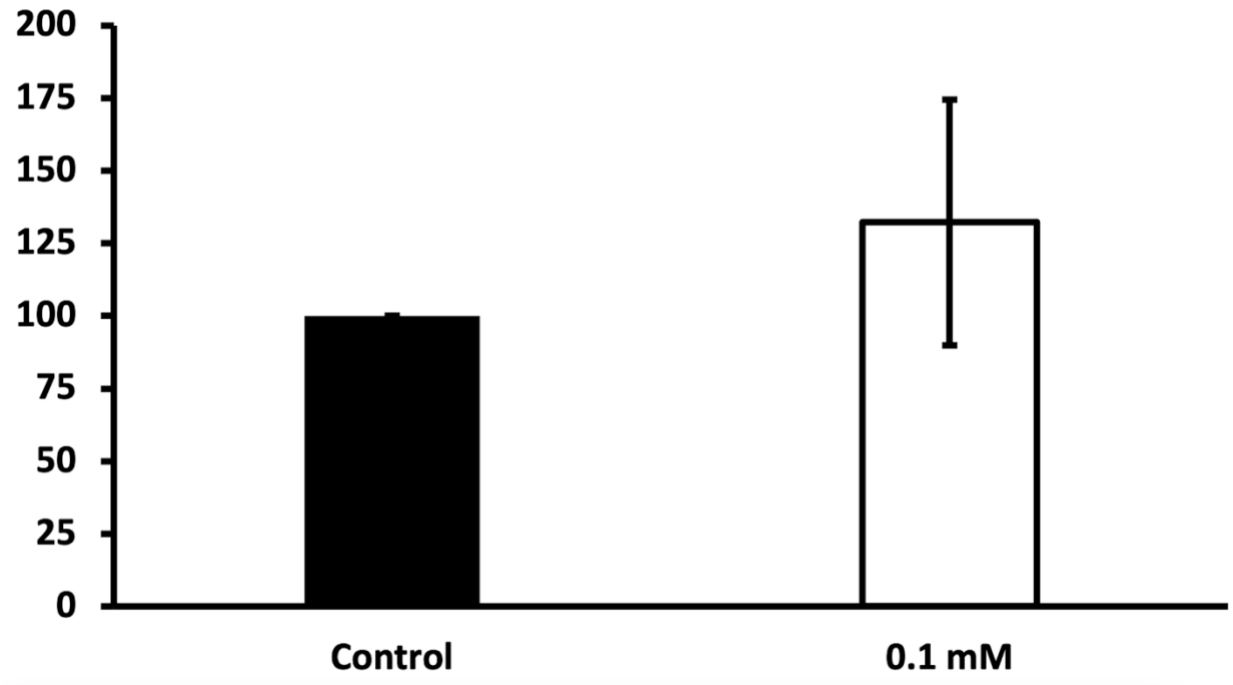

Figure 5. Quantitative analysis of PSD-95 palmitoylation with zinc treatment of PSD-95 H28A MT. Cells were transfected with PSD-95 (H28A) MT plasmid. After incubation for 48 hours, the cells were stimulated with 0.1 mM $ZnCl_2$ for 5 minutes. The cells were harvested and the palmitoylation of PSD- 95 MT was assessed using acyl-biotinyl exchange (ABE) method and Western blot. PBS was used in the control group in place of the $ZnCl_2$.

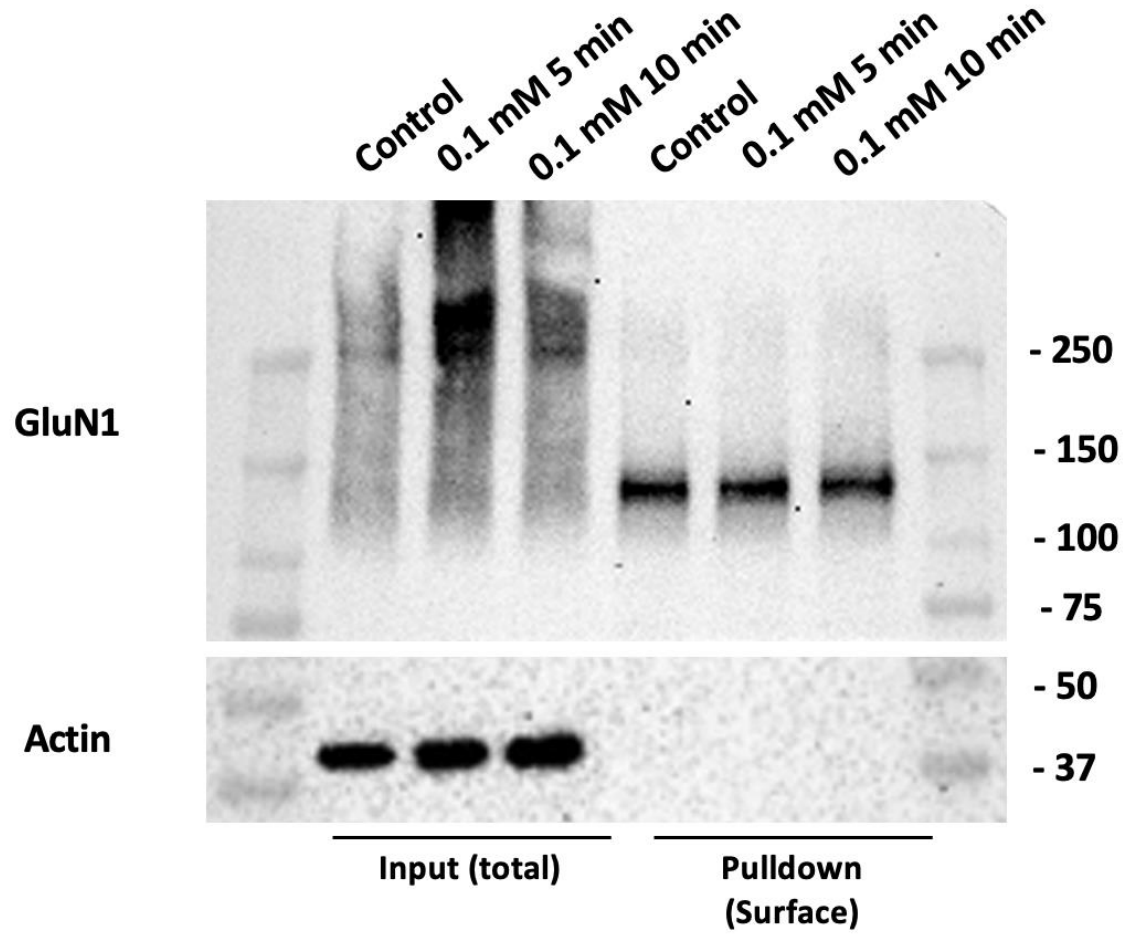

Figure 6. Western Blot analysis of Surface NMDA receptor Expression (GluN1) with 0.1 mM zinc treatment. The top blot was probed using anti-GluN1 antibody. HEK-293 Cells were transfected with GluN1 subunit, GluN2A subunit, and PSD-95 plasmids. After incubation for 48 hours, the cells were stimulated with 0.1 mM $ZnCl_2$ for 5 minutes and 10 minutes. Cells were harvested and the surface expression of NMDA receptor was assessed using surface expression assay method and Western blot. PBS was used in the control group in place of the $ZnCl_2$. The bottom blot was probed with anti-actin antibody.

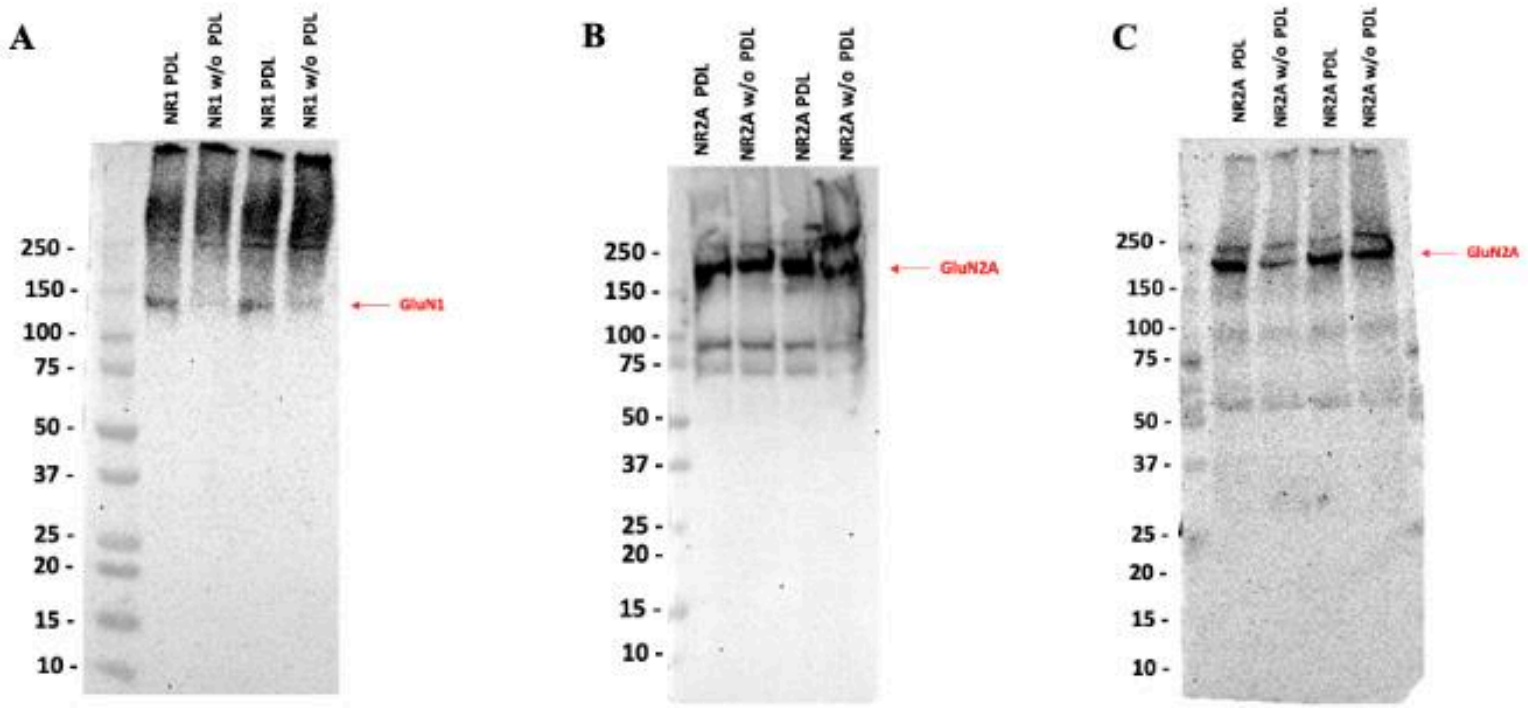

Figure 7. Western blot analysis of GluN1 and GluN2A troubleshooting antibodies. GluN1 (Fig. 7A), GluN2A C-terminal (Fig. 7B) and GluN2A N-terminal (Fig. 7C). Poly-D-Lysine (PDL) was used prior to seeding to facilitate better cell attachment. PDL-coated dishes showed better expression of NMDA receptors. Molecular weight, GluN1 subunit ~120 kDa, GluN2A subunit ~170 kDa.

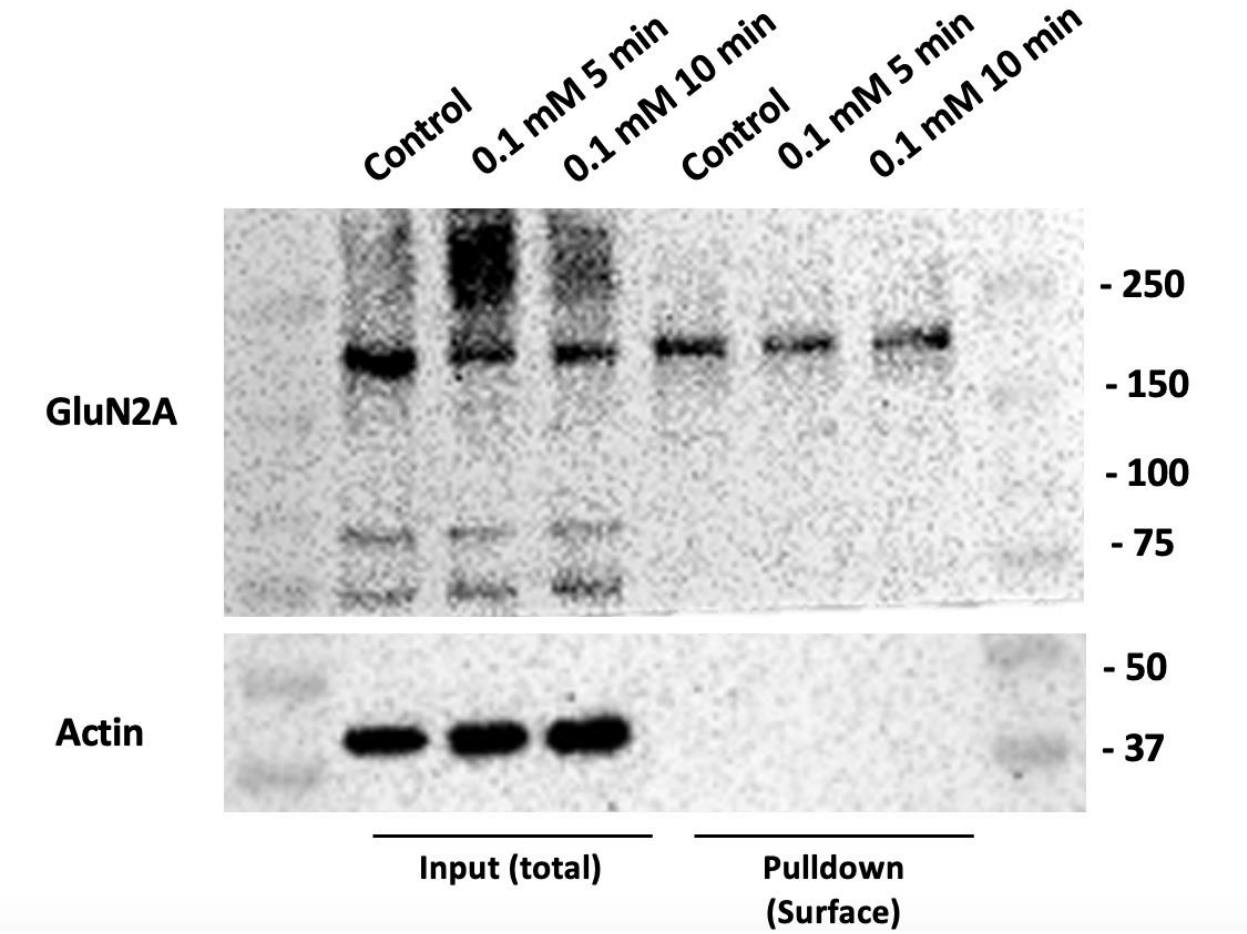

Figure 8. Western Blot analysis of Surface NMDA receptor Expression (GluN2A) with 0.1 mM zinc treatment the top blot was probed using anti-GluN2A C-terminal antibody. HEK-293 cells were transfected with GluN1 subunit, GluN2A subunit, and PSD-95 plasmids. After incubation for 48 hours, the cells were stimulated with 0.1 mM $ZnCl_2$ for 5 minutes and 10 minutes. Cells were harvested and the surface expression of NMDA receptors was assessed using surface expression assay method and Western blot. PBS was used in the control group in place of the $ZnCl_2$. The bottom blot was probed with anti-actin antibody.

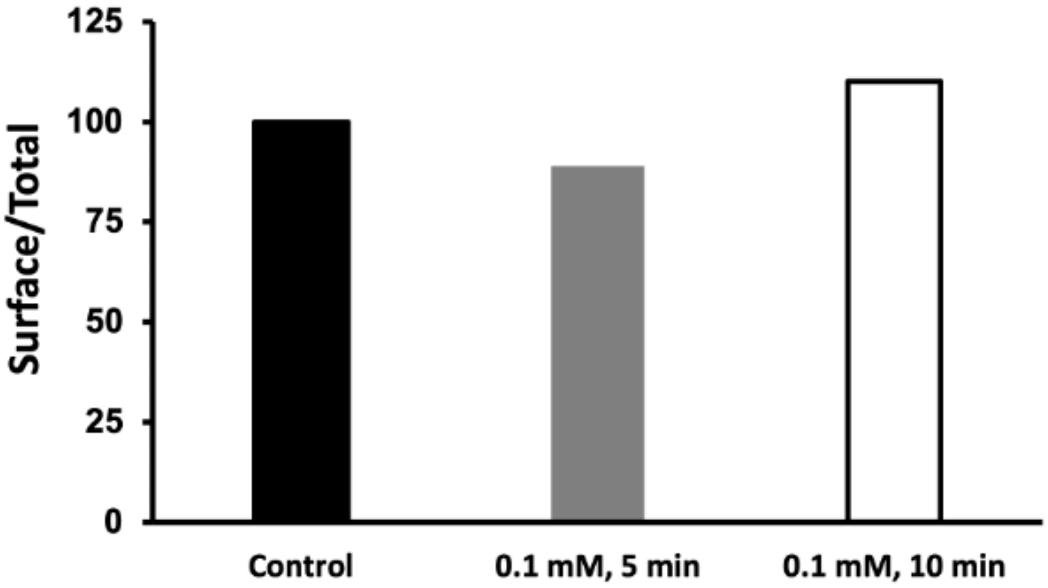

Figure 9. Quantitative analysis of surface expression assay of NMDA receptors after zinc treatment. The surface/total protein expression ratio was taken to determine the quantification of the western blot analysis.

BIOLOGICAL SKETCH

The author, Luis Fernando Acosta Gloria was born on May 20, 1998, in the state of Tamaulipas, Mexico. He is the middle child of his two siblings, who have played an essential role in his life.

Luis's family was all born in Mexico, except for his youngest sibling, and during the early 2000's, as a form of better education and job opportunities, his family moved to the United States. Throughout his life, Luis was always encouraged to focus on academic studies since both his parents did not complete high school. Being the first in the family to obtain a dual degree with an associated degree and high school diploma, and later graduating with a bachelor's degree was a huge accomplishment for him and his family. Luis attended South Texas College during his high school career and once finishing his associates degree in Biology, he later transferred to The University of Texas Rio Grande Valley (UTRGV) where he obtained his bachelor's degree in Biology with focus on Biological Sciences and a minor in Chemistry. Throughout Luis's life, he was always determined to attend medical school. He was an officer for a premedical biomedical society organization at UTRGV and continues to be a mentor for College Assistance Migrant Program at UTRGV. He also has been volunteering for the Young Center for Immigrant Children's Rights since his undergraduate career, as a way of giving back to his community. After obtaining his bachelor's degree, the author pursued his Master of Biochemistry and Molecular Biology and was awarded the Presidential Graduate Research Assistantship for funding. Since there was always an interest in pursuing medical school, Luis joined Dr. Xiaoqian Fang research lab where he focused on Neurological biochemical mechanisms which would provide a great interest and appreciation for research and its role in medicine outside of patient care. During his graduate career, Luis gave a presented a poster on The Role of Zinc in PSD-95

Palmitoyl Modification and was one of the winners during the 2021 COS Annual Research Conference. He also was a collaborator in the published research paper of Zinc-chelating postsynaptic density-95 N-terminus impairs its palmitoyl modification by Dr. Yonghong Zhang.

Luis earned his Master of Science in Biochemistry at The University of Texas Rio Grande Valley on May 14, 2022 and will be applying to medical school in the upcoming year. Luis can be contacted through his email luis.f.acosta00@gmail.com.