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Abstract

L-proline is a non-essential amino acid and one of the major amino acid constituents of extracellular fluid. Specific mutations in genes that control proline metabolism lead to hyperprolinemia which is characterized by an increase in the level of blood and cerebrospinal fluid concentration of L-proline. In fact, deletion or mutation of the PRODH gene which codes for proline oxidase (POX) / proline dehydrogenase which is critical for proline metabolism leads to Type I hyperprolinemia and is strongly associated with schizophrenia, autism and mental retardation. Proline has previously been shown to be a low affinity agonist at glutamate and glycine receptors. Because schizophrenia is thought to arise from glutamate receptor hypofunction and L-proline can act at glutamate receptors, it is necessary to understand whether increase in L-proline can contribute to glutamate receptor hypofunction.

The aim of this research was to determine the pharmacological profile of physiological and pathophysiological relevant concentrations of L-proline at two recombinant AMPA receptors: homomeric GluR1 and heteromeric GluR1/2. Dose response to L-proline (concentrations of 3 μ M – 10 mM) at homomeric GluR1 and heteromeric GluR1/2 receptors were determined and compared to saturating concentration of glutamate (10 mM). The effect of tonically present physiological (3 μ M) or pathophysiological (30 μ M) levels of L-proline on glutamate-evoked responses at both receptor types were also determined. This research supports previous findings that L-proline is a low affinity agonist at glutamate receptors activating less than 5 % of current elicited by a saturating concentration of glutamate (10 mM). Tonically present pathophysiological concentration of L-proline was observed to lead to a decrease in glutamate activation of homomeric GluR1 but not heteromeric GluR1/2 receptors. This decrease in glutamate-induced current was blocked by the application of 0.1 mM cyclothiazide indicating that at pathophysiological concentrations L-proline leads to selective desensitization of GluR1 receptors. These actions of L-proline may be relevant for understanding how hyperprolinemia contributes to glutamate receptor hypofunction in schizophrenia.

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**THE EFFECT OF L-PROLINE ON TWO RECOMBINANT AMPA
GLUTAMATE RECEPTORS**

A Thesis
Presented for
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Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science
From The University of Tennessee

By
Adetutu Abiose Oyelami
May 2010

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DEDICATION

This thesis is dedicated to my mum,
Mrs. O. S. Oyelami
and to memories of my dad.

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ABSTRACT

L-proline is a non-essential amino acid and one of the major amino acid constituents of extracellular fluid. Specific mutations in genes that control proline metabolism lead to hyperprolinemia which is characterized by an increase in the level of blood and cerebrospinal fluid concentration of l-proline. In fact, deletion or mutation of the PRODH gene which codes for proline oxidase (POX) / proline dehydrogenase which is critical for proline metabolism leads to type I hyperprolinemia and is strongly associated with schizophrenia, autism and mental retardation. Proline has previously been shown to be a low affinity agonist at glutamate and glycine receptors. Because schizophrenia is thought to arise from glutamate receptor hypofunction and l-proline can act at glutamate receptors, it is necessary to understand whether increase in l-proline can contribute to glutamate receptor hypofunction.

The aim of this research was to determine the pharmacological profile of physiological and pathophysiological relevant concentrations of l-proline at two recombinant AMPA receptors: homomeric GluR1 and heteromeric GluR1/2. Dose response to l-proline (concentrations of 3 μ M – 10 mM) at homomeric GluR1 and heteromeric GluR1/2 receptors were determined and compared to saturating concentration of glutamate (10 mM). The effect of tonically present physiological (3 μ M) or pathophysiological (30 μ M) levels of l-proline on glutamate-evoked responses at both receptor types were also determined. This research supports previous findings that l-proline is a low affinity agonist at glutamate receptors activating less than 5 % of current elicited by a saturating concentration of glutamate (10 mM). Tonically present pathophysiological concentration of l-proline was observed to lead to a decrease in glutamate activation of homomeric GluR1 but not heteromeric GluR1/2 receptors. This decrease in glutamate-induced current was blocked by the application of 0.1 mM cyclothiazide indicating that at pathophysiological concentrations l-proline leads to selective desensitization of GluR1 receptors. These actions of l-proline may be relevant for understanding how hyperprolinemia contributes to glutamate receptor hypofunction in schizophrenia.

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CHAPTER 1. INTRODUCTION

Proline is classified as a non-essential amino acid in adults and full-term infants (Miller et al. 1995; Phang et al. 2001). It can be derived from glutamine in the liver, small intestine and the kidney (Bertolo and Burrin 2008). L-proline is one of the major amino acid constituent of extracellular fluid with a plasma concentration of 37-312 μM (Phang et al. 2001; Afenjar et al. 2007). The concentration of proline in the cerebrospinal fluid (CSF) is about a 1% of the serum concentration and ranges from 1-4 μM (Phang et al. 2001). Proline, like other known neurotransmitters in the central nervous system (CNS), is maintained in homeostasis by the activity of high affinity proline transporters. Thus suggesting that proline itself is a neurotransmitter (Renick et al. 1999; Phang et al. 2001).

Proline can be derived from glutamate or ornithine through the common precursors pyrroline-5-carboxylate (P5C) or glutamic- γ -semialdehyde (Phang et al. 2001). The conversion of glutamate and glutamine into the intermediate P5C is catalyzed by the enzyme P5C synthase (**Figure 1-1**) (Bertolo and Burrin 2008). This step which is central to the synthesis/metabolism of proline is restricted to the gut (Jones 1985; Bertolo and Burrin 2008). Mutation of enzymes in the metabolic pathways controlling the synthesis (anabolism) as well as the breakdown (catabolism) of proline, results in disease conditions which include hyperprolinemia (Phang et al. 2001).

1.1 Diseases of Proline Metabolism

The conditions resulting from alteration in the metabolism of proline include hyperprolinemia type I (HPI), hyperprolinemia type II (HPH), ornithine aminotransferase (OAT) deficiency, hydroxyprolinemia, iminoglycinuria and Δ -1-pyrroline-5-carboxylic acid (P5C) synthetase deficiency (**Table 1-1**) (Phang et al. 2001; Mitsubuchi et al. 2008). Each of these conditions is caused by a mutation or deletion in one of the genes coding for proteins in the metabolic pathways of proline (**Figure 1-1**) (Bertolo and Burrin 2008; Mitsubishi et al. 2008). Only three of these conditions, HPI, HPH and P5C synthase deficiency, however result in changes in plasma and CSF concentration of proline (Phang et al. 2001).

Disruption of proline catabolism which results in an increase in proline concentration beyond the physiological range is known as hyperprolinemia (Woody et al. 1969; Afenjar et al. 2007). Hyperprolinemia is an autosomal recessive condition caused by mutations in genes that code for one of two enzymes: proline oxidase (POX) / proline dehydrogenase (PRODH) and Δ -1-pyrroline-5-carboxylic dehydrogenase (P5CDH) (Woody et al. 1969; Phang et al. 2001). In hyperprolinemia, a 10-fold increase plasma proline concentration is accompanied by an equivalent 10-fold increase in the CSF (Phang et al. 2001). Hyperprolinemia has been associated with schizophrenia,

Figure 1-1 Metabolic Pathway of Proline and Related Amino Acids

Hyperprolinemia type I is caused by a mutation or deletion of the *PROD* gene which codes for the enzyme proline oxidase that catalyses the conversion of proline to Δ -1-proline-5-carboxylate. Hyperprolinemia type II is caused by a mutation or deletion in the Δ -1-proline-5-carboxylate acid dehydrogenase (P5CDH). Reprinted with permission from Bertolo RF, Burrin DG (2008). Comparative aspects of tissue glutamine and proline metabolism. *J Nutr* 138:2032S-2039S.

Table 1-1 Characteristics of human enzymes involved in proline metabolism and associated diseases

	P5C synthase	OAT	POX	P5C dehydrogenase	Prolidase
EC	None	EC26.1.13	None	EC1.5.1.12	EC3.4.13.9
Subcellular location	Mitochondria inner membrane	Mitochondrial matrix	Mitochondrial inner membrane	Mitochondrial matrix	Cytoplasm
Disease association	Hypoprolinemia Hypocitrullinemia Hypoornithinemia Hyperammonemia	Gyrate atrophy	HPI	HPH	Prolidase deficiency
Map location	10q24.3 to 24.6	0q26	22q11.2	1p36	19q12-13.11

Mutations in the enzymes P5C synthase, POX and P5C dehydrogenase lead to disruption in plasma proline concentration. Reprinted with permission from Mitsubuchi H, Nakamura K, Matsumoto S, Endo F (2008). Inborn errors of proline metabolism. J Nutr 138:2016S-2020S.

autism, epilepsy as well as neuronal disorders which include mental retardation, epilepsy and delays in psychomotor development, as well as aggression in some patients (Flynn et al. 1989, Afenjar et al. 2007, Zinkstok et al. 2008). Thus, it is important to understand the actions of proline, particularly within the CNS.

There are two different types of hyperprolinemia: Hyperprolinemia Type I (HPI) and Hyperprolinemia Type II (HPH). Although HPI and HPH result in elevation of proline levels, there are differences in the magnitude of proline elevation as well as in the mutations that cause these disorders (Valle et al. 1976; Phang et al. 2001; Jacquet et al. 2002).

1.1.1 Hyperprolinemia Type I (HPI)

HPI is characterized by a 5-10 fold increase in the normal concentration of plasma proline (Phang et al. 2001; Jacquet et al. 2002). The increase in plasma proline level in HPI is due to a deletion or mutation in the proline dehydrogenase (PRODH) gene (Jacquet et al. 2002; Bender et al. 2005; Jacquet et al. 2005). The PRODH gene lies on the q arm of chromosome 22 at band 11.2 of the human genome. It encodes for the enzyme proline oxidase, an inner membrane mitochondria protein, which catalyzes the first step in the catabolism of proline to glutamate (**Figure 1-1**) (Campbell et al. 1997; Jacquet et al. 2002; Jacquet et al. 2005).

22q11.2 deletion syndrome (22q11.2DS) also known as DiGeorge syndrome (DGS) or Velo-cardio-facial syndrome (VCFS) is associated with increases in plasma proline levels of individuals, higher risk for schizophrenia, as well as high prevalence of autism spectrum disorder (Murphy et al. 1999, Goodman et al. 2000). The syndrome, as the name suggests, is caused by a micro deletion of chromosome 22q11.2. Symptoms of 22q11.2DS include congenital heart defects, seizures, palatal abnormalities, learning difficulties, hearing loss and in some case renal dysfunction (Shprintzen et al. 1978, Lipson et al. 1991, Humbertclaude et al. 2001). The cardiac defects associated with the syndrome appear to be generated primarily through the loss of the TBX1 gene, on chromosome 22q11.2 (Yagi et al. 2003). Neuropsychiatric symptoms associated with HPI include mental retardation, epilepsy and cognitive impairment (Di Rosa et al. 2008). This suggests that elevations in brain proline levels may contribute to the psychiatric disturbances observed in patients with 22q11.2DS.

1.1.2 Hyperprolinemia Type II (HPH)

HPH is characterized by typically higher elevations in proline concentration than in HPI. Proline levels are elevated 10 -15 fold in severe HPH with plasma concentrations as high as 413-1883 μ M (Phang et al. 2001; Afenjar et al. 2007). The increase in proline in HPH is accompanied by an increase in the concentration of P5C, a by-product of proline degradation (Valle et al. 1976). HPH is due to a mutation or deletion in the

ALDH4A1 gene which codes for the enzyme P5CDH. P5CDH catalyzes the breakdown of P5C to the amino acid glutamate (Phang et al. 2001).

1.2 Pathophysiology of L-Proline

In rats, acute systemic administration of l-proline causes oxidative stress by reducing cytochrome c oxidase activity (Delwing et al. 2003; Delwing et al. 2007). When applied chronically l-proline has also been shown to lead to a decrease in membrane lipid content in the cerebellum of rats (Vianna et al. 2008), decrease in creatine kinase activity (Kessler et al. 2003), as well as decrease in the activities of $\text{Na}^+\text{-K}^+\text{-ATPase}$ and acetylcholinesterase (Pontes et al. 2001). The fact that l-proline administration leads to a deficit in learning and memory suggest that increase in l-proline levels affect the CNS (Davis et al, 1987).

High affinity Na^+ -and Cl^- -dependent proline transporters (PROT) are widely expressed within a subset of glutamate neurons in the CNS (Hauptmann et al. 1983; Velaz-Faircloth et al. 1995). This structural localization of proline transporter, as well as studies showing that l-proline affects excitatory synaptic transmission suggest that l-proline plays a role in the glutamate transmission (Cohen and Nadler 1997; Renick et al. 1999). Direct evidence of proline activity at synapses include the ability of l-proline to activate glutamate and glycine receptors at concentrations above 1 mM (Henzi et al. 1992). However, this concentration far exceeds the physiological or pathophysiological relevant concentrations of l-proline within the CNS (Henzi et al. 1992, Cohen and Nadler 1997; Phang et al. 2001). At physiological and pathophysiological concentrations l-proline potentiates field excitatory postsynaptic potential (fEPSP) in neuronal slices (Cohen and Nadler 1997). Due to the limited literature on the effects of l-proline at glutamate and glycine receptors, the spectrum of the pharmacological actions of l-proline towards glutamate receptors remains largely unknown. This study therefore focused on the effects of l-proline on two recombinant glutamate receptors.

1.3 Glutamate Receptors: Physiological and Pathological Importance

Ligand-gated glutamate receptors mediate the majority of excitatory neuronal transmission in the central nervous system (Dingledine et al. 1999). The amino acid glutamate binds to and activates a broad class of receptors collectively known as glutamate receptors (GluR) (Dingledine et al. 1999). The excitatory neurotransmitter glutamate and its receptors have been shown to be important in physiological processes such as neuronal plasticity as well as learning and memory (Tsien et al. 1996; Riedel et al. 2003).

Neuronal plasticity, the ability of the brain to be modified by experiences, is a phenomenon underpinning learning and memory (Alkon et al. 1982; Antonov et al. 2003). Examples of processes mediated by changes in the properties of neurons include long-term potentiation (LTP) (Bliss and Lomo 1973). Induction and maintenance of LTP,

a persistent increase in synaptic strength, is thought to be required for the formation of long term memories (Jones et al. 2001; Pastalkova et al. 2006; Whitlock et al. 2006).

Excessive glutamate in the synaptic cleft leads to over-excitation of neurons, often leading to neuronal death due to excitotoxicity (Tanaka et al. 1996). Specific transporters maintain glutamate homeostasis by clearing excessive glutamate at synaptic cleft thereby shaping glutamate transmission (Diamond 2001; Katagiri et al. 2001; Hires et al. 2008). Glutamate-induced excitotoxicity is thought to be mediated in part by the increase in intracellular Ca^{2+} concentration due to influx mediated by glutamate receptors (Schlaepfer and Bunge 1973, Tymianski et al. 1993). Many neurodegenerative disorders, such as Parkinson's, epilepsy Alzheimer's and Huntington's diseases have been attributed to glutamate-induced excitotoxicity (Tanaka 1996; Cha et al. 1998; Ozawa 1999).

Alteration in the composition, number, subtypes and/or activity of glutamate receptors have also been shown to be associated with mental illnesses, neurodegenerative diseases such as Alzheimer's and in other neurological disorders, such as epilepsy and ischemia (Meldrum 2000; Bernareggi et al. 2006; Borbely et al. 2009). Glutamate receptors are divided into two major groups based on the pathway that is activated upon binding of glutamate or other agonists to the receptors (Dingledine et al. 1999). The two major groups of glutamate receptors are metabotropic and ionotropic receptors as illustrated in **Figure 1-2** (Kew and Kemp 2005).

1.3.1 Metabotropic Glutamate Receptors

The metabotropic glutamate receptor family (mGluR) currently consists of eight different subunits and genes and is divided into three different groups based on pharmacology, as well as the second messenger activated downstream to the receptors (**Figure 1-1**) (Dingledine et al. 1999). The activation of Group I mGluR family results in activation of second messenger signaling pathways such as phosphoinositol and results in an increase in cytoplasmic concentration of Ca^{2+} (Abe et al. 1992). mGluRs are coupled to G-proteins which can be inhibitory or excitatory (Dingledine et al. 1999, Kew and Kemp 2005).

Group I and Group II have been shown to be predominantly coupled to phospholipase C (PLC) signal, with the former activating and the latter inhibiting this pathway (Abe et al. 1992). Members of Group III mGluRs predominately inhibit Ca^{2+} channels and cyclic adenosine monophosphate (cAMP) generation (Okamoto et al. 1994). Members of the Group I receptors can also lead to the activation of intracellular signaling cascades which are independent of G-proteins and mediated by a src-tyrosine kinase (Heuss et al. 1999).

Figure 1-2 Classification of Glutamate Receptors

Glutamate receptor family, showing classification of ionotropic and metabotropic glutamate receptors and subunits within each class. Reprinted with permission from Kew J, Kemp J (2005). Ionotropic and metabotropic glutamate receptor structure and pharmacology. *Psychopharmacology* 179:4-29.

1.3.2 Ionotropic Glutamate Receptors

Ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels concentrated in postsynaptic cells in clusters called postsynaptic densities (Petralia et al. 1994; Popratiloff et al. 1996). They are selectively permeable to Na^+ , K^+ and differentially permeable to Ca^{2+} (Burnashev et al. 1995). The ionotropic glutamate receptor is tetrameric. Based on the subunit composition a particular receptor is classified as homomeric or heteromeric. Each of the four subunits forming the receptors is a membrane protein with three transmembrane domains (M1, M3 and M4) with a re-entrant loop (M2), an intracellular C-terminus and an extracellular N-terminus as shown in **Figure 1-3** (Bennett and Dingledine 1995; Dingledine et al. 1999; Anand, 2000). The agonist binding site on each subunit is formed within two segments (S1 and S2) in two domains of the extracellular ligand binding core (Bennet and Dingledine 1995; Armstrong et al. 1998; Anand 2000; Naur et al. 2005). The binding of a ligand to iGluRs results in conformational changes and in the opening of the ion pore, allowing current to flow down its electrochemical gradient (Jin et al. 2003).

There are three major classes of ionotropic glutamate receptors: α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPA), kainate receptors, and N-methyl-D-aspartate receptors (NMDAR) (Dingledine et al. 1999).

1.3.2.1 Kainate receptors: The kainate glutamate receptor family is formed by the glutamate receptor subunits GluR5-7, KA1 and KA2 (Dingledine et al. 1999). The proteins for all five subunits are encoded for by the genes GRIK1-5 respectively (Dingledine et al. 1999). KA1 and KA2 subunits show a higher affinity for kainate than all other subunits, however they do not result in the formation of functional homomeric receptors (Ren et al. 2003). Kainate receptors have been implicated in diseases such as early onset Huntington's, autism, schizophrenia and epilepsy (Jane et al. 2009).

1.3.2.2 NMDA receptors: Unique among the iGluRs, NMDA receptors require glycine as a coagonist as well as their ligand, glutamate, for activation (Wafford et al. 1995). NMDA receptors also have a voltage-dependent Mg^{2+} block which is absent in other classes of iGluRs and are permeable to Ca^{2+} (Mayer and Westbrook 1987; Jahr and Stevens 1993). The current induced by activation of NMDA receptors has a longer duration than that induced by other types of ionotropic glutamate receptors due to prolonged opening time of the receptors and slower desensitization (Gibb and Colquhoun 1991). During desensitization the ligand remains bound to a receptor, however conformational changes in the receptor prevent the passage of current through the receptor. NMDA receptors unlike most ionotropic glutamate receptors are permeable to Ca^{2+} and therefore directly coupled to the build up of Ca^{2+} in the cytoplasm. Over activation of NMDA receptors therefore directly results in a Ca^{2+} -dependent excitotoxicity (Alford et al. 1993; Christie and Jahr 2008). NMDA receptor-dependent Ca^{2+} permeability has been shown to be directly involved in the induction of LTP and in learning and memory through the activity of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) (Sanhueza et al. 2007).

Figure 1-3 Structure of AMPA Receptor Subunit

Transmembrane topology of a single subunit of the receptor showing the three transmembrane domains (M1, M3 and M4), re-entrant loop (M2), the ligand-binding domains (S1 and S2), the intracellular C terminal and an extracellular N terminal. Reprinted with permission from Dingledine R, Borges K, Bowie D, Traynelis S (1999). The glutamate receptor ion channels. *Pharmacol Rev* 51:7-62.

1.3.2.3 AMPA receptors: AMPAR are tetrameric receptors composed of four subunits, GluR1-4 (Rosenmund et al. 1998). Each subunit can exist as a 'flip' or flop' which is a variant created by alternate splicing of a 115-base pair section of genes encoding the subunit. (Dingledine et al. 1999). Further posttranslational modifications can result in a great diversity of the receptors. Phosphorylation of amino acid residues on AMPA receptors by kinases such as protein kinase A (PKA), CaMKII and protein kinase C (PKC) provide a mechanism for controlling the activity of the receptors (Lee et al. 2003). The genes encoding the AMPA receptors are a single family including GRIA1, GRIA2, GRIA3 and GRIA4.

The different subunits can combine to form homomeric or heteromeric receptors which have different patterns of distribution within the brain. This suggests important differences in the properties of each subunit and the receptors they form (Wenthold et al. 1996; Mano and Teichberg 1998). AMPA receptors are generally non-selective cation channels being permeable to Na^+ and K^+ and, in general, largely impermeable to Ca^{2+} ion in the CNS (Burnashev et al. 1995). Ca^{2+} permeability of AMPA receptors is largely dependent on the amino acid residue on position 586 of the GluR2 protein/subunit. This site is present at the conduction site in the pore formed by the subunits (Verdoon et al. 1991). GluR2 subunits undergo subunit-specific RNA editing resulting in the conversion of the encoded glutamine codon (Q) to arginine (R) that determines the kinetics of the channel (Lomeli et al. 1994). The unedited, GluR2-Q, subunit has relatively the same permeability to Ca^{2+} as other AMPA receptor subunits, and the edited GluR2-R have a reduced permeability to Ca^{2+} (Burnashev et al. 1996). AMPAR properties including rectification and Ca^{2+} permeability differ based on subunits composition. The edited GluR2-R subunit is impermeable to Ca^{2+} , and co-expression of this subunit in a heteromeric receptor determines the Ca^{2+} permeability of the receptor (Burnashev et al. 1995; Geiger et al. 1995; Shi et al. 2001). GluR1 and GluR2 subunits of AMPA receptors are predominantly expressed in the hippocampus (Wenthold et al. 1996). There is a loss of both GluR1 and GluR2 subunit expression in the hippocampus in schizophrenia (Eastwood et al. 1995), therefore reductions in the activity of these receptors maybe important for the development of schizophrenia.

Glutamate receptor I (GluR1): Glutamate receptor I (GluR1) subunits can be expressed as homomers or heteromers with GluR2, GluR3 and/or GluR4 subunits (Boulter et al. 1990). Expression of GluR1 homomeric receptor varies throughout the CNS with preferential location in the cortex, striatum and spinal cord (Wenthold et al. 1996; Hermes et al. 2008; Lane et al. 2008). GluR1 is also necessary for the acquisition of short-term memory necessary, for acquisition of learning in a spatial memory task, as well as in the formation of LTP and LTD (Lee et al. 2003). Current generated by GluR1 homomers when expressed in human embryonic kidney (HEK) cell lines is mediated by the flow of Na^+ , K^+ and to a lesser extent Ca^{2+} (Dingledine et al. 1999). Homomeric GluR1 receptors are characterized by the ability to pass more current at a negative membrane potentials (-60 mV) than at positive membrane potentials (+60 mV) of the same magnitude, a property known as inward rectification (Burnashev et al. 1995). Homomeric GluR1 receptors are activated by agonists including AMPA, domoate, kainate and quisqualate (Dawson et al. 1990).

GluR1/2: GluR1/2 heteromeric receptors are composed of both GluR1 and GluR2 subunits expressed as a tetramer, known as a dimer of dimers (Ayalon and Stern-bach 2001). GluR1/2 heteromers are the major receptor type within the hippocampus and increases in activity and numbers of both subunits are associated with manic-like behavior (Wentholt et al. 1996; Du et al. 2008). When expressed in mammalian cell lines the homomeric GluR1/2 exhibits a non-rectifying current-voltage (I-V) relationship and differ from the GluR1 homomers in their lower permeability to Ca^{2+} . These differences in I-V relationship can therefore be used as a means of differentiating between different receptors subtypes (Verdoorn et al. 1991; Burnashev et al. 1995).

1.4 Pharmacology of AMPA Glutamate Receptors

An agonist is any substance that binds to and activates a given receptor (Watkins 2000). It can be a naturally occurring substance, or a substance that binds with as high affinity to a receptor as its known naturally occurring agonist (Li et al. 1997). Agonist binding sites present on AMPA-type glutamate receptors have been mapped using molecular techniques to disrupt the agonist binding sites, as well as by the analysis of crystalline structures of bound molecule to receptors (Hogner et al. 2002). The AMPA agonist binding site has been mapped to amino acids within the S1 and S2 segments in both AMPA and kainate receptors (Li et al. 1995; Hogner et al. 2002). Agonists can be broadly divided into either full or partial agonists. The classification of an agonist is based on the concentration of the agonist needed to elicit a response and the ability of that agonist to reach a concentration at which there is no further increase in the response generated from the receptor (Li et al. 1997; Traynor et al. 2001). Major known agonists at glutamate receptors include AMPA, NMDA and kainate.

A full agonist at a receptor is able to elicit a maximum affinity and/or efficacy at a receptor site, therefore producing a maximum response of the receptor or downstream to the activation of the receptor (Li et al. 1997; Traynor et al. 2001). When compared to a full agonist, a partial agonist elicits sub-maximal response at the receptor site (Jin et al. 2003). It is therefore possible to classify agonists based on the electrophysiological response of the receptor subtype of interest (Yakushiji et al. 1989). Decrease in the current elicited by a partial agonist, at ionotropic glutamate receptors, is a result of a decrease in the receptor opening probability of the receptor (Jin et al. 2003; Maltsev et al. 2008; Kussius and Popescu 2009). The currents produced at NMDA receptors with the application of a partial agonist such as alanine has been shown to desensitize faster, there is however no evidence that a partial agonist is able to promote the entry of the receptor into a desensitized state (Kussius and Popescu 2009).

1.5 Rationale

Schizophrenia a chronic mental disorder with symptoms classified into three broad categories: positive symptoms, negative symptoms and cognitive symptoms (NIMH). Over the years two major molecular basis have been proposed for

schizophrenia: glutamatergic hypofunction and dopaminergic hyperfunction (Mohn et al. 1999; Lang et al. 2007; Lisman et al. 2008). Glutamate hypofunction and in particular AMPA receptor hypofunction is backed by studies that have shown a disruption in AMPA receptor regulation is associated with schizophrenia. In particular a conditional knock-out of GluR1 receptors results in schizophrenic-like behavior in mice (Wiedholz et al. 2008). The association of 22q11.2DS and hyperprolinemia to schizophrenia point to a possible role of l-proline in the pathology of schizophrenia (Jacquet et al. 2002; Jacquet et al. 2005). Hyperprolinemia has also been associated to other disease conditions such as autism and mental retardation (Afenjar et al. 2007; Shprintzen 2008).

Proline transporters have been shown to be highly expressed in glutamatergic pathways in the CNS (Hauptmann et al. 1983; Renick et al. 1999). This structural localization of proline transporter have led to the suggestion that l-proline could play a role in the glutamate signal transmission pathways (Renick et al. 1999). Mutations in genes coding for enzymes in proline metabolism pathway results in conditions which include hyperprolinemia. Hyperprolinemia is also caused by errors in the catabolism of proline and is characterized by increases in the blood plasma levels of proline and its derivatives.

At concentrations greater than pathophysiologically relevant concentration in the CNS (1 mM) l-proline has two opposite effects on membrane potential of rat dorsal horn neurons (Henzi et al. 1992). These effects, excitatory and inhibitory, are mediated by two different receptor types, the glutamate receptors and glycine receptors (Henzi et al. 1992; Phang et al. 2001). L-proline activates glycine receptors, NMDA receptors and non-NMDA glutamate receptors (Henzi et al. 1992; Phang et al. 2001). Inhibitory effects of proline is also mediated by the d-proline isomer-activation of glycine receptors (Henzi et al. 1992). In spite of the low agonist affinity of l-proline for glutamate receptors, physiological (3 μ M) and pathophysiologically (30 μ M) concentrations have been shown to potentiate glutamatergic transmission in the hippocampus (Cohen and Nadler 1997). Suggesting that even at very low concentrations l-proline affects glutamate transmission.

These previous studies of the agonist activity of l-proline were carried out in native receptors in neurons where currents recorded were pharmacologically dissected. Thus the identity and receptor composition contributing to proline-evoked responses are unclear. Given the physiological and pharmacological variation in the different glutamate receptors, there is a need to understand the spectrum of the agonist activity of proline at specific glutamate receptors.

Thus the aims of this study were:

1. To determine the effect of subunit composition on the sensitivity of recombinant AMPA receptors to l-proline.
2. To determine if desensitization contributes to the sensitivity of AMPA receptors to l-proline.
3. To determine the effect of tonic l-proline levels on glutamate activation of AMPA receptors as a model for hyperprolinemia.

CHAPTER 2. MATERIALS AND METHODS

2.1 Expression Construct and Preparation

cDNA encoding for rat GluR1 and GluR2 were in the pRK5 vector. pEGFP vector (Clontech) was used as a transfection marker. Each construct was transformed into DH5 α cells and plated on Luria-Bertani (LB) agar plates supplemented with antibiotic to which the vectors had resistant genes. LB agar was supplemented with ampicillin (50 μ g/ml) for propagation of GluR1 and GluR2 or kanamycin (50 μ g/ml) for GFP. Plates were incubated at 37 °C overnight. A single colony was selected from each plate and grown in LB broth supplemented with ampicillin or kanamycin as noted above. The culture was allowed to grow overnight at 37 °C in a shaking incubator at 300 rpm for 16-18 hours. The culture was harvested by centrifugation at 6000 rpm 4 °C for 5 mins and plasmids were purified with the use of a Hi-speed maxi prep kit (Qiagen; Valencia, CA). UV absorbance (260 nm) was used to determine the concentration of the purified DNA. The plasmid DNA was stored at -20 °C and later used for transfection into HEK 293 cells.

2.2 Cell Cultures and Transfections

Human embryonic kidney cells lines (HEK 293) were obtained from ATCC (CRL-1573, Manassas, VA). Only cells from passages 36-42 were used for experiments. The cells were grown in culture in media consisting of Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen; Carlsbad, CA), 10 % fetal bovine serum (FBS) (Hyclone; Logan, UT) and penicillin (100 U/ml)/streptomycin (100 U/ml) (P/S) (Invitrogen; Carlsbad, CA). Cells were maintained in an incubator with a temperature of 37 °C; with an air to CO₂ ratio of 95:5 %. Cells were passaged every 4-5 days by trypsinization. The calcium phosphate precipitation technique was used for transfection of plasmids into HEK 293 cells (Chen and Okayama 1987). Transfection was performed using 1 μ g of each construct (GluR1 or GluR1 + GluR2). GFP (0.2 μ g) was included as a transfection marker. The cells were incubated after transfection at 37 °C and washed with phosphate buffered saline (PBS) (Invitrogen; Carlsbad, CA) 16-18 hours after transfection. The PBS was subsequently replaced with fresh media. Cells were returned to the incubator and electrophysiological recordings were performed 24 hours later.

2.3 Electrophysiology and Data Analysis

HEK cells for each experiment were selected by microscopic visualization of GFP epifluorescence. Whole-cell patch clamp recording were carried out using a Multiclamp 700A (Molecular devices; Union City, CA). Pipettes were fabricated from borosilicate glass (TW150F; WPI; Sarasota, FL) using a two-stage electrode puller (model PP830; Narshige; Tokyo, Japan). Only pipette with resistances of 2-4 M Ω were used. Pipettes were filled with intracellular solution containing 140 mM cesium methanesulfonate, 10 mM HEPES, 5 mM adenosine triphosphate (sodium salt), 5 mM MgCl₂, 2 mM CaCl₂ and 10 mM BAPTA at pH 7.40. The extracellular solution contained 150 mM NaCl, 5 mM

KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES and 10 mM glucose with a pH of 7.40 and was perfused into the recording chamber at a flow rate of 1-2 ml/min. Upon successful establishment of the whole-cell configuration, cells were lifted off the coverslip and positioned in the path of control flow line. Fast solution exchange was achieved by moving a series of flow pipes using a piezoelectric bimorph. Agonist-evoked currents were elicited every 10-30s. All experiments commenced by obtaining 5 consecutive stable currents in response to a saturating concentration of glutamate (10 mM; 500 ms).

To determine the l-proline dose-response for recombinant AMPA receptors, l-proline (3 μ M, 30 μ M, 300 μ M, 3 mM or 10 mM) was applied for 500 ms with an interval of 10s between sweeps. Peak current evoked in response to l-proline at each concentration was normalized to the peak current obtained in response to glutamate. To assess the effect of tonic l-proline concentrations on glutamate-evoked currents, l-proline (0 μ M, 3 μ M or 30 μ M) was added to both control and glutamate (10 mM) containing extracellular solution. Glutamate evoked current (10 mM; 500 ms) in the absence or presence of tonic l-proline were elicited every 30s. Peak current each for sweep was normalized to the peak of the initial glutamate-evoked response. For paired-pulse experiments, a protocol was created to deliver two pulses of glutamate (10 mM) in the tonic presence or absence of a pathophysiological relevant concentration of l-proline (30 μ M). The pulses were delivered for 25 ms with a delay of 200 ms between the first and second pulses.

All currents were recorded at -60 mV. For all cells used in data analysis, measured currents were normalized to the initial peak current. Currents were digitized at 5-10 kHz and filtered at 1-2 kHz with a Digidata 1322A board and Clampex 9 software (Axon Instruments; Foster City, CA). Only cells with currents greater than 100 pA induced by 10 mM glutamate and membrane resistance ≥ 1.0 G Ω were included in the data analysis. I-V relationships were generated over the range -60 mV to +40 mV, in 20 mV increments. I-V relationships were obtained at the onset of each experiment in order to confirm successful co-transfection of the cDNA for GluR1 and GluR2. GluR1/2 transfected HEK cells do not show the rectification observed in HEK cells transfected with GluR1 alone (**Figure 2-1**). Only cells with a ratio of current at +40 mV to -60 mV of approximately 0.4 or more were included in data analysis (Burnashev et al. 1995). Data analysis was performed using Clampfit (Axon Instruments; Foster City, CA), and Origin Pro 7.0 (Microcal; Northampton, MA). All data are displayed as mean $\pm$ s.e.m.

Figure 2-1 Current-Voltage Relationship for GluR1 and GluR1/2 Transfected Cells

HEK cells were transfected with GluR1 +/- GluR2. **(A)** Representative current traces for GluR1 at various holding potentials in response to I-V protocol (-60 mV to +40 mV). **(B)** Representative traces for GluR1/2. **(C)** Average I-V relationships are shown in response to the I-V protocol (-60 mV to +40 mV) for GluR1 (red) and GluR1/2 (black) in the absence (open) or presence (filled) of the desensitization blocker CTZ (100 μ M)

CHAPTER 3. RESULTS

3.1 L-Proline is a Low Affinity Agonist at Recombinant AMPA Receptors

Previous work suggests that l-proline is a very low affinity agonist at glutamate receptors in rat spinal cord neurons (Henzi et al. 1992). However l-proline can dramatically affect hippocampal excitatory transmission at much lower concentrations (Cohen and Nadler 1997). The pharmacological action of proline at glutamate receptors therefore remains unclear. This stems, in part, from the unknown receptor subtypes or stoichiometry of glutamate receptors that exist in these neurons. Thus, experiments were designed to determine the sensitivity of l-proline at recombinant AMPA-type glutamate receptors. Homomeric GluR1 and heteromeric GluR1/2 receptors were examined because these are the prevailing AMPA receptors in the hippocampus (Wenthold et al. 1996).

To determine the efficacy of l-proline as an agonist relative to glutamate, the dose-response relationship with l-proline was determined for GluR1 and GluR1/2 receptors and compared to a saturating concentration of glutamate (10 mM). The effectiveness of l-proline in inducing current at GluR1 and GluR1/2 is dose-dependent as shown by its ability to induce greater currents at higher concentrations. At a concentration of 10 mM, l-proline elicited a current of 0.559 ± 0.141 % and 1.166 ± 0.223 % of the initial current induced by the application of 10 mM glutamate at GluR1 and GluR1/2 receptors respectively (**Figure 3-1**). Because the currents elicited with the application of l-proline were small, cyclothiazide (CTZ), a blocker of desensitization (Yamada and Tang 1993), was applied in order to enhance detection of proline-evoked currents. Current evoked by 10 mM l-proline at heteromeric GluR1/2 receptors were significantly enhanced by the presence of 100 μ M CTZ (4.005 ± 0.38 %, $n = 5$, $p < 0.001$). However, even at 10 mM l-proline generated current was less than 5 % of the initial 10 mM glutamate-induced current at GluR1/2 receptors. Co-application of 10 mM l-proline and CTZ increased current generated at GluR1 receptor to 2.045 ± 0.583 % of initial 10 mM glutamate-induced current.

Consistent with the known actions of CTZ to increase current flow due to a reduction in desensitization (Yamada and Tang 1993), application of CTZ increased the current density in GluR1 and GluR1/2-expressing cells (**Figure 3-2**). Where current density is calculated as follows:

$$\text{Current density} = \frac{\text{Total Current (pA)}}{\text{Membrane capacitance (pF)}}$$

Application of CTZ was also shown to not alter the I-V relationship, a physiological property used in distinguishing between GluR1 and GluR1/2 receptors as shown in **Figure 2-1**. These results support previous study (Henzi et al. 1992) that have shown l-proline to be very low affinity agonist at glutamate receptors.

Figure 3-1 L-proline Dose Response for GluR1 and GluR1/2

Whole-cell recordings of GluR1 and GluR1/2 receptor currents expressed in HEK 293 cells. Currents are evoked by 500 ms application of 10 mM glutamate or 3 μ M, 30 μ M, 300 μ M, 3 mM and 10 mM L-proline. **(A)** Representative traces to the initial 10 mM glutamate application (black) and to application of 10 mM L-proline in GluR1 receptors (red). **(B)** Same as A, in the presence of CTZ. **(C)** Representative traces to the initial 10 mM glutamate application (black) and to application of 10 mM L-proline in GluR1/2 receptor. **(D)** Same as C, in the presence of CTZ. **(E)** Summary graph showing the dose-response of the L-proline peak current normalized to current induced with 10 mM Glutamate (not shown) for different concentration of L-proline (3 μ M to 10 mM) with GluR1 (open square), GluR1 in the presence of CTZ (filled square), GluR1/2 (open triangle) and GluR1/2 in the presence of CTZ (filled triangle) where n indicated in parenthesis is the number of observations.

Figure 3-2 Current Density of GluR1 and GluR1/2

HEK 293 cells were transfected with GluR1 $\pm$ GluR1/2. Current densities of GluR1 and GluR1/2 receptors in the presence or absence of CTZ measured using glutamate (10 mM) as an agonist at a holding potential -60 mV.

3.2 The Effect of Tonic L-Proline Concentrations on Recombinant AMPA Receptors

In hyperprolinemia l-proline concentrations are elevated 10-fold in the cerebrospinal fluid to approximately 30 μ M (Phang et al. 2001). This study hypothesized that the persistent increase in l-proline level may affect glutamate signaling at glutamate receptors. To test this hypothesis, the effect of tonic physiological (3 μ M) and pathophysiological (30 μ M) l-proline levels on glutamate-induced currents at GluR1 and GluR1/2 receptors were examined.

Application of 30 μ M l-proline was found to result in a statistically significant ($p < 0.05$) decrease in current generated by the application of 10 mM of glutamate (23.6 ± 10.0 %; $n = 5$) (**Figure 3-3**). In contrast, there was no significant decrease in current induced with the application of 10 mM glutamate in the presence of 3 μ M l-proline (1.8 ± 8.5 %; $n = 6$). Application of 30 μ M l-proline lead to a slight shift in the kinetics of the channel as shown by a delay in the activation rate with application of 10 mM of glutamate in the presence of 30 μ M of l-proline. These findings indicate that the tonic presence of pathophysiological but not physiological concentration of l-proline resulted in a disruption of the ability of glutamate to activate homomeric GluR1 receptors. This result suggests that l-proline, acting as a partial agonist, reduced the agonist effect of glutamate by competing with glutamate for the receptors. Alternatively, l-proline could be responsible for driving the GluR1 receptors into a state of desensitization. These findings were however consistent with the reduction in agonist activity when assessed in the presence of a partial agonist. Thus, tonic pathophysiological concentrations of l-proline as encountered in hyperprolinemia may contribute to glutamate receptor hypofunction.

With heteromeric GluR1/2 receptors, there was no significant change in the current with the application of physiological (-3.534 ± 4.9975 %; $n = 5$) or pathophysiological (6.4360 ± 4.8742 %; $n = 6$) concentrations of l-proline when compared to control (**Figure 3-4**). Co-application of pathophysiological 30 μ M l-proline with 10 mM glutamate was previously shown to lead to a slight shift in the kinetics of GluR1 homomeric receptors. However, under the same conditions no change in the kinetics of GluR1/2 heteromeric receptors was observed. Taken together, these findings suggest that although l-proline induced a greater current in GluR1/2 receptors than GluR1 receptors, tonic application of pathophysiological concentrations of l-proline selectively suppressed the ability of glutamate to activate homomeric GluR1 AMPA receptors. Given that GluR1 receptors desensitized more completely than heteromeric GluR1/2 receptors, l-proline may preferentially drive GluR1, but not GluR1/2, receptors into a state of desensitization.

3.3 CTZ Application Blocks the Effect of Tonic L-Proline on GluR1 Currents

To test if the reduction in glutamate-induced current in the tonic presence of pathophysiological l-proline is due to desensitization of the receptors, CTZ was applied through out the experiment. The aforementioned proline-induced decrease in the current

Figure 3-3 The Effect of Tonic L-Proline Levels on GluR1 Currents

HEK cells were transfected with GluR1 and GFP. Control whole cell recordings were made from cells exposed to control solution of 10 mM glutamate (black), with subsequent co-application of 3 μM l-proline or 30 μM l-proline (red and green respectively). (A-C) Representative traces during application of 10 mM glutamate (black) and application of 10 mM glutamate in the presence of control (0 μM), 3 μM and 30 μM l-proline respectively (red). (D) Summary graph showing the average time course of peak current normalized to the initial control pulse is plotted for coapplication of 3 μM or 30 μM l-proline concentrations with 10 mM glutamate at GluR1. Where n indicated in parenthesis is the number of observations.

Figure 3-4 The Effect of Tonic L-Proline Levels on GluR1/2 Currents

HEK cells were transfected with GluR1, GluR2 and GFP. Control whole cell recordings were made from cells exposed to control solution of 10 mM glutamate (black), 3 μM l-proline or 30 μM l-proline (red and green respectively). **(A)** Average I-V relationships are shown in response to I-V protocol (-60 mV to +40 mV). The I-V relationships were generated with the same cells used in the experiments. **(B)** Representative traces during co-application of 30 μM l-proline and 10 mM glutamate (red) and to initial 10 mM glutamate application (black) are shown. **(C)** Summary graph showing average time course for peak current normalized to the initial control pulse for co-application of 3 μM or 30 μM l-proline concentrations with 10 mM glutamate at GluR1/2 heteromeric receptors

produced by 10 mM glutamate at GluR1 receptors was abolished by the application of CTZ (-3.3188 ± 4.8352 %; $n = 5$) when compared with control in the absence of CTZ (-1.8860 ± 6.6879 % $n = 7$). Data generated for 3 μ M and 30 μ M l-proline are plotted on the same graph for visual comparison of the effect of 30 μ M l-proline on GluR1 homomeric receptors in the presence and absence of CTZ (**Figure 3-5**). No change in the kinetics of the receptor was detected upon the co-application of CTZ and pathophysiological concentration of l-proline. These findings suggest that the reduction of glutamate-evoked current by the tonic presence of a pathophysiological l-proline concentration was due to the ability of l-proline to drive homomeric GluR1 receptors into a desensitized state.

3.4 Effect of Pathophysiological L-Proline Concentration on Paired-Pulse-Ratio in GluR1

Previous studies have suggested that l-proline decreased glutamate transmission by reducing glutamate release in rat cerebral cortex and hippocampus (Keller et al. 1981; Cohen and Nadler 1997). The ability of proline to decrease glutamatergic transmission in the hippocampus was determined using a paired-pulse assay, in which an increase in the paired-pulse ratio is interpreted as a reduction in the presynaptic release (Cohen and Nadler 1997). However, if l-proline binds to glutamate receptors with a weak affinity, then a second pulse should compete off proline and generate a relatively larger current.

With glutamate a paired-pulse-ratio (ratio of second pulse to the first pulse) was computed at 0.34 ± 0.03 ($n = 5$) with the co-application of pathophysiological concentration of l-proline (30 μ M) there was a significant increase in the paired pulse ratio to 0.47 ± 0.04 ($p < 0.05$) (**Figure 3-6**). Therefore, an increase in the paired-pulse-ratio in the presence of pathophysiological concentration of l-proline at GluR1 receptors occurred. These findings suggest that the increase in paired-pulse-ratio previously observed in hippocampal neurons in response to pathophysiological concentrations of l-proline may not simply be a result of altered presynaptic function but may arise, in part, due to l-proline-induced desensitization of GluR1.

Figure 3-5 CTZ Application Blocks the Effect of Tonic L-Proline on GluR1 Currents

HEK cells were transfected with GluR1 and GFP. Control whole cell recordings were made from cells exposed to control solution of 10 mM glutamate (black), 3 μ M l-proline (green) or 30 μ M l-proline with CTZ present (blue). (A) Representative traces during co-application of 10 mM glutamate and 30 μ M l-proline application (red) to the initial glutamate application (black) and at recovery (gray) in the presence of CTZ are shown. (B) Summary of the average time course of peak current normalized to the initial control pulse for co-application of 10 mM glutamate and 30 μ M l-proline in the presence or absence of CTZ at GluR1 receptors. Where n indicated in parenthesis is the number of observations.

Figure 3-6 Effect of Pathophysiological L-Proline Concentration on Paired-Pulse-Ratio in GluR1

HEK cells were transfected with GluR1 and GFP exposed to 25 ms pulse of 10 mM glutamate (control) or 10 mM glutamate and 30 μ M l-proline. **(A)** Representative traces to the initial paired pulse in the presence of control (10 mM glutamate) (black), control and 30 μ M l-proline (red) and recovery (grey) are shown. **(B)** Average of ratio of second pulse to the first pulse plotted against test conditions; in the presence of 10 mM glutamate (control), 10 mM glutamate and 30 μ M l-proline and recovery.

CHAPTER 4. DISCUSSION

4.1 L-Proline as a Partial Agonist at Recombinant AMPA Receptors

Consistent with previous studies which suggested that l-proline is low affinity agonist at native NMDA and non-NMDA glutamate receptors (Henzi et al. 1992), the data presented here confirm that l-proline exhibits a low efficacy at recombinant AMPA receptors composed of homomeric GluR1 and heteromeric GluR1/2 receptors. Indeed, 10 mM l-proline elicited less than 2.5 % of the current evoked by the full agonist at the same concentration in either of the receptors. Thus, these data are consistent with an estimated EC₅₀ of above 50 mM for l-proline at native glutamate receptors and are consistent with l-proline acting as a partial agonist (Henzi et al. 1992). Given that l-proline generates less than 2.5 % of the maximum available current at a concentration of 10 mM, pathophysiological and physiological concentrations of l-proline will be expected to produce negligible direct excitation of native AMPA receptors. Rather, the actions of l-proline at pathophysiological levels may be to dampen glutamate-mediated activation of AMPA receptors which is also consistent with a partial agonist profile for proline.

4.2 L-Proline Disruption of Glutamate-Induced Current at Recombinant Glutamate Receptors

A previous study has shown that l-proline at both physiological and pathophysiological potentiates glutamate transmission (Cohen and Nadler 1997). This effect could have been as a result of the wide repertoire of glutamate receptors expressed by the brain slices or the possibility that the l-proline induced potentiation of the fEPSP was mediated through other targets (Henzi et al. 1992; Cohen and Nadler 1997). Although l-proline exhibited negligible activation of the recombinant AMPARs in the pathophysiological to pathophysiological range, the tonic presence of pathophysiological l-proline selectively disrupted the ability of glutamate to activate homomeric GluR1 receptors. Data generated from this study show a significant decrease of approximately 24% of glutamate-induced current at GluR1 receptors with the application of a pathophysiological relevant concentration of l-proline. This decrease in glutamate-induced current was however not observed in the presence of physiological concentration of l-proline. This suggests that the increases in l-proline level observed in hyperprolinemia leads to a decrease in glutamate-induced current at GluR1 receptors and could be relevant in the hypoglutamatergic theory of schizophrenia. Indeed, this is consistent with the schizophrenic behavior in animals lacking GluR1 (Wiedholz et al. 2008).

Interestingly l-proline acts differently at homomeric GluR1 and heteromeric GluR1/2 receptors, indicating a pharmacological difference between the two receptors. Moreover, the ability of homomeric GluR1 receptors to be selectively targeted may be particularly relevant for schizophrenia. Indeed these receptors tend to be preferentially expressed on interneurons (Geiger et al. 1995; Rossi et al. 2008). Reduction in the

activity of these neurons has been proposed to be critical for schizophrenia (Belforte et al. 2010).

4.3 L-Proline Desensitization of GluR1 Receptors

The proline-induced reduction in glutamate-induced current observed at GluR1 receptors was abolished by the application of CTZ. These findings, as well as data generated from the paired-pulse ratio experiment suggest l-proline acts by binding at homomeric GluR1 receptors and at least at pathophysiological concentration by inducing significant desensitization of the receptor. Interestingly, the increase in Paired-pulse-ratio due to pathophysiological l-proline described by Cohen and Nadler (1997) was approximately 50 %, which is similar to the approximately 38 % increased observed in response to a similar protocol in the recombinant system employed. Thus, contrary to a previous study suggesting that the l-proline-induced increase in paired-pulse ratio observed on native receptors in hippocampal slices is through a reduction in pre-synaptic glutamate release; the data presented here indicate a postsynaptic mechanism by which l-proline induces a decrease in glutamate-induced current at GluR1 receptor through desensitization of the receptor. This is particularly interesting as a recent study has suggested that Ca^{2+} -permeable AMPA receptors (which are presumably GluR1 homomers) reside in the perisynaptic space (He et al. 2009). The presence of these Ca^{2+} -permeable AMPA receptors are preferentially revealed by paired pulse stimulation protocols (He et al. 2009). Thus, the ability of l-proline to induce an increase in paired pulse ratio is consistent with the selective regulation of homomeric GluR1 AMPA receptors described here.

As shown in this study l-proline has differential effect on glutamate-induced current at two different AMPA receptors, thus the possibility exists that l-proline acts at other types of glutamate receptors including NMDA receptors which are important in a subunit specific manner for learning and memory. Indeed, one can expect these findings to be extended to other glutamate receptors and thus, the possibility that l-proline could also account for glutamate hypofunction at these receptors.

CHAPTER 5. CONCLUSION

The experiments carried out in this study were designed with a view to determining the role of l-proline as an agonist, as well as the implications of hyperprolinemia at two recombinant AMPA receptors. Specifically, to determine the differential effect of tonically present physiological versus pathophysiological concentration of l-proline on the ability of glutamate to bind to and activate GluR1 homomeric and GluR1/2 heteromeric receptors. A decrease in glutamate-induced current was observed with the application of pathophysiological concentration of l-proline (30 μM) when compared to the physiological concentration (3 μM). This decrease was not observed with the same concentration in GluR1/2 heteromeric receptors. L-proline was shown to induce a larger current at GluR1/2 heteromeric receptors than at GluR1 homomeric receptors, however the current induced at 30 μM (pathophysiological concentration) of l-proline is shown to be statistically insignificant.

The reduction in glutamate-induced current in the tonic presence of pathophysiological concentration of l-proline was shown to be due to desensitization of the receptor. The differences in the ability of l-proline to induce desensitization at GluR1 homomeric receptors and its ability to induce currents at GluR1/2 heteromeric and GluR1 homomeric receptors may provide a foundation to the understanding of how l-proline affects glutamate transmission. The ability of pathophysiological concentrations of l-proline to induce a decrease in glutamate-induced current at homomeric GluR1 receptors may be a relevant mechanism by which hyperprolinemia contributes to schizophrenia.

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VITA

Adetutu Abiose Oyelami was born in Lagos, Nigeria, on November 9, 1980. She attended high school at Suleja (Federal) Academy, school for the gifted and talented, Niger State, Nigeria, in 1993 and graduated in 1998. She matriculated at Obafemi Awolowo University (OAU) in September 2000 and completed a Bachelor of Science degree in Microbiology in 2006. She joined the intergrated program in biomedical science at the University of Tennessee Health Science Center in August of 2007 and graduates with a Master's degree in Neuroscience in May 2010.