DIFFERENTIAL MODULATION OF GLUTAMATERGIC SYNAPTIC

TRANSMISSION BY POLYSIALIC ACID

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DIFFERENTIAL MODULATION OF GLUTAMATERGIC SYNAPTIC TRANSMISSION BY POLYSIALIC ACID

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DIFFERENTIAL MODULATION OF GLUTAMATERGIC SYNAPTIC

TRANSMISSION BY POLYSIALIC ACID

Catrina Sims-Robinson

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VITA

Catrina Sims, daughter of Elizabeth Sims, was born on November 19, 1982, in Opelika, Alabama. She graduated from Saint Jude Educational Institute with honors in May of 2000. She attended Xavier University of Louisiana for two years working towards a Bachelor of Science degree in Biology. She transferred to Auburn University and graduated cum laude with a Bachelor of Science degree in Laboratory Technology in December of 2004. She joined the graduate program at Auburn University in January of 2005, in the Pharmacal Sciences Department. As a dedicated graduate student she worked as a Graduate Teaching Assistant and a Facilitator of Pharmacotherapy for the Auburn University Harrison School of Pharmacy and as a tutor for the Auburn University Athletic Department. The Auburn University Graduate School, Pharmacy School, and Multicultural and Diversity department in 2006 selected her as the "Outstanding Graduate Student."

DISSERTATION ABSTRACT

DIFFERENTIAL MODULATION OF GLUTAMATERGIC SYNAPTIC TRANSMISSION BY POLYSIALIC ACID

Catrina Sims-Robinson

Doctor of Philosophy, December 17, 2007
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Controlled modulation and regulation of glutamate receptors are essential for synaptogenesis and synaptic plasticity. The two major types of excitatory glutamate receptors found at the synapse are AMPA and NMDA, which during over-activation leads to glutamate induced excitotoxicity. The functional properties of single synaptic receptors specifically NMDA receptors was characterized in detail in this study. The neural cell adhesion molecule (NCAM) is a glycoprotein that modulates synaptic plasticity. NCAM is covalently linked to polysialic acid (PSA), a negatively charged carbohydrate which is a polymer of sialic acid with an unusual α2, 8-linkage containing up to 200 residues. PSA-NCAM plays vital roles in the development of the nervous system and NMDA receptor-dependent synaptic plasticity in the adult. It is well known that carbohydrates play a vital role in cellular recognition and adhesive processes. There are several studies that established a link between PSA-NCAM and glutamate

receptors. However, the mechanisms whereby PSA-NCAM modulates glutamate receptors have not been studied. Therefore, this study will investigate the effects of endogenous and soluble PSA-NCAM on synaptic glutamate receptors. Elucidating the functional properties of single synaptic glutamate receptors and the modulation of these receptors by PSA will be a step towards understanding many neurodegenerative disorders where PSA expression is altered.

Synaptosomes and membrane fractions from rat hippocampi and recombinant receptors, respectively, were used in this study to evaluate the mechanistic effects of PSA on AMPA receptors and the NR2 subunit containing NMDA receptor subtypes—NR2A, NR2B, and NR2A/NR2B, specifically. The hippocampi were isolated from 7-10 days old Sprague Dawley rats for synaptosomal preparation for synaptic AMPA receptor and NMDA receptor electrical recordings in the presence of specific receptor antagonist and agonists. Our results indicate that PSA potentiates synaptic AMPA receptor properties and that cyclothiazide, a known inhibitor of AMPA receptor desensitization does not occlude PSA effects on AMPA receptor activity. This indicates that PSA, in addition to decreasing the desensitization of AMPA receptors may also act through another mechanism. PSA also inhibited with the binding of glutamate to NR2B subunit-containing NMDA receptors. Furthermore, it is specific for the NR2B subunit and unlike other NR2B specific antagonists; PSA also inhibits NR2A/NR2B channels. PSA decreased the open probability and not the conductance of the NR2B subunit containing NMDA receptors. The results of our study reveal that 1) PSA potentiates AMPA receptor function and thereby possibly enhance synaptic plasticity, 2) inhibits NR2B containing NMDA receptors and thereby decreasing glutamate induced excitotoxicity.

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1. INTRODUCTION

Glutamate, the major excitatory neurotransmitter in the mammalian brain, plays a major role in maintenance of synaptic plasticity, neuronal proliferation, migration, and differentiation or survival during neural development (Nguyen et al., 2001; Lujan et al., 2005). Glutamate activity is mediated by activation of synaptic ionotropic glutamate receptors or metabotropic glutamate receptors. Excess glutamate in the synapse is capable of causing neuronal cell death by excitotoxicity of these receptor subtypes, which plays a major role in many neurodegenerative disorders. In addition to glutamate, the neural cell adhesion molecules (NCAMs) are also involved in brain development by building and maintaining synaptic structure. Synaptic carbohydrates such as polysialic acid play a critical role in cellular recognition and adhesive processes (Benson et al., 2000).

Ionotropic glutamate receptors also referred to as ligand-gated ion channels, which have an extracellular recognition site for neurotransmitters, open to form a pore for ions upon interaction with glutamate. They are further subdivided into three types of receptors—alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, *N*-methyl-D-aspartic acid (NMDA) receptor, and Kainate (KA) receptor. Metabotropic receptors use an intermediate molecule, G-protein, for their function. This

second messenger system either directly or indirectly acts via enzymatic interactions causes the opening or closing of channels elsewhere on the membrane.

NMDA and AMPA, the two major subtypes of ionotropic glutamate receptors responsible for excitatory neurotransmission, are necessary for the induction and maintenance of long-term potentiation (LTP), a form of synaptic plasticity which is the widely accepted cellular and molecular mechanism of learning and memory.

The AMPA receptor is ligand-gated, whereas the NMDA receptor is both ligand-gated and voltage-dependent. The NMDA receptor has a slower excitatory transmission due the voltage-dependent magnesium block (Mayer et al., 1984; Nowak et al., 1984), compared to AMPA receptors. However, NMDA receptors have a longer duration of action (Lester and Jahr, 1992) than AMPA receptors due to its higher affinity for glutamate. NMDA receptors are nonselective to cations. Unlike AMPA receptors, NMDA receptors require glycine in addition to glutamate for activation (Benveniste and Mayer, 1991; Clements and Westbrook, 1991). NMDA receptors are heteromulitmers composed of at least one NR1 subunit, which binds glycine, and one or more regionally localized NR2 subunits, which binds glutamate. The NR2 subunit, which has four different isoforms (NR2A-NR2D), are developmentally regulated (Monyer et al., 1994; Sheng et al., 1994; Zhong et al., 1995) and alter the NMDA receptor's permeability and kinetic properties (Kutsuwada et al., 1992; Monyer et al., 1992; Stern et al., 1992; Monyer et al., 1994; Kuner and Schoepfer, 1996). It has been reported that multimers NR1/NR2A and NR1/NR2A/NR2B containing NMDA receptors are located at mature synapses. Previous data has reported that multimer NR1/NR2B containing NMDA receptors are located extrasynaptically (Tovar and Westbrook, 1999). However, recent

data shows that NR1/NR2B may be located either synaptically or extrasynaptically (Thomas et al., 2006). Since synaptic and extrasynaptic NMDA receptors may activate opposing pathways to prevent or induce glutamate-induced cell death, respectively (Hardingham et al., 2002), this study will characterize the functional properties of synaptic NMDA receptors, specifically, NR2A, NR2B, and NR2A/NR2B subunit containing receptors. The AMPA receptors are composed of glutamate receptor subunits GluR 1-4 (Hollmann and Heinemann, 1994). The channel is made up of four subunits with different subunit composition. The subunit composition determines the kinetic properties of this receptor (Greger et al., 2007). When an AMPA receptor channel is composed of the GluR2 subunit, it is not permeable to calcium ions due the presence of arginine residues in the pore forming segment of the ion channel (Isaac et al., 2007).

Cell adhesion molecules (CAMs), proteins that extend across the cell surface membrane, are responsible for activity-dependent long-term changes in synaptic strength and morphology (Ronn et al., 1998; Benson et al., 2000). Integrins, cadherins, neurexins, and neuroligins are the four major groups of CAMs. The neural cell adhesion molecule (NCAM), a member of the Ig superfamily, is involved in cell-cell adhesion, neurite outgrowth, synaptic plasticity, and learning and memory and is expressed on the surface of most cells. It promotes cell-cell adhesion via homophilic binding (Doherty et al., 1990; Hall et al., 1990). Since NCAM is involved in synaptic plasticity, this study will investigate the effects of endogenous and soluble NCAM on synaptic activity.

Polysialic acid (PSA) is a negatively charged carbohydrate composed of 8-200 sialic acid residues. PSA orchestrates dynamic changes in the shape and movement of cells and their processes by decreasing adhesion forces and modulating cell surface

interactions. In NCAM deficient mice PSA was almost completely absent (Tomasiewicz et al., 1993; Cremer et al., 1994; Ono et al., 1994). Thus, PSA in mammals is found exclusively attached to NCAM in an unusual α-2,8 linkage in its fifth immunoglobulin-like domain. PSA is thought to modulate homophilic and heterophilic binding of the NCAM glycoprotein backbone by steric interference, thus promoting plastic changes in the CNS (Weinhold et al., 2005). Therefore, the effects of PSA modulation on synaptic receptors will be carried out in this study.

PSA-NCAM plays important roles in the development of the nervous system and NMDA receptor-dependent synaptic plasticity in the adult including synaptic remodeling and LTP (Muller et al., 1994). Activity-dependent synaptic plasticity is crucial for neural circuitry during brain development (Katz and Shatz, 1996). PSA-NCAM allows/prevents neurons from moving or changing morphology, thereby participating in axonal growth (Zhang et al., 1992) and synaptic reorganization (Seki and Rutishauser, 1998). The decrease in postnatal PSA expression is associated with stabilization of cellular interactions and synapse formation (Szele et al., 1994). The expression of PSA is high during development and low in the adult brain. However, brain regions retaining PSA-expression show up-regulation during neuronal activity and learning-induced neuroplasticity in the hippocampus (Murphy et al., 1996). Removal of PSA by endosialidase-N, an enzyme which specifically cleaves endogenous PSA-NCAM, disturbs neuronal migration and axonal sprouting, branching and fasciculation, synaptogenesis, synaptic plasticity, and spatial memory (Rutishauser and Landmesser, 1996; Kiss and Rougon, 1997; Kleene and Schachner, 2004). Therefore, this study will

evaluate the effects of both endogenous and exogenous or soluble PSA-NCAM on synaptic activity.

AMPA and NMDA receptors have been associated with many abnormal disease states such as epilepsy, stroke, traumatic brain injury, Alzheimer's disease, Parkinson's disease, Huntington's disease, and neuropathic pain, and psychiatric disorders such as Schizophrenia (Hugon et al., 1996; Parsons et al., 1998; Meldrum et al., 1999). Positive modulators of AMPA receptors have been proven to enhance cognitive function, specifically memory (Lynch, 2004) and improve some disease states. Cyclothiazide (CTZ), a positive modulator of AMPA receptors, has been shown to inhibit desensitization in hippocampal neurons (Wong and Mayer, 1993) and single AMPA receptor channels (Fucile et al., 2006). Since PSA has been shown to increase the single channel open probability of purified AMPA receptors (Vaithianathan et al., 2004), this study will evaluate the mechanism of action of PSA with CTZ.

NMDA receptors play a crucial role in plasticity during development (Contestabile, 2000), learning, memory, synaptic plasticity (Hrabetova et al., 2000), and regulation of PSA-NCAM expression (Wang et al., 1996). Previous studies indicate that NMDA receptors are co-redistributed with NCAM after induction of LTP (Fux et al., 2003) and pharmacological blockade of NMDA receptor activity prevents PSA-NCAM stimulated synaptogenesis (Dityatev et al., 2004). In addition, NCAM associates with the spectrin based scaffold cross-linking NCAM with NMDA receptor calmodulin kinase II alpha (Sytnyk et al., 2006). Controlled modulation of both AMPA and NMDA receptors are essential for synaptogenesis and synaptic plasticity. Although excessive activation of both AMPA and NMDA receptors may lead to excitotoxicity (Yamada, 2000), NMDA

receptor activation contributes to cell death more rapidly than AMPA receptors (Choi, 1992; McDonald and Johnston, 1993). Therefore, since NMDA receptor targeting, clustering, and surface expression at the synapse are important processes in synaptogenesis and synaptic plasticity (Bolton et al., 2000), it is of great pharmacological importance to understand the role of PSA, which is widely expressed in the synapse, in regulating NMDA receptor function and neuronal excitability.

In conclusion, there is evidence that supports a link between PSA and glutamate receptors. However, the mechanism of action of PSA on AMPA and NMDA receptors has not been investigated. The method of incorporating isolated synaptosomes into an artificial phospholipid bilayer will serve as a valuable tool to evaluate PSA modulation of single channel properties of glutamate receptors. Since positive AMPA modulators can have detrimental effects in some disease states without a neuro-protective mechanism in place (Yamada, 2000), this study will evaluate whether or not PSA provides neuro-protection through its influence on glutamate receptors. An understanding of the functional properties of single synaptic glutamate receptors and the effects of PSA on glutamate receptors will be a step towards the challenge of understanding mechanistic basis of many neurodegenerative disorders and possibly lead to appropriate therapeutic measures.

REFERENCES

Benson DL, Schnapp LM, Shapiro L, Huntley GW (2000) Making memories stick: cell-adhesion molecules in synaptic plasticity. Trends Cell Biol 10:473-482.

Benveniste M, Mayer ML (1991) Kinetic analysis of antagonist action at N-methyl-D-aspartic acid receptors. Two binding sites each for glutamate and glycine. Biophys J 59:560-573.

Bolton MM, Blanpied TA, Ehlers MD (2000) Localization and stabilization of ionotropic glutamate receptors at synapses. Cell Mol Life Sci 57:1517-1525.

Choi DW (1992) Excitotoxic cell death. J Neurobiol 23:1261-1276.

Clements JD, Westbrook GL (1991) Activation kinetics reveal the number of glutamate and glycine binding sites on the N-methyl-D-aspartate receptor. Neuron 7:605-613.

Contestabile A (2000) Roles of NMDA receptor activity and nitric oxide production in brain development. Brain Res Brain Res Rev 32:476-509.

Cremer H, Lange R, Christoph A, Plomann M, Vopper G, Roes J, Brown R, Baldwin S, Kraemer P, Scheff S, et al. (1994) Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. Nature 367:455-459.

Dityatev A, Dityateva G, Sytnyk V, Delling M, Toni N, Nikonenko I, Muller D, Schachner M (2004) Polysialylated neural cell adhesion molecule promotes remodeling and formation of hippocampal synapses. J Neurosci 24:9372-9382.

Doherty P, Fruns M, Seaton P, Dickson G, Barton CH, Sears TA, Walsh FS (1990) A threshold effect of the major isoforms of NCAM on neurite outgrowth. Nature 343:464-466.

Fucile S, Miledi R, Eusebi F (2006) Effects of cyclothiazide on GluR1/AMPA receptors. Proc Natl Acad Sci U S A 103:2943-2947.

Fux CM, Krug M, Dityatev A, Schuster T, Schachner M (2003) NCAM180 and glutamate receptor subtypes in potentiated spine synapses: an immunogold electron microscopic study. Mol Cell Neurosci 24:939-950.

Greger IH, Ziff EB, Penn AC (2007) Molecular determinants of AMPA receptor subunit assembly. Trends Neurosci 30:407-416.

Hall AK, Nelson R, Rutishauser U (1990) Binding properties of detergent-solubilized NCAM. J Cell Biol 110:817-824.

Hardingham GE, Fukunaga Y, Bading H (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci 5:405-414.

Hollmann M, Heinemann S (1994) Cloned glutamate receptors. Annu Rev Neurosci 17:31-108.

Hrabetova S, Serrano P, Blace N, Tse HW, Skifter DA, Jane DE, Monaghan DT, Sacktor TC (2000) Distinct NMDA receptor subpopulations contribute to long-term potentiation and long-term depression induction. J Neurosci 20:RC81.

Hugon J, Vallat JM, Dumas M (1996) [Role of glutamate and excitotoxicity in neurologic diseases]. Rev Neurol (Paris) 152:239-248.

Isaac JT, Ashby M, McBain CJ (2007) The role of the GluR2 subunit in AMPA receptor function and synaptic plasticity. Neuron 54:859-871.

Katz LC, Shatz CJ (1996) Synaptic activity and the construction of cortical circuits. Science 274:1133-1138.

Kiss JZ, Rougon G (1997) Cell biology of polysialic acid. Curr Opin Neurobiol 7:640-646.

Kleene R, Schachner M (2004) Glycans and neural cell interactions. Nat Rev Neurosci 5:195-208.

Kuner T, Schoepfer R (1996) Multiple structural elements determine subunit specificity of Mg2+ block in NMDA receptor channels. J Neurosci 16:3549-3558.

Kutsuwada T, Kashiwabuchi N, Mori H, Sakimura K, Kushiya E, Araki K, Meguro H, Masaki H, Kumanishi T, Arakawa M, et al. (1992) Molecular diversity of the NMDA receptor channel. Nature 358:36-41.

Lester RA, Jahr CE (1992) NMDA channel behavior depends on agonist affinity. J Neurosci 12:635-643.

Lujan R, Shigemoto R, Lopez-Bendito G (2005) Glutamate and GABA receptor signalling in the developing brain. Neuroscience 130:567-580.

Lynch G (2004) AMPA receptor modulators as cognitive enhancers. Curr Opin Pharmacol 4:4-11.

Mayer ML, Westbrook GL, Guthrie PB (1984) Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones. Nature 309:261-263.

McDonald JW, Johnston MV (1993) Excitatory amino acid neurotoxicity in the developing brain. NIDA Res Monogr 133:185-205.

Meldrum BS, Akbar MT, Chapman AG (1999) Glutamate receptors and transporters in genetic and acquired models of epilepsy. Epilepsy Res 36:189-204.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12:529-540.

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, Seeburg PH (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science 256:1217-1221.

Muller D, Stoppini L, Wang C, Kiss JZ (1994) A role for polysialylated neural cell adhesion molecule in lesion-induced sprouting in hippocampal organotypic cultures. Neuroscience 61:441-445.

Murphy KJ, O'Connell AW, Regan CM (1996) Repetitive and transient increases in hippocampal neural cell adhesion molecule polysialylation state following multitrial spatial training. J Neurochem 67:1268-1274.

Nguyen L, Rigo JM, Rocher V, Belachew S, Malgrange B, Rogister B, Leprince P, Moonen G (2001) Neurotransmitters as early signals for central nervous system development. Cell Tissue Res 305:187-202.

Nowak L, Bregestovski P, Ascher P, Herbet A, Prochiantz A (1984) Magnesium gates glutamate-activated channels in mouse central neurones. Nature 307:462-465.

Ono K, Tomasiewicz H, Magnuson T, Rutishauser U (1994) N-CAM mutation inhibits tangential neuronal migration and is phenocopied by enzymatic removal of polysialic acid. Neuron 13:595-609.

Parsons CG, Danysz W, Quack G (1998) Glutamate in CNS disorders as a target for drug development: an update. Drug News Perspect 11:523-569.

Ronn LC, Hartz BP, Bock E (1998) The neural cell adhesion molecule (NCAM) in development and plasticity of the nervous system. Exp Gerontol 33:853-864.

Rutishauser U, Landmesser L (1996) Polysialic acid in the vertebrate nervous system: a promoter of plasticity in cell-cell interactions. Trends Neurosci 19:422-427.

Seki T, Rutishauser U (1998) Removal of polysialic acid-neural cell adhesion molecule induces aberrant mossy fiber innervation and ectopic synaptogenesis in the hippocampus. J Neurosci 18:3757-3766.

Sheng M, Cummings J, Roldan LA, Jan YN, Jan LY (1994) Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. Nature 368:144-147.

Stern P, Behe P, Schoepfer R, Colquhoun D (1992) Single-channel conductances of NMDA receptors expressed from cloned cDNAs: comparison with native receptors. Proc Biol Sci 250:271-277.

Sytnyk V, Leshchyns'ka I, Nikonenko AG, Schachner M (2006) NCAM promotes assembly and activity-dependent remodeling of the postsynaptic signaling complex. J Cell Biol 174:1071-1085.

Szele FG, Dowling JJ, Gonzales C, Theveniau M, Rougon G, Chesselet MF (1994) Pattern of expression of highly polysialylated neural cell adhesion molecule in the developing and adult rat striatum. Neuroscience 60:133-144.

Thomas CG, Miller AJ, Westbrook GL (2006) Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons. J Neurophysiol 95:1727-1734.

Tomasiewicz H, Ono K, Yee D, Thompson C, Goridis C, Rutishauser U, Magnuson T (1993) Genetic deletion of a neural cell adhesion molecule variant (N-CAM-180) produces distinct defects in the central nervous system. Neuron 11:1163-1174.

Tovar KR, Westbrook GL (1999) The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. J Neurosci 19:4180-4188.

Vaithianathan T, Matthias K, Bahr B, Schachner M, Suppiramaniam V, Dityatev A, Steinhauser C (2004) Neural cell adhesion molecule-associated polysialic acid potentiates alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor currents. J Biol Chem 279:47975-47984.

Vaithianathan T, Manivannan K, Kleene R, Bahr BA, Dey MP, Dityatev A, Suppiramaniam V (2005) Single channel recordings from synaptosomal AMPA receptors. Cell Biochem Biophys 42:75-85.

Wang C, Pralong WF, Schulz MF, Rougon G, Aubry JM, Pagliusi S, Robert A, Kiss JZ (1996) Functional N-methyl-D-aspartate receptors in O-2A glial precursor cells: a critical role in regulating polysialic acid-neural cell adhesion molecule expression and cell migration. J Cell Biol 135:1565-1581.

Weinhold B, Seidenfaden R, Rockle I, Muhlenhoff M, Schertzinger F, Conzelmann S, Marth JD, Gerardy-Schahn R, Hildebrandt H (2005) Genetic ablation of polysialic acid causes severe neurodevelopmental defects rescued by deletion of the neural cell adhesion molecule. J Biol Chem 280:42971-42977.

Wong LA, Mayer ML (1993) Differential modulation by cyclothiazide and concanavalin A of desensitization at native alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid- and kainate-preferring glutamate receptors. Mol Pharmacol 44:504-510.

Yamada KA (2000) Therapeutic potential of positive AMPA receptor modulators in the treatment of neurological disease. Expert Opin Investig Drugs 9:765-778.

Zhang H, Miller RH, Rutishauser U (1992) Polysialic acid is required for optimal growth of axons on a neuronal substrate. J Neurosci 12:3107-3114.

Zhong J, Carrozza DP, Williams K, Pritchett DB, Molinoff PB (1995) Expression of mRNAs encoding subunits of the NMDA receptor in developing rat brain. J Neurochem 64:531-539.

2. REVIEW OF LITERATURE

2.1. Synaptic Transmission

Information is carried within and between neurons by electrical and chemical signals. The synapse is the point at which one neuron communicates with another neuron. The average neuron forms approximately 1000 synaptic connections and receives approximately 10,000 connections. The communication between neurons is known as synaptic transmission. Synaptic transmission can be either electrical or chemical and the neurons may receive either an excitatory or inhibitory signal.

There is an approximate 3.5 nm distance between the pre- and post- synaptic membrane in electrical synapses. It has bidirectional transmission and virtually no synaptic delay across the gap junction, which is composed of specialized protein structures that conduct the flow of ionic current from the presynaptic to the postsynaptic called gap-junction channels. Electrical synapses are used for simple depolarizing signals. They neither produce inhibitory actions nor are involved in long-lasting changes to the electrical properties of the postsynaptic cell.

There is an approximate 20-40 nm distance between the pre- and post-synaptic membranes in chemical synapses. It has unidirectional transmission with a significant synaptic delay of at least 0.3 ms but usually 1-5 ms or longer. A chemical transmitter compared to ion current in electrical synapses is responsible for transmission. The small

space that separates the pre- and post- synaptic neurons at chemical synapses is called the synaptic cleft. In chemical synapses the neurotransmitter is released from presynaptic terminals, which contain discrete collections of synaptic vesicles each filled with several thousand molecules, into the synaptic cleft to bind to specific receptors located in the postsynaptic membrane. The neurotransmitter vesicles cluster at active zones for release.

The propagation of an action potential causes an influx of calcium through voltage-gated calcium channels at the active zones causing the vesicles to fuse with the presynaptic membrane, a process known as exocytosis. The binding of the neurotransmitter leads to opening and closing of ion channels. Ionic flux can alter membrane conductance and potential. Thus, chemical synapses can produce more complex behaviors compared to electrical synapses. Since the activation of one vesicle can lead to thousands of ion channels openings, chemical synapses serve to amplify neuronal signals so that even a small presynaptic nerve terminal can alter the response of a large postsynaptic cell. In addition chemical synapses can mediate either excitatory or inhibitory actions in postsynaptic cells and produce long lasting changes. Whether a synapse is excitatory or inhibitory depends on the type of transmitter released. The major excitatory synapse is known as glutamatergic synapses whereas the major inhibitory synapse is known as GABA-ergic (gamma-aminobutyric acid) synapses.

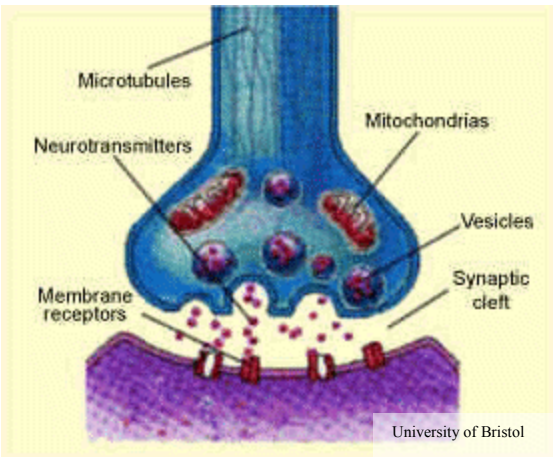

Figure 2.1.: Synaptic Transmission

Presynaptic release of neurotransmitter (chemical synapse) into the synaptic cleft activates postsynaptic receptor channels.

2.1.1. Ion Channels

Ion channels, which are embedded within a phospholipid bilayer, are a class of integral proteins located in all cells of the body that mediate rapid changes in membrane potential. There are currently two widely accepted methods of investigating ion channel structure and function: x-ray crystallography for structural analysis and single channel recordings utilizing patch clamp technique for functional analysis. The single channel recordings in lipid bilayers allows to record from channels that are inaccessible in patch clamp technique and allows to study how membrane composition affects channel function. The three types of ion channels are voltage-gated, ligand-gated, and mechanically-gated channels. Ion channels are selective to specific ions and they open and close in response to specific signals such as electrical, mechanical, or chemical.

Ion channels are passive in that it requires no metabolic energy. The gating of ion channels involves conformational changes. Ligand-gated channels can enter a refractory

state when exposed to a ligand for a prolonged period called desensitization. This can be due to either an intrinsic interaction between the ligand and the channel or due to the phosphorylation of the channel by protein kinase. Voltage-gated channels may undergo inactivation which is a refractory state after activation which results from an intrinsic conformational change controlled by a subunit or region of the channel that is separate from the activation region.

Some ion channels have complex ways of opening and closing. However, in all cases where the channels open or close, a conformational change is involved whether passive or with energy expenditure. This conformational change may involve a physical change or a change in charge distribution inside the pore. A channels gating may be controlled by the membrane potential, ligand binding, or phosphorylation. Phosphorylated proteins have more energy and can therefore undergo changes in shape and conformation.

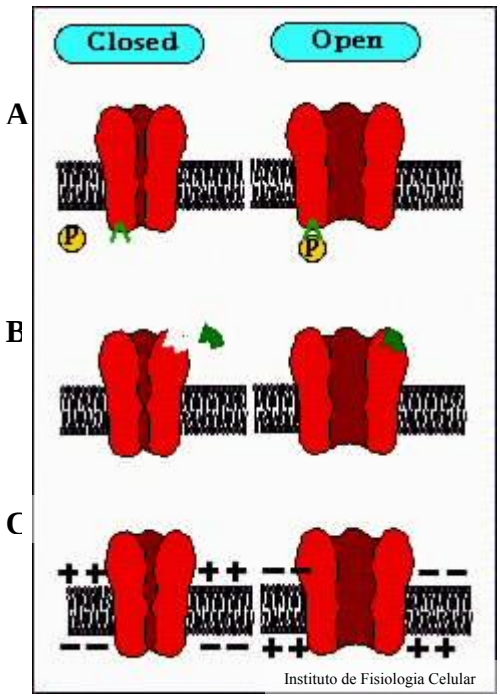

Figure 2.2.: Diagram of Three Mechanisms of Ion Channel Opening and Closing.

Three different mechanisms for the opening and closing of ion channels. **A)** Phosphorylation and dephosphorylation of the protein leads to conformational changes of the channel. These are mechanically-gated channels. **B)** The binding of a ligand, neurotransmitter, to the receptor causes a conformational change leading to the channel opening. These are ligand-gated channels. **C)** A change in membrane potential causes a conformational change. These are voltage-gated channels.

2.1.2. Glutamate Receptors

Glutamate receptors are a type of ligand-gated ion channel along with nicotinic acetylcholine receptors (nAChR), GABA and glycine receptors, and serotonin-gated ion channels (5-HT). nAChR are found in nerve and muscle cells. GABA and glycine receptors are families of chloride channels. 5-HT is activated by hydroxytryptamine. Glutamate receptors include AMPA, NMDA, and Kainate.

Glutamate receptors, mostly present in the central nervous system, have been found in other regions such as pancreatic islet cells, osteoclasts, mast cells, cardiac ganglia, and taste buds (Dingledine et al., 1999). However, in the CNS glutamate receptors are abundant in the cortex, basal ganglia, and sensory pathways (Rang et al., 1999). Glutamate receptors are all multimeric proteins composed each of 4 subunits, which contain only three transmembrane α-helices. The channel pore may be formed by a loop connecting the first and second transmembrane segments. A single amino acid residue in the pore-forming M2 region attributes to the pore property differences in AMPA and NMDA receptors. Glutamate receptors cluster at postsynaptic sites in the membrane opposed to glutamatergic presynaptic terminals.

The abundance of glutamate receptors subtypes at excitatory synapses is both developmentally regulated and activity-dependent. The majority of excitatory synapses contain both NMDA and AMPA receptors. However, some have NMDA receptors and lack functional AMPA receptors, whereas others contain no NMDA receptors. During early development synapses contain only NMDA receptors.

Activity-dependent changes in glutamate receptor localization were first demonstrated to occur over many days (Craig, 1998). Blockade of NMDA receptor activity ranging from days to weeks increased the number of synaptic NMDA receptor clusters without affecting the total number of synapses or the clustering of the receptor protein PSD-95 in cultured hippocampal neurons (Rao and Craig, 1997; Lissin et al., 1998; Liao et al., 1999). Using an antagonist or treating cultures with tetrodotoxin (ttx), sodium channel blocker which blocks synaptic activity, induces upregulation of synaptic NMDA receptors (Rao and Craig, 1997; Liao et al., 1999). This activity-dependent

regulation of NMDA receptor localization could function to reset the synaptic modification threshold for inducing LTP or LTD (Bear, 1996) or to maintain neuronal sensitivity to other NMDA receptor dependent signaling cascades.

2.1.3. Neurotransmitters

There are many neurotransmitters in the brain because of its non-uniform functions. For example, there are both excitatory and inhibitory responses in the brain. Not only are neurotransmitters excitatory or inhibitory, but also the receptors may also determine whether a response is excitatory or inhibitory (for example, dopamine). The three major categories of neurotransmitters are amino acids, peptides, and monoamines. Amino acid neurotransmitters include glutamic acid (glutamate), GABA, aspartic acid, and glycine. Vasopressin, somatostatin, and neurotensin are referred to as peptide neurotransmitters. Monoamines include norepinephrines, dopamine, and serotonin. The two major types of neurotransmitters in the brain are glutamate and GABA. Peptides either function in the hypothalamus or act as co-factors in other areas of the brain. Monoamines perform specialized modulating functions.

The simplest amino acid is glycine, which consist of an amino group and acidic group. Glycine is a neurotransmitter only in vertebrate animals. In the spinal cord, brainstem, and retina, glycine is an inhibitory neurotransmitter. However, glycine is a co-agonist with glutamate for NMDA receptors during excitatory neurotransmission.

The excitatory action of glutamate, the most common excitatory neurotransmitter, in mammalian brain has been known since the 1950s. It plays an important role in synaptogenesis, synaptic plasticity, and neurological disorders (Conti and Weinberg,

1999). Glutamate does not cross the blood brain barrier. When released into the synapse, glutamate is either reabsorbed directly into neurons via transport system or soaked up by astrocytes, which convert glutamate into glutamine, less toxic form. Glutamine can then be transported back to neurons for re-conversion into glutamate. Although it is thought that calcium entry is more of cause, glutamate, due to its acidity, has the potential to destroy neurons when released in excessive amounts.

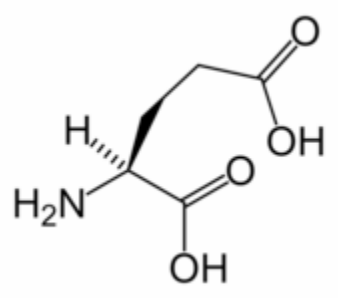

Figure 2.3.: Glutamate

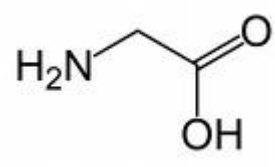

Figure 2.4.: Glycine

2.1.4. Postsynaptic Density Proteins

Postsynaptic density of 95 kDa molecular weight (PSD-95) is a large protein cytoskeleton complex on the postsynaptic side of the neuronal junction that aids in signaling via protein-protein interaction. PSD-95 contains 3 repeated domains called PDZ, which bind a number of cellular proteins. PDZ is named for the 3 proteins that were first identified—PSD-95, DLG (Drosophila disc large tumor suppressor protein), and ZO-1 (zonula occludens-1). They are composed of glutamate neurotransmitter

receptors, molecular scaffolding molecules, cell adhesion molecules, and other diverse signaling proteins. Proteins located with the PSD interact for glutamatergic signal transduction and are responsible for receptor targeting and trafficking (Steigerwald et al., 2000; Roche et al., 2001; Sans et al., 2001; Scott et al., 2001).

2.1.4.1. NR1

The NR1 subunit gates calcium through interactions with cytoplasmic proteins such as α-actinin and F-actin. α-actinin is a microfilament protein that cross links the actin cytoskeleton to the NR1 C-terminus subunit (Wyszynski et al., 1997). A previous study suggests that α-actinin plays more of a modulatory role in the regulation of NMDA receptor function and perhaps localization (Allison et al., 1998), competitively with calmodulin (Ehlers et al., 1996; Wyszynski et al., 1997). Calcium calmodulin-dependent protein kinase II (CaMKII) has been found to bind to α-actinin (Walikonis et al., 2001). F-actin is a subunit of actin called the filamentous polymer. Because in the absence of F-actin, many NR1 clusters were released from the postsynaptic sites, it is believed that F-actin is important for synaptic localization and not NMDA receptor clustering (Allison et al., 1998). In addition, evidence shows that CaMKII interaction with NMDA receptors, α-actinin, and F-actin may increase anchoring sites for AMPA receptors at the synapse, thereby enhancing synaptic transmission (Lisman et al., 2002).

Calmodulin, a small acidic ubiquitous calcium-binding protein, can bind to and regulate various protein targets and affect cellular functions. It can be used as a calcium sensor or a signal transducer. Calcium binding to calmodulin induces a conformational change in calmodulin. It can bind up to four calcium ions. The calcium regulation of

NMDA receptors by calmodulin is thought to involve a linkage between the receptor and cytoskeleton (Rosenmund and Westbrook, 1993). Inactivation of NMDA receptors from a reduction in the probability of channel openings occurs after elevation of intracellular calcium (Legendre et al., 1993). It has been postulated that this inactivation is due to calcium binding of calmodulin dependent release of the NMDA receptor from the cytoskeleton (Zhang et al., 1998).

Yotiao, a NMDA receptor associated protein, binds to PKA (Lin et al., 1998) and Protein Phosphatase 1 (PP1) (Westphal et al., 1999). Yotiao is a scaffold protein that regulates channel activity via the anchoring of PKA and PP1 to NMDA receptors (Arino, 2004). Hippocampal NMDA receptor dependent LTD requires the activation of PP1, which is abundant in the PSD (Mulkey et al., 1994). In addition peptides that interfere with the binding of PP1 to its postsynaptic anchoring proteins block NMDA receptor dependent LTD (Morishita et al., 2001). Since PKA binds to some of the same anchoring proteins that interact with protein phosphatases, signaling complexes containing both kinases and phosphatases are targeted to glutamate receptors, thereby facilitating regulation during synaptic plasticity (Westphal et al., 1999; Tavalin et al., 2002).

2.1.4.2. NR2/PSD-95

PSD components are responsible for the clustering of NMDA receptors at the synapse (Sheng and Kim, 1996) via binding of scaffolding proteins, which are responsible for protein-protein interactions and coordination of signal transduction cascades, PSD-95 and α-actinin. PSD-95, a synaptic protein, is important in coupling the NMDA receptor to pathways that control synaptic plasticity and learning. The NR2

subunit C-terminal binds to the first PSD-95 PDZ domain (Garner et al., 2000; Scannevin and Huganir, 2000; Sheng and Pak, 2000). The two major functions of the NR2-PSD-95 complex are targeting of NMDA receptors to the synapse and coupling NMDA receptors to signaling proteins.

The NR2-PSD-95 complex also binds to nNOS, neuronal nitric oxide synthase. Neuronal nitric oxide synthase is a soluble calcium calmodulin regulatory enzyme in the cytosol that is activated by calcium of NMDA receptors. It produces nitric oxide in the central and peripheral nervous system. Neuronal NOS plays a role in cellular communication.

Synaptic GTPase activating protein (SynGAP) is an activating protein for Ras that binds to PSD-95 (Chen et al., 1998; Kim et al., 1998). It decreases the downstream genetic signaling pathways. Phosphorylation of calcium calmodulin-dependent kinase II (CaMKII) via calcium entry through NMDA receptor leads to inhibition of SynGAP which accelerates gene expression downstream. SynGAP also plays a crucial role in early development of the brain and synaptic plasticity in the adult brain (Komiyama et al., 2002; Kim et al., 2003). It has been shown that newborn mice with a SynGAP deletion die a few days after birth whereas adult mice, which are heterozygous for the deletion, have altered synaptic plasticity (Komiyama et al., 2002).

The Ras-mitogen-activated protein kinase (MAPK) is an important postsynaptic signaling mechanism in synaptic plasticity (Adams and Sweatt, 2002). Although the underlying mechanism is unclear, Ras is stimulated by NMDA receptor activation (Yun et al., 1998). A previous study indicates that Ca^{++} from calcium CaM activated Ras-guanine-nucleotide releasing factor (Ras-GRF), which is a guanine nucleotide exchange

factor (GEF) for Ras (Cullen and Lockyer, 2002). Since in hippocampal tissue Ras activation seems to be required for NMDA receptor dependent LTP (Zhu et al., 2002), Ras signal transduction is important for synaptic plasticity and/or learning and memory.

MAPK and phosphoinositide 3'-kinase (PI3K) are two effector pathways that have been characterized for Ras (Cullen and Lockyer, 2002). The sequential protein kinases of the MAPK pathway, which is a major output for postsynaptic Ras signaling, are present in the NMDA receptor complex (Husi et al., 2000). Inhibitors of an upstream activator of MAPK, which is activated in the hippocampus by LTP-inducing stimuli, impairs LTP (Adams and Sweatt, 2002).

Fyn, a member of the Src family of non-receptor protein tyrosine kinases, plays a role in the phosphorylation of the NR2 subunit containing NMDA receptors (Wang and Salter, 1994; Yu et al., 1997). It plays a role in synaptic transmission and plasticity at excitatory synapses. Fyn is necessary for induction of LTP (Grant et al., 1992; Kojima et al., 1997; Lu et al., 1998).

The guanylate kinase domain associate protein, GKAP, bind to the guanylate kinase-like domain of PSD-95 (Kim et al., 1997; Naisbitt et al., 1997; Satoh et al., 1997; Takeuchi et al., 1997) and the synaptic scaffolding molecule (S-SCAM), a postsynaptic protein related to PSD-95 (Hirao et al., 1998). GKAP also provides a link to inositol triphosphate (IP3) receptor and intracellular calcium stores. GKAP is also known as synapse associated protein associated protein (SAPAP) and discs-large associated protein (DAP). Although GKAP is highly enriched and specifically localized in the PSD, its function remains a mystery. It may function as a scaffolding protein. GKAP interacts

with the Shank 1-3 set of PSD proteins that are important in linking NMDA receptor and PSD-95 complex (Naisbitt et al., 1999).

The NMDA receptor is linked to the cytoskeleton via interaction with the NR2 subunits with PSD-95 (Niethammer et al., 1998). The microtubule-associated protein 1A (MAP1A) is a GK domain interacting molecule. It interacts with the GK domain of PSD-93/ chapsyn-110 (Brenman et al., 1998). The cysteine-rich interactor of PDZ3 (CRIPT) is a polypeptide which binds the third PDZ domain of PSD-95/SAP 90 and interacts with tubulin (Niethammer et al., 1998).

2.1.4.3. AMPA

AMPA receptors clustered in the PSD at the synapse provide structurally organized spatially restricted signaling complex associated with scaffolding and adapter proteins (Carroll and Zukin, 2002). This clustered arrangement facilitates rapid and efficient transmission. AMPA receptors are loosely attached to the PSD compared to NMDA receptors (Malenka and Nicoll, 1999). AMPA receptors interact with a number of proteins that aid transport to and from the cell surface. Subunit-interacting proteins such as the glutamate-receptor-interacting-protein (GRIP), protein interacting with C kinase (PICK), and the N-ethylmaleimide sensitive factor (NSF) play important roles in regulation of the number of synaptic AMPA receptors.

GRIP, which is also known as the AMPA-receptor-binding protein (ABP), contains a PDZ domain that functions as a motif for protein-protein interaction. GRIP promotes synaptic accumulation of AMPA receptors by either promoting receptor insertion into the synaptic membrane or anchoring receptor to cytoskeleton through PDZ

domains (Song and Huganir, 2002). Thus far only two variants of the GRIP protein, GRIP1 and GRIP2, have been identified (Srivastava et al., 1998; Dong et al., 1999). GRIP proteins interact with the C-terminal tails of GluR2 and GluR3 in vitro (Dong et al., 1997; Srivastava et al., 1998) and in the brain (Dong et al., 1999; Wyszynski et al., 1999).

PICK1 interacts with the C-terminal tail of GluR2, GluR3, and GluR4c. It also has a central coiled-coil domain thought to mediate dimerization. Cultured hippocampal neurons have shown that PICK1 colocalizes with AMPA receptors and protein kinase C alpha (PKCα) at excitatory synapses using immunofluorescent localization (Xia et al., 1999). PICK1 also induces clustering of AMPA receptors in transfected cells (Xia et al., 1999).

AMPA receptor interacting protein	**AMPA receptor subunit interaction**	**Function to AMPA receptor**
GRIP	GluR2, GluR3, GluR4c	Promotes synaptic accumulation
PICK1	GluR2, GluR3, GluR4c	Promotes internalization
NSF	GluR2	Increases incidence at cell surface
SAP97	GluR1	Promotes synaptic delivery during LTP
4.IN	GluR1	Stabilizes at surface
KIF5-GRIP1 complex	GluR2	Transports GluR2 to dendrites
Stargazin	GluR1-GluR4	Promotes synaptic targeting and surface delivery
NARP	GluR1-GluR4	Induces or helps stabilize synaptic clusters

Table 2.1.: AMPA Receptor and Postsynaptic Density Proteins

Table of AMPA receptors interacting proteins of the PSD and the specific subunits involved in the interaction. The table also outlines that function that each interacting protein has on AMPA receptors.

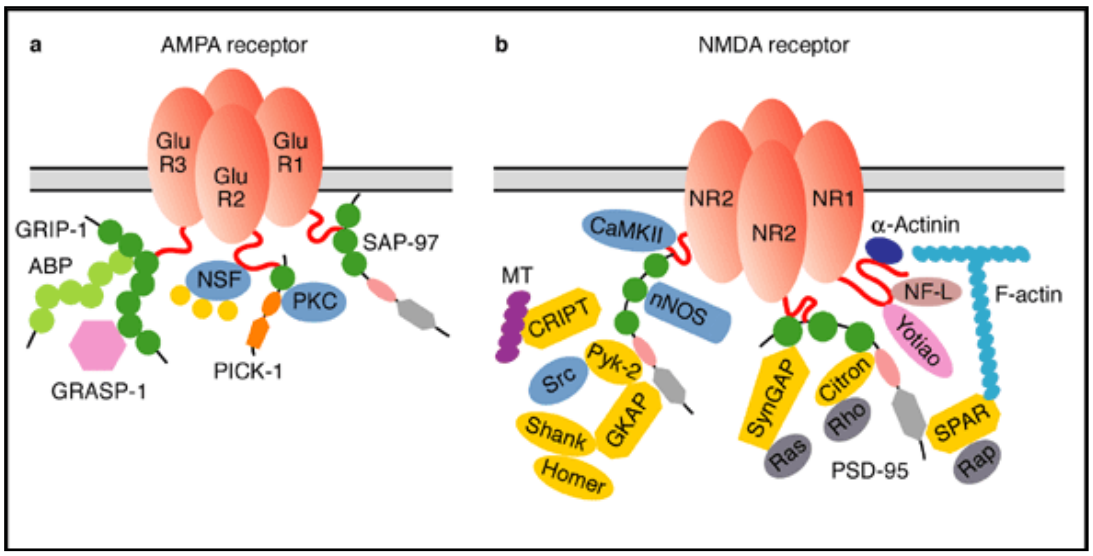

Figure 2.5.: Interaction of Glutamate Receptors with PSDs

Diagram of the various postsynaptic density proteins and their interactions with glutamate receptors ((a) AMPA; (b) NMDA receptors). These proteins are involved in glutamate receptor regulation and function.

2.1.5. Synaptic Plasticity

The activity dependent changes in the strength of synapses, which underlie behavior, cognition, and learning and memory, is known as synaptic plasticity. The NMDA receptor plays a major role in learning and memory by initiating synaptic plasticity and the formation of new neural networks because of its coincidence detection (Dingledine et al., 1999). Active insertion or removal of AMPA receptors at the synapse is triggered by calcium influx through the NMDA receptor. Various patterns of NMDA receptor activation leads to either an increase in synaptic plasticity, known as long-term potentiation (LTP), or a decrease synaptic strength, known as long-term depression (LTD). Alterations in synaptic strength may be due to a presynaptic or postsynaptic mechanism. Altered neurotransmitter release (Choi et al., 2000; Renger et al., 2001;

Zakharenko et al., 2001) is an example of a presynaptic mechanism change. Whereas, changes in the activity and/or abundance of postsynaptic receptors is the primary means of modulating synaptic transmission is an example of a postsynaptic mechanism.

Studies have shown that NMDA receptors are trafficked in mobile transport packets to developing synaptic sites (Washbourne et al., 2002). Since NMDA receptors are integral membrane proteins, they must be transported to the synaptic membrane via a vesicular intermediate. Rapid delivery of NMDA receptors into the post-synaptic membrane occurs by PKC activated soluble N-ethylmaleimide-sensitive factor attachments protein receptors (SNARE)-dependent exocytosis (Lan et al., 2001). Live imagina of GFP-NMDA receptor proteins shows that the NR2B subunits are recruited at the early post-synapse and then are internalized and replaced by the NR2A subunit as development progresses. This synaptic trafficking and insertion of NMDA receptors into the synapse is tightly regulated.

Since AMPA receptors show dynamic changes in their subcellular distribution, the major mechanism underlying NMDA receptor dependent LTP could be the physical delivery of AMPA receptors to the postsynaptic membrane (Sheng and Lee, 2001; Malinow and Malenka, 2002). Synaptic potentiation is induced by delivery of GluR1-containing AMPA receptors to the synapse by activation of NMDA receptors and CaMKII (Hayashi et al., 2000). On the other hand, incorporation of GluR2, which is activity-independent, occurs by exchange with existing synaptic AMPA receptors, with no net change in synaptic strength (Shi et al., 2001). Although the delivery of GluR1 to the neuronal surface is induced by NMDA receptor activation, this exocytosis of GluR2 is rapid (Passafaro et al., 2001). Previous studies indicate that hippocampal CA1 LTP is

lost in adult mutant mice lacking GluR1 (Zamanillo et al., 1999). However, this precise mechanism by which NMDA receptor activation stimulates exocytosis of AMPA receptors remains unclear. Indirect evidence indicated that GluR1 containing AMPA receptors first surface at extrasynaptic sites followed by a lateral translocation into synapses (Passafaro et al., 2001). However, since newly secreted GluR2 was found at synaptic sites, direct exocytosis at synapses in exchange for existing synaptic receptors is indicated (Passafaro et al., 2001).

Trafficking of AMPA receptors to the post-synaptic spine and then to post-synaptic membrane requires an interaction between AMPA receptor subunits and PSD proteins through PDZ domains (Kornau et al., 1997) (Montgomery et al., 2004; Kornau et al., 1997). Any mutations in stargazin, a protein which can bind to AMPA receptors, dissociates surface delivery from synaptic accumulation of AMPA receptors (Chen et al., 2000). There is evidence that suggest that surface translocation from nonsynaptic to synaptic sites require an additional step in synaptic targeting of AMPA receptors, since data show rapid lateral mobility in plasma membrane followed by periods of immobilization in vicinity of synapses (Borgdorff and Choquet, 2002).

The mechanism of synaptic depression in the CA1 and CA3 region of the hippocampus is due to the internalization of AMPA receptors from the neuronal surface (Luscher et al., 1999; Man et al., 2000; Wang and Linden, 2000; Carroll et al., 2001). NMDA receptor stimulation can induce synaptic depression which enhances AMPA receptor endocytosis (Beattie et al., 2000; Ehlers, 2000; Lin et al., 2000; Man et al., 2000). AMPA receptors have been shown to be rapidly recycled out of the synapse in the time course of minutes (Daw et al., 2000; Carroll et al., 2001; Lee et al., 2002). This

process is known to be linked to the endocytic proteins and PSD proteins GRIP and PICK (Chung et al., 2000; Carroll et al., 2001; Lee et al., 2002).

2.1.6. AMPA Receptors

AMPA receptors are tetramers composed of subunits GluR1-GluR4, which interact with a specific set of intracellular proteins (Kornau et al., 1997; Scannevin and Huganir, 2000; Sheng and Pak, 2000). However, in the hippocampus, AMPA receptors are composed mainly of GluR1-GluR2 and GluR2-GluR3 heteromers (Wenthold et al., 1996). The four homologous subunits have a large extracellular N-terminal domain and three transmembrane domains, and an intracellular C-terminal domain, which is vital for the regulation of AMPA receptor function. Altering the activity or number of AMPA receptors in the postsynaptic membrane will modify synaptic strength since AMPA receptors mediated fast excitatory neurotransmission. AMPA receptor activity is regulated by phosphorylation. Therefore, CaMKII phosphorylation of GluR1 increases the single channel conductance of AMPA receptors (Derkach et al., 1999).

Although the AMPA receptor subunits are highly homologous, both the functional properties of AMPA receptors and their trafficking depend upon the subunit composition (Dingledine et al., 1999; Malinow and Malenka, 2002; Collingridge et al., 2004). The carboxyl (C) termini, the most structurally and functionally divergent region of the AMPA receptor subunits, contain regulatory domains that are targeted by multiple intracellular signal transduction pathways. In addition the C termini interacts with scaffold proteins that bind signaling proteins such as kinases and phosphatases as well as cytoskeletal proteins such as actin (Collingridge et al., 2004; Kim and Sheng, 2004;

Nicoll et al., 2006). Not only does these multiprotein complexes influence gating, trafficking, and stabilization of AMPA receptors at the synapse, but also neuronal activity regulates the synthesis of AMPA receptors and their abundance at synapses (Ju et al., 2004; Sutton et al., 2006). Thus, multiple mechanisms contribute to the complexity of AMPA receptor functionality and regulation in synaptic strength.

In the adult brain the GluR2 undergoes RNA editing such that the genomic glutamine (Q) is replaced by arginine (R), which is known as the Q/R site. This change controls the AMPA receptor permeability to Ca^{++}, channel conductance, kinetics, and receptor affinity for glutamate, and subunit assembly into a functional receptor (Geiger et al., 1995; Swanson et al., 1997; Dingledine et al., 1999; Mansour et al., 2001; Greger et al., 2003; Oh and Derkach, 2005). The presence of absence of the GluR2 subunit alters AMPA receptor properties and synaptic transmission. RNA editing may also occur at the R/G site in which arginine is encoded to glycine. Thus is usually specific for the GluR2, GluR3, and GluR4 subunits (Seeburg, 1996). Adjacent to this site is the flip flop region, which contains 38 amino acids and is introduced in GluR subunits by alternative RNA splicing (Seeburg, 1996; Pellegrini-Giampietro et al., 1997). The function of AMPA receptors is greatly influenced by the sites of RNA editing and flip/flop variants. Specifically the flip/flop and the R/G site are associated with desensitization and recovery of the receptor, respectively (Jayakar and Dikshit, 2004).

AMPA receptor phosphorylation leads to modulation of receptor properties (Derkach et al., 1999; Banke et al., 2000). In addition, phosphorylation is considered an important functional readout for signaling pathways associated with synaptic plasticity and learning (Soderling and Derkach, 2000; Lisman et al., 2002; Malinow and Malenka,

2002; Lee et al., 2003; Whitlock et al., 2006). Deficits in LTD and LTP and spatial learning tasks were seen in mice lacking an important regulatory phosphorylation site in GluR1 (Lee et al., 2003). Due to enhancing the coupling efficiency between glutamate binding and channel opening (Erreger et al., 2004), the single channel conductance of homomeric GluR1 AMPA receptors was significantly increased by phosphorylation of Ser831 in the intracellular C terminus by calcium calmodulin dependent protein kinase II (CAMKII) (Barria et al., 1997; Lee et al., 2000). Despite the fact that CAMKII still phosphorylates Ser831 of GluR1 in heteromers, the enhancement of the channel conductance is absent in heteromeric GluR1/GluR2 compared to homomeric GluR1 (Oh and Derkach, 2005). Therefore the presence of GluR2 suppresses the enhanced conductance of GluR1, which occurs during LTP and learning (Barria et al., 1997; Lee et al., 2000; Whitlock et al., 2006).

Early phase LTP is mediated by recruitment of AMPA receptors lacking the GluR2 subunit (Plant et al., 2006). Thereby altering the properties of synaptic AMPA receptors, such as the increase in channel conductance, which is associated with both hippocampal CA1 LTP and postsynaptic infusion of CAMKII (Benke et al., 1998; Poncer et al., 2002; Luthi et al., 2004). In addition AMPA receptors lacking GluR2 had an increased contribution in activity-dependent strengthening of hippocampal synapses (Ju et al., 2004; Thiagarajan et al., 2005). Experience-dependent strengthening of neocortical excitatory synapses was also associated with the increased contribution of AMPA receptors lacking the GluR2 subunit (Clem and Barth, 2006)

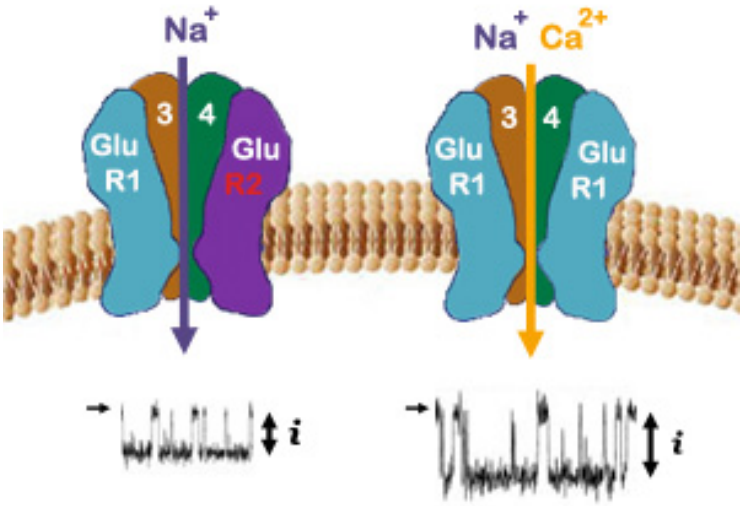

Figure 2.6.: AMPA Receptor Subunit Composition Ion Permeability

Diagram of the differences in ion permeability with the presence of GluR2 subunit, which is not permeable to calcium.

2.1.7. NMDA Receptors

The NMDA type of glutamate receptors contribute to a slow excitatory synaptic transmission in the mammalian central nervous system (Mayer et al., 1984). The influx of calcium through NMDA receptors play a vital role in synaptogenesis, synaptic remodeling, and long-lasting forms of synaptic plasticity such as LTP, the cellular model of learning and memory formation (Bliss and Collingridge, 1993; Constantine-Paton and Cline, 1998; Malenka and Nicoll, 1999; Lynch, 2004; Duguid and Sjostrom, 2006). These receptors are highly permeable to calcium (MacDermott et al., 1986; Ascher and Nowak, 1988) and therefore, overstimulation of NMDA receptors can lead to excitotoxicity.

The two major NMDA receptor subunits in rodents are NR1 and NR2 (Moriyoshi et al., 1991; Monyer et al., 1992; Ishii et al., 1993). Activation of NMDA receptors require the binding of glycine, which binds to the NR1 subunit, in addition to glutamate, which binds to the NR2 subunit (Reynolds et al., 1987; Henderson et al., 1990; Benveniste and Mayer, 1991; Clements and Westbrook, 1991). The NR2 subunit has four different isoforms (NR2A-NR2D), which are regulated developmentally (Monyer et al., 1994; Sheng et al., 1994; Zhong et al., 1995) and alter NMDA receptor permeability and kinetic properties (Kutsuwada et al., 1992; Monyer et al., 1992; Stern et al., 1992; Monyer et al., 1994; Kuner and Schoepfer, 1996). The NR2C subunit containing NMDA receptors are present at high levels only in cerebellar granule cells (Chazot et al., 1994; Thompson et al., 2000). There is currently no evidence of the NR2D subunit presence in the synapse but it is present in several different cell types extrasynaptically (Momiyama et al., 1996; Cull-Candy et al., 1998).

There is a developmental shift in functional properties and expression of the NR2A and NR2B subunit containing NMDA receptors in the cortex and hippocampus (Hestrin, 1992; Monyer et al., 1994; Sheng et al., 1994; Flint et al., 1997; van Zundert et al., 2004). The mRNA levels are more prevalent for NR1/NR2B when synapses are forming, whereas NR1/NR2A mRNA levels is low and then increase later in development in the hippocampus (McDonald and Johnston, 1990; Monyer et al., 1994). The NR2B subunit has a higher affinity for glutamate compared to that of NR2A (Laurie and Seeburg, 1994; Mori and Mishina, 1995; Priestley et al., 1995; Cull-Candy et al., 2001). Previous studies have shown that the NR2B subunit is located mainly extrasynaptic and NR2A subunit is mainly synaptic in the adult (Tovar and Westbrook,

1999). However, recent studies have reported that both subtypes may be located synaptic or extrasynaptic in mature cultured neurons (Thomas et al., 2006).

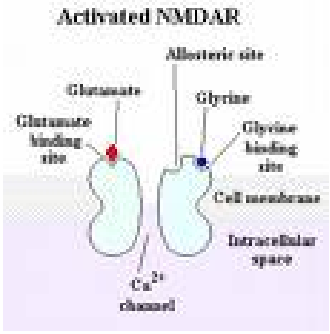

Figure 2.7.: Activated NMDA Receptor

Diagram of the two agonists required to activate NMDA receptors, glutamate and glycine, binding to two different subunits which are NR2 and NR1, respectively.

2.1.8. Hippocampus

The hippocampus is located in the medical temporal lobe posterior to the amygdala. It is composed of 6 parts: hippocampal fissure, parahippocampal gyrus, subicular cortex, entorhinal cortex, collateral sulcus, and perirhinal cortex. The Cornis Ammonis (CA) and dentate gyrus interlocks in a "C" shape forming the hippocampus. The CA has large excitatory pyramidal cells. There are 4 major regions that comprise the CA: CA1, CA2, CA3, and CA4. Polymorph, pyramidal, and molecular layers make up the three layers of the allocortex, which are GABAergic cells, pyramidal cells or glutamatergic cells, and basket cells or apical dendrites of pyramidal cells, respectively. The cortical sensory association area (entorhinal cortex) is one of six major inputs to the hippocampus. It is composed of 2 types of fibers: alvear and prefrontal. The septohippocampal pathway via fornix, ventral tegmental area, locus coeruleus, raphe nuclei, and contralateral hippocampus via hippocampal commissure are the other 5 inputs which are cholinergic

fivers, dopaminergic fibers, noradrenergic fibers, serotonergic fibers. The perforant pathway contains the glutamatergic system, which includes AMPA and NMDA subtype of glutamate receptors, and connects entorhinal cortex to dentate gyrus. Kainate, subtype of glutamate receptors, is in the mossy fibers which connect the dentate gyrus to CA3. Schaffer collaterals connect the CA3 to the CA1 region. The CA1 pyramidal cells send axons to subiculum. The neurons of the subiculum send input back to the main hippocampal output to the Entorhinal cortex.

2.1.9. AMPA and NMDA Desensitization

Desensitization is a receptor's entry into an inactive state, although the agonist remains tightly bound to the receptor. It is a functionally important phenomenon that occurs in fast ligand gated ion channels at chemical synapses. Furthermore desensitization is an important modulator of receptor function in that it determines the amplitude, duration and frequency of signaling between cells (Sun et al., 2002). Receptors that tend to desensitize more slowly allow larger currents (Choi, 1992) than receptors that desensitize quickly.

Previous studies have shown that neurotoxicity did not occur via calcium permeable AMPA receptors until the receptor desensitization was removed (Raymond et al., 1996). Exogenous NMDA and AMPA sensitivity of hippocampal slices during LTP in CA1 neurons was reduced (Youssef et al., 2000; Youssef et al., 2001). Desensitization of AMPA receptors appeared to be involved in the loss of response (Stone and Addae, 2002). Therefore, desensitization is protective to the neuron. The ligand-binding cleft formed by domain 1 and domain 2 of a receptor subunit or dimer interface, which is also

known as the "clamshell" ligand-binding cleft, and the gate of the ion channel play an important role in the process of receptor desensitization (Sun et al., 2002). During the resting state of a receptor, the configuration of the dimer is that of a closed channel and an open cleft or unbound ligand. Upon agonist binding, the ligand-binding domain closes, trapping the agonist at the dimer interface. Activation occurs when the interface remains intact and the conformational strain due to domain closure is transferred to channel gate, opening the channel—i.e. conformational change of channel occurs. Desensitization occurs when domain 1 breaks the dimer interface and decouples domain closure from channel gate opening. Thus, the channel remains closed although the agonist is bound to the receptor (Armstrong and Gouaux, 2000; Mayer et al., 2001; Madden, 2002; Sun et al., 2002).

Desensitization time constants for native AMPA receptors range from 1 to 10 milliseconds (Wheal and Thomson, 1995; Mosbacher et al., 1994). GluR3 and GluR4 homomeric AMPA receptors with the flop splice variants show desensitization constants of about 1 millisecond, whereas the flip variants are four times slower (Mosbacher et al., 1994). Therefore, since flip variants desensitize more slowly compared to flop (Dingledine et al., 1999), they carry larger currents (Choi, 1992), which is important in calcium permeable AMPA receptors. However, no difference in desensitization exists with GluR1 subunits between flip-flop variants. In addition, desensitization is not affected when GluR2 subunit is incorporated into homomeric receptors when GluR2 carried the flop splice variant. In contrast, if GluR2 carries the flip splice variant, an increase in the desensitization time constant is observed (Mosbacher et al., 1994).

RNA edited AMPA receptors at the R/G site recovered faster from desensitization than the unedited receptor. It is believed that these edited AMPA receptors desensitize to a lesser extent than the unedited with homomeric GluR4 being an exception (Seeburg, 1996). Receptors in which arginine replaced by glycine permits a faster recovery, can transmit trains of fast stimuli (Seeburg, 1996). However, this can be unfavorable for neurons containing calcium permeable channels, especially after neuronal insult (Jayakar and Dikshit, 2004).

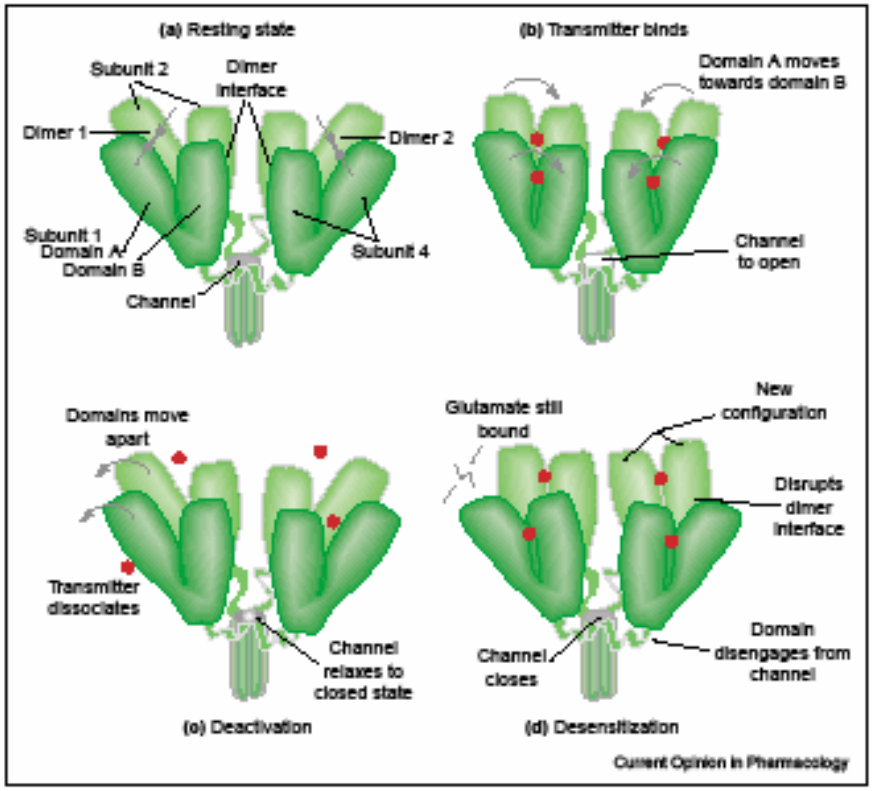

Figure 2.8.: AMPA Receptor Deactivation and Desensitization

Schematic version of AMPA receptor deactivation and desensitization as proposed by Sun et al., 2002. (a) The receptor is composed of four homologous subunits each with extracellular domains (A and B). Two subunits form a dimer. (b) Glutamate (red dots) binds to receptor, causing domain A to move toward domain B.

The channel closes when one of two events occurs: (c) Deactivation which involves the dissociation of glutamate followed by relaxation of the pore to its closed state. (d) Desensitization in which the transmitter remains bound but further domain movements disrupt the dimer interface and extracellular interactions with the transmembrane region. Thus the channel slows despite continued presence of agonist.

Desensitization of NMDA receptors are more complex than that interpreted for AMPA receptors. Weakening of agonist affinity when channels are activated and channels entering a long-lived non-conducting state are the two distinct forms of desensitization characterized for NMDA receptors (Nahum-Levy et al., 2001). Calcium and glycine independent desensitization occurs due to binding of glutamate. A high glutamate concentration is associated with a decrease in glycine affinity, which can be overcome with a high glycine concentration (Mayer et al., 1989; McBain and Mayer, 1994). This is not true desensitization but rather due to dissociation of glycine from its binding site triggered by the interaction of glutamate with the NMDA receptor (Benveniste et al., 1990; Lester et al., 1993). Desensitization may also be mediated by a rise in the activity of intracellular calcium, which is referred to as calcium-dependent inactivation, resulting form ion influx through NMDA receptor channels (Mayer and Westbrook, 1985; Mayer et al., 1987; Zorumski et al., 1989; Vyklicky et al., 1990; Legendre et al., 1993; Rosenmund and Westbrook, 1993). This form of NMDA receptor desensitization is thought to be modulated by second messenger systems and occur during synaptic transmission (Rosenmund and Westbrook, 1993; Tong et al., 1995; Raman et al., 1996). Since NMDA receptor responses in dialyzed small cells or excised membrane patches rapidly desensitized (Sather et al., 1990), NMDA receptors also have a glycine-sensitive desensitization (Mayer et al., 1989; Vyklicky et al., 1990). All forms

of NMDA receptor desensitization are subunit dependent. The calcium dependent desensitization is prominent for NR2A subunit containing NMDA receptors and not significant in NR2B or NR2C containing receptors (Medina et al., 1995; Krupp et al., 1996). The NR2A subunits have been showing fast deactivation and the most prominent in desensitization of all types. NMDA receptor deactivation is also dependent on subunit composition. There is a prolonged activation for NR2D subunit containing receptors early in development (Watanabe et al., 1992; Monyer et al., 1994) associated with a much slower deactivation, which is important for the formation, stabilization, or elimination of synapses during development.

2.1.10. Excitotoxicity

Increased intracellular calcium levels due to persistent NMDA receptors activation leads to the necrosis of both cortical and subcortical neurons, a phenomenon known as "excitotoxicity" (Dodd, 2002), which occurs due to excess exposure to endogenous toxins. Neurotoxicity occurs when neurons are exposed to exogenous neurotoxins disrupting normal activity. Both excitotoxicity and neurotoxicity may be involved in a large number of neurological disorders such as prolonged seizures, hypoglycemia, hypoxia, cerebral ischemia, microvasculature defects and, head traumas and chronic neurodegenerative disorders such as Alzheimer's disease, Huntington's chorea, Parkinson's disease, AIDS encephalopathy, ALS, epilepsy, schizophrenia, and dementia (Choi, 1992; Lipton and Rosenberg, 1994). However, the mechanism is not fully understood.

Calcium entry via the NMDA receptor is a key component to excitotoxic cell death (Tymianski et al., 1993). It has been shown that three phases of changes in intracellular calcium occurs prior to cell death (Randall and Thayer, 1992). The trigger phase is the phase in which the exposure to calcium occurs for at least 5 minutes leading to high intracellular levels. This is followed by a latent phase in which levels return to basal level. The latent phase may involve transient increases in intracellular calcium. The final phase is characterized as delayed calcium overload, which involves a gradual increase in calcium to a sustained plateau.

The activation of NMDA receptors and/or increase in neuronal calcium triggers other possible toxic events such as the activation of calpain, synthesis of nitric oxide (NO), production of protein kinase C, phospholipases, proteases, protein phosphatases, and reactive oxygen species (Dawson et al., 1991; Trout et al., 1993; Brorson et al., 1995; Ayata et al., 1997). Although inhibitors of calpain reduced ischemic neuronal loss in the hippocampus (Lee et al., 1991), an *in vitro* study did not show any neuroprotective effects of protein synthesis inhibitors against glutamate-induced neurotoxicity in cultured cortical neurons (Leppin et al., 1992). NO mediated glutamate-induced neurotoxicity in both cortical and striatal neurons (Dawson et al., 1991) and hippocampal slices (Izumi et al., 1992). Glutamate neurotoxicity in neuronal cultures has been shown to be mediated by a heteromeric NR1/NR2B receptor (Cheng et al., 1999).

Protein kinase C (PKC) activation modulates NMDA receptors (Ben-Ari et al., 1992; Kelso et al., 1992; Urushihara et al., 1992; Raymond et al., 1994; MacDonald et al., 1998; Xiong et al., 1998; Logan et al., 1999; Zheng et al., 1999) and calcium influx

through NMDA receptor channels (Grant et al., 1998). Thus elevation of PKC leads to neurotoxic NMDA receptor mediated activation (Wagey et al., 2001).

AMPA receptor antagonist have been found to more effective than NMDA receptor antagonist in preventing neuronal cell death in the CA1 region of the hippocampus following severe global ischemia (Nellgard and Wieloch, 1992; Sheardown et al., 1993; Choi, 1995). Thus, the role that AMPA receptors play in excitotoxicity is becoming more important. Since calcium permeable channels can be harmful to neurons on over-activation, under normal conditions in the hippocampus, GluR2 subunit containing AMPA receptors are delivered to the glutamatergic synapses, which lowers the probability of calcium permeable channel formation and thus protects neurons (Liu and Cull-Candy, 2000; Shi et al., 2001). AMPA receptor desensitization is a protective mechanism against this excess activity and excitotoxicity (Glazner et al., 2000). Therefore, altering the receptor recovery (slower) rate or the capacity to desensitize (faster) could be a way to modulate AMPA receptors to prevent excitotoxic damage to neurons.

2.1.11. Cyclothiazide

Cyclothiazide (CTZ), a benzothiadiazide diuretic compound, was initially developed as a drug to treat hypertension (Antlitz and Valle, 1967). CTZ was found to inhibit desensitization of AMPA receptors and prolong glutamatergic synaptic currents (Zorumski et al., 1993; Yamada and Tang, 1993; Patneau et al., 1993). It is the most potent and extensively studied positive modulation of AMPA receptors (Trussell et al., 1993; Diamond and Jahr, 1995; Clements et al., 1998; Ishikawa and Takahashi, 2001;

Mennerick and Zorumski, 1995; Barnes-Davies and Forsythe, 1995). Positive modulators of AMPA receptors facilitate LTP and improve learning and memory (Yamada, 1998; Staubli et al., 1994; Staubli et al., 1994; Lynch, 2002). However, CTZ might cause seizures and cell death (Yasuda et al., 2000; May and Robison, 1993; Moudy et al., 1994). Specifically, CTZ increases the frequency of ion channel openings and duration of spontaneous mEPSCs in rat hippocampus (Yamada and Tang, 1993), causes a slow increase in peak amplitude of whole cell currents (Yamada and Tang, 1993; Partin et al., 1994; Patneau et al., 1993), enhance current deactivation time (Patneau et al., 1993), prolongs single channel openings (Yamada and Tang, 1993), decrease agonist binding (Kessler et al., 1996; Hennegriff et al., 1997), and increase apparent affinity for agonists (Yamada and Tang, 1993; Partin et al., 1994; Patneau et al., 1993). In addition to its effects on AMPA receptors, CTZ strongly inhibits $GABA_A$ receptors diminishing GABAergic transmission (Deng and Chen, 2003).

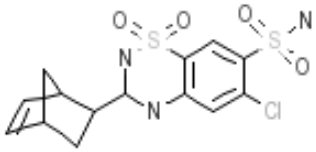

Figure 2.9.: CTZ

2.2. Cellular Recognition

2.2.1. Carbohydrates and Cellular Processes

Glycans or carbohydrates, consists of various chain lengths of simple sugars or monosaccharides. Carbohydrates play a critical role in cellular recognition and adhesive processes. They are involved in functions such as cell migration, neurite outgrowth and fasciculation, synapse formation and stabilization, and modulation of synaptic efficacy (Kleene and Schachner, 2004). Polysialic acid (PSA), a negatively charged carbohydrate composed of 8-200 sialic acid residues, is the most studied glycan in the nervous system. PSA orchestrates dynamic changes in the shape and movement of cells and their processes by decreasing adhesion forces and modulating cell surface interactions (Rutishauser, 1998). In NCAM deficient mice PSA was almost completely absent (Tomasiewicz et al., 1993; Cremer et al., 1994; Ono et al., 1994). Thus, PSA in mammals is found exclusively attached to NCAM in an unusual α-2, 8 linkage in its fifth immunoglobulin-like domain. PSA is thought to modulate homophilic and heterophilic binding of the NCAM glycoprotein backbone by steric interference, thus promoting plastic changes in the CNS (Weinhold et al., 2005).

2.2.2. Cell Adhesion Molecules

Cell adhesion molecules (CAMs), proteins that extend across the cell surface membrane, are responsible for activity-dependent long-term changes in synaptic strength and morphology (Ronn et al., 1998; Benson et al., 2000). They are good candidate molecules to take part in synaptic plasticity (Fields and Itoh, 1996; Ronn et al., 1999). The binding of CAMs have been shown to activate intracellular signal transduction

cascades, which initiate cellular responses including neurite outgrowth (Doherty and Walsh, 1996). The expression of certain CAMs are influenced by neuronal activity (Landmesser et al., 1990; Kiss et al., 1994; Muller et al., 1996; Holst et al., 1998). It has been well recognized that CAMs play important roles in the formation of neuronal connections during development (Edelman and Crossin, 1991) and synaptic plasticity (Doyle et al., 1992a, b; Scholey et al., 1993; Ronn et al., 1995; Luthi et al., 1994).

Integrins, cadherins, immunoglobulin-like, and selectins are the four major groups of CAMs. The neural cell adhesion molecule (NCAM), a member of the Ig superfamily, is involved in cell-cell adhesion, neurite outgrowth, synaptic plasticity, and learning and memory and is expressed on the surface of most cells. It promotes cell-cell adhesion via homophilic binding (Rutishauser, 1983; Doherty et al., 1990; Hall et al., 1990). Small changes in the expression of CAMs brought about changes in synaptic strength (Hoffman and Edelman, 1983; Mayford et al., 1992).

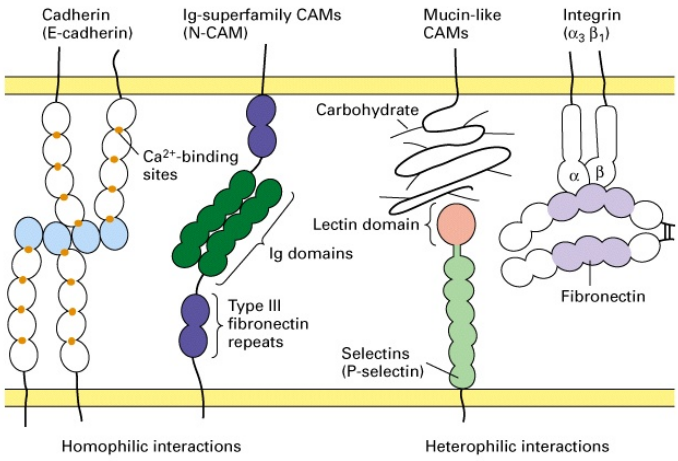

Figure 2.10.: Cell Adhesion Molecules

Diagram of the 4 types of cell adhesion molecules, their composition and type of interactions involved.

2.2.3. Neural Cell Adhesion Molecule

The neural cell adhesion molecule (NCAM) was the first cell adhesion molecule to be identified (Jorgensen et al., 1974; Rutishauser et al., 1976). NCAM is a member of the immunoglobulin (Ig) superfamily, which can be classified into four main groups. The extracellular portion of NCAM consists of five Ig homology modules, Ig 1-5, and two fibronectin type III (FnIII) homology modules. NCAM exists in three main forms—NCAM-180, NCAM-140, and NCAM -120, which differ in molecular weight, structural distribution, and functions (Schachner, 1997). NCAM is expressed in the central nervous system during neural tube closure and persists into adulthood on both neurons and glia. NCAM-140 is localized to migratory growth cones and axon shafts of developing neurons and mediates neurite outgrowth responses. However, NCAM-180 is enriched at site of cell contact and postsynaptic densities of mature neurons (Persohn et al., 1989). NCAM-120 is predominantly expresses by glial cells. Polysialic acid (PSA), a negatively charged carbohydrate, is found almost exclusively on NCAM in the vertebrate brain (Rougon et al., 1986; Cremer et al., 1994). PSA attaches to two asparagines in the Ig5 module of NCAM, which is catalyzed by one of the sialyltransferase enzymes (Kiss and Rougon, 1997). NCAM has been linked to disorders such as schizophrenia, bipolar disorder, and Alzheimer's disease and to learning and memory deficits in mice.

NCAM plays an important role in synaptic plasticity and is required for LTP of synaptic activity in the CA1 and CA3 regions of the hippocampus (Cremer et al., 1998). NCAM mediates homophilic cell adhesion via a calcium independent mechanism. It also binds to extracellular matrix components mediating heterophilic cell-substratum interactions. Upon homophilic binding an intracellular signaling cascade is activated,

which depends on a cis-interaction with the fibroblast growth factor receptor (FGR-R). Through activation of this intracellular cascade, NCAM promotes neurite outgrowth.

2.2.4. Sialic Acid / Polysialic Acid

Sialic Acid is a nine carbon monosaccharide that is found in animal tissues and bacteria, especially in glycoproteins and gangliosides. Sialic acid rich regions contribute to a negative charge on cell surfaces. Polysialic acid, a negatively charged carbohydrate, is a linear homopolymer glycan with an unusual α2, 8 linkage of up to 200 sialic acid residues. The two enzymes responsible for the formation of PSA identified in the brain are polysialyltransferase ST8siaIV and ST8siaII. ST8siaIV is predominantly expressed in the adult while ST8siaII is more highly expressed during development (Eckhardt et al., 2000). However, St8SiaII continues to be expressed in the adult brain mainly in neuroblasts and the innermost granule cell layer of the hippocampal dentate gyrus. The amount of PSA on the cell surface depends on two major regulatory mechanisms—the synthesis of PSA-NCAM and the turnover of the molecule at the cell surface (Kiss and Rougon, 1997).

PSA was originally found in 1982 in vertebrate brains by Finne (Finne et al., 1983) in associated with the neural cell adhesion molecule (NCAM). The cell biology of PSA indicates that it is a regulator of cell interactions via its physical properties rather than a specific affinity for a receptor. PSA orchestrates dynamic changes in the shape and movement of cells. Thus it is involved in neuronal cell migration, axonal guidance via the NCAM molecule, fasciculation, synaptogenesis, and synaptic plasticity. Both enzymes of PSA add sialic acid in an α2, 8 linkage to the terminal α2, 3 linked sialic acid

of N-glycans in the fifth immunoglobulin (Ig) link domain of all three isoforms of NCAM (von Der Ohe et al., 2002).

Purified PSA-NCAM *in vitro* studies show that PSA can decrease homophilic NCAM- mediated interactions (Cunningham et al., 1983; Sadoul et al., 1983). Therefore, it is an anti-adhesive component of the NCAM molecule due to its highly negative change and/or large hydration volume (Yang et al., 1994). The removal of PSA from cells can enhance the function of other cell adhesion molecules (CAMs) but has important consequences for neural development. In the developing brain, PSA is abundant and regulated on growing axons in many neural systems (Chuong and Edelman, 1984). It is highly expressed in the brain during the perinatal period, time frame in which most synapses are forming, but declines during postnatal development except in areas of postnatal neurogenesis (Rutishauser and Landmesser, 1996). PSA is not always present at synapses where NCAM is present and can be absent form NCAM-positive synapses as well (Seki and Arai, 1999).

Since AMPA receptors are required for LTP, the role of PSA in LTP may involve the modulation of AMPA receptors (Eckhardt et al., 2000). PSA increases AMPA receptor channel open time (Vaithianathan et al., 2004). In addition AMPA binding to its receptor is altered in desialyation of hippocampal membranes (Hoffman et al., 1997). Activity rapidly modulates PSA-NCAM and AMPA receptor expression on the cell surface (Isaac et al., 1995; Muller et al., 1996; Luscher et al., 1999). This rapid recycling may be essential for LTP (Isaac et al., 1995; Luscher et al., 1999).

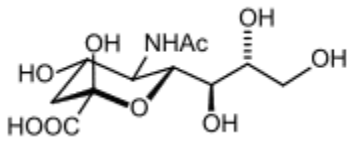

Figure 2.11.: Sialic Acid

2.2.5. PSA-NCAM

PSA-NCAM plays important roles in the development of the nervous system and NMDA receptor-dependent synaptic plasticity in the adult including synaptic remodeling and LTP (Tang and Landmesser, 1993; Muller et al., 1994). Activity-dependent synaptic plasticity is crucial for neural circuitry during brain development (Katz and Shatz, 1996). PSA-NCAM allows/prevents neurons from moving or changing morphology, thereby participating in axonal growth (Zhang et al., 1992) and synaptic reorganization (Seki and Rutishauser, 1998). The decrease in postnatal PSA expression is associated with a stabilization of cellular interactions and synapse formation (Szele et al., 1994). The expression of PSA is high during development and low in the adult brain. However, brain regions retaining PSA-NCAM is confined to areas of the brain characterized by a high level of structural remodeling such as the olfactory system and the mossy fiber system in the hippocampal formation (Ni Dhuill et al., 1999; Seki and Rutishauser, 1998). PSA-expression shows up-regulation during neuronal activity and learning-induced neuroplasticity in the hippocampus (Murphy et al., 1996). Biosynthesis of PSA-NCAM is regulated by cell activation (Kiss et al., 1994), NMDA-evoked increase in the intracellular calcium ions that can induce either exocytosis (Wang et al., 1996) or endocytosis (Bouzioukh et al., 2001) of PSA-NCAM with increased expression or

degradation, respectively. Removal of PSA by endosialidase-N, an enzyme which specifically cleaves endogenous PSA-NCAM, disturbs neuronal migration and axonal sprouting, branching and fasciculation, synaptogenesis, synaptic plasticity, and spatial memory (Rutishauser and Landmesser, 1996; Kiss and Rougon, 1997; Kleene and Schachner, 2004).

Glycosylation of NCAM with PSA results from the activity of ST8SiaII and ST8SiaIV, although ST8SiaII also has some activity (Angata et al., 2000). ST8SiaII or STX is highly expressed in embryonic development but diminishes rapidly in the early postnatal period, whereas ST8SiaIV or PST-1 remains high in the adult brain (Kurosawa et al., 1997; Ong et al., 1998). Mice that lacked PST-1 showed deficits in both LTP and LTD in the CA1 region of the hippocampus (Eckhardt et al., 2000). Earlier studies also confirmed that hippocampal slices treated with endoneuraminidase, which cleaves PSA from endogenous PSA-NCAM, inhibited LTP and LTD as well as spatial learning (Becker et al., 1996; Muller et al., 1996).

PSA-NCAM is necessary for structural remodeling of synaptic connections associated with long-term memory. Polysialylation of NCAM-180 was transiently increased during the acquisition and consolidation of a passive avoidance response in rats (Doyle et al., 1992b). Intraventricular injection of NCAM antibodies in rats and chicks impaired learning (Doyle et al., 1992a; Scholey et al., 1993) and inhibited the induction of early maintenance of LTP in the CA1 region of hippocampal slices in vitro (Olsen et al., 1995; Ronn et al., 1995). In addition, not only do NCAM-deficient mice have impairment in spatial learning tasks (Cremer et al., 1997), but also enzymatic removal of PSA inhibits spatial learning in rats in vivo (Becker et al., 1996) and inhibits LTP in the

hippocampal CA1 region in vitro (Becker et al., 1996; Muller et al., 1996). Thus the expression of PSA-NCAM is not only important for LTP but learning also. Since enzymatic removal of sialic acid from cortical or hippocampal membranes has been shown to modulate AMPA binding to AMPA receptors (Hoffman et al., 1997), the expression of PSA-NCAM may influence the activity of synaptic transmission mediated by glutamate receptors.

REFERENCES

Adams JP, Sweatt JD (2002) Molecular psychology: roles for the ERK MAP kinase cascade in memory. Annu Rev Pharmacol Toxicol 42:135-163.

Allison DW, Gelfand VI, Spector I, Craig AM (1998) Role of actin in anchoring postsynaptic receptors in cultured hippocampal neurons: differential attachment of NMDA versus AMPA receptors. J Neurosci 18:2423-2436.

Angata K, Suzuki M, McAuliffe J, Ding Y, Hindsgaul O, Fukuda M (2000) Differential biosynthesis of polysialic acid on neural cell adhesion molecule (NCAM) and oligosaccharide acceptors by three distinct alpha 2,8-sialyltransferases, ST8Sia IV (PST), ST8Sia II (STX), and ST8Sia III. J Biol Chem 275:18594-18601.

Arino JA, Denis R. (2004) Protein Phosphatases.

Armstrong N, Gouaux E (2000) Mechanisms for activation and antagonism of an AMPA-sensitive glutamate receptor: crystal structures of the GluR2 ligand binding core. Neuron 28:165-181.

Ascher P, Nowak L (1988) The role of divalent cations in the N-methyl-D-aspartate responses of mouse central neurones in culture. J Physiol 399:247-266.

Ayata C, Ayata G, Hara H, Matthews RT, Beal MF, Ferrante RJ, Endres M, Kim A, Christie RH, Waeber C, Huang PL, Hyman BT, Moskowitz MA (1997) Mechanisms of reduced striatal NMDA excitotoxicity in type I nitric oxide synthase knock-out mice. J Neurosci 17:6908-6917.

Banke TG, Bowie D, Lee H, Huganir RL, Schousboe A, Traynelis SF (2000) Control of GluR1 AMPA receptor function by cAMP-dependent protein kinase. J Neurosci 20:89-102.

Barria A, Derkach V, Soderling T (1997) Identification of the Ca2+/calmodulin-dependent protein kinase II regulatory phosphorylation site in the alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate-type glutamate receptor. J Biol Chem 272:32727-32730.

Bear MF (1996) Progress in understanding NMDA-receptor-dependent synaptic plasticity in the visual cortex. J Physiol Paris 90:223-227.

Beattie EC, Carroll RC, Yu X, Morishita W, Yasuda H, von Zastrow M, Malenka RC (2000) Regulation of AMPA receptor endocytosis by a signaling mechanism shared with LTD. Nat Neurosci 3:1291-1300.

Becker CG, Artola A, Gerardy-Schahn R, Becker T, Welzl H, Schachner M (1996) The polysialic acid modification of the neural cell adhesion molecule is involved in spatial learning and hippocampal long-term potentiation. J Neurosci Res 45:143-152.

Ben-Ari Y, Aniksztejn L, Bregestovski P (1992) Protein kinase C modulation of NMDA currents: an important link for LTP induction. Trends Neurosci 15:333-339.

Benke TA, Luthi A, Isaac JT, Collingridge GL (1998) Modulation of AMPA receptor unitary conductance by synaptic activity. Nature 393:793-797.

Benson DL, Schnapp LM, Shapiro L, Huntley GW (2000) Making memories stick: cell-adhesion molecules in synaptic plasticity. Trends Cell Biol 10:473-482.

Benveniste M, Mayer ML (1991) Kinetic analysis of antagonist action at N-methyl-D-aspartic acid receptors. Two binding sites each for glutamate and glycine. Biophys J 59:560-573.

Benveniste M, Clements J, Vyklicky L, Jr., Mayer ML (1990) A kinetic analysis of the modulation of N-methyl-D-aspartic acid receptors by glycine in mouse cultured hippocampal neurones. J Physiol 428:333-357.

Bliss TV, Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361:31-39.

Borgdorff AJ, Choquet D (2002) Regulation of AMPA receptor lateral movements. Nature 417:649-653.

Bouzioukh F, Tell F, Jean A, Rougon G (2001) NMDA receptor and nitric oxide synthase activation regulate polysialylated neural cell adhesion molecule expression in adult brainstem synapses. J Neurosci 21:4721-4730.

Brenman JE, Topinka JR, Cooper EC, McGee AW, Rosen J, Milroy T, Ralston HJ, Bredt DS (1998) Localization of postsynaptic density-93 to dendritic microtubules and interaction with microtubule-associated protein 1A. J Neurosci 18:8805-8813.

Brorson JR, Marcuccilli CJ, Miller RJ (1995) Delayed antagonism of calpain reduces excitotoxicity in cultured neurons. Stroke 26:1259-1266; discussion 1267.

Carroll RC, Zukin RS (2002) NMDA-receptor trafficking and targeting: implications for synaptic transmission and plasticity. Trends Neurosci 25:571-577.

Carroll RC, Beattie EC, von Zastrow M, Malenka RC (2001) Role of AMPA receptor endocytosis in synaptic plasticity. Nat Rev Neurosci 2:315-324.

Chazot PL, Coleman SK, Cik M, Stephenson FA (1994) Molecular characterization of N-methyl-D-aspartate receptors expressed in mammalian cells yields evidence for the coexistence of three subunit types within a discrete receptor molecule. J Biol Chem 269:24403-24409.

Chen HJ, Rojas-Soto M, Oguni A, Kennedy MB (1998) A synaptic Ras-GTPase activating protein (p135 SynGAP) inhibited by CaM kinase II. Neuron 20:895-904.

Chen L, Chetkovich DM, Petralia RS, Sweeney NT, Kawasaki Y, Wenthold RJ, Bredt DS, Nicoll RA (2000) Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. Nature 408:936-943.

Cheng C, Fass DM, Reynolds IJ (1999) Emergence of excitotoxicity in cultured forebrain neurons coincides with larger glutamate-stimulated [Ca(2+)](i) increases and NMDA receptor mRNA levels. Brain Res 849:97-108.

Choi DW (1992) Bench to bedside: the glutamate connection. Science 258:241-243.

Choi DW (1995) Calcium: still center-stage in hypoxic-ischemic neuronal death. Trends Neurosci 18:58-60.

Choi S, Klingauf J, Tsien RW (2000) Postfusional regulation of cleft glutamate concentration during LTP at 'silent synapses'. Nat Neurosci 3:330-336.

Chung HJ, Xia J, Scannevin RH, Zhang X, Huganir RL (2000) Phosphorylation of the AMPA receptor subunit GluR2 differentially regulates its interaction with PDZ domain-containing proteins. J Neurosci 20:7258-7267.

Chuong CM, Edelman GM (1984) Alterations in neural cell adhesion molecules during development of different regions of the nervous system. J Neurosci 4:2354-2368.

Clem RL, Barth A (2006) Pathway-specific trafficking of native AMPARs by in vivo experience. Neuron 49:663-670.

Clements JD, Westbrook GL (1991) Activation kinetics reveal the number of glutamate and glycine binding sites on the N-methyl-D-aspartate receptor. Neuron 7:605-613.

Collingridge GL, Isaac JT, Wang YT (2004) Receptor trafficking and synaptic plasticity. Nat Rev Neurosci 5:952-962.

Constantine-Paton M, Cline HT (1998) LTP and activity-dependent synaptogenesis: the more alike they are, the more different they become. Curr Opin Neurobiol 8:139-148.

Conti F, Weinberg RJ (1999) Shaping excitation at glutamatergic synapses. Trends Neurosci 22:451-458.

Craig AM (1998) Activity and synaptic receptor targeting: the long view. Neuron 21:459-462.

Cremer H, Chazal G, Goridis C, Represa A (1997) NCAM is essential for axonal growth and fasciculation in the hippocampus. Mol Cell Neurosci 8:323-335.

Cremer H, Chazal G, Carleton A, Goridis C, Vincent JD, Lledo PM (1998) Long-term but not short-term plasticity at mossy fiber synapses is impaired in neural cell adhesion molecule-deficient mice. Proc Natl Acad Sci U S A 95:13242-13247.

Cremer H, Lange R, Christoph A, Plomann M, Vopper G, Roes J, Brown R, Baldwin S, Kraemer P, Scheff S, et al. (1994) Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. Nature 367:455-459.

Cull-Candy S, Brickley S, Farrant M (2001) NMDA receptor subunits: diversity, development and disease. Curr Opin Neurobiol 11:327-335.

Cull-Candy SG, Brickley SG, Misra C, Feldmeyer D, Momiyama A, Farrant M (1998) NMDA receptor diversity in the cerebellum: identification of subunits contributing to functional receptors. Neuropharmacology 37:1369-1380.

Cullen PJ, Lockyer PJ (2002) Integration of calcium and Ras signalling. Nat Rev Mol Cell Biol 3:339-348.

Cunningham BA, Hoffman S, Rutishauser U, Hemperly JJ, Edelman GM (1983) Molecular topography of the neural cell adhesion molecule N-CAM: surface orientation and location of sialic acid-rich and binding regions. Proc Natl Acad Sci U S A 80:3116-3120.

Daw MI, Chittajallu R, Bortolotto ZA, Dev KK, Duprat F, Henley JM, Collingridge GL, Isaac JT (2000) PDZ proteins interacting with C-terminal GluR2/3 are involved in a PKC-dependent regulation of AMPA receptors at hippocampal synapses. Neuron 28:873-886.

Dawson VL, Dawson TM, London ED, Bredt DS, Snyder SH (1991) Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. Proc Natl Acad Sci U S A 88:6368-6371.

Derkach V, Barria A, Soderling TR (1999) Ca2+/calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. Proc Natl Acad Sci U S A 96:3269-3274.

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

Dodd PR (2002) Excited to death: different ways to lose your neurones. Biogerontology 3:51-56.

Doherty P, Walsh FS (1996) CAM-FGF receptor interactions: a model for axonal growth. Mol Cell Neurosci 8:99-111.

Doherty P, Fruns M, Seaton P, Dickson G, Barton CH, Sears TA, Walsh FS (1990) A threshold effect of the major isoforms of NCAM on neurite outgrowth. Nature 343:464-466.

Dong H, Zhang P, Liao D, Huganir RL (1999) Characterization, expression, and distribution of GRIP protein. Ann N Y Acad Sci 868:535-540.

Dong H, O'Brien RJ, Fung ET, Lanahan AA, Worley PF, Huganir RL (1997) GRIP: a synaptic PDZ domain-containing protein that interacts with AMPA receptors. Nature 386:279-284.

Doyle E, Nolan PM, Bell R, Regan CM (1992a) Intraventricular infusions of anti-neural cell adhesion molecules in a discrete posttraining period impair consolidation of a passive avoidance response in the rat. J Neurochem 59:1570-1573.

Doyle E, Nolan PM, Bell R, Regan CM (1992b) Hippocampal NCAM180 transiently increases sialylation during the acquisition and consolidation of a passive avoidance response in the adult rat. J Neurosci Res 31:513-523.

Duguid I, Sjostrom PJ (2006) Novel presynaptic mechanisms for coincidence detection in synaptic plasticity. Curr Opin Neurobiol 16:312-322.

Eckhardt M, Bukalo O, Chazal G, Wang L, Goridis C, Schachner M, Gerardy-Schahn R, Cremer H, Dityatev A (2000) Mice deficient in the polysialyltransferase ST8SiaIV/PST-1 allow discrimination of the roles of neural cell adhesion molecule protein and polysialic acid in neural development and synaptic plasticity. J Neurosci 20:5234-5244.

Edelman GM, Crossin KL (1991) Cell adhesion molecules: implications for a molecular histology. Annu Rev Biochem 60:155-190.

Ehlers MD (2000) Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting. Neuron 28:511-525.

Ehlers MD, Zhang S, Bernhadt JP, Huganir RL (1996) Inactivation of NMDA receptors by direct interaction of calmodulin with the NR1 subunit. Cell 84:745-755.

Erreger K, Chen PE, Wyllie DJ, Traynelis SF (2004) Glutamate receptor gating. Crit Rev Neurobiol 16:187-224.

Fields RD, Itoh K (1996) Neural cell adhesion molecules in activity-dependent development and synaptic plasticity. Trends Neurosci 19:473-480.

Finne J, Finne U, Deagostini-Bazin H, Goridis C (1983) Occurrence of alpha 2-8 linked polysialosyl units in a neural cell adhesion molecule. Biochem Biophys Res Commun 112:482-487.

Flint AC, Maisch US, Weishaupt JH, Kriegstein AR, Monyer H (1997) NR2A subunit expression shortens NMDA receptor synaptic currents in developing neocortex. J Neurosci 17:2469-2476.

Garner CC, Nash J, Huganir RL (2000) PDZ domains in synapse assembly and signalling. Trends Cell Biol 10:274-280.

Geiger JR, Melcher T, Koh DS, Sakmann B, Seeburg PH, Jonas P, Monyer H (1995) Relative abundance of subunit mRNAs determines gating and Ca2+ permeability of AMPA receptors in principal neurons and interneurons in rat CNS. Neuron 15:193-204.

Glazner GW, Chan SL, Lu C, Mattson MP (2000) Caspase-mediated degradation of AMPA receptor subunits: a mechanism for preventing excitotoxic necrosis and ensuring apoptosis. J Neurosci 20:3641-3649.

Grant ER, Bacskai BJ, Anegawa NJ, Pleasure DE, Lynch DR (1998) Opposing contributions of NR1 and NR2 to protein kinase C modulation of NMDA receptors. J Neurochem 71:1471-1481.

Grant SG, O'Dell TJ, Karl KA, Stein PL, Soriano P, Kandel ER (1992) Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice. Science 258:1903-1910.

Greger IH, Khatri L, Kong X, Ziff EB (2003) AMPA receptor tetramerization is mediated by Q/R editing. Neuron 40:763-774.

Hall AK, Nelson R, Rutishauser U (1990) Binding properties of detergent-solubilized NCAM. J Cell Biol 110:817-824.

Hayashi Y, Shi SH, Esteban JA, Piccini A, Poncer JC, Malinow R (2000) Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. Science 287:2262-2267.

Henderson G, Johnson JW, Ascher P (1990) Competitive antagonists and partial agonists at the glycine modulatory site of the mouse N-methyl-D-aspartate receptor. J Physiol 430:189-212.

Hestrin S (1992) Developmental regulation of NMDA receptor-mediated synaptic currents at a central synapse. Nature 357:686-689.

Hirao K, Hata Y, Ide N, Takeuchi M, Irie M, Yao I, Deguchi M, Toyoda A, Sudhof TC, Takai Y (1998) A novel multiple PDZ domain-containing molecule interacting with N-methyl-D-aspartate receptors and neuronal cell adhesion proteins. J Biol Chem 273:21105-21110.

Hoffman KB, Kessler M, Lynch G (1997) Sialic acid residues indirectly modulate the binding properties of AMPA-type glutamate receptors. Brain Res 753:309-314.

Hoffman S, Edelman GM (1983) Kinetics of homophilic binding by embryonic and adult forms of the neural cell adhesion molecule. Proc Natl Acad Sci U S A 80:5762-5766.

Holst BD, Vanderklish PW, Krushel LA, Zhou W, Langdon RB, McWhirter JR, Edelman GM, Crossin KL (1998) Allosteric modulation of AMPA-type glutamate receptors increases activity of the promoter for the neural cell adhesion molecule, N-CAM. Proc Natl Acad Sci U S A 95:2597-2602.

Husi H, Ward MA, Choudhary JS, Blackstock WP, Grant SG (2000) Proteomic analysis of NMDA receptor-adhesion protein signaling complexes. Nat Neurosci 3:661-669.

Isaac JT, Nicoll RA, Malenka RC (1995) Evidence for silent synapses: implications for the expression of LTP. Neuron 15:427-434.

Ishii T, Moriyoshi K, Sugihara H, Sakurada K, Kadotani H, Yokoi M, Akazawa C, Shigemoto R, Mizuno N, Masu M, et al. (1993) Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits. J Biol Chem 268:2836-2843.

Izumi Y, Benz AM, Clifford DB, Zorumski CF (1992) Nitric oxide inhibitors attenuate N-methyl-D-aspartate excitotoxicity in rat hippocampal slices. Neurosci Lett 135:227-230.

Jayakar SS, Dikshit M (2004) AMPA receptor regulation mechanisms: future target for safer neuroprotective drugs. Int J Neurosci 114:695-734.

Jorgensen OS, Bock E (1974) Brain specific synaptosomal membrane proteins demonstrated by crossed immunoelectrophoresis. J Neurochem 23:879-880.

Ju W, Morishita W, Tsui J, Gaietta G, Deerinck TJ, Adams SR, Garner CC, Tsien RY, Ellisman MH, Malenka RC (2004) Activity-dependent regulation of dendritic synthesis and trafficking of AMPA receptors. Nat Neurosci 7:244-253.

Katz LC, Shatz CJ (1996) Synaptic activity and the construction of cortical circuits. Science 274:1133-1138.

Kelso SR, Nelson TE, Leonard JP (1992) Protein kinase C-mediated enhancement of NMDA currents by metabotropic glutamate receptors in Xenopus oocytes. J Physiol 449:705-718.

Kim E, Naisbitt S, Hsueh YP, Rao A, Rothschild A, Craig AM, Sheng M (1997) GKAP, a novel synaptic protein that interacts with the guanylate kinase-like domain of the PSD-95/SAP90 family of channel clustering molecules. J Cell Biol 136:669-678.

Kim JH, Liao D, Lau LF, Huganir RL (1998) SynGAP: a synaptic RasGAP that associates with the PSD-95/SAP90 protein family. Neuron 20:683-691.

Kim JH, Lee HK, Takamiya K, Huganir RL (2003) The role of synaptic GTPase-activating protein in neuronal development and synaptic plasticity. J Neurosci 23:1119-1124.

Kim E, Sheng M (2004) PDZ domain proteins of synapses. Nat Rev Neurosci 5:771-781.

Kiss JZ, Rougon G (1997) Cell biology of polysialic acid. Curr Opin Neurobiol 7:640-646.

Kiss JZ, Wang C, Olive S, Rougon G, Lang J, Baetens D, Harry D, Pralong WF (1994) Activity-dependent mobilization of the adhesion molecule polysialic NCAM to the cell surface of neurons and endocrine cells. Embo J 13:5284-5292.

Kleene R, Schachner M (2004) Glycans and neural cell interactions. Nat Rev Neurosci 5:195-208.

Kojima N, Wang J, Mansuy IM, Grant SG, Mayford M, Kandel ER (1997) Rescuing impairment of long-term potentiation in fyn-deficient mice by introducing Fyn transgene. Proc Natl Acad Sci U S A 94:4761-4765.

Komiyama NH, Watabe AM, Carlisle HJ, Porter K, Charlesworth P, Monti J, Strathdee DJ, O'Carroll CM, Martin SJ, Morris RG, O'Dell TJ, Grant SG (2002) SynGAP regulates ERK/MAPK signaling, synaptic plasticity, and learning in the complex with postsynaptic density 95 and NMDA receptor. J Neurosci 22:9721-9732.

Kornau HC, Seeburg PH, Kennedy MB (1997) Interaction of ion channels and receptors with PDZ domain proteins. Curr Opin Neurobiol 7:368-373.

Krupp JJ, Vissel B, Heinemann SF, Westbrook GL (1996) Calcium-dependent inactivation of recombinant N-methyl-D-aspartate receptors is NR2 subunit specific. Mol Pharmacol 50:1680-1688.

Kuner T, Schoepfer R (1996) Multiple structural elements determine subunit specificity of Mg2+ block in NMDA receptor channels. J Neurosci 16:3549-3558.

Kurosawa N, Yoshida Y, Kojima N, Tsuji S (1997) Polysialic acid synthase (ST8Sia II/STX) mRNA expression in the developing mouse central nervous system. J Neurochem 69:494-503.

Kutsuwada T, Kashiwabuchi N, Mori H, Sakimura K, Kushiya E, Araki K, Meguro H, Masaki H, Kumanishi T, Arakawa M, et al. (1992) Molecular diversity of the NMDA receptor channel. Nature 358:36-41.

Lan JY, Skeberdis VA, Jover T, Grooms SY, Lin Y, Araneda RC, Zheng X, Bennett MV, Zukin RS (2001) Protein kinase C modulates NMDA receptor trafficking and gating. Nat Neurosci 4:382-390.

Landmesser L, Dahm L, Tang JC, Rutishauser U (1990) Polysialic acid as a regulator of intramuscular nerve branching during embryonic development. Neuron 4:655-667.

Laurie DJ, Seeburg PH (1994) Ligand affinities at recombinant N-methyl-D-aspartate receptors depend on subunit composition. Eur J Pharmacol 268:335-345.

Lee CJ, Bardoni R, Tong CK, Engelman HS, Joseph DJ, Magherini PC, MacDermott AB (2002) Functional expression of AMPA receptors on central terminals of rat dorsal root ganglion neurons and presynaptic inhibition of glutamate release. Neuron 35:135-146.

Lee HK, Barbarosie M, Kameyama K, Bear MF, Huganir RL (2000) Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity. Nature 405:955-959.

Lee HK, Takamiya K, Han JS, Man H, Kim CH, Rumbaugh G, Yu S, Ding L, He C, Petralia RS, Wenthold RJ, Gallagher M, Huganir RL (2003) Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. Cell 112:631-643.

Lee KS, Frank S, Vanderklish P, Arai A, Lynch G (1991) Inhibition of proteolysis protects hippocampal neurons from ischemia. Proc Natl Acad Sci U S A 88:7233-7237.

Legendre P, Rosenmund C, Westbrook GL (1993) Inactivation of NMDA channels in cultured hippocampal neurons by intracellular calcium. J Neurosci 13:674-684.

Leppin C, Finiels-Marlier F, Crawley JN, Montpied P, Paul SM (1992) Failure of a protein synthesis inhibitor to modify glutamate receptor-mediated neurotoxicity in vivo. Brain Res 581:168-170.

Lester RA, Tong G, Jahr CE (1993) Interactions between the glycine and glutamate binding sites of the NMDA receptor. J Neurosci 13:1088-1096.

Liao D, Zhang X, O'Brien R, Ehlers MD, Huganir RL (1999) Regulation of morphological postsynaptic silent synapses in developing hippocampal neurons. Nat Neurosci 2:37-43.

Lin JW, Wyszynski M, Madhavan R, Sealock R, Kim JU, Sheng M (1998) Yotiao, a novel protein of neuromuscular junction and brain that interacts with specific splice variants of NMDA receptor subunit NR1. J Neurosci 18:2017-2027.

Lin JW, Ju W, Foster K, Lee SH, Ahmadian G, Wyszynski M, Wang YT, Sheng M (2000) Distinct molecular mechanisms and divergent endocytotic pathways of AMPA receptor internalization. Nat Neurosci 3:1282-1290.

Lipton SA, Rosenberg PA (1994) Excitatory amino acids as a final common pathway for neurologic disorders. N Engl J Med 330:613-622.

Lisman J, Schulman H, Cline H (2002) The molecular basis of CaMKII function in synaptic and behavioural memory. Nat Rev Neurosci 3:175-190.

Lissin DV, Gomperts SN, Carroll RC, Christine CW, Kalman D, Kitamura M, Hardy S, Nicoll RA, Malenka RC, von Zastrow M (1998) Activity differentially regulates the surface expression of synaptic AMPA and NMDA glutamate receptors. Proc Natl Acad Sci U S A 95:7097-7102.

Liu SQ, Cull-Candy SG (2000) Synaptic activity at calcium-permeable AMPA receptors induces a switch in receptor subtype. Nature 405:454-458.

Logan SM, Rivera FE, Leonard JP (1999) Protein kinase C modulation of recombinant NMDA receptor currents: roles for the C-terminal C1 exon and calcium ions. J Neurosci 19:974-986.

Lu YM, Roder JC, Davidow J, Salter MW (1998) Src activation in the induction of long-term potentiation in CA1 hippocampal neurons. Science 279:1363-1367.

Luscher C, Xia H, Beattie EC, Carroll RC, von Zastrow M, Malenka RC, Nicoll RA (1999) Role of AMPA receptor cycling in synaptic transmission and plasticity. Neuron 24:649-658.

Luthi A, Chittajallu R, Duprat F, Palmer MJ, Benke TA, Kidd FL, Henley JM, Isaac JT, Collingridge GL (1999) Hippocampal LTD expression involves a pool of AMPARs regulated by the NSF-GluR2 interaction. Neuron 24:389-399.

Luthi A, Wikstrom MA, Palmer MJ, Matthews P, Benke TA, Isaac JT, Collingridge GL (2004) Bi-directional modulation of AMPA receptor unitary conductance by synaptic activity. BMC Neurosci 5:44.

Lynch G (2004) AMPA receptor modulators as cognitive enhancers. Curr Opin Pharmacol 4:4-11.

MacDermott AB, Mayer ML, Westbrook GL, Smith SJ, Barker JL (1986) NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurones. Nature 321:519-522.

MacDonald JF, Xiong XG, Lu WY, Raouf R, Orser BA (1998) Modulation of NMDA receptors. Prog Brain Res 116:191-208.

Madden DR (2002) The inner workings of the AMPA receptors. Curr Opin Drug Discov Devel 5:741-748.

Malenka RC, Nicoll RA (1999) Long-term potentiation--a decade of progress? Science 285:1870-1874.

Malinow R, Malenka RC (2002) AMPA receptor trafficking and synaptic plasticity. Annu Rev Neurosci 25:103-126.

Man HY, Lin JW, Ju WH, Ahmadian G, Liu L, Becker LE, Sheng M, Wang YT (2000) Regulation of AMPA receptor-mediated synaptic transmission by clathrin-dependent receptor internalization. Neuron 25:649-662.

Mansour M, Nagarajan N, Nehring RB, Clements JD, Rosenmund C (2001) Heteromeric AMPA receptors assemble with a preferred subunit stoichiometry and spatial arrangement. Neuron 32:841-853.

Mayer ML, Westbrook GL (1985) The action of N-methyl-D-aspartic acid on mouse spinal neurones in culture. J Physiol 361:65-90.

Mayer ML, Westbrook GL, Guthrie PB (1984) Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones. Nature 309:261-263.

Mayer ML, Vyklicky L, Jr., Clements J (1989) Regulation of NMDA receptor desensitization in mouse hippocampal neurons by glycine. Nature 338:425-427.

Mayer ML, Olson R, Gouaux E (2001) Mechanisms for ligand binding to GluR0 ion channels: crystal structures of the glutamate and serine complexes and a closed apo state. J Mol Biol 311:815-836.

Mayer ML, MacDermott AB, Westbrook GL, Smith SJ, Barker JL (1987) Agonist- and voltage-gated calcium entry in cultured mouse spinal cord neurons under voltage clamp measured using arsenazo III. J Neurosci 7:3230-3244.

Mayford M, Barzilai A, Keller F, Schacher S, Kandel ER (1992) Modulation of an NCAM-related adhesion molecule with long-term synaptic plasticity in Aplysia. Science 256:638-644.

McBain CJ, Mayer ML (1994) N-methyl-D-aspartic acid receptor structure and function. Physiol Rev 74:723-760.

McDonald JW, Johnston MV (1990) Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. Brain Res Brain Res Rev 15:41-70.

Medina I, Filippova N, Charton G, Rougeole S, Ben-Ari Y, Khrestchatisky M, Bregestovski P (1995) Calcium-dependent inactivation of heteromeric NMDA receptor-channels expressed in human embryonic kidney cells. J Physiol 482 (Pt 3):567-573.

Momiyama A, Feldmeyer D, Cull-Candy SG (1996) Identification of a native low-conductance NMDA channel with reduced sensitivity to Mg2+ in rat central neurones. J Physiol 494 (Pt 2):479-492.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12:529-540.

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, Seeburg PH (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science 256:1217-1221.

Mori H, Mishina M (1995) Structure and function of the NMDA receptor channel. Neuropharmacology 34:1219-1237.

Morishita W, Connor JH, Xia H, Quinlan EM, Shenolikar S, Malenka RC (2001) Regulation of synaptic strength by protein phosphatase 1. Neuron 32:1133-1148.

Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno N, Nakanishi S (1991) Molecular cloning and characterization of the rat NMDA receptor. Nature 354:31-37.

Mosbacher J, Schoepfer R, Monyer H, Burnashev N, Seeburg PH, Ruppersberg JP (1994) A molecular determinant for submillisecond desensitization in glutamate receptors. Science 266:1059-1062.

Mulkey RM, Endo S, Shenolikar S, Malenka RC (1994) Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. Nature 369:486-488.

Muller D, Stoppini L, Wang C, Kiss JZ (1994) A role for polysialylated neural cell adhesion molecule in lesion-induced sprouting in hippocampal organotypic cultures. Neuroscience 61:441-445.

Muller D, Wang C, Skibo G, Toni N, Cremer H, Calaora V, Rougon G, Kiss JZ (1996) PSA-NCAM is required for activity-induced synaptic plasticity. Neuron 17:413-422.

Murphy KJ, O'Connell AW, Regan CM (1996) Repetitive and transient increases in hippocampal neural cell adhesion molecule polysialylation state following multitrial spatial training. J Neurochem 67:1268-1274.

Nahum-Levy R, Lipinski D, Shavit S, Benveniste M (2001) Desensitization of NMDA receptor channels is modulated by glutamate agonists. Biophys J 80:2152-2166.

Naisbitt S, Kim E, Weinberg RJ, Rao A, Yang FC, Craig AM, Sheng M (1997) Characterization of guanylate kinase-associated protein, a postsynaptic density protein at excitatory synapses that interacts directly with postsynaptic density-95/synapse-associated protein 90. J Neurosci 17:5687-5696.

Naisbitt S, Kim E, Tu JC, Xiao B, Sala C, Valtschanoff J, Weinberg RJ, Worley PF, Sheng M (1999) Shank, a novel family of postsynaptic density proteins that binds to the NMDA receptor/PSD-95/GKAP complex and cortactin. Neuron 23:569-582.

Nellgard B, Wieloch T (1992) Postischemic blockade of AMPA but not NMDA receptors mitigates neuronal damage in the rat brain following transient severe cerebral ischemia. J Cereb Blood Flow Metab 12:2-11.

Nicoll RA, Tomita S, Bredt DS (2006) Auxiliary subunits assist AMPA-type glutamate receptors. Science 311:1253-1256.

Ni Dhuill CM, Fox GB, Pittock SJ, O'Connell AW, Murphy KJ, Regan CM (1999) Polysialylated neural cell adhesion molecule expression in the dentate gyrus of the human hippocampal formation from infancy to old age. J Neurosci Res 55:99-106.

Niethammer M, Valtschanoff JG, Kapoor TM, Allison DW, Weinberg RJ, Craig AM, Sheng M (1998) CRIPT, a novel postsynaptic protein that binds to the third PDZ domain of PSD-95/SAP90. Neuron 20:693-707.

Oh MC, Derkach VA (2005) Dominant role of the GluR2 subunit in regulation of AMPA receptors by CaMKII. Nat Neurosci 8:853-854.

Olsen M, Zuber C, Roth J, Linnemann D, Bock E (1995) The ability to re-express polysialylated NCAM in soleus muscle after denervation is reduced in aged rats compared to young adult rats. Int J Dev Neurosci 13:97-104.

Ong E, Nakayama J, Angata K, Reyes L, Katsuyama T, Arai Y, Fukuda M (1998) Developmental regulation of polysialic acid synthesis in mouse directed by two polysialyltransferases, PST and STX. Glycobiology 8:415-424.

Ono K, Tomasiewicz H, Magnuson T, Rutishauser U (1994) N-CAM mutation inhibits tangential neuronal migration and is phenocopied by enzymatic removal of polysialic acid. Neuron 13:595-609.

Passafaro M, Piech V, Sheng M (2001) Subunit-specific temporal and spatial patterns of AMPA receptor exocytosis in hippocampal neurons. Nat Neurosci 4:917-926.

Pellegrini-Giampietro DE, Gorter JA, Bennett MV, Zukin RS (1997) The GluR2 (GluR-B) hypothesis: Ca(2+)-permeable AMPA receptors in neurological disorders. Trends Neurosci 20:464-470.

Persohn E, Pollerberg GE, Schachner M (1989) Immunoelectron-microscopic localization of the 180 kD component of the neural cell adhesion molecule N-CAM in postsynaptic membranes. J Comp Neurol 288:92-100.

Plant K, Pelkey KA, Bortolotto ZA, Morita D, Terashima A, McBain CJ, Collingridge GL, Isaac JT (2006) Transient incorporation of native GluR2-lacking AMPA receptors during hippocampal long-term potentiation. Nat Neurosci 9:602-604.

Poncer JC, Esteban JA, Malinow R (2002) Multiple mechanisms for the potentiation of AMPA receptor-mediated transmission by alpha-Ca2+/calmodulin-dependent protein kinase II. J Neurosci 22:4406-4411.

Priestley T, Laughton P, Myers J, Le Bourdelles B, Kerby J, Whiting PJ (1995) Pharmacological properties of recombinant human N-methyl-D-aspartate receptors comprising NR1a/NR2A and NR1a/NR2B subunit assemblies expressed in permanently transfected mouse fibroblast cells. Mol Pharmacol 48:841-848.

Raman IM, Tong G, Jahr CE (1996) Beta-adrenergic regulation of synaptic NMDA receptors by cAMP-dependent protein kinase. Neuron 16:415-421.

Randall RD, Thayer SA (1992) Glutamate-induced calcium transient triggers delayed calcium overload and neurotoxicity in rat hippocampal neurons. J Neurosci 12:1882-1895.

Rao A, Craig AM (1997) Activity regulates the synaptic localization of the NMDA receptor in hippocampal neurons. Neuron 19:801-812.

Raymond LA, Moshaver A, Tingley WG, Huganir RL (1996) Glutamate receptor ion channel properties predict vulnerability to cytotoxicity in a transfected nonneuronal cell line. Mol Cell Neurosci 7:102-115.

Raymond LA, Tingley WG, Blackstone CD, Roche KW, Huganir RL (1994) Glutamate receptor modulation by protein phosphorylation. J Physiol Paris 88:181-192.

Renger JJ, Egles C, Liu G (2001) A developmental switch in neurotransmitter flux enhances synaptic efficacy by affecting AMPA receptor activation. Neuron 29:469-484.

Reynolds IJ, Murphy SN, Miller RJ (1987) 3H-labeled MK-801 binding to the excitatory amino acid receptor complex from rat brain is enhanced by glycine. Proc Natl Acad Sci U S A 84:7744-7748.

Roche KW, Standley S, McCallum J, Dune Ly C, Ehlers MD, Wenthold RJ (2001) Molecular determinants of NMDA receptor internalization. Nat Neurosci 4:794-802.

Ronn LC, Hartz BP, Bock E (1998) The neural cell adhesion molecule (NCAM) in development and plasticity of the nervous system. Exp Gerontol 33:853-864.

Ronn LC, Bock E, Linnemann D, Jahnsen H (1995) NCAM-antibodies modulate induction of long-term potentiation in rat hippocampal CA1. Brain Res 677:145-151.

Ronn LC, Olsen M, Ostergaard S, Kiselyov V, Berezin V, Mortensen MT, Lerche MH, Jensen PH, Soroka V, Saffell JL, Doherty P, Poulsen FM, Bock E, Holm A (1999) Identification of a neuritogenic ligand of the neural cell adhesion molecule using a combinatorial library of synthetic peptides. Nat Biotechnol 17:1000-1005.

Rosenmund C, Westbrook GL (1993) Rundown of N-methyl-D-aspartate channels during whole-cell recording in rat hippocampal neurons: role of Ca2+ and ATP. J Physiol 470:705-729.

Rougon G, Dubois C, Buckley N, Magnani JL, Zollinger W (1986) A monoclonal antibody against meningococcus group B polysaccharides distinguishes embryonic from adult N-CAM. J Cell Biol 103:2429-2437.

Rutishauser U (1983) Molecular and biological properties of a neural cell adhesion molecule. Cold Spring Harb Symp Quant Biol 48 Pt 2:501-514.

Rutishauser U, Thiery JP, Brackenbury R, Sela BA, Edelman GM (1976) Mechanisms of adhesion among cells from neural tissues of the chick embryo. Proc Natl Acad Sci U S A 73:577-581.

Rutishauser U (1998) Polysialic acid at the cell surface: biophysics in service of cell interactions and tissue plasticity. J Cell Biochem 70:304-312.

Rutishauser U, Landmesser L (1996) Polysialic acid in the vertebrate nervous system: a promoter of plasticity in cell-cell interactions. Trends Neurosci 19:422-427.

Sadoul R, Hirn M, Deagostini-Bazin H, Rougon G, Goridis C (1983) Adult and embryonic mouse neural cell adhesion molecules have different binding properties. Nature 304:347-349.

Sans N, Racca C, Petralia RS, Wang YX, McCallum J, Wenthold RJ (2001) Synapse-associated protein 97 selectively associates with a subset of AMPA receptors early in their biosynthetic pathway. J Neurosci 21:7506-7516.

Sather W, Johnson JW, Henderson G, Ascher P (1990) Glycine-insensitive desensitization of NMDA responses in cultured mouse embryonic neurons. Neuron 4:725-731.

Satoh K, Yanai H, Senda T, Kohu K, Nakamura T, Okumura N, Matsumine A, Kobayashi S, Toyoshima K, Akiyama T (1997) DAP-1, a novel protein that interacts with the guanylate kinase-like domains of hDLG and PSD-95. Genes Cells 2:415-424.

Scannevin RH, Huganir RL (2000) Postsynaptic organization and regulation of excitatory synapses. Nat Rev Neurosci 1:133-141.

Schachner M (1997) Neural recognition molecules and synaptic plasticity. Curr Opin Cell Biol 9:627-634.

Scholey AB, Rose SP, Zamani MR, Bock E, Schachner M (1993) A role for the neural cell adhesion molecule in a late, consolidating phase of glycoprotein synthesis six hours following passive avoidance training of the young chick. Neuroscience 55:499-509.

Scott DB, Blanpied TA, Swanson GT, Zhang C, Ehlers MD (2001) An NMDA receptor ER retention signal regulated by phosphorylation and alternative splicing. J Neurosci 21:3063-3072.

Seeburg PH (1996) The role of RNA editing in controlling glutamate receptor channel properties. J Neurochem 66:1-5.

Seki T, Rutishauser U (1998) Removal of polysialic acid-neural cell adhesion molecule induces aberrant mossy fiber innervation and ectopic synaptogenesis in the hippocampus. J Neurosci 18:3757-3766.

Seki T, Arai Y (1999) Different polysialic acid-neural cell adhesion molecule expression patterns in distinct types of mossy fiber boutons in the adult hippocampus. J Comp Neurol 410:115-125.

Sheardown MJ, Suzdak PD, Nordholm L (1993) AMPA, but not NMDA, receptor antagonism is neuroprotective in gerbil global ischaemia, even when delayed 24 h. Eur J Pharmacol 236:347-353.

Sheng M, Kim E (1996) Ion channel associated proteins. Curr Opin Neurobiol 6:602-608.

Sheng M, Pak DT (2000) Ligand-gated ion channel interactions with cytoskeletal and signaling proteins. Annu Rev Physiol 62:755-778.

Sheng M, Lee SH (2001) AMPA receptor trafficking and the control of synaptic transmission. Cell 105:825-828.

Sheng M, Cummings J, Roldan LA, Jan YN, Jan LY (1994) Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. Nature 368:144-147.

Shi S, Hayashi Y, Esteban JA, Malinow R (2001) Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. Cell 105:331-343.

Soderling TR, Derkach VA (2000) Postsynaptic protein phosphorylation and LTP. Trends Neurosci 23:75-80.

Song I, Huganir RL (2002) Regulation of AMPA receptors during synaptic plasticity. Trends Neurosci 25:578-588.

Srivastava S, Osten P, Vilim FS, Khatri L, Inman G, States B, Daly C, DeSouza S, Abagyan R, Valtschanoff JG, Weinberg RJ, Ziff EB (1998) Novel anchorage of GluR2/3 to the postsynaptic density by the AMPA receptor-binding protein ABP. Neuron 21:581-591.

Steigerwald F, Schulz TW, Schenker LT, Kennedy MB, Seeburg PH, Kohr G (2000) C-Terminal truncation of NR2A subunits impairs synaptic but not extrasynaptic localization of NMDA receptors. J Neurosci 20:4573-4581.

Stern P, Behe P, Schoepfer R, Colquhoun D (1992) Single-channel conductances of NMDA receptors expressed from cloned cDNAs: comparison with native receptors. Proc Biol Sci 250:271-277.

Stone TW, Addae JI (2002) The pharmacological manipulation of glutamate receptors and neuroprotection. Eur J Pharmacol 447:285-296.

Sun Y, Olson R, Horning M, Armstrong N, Mayer M, Gouaux E (2002) Mechanism of glutamate receptor desensitization. Nature 417:245-253.

Sutton MA, Ito HT, Cressy P, Kempf C, Woo JC, Schuman EM (2006) Miniature neurotransmission stabilizes synaptic function via tonic suppression of local dendritic protein synthesis. Cell 125:785-799.

Swanson GT, Kamboj SK, Cull-Candy SG (1997) Single-channel properties of recombinant AMPA receptors depend on RNA editing, splice variation, and subunit composition. J Neurosci 17:58-69.

Szele FG, Dowling JJ, Gonzales C, Theveniau M, Rougon G, Chesselet MF (1994) Pattern of expression of highly polysialylated neural cell adhesion molecule in the developing and adult rat striatum. Neuroscience 60:133-144.

Takeuchi M, Hata Y, Hirao K, Toyoda A, Irie M, Takai Y (1997) SAPAPs. A family of PSD-95/SAP90-associated proteins localized at postsynaptic density. J Biol Chem 272:11943-11951.

Tang J, Landmesser L (1993) Reduction of intramuscular nerve branching and synaptogenesis is correlated with decreased motoneuron survival. J Neurosci 13:3095-3103.

Tavalin SJ, Colledge M, Hell JW, Langeberg LK, Huganir RL, Scott JD (2002) Regulation of GluR1 by the A-kinase anchoring protein 79 (AKAP79) signaling complex shares properties with long-term depression. J Neurosci 22:3044-3051.

Thiagarajan TC, Lindskog M, Tsien RW (2005) Adaptation to synaptic inactivity in hippocampal neurons. Neuron 47:725-737.

Thomas CG, Miller AJ, Westbrook GL (2006) Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons. J Neurophysiol 95:1727-1734.

Thompson CL, Drewery DL, Atkins HD, Stephenson FA, Chazot PL (2000) Immunohistochemical localization of N-methyl-D-aspartate receptor NR1, NR2A, NR2B and NR2C/D subunits in the adult mammalian cerebellum. Neurosci Lett 283:85-88.

Tomasiewicz H, Ono K, Yee D, Thompson C, Goridis C, Rutishauser U, Magnuson T (1993) Genetic deletion of a neural cell adhesion molecule variant (N-CAM-180) produces distinct defects in the central nervous system. Neuron 11:1163-1174.

Tong G, Shepherd D, Jahr CE (1995) Synaptic desensitization of NMDA receptors by calcineurin. Science 267:1510-1512.

Tovar KR, Westbrook GL (1999) The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. J Neurosci 19:4180-4188.

Trout JJ, Koenig H, Goldstone AD, Iqbal Z, Lu CY, Siddiqui F (1993) N-methyl-D-aspartate receptor excitotoxicity involves activation of polyamine synthesis: protection by alpha-difluoromethylornithine. J Neurochem 60:352-355.

Tymianski M, Charlton MP, Carlen PL, Tator CH (1993) Source specificity of early calcium neurotoxicity in cultured embryonic spinal neurons. J Neurosci 13:2085-2104.

Urushihara H, Tohda M, Nomura Y (1992) Selective potentiation of N-methyl-D-aspartate-induced current by protein kinase C in Xenopus oocytes injected with rat brain RNA. J Biol Chem 267:11697-11700.

Vaithianathan T, Matthias K, Bahr B, Schachner M, Suppiramaniam V, Dityatev A, Steinhauser C (2004) Neural cell adhesion molecule-associated polysialic acid potentiates alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor currents. J Biol Chem 279:47975-47984.

van Zundert B, Yoshii A, Constantine-Paton M (2004) Receptor compartmentalization and trafficking at glutamate synapses: a developmental proposal. Trends Neurosci 27:428-437.

von Der Ohe M, Wheeler SF, Wuhrer M, Harvey DJ, Liedtke S, Muhlenhoff M, Gerardy-Schahn R, Geyer H, Dwek RA, Geyer R, Wing DR, Schachner M (2002) Localization and characterization of polysialic acid-containing N-linked glycans from bovine NCAM. Glycobiology 12:47-63.

Vyklicky L, Jr., Benveniste M, Mayer ML (1990) Modulation of N-methyl-D-aspartic acid receptor desensitization by glycine in mouse cultured hippocampal neurones. J Physiol 428:313-331.

Wagey R, Hu J, Pelech SL, Raymond LA, Krieger C (2001) Modulation of NMDA-mediated excitotoxicity by protein kinase C. J Neurochem 78:715-726.

Walikonis RS, Oguni A, Khorosheva EM, Jeng CJ, Asuncion FJ, Kennedy MB (2001) Densin-180 forms a ternary complex with the (alpha)-subunit of Ca2+/calmodulin-dependent protein kinase II and (alpha)-actinin. J Neurosci 21:423-433.

Wang C, Pralong WF, Schulz MF, Rougon G, Aubry JM, Pagliusi S, Robert A, Kiss JZ (1996) Functional N-methyl-D-aspartate receptors in O-2A glial precursor cells: a critical role in regulating polysialic acid-neural cell adhesion molecule expression and cell migration. J Cell Biol 135:1565-1581.

Wang YT, Salter MW (1994) Regulation of NMDA receptors by tyrosine kinases and phosphatases. Nature 369:233-235.

Wang YT, Linden DJ (2000) Expression of cerebellar long-term depression requires postsynaptic clathrin-mediated endocytosis. Neuron 25:635-647.

Washbourne P, Bennett JE, McAllister AK (2002) Rapid recruitment of NMDA receptor transport packets to nascent synapses. Nat Neurosci 5:751-759.

Watanabe M, Inoue Y, Sakimura K, Mishina M (1992) Developmental changes in distribution of NMDA receptor channel subunit mRNAs. Neuroreport 3:1138-1140.

Weinhold B, Seidenfaden R, Rockle I, Muhlenhoff M, Schertzinger F, Conzelmann S, Marth JD, Gerardy-Schahn R, Hildebrandt H (2005) Genetic ablation of polysialic acid causes severe neurodevelopmental defects rescued by deletion of the neural cell adhesion molecule. J Biol Chem 280:42971-42977.

Wenthold RJ, Petralia RS, Blahos J, II, Niedzielski AS (1996) Evidence for multiple AMPA receptor complexes in hippocampal CA1/CA2 neurons. J Neurosci 16:1982-1989.

Westphal RS, Tavalin SJ, Lin JW, Alto NM, Fraser ID, Langeberg LK, Sheng M, Scott JD (1999) Regulation of NMDA receptors by an associated phosphatase-kinase signaling complex. Science 285:93-96.

Wheal HV TA (1995) Excitatory amino acids and synaptic transmission: London: Academic Press.

Whitlock JR, Heynen AJ, Shuler MG, Bear MF (2006) Learning induces long-term potentiation in the hippocampus. Science 313:1093-1097.

Wyszynski M, Lin J, Rao A, Nigh E, Beggs AH, Craig AM, Sheng M (1997) Competitive binding of alpha-actinin and calmodulin to the NMDA receptor. Nature 385:439-442.

Wyszynski M, Valtschanoff JG, Naisbitt S, Dunah AW, Kim E, Standaert DG, Weinberg R, Sheng M (1999) Association of AMPA receptors with a subset of glutamate receptor-interacting protein in vivo. J Neurosci 19:6528-6537.

Xia J, Zhang X, Staudinger J, Huganir RL (1999) Clustering of AMPA receptors by the synaptic PDZ domain-containing protein PICK1. Neuron 22:179-187.

Xiong ZG, Raouf R, Lu WY, Wang LY, Orser BA, Dudek EM, Browning MD, MacDonald JF (1998) Regulation of N-methyl-D-aspartate receptor function by constitutively active protein kinase C. Mol Pharmacol 54:1055-1063.

Yang P, Major D, Rutishauser U (1994) Role of charge and hydration in effects of polysialic acid on molecular interactions on and between cell membranes. J Biol Chem 269:23039-23044.

Youssef F, Stone TW, Addae JI (2000) Interactions of glutamate receptor agonists with long-term potentiation in the rat hippocampal slice. Eur J Pharmacol 398:349-359.

Youssef FF, Addae JI, McRae A, Stone TW (2001) Long-term potentiation protects rat hippocampal slices from the effects of acute hypoxia. Brain Res 907:144-150.

Yu XM, Askalan R, Keil GJ, 2nd, Salter MW (1997) NMDA channel regulation by channel-associated protein tyrosine kinase Src. Science 275:674-678.

Yun HY, Gonzalez-Zulueta M, Dawson VL, Dawson TM (1998) Nitric oxide mediates N-methyl-D-aspartate receptor-induced activation of p21ras. Proc Natl Acad Sci U S A 95:5773-5778.

Zakharenko SS, Zablow L, Siegelbaum SA (2001) Visualization of changes in presynaptic function during long-term synaptic plasticity. Nat Neurosci 4:711-717.

Zamanillo D, Sprengel R, Hvalby O, Jensen V, Burnashev N, Rozov A, Kaiser KM, Koster HJ, Borchardt T, Worley P, Lubke J, Frotscher M, Kelly PH, Sommer B, Andersen P, Seeburg PH, Sakmann B (1999) Importance of AMPA receptors for hippocampal synaptic plasticity but not for spatial learning. Science 284:1805-1811.

Zhang H, Miller RH, Rutishauser U (1992) Polysialic acid is required for optimal growth of axons on a neuronal substrate. J Neurosci 12:3107-3114.

Zhang S, Ehlers MD, Bernhardt JP, Su CT, Huganir RL (1998) Calmodulin mediates calcium-dependent inactivation of N-methyl-D-aspartate receptors. Neuron 21:443-453.

Zheng X, Zhang L, Wang AP, Bennett MV, Zukin RS (1999) Protein kinase C potentiation of N-methyl-D-aspartate receptor activity is not mediated by phosphorylation of N-methyl-D-aspartate receptor subunits. Proc Natl Acad Sci U S A 96:15262-15267.

Zhong J, Carrozza DP, Williams K, Pritchett DB, Molinoff PB (1995) Expression of mRNAs encoding subunits of the NMDA receptor in developing rat brain. J Neurochem 64:531-539.

Zhu JJ, Qin Y, Zhao M, Van Aelst L, Malinow R (2002) Ras and Rap control AMPA receptor trafficking during synaptic plasticity. Cell 110:443-455.

Zorumski CF, Yang J, Fischbach GD (1989) Calcium-dependent, slow desensitization distinguishes different types of glutamate receptors. Cell Mol Neurobiol 9:95-104.

3. SINGLE CHANNEL RECORDINGS FROM SYNAPTOSOMAL NMDA RECEPTORS

ABSTRACT

The majority of excitatory neurotransmission in the mammalian central nervous system is mediated through ionotropic glutamate receptors. There are currently no techniques available to directly measure the single channel properties of synaptic glutamate receptors. Therefore, we have utilized biochemical isolation of synaptosomes and subsequent reconstitution of these synaptosomes into artificial phospholipid bilayers to characterize the single channel properties of synaptic NMDA (N-methyl-D-aspartate) receptors under controlled conditions. The single synaptic NMDA receptor exhibited channel conductances of approximately 25-50 pS depending on its subunit composition. The single channel open time for NMDA receptors were constructed with one to two exponential fits ranging from 1.3 to 8.3 milliseconds. The closed times were fitted with one or two exponentials ranging from 10 to 86 milliseconds. The results indicate that the single channel properties of synaptic NMDA receptors are influenced by their subunit composition.

INTRODUCTION

Excitatory neurotransmission is the result of the presynaptic terminal release of the neurotransmitter, glutamate, from vesicles activating the postsynaptic glutamate receptors (Dingledine et al., 1999). NMDA receptors require the binding of glycine in addition to glutamate for activation (Reynolds et al., 1987; Henderson et al., 1990;

Benveniste and Mayer, 1991; Clements and Westbrook, 1991). Unlike the α amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) subtype of glutamate receptors, the NMDA subtype contribute to a slower excitatory synaptic transmission at sites throughout the brain and spinal cord, due to the voltage-dependent magnesium block (Mayer et al., 1984; Nowak et al., 1984). However, due to its higher affinity for glutamate (Patneau and Mayer, 1990), NMDA receptor currents exhibit slow decay (Lester and Jahr, 1992). NMDA receptors also are involved in long-term potentiation (LTP) through pre- and postsynaptic coincidence detection (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Lynch, 2004; Duguid and Sjostrom, 2006) and are highly permeable to calcium (MacDermott et al., 1986; Ascher and Nowak, 1988). NMDA receptors play a critical role in learning and some forms of associative memory (Tsien et al., 1996). Previous findings have proposed NMDA receptors to be involved in certain pathophysiological conditions of the nervous system such as AIDS-related dementia, epilepsy, neuronal cell death following stroke, trauma, or ischemia, and ethanol intoxication (Collingridge and Lester, 1989; Lipton, 1992; Grant, 1994; Lipton and Rosenberg, 1994).

The two major families of NMDA receptor subunits in the rat are NR1 and NR2 (Moriyoshi et al., 1991; Monyer et al., 1992; Ishii et al., 1993). Glycine binds to NR1 whereas glutamate binds to the NR2 subunit. The NR2 subunit has four different isoforms (NR2A-NR2D), which are regulated developmentally (Monyer et al., 1994; Sheng et al., 1994; Zhong et al., 1995) and alter NMDA receptor permeability and kinetic properties (Kutsuwada et al., 1992; Monyer et al., 1992; Stern et al., 1992; Monyer et al., 1994; Kuner and Schoepfer, 1996). The NR2C subunit containing NMDA receptors are

present at high levels only in cerebellar granule cells (Chazot et al., 1994; Thompson et al., 2000). There is currently no evidence of the NR2D subunit presence in the synapse but it is present in several different cell types extrasynaptically (Momiyama et al., 1996; Cull-Candy et al., 1998). Although there is a developmental shift in functional properties, the NR2A and NR2B subunit containing NMDA receptors have been studied extensively in the cortex and hippocampus (Hestrin, 1992; Monyer et al., 1994; Sheng et al., 1994; Flint et al., 1997; van Zundert et al., 2004). The mRNA levels are more prevalent for NR1 and NR2B during synaptogenesis, whereas NR2A mRNA levels are low and increases later during development in the hippocampus (Monyer et al., 1994). The NR2B subunit has been shown to have a higher affinity for glutamate compared to that of NR2A (Laurie and Seeburg, 1994; Mori and Mishina, 1995; Priestley et al., 1995; Cull-Candy et al., 2001). Previous studies have shown that the NR2B subunits are located mainly in the extrasynaptic region and the NR2A subunits predominantly in the synaptic region of the adult brain (Tovar and Westbrook, 1999). However, recent studies have reported that both subtypes may be located synaptically or extrasynaptically in mature cultured neurons (Thomas et al., 2006).

The kinetics of NMDA receptors are complex and vary with extracellular calcium and intracellular magnesium. Extracellular calcium affects the conductance of single channel currents (Ascher and Nowak, 1988; Gibb and Colquhoun, 1992; Jahr and Stevens, 1993; Premkumar and Auerbach, 1996; Clark et al., 1997) whereas intracellular magnesium inhibits single channel currents (Li-Smerin and Johnson, 1996). Therefore, we will characterize the kinetic properties of NMDA receptors in the presence of low extracellular calcium and intracellular magnesium concentrations. We will use isolated

synaptosomes reconstituted in lipid bilayers to measure the single channel properties of synaptic NMDA receptors, more specifically compare the kinetic properties of NR2A and NR2B subunit containing synaptic NMDA receptor channels.

MATERIALS AND METHODS

Synaptosomes Preparation:

The synaptosomes were prepared as described elsewhere (Johnson et al., 1997) with minor modifications. The hippocampus was isolated from 3-5 days old, for NR2B recordings, and 1 month old, for NR2A recordings, Sprague Dawley rats and homogenized in an Eppendorf tube with 400 μl of ice cold Krebs-Henseleit buffer, which consist of: 118.5 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.18 mM KH_2PO_4, 24.9 mM $NaHCO_3$, 10 mM dextrose, 10 mg/ml adenosine deaminase and pH adjusted to 7.4 by bubbling with O_2:CO_2 (95:5). Leupeptin (0.01 mg/ml), pepstatin A (0.005 mg/ml), aprotinin (0.10 mg/ml), and benzamide (5 mM) were added to the buffer to minimize proteolysis. The homogenate was diluted with 1.60 ml of additional Krebs buffer, after being homogenized with five turns of a hand-held pestle. The mixture was filtered using a 13 mm diameter Millipore syringe filter holder with a 1 cc Tuberculin syringe. The diluted filtrate was forced through three layers of nylon (Tetko, 100 μm pore size) pre-wet with 150 μl of Krebs buffer, and collected in an Eppendorf tube. Upon filtering again with a pre-wet 5 mm Millipore nitrocellulose filter, the filtrate was centrifuged at 1000 x *g* for 15 minutes in a microcentrifuge at 4^{o}C. After removing the supernatant, the pellet, which contains the synaptoneurosomes, was resuspended in 100 μl of Krebs buffer for electrophysiological recordings.

Reconstitution of Synaptosomal NMDA Receptors in Lipid Bilayers:

Incorporation of synaptosomal NMDA receptors in artificial lipid bilayers was carried out using 'tip-dip" method (Coronado and Latorre, 1983; Suppiramaniam et al., 2001). The lipid phospholipid bilayer was formed at the tip of a polished borosilicate glass pipette (World Precision Instruments Inc., Sarasota, FL). The P-2000 laser micropipette puller (Sutter Instrument Company, Novato, CA) was used to pull pipettes with 100 MΩ resistance. The synthetic phospholipids were prepared by dissolving 1,2 – diphytanoyl-sn-glycero-3-phosphocholine (Avanti Polar-Lipids Inc., Alabaster, AL) in anhydrous at hexane (Aldrich Chemical Co., Milwaukee, WI) to obtain a concentration of 1 mg/mL. Approximately 3-5 μl of synthetic phospholipids was delivered into 300 μl of bath solution. The bilayer formation was initiated by successive transfer of two monolayers onto the tip of the patch pipette in an asymmetric saline condition with "out side-out" configuration. The bath solution contained 300 μl of pseudo-extracellular fluid (ECF) composed of 125 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, and 5 mM Tris HCl. The pseudo-intracellular fluid (ICF) consisted of 110 mM KCl, 4 mM NaCl, 2 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 2 mM 3-N-Morpholino propanesulfonic acid (MOPS) was used as the pipette solution (pH adjusted to 7.4). After forming a stable membrane, 3-5 μl suspension of synaptosomes was delivered to the ECF. Gentle stirring using an air driven magnetic stir bar placed at the bottom of the microbeaker helps fusion of synaptosomal fragments into the bilayer.

Single Channel Recording and Analysis:

Single synaptosomal NMDA receptor channel currents were obtained by the addition of 2 μM glutamate (L-glutamic acid, Sigma-Aldrich) and 1 μM glycine (amino acetic acid, Sigma-Aldrich) in the presence of channel blockers 1 μM SYM 2206 (4-(4-aminophenyl)-1,2-dihydro-1-methyl-2-propylcarbamoyl-6, 7-methylenedioxyphthalazine, Tocris), 1 μM SYM 2081 ((2S, 4R)-4-methylglutamic acid, Sigma-Aldrich), 100 μM picrotoxin (cocculin, Sigma-Aldrich), 2 μM TEA (tetraethyl ammonium chloride, Sigma-Aldrich), and 1 μM TTX (tetrodotoxin, Sigma-Aldrich), which are AMPA, Kainate, and GABA receptors and sodium and potassium channels, respectively. The resulting single channel currents were digitized using a PCM interface (VR-10B Digital Data Recorder, Instrutech. Corp., Elmont, NY) and recorded on a VHS tape (Sony Corp., New York). For off line analysis, the data were filtered at 2 kHz and digitized between 5-25 kHz with pClamp6 software. The data processing of single channel recordings from synaptosomal receptors was similar to the one described elsewhere (Vaithianathan et al., 2005).

Only the data exhibiting long stretches of single channel current transition without base line drifts were chosen for quantitative analysis. The all points-current amplitude histograms were constructed and fitted with Gaussian curves to identify the individual conductances. The single channel conductances of NMDA receptors were obtained by plotting current as a function of membrane voltage and the conductance were determined according to the equation $g = I/(V-V_0)$, where I is the single channel current, V is the voltage and V_0 is the reversal potential. The single channel open and closed probabilities were extracted from the area under the current-amplitude histogram. If the areas under the closed and open regions are R_c and R_o respectively, the single channel probabilities

can be calculated by the straightforward relationships $P_c = R_c/ (R_c + R_o)$ and $P_o = R_o/ (R_c + R_o)$.

RESULTS

Concentration Dependent Effects of Glutamate on Single Synaptosomal NR2A-Containing NMDA Receptor Channel

Blockers for sodium channels, potassium channels, AMPA receptors, kainate receptors, $GABA_A$ receptors, and NR2B subunit containing specific antagonist, Ro 25-6981, were added to the extracellular solution to isolate the single channel activity of NR2A subunit containing NMDA receptors. Figure 1A shows the lack of channel activity, in the absence of NMDA receptor agonist glutamate (glycine was previously added to the extracellular fluid), of NR2A subunit containing NMDA receptors. Figure 1B-D shows that after addition of 2 μM, 4 μM, and 6 μM glutamate respectively. The channel activity elicited were completely blocked by 10 μM – 30 μM of the specific NMDA receptor antagonist D-AP5 (D-2-Amino-5-phosphonovaleric acid, Sigma Aldrich). The corresponding amplitude histograms in Figure 1 A show a single peak at 0 pA in the absence of glutamate and after the application of AP5 (Figure 1 E). An additional peak representing the open state is observed upon the addition of 2, 4, and 6 μM glutamate (Figure 1 B-D, respectively). The open probability increases in a dose-dependent manner from 0 in the absence of glutamate to 0.27, 0.45, and 0.63 at 2, 4, and 6 μM glutamate, respectively.

Concentration Dependent Effects of Glutamate on Single Synaptosomal NR2B-Containing NMDA Receptor Channel

To isolate the single channel activity of NR2B subunit containing NMDA receptors, blockers for sodium channels, potassium channels, AMPA receptors, kainate receptors, and $GABA_A$ receptors were added to the extracellular solution. Figure 2 A shows the lack of channel activity in the absence of NMDA receptor agonist, glutamate (glycine was previously added to the extracellular fluid). However, Figure 2 E shows that after addition of 2 μM, 4 μM, and 6 μM glutamate (Figures 2 B-D, respectively) channel activity was elicited and complete blockade with 1 μM – 3 μM of Ro 25-6981, a NR2B subunit specific antagonist. The corresponding amplitude histograms in Figure 2 A show a single peak at 0 pA in the absence of glutamate and after the application of Ro 25-6981 (Figure 2 E). An additional peak is observed upon the addition of 2, 4, and 6 μM glutamate (Figure 2 B-D, respectively). The open probability increases in a dose-dependent manner from 0 in the absence of glutamate to 0.45, 0.54, and 0.64 at 2, 4, and 6 μM glutamate, respectively.

Single Channel Properties of Synaptosomal NMDA Receptors

Multiple experiments proved that the open probability not the conductance has a dose dependent response to increasing concentrations of glutamate in NR2A and NR2B subunit containing NMDA receptors. The average conductance level observed for NR2A subunit containing NMDA receptors was 43 pS $\pm$ 3 and 30 $\pm$ 3 for NR2B subunit containing NMDA receptors. Table 1 displays the comparison of single channel NR2A open probability with that of NR2B along with the number of experiments (n) and the

average conductance (pS) observed at the varying glutamate concentrations and open and closed dwell time at 2 μM glutamate. One exponential fit was used for the open and two exponential fits for the closed dwell time histograms for NR2A subunit containing NMDA receptors (Figure 5C and 5D). Whereas, one exponential gave the best fit for both the open and closed time histograms for NR2B subunit containing NMDA receptors (Figure 6C and 6D).

DISCUSSION

The single channel properties of synaptic NMDA receptors can be directly studied under controlled conditions utilizing the unique technique of incorporating isolated synaptosomes into artificial phospholipid bilayers and subsequently recording the channel activity. The single channel synaptosomal NR2A subunit containing NMDA receptors exhibited similar characteristics previously described in outside-out patch recordings.

Synaptosomal NR2A subunit containing NMDA receptors were activated and blocked by micromolar concentrations of glutamate and AP5, respectively. Our results indicates that increases in glutamate concentration leads to an increase in the probability of opening of NR2A subunit containing NMDA receptors, in which the maximal open probability of 0.63 was observed in the presence of 6 μM glutamate. Similarly, the probability of open at the same concentration for NR2B subunit containing NMDA receptors was 0.64. Interestingly, in the presence of 2 μM glutamate the probability of opening for NR2A subunit containing NMDA receptors is 0.27 whereas NR2B subunit containing NMDA receptors is 0.44. This difference can be attributed to the fact that

NR2B subunit containing NMDA receptors have a higher affinity for glutamate than NR2A subunit containing NMDA receptors (Neyton and Paoletti, 2006). A recent report suggests a major role for NR2B containing NMDA receptors in excitoxicity (Hammond et al., 2006). It is interesting to note that in our experiments NR2B containing receptors exhibited a higher probability of channel openings at 2 μM glutamate compared to NR2A-NMDRA receptors. The higher probability of channel opening of NR2B-NMDA receptors can lead excessive calcium influxes and possibly contribute to cell death.

Due to the differences in the subunit-dependent channel properties NR2A and NR2B subunits have high single channel conductances whereas, NR2C and NR2D exhibit low conductances. In our study, only one conductance level was observed for both NR2A and NR2B subunit containing NMDA receptor channels, whereas previous studies have reported a subconductance state. The conductance of 43 pS for NR2A subunit containing synaptosomal NMDA receptors is same as the previously reported value for somatic NMDA receptors (Ascher and Nowak, 1988; Cull-Candy and Usowicz, 1989; Lieberman and Mody, 1999). In addition, the conductance of the NR2B subunit containing synaptosomal NMDA receptor of 30 pS is similar to the previously reported values for somatic receptors (Erreger et al., 2005).

The mean open times reported in our experiments were 2.13 and 8.26 for NR2A subunit containing and 1.32 and 6.49 for NR2B subunit containing NMDA receptors. However, although the dwell open time was similar to that of previously described values (Lieberman and Mody, 1998; Rycroft and Gibb, 2002), the smaller open time is not observed in single synaptosomal recordings. The above findings indicate though synaptic NMDA receptors share similar channel properties with that of somatic receptors they

vary in certain kinetic properties suggesting synaptic receptors possibly posses unique characteristics based on their subunit variation and the influence of the microenvironment of the synapse.

REFERENCES

Ascher P, Nowak L (1988) The role of divalent cations in the N-methyl-D-aspartate responses of mouse central neurones in culture. J Physiol 399:247-266.

Benveniste M, Mayer ML (1991) Kinetic analysis of antagonist action at N-methyl-D-aspartic acid receptors. Two binding sites each for glutamate and glycine. Biophys J 59:560-573.

Bliss TV, Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361:31-39.

Chazot PL, Coleman SK, Cik M, Stephenson FA (1994) Molecular characterization of N-methyl-D-aspartate receptors expressed in mammalian cells yields evidence for the coexistence of three subunit types within a discrete receptor molecule. J Biol Chem 269:24403-24409.

Clark KD, Hennessey TM, Nelson DL, Preston RR (1997) Extracellular GTP causes membrane-potential oscillations through the parallel activation of Mg2+ and Na+ currents in Paramecium tetraurelia. J Membr Biol 157:159-167.

Clements JD, Westbrook GL (1991) Activation kinetics reveal the number of glutamate and glycine binding sites on the N-methyl-D-aspartate receptor. Neuron 7:605-613.

Collingridge GL, Lester RA (1989) Excitatory amino acid receptors in the vertebrate central nervous system. Pharmacol Rev 41:143-210.

Coronado R, Latorre R (1983) Phospholipid bilayers made from monolayers on patch-clamp pipettes. Biophys J 43:231-236.

Cull-Candy S, Brickley S, Farrant M (2001) NMDA receptor subunits: diversity, development and disease. Curr Opin Neurobiol 11:327-335.

Cull-Candy SG, Brickley SG, Misra C, Feldmeyer D, Momiyama A, Farrant M (1998) NMDA receptor diversity in the cerebellum: identification of subunits contributing to functional receptors. Neuropharmacology 37:1369-1380.

Davis LG, Brunngraber EG, Routtenberg A (1979) A regional study of electrophoretically separated proteins from the crude synaptosomal fractions of the developing rat brain. J Neurosci Res 4:25-34.

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

Duguid I, Sjostrom PJ (2006) Novel presynaptic mechanisms for coincidence detection in synaptic plasticity. Curr Opin Neurobiol 16:312-322.

Flint AC, Maisch US, Weishaupt JH, Kriegstein AR, Monyer H (1997) NR2A subunit expression shortens NMDA receptor synaptic currents in developing neocortex. J Neurosci 17:2469-2476.

Gibb AJ, Colquhoun D (1992) Activation of N-methyl-D-aspartate receptors by L-glutamate in cells dissociated from adult rat hippocampus. J Physiol 456:143-179.

Grant KA (1994) Emerging neurochemical concepts in the actions of ethanol at ligand-gated ion channels. Behav Pharmacol 5:383-404.

Henderson G, Johnson JW, Ascher P (1990) Competitive antagonists and partial agonists at the glycine modulatory site of the mouse N-methyl-D-aspartate receptor. J Physiol 430:189-212.

Hestrin S (1992) Developmental regulation of NMDA receptor-mediated synaptic currents at a central synapse. Nature 357:686-689.

Ishii T, Moriyoshi K, Sugihara H, Sakurada K, Kadotani H, Yokoi M, Akazawa C, Shigemoto R, Mizuno N, Masu M, et al. (1993) Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits. J Biol Chem 268:2836-2843.

Jahr CE, Stevens CF (1993) Calcium permeability of the N-methyl-D-aspartate receptor channel in hippocampal neurons in culture. Proc Natl Acad Sci U S A 90:11573-11577.

Johnson MW, Chotiner JK, Watson JB (1997) Isolation and characterization of synaptoneurosomes from single rat hippocampal slices. J Neurosci Methods 77:151-156.

Kuner T, Schoepfer R (1996) Multiple structural elements determine subunit specificity of Mg2+ block in NMDA receptor channels. J Neurosci 16:3549-3558.

Kutsuwada T, Kashiwabuchi N, Mori H, Sakimura K, Kushiya E, Araki K, Meguro H, Masaki H, Kumanishi T, Arakawa M, et al. (1992) Molecular diversity of the NMDA receptor channel. Nature 358:36-41.

Laurie DJ, Seeburg PH (1994) Ligand affinities at recombinant N-methyl-D-aspartate receptors depend on subunit composition. Eur J Pharmacol 268:335-345.

Lester RA, Jahr CE (1992) NMDA channel behavior depends on agonist affinity. J Neurosci 12:635-643.

Lipton SA (1992) Models of neuronal injury in AIDS: another role for the NMDA receptor? Trends Neurosci 15:75-79.

Lipton SA, Rosenberg PA (1994) Excitatory amino acids as a final common pathway for neurologic disorders. N Engl J Med 330:613-622.

Li-Smerin Y, Johnson JW (1996) Effects of intracellular Mg2+ on channel gating and steady-state responses of the NMDA receptor in cultured rat neurons. J Physiol 491 (Pt 1):137-150.

Lynch G (2004) AMPA receptor modulators as cognitive enhancers. Curr Opin Pharmacol 4:4-11.

MacDermott AB, Mayer ML, Westbrook GL, Smith SJ, Barker JL (1986) NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurones. Nature 321:519-522.

Malenka RC, Nicoll RA (1999) Long-term potentiation--a decade of progress? Science 285:1870-1874.

Mayer ML, Westbrook GL, Guthrie PB (1984) Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones. Nature 309:261-263.

Momiyama A, Feldmeyer D, Cull-Candy SG (1996) Identification of a native low-conductance NMDA channel with reduced sensitivity to Mg2+ in rat central neurones. J Physiol 494 (Pt 2):479-492.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12:529-540.

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, Seeburg PH (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science 256:1217-1221.

Mori H, Mishina M (1995) Structure and function of the NMDA receptor channel. Neuropharmacology 34:1219-1237.

Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno N, Nakanishi S (1991) Molecular cloning and characterization of the rat NMDA receptor. Nature 354:31-37.

Nowak L, Bregestovski P, Ascher P, Herbet A, Prochiantz A (1984) Magnesium gates glutamate-activated channels in mouse central neurones. Nature 307:462-465.

Patneau DK, Mayer ML (1990) Structure-activity relationships for amino acid transmitter candidates acting at N-methyl-D-aspartate and quisqualate receptors. J Neurosci 10:2385-2399.

Premkumar LS, Auerbach A (1996) Identification of a high affinity divalent cation binding site near the entrance of the NMDA receptor channel. Neuron 16:869-880.

Priestley T, Laughton P, Myers J, Le Bourdelles B, Kerby J, Whiting PJ (1995) Pharmacological properties of recombinant human N-methyl-D-aspartate receptors comprising NR1a/NR2A and NR1a/NR2B subunit assemblies expressed in permanently transfected mouse fibroblast cells. Mol Pharmacol 48:841-848.

Rang H, Dale, MM, & Ritter, JM (1999) Pharmacology: Edinburgh: Churchill Livingstone.

Reynolds IJ, Murphy SN, Miller RJ (1987) 3H-labeled MK-801 binding to the excitatory amino acid receptor complex from rat brain is enhanced by glycine. Proc Natl Acad Sci U S A 84:7744-7748.

Sheng M, Cummings J, Roldan LA, Jan YN, Jan LY (1994) Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. Nature 368:144-147.

Stern P, Behe P, Schoepfer R, Colquhoun D (1992) Single-channel conductances of NMDA receptors expressed from cloned cDNAs: comparison with native receptors. Proc Biol Sci 250:271-277.

Suppiramaniam V, Bahr BA, Sinnarajah S, Owens K, Rogers G, Yilma S, Vodyanoy V (2001) Member of the Ampakine class of memory enhancers prolongs the single channel open time of reconstituted AMPA receptors. Synapse 40:154-158.

Thomas CG, Miller AJ, Westbrook GL (2006) Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons. J Neurophysiol 95:1727-1734.

Thompson CL, Drewery DL, Atkins HD, Stephenson FA, Chazot PL (2000) Immunohistochemical localization of N-methyl-D-aspartate receptor NR1, NR2A, NR2B and NR2C/D subunits in the adult mammalian cerebellum. Neurosci Lett 283:85-88.

Tovar KR, Westbrook GL (1999) The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. J Neurosci 19:4180-4188.

Tsien JZ, Huerta PT, Tonegawa S (1996) The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. Cell 87:1327-1338.

Vaithianathan T, Manivannan K, Kleene R, Bahr BA, Dey MP, Dityatev A, Suppiramaniam V (2005) Single channel recordings from synaptosomal AMPA receptors. Cell Biochem Biophys 42:75-85.

van Zundert B, Yoshii A, Constantine-Paton M (2004) Receptor compartmentalization and trafficking at glutamate synapses: a developmental proposal. Trends Neurosci 27:428-437.

White WF, Snodgrass SR (1983) Isoguvacine binding, uptake, and release: relation to the GABA system. J Neurochem 40:1701-1708.

Zhong J, Carrozza DP, Williams K, Pritchett DB, Molinoff PB (1995) Expression of mRNAs encoding subunits of the NMDA receptor in developing rat brain. J Neurochem 64:531-539.

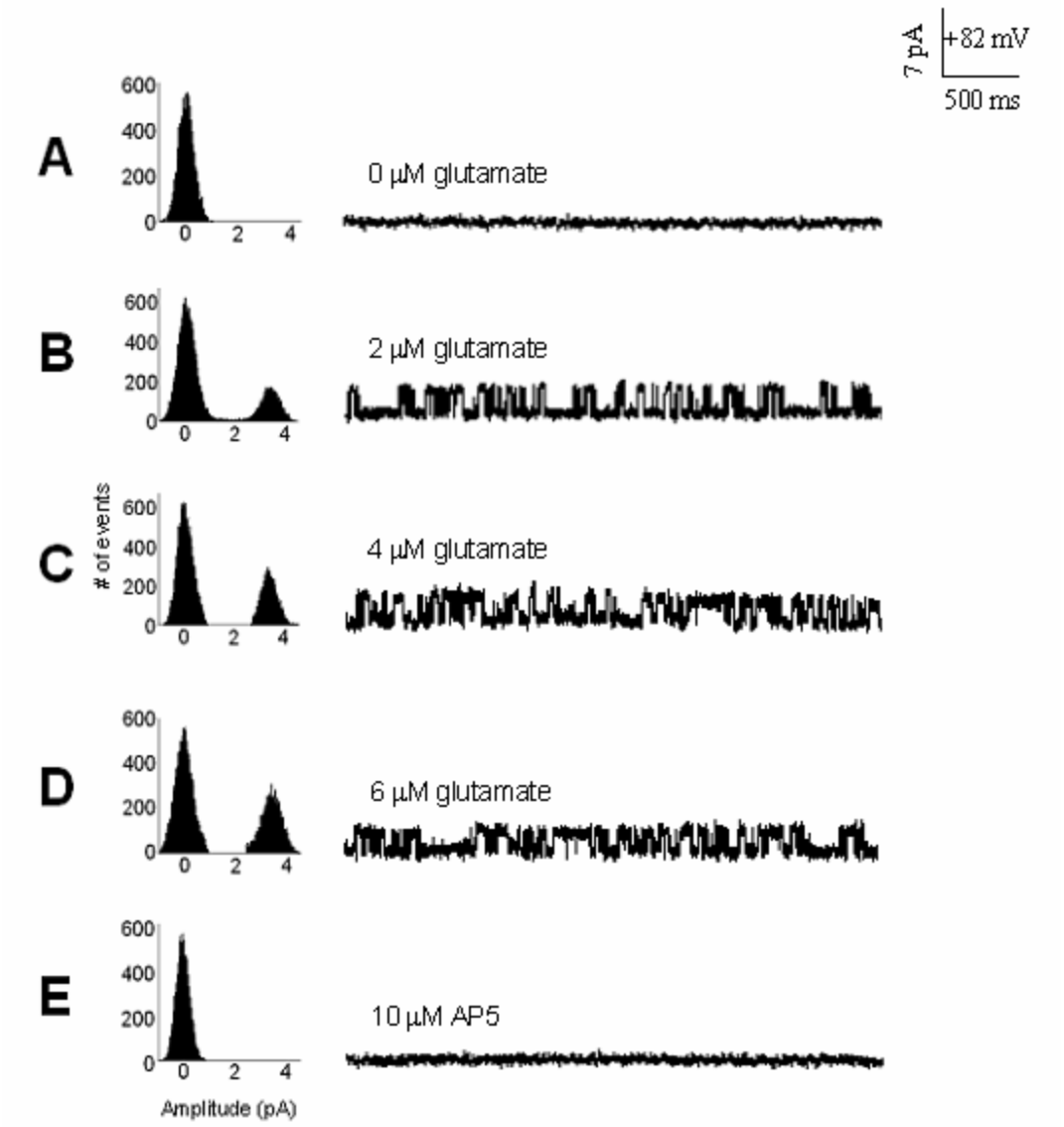

Figure 3.1: Concentration Dependent Effects of Glutamate on NR2A-containing Synaptosomal NMDA Receptors

Channel Openings are indicated by upward current deflections recorded in the presence of 1μM Ro 25-6981, (A-E) represents traces (right) and amplitude histograms (left) recorded in the absence (A) or presence of 2 μM (B), 4 μM (C), and 6 μM (D) glutamate. Activity elicited by 6 μM glutamate in (E) was blocked with 10 μM AP5. All recordings were done at +85 mV. The amplitude histograms show bimodal distributions with peaks corresponding to the stationary levels (i.e. open and closed states). The maximum unitary current was 3.5 pA. The channel conductance was 43 pS.

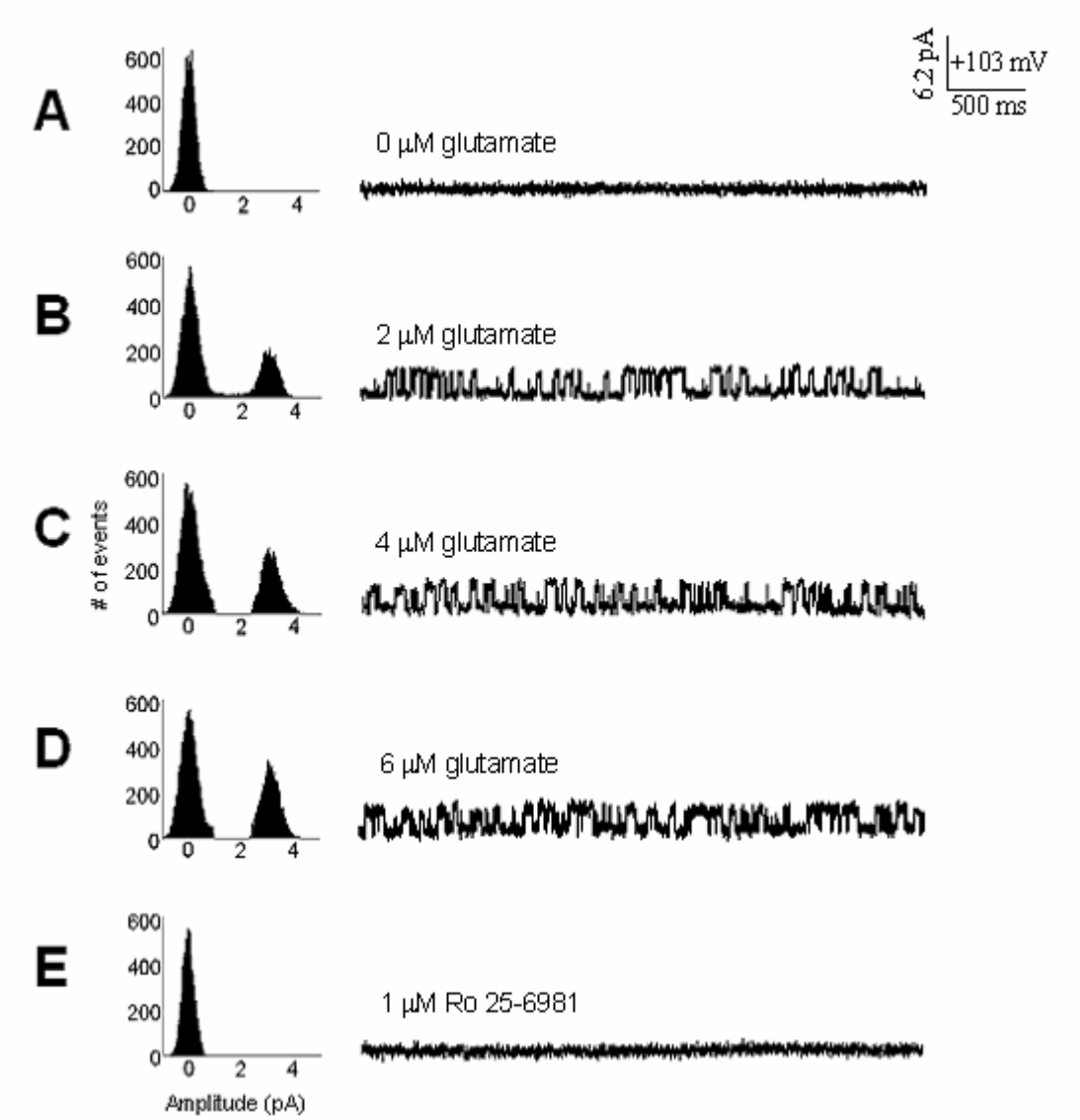

Figure 3.2: Concentration Dependent Effects of Glutamate on NR2B-containing Synaptosomal NMDA Receptors.

Channel openings are indicated by upward current deflections, (A-E) represents traces (right) and amplitude histograms (left) recorded in the absence (A) or presence of 2 μM (B), 4 μM (C), and 6 μM (D) glutamate. Activity elicited by 6 μM glutamate in (E) was blocked with the specific NR2B subunit containing NMDA receptor antagonist Ro 25-6981 (1 μM). All recordings were done at +103 mV. The amplitude histograms show bimodal distributions with peaks corresponding to the stationary levels (i.e. open and closed states). The maximum unitary current was 3.1 pA. The channel conductance was 30 pS.

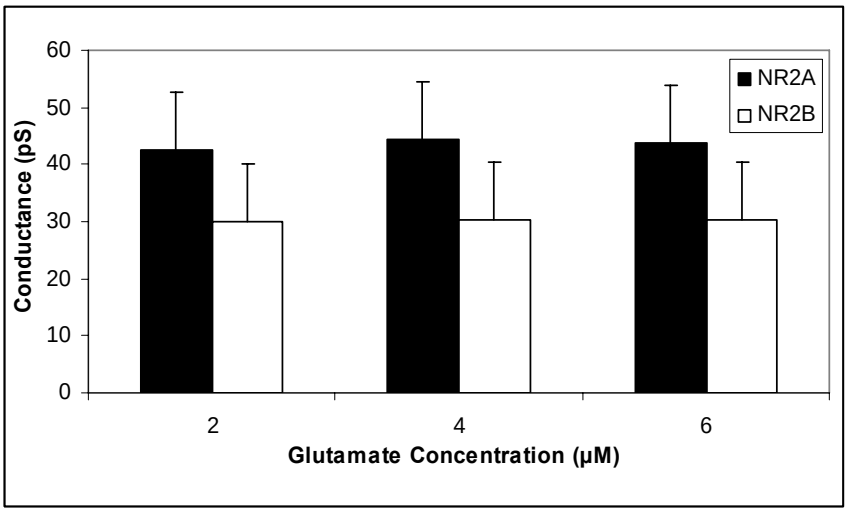

Figure 3.3: Concentration Dependent Effects of Glutamate on Conductance of Synaptosomal NMDA Receptors.

Increasing glutamate concentration does not alter the single channel conductance of synaptosomal NR2A nor NR2B subunit containing NMDA receptors. Bars represent mean ± SEM.

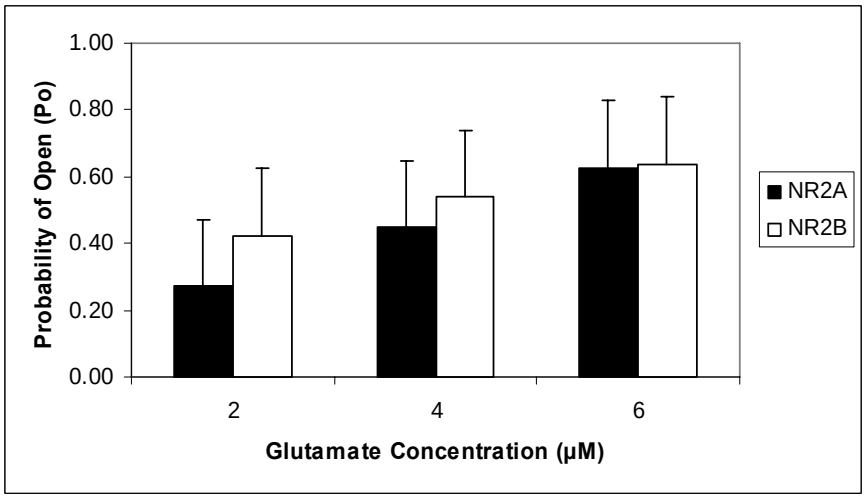

Figure 3.4: Concentration Dependent Effects of Glutamate on Open Probability of Synaptosomal NMDA Receptors.

Dose-dependent response of glutamate on Po of synaptosomal NR2A subunit containing NMDA receptors. Bars represent mean ± SEM.

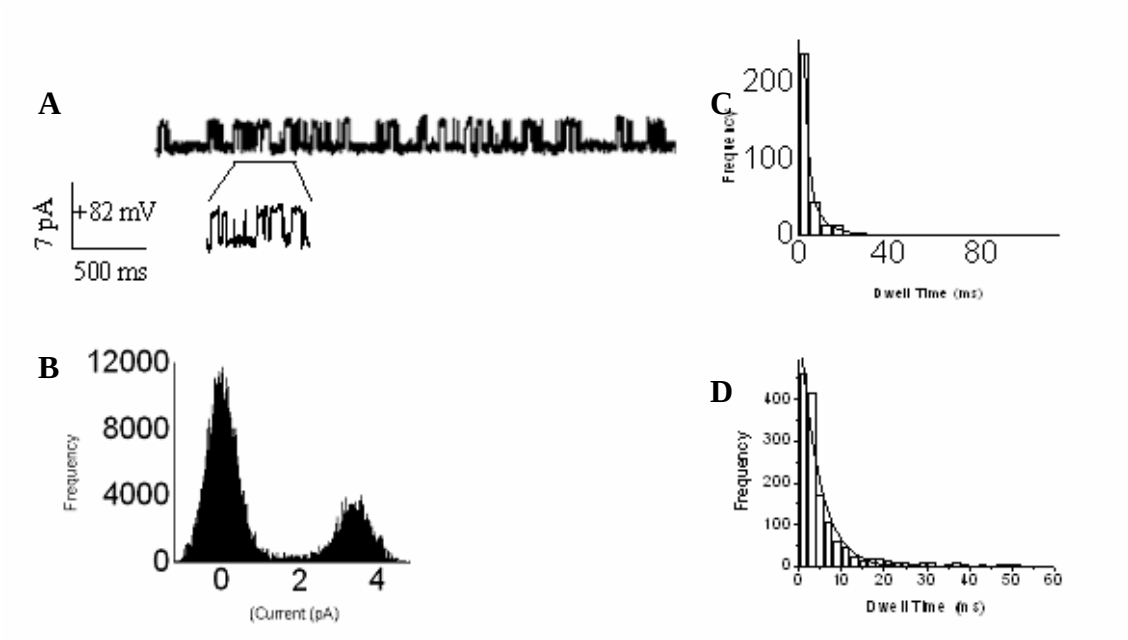

Figure 3.5**:** Single Channel Kinetic Properties of Synaptosomal NR2A-containing NMDA Receptors

(A) Average Sample Trace: Single channel currents of synaptosomal NR2A subunit containing NMDA receptors were elicited by the addition of 2 μM glutamate at the holding potential of +82 mV. (B) Average Histogram: The amplitude distribution shows major conductance of 43 pS fitted by a bimodal distribution, channel closed current level, and the channel open current level. (C) Average Open time: Histograms of duration of openings and (D) Average Closed Time: closings of single channel currents were analyzed using origin 6.0 with one exponential fit for the opening and two exponential fits for the closing. Time constants and relative occurrence of the closed state was τ_{1c} = 10.6 ms and τ_{2c} = 86.7. Time constants and relative occurrences of the closed states were τ_{1o} = 2.13 ms and τ_{2o} = 8.26 ms. Table 1 summarizes the average time constants obtained from analysis.

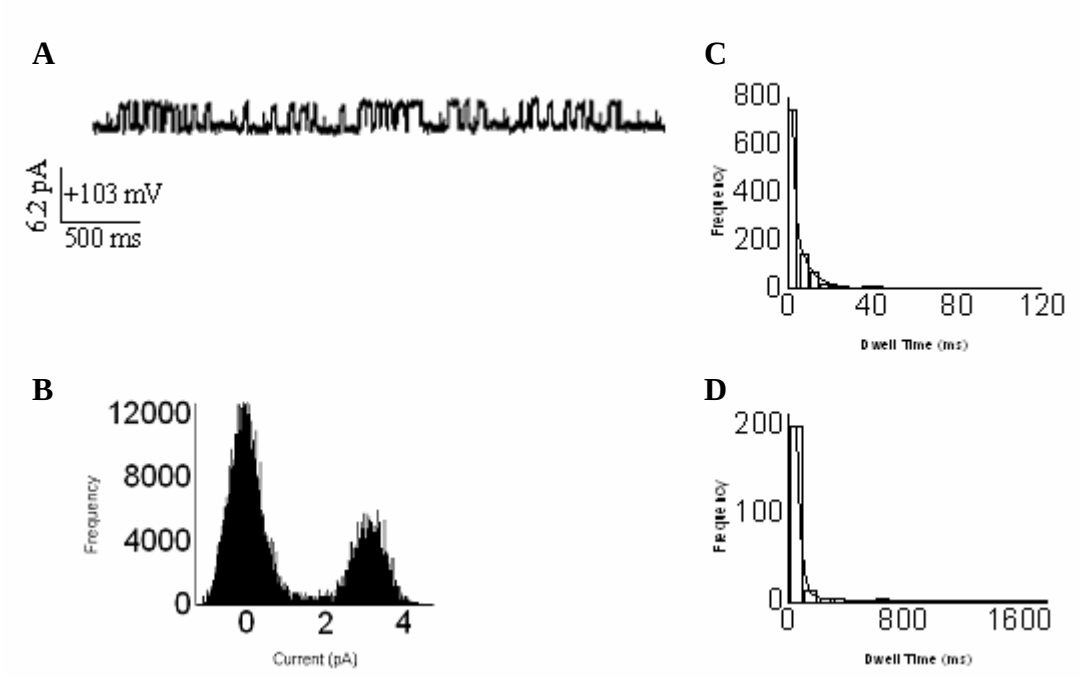

Figure 3.6: Single Channel Kinetic Properties of Synaptosomal NR2B Subunit Containing NMDA Receptors

(A) Average Sample Trace: Single channel currents of synaptosomal NR2B subunit containing NMDA receptors were elicited by the addition of 2 μM glutamate at the holding potential of +103 mV. (B) Average Histogram: The amplitude distribution shows major conductance of 30 pS fitted by a bimodal distribution, channel closed current level, and the channel open current level. (C) Average Open Time: Histograms of duration of openings and (D) Average Closed time: closings of single channel currents were analyzed using origin 6.0 with one exponential fit for the opening and the closing. Time constants and relative occurrences of the open state was τ_{1o} = 1.32 ms and τ_{2o} = 6.49 ms and τ_{1c} = 36 ms for the closed state.

	NR2A				NR2B		
2 μM glutamate		**n**	**SD**			**n**	**SD**
Conductance (pS)	42.53768	5	3.917587		30.25619	13	0.998415
Open state probability	0.271691	5	0.07473		0.446729	13	0.047329
Open time τ_{1o}(ms)	2.13	5	0.33213		1.32	7	0.72501
Open time τ_{2o}(ms)	8.26		2.06393		6.49	7	0.98369
Closed time τ_{1c}(ms)	10.5691	5	0.46038		36.0023	7	1.67144
Closed time τ_{2c}(ms)	86.72517	5	3.65368				
4 μM glutamate							
Conductance (pS)	44.3846	5	4.6818		30.30239	5	1.52389
Open state probability	0.4485	5	0.074314		0.539812	5	0.03875
6 μM glutamate							
Conductance (pS)	43.8112	5	4.7598		30.28526	5	0.989346
Open state probability	0.6283	5	0.021849		0.638480	5	0.043279

Table 3.1: Summary of Single Channel Properties of Synaptosomal NMDA Receptors

Comparison of the conductance, open state probability, and number of experiments (n) for the varying increasing concentrations of glutamate and Tau open and closed (dwell time) values in synaptosomal NR2A and NR2B subunit containing NMDA receptors.

4. NEURAL CELL ADHESION MOLECULE-ASSOCIATED POLYSIALIC ACID INHIBITS NR2B-CONTAINING N-METHYL-D-ASPARTATE RECEPTORS AND PREVENTS GLUTAMATE-INDUCED CELL DEATH

ABSTRACT

The neural cell adhesion molecule (NCAM) and its associated polysialic acid (PSA) play important roles in the development of the nervous system and N-methyl-D-aspartate receptor (NMDA receptor)-dependent synaptic plasticity in the adult. Here, we investigated the influence of PSA on NMDA receptor activity. We found that glutamate-elicited NMDA receptor currents in cultured hippocampal neurons were reduced by ≈30% with the application of PSA or PSA-NCAM, but not by the sialic acid monomer, chondroitin sulfate or NCAM. PSA inhibited NMDA receptor currents elicited by 3 μM glutamate, but not by 30 μM glutamate, suggesting that PSA acts as a competitive antagonist, possibly at the glutamate binding site. The PSA induced effects were mimicked and fully occluded by the NR2B subunit specific antagonist, ifenprodil. These effects were also observed in CHO cells co-transfected with vectors for expression of the NR1 and NR2B, but not with the NR1 and NR2A subunits. Recordings of single-channel synaptosomal NMDA receptors reconstituted in lipid bilayers revealed that PSA reduced open-probability but not conductance of NR2B-containing NMDA receptors in a PSA and glutamate concentration-dependent manner. Application of PSA to hippocampal

cultures reduced excitotoxic cell death induced by low micro molar concentration of glutamate via activation of NR2B-containing NMDA receptors, whereas enzymatic removal of PSA resulted in increased cell death that occluded glutamate-induced excitotoxicity. These observations indicate that the cell adhesion molecule-associated glycan PSA is able to prevent excitotoxicity via inhibition of NR2B subunit-containing NMDA receptors.

INTRODUCTION

Cell interactions play important roles during development, and in maintenance and modification of synaptic functions in the adult. The question is whether recognition molecules that specify and modulate contacts between neural cells may influence other parameters essential for nervous system function, such as neurotransmitter release and receptor activity. Among the recognition molecules with widespread functions is the neural cell adhesion molecule (NCAM) that starts to be expressed at the time of neural tube closure and remains detectable at lower levels in the adult. Its importance in shaping synaptic functions in the adult has long been recognized (Persohn et al., 1989; Luthl et al., 1994). NCAM is unique among recognition molecules in that its adhesive and concomitant signal transduction functions are modified by an unusual glycan, polysialic acid a highly negatively charged and voluminous carbohydrate, which is regulated by its attachment to the protein backbone (Rutishauser and Landmesser, 1996). PSA is a polymer of α2,8 linked sialic acid residues with chain lengths of up to 200 sialic acid residues. It has not been detected on other recognition molecules. It is synthesized by two

sialyltransferases, ST8Sia-II and ST8Sia-IV and attached to the fifth immunoglobulin-like domain of NCAM (Angata and Fukuda, 2003). Even more so than the protein backbone of NCAM, expression of PSA is developmentally regulated, with high expression during embryonic stages and gradual reduction as development proceeds. However, it remains expressed in some areas of the brain during adulthood, including the hippocampus, which undergoes functional changes underlying synaptic plasticity. In particular, PSA-NCAM is required for NMDA receptor-dependent long-term potentiation and spatial learning (Becker et al., 1996; Muller et al., 1996; Eckhardt et al., 2000; Kiss et al., 2001; Kleene and Schachner, 2004).

The NMDA receptors are subtype of ionotropic glutamate receptors which are found widely throughout the brain. NMDA receptors are heteromers assembled from the NMDA receptor subunit NR1 and at least one type of NR2 subunit (Seeburg, 1993). CA1 pyramidal cells, for instance, express mostly two different NR2 subunits, NR2A and NR2B, and perinatally NR2D (Kirson et al., 1999). It is believed that the different NR2 subunits confer distinct gating and pharmacological properties to NMDA receptors (Monyer et al., 1994) and couple them to distinct intracellular signaling machineries (Sheng and Pak, 2000) which may shape their characteristic roles in synaptic plasticity (Kohr et al., 2003; Liu et al., 2004; Massey et al., 2004). NMDA receptors are expressed both synaptically and extra-synaptically. The subunit composition at these locations is not uniform: synaptic NMDA receptors predominantly contain NR2A, whereas extra-synaptic NMDA receptors contain mostly the NR2B subunit (Tovar and Westbrook, 1999), although recent data in mature cultured hippocampal neurons support a view that both subtypes can be located in either synaptic or extrasynaptic compartments (Thomas et

al., 2006). Importantly, synaptic and extra-synaptic NMDA receptors may activate opposing pathways to prevent or induce glutamate induced excitotoxic cell death, respectively (Hardingham et al., 2002).

Several lines of evidence indicate that there are intriguing links between PSA-NCAM and glutamate receptors. NMDA receptors are co-redistributed with NCAM after induction of LTP (Fux et al., 2003). Activity of NMDA receptors is required for PSA-NCAM stimulated synaptogenesis (Dityatev et al., 2004), and PSA directly increases the probability of the open state of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate subtype of glutamate receptors (AMPARs) (Vaithianathan et al., 2004). To address the question whether PSA may also modulate NMDA receptors, we examined here the effects of PSA on glutamate-evoked NMDA receptor currents in cultured hippocampal neurons, CHO cells transiently expressing different NMDA receptor subunits, and synaptosomal NMDA receptors incorporated in artificial lipid bilayers.

MATERIALS AND METHODS

Plasmids and Chemicals:

The GluR4 subunit with an N-terminal flag tag in the pcDNA3.1 expression vector (Pasternack et al., 2002) was a kind gift of Kari Keinänen. NR1-1a with an HA tag in the pEGFP-N1 vector (Holmes et al., 2002) was a kind gift of Kevin Holmes and Gregory Dekaban. GluR6 in the pRK5 vector (Monyer et al., 1992), NR2A in the pRK5 vector and NR2B in the pRK7 vector (Kohler et al., 1993) were kind gifts of Peter Seeburg.

Polymers containing 25-50 sialic acid residues were purified from colominic acid (Fluka, Buchs, Switzerland) using anion exchange chromatography on a Hamilton PRPX column, dissolved in 20 mM Tris, pH 7.4, with 0 to 500 mM NaCl and detected at 214 nm (Hallenbeck et al., 1987). The concentration was determined using a colorimetric resorcinol method adapted to micro titer plate format (Bhavanandan and Sheykhnazari, 1993).

Mouse PSA-NCAM-Fc, containing the extracellular domain of NCAM and the Fc portion of human IgG, was produced according to Vutskits and colleagues (Vutskits et al., 2001) using a stably transfected TE671 cell line kindly provided by Genevieve Rougon. Mouse NCAM-Fc was produced using stably transfected CHO cells as described (Chen et al., 1999). Polysialylation of PSA-NCAM-Fc and PSA-negativity of NCAM-Fc were checked by Western blotting using a monoclonal antibody (clone 735) to PSA (Frosch et al., 1985; Dityatev et al., 2004). Recombinant endoneuraminidase-N (Endo-N) (0.2 μg/ml) that specifically cleaves the long-chain PSA residues from the NCAM protein backbone (Gerardy-Schahn et al., 1995) was used to remove PSA from cultured neurons. The efficacy of this enzymatic treatment was confirmed by the loss of PSA immunoreactivity using the monoclonal antibody to PSA.

Electrophysiological Recordings in Cultured Cells:

All recordings were performed in the whole-cell patch clamp mode at room temperature in extracellular solution containing (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 0.5 MgCl2, 10 HEPES, 30 D-glucose, 12 D-sucrose, adjusted to pH 7.3 with NaOH. Patch pipettes were filled with (in mM): 135 CsCl, 10 KCl, HEPES 10, 0.2 EGTA, 2 Mg-ATP,

0.2 Na-GTP, 10 D-glucose, adjusted to pH 7.2 with CsOH with an osmolarity of 290-320 mOsm. Fire-polished patch pipettes with a resistance of 2-4MΩ were pulled on a DMZ-Universal puller (Zeitz, Munich, Germany) using GB150F-8P borosilicate glass (Science Products, Hofheim, Germany). Dissociated hippocampal cultures used for recordings were prepared from 1- to 3-day-old mice (Dityatev et al., 2000) and maintained *in vitro* for 12-20 days. In these relatively mature neurons, the composition of NMDA receptors appears to be stable (Thomas et al., 2006). CHO cells used for recordings were transiently transfected with recombinant glutamate receptors 24-72 hours before experiments.

To isolate NMDA receptor currents in neurons, tetrodotoxin (TTX, 1μM, Alomone Labs, Jerusalem, Israel), tetraethyl-ammonium chloride (TEA, 10mM, Sigma-Aldrich, Steinheim, Germany), 1,2,3,4-tetrahydrobenzo[f] quinoxaline-7-sulfonamide (NBQX, 5μM, Tocris, Bristol UK), (RS)-Methyl-4-carboxyphenylglycine (MCPG, 100μM, Tocris) and picrotoxin (PiTx, 100μM, Tocris) were added to the extracellular solution. These compounds were applied to block Na^+ and K^+ voltage-dependent channels and $GABA_A$, glycine and non-NMDA glutamate receptors. Cells were voltage-clamped, using an EPC9 amplifier (HEKA, Lambrecht, Germany), at -60mV with a series of voltage steps from -100 to +60mV every 3 seconds, with each step lasting 100ms. NMDA receptor currents were evoked by glutamate (3, 5 or 30μM), which was applied using a multiple input, single barrel application system that allowed direct application onto the patched cell. Glutamate rather than NMDA was used in our experiments since it is a natural ligand of NMDA receptors and, thus, the data obtained are easier to relate to physiological processes in the brain. Control experiments were

performed to verify that solutions coming from different inputs did not interfere with each other. Compounds were applied for 10 seconds with a 60 second washout period between applications. The current/voltage relationships were plotted in Sigma Plot 5.0 (SPSS Inc., Chicago, IL, USA) after subtraction of the leak current and averaging currents elicited by four voltage steps during the application of glutamate. The leak current was the average of the five voltage steps before agonist application. Currents were normalized using the value recorded in the presence of glutamate at +60mV. Statistical comparisons of the current/voltage curves were done in Statistica 5.0 (StatSoft Inc., OK, USA) using ANOVA with "voltage" and "treatment" as repeated measures. To evaluate whether PSA induces changes in the conductance or gating of NMDA receptor channels in cultures, the fluctuation analysis of glutamate-activated NMDA receptor currents was performed (Heinemann and Conti, 1992; Tsvetkov et al., 2002). The coefficient of variation was calculated for glutamate-evoked NMDA receptor currents at –60 mV as the following ratio: CV = [Variance (glutamate response)-Variance (baseline)]$^{1/2}$ / [Mean (glutamate response)-Mean (baseline)].

Synaptosomal Preparation:

The synaptosomes were prepared as described elsewhere (Johnson et al., 1997) with minor modifications. The hippocampi were isolated from 7-10 days old Sprague Dawley rats and homogenized in an Eppendorf tube with 400 µl of ice cold 95%O_2:5%CO_2 aerated Krebs-Henseleit buffer that consisted of: 118.5 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.18 mM KH_2PO_4, 24.9 mM $NaHCO_3$, 10 mM dextrose, and pH adjusted to 7.4. Leupeptin (0.01 mg/ml), pepstatin A (0.005

mg/ml), aprotinin (0.10 mg/ml), adenosine deaminase (10 mg/ml) and benzamide (5 mM) were added to the buffer to minimize proteolysis. The homogenate was diluted with 1.60 ml of additional Krebs buffer, after being homogenized with five turns of a hand-held pestle. The mixture loaded into the 1 cc tuberculin syringe was forced through three layers of nylon (Tetko, 100 μm pore size) pre-wet with 150 μl of Krebs buffer, and collected in an Eppendorf tube. Following another filtering with a pre-wet Millex filter (5μm pore size PVDF Millipore filter), the filtrate was centrifuged at 1000 x *g* for 15 minutes in a microcentrifuge at 4^{o}C. After removing the supernatant, the pellet, which contained the synaptosomes, was resuspended in 100 μl of Krebs buffer for electrophysiological recordings.

Reconstitution of Synaptosomal NMDA Receptors in Lipid Bilayers:

Incorporation of synaptosomal NMDA receptors in artificial lipid bilayers was carried out using the "tip-dip" method (Vaithianathan et al., 2005). The phospholipid bilayer was formed at the tip of a polished glass pipette (World Precision Instruments Inc., Sarasota, FL). The P-2000 laser micropipette puller (Sutter Instrument Company, Novato, CA) was used to pull pipettes with 100 MΩ resistance. The synthetic phospholipids were prepared by dissolving 1,2–diphytanoyl,-sn-glycero-3-phosphocholine (Avanti Polar-Lipids Inc., Alabaster, AL) in hexane (Aldrich Chemical Co., Milwaukee, WI) to obtain a concentration of 1 mg/mL. About 3-5 μl of this phospholipid preparation was delivered into 300 μl of bath solution. The bilayer formation was initiated by successive transfer of two monolayers onto the tip of the patch pipette in an asymmetric saline condition with "outside-out" configuration. The bath

solution contained pseudoextracellular fluid (ECF) composed of 125 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, and 5 mM Tris HCl. The pseudointracellular fluid (ICF) consisting of 110 mM KCl, 4 mM NaCl, 2 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 2 mM 3-N-morpholino propanesulfonic acid (MOPS) was used as the pipette solution. After forming a stable membrane, 3-5 μl suspension of synaptosomes was transferred to the ECF. Gentle stirring facilitated fusion of synaptosomal fragments into the bilayer.

Single Channel Recording and Analysis:

Single synaptosomal NMDA receptor channel currents were elicited by application of glutamate (Sigma-Aldrich) and 1 μM glycine (Sigma-Aldrich) in the presence 1μM4-(4-aminophenyl)-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7 methylenedioxyphtha-lazine (SYM 2206, Tocris), 1 μM (2S, 4R)-4-methylglutamic acid (SYM 2081, Sigma-Aldrich), 100 μM picrotoxin (Sigma-Aldrich), 2 μM TEA (Sigma-Aldrich), and 1 μM TTX (Sigma-Aldrich) which block AMPA, kainate, GABA and glycine receptors, and potassium and sodium channels, respectively. PSA (colominic acid) was delivered to the ECF in increasing concentrations ranging from 1 μg/ml to 3 μg/ml. Chondroitin sulfate A (Sigma-Aldrich), was used as a control at a concentration of 10 μg/ml. The single channel currents were amplified (Axopatch 200B, Molecular Devices., CA), digitized using a PCM interface (VR-10B Digital Data Recorder, Instrutech. Corp., Elmont, NY), and stored on VHS tape. For off-line analysis, the data were filtered at 2 kHz and digitized between 5-25 kHz with PClamp6 software. The

channel currents, which were sensitive to NR2B specific antagonist 1 μM Ro (Ro 25-6981 maleate, Tocris), were included in the analysis. The data processing of single channel recordings from synaptosomal receptors were similar to the one described elsewhere (Vaithianathan et al., 2005).

Only the data exhibiting long stretches of single channel current transition without base line drifts were chosen for quantitative analysis. All point current- amplitude histograms were constructed and fitted with Gaussian curves to identify the individual conductances. The single channel conductance of NMDA receptors were obtained by plotting current as a function of membrane voltage and the conductance was determined according to the equation $g = I/(V-V_0)$, where I is the single channel current, V is the voltage and V_0 is the reversal potential. The single channel open probability was estimated as $P_o = R_o/ (R_c + R_o)$, where R_c and R_o stand for the areas under the current-amplitude histogram corresponding to close and open states, respectively.

Excitotoxicity Assay:

Cell survival assays were performed using dissociated hippocampal cultures maintained *in vitro* for 7 days in glass bottomed 96-well plates. The conditioned culture medium (70μl per well) was removed from wells and saved for the later use (see below). Compounds used during the induction of excitotoxicity were applied in Lockes buffer (in mM): 154 NaCl, 5.6 KCl, 10 $CaCl_2$, 3.6 $NaHCO_3$, 5 HEPES and 5.5 glucose, pH 7.4. Lockes buffer (70μl) alone or Lockes buffer containing the compounds of interest was then added to and immediately removed from the cells twice. A further 70μl of the respective solutions was then added, and the cells were returned to the incubator for ten

minutes. For washing, solutions were exchanged three times by replacing 70% of the total volume at each step to avoid drying out the cells during the washing procedures and to obtain a >95% solution exchange after the last step. Following this, the conditioned medium (saved as culture supernatant) was returned to the cells. Cell survival was measured 20-24 hours after treatment using the live cell marker, calcein (500nM, Molecular Probes, Eugene, Oregon, USA) and the dead cell marker, propidium iodide (3μM, Sigma-Aldrich). Three visual fields were counted per well and there were three wells per treatment group per culture preparation. Excitotoxicity was calculated as the ratio of dead cells to total cells. The t-test was used to determine the statistical differences between the experimental groups.

RESULTS

Reduction of NMDA receptor Currents by PSA in Cultures of Dissociated Hippocampal Neurons

NMDA receptor currents were evoked by the application of glutamate (3μM) and recorded at different membrane potentials in pyramidal-like neurons from 12-to 20-day-old cultures (Figure 4.1 A). Since previous experiments showed an effect of 40μg/ml bacterially produced PSA, colominic acid, on AMPAR currents in hippocampal neurons (Vaithianathan et al., 2004), we first tested whether this concentration of colominic acid would affect NMDA receptor activity. Co-application of colominic acid with glutamate reduced the NMDA receptor currents by ~30% ($n=40$, $F_{8,312}=60.34$, $p<0.000001$) at all tested membrane potentials (Figure 4.1 B). After wash-out of colominic acid the NMDA receptor currents in seconds returned to control levels (Figure 4.1 A).

To verify that the reduction of NMDA receptor currents was specific for colominic acid, a series of control experiments was performed. First, to show that the effect was due to PSA and not impurities in the sample, polymers of sialic acid with 25-50 residues were purified. Co-application of the purified PSA (10μg/ml) with glutamate produced a reduction of NMDA receptor currents of ~20% (n=4, $F_{8,24}$=4.28, p<0.005) in comparison to glutamate alone. This effect was not different from the effect of unpurified colominic acid (Figure 4.2).

Since PSA is a polymer of sialic acid, there was the possibility that the effect was not specific for the polymeric and highly negatively charged PSA but could also be induced by equally overall negatively charged monomers. To investigate this possibility, sialic acid monomers (40μg/ml) were co-applied with glutamate. This, however, did not produce any significant change in the glutamate induced NMDA receptor currents compared to glutamate alone (n=4, $F_{8,24}$=2.12, p>0.05, Figure 4.2). As another control, colominic acid was treated with the enzyme endoneuraminidase (endo-N), which is known to specifically cleave α2,8-linked PSA. Endo-N treated colominic acid did not change NMDA receptor currents when co-applied with glutamate (n=6, $F_{8,56}$=0.98, p>0.05, Figure 4.2).

Next, we examined the effect of another negatively charged carbohydrate polymer, chondroitin sulfate. Co-application of chondroitin sulfate (40μg/ml) with glutamate resulted in NMDA receptor currents that were not different from those induced by glutamate alone (n=7, $F_{8,48}$=0.35, p>0.05, Figure 4.2). Thus, the influence of colominic acid on NMDA receptors is specific to the unique PSA polymer of sialic acid.

Since these experiments were performed using a bacterially produced PSA we next addressed the question whether mammalian PSA associated with NCAM would affect NMDA receptors. Co-application of glutamate with mouse PSA-NCAM-Fc (10 μg/ml), containing the extracellular domain of NCAM in fusion with the Fc portion of human IgG, reduced NMDA receptor currents in the same manner as PSA (n=7, $F_{8,48}$=19.38, p<0.000001). Application of non-polysialylated NCAM-Fc (10 μg/ml) did not affect NMDA receptor currents (n=3, $F_{8,16}$=2.41, p>0.05). Thus, PSA carried by NCAM, but not the NCAM protein backbone that carries glycan chains not related to PSA, inhibits NMDA receptor currents.

Pharmacological and Fluctuation Analyses of the Effects of PSA on NMDA receptors

Since divalent cations - forming a complex with PSA – may modify properties of the PSA chain (Shimoda et al., 1994; Hayrinen et al., 2002) and regulate the activity of NMDA receptors (Monyer et al., 1994; Rock and Macdonald, 1995), we tested the influence of colominic acid on NMDA receptor currents at different concentrations of Ca^{2+} and Mg^{2+}. Changes in the concentration of extracellular Ca^{2+} (from 2mM to 0 mM) or Mg^{2+} (from 0.5mM to 1.5mM) did not influence the effect of colominic acid on NMDA receptor currents (Figure 4.3 A). These experiments suggest that association of the negatively-charged PSA with positively-charged divalent cations is not critical for the effects of PSA on NMDA receptors. Also addition of the NMDA receptor agonist spermidine (100 μM) or omitting 2 μM glycine normally included in the perfusion solution did not alter the inhibition of NMDA receptor currents by colominic acid (data

not shown). However, increasing the concentration of glutamate from 3 to 30 μM abolished the inhibition of NMDA receptor currents by colominic acid (Figure 4.3 A), suggesting that PSA acts as a competitive antagonist, possibly at the glutamate binding site.

To investigate whether PSA reduces the conductance of NMDA receptors or number of functional channels and probability of NMDA receptor channel opening, a fluctuation analysis was performed. Only recordings with a low level of background noise (SD<2.5 pA) were selected for this analysis to provide a reliable estimate of the coefficient of current variation. Analysis of colominic acid- and PSA-NCAM-Fc induced changes in the glutamate-induced current (I; recorded at -60 mV) and CV^{-2}, where CV is the coefficient of current variation (Figure 4.3 B), revealed a linear relationship between these parameters, suggesting that colominic acid reduces the probability of NMDA receptor channel openings and/or completely closes a subset of NMDA receptor channels.

The NR2B Subunit is Necessary for Inhibition of NMDA Receptors by PSA

The results derived by the fluctuation analysis and the fact that PSA only partially inhibited NMDA receptor currents at all tested concentrations suggest that PSA inhibits a fraction of NMDA receptors at the cell surface. To test whether NMDA receptors containing NR2B subunit are affected by PSA, the NR2B specific antagonist, ifenprodil (10 μM), was applied to cultures of hippocampal neurons. Ifenprodil reduced NMDA receptor currents by ~40% (n=6, $F_{8,40}$=11.42, p<0.000001), which is a level of inhibition similar to that generated by PSA when applied to these cells. Furthermore, ifenprodil

fully occluded the inhibition of NMDA receptor currents elicited by colominic acid (Figure 4.4 A). Similarly, another potent antagonist of NR2B-containing NMDA receptors, RO 25-6981 (0.5 μM), mimicked the effects of colominic acid (Figure 4.4 C).

Since our data provided evidence that PSA and PSA-NCAM inhibit NMDA receptors, we next asked whether PSA-NCAM or NCAM, which are expressed at the cell surface of hippocampal neurons, may regulate the activity of NMDA receptors. We thus treated wild type neurons with endo-N or used neurons derived from NCAM deficient mice (Cremer et al., 1994) to estimate the total, ifenprodil-sensitive (NR2B-subunit mediated) and insensitive (NR2A-subunit mediated) NMDA receptor current components in neurons deficient in PSA and/or NCAM. There was no difference between control and endo-N treated neurons in the amplitude of the total, ifenprodil sensitive and insensitive components of NMDA receptor currents (Figure 4.4 B). However, the magnitudes of the total NMDA receptor current and its components were increased two-fold in NCAM deficient neurons. Enzymatic removal of PSA or ablation of NCAM did not affect the response of NMDA receptors to PSA (Figure 4.4 C). Thus, our data show that (1) only PSA and PSA-NCAM added to the cultures as soluble compounds, but not PSA associated with NCAM at the cell surface of neurons affect NR2B-containing NMDA receptors and (2) expression of NCAM at the neuronal cell surface is required for normal activity of all subtypes of NMDA receptors via additional PSA-independent mechanisms.

To confirm that PSA specifically inhibits NMDA receptors containing the NR2B subunit, we examined recombinant NMDA receptors heterologously expressed in CHO cells. Co-expression of the NR1 and NR2A subunits produced NMDA receptor currents that were not inhibited by colominic acid ($n=9$, $F_{8,64}=0.56$, $p>0.05$; Figure 4.5).

Colominic acid also did not affect glutamate induced currents mediated by homomeric AMPARs composed of subunit GluR4 (n=5, $F_{8,32}$=0.72, p>0.05; Figure 4.5). There was also no effect of colominic acid on the amplitude and half-width of currents mediated by homomeric kainate receptors composed of GluR6 (recorded only at -60 mV due to fast desensitization; n=10, p>0.05, t-test; Figure 4.5). However, co-expression of NR1 and NR2B produced NMDA receptor currents that were reduced by colominic acid (n=20, $F_{8,152}$=18.05, p<0.000001; Figure 4.5). These results demonstrate that the PSA specifically inhibits NMDA receptors containing the NR2B subunit.

PSA Reduces Open Probability of NR2B-containing Synaptosomal NMDA Receptors Reconstituted in Lipid Bilayers

We have previously developed a method for recording single synaptosomal glutamate receptors (Vaithianathan et al., 2005) and here utilized it to study the modulation of NMDA receptors by PSA. Single-channel currents mediated by NMDA receptors were isolated pharmacologically (Figure 4.6 A_0 and 4.6 B_0). Their amplitudes were approximately of 4 pA. To verify their identity, the specific antagonist of NR2B-containing NMDA receptors, RO 25-6981, was applied at the end of each experiment and only channels that were fully blocked by Ro 25-6981 were included in the analysis (Figure 4.6 A_4 and 4.6 B_2).

Application of colominic acid to a single NMDA receptor at increasing concentrations (1, 2, and 3 μg/ml) produced a progressive inhibition of NMDA receptor activity (Figure 4.6 A_1-A_3). Analysis of amplitude histograms showed that colominic acid affected exclusively open probability rather than single-channel conductance (Figure 4.7

A). When colominic acid was applied to NMDA receptors at 0.01 and 0.1 μg/ml, it produced no significant effects (Figure 4.7 A). Also chondroitin sulfate (even at 10 μg/ml) did not affect activity of NMDA receptors, confirming the specificity of PSA action.

Since experiments with hippocampal neurons revealed that PSA inhibits only NMDA receptors activated by low glutamate concentrations, we investigated this aspect also using single-channel recordings. Application of glutamate at increasing concentrations (2, 4 and 6 μM) to a single channel in the presence of colominic acid (3 μg/ml) resulted in restoration of channel openings (Figure 4.7 B). The single channel conductance was not affected at any tested concentration of glutamate (Figure 4.7 B). In summary, these data demonstrate that PSA affects gating of NR2B-containing NMDA receptor at low glutamate concentrations.

PSA Reduces Excitotoxic Cell Death Induced by Low Micromolar Concentrations of Glutamate

Exposure of hippocampal neurons to 5 or 30 μM glutamate for 10 min induced, a 20% and 40% increase, respectively, in cell death of cultured hippocampal neurons within 24 hours (Figure 4.8 A-C). We chose these concentrations of glutamate since colominic acid significantly inhibited NMDA receptor currents activated by 5 μM glutamate but failed to inhibit NMDA receptor currents activated by 30 μM glutamate (Figure 4.3 A). Co-application of colominic acid with the general NMDA receptor antagonist APV or the highly potent and specific antagonist of NR2B-containing NMDA

receptors, RO 25-6981, abolished the excitotoxic effect of 5 μM glutamate. While both APV and RO 25-6981 also abolished the excitotoxic effect of 30μM glutamate, colominic acid failed to do so. These results show that glutamate-induced excitotoxic cell death depends on NMDA receptors containing the NR2B subunit and that colominic acid is able to prevent this effect at low glutamate concentrations. The failure of colominic acid to inhibit the excitotoxicity induced by a higher concentration (30μM) of glutamate is in accordance with the electrophysiological data showing that NMDA receptor currents elicited by this concentration of glutamate are insensitive to colominic acid.

To investigate the role of PSA expressed at the cell surface in neuronal survival, neurons were pre-treated with endo-N for three hours before exposure to glutamate. Endo-N pre-treatment increased cell death to a level similar to that observed after application of 5μM glutamate ($n=5$, $p>0.05$; Figure 4.8 D). Pre-treatment of neurons with endo-N occluded the induction of further cell death by 5μM glutamate and its block by co-application of PSA. To investigate if cell surface-expressed PSA inhibits the activity of NR2B-containing NMDA receptors, we tested whether RO 25-6981 would reverse the effect of endo-N pre- treatment. In accordance with this notion, co-treatment of neurons with endo-N and RO 25-6981 reversed neuronal cell survival to control levels, which was the survival seen in the absence of additives ($n=3$, $p>0.05$ compared to control).

DISCUSSION

Our study shows that both bacterially produced PSA (colominic acid) and eukaryotically produced PSA-NCAM specifically inhibit the NR2B subunit-containing NMDA receptors of cultured hippocampal neurons. Furthermore PSA inhibits the activity

of single NR2B-containing NMDA receptors reconstituted in lipid bilayers and whole-cell NMDA receptor currents in CHO cells transfected with NR1 and NR2B subunits. Since 10 μM ifenprodil occluded effects of PSA in hippocampal neurons, it is likely that PSA acts on NR1/NR2B receptors rather than on triheteromeric NR1/NR2A/NR2B receptors that are more resistant to ifenprodil (Hatton and Paoletti, 2005). Effects of PSA are prominent at the rather low concentration of 1 μg/ml that corresponds to 44 nM, assuming a chain length of 73 sialic acid residues which is the estimated average size of colominic acid used in this study. Since the inhibition of NMDA receptors by PSA is immediate and similar in hippocampal neurons, transfected CHO cells, and lipid bilayers we take this as an indication that PSA may act directly on NMDA receptors. The same concentrations of PSA as used in the present study have been previously shown to potentiate purified AMPARs reconstituted in lipid bilayers and AMPAR-mediated currents in cultured astrocytes and immature hippocampal neurons (Vaithianathan et al., 2004). The fact that PSA inhibited NMDA receptor currents at lower but not at higher concentrations of glutamate suggests that PSA competes with glutamate in binding to positively charged amino acids in the S1 and S2 extracellular domains of NR2 subunits which form the glutamate binding site of NMDA receptors (Laube et al., 1997). Computer modeling predicts that there are only 4-6 NR2 subunit-specific amino acid residues exposed to the glutamate-binding pocket of NR2 subunits (Kinarsky et al., 2005). These residues are located at the edge of the glutamate-binding pocket, indicating that only larger sized antagonists may provide subtype-specific inhibition of NMDA receptors. This notion is in line with our observation that only polymers but not monomers of sialic acid inhibited NR2B-containing NMDA receptors. Although the data

obtained, particularly in artificial lipid bilayers, suggest that PSA directly interferes with binding of glutamate to NMDA receptors, we can not exclude the possibility that this influence is indirect, for instance, via interaction with a lipid membrane nearby the receptor that could modify its configuration and, thus, indirectly affect ligand binding.

An increase in total, ifenprodil-sensitive and –insensitive NMDA receptor currents in NCAM deficient neurons as compared to wild type neurons suggests that NCAM is involved in regulation of NR2B- and NR2A-containing NMDA receptor activity. Since endo-N pre-treated neurons have normal NMDA receptor currents, this regulation appears to be PSA-independent but NCAM glycoprotein backbone dependent. The related important question is whether synaptic or extrasynaptic NMDA receptor currents are increased in NCAM deficient mice. Because impaired LTP in NCAM deficient mice (Muller et al., 1996) can be normalized via elevation of extracellular Ca^{2+} (Bukalo et al., 2004), we have previously suggested a deficit in the activity of synaptic NMDA receptors and synaptic Ca^{2+} influx in NCAM deficient mice. These previous and the present findings are compatible, assuming that the absence of NCAM leads to (i) a mistargeting of NMDA receptors from synaptic to extrasynaptic sites and (ii) a compensatory increase in the total number of NMDA receptors at the cell surface. Since bath application of glutamate in our experiments likely activates both extrasynaptic and synaptic NMDA receptors and PSA inhibited NMDA receptors as strongly as the NR2B-specific antagonists, ifenprodil and RO 25-6981 (i.e., inhibited all NR2B-containing NMDA receptors), it is conceivable that both extrasynaptic and synaptic NR2B-containing NMDA receptors can be inhibited by PSA. However, since PSA inhibits only currents activated by low concentration of glutamate that is much below the

concentration of glutamate released in the synaptic cleft in response to presynaptic stimulation, PSA action in situ is likely to be mostly extrasynaptic. Thus, on the basis of the present study, one can expect an increase in NR2B-mediated (mostly extrasynaptic) NMDA receptor currents in brains from PSA/NCAM-deficient mice. Dysregulation of extrasynaptic versus synaptic Ca^{2+} signaling may lead to drastic changes in synaptic plasticity (Nishiyama et al., 2000; Massey et al., 2004). Inhibition of NR2B-containing NMDA receptors by PSA in wild type animals may thus affect synaptic plasticity and the absence of this regulation in PSA-deficient slices may lead to synaptic abnormalities. Consistently with this hypothesis, we found that (i) injection of PSA restore CA1 LTP and contextual memory in NCAM deficient mice (Senkov et al., 2006) and (ii) inhibitors of NR2B-containing NMDA receptors fully rescue CA1 LTP in NCAM deficient and endo-N treated hippocampal slices (Kochlamazaschvili G., unpublished).

The observation that pre-treatment of neurons with endo-N did not lead to changes in NMDA receptor currents demonstrates that only PSA added to neurons in the form of soluble PSA – as colominic acid or as recombinant PSA-NCAM - has access to NMDA receptors. Although we did not observe any influence of endo-N treatment on currents mediated by NR2B subunits in electrophysiological recordings, endo-N treatment promoted NR2B-mediated excitotoxicity. This difference between the electrophysiological and excitotoxicity assays can be accounted for by the following argument: soluble molecules are likely to be washed out in the perfusion chamber in which electrophysiological recordings are performed, whereas soluble NCAM remains present in the neuronal culture supernatant (Bock et al., 1987) and thus may interfere with glutamate-induced cell death in the closed volumes of small wells used for the

excitotoxicity assay. Since the effects of added PSA were observed in endo-N treated and NCAM deficient neurons, they are not mediated by neuronal cell surface- expressed PSA-NCAM or NCAM. This observation is noteworthy since soluble PSA-NCAM has been detected in brain tissue and cerebrospinal fluid (Endo et al., 1998; Strekalova et al., 2006). Furthermore, it is interesting in the context of the biological significance of our present observations that expression and cleavage of the extracellular domain of NCAM/PSA-NCAM is regulated by metalloproteinase activity (Kalus et al., 2006) and activation of NMDA receptors and the plasmin/tissue plasminogen activator system (Endo et al., 1998; Hoffman, 1998). Thus, inhibition of NMDA receptor by PSA-NCAM released by neuronal activity from the cell surface may provide a feedback to reduce NMDA receptor currents and excitotoxic damage of the activated neurons.

Our analysis of glutamate induced excitotoxicity revealed that application of PSA prevents cell death, whereas removal of neuronal cell surface expressed PSA promotes cell death, occluding the excitotoxic effects of glutamate. These results suggest that it is the same population of hippocampal neurons that is susceptible to 5 μM glutamate and endo-N induced cell death. Excitotoxicity contributes to neuronal degeneration in many traumatic insults to the nervous system, such as ischemia, amyotrophic lateral sclerosis and epilepsy (Arundine and Tymianski, 2003). Excitotoxicity appears to be strongly linked to activation of extrasynaptic NMDA receptors since Ca^{2+} entry through these receptors, triggered by glutamate exposure or hypoxic/ischemic conditions, activates a general and dominant cyclic AMP response element-binding protein shut-off pathway and reduction in brain-derived neurotrophic factor (BDNF) expression. In contrast, Ca^{2+} entry through synaptic NMDA receptors promotes cyclic AMP response element-binding

protein activity, cell survival and expression of BDNF (Hardingham et al., 2002). It is noteworthy in this respect that removal of PSA with endo-N has previously been shown to reduce survival of cultured cortical neurons due to reduced BDNF-mediated signaling (Vutskits et al., 2001). Supplementation of culture medium with an excess of BDNF in these experiments was able to rescue survival of endo-N treated cortical neurons. Under our experimental conditions, however, addition of BDNF to endo-N treated hippocampal neurons did not affect endo-N induced cell death (Kochlamazaschvili G., unpublished), suggesting that other and/or additional factors are necessary for hippocampal neurons to survive in the absence of PSA.

Excitotoxic activity of NR2B-containing NMDA receptors is likely to mediate selective neurodegeneration of striatal medium-sized spiny projection neurons in Huntington's disease (Li et al., 2003), neurotoxicity associated with alcohol-withdrawal (Nagy, 2004), and neuronal cell death after transient cerebral ischemia (Kundrotiene et al., 2004). Since PSA inhibits NR2B-containing NMDA receptors and is highly expressed during early development and up-regulated during synaptic activity (Rutishauser and Landmesser, 1996; Bouzioukh et al., 2001), our present results suggest that PSA is well in place to prevent excitotoxic neuronal cell death during development and under pathological conditions resulting in high glutamate release, at least in cases when glutamate is accumulated in the extracellular space at low micromolar concentrations. These concentrations are physiological and found in normal and epileptic brains, but may be far exceeded during transient cerebral ischemia (Sherwin, 1999; Ueda et al., 2001; Berger et al., 2004; Ritz et al., 2004).

REFERENCES

Angata K, Fukuda M (2003) Polysialyltransferases: major players in polysialic acid synthesis on the neural cell adhesion molecule. Biochimie 85:195-206.

Arundine M, Tymianski M (2003) Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. Cell Calcium 34:325-337.

Becker CG, Artola A, Gerardy-Schahn R, Becker T, Welzl H, Schachner M (1996) The polysialic acid modification of the neural cell adhesion molecule is involved in spatial learning and hippocampal long-term potentiation. J Neurosci Res 45:143-152.

Berger C, Dohmen C, Maurer MH, Graf R, Schwab S (2004) [Cerebral microdialysis in stroke]. Nervenarzt 75:113-123.

Bhavanandan VP, Sheykhnazari M (1993) Adaptation of the periodate-resorcinol method for determination of sialic acids to a microassay using microtiter plate reader. Anal Biochem 213:438-440.

Bock E, Edvardsen K, Gibson A, Linnemann D, Lyles JM, Nybroe O (1987) Characterization of soluble forms of NCAM. FEBS Lett 225:33-36.

Bouzioukh F, Tell F, Jean A, Rougon G (2001) NMDA receptor and nitric oxide synthase activation regulate polysialylated neural cell adhesion molecule expression in adult brainstem synapses. J Neurosci 21:4721-4730.

Bukalo O, Fentrop N, Lee AY, Salmen B, Law JW, Wotjak CT, Schweizer M, Dityatev A, Schachner M (2004) Conditional ablation of the neural cell adhesion molecule reduces precision of spatial learning, long-term potentiation, and depression in the CA1 subfield of mouse hippocampus. J Neurosci 24:1565-1577.

Chen S, Mantei N, Dong L, Schachner M (1999) Prevention of neuronal cell death by neural adhesion molecules L1 and CHL1. J Neurobiol 38:428-439.

Cremer H, Lange R, Christoph A, Plomann M, Vopper G, Roes J, Brown R, Baldwin S, Kraemer P, Scheff S, et al. (1994) Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. Nature 367:455-459.

Dityatev A, Dityateva G, Schachner M (2000) Synaptic strength as a function of post- versus presynaptic expression of the neural cell adhesion molecule NCAM. Neuron 26:207-217.

Dityatev A, Dityateva G, Sytnyk V, Delling M, Toni N, Nikonenko I, Muller D, Schachner M (2004) Polysialylated neural cell adhesion molecule promotes remodeling and formation of hippocampal synapses. J Neurosci 24:9372-9382.

Eckhardt M, Bukalo O, Chazal G, Wang L, Goridis C, Schachner M, Gerardy-Schahn R, Cremer H, Dityatev A (2000) Mice deficient in the polysialyltransferase ST8SiaIV/PST-1 allow discrimination of the roles of neural cell adhesion molecule protein and polysialic acid in neural development and synaptic plasticity. J Neurosci 20:5234-5244.

Endo A, Nagai N, Urano T, Ihara H, Takada Y, Hashimoto K, Takada A (1998) Proteolysis of highly polysialylated NCAM by the tissue plasminogen activator-plasmin system in rats. Neurosci Lett 246:37-40.

Frosch M, Gorgen I, Boulnois GJ, Timmis KN, Bitter-Suermann D (1985) NZB mouse system for production of monoclonal antibodies to weak bacterial antigens: isolation of an IgG antibody to the polysaccharide capsules of Escherichia coli K1 and group B meningococci. Proc Natl Acad Sci U S A 82:1194-1198.

Fux CM, Krug M, Dityatev A, Schuster T, Schachner M (2003) NCAM180 and glutamate receptor subtypes in potentiated spine synapses: an immunogold electron microscopic study. Mol Cell Neurosci 24:939-950.

Gerardy-Schahn R, Bethe A, Brennecke T, Muhlenhoff M, Eckhardt M, Ziesing S, Lottspeich F, Frosch M (1995) Molecular cloning and functional expression of bacteriophage PK1E-encoded endoneuraminidase Endo NE. Mol Microbiol 16:441-450.

Hallenbeck PC, Yu F, Troy FA (1987) Rapid separation of oligomers of polysialic acid by high-performance liquid chromatography. Anal Biochem 161:181-186.

Hardingham GE, Fukunaga Y, Bading H (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci 5:405-414.

Hatton CJ, Paoletti P (2005) Modulation of triheteromeric NMDA receptors by N-terminal domain ligands. Neuron 46:261-274.

Hayrinen J, Haseley S, Talaga P, Muhlenhoff M, Finne J, Vliegenthart JF (2002) High affinity binding of long-chain polysialic acid to antibody, and modulation by divalent cations and polyamines. Mol Immunol 39:399-411.

Heinemann SH, Conti F (1992) Nonstationary noise analysis and application to patch clamp recordings. Methods Enzymol 207:131-148.

Hoffman KB (1998) The relationship between adhesion molecules and neuronal plasticity. Cell Mol Neurobiol 18:461-475.

Holmes KD, Mattar PA, Marsh DR, Weaver LC, Dekaban GA (2002) The N-methyl-D-aspartate receptor splice variant NR1-4 C-terminal domain. Deletion analysis and role in subcellular distribution. J Biol Chem 277:1457-1468.

Johnson MW, Chotiner JK, Watson JB (1997) Isolation and characterization of synaptoneurosomes from single rat hippocampal slices. J Neurosci Methods 77:151-156.

Kalus I, Bormann U, Mzoughi M, Schachner M, Kleene R (2006) Proteolytic cleavage of the neural cell adhesion molecule by ADAM17/TACE is involved in neurite outgrowth. J Neurochem 98:78-88.

Kinarsky L, Feng B, Skifter DA, Morley RM, Sherman S, Jane DE, Monaghan DT (2005) Identification of subunit- and antagonist-specific amino acid residues in the N-Methyl-D-aspartate receptor glutamate-binding pocket. J Pharmacol Exp Ther 313:1066-1074.

Kirson ED, Schirra C, Konnerth A, Yaari Y (1999) Early postnatal switch in magnesium sensitivity of NMDA receptors in rat CA1 pyramidal cells. J Physiol 521 Pt 1:99-111.

Kiss JZ, Troncoso E, Djebbara Z, Vutskits L, Muller D (2001) The role of neural cell adhesion molecules in plasticity and repair. Brain Res Brain Res Rev 36:175-184.

Kleene R, Schachner M (2004) Glycans and neural cell interactions. Nat Rev Neurosci 5:195-208.

Kochlamazaschvili G. SM, and Dityatev A. (unpublished) In.

Kohler M, Burnashev N, Sakmann B, Seeburg PH (1993) Determinants of Ca2+ permeability in both TM1 and TM2 of high affinity kainate receptor channels: diversity by RNA editing. Neuron 10:491-500.

Kohr G, Jensen V, Koester HJ, Mihaljevic AL, Utvik JK, Kvello A, Ottersen OP, Seeburg PH, Sprengel R, Hvalby O (2003) Intracellular domains of NMDA receptor subtypes are determinants for long-term potentiation induction. J Neurosci 23:10791-10799.

Kundrotiene J, Cebers G, Wagner A, Liljequist S (2004) The NMDA NR2B subunit-selective receptor antagonist, CP-101,606, enhances the functional recovery the NMDA NR2B subunit-selective receptor and reduces brain damage after cortical compression-induced brain ischemia. J Neurotrauma 21:83-93.

Laube B, Hirai H, Sturgess M, Betz H, Kuhse J (1997) Molecular determinants of agonist discrimination by NMDA receptor subunits: analysis of the glutamate binding site on the NR2B subunit. Neuron 18:493-503.

Li L, Fan M, Icton CD, Chen N, Leavitt BR, Hayden MR, Murphy TH, Raymond LA (2003) Role of NR2B-type NMDA receptors in selective neurodegeneration in Huntington disease. Neurobiol Aging 24:1113-1121.

Liu L, Wong TP, Pozza MF, Lingenhoehl K, Wang Y, Sheng M, Auberson YP, Wang YT (2004) Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. Science 304:1021-1024.

Luthl A, Laurent JP, Figurov A, Muller D, Schachner M (1994) Hippocampal long-term potentiation and neural cell adhesion molecules L1 and NCAM. Nature 372:777-779.

Massey PV, Johnson BE, Moult PR, Auberson YP, Brown MW, Molnar E, Collingridge GL, Bashir ZI (2004) Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. J Neurosci 24:7821-7828.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12:529-540.

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, Seeburg PH (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science 256:1217-1221.

Muller D, Wang C, Skibo G, Toni N, Cremer H, Calaora V, Rougon G, Kiss JZ (1996) PSA-NCAM is required for activity-induced synaptic plasticity. Neuron 17:413-422.

Nagy J (2004) Renaissance of NMDA receptor antagonists: do they have a role in the pharmacotherapy for alcoholism? IDrugs 7:339-350.

Nishiyama M, Hong K, Mikoshiba K, Poo MM, Kato K (2000) Calcium stores regulate the polarity and input specificity of synaptic modification. Nature 408:584-588.

Pasternack A, Coleman SK, Jouppila A, Mottershead DG, Lindfors M, Pasternack M, Keinanen K (2002) Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor channels lacking the N-terminal domain. J Biol Chem 277:49662-49667.

Persohn E, Pollerberg GE, Schachner M (1989) Immunoelectron-microscopic localization of the 180 kD component of the neural cell adhesion molecule N-CAM in postsynaptic membranes. J Comp Neurol 288:92-100.

Ritz MF, Schmidt P, Mendelowitsch A (2004) Acute effects of 17beta-estradiol on the extracellular concentration of excitatory amino acids and energy metabolites during transient cerebral ischemia in male rats. Brain Res 1022:157-163.

Rock DM, Macdonald RL (1995) Polyamine regulation of N-methyl-D-aspartate receptor channels. Annu Rev Pharmacol Toxicol 35:463-482.

Rutishauser U, Landmesser L (1996) Polysialic acid in the vertebrate nervous system: a promoter of plasticity in cell-cell interactions. Trends Neurosci 19:422-427.

Seeburg PH (1993) The TINS/TiPS Lecture. The molecular biology of mammalian glutamate receptor channels. Trends Neurosci 16:359-365.

Senkov O, Sun M, Weinhold B, Gerardy-Schahn R, Schachner M, Dityatev A (2006) Polysialylated neural cell adhesion molecule is involved in induction of long-term potentiation and memory acquisition and consolidation in a fear-conditioning paradigm. J Neurosci 26:10888-109898.

Sheng M, Pak DT (2000) Ligand-gated ion channel interactions with cytoskeletal and signaling proteins. Annu Rev Physiol 62:755-778.

Sherwin AL (1999) Neuroactive amino acids in focally epileptic human brain: a review. Neurochem Res 24:1387-1395.

Shimoda Y, Kitajima K, Inoue S, Inoue Y (1994) Calcium ion binding of three different types of oligo/polysialic acids as studied by equilibrium dialysis and circular dichroic methods. Biochemistry 33:1202-1208.

Strekalova H, Buhmann C, Kleene R, Eggers C, Saffell J, Hemperly J, Weiller C, Muller-Thomsen T, Schachner M (2006) Elevated levels of neural recognition molecule L1 in the cerebrospinal fluid of patients with Alzheimer disease and other dementia syndromes. Neurobiol Aging 27:1-9.

Thomas CG, Miller AJ, Westbrook GL (2006) Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons. J Neurophysiol 95:1727-1734.

Tovar KR, Westbrook GL (1999) The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. J Neurosci 19:4180-4188.

Tsvetkov E, Carlezon WA, Benes FM, Kandel ER, Bolshakov VY (2002) Fear conditioning occludes LTP-induced presynaptic enhancement of synaptic transmission in the cortical pathway to the lateral amygdala. Neuron 34:289-300.

Ueda Y, Doi T, Tokumaru J, Yokoyama H, Nakajima A, Mitsuyama Y, Ohya-Nishiguchi H, Kamada H, Willmore LJ (2001) Collapse of extracellular glutamate regulation during epileptogenesis: down-regulation and functional failure of glutamate transporter function in rats with chronic seizures induced by kainic acid. J Neurochem 76:892-900.

Vaithianathan T, Matthias K, Bahr B, Schachner M, Suppiramaniam V, Dityatev A, Steinhauser C (2004) Neural cell adhesion molecule-associated polysialic acid potentiates alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor currents. J Biol Chem 279:47975-47984.

Vaithianathan T, Manivannan K, Kleene R, Bahr BA, Dey MP, Dityatev A, Suppiramaniam V (2005) Single channel recordings from synaptosomal AMPA receptors. Cell Biochem Biophys 42:75-85.

Vutskits L, Djebbara-Hannas Z, Zhang H, Paccaud JP, Durbec P, Rougon G, Muller D, Kiss JZ (2001) PSA-NCAM modulates BDNF-dependent survival and differentiation of cortical neurons. Eur J Neurosci 13:1391-1402.

FOOTNOTES

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The abbreviations used are: AMPAR, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor; BDNF, brain-derived neurotrophic factor; CA, colominic acid; CS, chondroitin sulfate; endo-N, endoneuraminidase-N; LTP, long-term potentiation; NCAM, neural cell adhesion molecule; NMDAR, N-methyl-D-aspartate subtype of glutamate receptors; PSA, polysialic acid; PSA-NCAM, polysialylated NCAM; SA, sialic acid

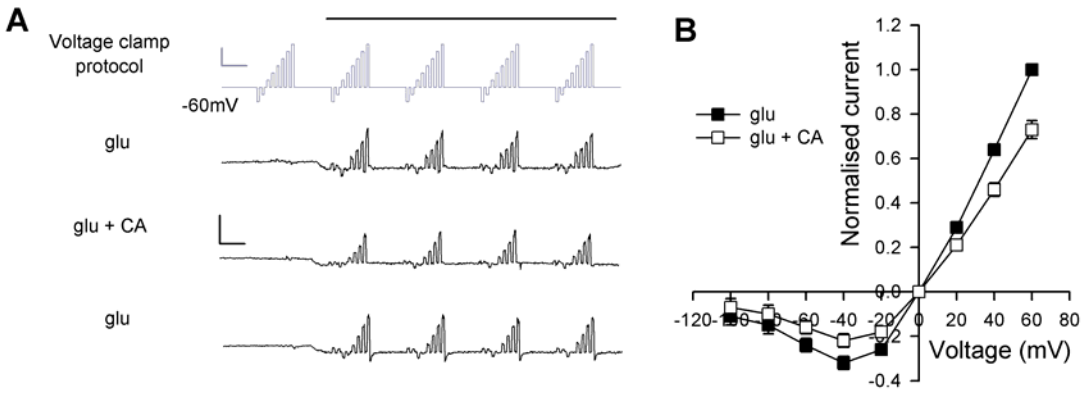


Figure 4.1: NMDA Receptor Currents in Dissociated Hippocampal Neurons are Inhibited by PSA.

(A) Voltage clamp protocol used to record the NMDA receptor currents (V_H=-60mV with voltage steps from –100 to +60 mV; upper trace) and representative recordings showing glutamate (glu) evoked NMDA receptor currents before, during and after, co-application of colominic acid (CA). Glutamate induced currents (second trace) are reduced by CA (third trace) in a fully reversible manner (fourth trace shows currents in 5 sec after washout of CA). Horizontal bar indicates the application of glutamate. Scale bars are 50mV or 500pA and 1 second. (B) Current/voltage relationship of NMDA receptor currents evoked by glutamate (3μM, filled squares). NMDA receptor currents are inhibited by ~30% by colominic acid (40μg/ml, open squares) at the all voltages tested. Data represent mean ± SEM, n=40, $F_{8,312}$=60.34, p<0.000001.

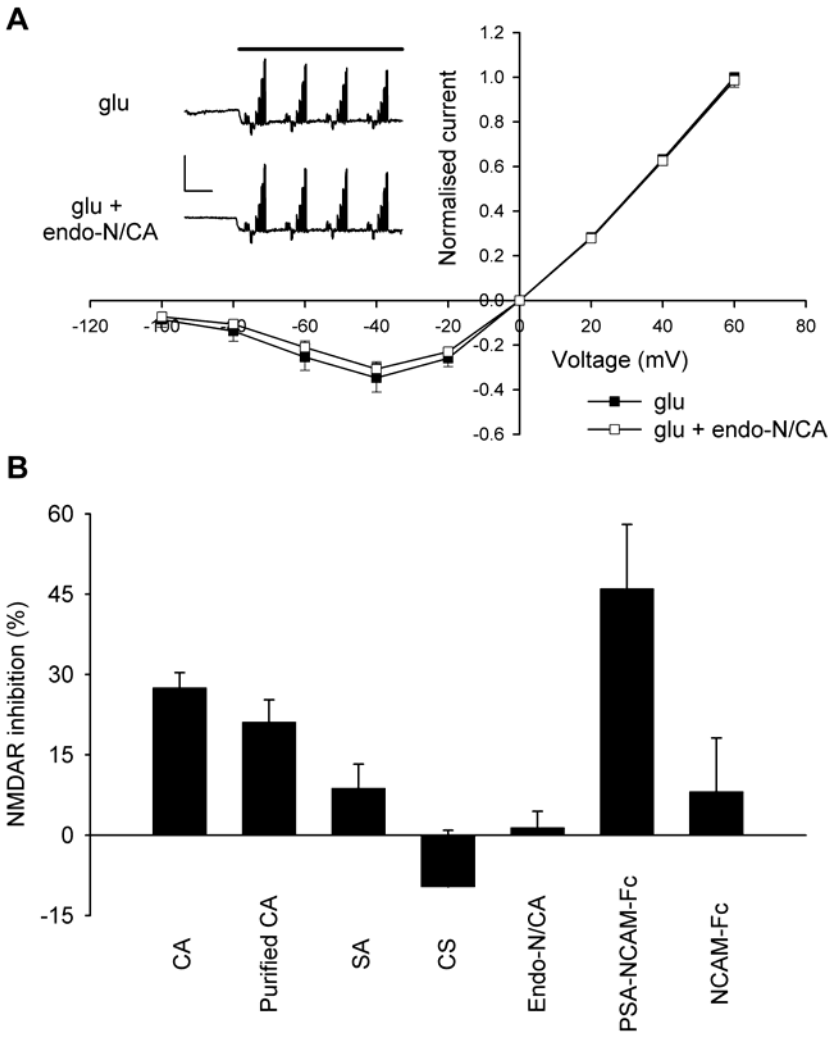


Figure 4.2: NMDA Receptor Currents in Dissociated Hippocampal Neurons are Inhibited Specifically by PSA and PSA-NCAM.

(A) Current/voltage relationship of NMDA receptor currents evoked by glutamate (3µM, filled squares). NMDA receptor currents are not inhibited by endo-N digested colominic acid (40µg/ml, open squares). Data represent mean ± SEM. Inset: Representative recordings showing glutamate evoked NMDA receptor currents in neurons (glu). Co-application of endo-N digested colominic acid with glutamate does not alter the NMDA receptor current (glu + endo-N/CA). Horizontal bar indicates the application of glutamate. Scale bars are 200pA and 2 seconds. (B) Inhibition of NMDA receptor currents at +60mV by colominic acid (CA, 40µg/ml, n=40, $F_{8,312}$= 60.34, p<0.000001), HPLC purified colominic acid (Purified CA, 10µg/ml, n=4, $F_{8,24}$=4.28, p<0.005), sialic acid (SA, 40µg/ml, n=4, $F_{8,24}$=2.12, p>0.05), chondroitin sulfate (40µg/ml, n=7, $F_{8,48}$=0.35, p>0.05, CS), endo-N digested colominic acid (Endo-N/CA, 40µg/ml, n=8, $F_{8,56}$=0.98, p>0.05), PSA-NCAM-Fc (10µg/ml, n=7, $F_{8,48}$=19.38, p<0.000001) and NCAM-Fc (10 µg/ml, n=3, $F_{8,16}$=2.41, p>0.05).

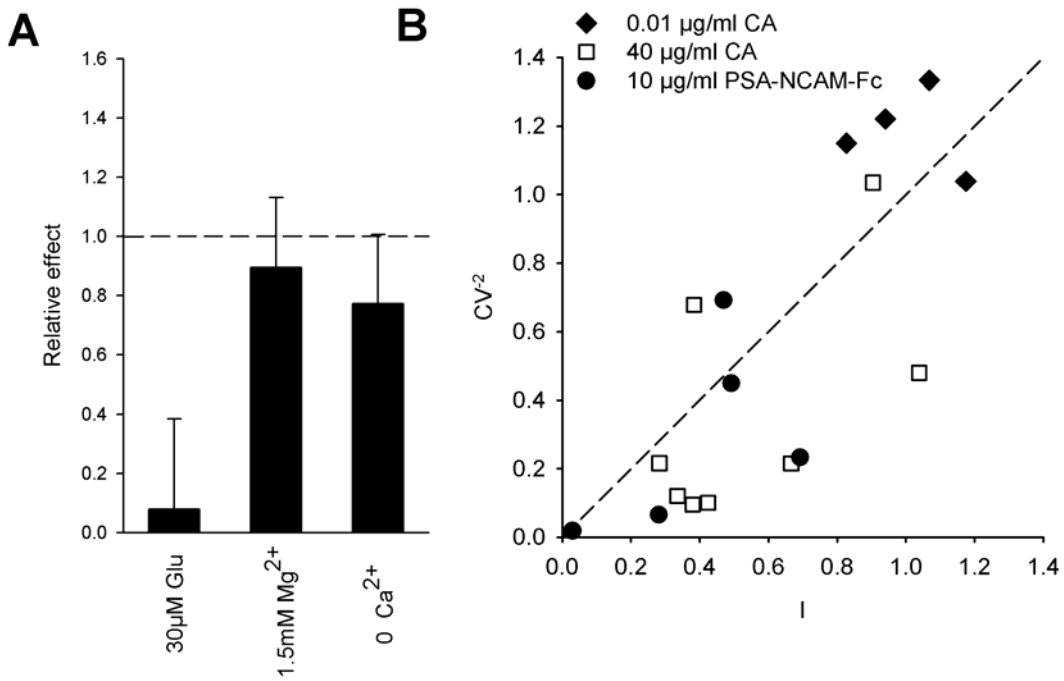


Figure 4.3: Characterization of PSA Action on NMDA Receptors

(A) Relative inhibition of NMDA receptor currents by colominic acid at +60mV by PSA (40μg/ml) under different experimental conditions: increased glutamate concentration (30μM, n=5, $p<0.05$), in the presence of an increased concentration of extracellular Mg^{2+} (1.5mM, n=5, $p>0.05$), and in nominally Ca^{2+} free solution (n=8, $p>0.05$). The effects of PSA under each of these conditions were normalized to the effects of PSA recorded in the same cell under standard conditions, i.e. when NMDA receptor currents were elicited by 3μM glutamate in the presence of 0.5 mM Mg^{2+} and 2 mM Ca^{2+}. (B) Relationship between PSA-induced changes in the current amplitude (I) and the coefficient of variation (CV) of glutamate-evoked NMDA receptor currents at +60 mV. Each point corresponds to one experiment. Note that the values are grouped around the line of identity y=x.

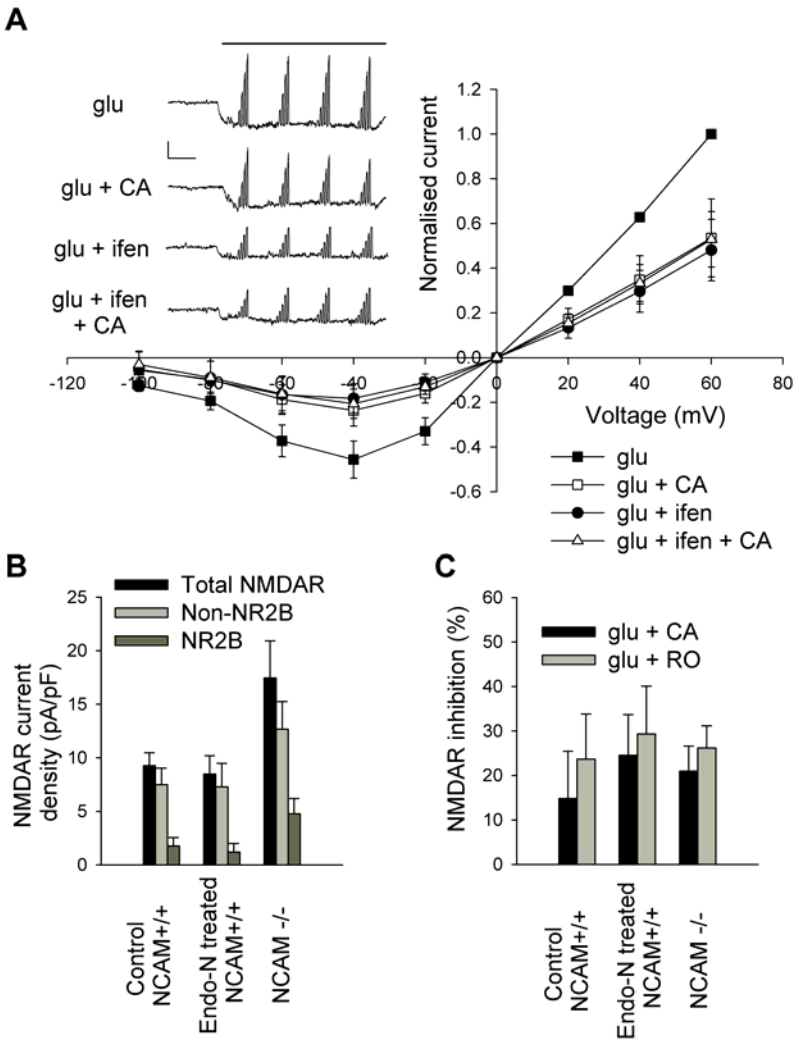


Figure 4.4: Inhibition of NR2B-containing NMDA receptor by Application of PSA, but not by Cell Surface-associated PSA.

(A) NMDA receptor currents evoked by glutamate (3µM, n=6, filled squares) are inhibited by colominic acid (40µg/ml, $F_{8,40}$=7.94, p<0.00001, open squares) and ifenprodil (10µM, $F_{8,40}$=11.42, p<0.000001, filled circle). Furthermore, ifenprodil occludes the inhibition by colominic acid ($F_{8,40}$=1.49, p>0.05, open triangles). Data represent mean ± SEM. Insets: Representative recordings showing glutamate evoked NMDA receptor currents in neurons (glu). Co-application of colominic acid (glu + CA), ifenprodil (glu + ifen) and both ifenprodil and colominic acid (glu + ifen + CA) inhibits the NMDA receptor currents. Horizontal bar indicates application of glutamate. Scale bars are 200pA and 2 seconds. (B) Current densities for total NMDA receptor currents, non-NR2B NMDA receptor currents and NR2B NMDA receptor currents in control, wild type (NCAM+/+) (n=14), endo-N treated NCAM+/+ (n=12, $F_{8,192}$=0.26, p>0.05,

$F_{8,192}$=0.07, p>0.05, $F_{8,192}$=0.25, p>0.05) and NCAM-deficient (NCAM-/-) neurons (n=8, $F_{8,160}$=6.26, p<0.00001, $F_{8,160}$=2.47, p<0.05, $F_{8,160}$=4.24, p<0.001). NMDA currents are normal in endo-N treated cultures but are increased in NCAM-/- neurons. (C) Similar levels of inhibition of NMDA receptor currents at +60mV in NCAM+/+, endo-N treated NCAM+/+ and NCAM-/- neurons by colominic acid (40μg/ml, $F_{16,248}$=0.25, p>0.05) and RO 25-6981 (0.5 μM, $F_{16,248}$=0.16, p>0.05).

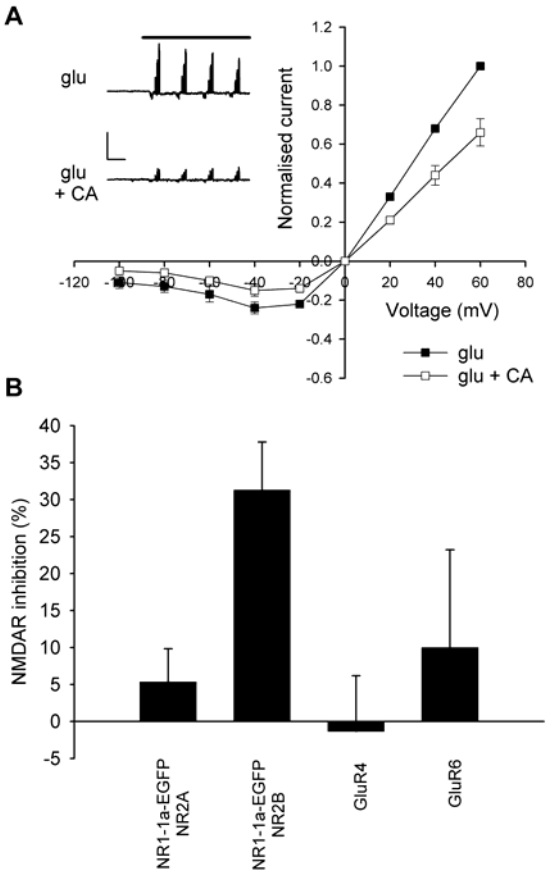


Figure 4.5: Recombinant Glutamate Receptors Containing the NR2B Subunit, but not NMDA Receptor Subunit NR2A or Other Glutamate Receptors, are Inhibited by PSA.

(A) Recombinant NR1-1/NR2B currents evoked by glutamate (10μM, filled squares) are inhibited by colominic acid (40μg/ml, open squares). Data represent mean ± SEM. Inset: Representative recordings showing that glutamate evoked currents in NR1-1/NR2B transfected CHO cells (glu). Co-application of colominic acid with glutamate reduces the NR1-1/NR2B currents (glu + CA). Horizontal bar indicates application of glutamate. Scale bars are 200pA and 2 seconds. (B) Inhibition of recombinant glutamate receptor currents by colominic acid at +60mV for NR1-1a-EGFP/NR2A (n=9 ,$F_{8,64}$=0.56, p>0.05), NR1-1a-EGFP/NR2B (n=20, $F_{8,152}$=18.05, p<0.000001), at –100mV for GluR4 (n=5, $F_{8,32}$=0.72, p>0.05) and at –60mV for GluR6 (n=10, p>0.05). To activate GluR4 and GluR6 receptors, 10μM and 5μM glutamate were used, respectively. These concentrations proved to be non-saturating in our conditions.

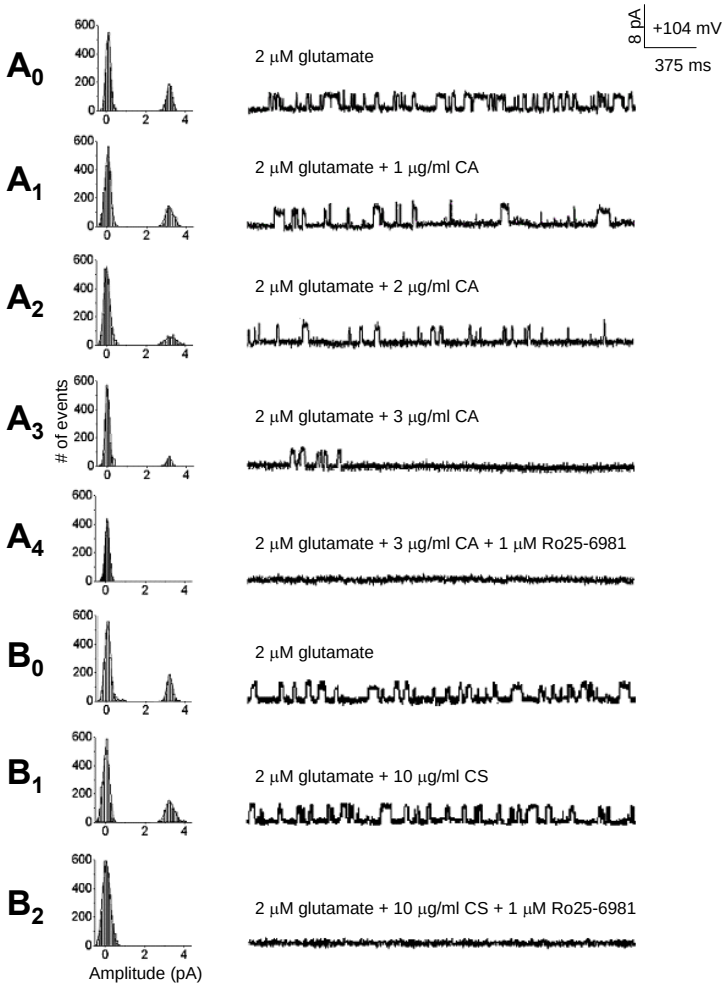


Figure 4.6: PSA Reduces Open Probability of NR2B-containing Synaptosomal NMDA Receptors

Sample traces show currents from NMDA receptors reconstituted in lipid bilayers, activated by 2μM glutamate, and voltage clamped at + 104 mV. Channel activity is evident by upward transitions of the current representing the open state. Respective amplitude histograms, with peaks corresponding to the closed and open states are shown to the left of each of sample traces. (A_0) Sampled recordings of single channel activity in the presence of 2 μM glutamate. (A_1-A_3) Significant decline in single channel open probability in the presence of 1, 2, and 3 μg/ml colominic acid, respectively. (A_4) A full blockade of residual activity of the NMDA receptor channel by the NR2B subunit specific antagonist Ro 25-6981. (B_0) Sampled recordings of single channel activity in the presence of 2 μM glutamate. (B_1) Neither single channel open probability nor conductance of NR2B subunit containing NMDA receptors was significantly altered in the presence of 10 μg/ml chondroitin sulfate (CS). (B_2) Ro 25-6981 completely blocked the NMDA receptor currents that were not affected by CS, confirming that the recorded channel contained the NR2B subunit.

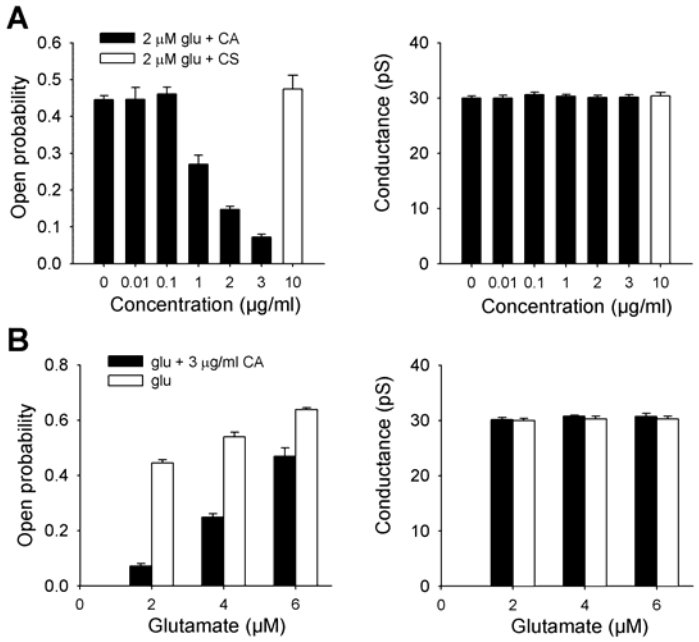


Figure 4.7: Inhibitory Effects of PSA on Open Probability of NR2B-containing Synaptosomal NMDA Receptors is PSA and Glutamate Concentration Dependent.

(A) Concentration dependent effect of colominic acid (CA) on open probability (Po) of NR2B-containing synaptosomal NMDA receptors in the presence of 2 μM glutamate. Bars represent mean ± SEM. CA significantly reduces Po at concentrations ≥ 1μg/ml ($P<0.01$; n=10). There is no change in Po in the presence of lower concentrations of CA ($P>0.05$, n=5) or chondroitin sulfate (CS; $P>0.05$; n=5). (B) CA and CS did not significantly alter the single channel conductance of NR2B subunit-containing NMDA receptors ($P>0.05$; n=10 for CA and n=5 for CS). (C) Inhibitory effect of PSA on Po of NR2B-containing synaptosomal NMDA receptors depends on glutamate concentration: the reduction of Po is many times smaller in the presence of 6 μM glutamate than at the presence of 2 μM glutamate ($P<0.01$, n=5). (D) PSA did not alter single channel conductance of NR2B subunit-containing NMDA receptors in the presence of 2, 4, and 6 μM glutamate ($P>0.05$; n=5).

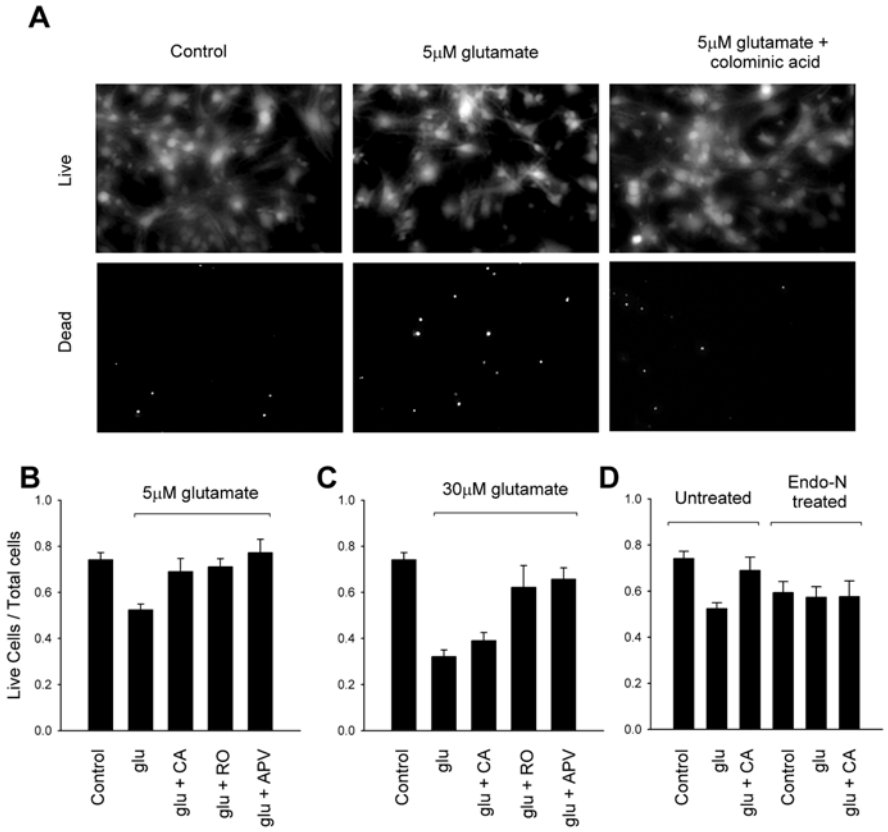


Figure 4.8: Application of PSA and Cell Surface Expressed PSA Inhibit Excitotoxicity.

(A) Representative images showing live cells marked by calcein (upper row) and dead cells by propidium iodide (lower row) under non-treated conditions (control), after treatment with glutamate (5μM) and after co-treatment with glutamate and colominic acid (5μM glutamate, 40μg/ml colominic acid). Images in one column were taken at the same position. (B) Neuronal cell survival under control conditions (number of culture preparations n=7, control) and in the presence of glutamate (5μM, n=7, $p<0.05$, glu), glutamate and colominic acid (40μg/ml, n=5, $p>0.05$, glu + CA), glutamate and RO 25-6981 (500nM, n=4, $p>0.05$, glu + RO) and glutamate and DL-2-amino-5-phosphonopentanoic acid (APV, 50μM, n=3, $p>0.05$, glu + APV). Note that colominic acid rescued neurons as well as APV or RO 25-6981. (C) Neuronal cell survival under control conditions (n=7, control), in the presence of glutamate (30μM, n=9, $p<0.05$, glu), glutamate and colominic acid (40μg/ml, n=5, $p<0.05$, glu + CA), glutamate and RO 25-6981 (50μM, n=3, $p>0.05$, glu + RO), and glutamate and DL-2-amino-5-phosphonopentanoic acid (APV, 50μM, n=3, $p>0.05$, glu + APV). Colominic acid failed to rescue neurons at this concentration of glutamate. (D) Neuronal cell survival in untreated (n=5) and endo-N treated (n=5) cell cultures under control conditions (control), in the presence of glutamate (5μM, glu), and glutamate and colominic acid (40μg/ml, glu + CA).

5. MODULATION OF SYNAPTOSOMAL AMPA RECEPTORS BY POLYSIALIC ACID

ABSTRACT

The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) subtype of glutamate receptors mediates fast excitatory neurotransmission in the mammalian central nervous system. Carefully controlled modulation of AMPA receptors is critical for normal synaptic transmission. Several endogenous molecules play a vital role in regulating the functional properties of AMPA receptors. Polysialic acid (PSA), a highly negatively charged, carbohydrate, covalently attached to the neural cell adhesion molecule (NCAM), is highly expressed in hippocampal synapses and have been recently shown to alter the single channel properties of purified and reconstituted AMPA receptors. However, the effects of PSA on native synaptic AMPA receptors have never been investigated. This study utilized biochemical isolation and functional reconstitution of synaptosomal AMPA receptors in lipid bilayers to elucidate the effects of PSA on synaptic AMPA receptors. PSA, in a concentration dependent manner, increases the single channel open probability and mean open time, and decreased the mean closed time without modifying the conductance. Cyclothiazide, a known inhibitor of AMPA receptor desensitization failed to occlude the effects of PSA. The results indicate that PSA potently modulates synaptic AMPA receptors. PSA in addition to decreasing the rate of desensitization appears to potentiate AMPA receptors by another mechanism.

INTRODUCTION

The glutamatergic system mediates most of the excitatory neuronal transmission in the mammalian brain (Barnard, 1992; Sprengel and Seeburg, 1993). Furthermore, fast excitatory synaptic transmission primarily involves the activation of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subtype of glutamate receptors (Michaelis, 1998). Regulation of these receptors is essential for synaptogenesis, synaptic plasticity, and memory consolidation (Bolton et al., 2000; Cottrell et al., 2000; Andrasfalvy and Magee, 2004; Vaithianathan et al., 2004). Alterations in the single channel properties of AMPA receptors such as conductance, open/close rate constants, and burst behavior have been shown to enhance glutamatergic transmission (Ambros-Ingerson and Lynch, 1993; Benke et al., 1998; Lin et al., 2002); moreover, recent findings have indicated that extracellular matrix molecules such as polysialic acid (PSA) play a role in enhancing synaptic transmission by interacting with AMPA receptors (Vaithianathan et al., 2004).

Desensitization and deactivation rates of AMPA receptors control the magnitude and time course of synaptic transmission; therefore, these processes are of particular interest in modulating synaptic plasticity mechanisms in the hippocampus. The desensitization rate refers to the decay of current in the continuous presence of the agonist, while the deactivation rate pertains to the decay of current after the agonist is removed (Mitchell and Fleck, 2007). Together these parameters indicate the decreased sensitivity of the receptor that occurs due to the prolonged presence of glutamate (Atassi and Glavinovic, 1999); furthermore, decreasing the rate of desensitization and

deactivation would have positive effects on AMPA receptor mediated synaptic transmission.

The present study aims to characterize polysialic acid (PSA) modulation of AMPA receptors in the hippocampus. PSA is a highly negatively charged homomeric polymer of sialic acid which is predominantly carried by the neural cell adhesion molecule (NCAM), and modulates its functions during cell migration and axonal outgrowth (Kiss and Rougon, 1997). Electron microscopy has revealed that PSA-NCAM is expressed on the neuronal membrane of pyramidal cells in the hippocampus on both presynaptic and postsynaptic sites (Schuster et al., 2001). Moreover, this expression is activity dependent (Kiss et al., 1994), and increases ten-fold following the induction of long-term potentiation in the CA1 region of the hippocampus (Becker et al., 1996; Eckhardt et al., 2000).

To study the effects of PSA on synaptic AMPA receptor-mediated neurotransmission, we utilized a technique developed in our laboratory utilizing isolated hippocampal synaptosomes reconstituted in lipid bilayers. Through this technique we investigated the direct modulation of synaptic AMPA receptor activity by PSA. In addition, we investigated whether cyclothiazide, a compound which is known to slow the rate of deactivation and desensitization of AMPA receptors (Partin et al., 1996; Atassi and Glavinovic, 1999; Mitchell and Fleck, 2007), can occlude the effects of PSA. Our findings demonstrate that PSA potentiates single channel properties of synaptic AMPA receptors and these effects are not occluded by cyclothiazide.

MATERIALS AND METHODS

Synaptosomal Preparation:

The synaptosomes were prepared as described elsewhere (Johnson et al., 1997) with minor modifications. The hippocampus was isolated from 7-10 days old Sprague Dawley rats and homogenized in an Eppendorf tube with 400 µl of ice cold Krebs-Henseleit buffer, which consist of: 118.5 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.18 mM KH_2PO_4, 24.9 mM $NaHCO_3$, 10 mM dextrose, 10 mg/ml adenosine deaminase and pH adjusted to 7.4 by bubbling with O_2:CO_2 (95:5). Leupeptin (0.01 mg/ml), pepstatin A (0.005 mg/ml), aprotinin (0.10 mg/ml), and benzamide (5 mM) were added to the buffer to minimize proteolysis. The homogenate was diluted with 1.60 ml of additional Krebs buffer, after being homogenized with five turns of a hand-held pestle. The mixture was filtered using a 13 mm diameter Millipore syringe filter holder with a 1 cc Tuberculin syringe. The diluted filtrate was forced through three layers of nylon (Tetko, 100 µm pore size) pre-wet with 150 µl of Krebs buffer, and collected in an Eppendorf tube. Upon filtering again with a pre-wet 5 mm Millipore nitrocellulose filter, the filtrate was centrifuged at 1000 x *g* for 15 minutes in a microcentrifuge at 4^{o}C. After removing the supernatant, the pellet, which contains the synaptoneurosomes, was resuspended in 100 µl of Krebs buffer for electrophysiological recordings.

Reconstitution of Synaptosomal AMPA Receptors in Lipid Bilayers:

Incorporation of synaptosomal AMPA receptors in artificial lipid bilayers is carried out using 'tip-dip" method (Suppiramaniam et al., 2001). The lipid phospholipid bilayer is formed at the tip of a polished borosilicate glass pipette (World Precision

Instruments Inc., Sarasota, FL). The P-2000 laser micropipette puller (Sutter Instrument Company, Novato, CA) is used to pull pipettes with100 MΩ resistance. The synthetic phospholipids were prepared by dissolving 1,2 –diphytanoyl-sn-glycero-3-phosphocholine (Avanti Polar-Lipids Inc., Alabaster, AL) in anhydrous at hexane (Aldrich Chemical Co., Milwaukee, WI) to obtain a concentration of 1 mg/mL. Approximately 3-5 μl of synthetic phospholipids is delivered into 300 μl of bath solution. The bilayer formation is initiated by successive transfer of two monolayers onto the tip of the patch pipette in an asymmetric saline condition with "out side-out" configuration. The bath solution contained 300 μl of pseudoextracellular fluid (ECF) composed of 125 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, and 5 mM Tris HCl. The pseudointracellular fluid (ICF) consisting of 110 mM KCl, 4 mM NaCl, 2 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 2 mM 3-N-Morpholino propanesulfonic acid (MOPS) is used as the pipette solution (pH adjusted to 7.4). After forming a stable membrane, 3-5 μl suspension of synaptosomes is delivered to the ECF. Gentle stirring using an air driven magnetic stir bar placed at the bottom of the microbeaker helps fusion of synaptosomal fragments into the bilayer.

Single Channel Recording and Analysis:

Single synaptosomal AMPA receptor channel currents were obtained by the addition of 290nM AMPA ((S)-alpha-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid, Sigma-Aldrich) in the presence of 1 μM AP5 (DL-2-Amino-5-phosphonopentanoic acid, Tocris), 1 μM SYM 2081 ((2S, 4R)-4-methylglutamic acid, Sigma-Aldrich), 100 μM picrotoxin (cocculin, Sigma-Aldrich), 2 μM TEA (tetraethyl ammonium chloride,

Sigma-Aldrich), and 1 μM TTX (tetrodotoxin, Sigma-Aldrich), which are NMDA, Kainate, and GABA receptors and sodium and potassium channels, respectively.

PSA (colominic acid) was delivered to the ECF in increasing concentrations ranging from 1 μg/ml to 3 μg/ml. In one set of experiments, since 2 μg/ml of PSA showed the maximum activity, cyclothiazide (CTZ), 5μM, was added to ECF. In another set of experiments CTZ was added first at its maximum channel activity concentration, 10 μM, and 1 μg/ml of PSA was added to ECF. The resulting single channel currents were digitized using a PCM interface (VR-10B Digital Data Recorder, Instrutech. Corp., Elmont, NY) and recorded on a VHS tape (Sony Corp., New York). The data were filtered at 2 kHz and digitized between 5-25 kHz with PClamp6 software for off line analysis. The data processing of single channel recordings from synaptosomal receptors was similar to the one described elsewhere (Vaithianathan et al., 2005).

Only the data exhibiting long stretches of single channel current transition without base line drifts were chosen for quantitative analysis. The all points-current amplitude histograms were constructed and fitted with Gaussian curves to identify the individual conductance. The single channel conductance of AMPA receptors were obtained by plotting current as a function of membrane voltage and the conductance were determined according to the equation $g = I/(V-V_0)$, where I is the single channel current, V is the voltage and V_0 is the reversal potential. The single channel open and closed probabilities were extracted from the area under the current-amplitude histogram. If the areas under the closed and open regions are R_c and R_o respectively, the single channel probabilities can be calculated by the straightforward relationships $P_c = R_c/(R_c + R_o)$ and $P_o = R_o/(R_c + R_o)$.

RESULTS

Effects of PSA on Single Channel Synaptosomal AMPA Receptors

To investigate the effects of PSA on synaptosomal AMPA receptors function, the receptors were reconstituted in a lipid bilayer activated by 290 nM of AMPA (Figure 5.1 A). This activity was blocked by the antagonist of the AMPA/kainate glutamate receptors, SYM 2206 (10 μM). The addition of PSA (or colominic acid, CA) at 1, 2, and 3 (data not shown) μg/ml, to the activated receptors, an increase in channel activity was observed (Figure 5.1 B and 5.1 C, respectively). The open probability increased from 0.227 $\pm$ 0.029 (control) to 0.43 $\pm$ 0.012 (1 μg/ml PSA), 0.735 $\pm$ 0.014 (2 μg/ml PSA) and 0.741 $\pm$ 0.016 (3 μg/ml PSA, data not shown). PSA induced no significant changes in the single channel conductance, but there was an increase in the channel open probability. Open probability, burst duration, and interburst intervals were saturated at concentrations 3 μg/ml PSA and greater (data not shown).

Similar to purified AMPA receptors (Vaithianathan et al., 2004), the modulation the open times of synaptosomal AMPA receptors was voltage-dependent (data not shown). The histograms were best fitted by two and three exponentials for open and closed durations (Figure 5.2 – 5.4 and Table 5.1). PSA increased the duration of all open states in a dose-dependent manner. In addition, it increased the small and decreased the larger closed state. Interestingly upon addition of PSA another smaller closed state was observed (Figure 5.3).

PSA Fully Occludes the Effects of CTZ

There was no significant change observed with the addition of 5 μM CTZ to 2 μg/ml PSA. The probability of channel open was 0.722 ± 0.022 for PSA + CTZ (Figure 5.1 D). The open times and closed times were not significantly different between 2 μg/ml PSA and 2 μg/ml PSA + 5 μM CTZ. The histograms were fitted for two and three exponentials for the open and closed durations (Figure 5.5).

Effects of CTZ on Single Channel Synaptosomal AMPA Receptors

To investigate the effects of CTZ on synaptosomal AMPA receptors function, the receptors were reconstituted in a lipid bilayer activated by 290 nM of AMPA (Figure 5.6). This activity was blocked by the antagonist of the AMPA/kainate glutamate receptors, CNQX (1 μM). The addition of CTZ 5, 10, and 15 (data not shown) μM, to the activated receptors, an increase in channel activity was observed (Figure 5.6 B and 5.6 C, respectively). The open probability increased from 0.229 ± 0.023 (control) to 0.429 ± 0.198 (5 μM), 0.637 ± 0.225 (10 μM) and 0.642 ± 0.021 (15 μM, data not shown). CTZ induced no significant changes in the single channel conductance, but there was an increase in the channel open probability. Open probability, burst duration, and interburst intervals were saturated at concentrations 10 μM and greater (data not shown).

The modulation the open times of synaptosomal AMPA receptors was voltage-dependent (data not shown). The histograms were best fitted by two and three exponentials for open and closed durations (Figure 5.7 – 5.9 and Table 5.2). CTZ increased the duration of all open states in a dose-dependent manner. In addition, it

increased the small and decreased the larger closed state. Interestingly upon addition of CTZ another smaller closed state was observed (Figure 5.8).

CTZ did not Occlude Effects of PSA

There was a significant change observed with the addition of 1 μg/ml PSA to 10 μM CTZ. The probability of channel open was 0.892 $\pm$ 0.212 for CTZ+ PSA (Figure 5.6 D). The open times and closed times were significantly different between 2 μg/ml PSA and 2 μg/ml PSA + 5 μM CTZ. The histograms were fitted for three exponentials for the open and closed durations (Figure 5.10).

DISCUSSION

Mechanisms of Interaction Between PSA and AMPA Receptors

In our single channel study of synaptosomal AMPA receptors, 290 nM elicited channel activity with a 28 pS conductance as previously reported for synaptosomal AMPA receptors (Vaithianathan et al., 2005). PSA increased the burst duration and the mean open time within bursts. In addition it decreased the interburst intervals and mean close time in a concentration-dependent manner similar to that of purified AMPA receptors (Vaithianathan et al., 2004). It has been speculated that purified and native AMPA receptors in lipid bilayers are modulated by the same mechanism.

Since it is know that CTZ decreases desensitization of AMPA receptors, one method of uncovering PSA mechanism of action is to determine if CTZ will fully occlude the effects of PSA. In the first set of experiments PSA was added first followed by CTZ. If indeed PSA was not acting by decreasing desensitization of AMPA receptors, then

upon addition of CTZ one would expect a significant change in the open probability. However, we found that CTZ did not produce any significant effects upon addition after PSA. This is because receptors in an open state cannot readily desensitize (Vyklicky et al., 1991; Ambros-Ingerson and Lynch, 1993). In addition reports previously showed that the number of receptors that can be activated and the rate of onset of desensitization is decreased in the presence of agonist (Trussell and Fischbach, 1989; Patneau and Mayer, 1991). Thus, PSA fully occludes the effects of CTZ. Therefore, PSA is acting in the same mechanism and possibly may keep AMPA receptors in a low affinity sensitized state.

Mechanisms of Interaction Between PSA in Presence of CTZ on AMPA Receptors

However, it is not known whether there may be an additional mechanism of action. Therefore, we conducted experiments where CTZ was added first followed by PSA initially to determine whether CTZ effects would fully occlude the effects of PSA. We found that PSA did produce additional significant changes in the open probability of single channel synaptosomal AMPA receptors after the addition of CTZ. This indicates that PSA acts in another mechanism other than desensitization.

Since PSA does not activate AMPA receptors in the absence of AMPA agonist, an open channel conformation is required for PSA interaction (Vaithianathan et al., 2004). This data proves that negatively charged PSA is more than likely interacting with positively charged amino acid residues of the open channel pore of synaptosomal AMPA receptors. This mechanism has been previously suggested for another polyanionic carbohydrate, heparin (Hall et al., 1996; Sinnarajah et al., 1999). Thus, PSA plays an

important role in stabilizing the open confirmation state of synaptosomal AMPA receptors by interacting with the channel pore.

Positive modulators of AMPA receptors potentially enhance cognition by offsetting losses of glutamatergic synapses and promoting synaptic plasticity (Lynch, 2004). PSA reduction of AMPA receptors entry into the desensitized state, results in a decrease in interburst interval and an increase in burst duration. This data gives great insight to the mechanism in which PSA modulates AMPA receptors. Since regulation of AMPA receptors are essential for synaptic plasticity and synaptogenesis, the role that PSA has in regulation of these receptors is still largely unknown. However, this study reveals that PSA not only decreases the desensitization but also interacts directly with the channel pore thereby stabilizing the receptor in an open state. This can be used for future therapeutic interventions to enhance synaptic activity by the development of a drug with similar structural and physiological properties of PSA.

REFERENCES

Ambros-Ingerson J, Lynch G (1993) Channel gating kinetics and synaptic efficacy: a hypothesis for expression of long-term potentiation. Proc Natl Acad Sci U S A 90:7903-7907.

Andrasfalvy BK, Magee JC (2004) Changes in AMPA receptor currents following LTP induction on rat CA1 pyramidal neurones. J Physiol 559:543-554.

Atassi B, Glavinovic MI (1999) Effect of cyclothiazide on spontaneous miniature excitatory postsynaptic currents in rat hippocampal pyramidal cells. Pflugers Arch 437:471-478.

Barnard EA (1992) Receptor classes and the transmitter-gated ion channels. Trends Biochem Sci 17:368-374.

Becker CG, Artola A, Gerardy-Schahn R, Becker T, Welzl H, Schachner M (1996) The polysialic acid modification of the neural cell adhesion molecule is involved in spatial learning and hippocampal long-term potentiation. J Neurosci Res 45:143-152.

Benke TA, Luthi A, Isaac JT, Collingridge GL (1998) Modulation of AMPA receptor unitary conductance by synaptic activity. Nature 393:793-797.

Bolton MM, Blanpied TA, Ehlers MD (2000) Localization and stabilization of ionotropic glutamate receptors at synapses. Cell Mol Life Sci 57:1517-1525.

Cottrell JR, Dube GR, Egles C, Liu G (2000) Distribution, density, and clustering of functional glutamate receptors before and after synaptogenesis in hippocampal neurons. J Neurophysiol 84:1573-1587.

Eckhardt M, Bukalo O, Chazal G, Wang L, Goridis C, Schachner M, Gerardy-Schahn R, Cremer H, Dityatev A (2000) Mice deficient in the polysialyltransferase ST8SiaIV/PST-1 allow discrimination of the roles of neural cell adhesion molecule protein and polysialic acid in neural development and synaptic plasticity. J Neurosci 20:5234-5244.

Hall RA, Vodyanoy V, Quan A, Sinnarajah S, Suppiramaniam V, Kessler M, Bahr BA (1996) Effects of heparin on the properties of solubilized and reconstituted rat brain AMPA receptors. Neurosci Lett 217:179-183.

Hollmann M, Hartley M, Heinemann S (1991) Ca2+ permeability of KA-AMPA--gated glutamate receptor channels depends on subunit composition. Science 252:851-853.

Johnson MW, Chotiner JK, Watson JB (1997) Isolation and characterization of synaptoneurosomes from single rat hippocampal slices. J Neurosci Methods 77:151-156.

Jonas P (1993) AMPA-type glutamate receptors--nonselective cation channels mediating fast excitatory transmission in the CNS. Exs 66:61-76.

Kiss JZ, Rougon G (1997) Cell biology of polysialic acid. Curr Opin Neurobiol 7:640-646.

Kiss JZ, Wang C, Olive S, Rougon G, Lang J, Baetens D, Harry D, Pralong WF (1994) Activity-dependent mobilization of the adhesion molecule polysialic NCAM to the cell surface of neurons and endocrine cells. Embo J 13:5284-5292.

Lin B, Brucher FA, Colgin LL, Lynch G (2002) Long-term potentiation alters the modulator pharmacology of AMPA-type glutamate receptors. J Neurophysiol 87:2790-2800.

Lynch G (2004) AMPA receptor modulators as cognitive enhancers. Curr Opin Pharmacol 4:4-11.

Michaelis EK (1998) Molecular biology of glutamate receptors in the central nervous system and their role in excitotoxicity, oxidative stress and aging. Prog Neurobiol 54:369-415.

Mitchell NA, Fleck MW (2007) Targeting AMPA receptor gating processes with allosteric modulators and mutations. Biophys J 92:2392-2402.

Partin KM, Fleck MW, Mayer ML (1996) AMPA receptor flip/flop mutants affecting deactivation, desensitization, and modulation by cyclothiazide, aniracetam, and thiocyanate. J Neurosci 16:6634-6647.

Patneau DK, Mayer ML (1991) Kinetic analysis of interactions between kainate and AMPA: evidence for activation of a single receptor in mouse hippocampal neurons. Neuron 6:785-798.

Schuster T, Krug M, Stalder M, Hackel N, Gerardy-Schahn R, Schachner M (2001) Immunoelectron microscopic localization of the neural recognition molecules L1, NCAM, and its isoform NCAM180, the NCAM-associated polysialic acid, beta1 integrin and the extracellular matrix molecule tenascin-R in synapses of the adult rat hippocampus. J Neurobiol 49:142-158.

Sinnarajah S, Suppiramaniam V, Kumar KP, Hall RA, Bahr BA, Vodyanoy V (1999) Heparin modulates the single channel kinetics of reconstituted AMPA receptors from rat brain. Synapse 31:203-209.

Sprengel R, Seeburg PH (1993) The unique properties of glutamate receptor channels. FEBS Lett 325:90-94.

Suppiramaniam V, Bahr BA, Sinnarajah S, Owens K, Rogers G, Yilma S, Vodyanoy V (2001) Member of the Ampakine class of memory enhancers prolongs the single channel open time of reconstituted AMPA receptors. Synapse 40:154-158.

Trussell LO, Fischbach GD (1989) Glutamate receptor desensitization and its role in synaptic transmission. Neuron 3:209-218.

Vaithianathan T, Matthias K, Bahr B, Schachner M, Suppiramaniam V, Dityatev A, Steinhauser C (2004) Neural cell adhesion molecule-associated polysialic acid potentiates alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor currents. J Biol Chem 279:47975-47984.

Vaithianathan T, Manivannan K, Kleene R, Bahr BA, Dey MP, Dityatev A, Suppiramaniam V (2005) Single channel recordings from synaptosomal AMPA receptors. Cell Biochem Biophys 42:75-85.

Vyklicky L, Jr., Patneau DK, Mayer ML (1991) Modulation of excitatory synaptic transmission by drugs that reduce desensitization at AMPA/kainate receptors. Neuron 7:971-984.

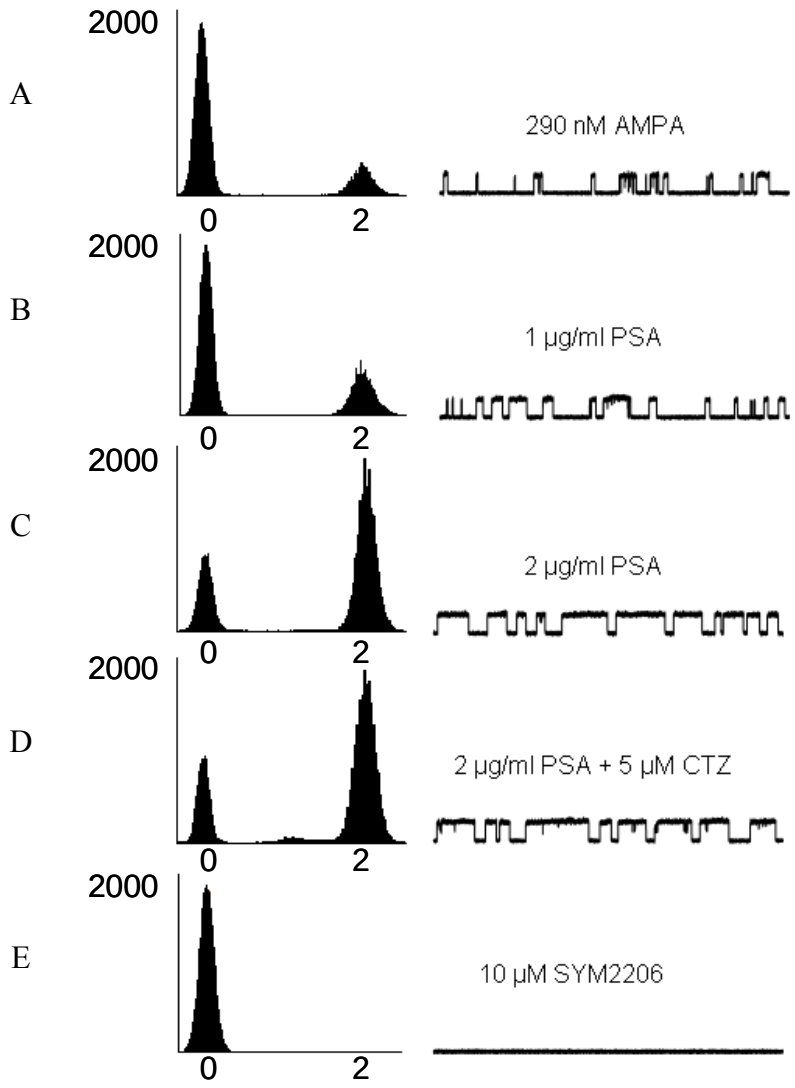
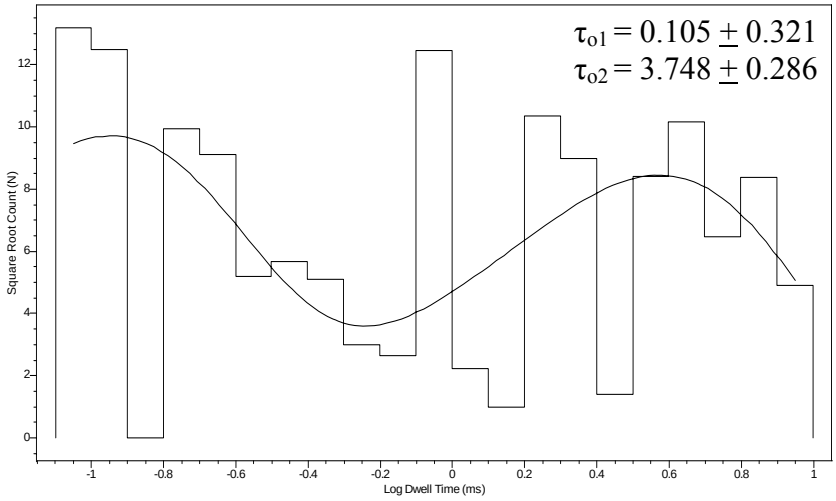


Figure 5.1: Modulation of Synaptosomal AMPA Receptors by PSA

Channel openings are indicated by upward transitions of the current. (A-E) represents average amplitude histograms (left) and the respective average trace (right) recorded in the presence of 290 nM AMPA (A), addition of 1 µg/ml PSA (B), 2 µg/ml PSA (C), and 2 µg/ml PSA + 5 µM CTZ (D). In (E) activity was blocked with the specific AMPA receptor antagonist, SYM2206 (10 µM). All recordings were done at +75 mV. The amplitude histograms show bimodal distributions with peaks corresponding to the stationary current levels (i.e. open and close states). The maximum unitary current was 2.1 pA. The channel conductance was 28 pS.

A



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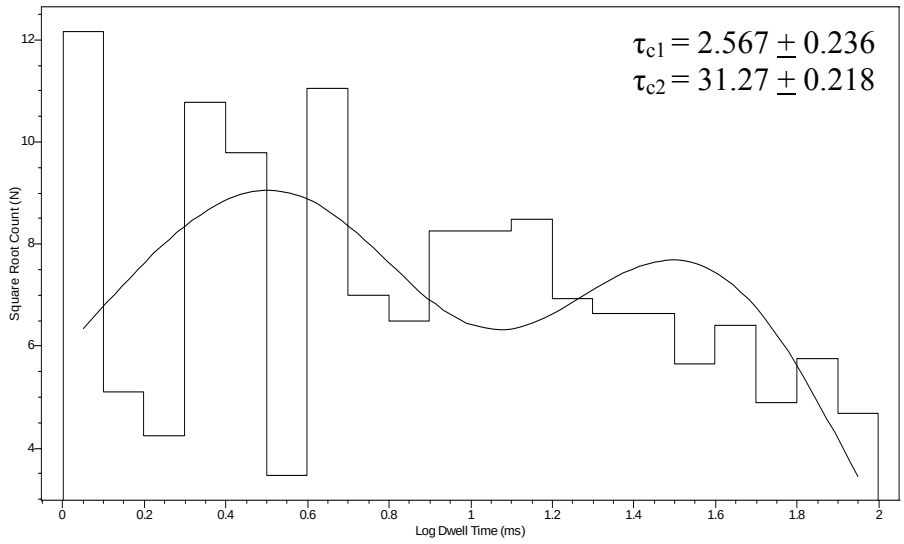


Figure 5.2: Distribution of Open and Closed Time for Single Channel Synaptosomal AMPA Receptors in the Presence of 290 nM AMPA.

Representative histograms of duration of openings (A) and closings (B) were analyzed using pClamp 9 software with two exponentials. Estimated time constants obtained from best fit of the distributions are indicated within the figure. See table 5.1 for comparisons.

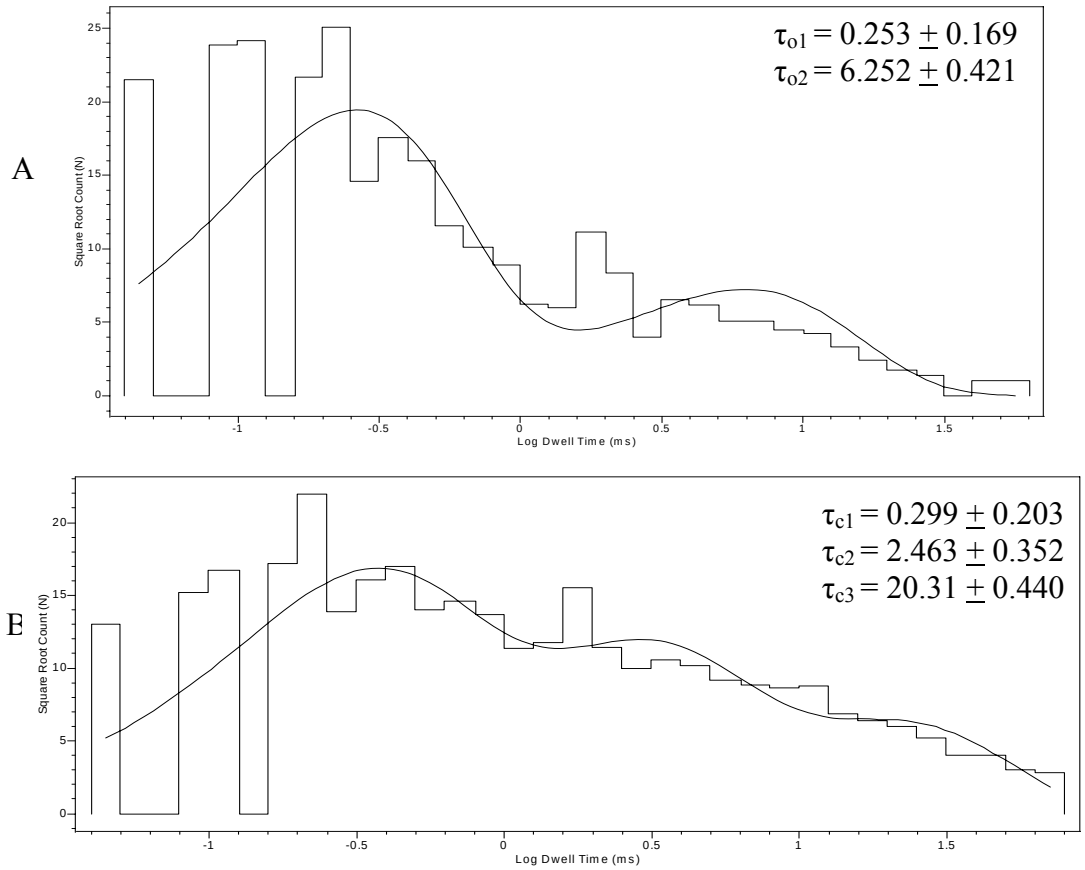


Figure 5.3: Distribution of Open and Closed Time for Single Channel Synaptosomal AMPA Receptors in the Presence of 1 µg/ml PSA.

Representative histograms of duration of openings (A) and closings (B) were analyzed using pClamp 9 software with two exponentials. Estimated time constants obtained from best fit of the distributions are indicated within the figure. See table 5.1 for comparisons. Interestingly, a smaller closed state is now detected as the intermediate state increases and the longest state decreases.

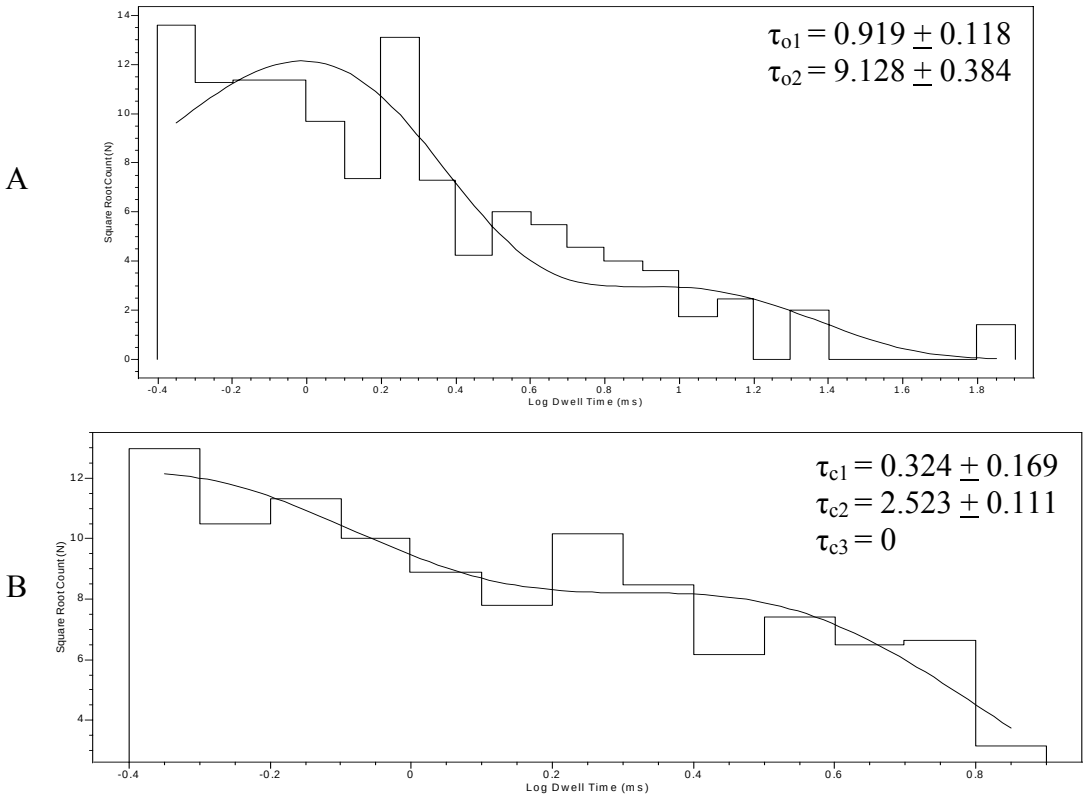
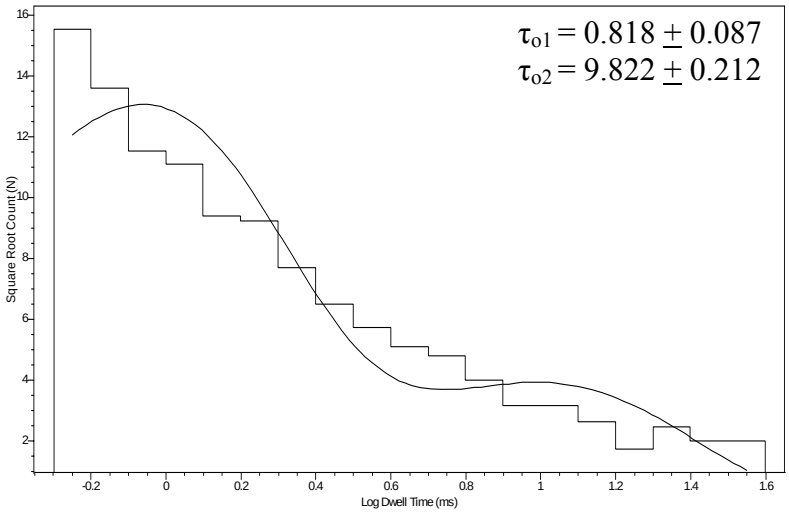


Figure 5.4: Distribution of Open and Closed Time for Single Channel Synaptosomal AMPA Receptors in the Presence of 2 μg/ml PSA.

Representative histograms of duration of openings (A) and closings (B) were analyzed using pClamp 9 software with two exponentials. Estimated time constants obtained from best fit of the distributions are indicated within the figure. See table 5.1 for comparisons. Notice that the longer closed state is now not detectable.

A



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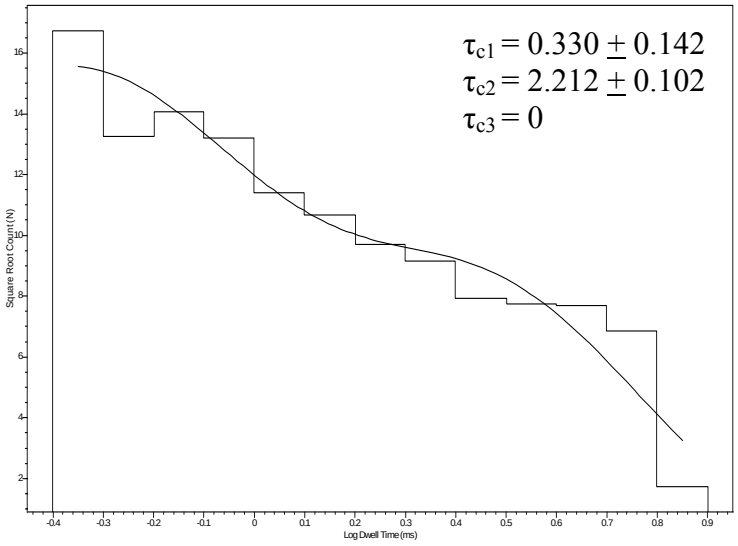


Figure 5.5: Distribution of Open and Closed Time for Single Channel Synaptosomal AMPA Receptors in the Presence of 2 μg/ml PSA and 5μM CTZ.

Representative histograms of duration of openings (A) and closings (B) were analyzed using pClamp 9 software with two exponentials. Estimated time constants obtained from best fit of the distributions are indicated within the figure. See table 5.1 for comparisons. There is not a significant difference between the open and close values here compared with the previous group.

Group	Po	Open time (ms)	Closed time (ms)
Control	0.227 ± 0.029	0.105 ± 0.321 3.748 ± 0.286	2.567 ± 0.236 31.27 ± 0.218
PSA 1	*0.43 ± 0.012	*0.253 ± 0.169 *6.252 ± 0.421	0.299 ± 0.203 2.463 ± 0.352 *20.31 ± 0.440
PSA 2	#0.735 ± 0.014	#0.919 ± 0.118 #9.128 ± 0.384	0.324 ± 0.169 2.523 ± 0.111 #0
PSA 2 + CTZ	0.722 ± 0.022	0.818 ± 0.086 9.822 ± 0.212	0.330 ± 0.142 2.212 ± 0.102 0

Table 5.1: Modulation of the Single Channel Properties of Synaptosomal AMPA Receptors by PSA and CTZ

Values represent mean ± SEM which were calculated form channel open probability (Po), channel open time, and channel closed time. Asterisk indicates significant differences with the corresponding values in control (*) and 1 μg/ml PSA (PSA 1, #). ($p<0.01$; One-way ANOVA; n=5-11).

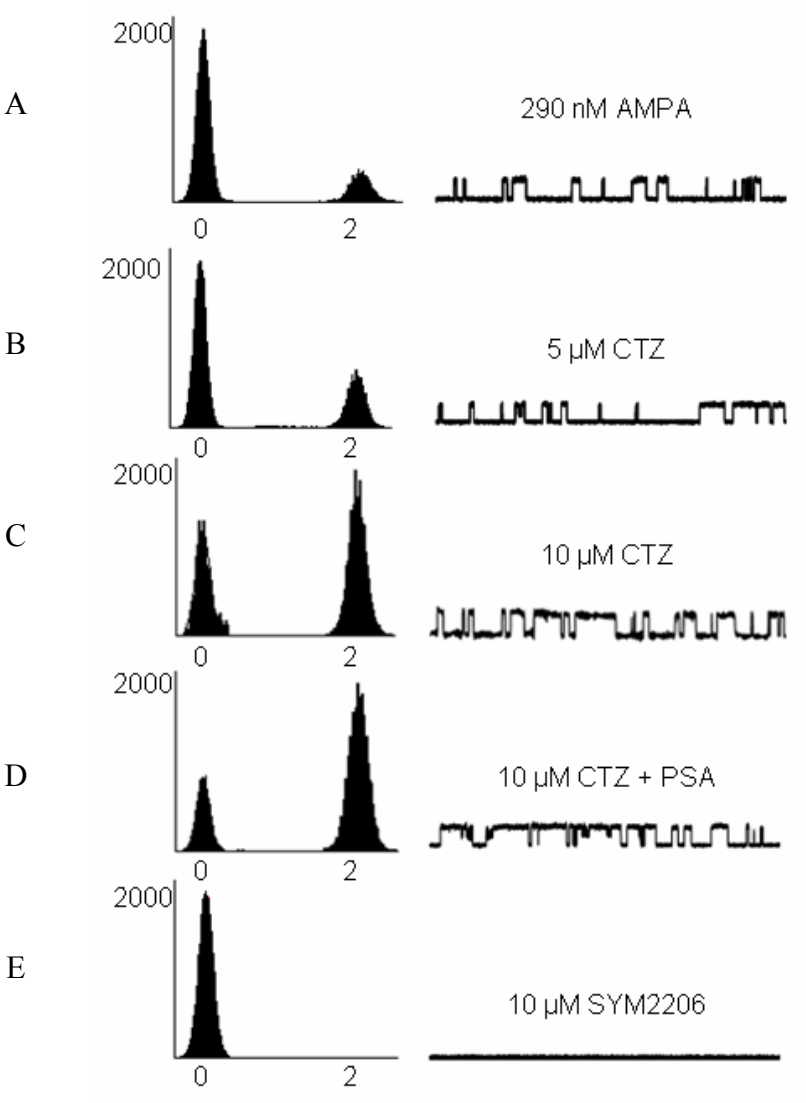
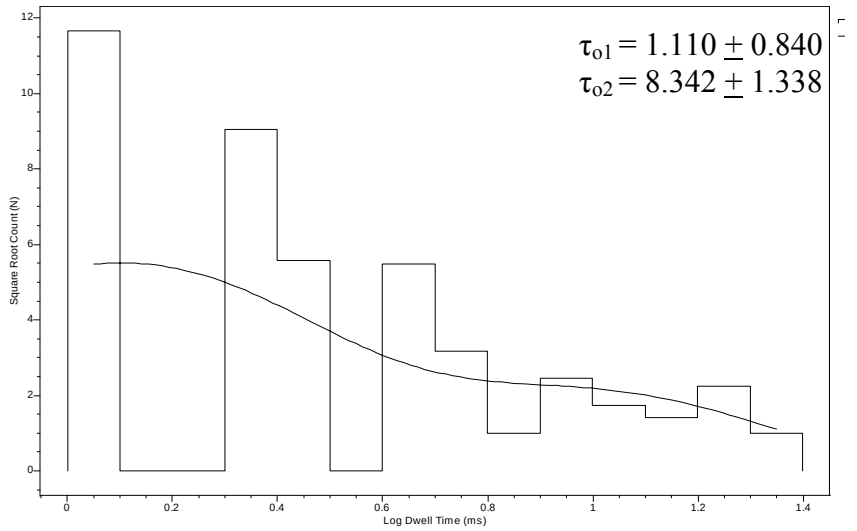


Figure 5.6: Modulation of Synaptosomal AMPA Receptors by CTZ

Channel openings are indicated by upward transitions of the current. (A-E) represents average amplitude histograms (left) and the respective average trace (right) recorded in the presence of 290 nM AMPA (A), addition of 5 µM CTZ (B), 10 µM CTZ (C), and 10 µM CTZ and 1 µg/ml PSA (D). In (E) activity was blocked with the specific AMPA receptor antagonist, SYM2206 (10 µM). All recordings were done at +75 mV. The amplitude histograms show bimodal distributions with peaks corresponding to the stationary current levels (i.e. open and close states). The maximum unitary current was 2.1 pA. The channel conductance was 28 pS.

A



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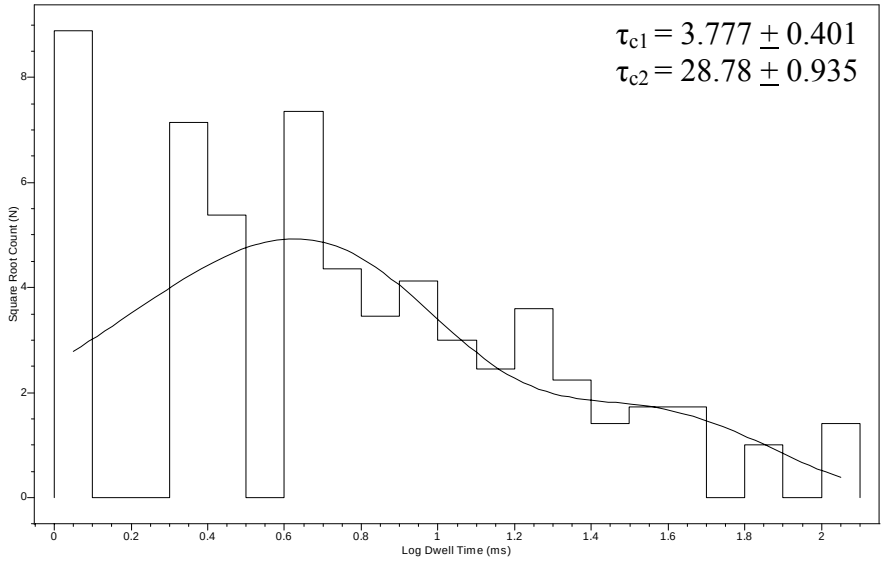


Figure 5.7: Distribution of Open and Closed Time for Single Channel Synaptosomal AMPA Receptors in the Presence of 290 nM AMPA.

Representative histograms of duration of openings (A) and closings (B) were analyzed using pClamp 9 software with two exponentials. Estimated time constants obtained from best fit of the distributions are indicated within the figure. See table 5.2 for comparisons.

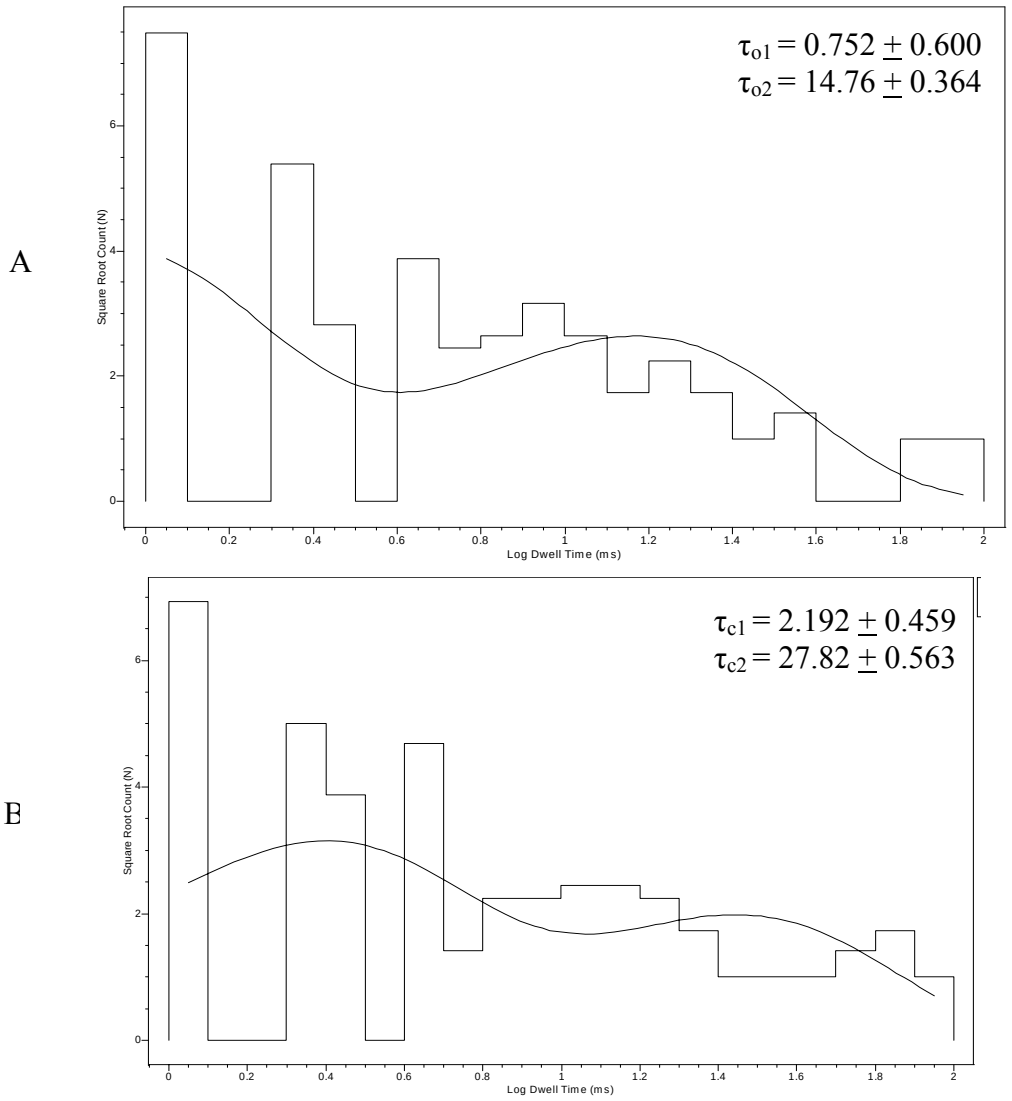
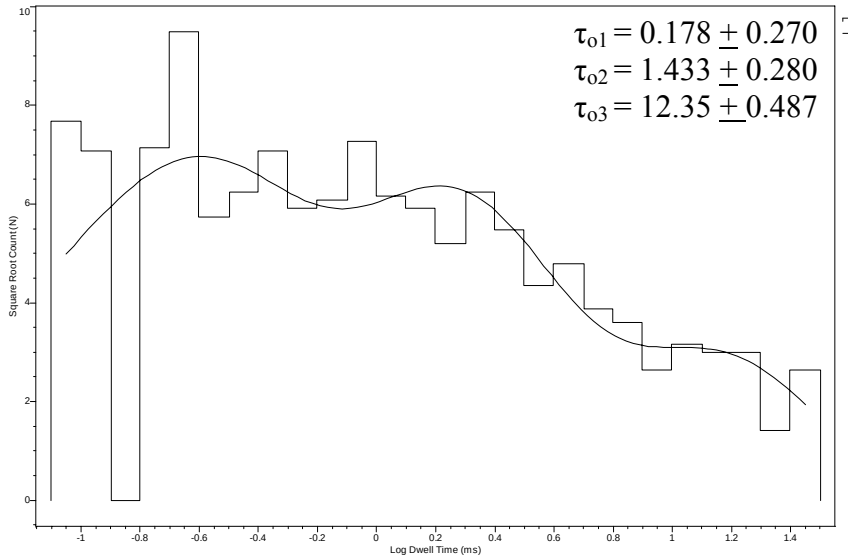


Figure 5.8: Distribution of Open and Closed Time for Single Channel Synaptosomal AMPA Receptors in the Presence of 5 μM CTZ.

Representative histograms of duration of openings (A) and closings (B) were analyzed using pClamp 9 software with two exponentials. Estimated time constants obtained from best fit of the distributions are indicated within the figure. See table 5.2 for comparisons. Interestingly, a smaller closed state is now detected as the intermediate state increases and the longest state decreases.

A



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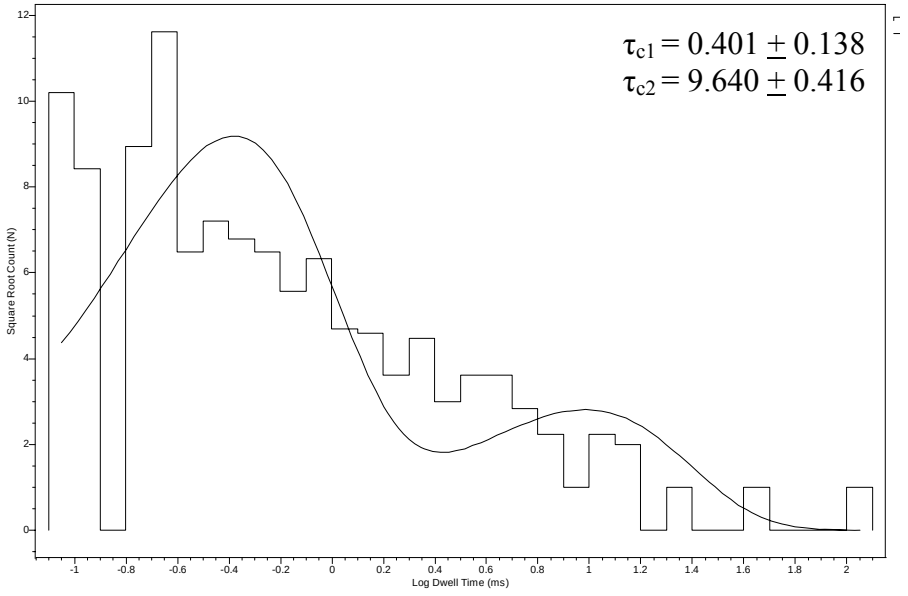
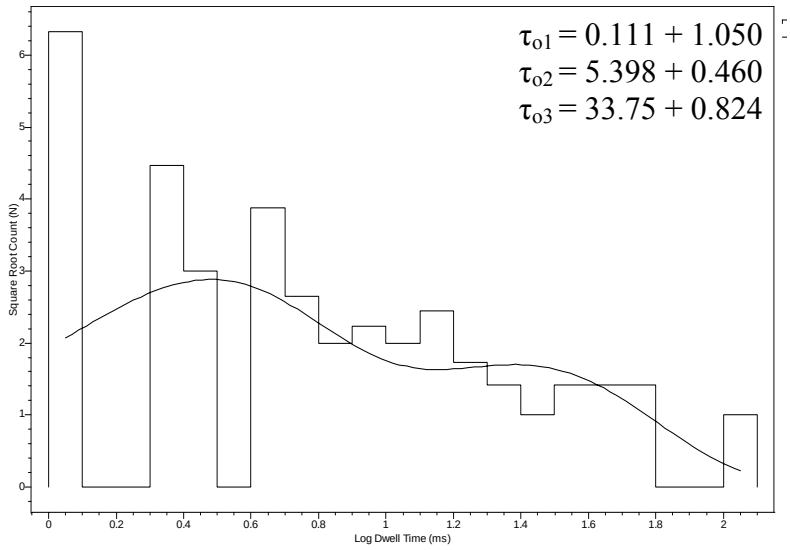


Figure 5.9: Distribution of Open and Closed Time for Single Channel Synaptosomal AMPA Receptors in the Presence of 10 μM CTZ.

Representative histograms of duration of openings (A) and closings (B) were analyzed using pClamp 9 software with two exponentials. Estimated time constants obtained from best fit of the distributions are indicated within the figure. See table 5.2 for comparisons. Notice that the longer closed state is now not detectable.

A



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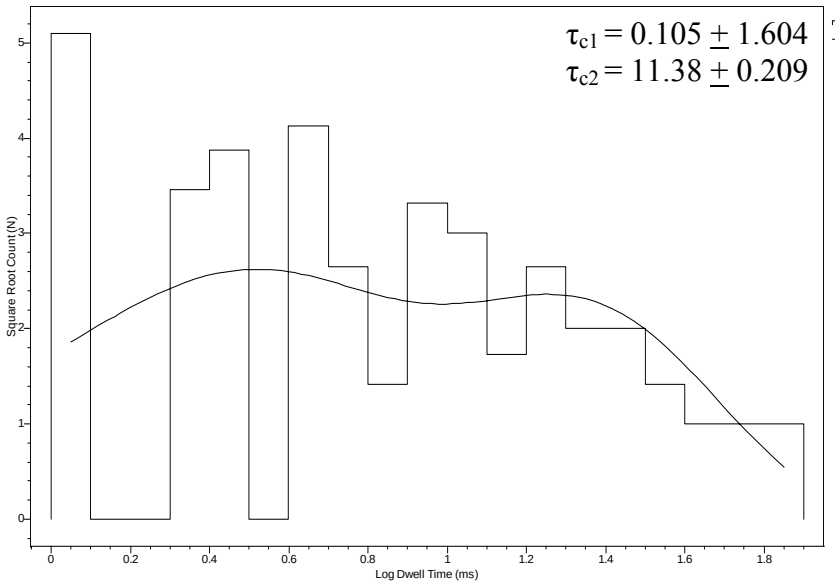

Figure 5.10: Distribution of Open and Closed Time for Single Channel Synaptosomal AMPA Receptors in the Presence of 10 µM CTZ and 1 µg/ml PSA.

Representative histograms of duration of openings (A) and closings (B) were analyzed using pClamp 9 software with two exponentials. Estimated time constants obtained from best fit of the distributions are indicated within the figure. See table 5.2 for comparisons. There is not a significant difference between the open and close values here compared with the previous group.

Group	Po	Open time (ms)	Closed time (ms)
Control	0.229 ± 0.023	1.110 ± 0.840 8.342 ± 1.338	3.777 ± 0.401 28.78 ± 0.935
CTZ 1	*0.429 ± 0.198	*0.752 ± 0.600 *14.76 ± 0.364	2.192 ± 0.459 27. 82 ± 0.563
CTZ 2	#0.637 ± 0.225	#0.178 ± 0.270 #1.433 ± 0.280 #12.35 ± 0.487	#0.401 ± 0.138 #9.640 ± 0.416 #0
CTZ 2 + PSA	*#0.892 ± 0.212	0.111 ± 1.050 *#5.398 ± 0.460 *#33.76 ± 0.824	0.105 ± 1.604 11.38 ± 0.209 0

Table 5.2: Modulation of the Single Channel Properties of Synaptosomal AMPA Receptors by CTZ and PSA

Values represent mean ± SEM which were calculated form channel open probability (Po), channel open time, and channel closed time. Asterisk indicates significant differences with the corresponding values in control (*), 5 μM CTZ (CTZ 1, #), and 10 μM CTZ (CTZ 2, *#). ($p<0.01$; One-way ANOVA; n = 5-11).

6. SUMMARY AND CONCLUSIONS

Functional alterations in excitatory neurotransmission, such as glutamatergic synaptic transmission, are implicated in synaptic plasticity and many neurodegenerative disorders. Cell adhesion molecules (CAMs) and carbohydrates, such as PSA, play a vital role in cell-cell interactions and recognition, respectively. Previously, several studies have indicated that there is a link between PSA-NCAM and glutamate receptors. (1) NMDA receptors have been shown to be co-redistributed with NCAM after the induction of long-term potentiation (LTP) (Fux et al., 2003). (2) NCAM associations with the postsynaptic spectrin-based scaffold, crosslinks NCAM with the NMDA receptor and calmodulin kinase IIα (CAMKIIα) (Sytnyk et al., 2006). (3) PSA-NCAM stimulated synaptogenesis requires the activity of NMDA receptors (Dityatev et al., 2004). (4) PSA has been shown to increase the open probability of purified single channel AMPA receptors in lipid bilayers (Vaithianathan et al., 2004). Elucidating the mechanism of interaction between single synaptic glutamate receptor and carbohydrates that are highly expressed in the synapse will help uncover the plasticity mechanism responsible for learning and memory and possibly aid in the development of therapies for neurodegenerative disorders involving cognitive deficits.

The first part of our study characterized the single channel kinetic properties of the NMDA subtype of glutamate receptors. We utilized biochemical isolation of synaptosomes and the tip-dip bilayer technique to investigate the single channel

properties of synaptic NMDA receptors and also recombinant expressed NR2 subunit specific receptors. This study characterized the differences in the kinetic properties of the NR2A and NR2B subunit containing synaptosomal NMDA receptors. The NR2A subunit containing NMDA receptors have a higher conductance and open time values than does the NR2B subunit containing NMDA receptors. In addition because the NR2B subunit containing NMDA receptors have a higher affinity for glutamate (Neyton and Paoletti, 2006), it has a higher open probability than the NR2A subunit containing NMDA receptors at the same concentration of glutamate. These findings are similar to the recordings from recombinant NMDA receptors. However, the conductance and probability of channel opening for the heterotrimeric NR1/NR2A/NR2B subunit containing NMDA receptor is higher than NR2A but lower than NR2B. Increasing glutamate concentration of NMDA receptors increases the frequency of channels or open probability without affecting the conductance.

The second portion of this study involved the modulation of NMDA receptors by the negatively charged carbohydrate, PSA. We found that exogenous or soluble PSA and endogenous PSA-NCAM-Fc inhibits NMDA receptor currents in dissociated hippocampal neurons. However, endo-N treated PSA-NCAM-Fc does not inhibit NMDA receptor currents. We found that the inhibition associated with PSA is through the NR2B subunit containing NMDA receptors. This inhibition of the NR2B subunit containing NMDA receptor occurred with the application of PSA but not with cell-surface associated PSA. Our recordings from synaptosomal NMDA receptors revealed that PSA does not affect the NR2A subunit containing NMDA receptors. In addition the inhibition of PSA through the NR2B subunit is dependent upon the glutamate concentration. Our

data shows that PSA inhibits the NR2B subunit at lower concentrations and not higher concentration suggesting that PSA is a competitive antagonist of glutamate. In addition the results from the excitotoxicity assay revealed that PSA is capable of preventing neuronal cell death at low concentrations of glutamate but not at high concentrations of glutamate. Thus, unlike other known antagonist APV and Ro, PSA effects are dependent upon glutamate concentration.

The Final portion of this study involved the determination of the mechanism of action for PSA enhancement of synaptic AMPA receptors. We found that PSA increases the open probability but not the conductance of synaptosomal AMPA receptors, this is similar to that observed with purified receptors (Vaithianathan et al., 2004). PSA, in a concentration dependent manner, decreases the mean closed time of synaptosomal AMPA receptors and increased the mean open time of AMPA receptors. Saturation is observed at concentrations greater than 3 μg/ml of PSA. We also found that upon addition of CTZ no changes were observed. This suggests that one of the mechanisms of action is that PSA decreases AMPA receptor desensitization, since this is a known mechanism of CTZ.

This study also shows the effects of CTZ on single channel synaptosomal receptors. CTZ increases the probability of channel openings and has no effects on channel conductance of single channel AMPA receptors. CTZ increases the open time and decreases the larger closed time. Both CTZ and PSA increase the burst activity of AMPA receptors. Upon addition of PSA in the presence of CTZ, an additional increase in channel activity is observed. This suggests that PSA has another mechanism of action in addition to decreasing desensitization. We suspect that the negatively charged PSA

interacts directly with the positively charged channel pore leading to an open state of the receptor.

Despite years of research the treatments for some neurodegenerative disorders are still ineffective or are associated with various side effects. In order to develop an effective treatment and possibly a cure for disorders associated with synaptic plasticity deficits, one must understand the mechanism of interactions among neuronal cells and molecules involved in the interactions. The glutamatergic system is responsible for fast excitatory neurotransmission and has been associated with many neurodegenerative disorders including schizophrenia, Alzheimer's disease, and Huntington's disease. This research has revealed the effects that PSA has on single channel glutamate receptors. Furthermore, while PSA potentiates AMPA subtype of glutamate receptors and enhances synaptic plasticity, it is also protective in that it prevents excitotoxicity by specifically inhibiting the NR2B subunit containing NMDA receptor.

In schizophrenia the expression of PSA-NCAM is decreased (Barbeau et al., 1995). Schizophrenia is also characterized by a polymorphism in the promoter region of the polysialyltransferase ST8SiaII, one of two enzymes involved in the polysialylation of NCAM (Arai et al., 2006; Tao et al., 2007). A recent study highlights NCAM as the candidate gene for the neurocognitive changes associated with schizophrenia (Sullivan et al., 2007). Therefore, the future direction and importance of this study involves understanding and revealing the mechanisms by which PSA-dependent NCAM regulates synaptic plasticity. Since PSA prevents activation of NR2B-subunit containing NMDA receptors activated by low concentrations of glutamate (Hammond et al., 2006), NCAM-

associated PSA may regulate synaptic plasticity and learning by restraining the signaling of extrasynaptic NR2B-subunit containing NMDA receptors.

REFERENCES

Arai M, Yamada K, Toyota T, Obata N, Haga S, Yoshida Y, Nakamura K, Minabe Y, Ujike H, Sora I, Ikeda K, Mori N, Yoshikawa T, Itokawa M (2006) Association between polymorphisms in the promoter region of the sialyltransferase 8B (SIAT8B) gene and schizophrenia. Biol Psychiatry 59:652-659.

Barbeau D, Liang JJ, Robitalille Y, Quirion R, Srivastava LK (1995) Decreased expression of the embryonic form of the neural cell adhesion molecule in schizophrenic brains. Proc Natl Acad Sci U S A 92:2785-2789.

Dityatev A, Dityateva G, Sytnyk V, Delling M, Toni N, Nikonenko I, Muller D, Schachner M (2004) Polysialylated neural cell adhesion molecule promotes remodeling and formation of hippocampal synapses. J Neurosci 24:9372-9382.

Fux CM, Krug M, Dityatev A, Schuster T, Schachner M (2003) NCAM180 and glutamate receptor subtypes in potentiated spine synapses: an immunogold electron microscopic study. Mol Cell Neurosci 24:939-950.

Hammond MS, Sims C, Parameshwaran K, Suppiramaniam V, Schachner M, Dityatev A (2006) Neural cell adhesion molecule-associated polysialic acid inhibits NR2B-containing N-methyl-D-aspartate receptors and prevents glutamate-induced cell death. J Biol Chem 281:34859-34869.

Neyton J, Paoletti P (2006) Relating NMDA receptor function to receptor subunit composition: limitations of the pharmacological approach. J Neurosci 26:1331-1333.

Sullivan PF, Keefe RS, Lange LA, Lange EM, Stroup TS, Lieberman J, Maness PF (2007) NCAM1 and neurocognition in schizophrenia. Biol Psychiatry 61:902-910.

Sytnyk V, Leshchyns'ka I, Nikonenko AG, Schachner M (2006) NCAM promotes assembly and activity-dependent remodeling of the postsynaptic signaling complex. J Cell Biol 174:1071-1085.

Tao R, Li C, Zheng Y, Qin W, Zhang J, Li X, Xu Y, Shi YY, Feng G, He L (2007) Positive association between SIAT8B and schizophrenia in the Chinese Han population. Schizophr Res 90:108-114.

Vaithianathan T, Matthias K, Bahr B, Schachner M, Suppiramaniam V, Dityatev A, Steinhauser C (2004) Neural cell adhesion molecule-associated polysialic acid potentiates alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor currents. J Biol Chem 279:47975-47984.