Neuronal and Glial Expression of GluN2C and GluN2D NMDA Receptor Subunit mRNA in Different Regions of the Mouse Forebrain

Hassan Alsaad
*University of Nebraska Medical Center*

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NEURONAL AND GLIAL EXPRESSION OF GluN2C AND GluN2D NMDA RECEPTOR SUBUNIT mRNA IN DIFFERENT REGIONS OF THE MOUSE FOREBRAIN

By

Hassan A. Alsaad
NEURONAL AND GLIAL EXPRESSION OF GluN2C AND GluN2D
NMDF RECEPTOR SUBUNIT mRNA IN DIFFERENT REGIONS OF
THE MOUSE FOREBRAIN

by

Hassan A. Alsaad

A DISSERTATION

Presented to the Faculty of
the University of Nebraska Graduate College
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

Pharmacology & Experimental Neuroscience
Graduate Program

Under the Supervision of Professor Daniel T. Monaghan

University of Nebraska Medical Center
Omaha, Nebraska

November, 2018

Supervisory Committee:
Jyothi Arikkath, Ph.D.       Myron Toews, Ph.D.
George Rozanski, Ph.D.     Andrew T. Dudley, Ph. D.
This dissertation is dedicated with all love to my family
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ABSTRACT

Neuronal and Glial Expression of GluN2C and GluN2D NMDA Receptor Subunit mRNA in Different Regions of the Mouse Forebrain

Hassan A. Alsaad, Ph.D.

University of Nebraska, 2018

Supervisor: Daniel T. Monaghan, Ph.D.

N-methyl-D-aspartate (NMDA) receptors are a subtype of glutamate receptors that are widely expressed in the brain, where they mediate critical functions. While the actions of the predominate GluN2 NMDAR subunits, GluN2A and GluN2B are relatively well understood, the function of GluN2C and GluN2D in the telencephalon is largely unknown. To better understand the possible role of GluN2C and GluN2D subunits, we used fluorescence in situ hybridization together with multiple cell markers to define the distribution and type of cells expressing GluN2C and GluN2D mRNA. Also, a GluN2C-KO mouse was used as a negative control.

GluN2C mRNA expression was only found in non-neuronal cells in the telencephalon (except for globus pallidus). In addition, a significant fraction of astrocytes expressed GluN2C mRNA. In contrast to GluN2C, GluN2D subunit mRNA colocalized with neuronal and not astrocyte markers in the telencephalon. In the thalamus, GluN2C and GluN2D mRNA showed region-specific distributions as previously reported, and both subunits mRNA were expressed in neurons. Consistent with these findings, cellular colocalization of GluN2C and GluN2D mRNA was found in the thalamus but not in the
telencephalon, except for the globus pallidus. Furthermore, the type of GluN2D-expressing neurons was examined by colocalization with GAD67 mRNA and found to be GAD67-positive interneurons.

Among GluN2 NMDA receptor subunits, GluN2C expression was selective to astrocytes in the telencephalon. This specific pattern of distribution could indicate a distinct function. Since glutamate and intracellular Ca\(^{2+}\) signaling are linked to glucose uptake in astrocytes, a potential role of GluN2C-containing NMDA receptors in regulating astrocytic glucose uptake was investigated using autoradioactive analysis. Compared to wild-type mice, GluN2C-KO mice were found to have less glucose uptake after ketamine-induced neuronal activation. These data suggest that GluN2C play a role in stimulating glucose transport into astrocytes.

Taken together, defining the cell-type distribution of GluN2C and GluN2D helps in understanding their functions in the brain and discovering therapeutic targets to treat neurological diseases and psychiatric disorders.
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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>[(^{14}\text{C})]-2DG</td>
<td>(^{14}\text{C})2-deoxy glucose</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AMPA</td>
<td>(\alpha)-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid</td>
</tr>
<tr>
<td>ATD</td>
<td>amino-terminal domain</td>
</tr>
<tr>
<td>A(\beta)</td>
<td>amyloid-(\beta)</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CTD</td>
<td>carboxyl-terminal domain</td>
</tr>
<tr>
<td>DIG</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>EPSC</td>
<td>excitatory postsynaptic current</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescence in situ hybridization</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GAD67</td>
<td>glutamate decarboxylase 67</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>iGluR</td>
<td>ionotropic glutamate receptors</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IUPHAR</td>
<td>International Union of Basic and Clinical Pharmacology</td>
</tr>
<tr>
<td>LBD</td>
<td>ligand binding domain</td>
</tr>
<tr>
<td>LTD</td>
<td>long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptors</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NeuN</td>
<td>neuronal nuclear protein/RNA binding protein fox-1 homolog 3</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>RTN</td>
<td>reticular thalamic nucleus</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>TBI</td>
<td>traumatic brain injury</td>
</tr>
<tr>
<td>TMD</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>TSA</td>
<td>tyramide signal amplification</td>
</tr>
<tr>
<td>VGlut1</td>
<td>vesicular glutamate transporter 1</td>
</tr>
<tr>
<td>VGlut2</td>
<td>vesicular glutamate transporter 2</td>
</tr>
<tr>
<td>VPL</td>
<td>ventral posteromedial nucleus</td>
</tr>
<tr>
<td>VPM</td>
<td>ventral posterolateral nucleus</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside</td>
</tr>
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</table>
CHAPTER 1

Introduction
1. Glutamate Receptors: Overview

Glutamate receptors are widely expressed in the central nervous system (CNS). These receptors mediate the vast majority of excitatory synaptic transmission upon their activation by the principal excitatory neurotransmitter in the CNS, L-glutamate. Under physiological conditions, glutamate receptors play critical roles in a variety of brain functions during development and adulthood such as synaptic plasticity, learning, and memory (Niswender and Conn, 2010; Traynelis, et al., 2010). However, dysfunction of glutamate receptors is implicated in many neurological diseases (e.g., Alzheimer’s disease, Parkinson’s disease, epilepsy, and ischemic stroke), and psychiatric disorders (e.g., schizophrenia and depression) (Bowie, 2008; Lau and Tymianski, 2010; Vaidya, et al., 2013).

Glutamate receptors are transmembrane proteins that function as ligand-gated ion channels and G-protein-coupled receptors. Therefore, glutamate receptors are divided into two major types: ionotropic glutamate receptors (iGluR) and metabotropic glutamate receptors (mGluR) (Willard and Koochekpour, 2013).

1.1. Ionotropic Glutamate Receptors

Receptors in this division are ligand-gated ion channels that are permeable to cations (Na⁺, K⁺, and Ca²⁺). The permeability of these receptors is varied depending on the class and the subunit composition of the receptor. Ionotropic GluRs are divided into four distinct classes: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, kainate receptors, N-methyl-D-aspartate (NMDA) receptors, and delta receptors. These four broad iGluR classes share common receptor morphology, yet they are differentiated based on their subunit composition. Each iGluR class consists of a
group of subunits that have similar homology, functional properties, and pharmacology (Traynelis, et al., 2010) (Figure 1). Interestingly, the delta receptors do not appear to form functional ion channels; however, these receptors are classified as iGluRs because they have a similar sequence identity to AMPA receptors, kainate receptors, and NMDA receptors (Yamazaki, et al., 1992; Lomeli, et al., 1993). The function of the delta receptors is not fully understood (Schmid and Hollmann, 2008). However, these receptors appear to have critical roles in synapse organization and synaptic plasticity (Matsuda, et al., 2010; Elegheert, et al., 2016).

Historically, the naming and classification of AMPA receptors, kainate receptors, and NMDA receptors were based on their selective pharmacological activating agonist. In the era of receptor cloning, a strong correlation was demonstrated between the sequence identity and the pharmacological properties of subunits in these functional classes (Traynelis, et al., 2010).

Ionotropic GluRs are a tetrameric structure composed of subunits within the same receptor class. However, delta receptors appear to form receptors that contain subunits from other iGluR subtypes. The composition of the receptor subunits determines its physiological and pharmacological properties. Upon L-glutamate binding and receptor activation, iGluRs induce cellular responses by allowing cations to cross the cell membrane. The influx of high concentrations of cations can result in an action potential and fast synaptic transmission (Willard and Koochekpour, 2013). Ionotropic GluRs can also lead to downstream signaling in the cells by increasing the intracellular Ca$^{2+}$ concentration.
Figure 1. Classification of ionotropic glutamate receptors. Four classes of iGluRs are identified and each class consists of multiple subunits.
1.2. Metabotropic Glutamate Receptors

Metabotropic GluRs are G-protein-coupled receptors that bind L-glutamate and modulate synaptic transmission in the CNS. Compared to iGluRs, mGluRs mediate prolonged effects in cells, which last hundreds of milliseconds to several seconds. Metabotropic GluRs share a partially common structure with the other G-protein-coupled receptors; their structure is divided into three main domains: an extracellular amino-terminal domain, a seven-transmembrane helices domain, and an intracellular carboxyl-terminal domain. However, the sequence identity of mGluRs shows little homology to the other G-protein-coupled receptors, except to gamma-aminobutyric acid (GABA) receptors. The major difference between mGluRs and classic GPCRs is that the ligand binding site of mGluRs is in the extracellular N-terminal rather than within the seven transmembrane domains portion of the receptor (Niswender and Conn, 2010; Unal and Karnik, 2012; Sherman, 2014). Like iGluRs, mGluRs are activated by the binding of L-glutamate to the extracellular amino-terminal of the receptor. The function of mGluRs is mediated by transmitting signals through the receptor protein to intracellular signaling partners (second messenger pathways), which in turn lead to downstream responses in cells. Metabotropic GluRs are divided into three groups based on their sequence homology, pharmacology, and signal transduction mechanisms of the composing receptor subunits. These groups are group I (mGluR1 and mGluR5) which activates phospholipase C, group II (mGluR2 and mGluR3) which inhibits adenylate cyclase, and group III (mGluR4 and mGluR6-8) which also inhibits adenylate cyclase (Conn and Pin, 1997; Yin and Niswender, 2014) (Figure 2).
Figure 2. Classification of metabotropic glutamate receptors. Multiple subunits of mGluRs are identified and divided into three groups.
2. NMDA Receptors: Structure and Nomenclature, Physiological Properties, and Distribution in the Brain

2.1. Structure and Nomenclature of NMDA Receptors

NMDA receptors are ligand-gated ion channels that mediate a major component of excitatory neurotransmission in the CNS. These receptors are hetero-tetrameric assemblies that must contain two GluN1 subunits and a combination of two subunits from the GluN2 (GluN2A-GluN2D) and/or GluN3 (GluN3A, GluN3B) subunit families (Hansen, et al., 2017). In this dissertation, the used nomenclature of NMDA receptor subunits followed the systemic name established by International Union of Basic and Clinical Pharmacology (IUPHAR). Other common names for the NMDA receptors subunits, which were mentioned in the cited literature, along with the IUPHAR names and the gene names are listed in table 1 (Collingridge, et al., 2009).

Seven NMDA receptor subunits have been identified which impose NMDA receptors diverse functional roles across the brain and enable precise tuning of synaptic transmission. This functional heterogeneity of NMDA receptors is accomplished by 1) the distinct physiological and pharmacological properties, 2) the distinct spatiotemporal distribution, and 3) specific cell-type expression (neuronal vs. glial cells) and subcellular localization (synaptic vs. extrasynaptic) for each receptor subunit (Paoletti, et al., 2013).

NMDA receptor subunits share a common structural topology. They consist of a large extracellular amino-terminal domain (ATD), a ligand binding domain (LBD), a transmembrane domain (TMD), and an intracellular carboxyl-terminal domain (CTD) (Figure 3). The ATD is a clamshell-like structure which plays a role in the assembly of the NMDA receptor. By binding to the ATD, some endogenous compounds (e.g.,
Table 1. Nomenclature and gene symbols of NMDA receptors

<table>
<thead>
<tr>
<th>IUPHAR Names</th>
<th>Common Names</th>
<th>Gene Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>GluN1</td>
<td>NMDA Receptor ζ1, NR1, NMDAR1</td>
<td><em>Grin1</em></td>
</tr>
<tr>
<td>GluN2A</td>
<td>NMDA Receptor ε1, NR2A, NMDAR2A</td>
<td><em>Grin2a</em></td>
</tr>
<tr>
<td>GluN2B</td>
<td>NMDA Receptor ε2, NR2B, NMDAR2B,</td>
<td><em>Grin2b</em></td>
</tr>
<tr>
<td>GluN2C</td>
<td>NMDA Receptor ε3, NR2C, NMDAR2C,</td>
<td><em>Grin2c</em></td>
</tr>
<tr>
<td>GluN2D</td>
<td>NMDA Receptor ε4, NR2D, NMDAR2D,</td>
<td><em>Grin2d</em></td>
</tr>
<tr>
<td>GluN3A</td>
<td>NR3A</td>
<td><em>Grin3a</em></td>
</tr>
<tr>
<td>GluN3B</td>
<td>NR3B</td>
<td><em>Grin3b</em></td>
</tr>
</tbody>
</table>
Figure 3. Schematic illustration of an NMDA receptor subunit topology. NMDA receptor subunit is composed of four domains: amino-terminal domain (ATD), ligand binding domain (LBD), transmembrane domain (TMD), and carboxyl-terminal domain (CTD).
extracellular Zn\(^{2+}\) and polyamines), as well as pharmacological reagents, can modulate the function of NMDA receptors. The TMD consists of four segments: three transmembrane helices (M1, M3, and M4) and a membrane re-entrant loop (M2). The TMD from each composing NMDA receptor subunit arranges to form an ion channel pore. The residues lining the ion channel pore, particularly the membrane re-entrant loop M2, determine the basic ion permeation properties (e.g., Ca\(^{2+}\) permeability and Mg\(^{2+}\) block). The LBD is a kidney-shaped structure which consists of two polypeptide segments. These segments fold to an upper lobe and a lower lobe form a cleft, i.e., ligand binding site. The LBD of each subunit forms a ligand binding site. The GluN1 and GluN3 subunits bind glycine and D-serine, whereas the GluN2 subunits bind L-glutamate for receptor activation. The CTD is suggested to have a role in receptor localization, trafficking, regulation, and signaling since the CTD contains numerous sites for protein-protein interactions and posttranslational modifications (Traynelis, et al., 2010; Vyklicky, et al., 2014).

The function and pharmacology of NMDA receptors and their correlations to the intra- and inter-subunit interactions have been extensively studied for more than two decades. Recently, crystallographic studies of NMDA receptors have provided important insight into the overall receptor structure, the domains organization, and the binding sites of allosteric modulators. These crystallographic structures have shown that the arrangement of the receptor subunits in GluN1/GluN2B receptors is in an alternating pattern (i.e., GluN1-GluN2B-GluN1-GluN2B) (Karakas and Furukawa, 2014; Lee, et al., 2014a). More recently, electron cryomicroscopy (cryo-EM) has provided receptor structures with higher resolution which can display dynamic pictures of the
conformational changes that happen in intact NMDA receptors and provide insights into the structural mechanism of ion channel gating (i.e., receptor activation) and mechanisms by which allosteric modulators influence NMDA receptor function (Lu, et al., 2017).

2.2. Physiological Properties of NMDA Receptors

NMDA receptors have distinct physiological and pharmacological properties compared to the other iGluRs, AMPA receptors and kainate receptors. Unlike other iGluRs, GluN2-containing NMDA receptors exhibit voltage-dependent Mg$^{2+}$-block, require simultaneous binding of two co-agonists for activation, and show high permeability to Ca$^{2+}$. These characteristics have a large impact on the distinguished functional roles of NMDA receptors in the CNS (Traynelis, et al., 2010).

Physiological concentrations of extracellular Mg$^{2+}$ can block NMDA receptors at the resting membrane potential but not at depolarized membrane potentials (Nowak, et al., 1984). This mechanism enables NMDA receptors to play a role in excitatory postsynaptic currents (EPSCs) that is distinct from AMPA receptors and kainate receptors. EPSCs typically consist of two main components: early-fast phase and late-slow phase. Initially, presynaptic glutamate release activates postsynaptic AMPA receptors which result in depolarization of membrane potential and the early-fast phase of the EPSCs. Because of the Mg$^{2+}$ block of NMDA receptors at the resting membrane potential, a single action potential leads to AMPA receptor-mediated currents without activation of an NMDA receptor current. If however the postsynaptic cell was already depolarized by a prior impulse or by a converging excitatory pathway, then another AMPA receptor synaptic response would activate an NMDA receptor current, which is the late-slow phase of the EPSCs (Hestrin, et al., 1990). Because NMDA receptors
require 1) simultaneous presynaptic glutamate release and 2) membrane potential
depolarization to produce the late-slow phase of the EPSCs, NMDA receptors are known
as coincidence detectors.

AMPA receptors and kainate receptors only require the binding of L-glutamate
for activation, whereas GluN2-containing NMDA receptors require the binding of two
agonists (L-glutamate and glycine/D-serine), simultaneously (Kleckner and Dingledine,
1988; Wolosker, 2007). NMDA receptors have a higher affinity for L-glutamate than
AMPA receptors. This higher glutamate affinity is one of the factors that contribute to the
prolonged time course of EPSCs induced by NMDA receptors (lasting for tens to
hundreds of milliseconds) (Traynelis, et al., 2010). The activity of iGluRs can be
regulated by changing L-glutamate concentration. However, the activity of NMDA
receptors can also be regulated by changing the concentration of glycine/D-serine, which
modulates the magnitude of NMDA receptors activation. In the brain, whether glycine or
D-serine serves as the endogenous co-agonist is thought to be dependent on brain region
and the synaptic/extrasynaptic NMDA receptor location (Mothet, et al., 2015).

The high Ca\(^{2+}\) permeability and the prolonged time course of activity allow
NMDA receptors to mediate large Ca\(^{2+}\)-influx during the EPSC, which in turn can affect
synaptic plasticity and multiple downstream signaling pathways in cells. Robust short-
term Ca\(^{2+}\)-influx can produce long-term potentiation (LTP), whereas less pronounced
long-term Ca\(^{2+}\)-influx can produce long-term depression (LTD) of the synaptic plasticity.
These varied synaptic responses which depend on the pattern of NMDA receptors activity
are thought to represent cellular mechanisms that underlie memory and learning (Paoletti,
et al., 2013).
Each NMDA receptor family (GluN1, GluN2, and GluN3 subunits) has distinct physiological and pharmacological properties compared to the other subunits, which differentially impact the NMDA receptor functional properties. Furthermore, different GluN1 isoforms, GluN2 subunits, and GluN3 subunits import different functional properties to NMDA receptors (Hansen, et al., 2017). Unlike GluN2 subunits, the functional significance of GluN1 isoforms in the CNS is still not well characterized and unclear.

NMDA receptors with different GluN2 subunits show distinct functional properties in terms of voltage-dependency, agonist affinity, opening probability, deactivation kinetics, and desensitization properties (Ishii, et al., 1993; Mishina, et al., 1993; Buller, et al., 1994; Monyer, et al., 1994; Vicini, et al., 1998) (Figure 4). This heterogeneity of NMDA receptor subtypes enables GluN2-containing NMDA receptors to have varied effects on the time course of the EPSC and other physiological properties in different cell types of the brain throughout development and adulthood (Paoletti, et al., 2013). For example, GluN2C- and GluN2D-containing NMDA receptors display a higher affinity for the co-agonists (L-glutamate and glycine/D-serine) than GluN2A- or GluN2B-containing NMDA receptors so they can be activated by lower concentrations of L-glutamate and glycine. GluN2C- and GluN2D-containing NMDA receptors are also less sensitive to Mg\(^{2+}\) block than GluN2A- or GluN2B-containing NMDA receptors, so they do not require coincident membrane depolarization (Traynelis, et al., 2010). Taken together, these features allow GluN2C- and GluN2D-containing NMDA receptors to have a distinct physiological role compared to GluN2A or GluN2B-containing NMDA receptors.
Figure 4. Physiological properties of recombinant NMDA receptor subtypes.

Recordings of current responses from human embryonic kidney 293 cells that express different subtypes of NMDA receptors. Application of L-Glutamate (1 mM) for one millisecond is indicated by the open tip current in the upper traces. Used with permission from (Vicini, et al., 1998).
GluN3A and GluN3B NMDA receptor subunits were cloned based on their similarity to GluN1 and GluN2 subunits (Ciabarra, et al., 1995; Sucher, et al., 1995; Nishi, et al., 2001). NMDA receptors can contain GluN3A and GluN3B subunits which bind glycine and D-serine as agonists (Nilsson, et al., 2007). Thus, unlike GluN2-containing NMDA receptors, GluN1/GluN3 NMDA receptors can be activated without the requirement for L-glutamate binding. Compared to GluN2-containing NMDA receptors, the GluN3-containing NMDA receptors show a reduced sensitivity to Mg\(^{2+}\) inhibition, Ca\(^{2+}\)-permeability, and response amplitudes. When GluN3 subunits are incorporated into GluN2-containing NMDA receptors, they appear to decrease the NMDA receptors responses (Dingledine, et al., 1999; Matsuda, et al., 2002). So, the ability of NMDA receptors to form tri-heteromeric structures by the assembly of GluN1, GluN2, and GluN3 subunits offers more diverse receptor properties. However, more investigations are needed to better understand the physiological roles of GluN3-containing NMDA receptors in the brain.

In conclusion, NMDA receptors have distinct pharmacological and physiological features compared to the other iGluRs. Likewise, NMDA receptor subunits (GluN1, GluN2, and GluN3) show subunit-specific pharmacological and physiological properties. Importantly, the subunit composition of the NMDA receptors primarily determines the variation of NMDA receptor responses in different cell types and brain regions.

2.3. Regional and Cellular Expression of NMDA Receptor Subunits in the Brain

Based on the subunit composition, NMDA receptors can perform a variety of functions in the CNS because different GluN2 and GluN3 subunits show different pharmacology, physiology, mechanism of regulation, and anatomical distribution
NMDA receptor subunits show distinct distributions between brain regions (Figure 5), cell types, and subcellular localizations (synaptic versus extrasynaptic sites), which allow more functional diversity. Thus, expressing different types of GluN2 subunits allows neuronal and glial cells to precisely tune their functional properties in different brain regions. Moreover, the expression patterns of the NMDA receptor subtypes undergo dramatic changes during development (Figure 5), which enable NMDA receptors to modify neuronal function during early development and maturation of the CNS.

2.3.1. Expression of GluN1, GluN2A, and GluN2B NMDA Receptor Subunits

Overall, the GluN1 subunit is ubiquitously expressed in the CNS during development and in adults (Akazawa, et al., 1994a; Monyer, et al., 1994) (Figure 5). This pattern of expression is consistent with the inclusion of the GluN1 subunits in all NMDA receptor subtypes (Ishii, et al., 1993). GluN1 subunit has eight different isoforms that arise from alternative splicing of three exons within a single gene product. These GluN1 subunit isoforms show some differences in the regional and developmental distribution in the brain and are likely to have a functional significance that could impact NMDA receptor functional properties (Paupard, et al., 1997). Nevertheless, the GluN2 subunits (A, B, C, and D subunits) are more likely to be responsible for the heterogeneity of the various NMDA receptor subtypes because of their highly varied physiological properties and more distinct distributions compared to GluN1 isoforms.

The expressions of GluN2A and GluN2B subunits display distinct temporal patterns during the early postnatal development in the brain (Figure 5). In rodents, the
Figure 5. Distribution of NMDA receptor subunits in parasagittal rat brain sections.

Autoradiograms of radioactive in situ hybridizations show regional and developmental expression of GluN1 and GluN2 NMDA receptor subunit mRNA. Used with permission from (Akazawa, et al., 1994a)
expression of the GluN2A subunit is very weak at birth. However, GluN2A expression increases dramatically during the second postnatal week. In adults, the GluN2A subunit is expressed in different brain regions and shows a high abundance in the cerebellum, cortex, and hippocampus. On the other hand, the expression of GluN2B subunit is high in the embryonic brain and throughout the early developmental stage. In the adult brain, the GluN2B expression is restricted to the forebrain (Watanabe, et al., 1992; Ishii, et al., 1993; Watanabe, et al., 1993a; Akazawa, et al., 1994a; Monyer, et al., 1994). Therefore, the GluN2A and GluN2B expression show a developmental switch in some brain regions (e.g., cerebellum, cortex, and hippocampus), where NMDA receptors change from primarily containing GluN2B subunits early in life to GluN2A-containing receptors.

The GluN2B-GluN2A developmental switch is suggested to include the assembly of tri-heteromeric NMDA receptors at some stages. Initially, cells may start expressing GluN2A subunits along with the GluN2B subunits, forming the tri-heteromeric NMDA receptor, which consist of two GluN1, one GluN2A, and one GluN2B subunit (Sheng, et al., 1994; Rauner and Kohr, 2011). However, it has also been suggested that the GluN2B-GluN2A developmental switch may involve the formation of new synapses that contain GluN2A subunits and lack GluN2B subunits (Liu, et al., 2004). It is suggested that GluN2B-GluN2A developmental switch is a critical step for the maturation and refinement of synapses, development of synaptic plasticity, and fine-tuning of neuronal circuits (Feldman, et al., 1998; Tang, et al., 1999). The mechanism by which GluN2B-GluN2A developmental switch is triggered is still not fully understood and remains an area for investigation. GluN2A and GluN2B subunit mRNA are highly expressed by a
variety of excitatory and inhibitory neurons (Fritschy, et al., 1998; Cserep, et al., 2012; Zhang, et al., 2014; Mancarci, et al., 2017).

2.3.2. Expression of GluN2C and GluN2D NMDA Receptor Subunits

Expression of GluN2C subunit is undetectable at birth, however, expression starts to appear in the cerebellum and the olfactory bulb in the second postnatal week. By adulthood, GluN2C subunit mRNA is highly expressed in cerebellar granular cells, moderately expressed in various lateral thalamic nuclei and the mitral cells and glomerular layer of the olfactory bulb, and weakly expressed in the telencephalon (cortex, hippocampus, amygdala, and striatum) (Figure 5). In the rodent telencephalon, GluN2C have a diffuse, non-laminar distribution pattern (Watanabe, et al., 1992; Watanabe, et al., 1993a; Akazawa, et al., 1994a; Monyer, et al., 1994). However, the identity of the GluN2C-expressing cells (neurons vs. glia) in the telencephalon is controversial.

Unlike GluN2C subunit, the GluN2D subunit is widely expressed in the brain during early development. However, GluN2D expression drops dramatically in the second postnatal week. In adulthood, GluN2D expression maintains the highest abundance in the diencephalon and brainstem. In the adult telencephalon, the signal of GluN2D mRNA is faint (Watanabe, et al., 1992; Akazawa, et al., 1994a) (Figure 5). Additionally, based on the pattern of the GluN2D signal, it was proposed that GluN2D mRNA is expressed by the inhibitory interneurons in the cortex and hippocampus (Watanabe, et al., 1993a; Monyer, et al., 1994). Interestingly, although the overall expression of GluN2C and GluN2D subunits appear to be low in the cortex and hippocampus, their expression can be highly colocalized to a subpopulation of neurons or glial cells in these regions.
Similar to GluN2A and GluN2B subunits, the expression of GluN2C and GluN2D subunits display profound changes during the early development. In addition, some changes during the development of synaptic plasticity appear not to include GluN2B-GluN2A switch (Barth and Malenka, 2001). Therefore, GluN2C and GluN2D subunits are likely to have roles in the development of synaptic plasticity and fine tuning of some neuronal circuits.

2.3.3. Expression of GluN3A and GluN3B NMDA Receptor Subunits

Similar to the GluN2A-GluN2B developmental switch, the expression of GluN3A and GluN3B subunits in the brain appears to undergo a GluN3A to GluN3B developmental switch during the first two postnatal weeks. In the embryonic brain, several brain regions show weak GluN3A expression. However, in the early postnatal development, the brain appears to have increasing expression of GluN3A subunits. In adults, the brain has weakly and widely distributed expression of GluN3A subunit (Ciabarra, et al., 1995; Sucher, et al., 1995). On the other hand, the GluN3B subunit is weakly expressed during the early stages of postnatal development. However, the expression increases in the late postnatal development, and reaches the peak in the adult brain (Matsuda, et al., 2002; Fukaya, et al., 2005; Wee, et al., 2008).

GluN3A and GluN3B subunits appear to have distinct subcellular localizations. Recent studies have reported that GluN3A subunit is primarily expressed at extrasynaptic and presynaptic sites, whereas GluN3B is mostly expressed in the postsynaptic membrane (Wee, et al., 2016). The differential spatiotemporal expression of GluN3A and GluN3B subunits is very likely to import distinct physiological roles for these subunits in
the CNS. Compared to primates, rodents are thought to have a different pattern of GluN3 subunits expression.

2.3.4. Conclusion

The different GluN2 subunits have profoundly distinct regional and developmental expression profiles in the brain. In addition to that, the different GluN2 subunits can be expressed by distinct neuronal populations or glial cells in a region-specific manner. Overall, GluN2A and GluN2B subunits are widely expressed in the adult brain. In contrast, the GluN2C and GluN2D subunits display less profound expression in adults. GluN2C appears predominantly in the cerebellum and olfactory bulb, whereas the GluN2D subunit is highly expressed in the diencephalon and the brainstem. Consistent with being an obligatory component to form functional NMDA receptors, the GluN1 subunits is ubiquitously expressed in the brain.

In addition to their distinct anatomical distribution, the NMDA receptor subunits exhibit a wide range of functional and pharmacological properties. Thus, the diversity in subunit composition of NMDA receptors imposes the different receptor subtypes with distinct physiological roles across neuronal and glial cells and enables precise synaptic transmission.

3. Functions of NMDA Receptors in the CNS

NMDA-type glutamate receptors mediate a major component of excitatory neurotransmission in the CNS. NMDA receptors are expressed by different types of cells and widely distributed at all developmental stages in the brain. These receptors are critically involved in normal brain functions, including brain development, synaptic
plasticity, learning, memory, and somatosensory and visual perception. When activated, the NMDA receptors can mediate large Ca\textsuperscript{2+}-influx into the postsynaptic cells. Increasing the intracellular concentrations of Ca\textsuperscript{2+} can trigger downstream signaling pathways, which are critical to mediate some of the NMDA receptor functions. Additionally, it appears that NMDA receptors can mediate cellular responses without the requirement of ion influx. This can occur by changing the receptor conformation, and interactions between the carboxyl-terminal domains of the NMDA receptor with intracellular proteins.

3.1. Role of NMDA Receptors in Synaptic Plasticity, Learning, and Memory

LTP and LTD are two forms of synaptic plasticity that are thought to underlie learning and memory (Castillo, 2012; Nabavi, et al., 2014). Synaptic plasticity is the ability of a synapse to change its neuronal morphology and strengthen/weaken synaptic efficacy (Holtmaat and Svoboda, 2009). Activation of neuronal NMDA receptors causes Ca\textsuperscript{2+}-influx which increases the intracellular Ca\textsuperscript{2+} levels during EPSC. This can induce short-term and long-term changes in the neuronal synapse depending on the frequency and duration of synaptic NMDA receptor activation. NMDA receptor can mediate robust Ca\textsuperscript{2+}-influx for a short duration or less pronounced Ca\textsuperscript{2+}-influx for a longer duration which can lead to LTP and LTD of synaptic efficacy, respectively (Citri and Malenka, 2008; Luscher and Malenka, 2012; Granger and Nicoll, 2013). Thus, NMDA receptors play a central role in memory and learning by affecting the physiology of both potentiation and depression of synaptic efficacy.
3.2. Neuroprotection

As described earlier, GluN2A and GluN2B subunits are highly expressed in the cortex and hippocampus. The expression of GluN2A and GluN2B subunits is developmentally regulated. In mature neurons, studies have shown that synapses are enriched with GluN2A-containing NMDA receptors, while extrasynaptic sites are predominantly express GluN2B-containing NMDA receptors (Steigerwald, et al., 2000). However, GluN2A- and GluN2B-containing NMDA receptors have also been observed at extrasynaptic and synaptic sites, respectively (Thomas, et al., 2006). The subunit composition of NMDA receptors can be critical for CNS function; for example, knocking out GluN2B subunits in mice at the embryonic stage is lethal (Kutsuwada, et al., 1996).

Using pharmacological antagonists to selectively block GluN2A and GluN2B NMDA receptor subtypes, GluN2A-containing NMDA receptors have been reported to promote neuronal survival, whereas activation of GluN2B-containing NMDA receptors can lead to neuronal death (Liu, et al., 2007). However, the selectivity of the GluN2A antagonist used in these studies has been controversial (Neyton and Paoletti, 2006).

C-terminal domains of NMDA receptors can interact with cytosolic regulatory proteins and trigger various intracellular signaling pathways. Survival signaling pathways (anti-apoptotic effects of phosphatidyl inositol 3-kinase) have been linked to activation of GluN2A-containing NMDA receptors. In contrast, neuronal death signaling can be interrupted by disrupting the interaction of GluN2B-containing NMDA receptors with PSD-95 (Aarts, et al., 2002; Lee, et al., 2002). However, more selective drugs and approaches are required to better understand the subunit-specific role in neuronal survival and death.
3.3. Synaptogenesis and Synaptic Refinement

NMDA receptors play an important role in synaptogenesis and spine formation. It appears that the function of NMDA receptors in synapse formation and maturation is subunit-specific. The developmental switch of NDMA receptor subunits from one to another seems to be critical for synapses to change from synaptogenesis and refinement state to mature state (Gambrill and Barria, 2011). For example, high expression of GluN3A subunit seems to be critical in synaptogenesis and spine formation in early development (Perez-Otano, et al., 2006). Subsequently, there is a reduction in GluN3A expression which is important for synapse maturation (Roberts, et al., 2009).

3.5 Roles of NMDA Receptors in Astrocytes

Astrocytes are a type of glial cells that are responsive to neuronal activity and L-glutamate. Following the presynaptic neuronal release of L-glutamate into the synapse, L-glutamate can activate a variety of iGluRs and mGluRs in postsynaptic and presynaptic neurons as well as in glial cells. Through elevating intracellular Ca\(^{2+}\) concentration in astrocytes, L-glutamate can lead to release of gliotransmitters (e.g., L-glutamate and D-serine) into the synaptic cleft (Volterra and Steinhauser, 2004; Perez-Alvarez and Araque, 2013). In this way, astrocytes can cause feedback regulation to neuronal activity and synaptic efficacy, which is known as neuro-glial communication.

Stimulation of NMDA receptors in astrocytes is suggested to cause glutamate release and subsequent extrasynaptic NMDA receptor neuronal currents. These long-lasting neuronal NMDA receptor currents are seen upon short burst stimulation in the hippocampus (Lozovaya, et al., 2004; Costa, et al., 2009). However, short burst stimulation by itself cannot lead to the long-lasting neuronal NMDA receptor currents.
Thus, glutamate release from astrocytes during repetitive synaptic stimulation in the CA3-CA1 hippocampal synapse might account for the extrasynaptic NMDA receptor currents.

The synaptic release of L-glutamate is suggested to couple neuronal activity to glucose uptake and utilization by astrocytes. Also, L-glutamate is reported to stimulate glucose transporter in astrocytes (Magistretti, 2009). Na\(^{2+}\) and Ca\(^{2+}\) co-signaling, which can be mediated by NMDA receptors, is reported to be essential for the stimulation of glucose transporter in cultured astrocytes (Porras, et al., 2008). Thus, a potential role of NMDA receptors in astrocytes is to mediate cations influx and subsequently stimulation of glucose transporter. This enhancement of the glucose transporter then supports activity-induced increases in oxidative metabolism and the generation of lactate which is then shuttled to neurons to provide an energy source for active neurons (Pellerin and Magistretti, 1994).

### 3.6 Roles of NMDA Receptors in Inhibitory Interneurons

Ionotropic GluRs play a critical role in the development and function of GABAergic inhibitory interneurons. The loss of specific iGluR subunits causes an imbalance of inhibitory–excitatory circuits and sometimes lethality (Akgul and McBain, 2016). GABAergic inhibitory interneurons are a critical component of brain network processing. By activating the GABAergic NMDA receptors, excitatory neurons can recruit the inhibitory interneurons in network processing and synchrony of the neuronal activity (Carlen, et al., 2012), which is a fundamental mechanism to process information and drive behavioral responses.
GABAergic interneurons can sense and respond to the activity of local neuronal circuits. Several excitatory outputs can converge when recruiting cortical or hippocampal interneurons. For example, interneurons in the cortex receive glutamatergic input from many different sources (e.g., local principal neurons and subcortical afferent pathways). Upon activation, basket cell interneurons provide a strong simultaneous inhibition of excitatory neurons in their regions. This action serves to synchronize excitatory activity that follows this synchronous inhibition (Klausberger and Somogyi, 2008). Thus, inhibitory interneurons can regulate and synchronize the activity of neural circuits with high spatiotemporal precision.

4. Pathology of NMDA Receptors Dysfunction

As described above, NMDA receptors play a central role in normal brain function. However, dysregulation of NMDA receptors can lead to many pathophysiological conditions (Hansen, et al., 2017; Zhou and Sheng, 2013). After identifying glutamate as a neurotransmitter, it was observed that high amounts of L-glutamate could induce toxic cellular signaling pathways, resulting in the impairment of neuronal health and neural cell death (i.e., excitotoxicity) (Choi, 1987; Rothman and Olney, 1995).

In acute pathological conditions (e.g., ischemia, and traumatic brain injury), there is a large increase in extracellular glutamate concentration and impaired glutamate re-uptake which can cause high Ca\(^{2+}\)-influx through NMDA receptors and neuronal death (Bullock, et al., 1998). Under chronic disease conditions (e.g., Parkinson’s disease, Alzheimer’s diseases, and Huntington's disease), neurons are more susceptible to the NMDA receptor-mediated excitotoxicity, which could impair the neuronal health over many years (Milnerwood and Raymond, 2010). Also, NMDA receptors are implicated in
the pathophysiology of some psychiatric disorders, such as schizophrenia and mood disorders (Yamamoto, et al., 2015). Thus, NMDA receptors are considered to be a potential causative feature, exacerbating component, or therapeutic target for numerous neurological conditions.

4.1. Schizophrenia

Schizophrenia has been hypothesized to be a result of enhanced dopamine activity; however, dopamine receptor antagonists are only effective to treat positive symptoms (e.g., hallucinations and delusions). Unfortunately, dopamine antagonists are not effective to treat negative symptoms (e.g., social withdrawal and lack of emotions) and cognitive deficits (Laruelle, 2014).

NMDA receptor dysfunction in schizophrenia has been proposed based on the clinical observation that NMDA receptor channel blockers, phencyclidine (PCP) and ketamine, produce side effects that strikingly resemble positive, negative, and cognitive symptoms of schizophrenia (Krystal, et al., 1994). Genetic studies in humans and animal models of schizophrenia have provided further support for the NMDA receptor hypofunction hypothesis of schizophrenia (Sodhi, et al., 2011; Tarabeux, et al., 2011; Ayalew, et al., 2012). Thus, NMDA receptor hypofunction model of psychosis has the potential to better explain schizophrenia than dopamine hyperactivity. This model hypothesizes that schizophrenia symptoms are caused by lower than normal glutamatergic activity in some brain circuits due to reduced NMDA receptors signaling (Hasan, et al., 2014). Consequently, selective NMDA receptor modulation in key brain circuits may potentially treat the spectrum of schizophrenia symptoms. The NMDA receptor hypofunction hypothesis for schizophrenia has driven research to discover
NMDA receptor modulators that can overcome the schizophrenia symptoms. Subunit-selective potentiators of NMDA receptors are suggested only to affect cells and brain regions that are associated with the disorder, reducing the possibility of developing side effects by affecting normal brain function.

4.2. Stroke and Traumatic Brain Injury

Stroke and traumatic brain injury (TBI) appear to cause sustained elevated levels of the extracellular L-glutamate which results in excitotoxicity and acute neuronal death. The high extracellular L-glutamate concentrations induce high Ca\(^{2+}\) influx and NMDA receptor-mediated excitotoxicity. Consistently, using NMDAR blockers have shown neuronal protection against ischemia in vitro and in vivo (Simon, et al., 1984). However, NMDA receptor subtypes seem to play different roles in excitotoxicity and neuronal death. Selective GluN2B antagonists are reported to block ischemic cell death, whereas activation of GluN2A-containing NMDA receptors is suggested to promote recovery after the ischemia (Liu, et al., 2007; Huang, et al., 2017; Tang, et al., 2018). Using GluN2C-deficient mice, few studies are done to investigate the role of GluN2C receptor subtype in ischemic stroke (Kadotani, et al., 1998; Chung, et al., 2016; Holmes, et al., 2018). However, whether GluN2C receptor subtype mediates beneficial or deleterious effects in ischemic stroke is controversial. Taken together, these distinct roles of NMDA receptor subtypes in ischemic stroke may explain the failure of NMDA receptor antagonists to protect against neuronal death in the clinical setting, and show the need to use a combination of NMDA receptor subtype-selective blockers and potentiators (Beauchamp, et al., 2008; Lai, et al., 2011). The effects of NMDA receptors in stroke and TBI seem to involve two phases: a short phase of excessive NMDA receptors activation
which contributes to neuronal death and a subsequent longer phase where adequate activation of NMDA receptors is critical for recovery brain (Biegon, et al., 2004). This later finding may help account for the failure of NMDA receptor antagonists in clinical trials for neuroprotection in stroke.

4.3. Alzheimer's Disease

NMDA receptors play a critical role in synaptic plasticity, learning, and memory. On the other hand, soluble oligomeric forms of amyloid-β (Aβ) peptides appear to perturb synaptic function (Pozueta, et al., 2013; Mota, et al., 2014). Therefore, it is suggested that effects of Aβ peptides on NMDA receptors cause dysfunction of the receptors leading to Alzheimer's disease (AD). For example, Aβ peptides are reported to impair LTP and facilitate LTD (Ondrejcak, et al., 2010). Recent studies have shown that Aβ requires the activation of GluN2B-containing NMDA receptors to affect synaptic function and plasticity, as GluN2B antagonists corrected the Aβ-induced changes in LTP and LTD. The extrasynaptic NMDA receptors, which are largely GluN2B-containing, are suggested to mediate the effects of Aβ peptides in the brain (Li, et al., 2011). However, the involvement of the other NMDA receptor subtypes (other than GluN2B) in AD and synaptic dysfunction is also suggested, because blocking GluN2C/GluN2D NMDA receptors by PPDA is reported to eliminate Aβ-induced LTP deficits in the hippocampal slices of rats (Zhang, et al., 2013).

4.4. Conclusions

Dysfunction of NMDA receptors is largely implicated in several major neurological disorders. The composition of NMDA receptor subunits could contribute
differentially to CNS disorders. To better understand the pathophysiology and the potential treatment for the NMDA receptor-related disorders, it is critical to determine which receptor subtypes compose NMDA receptors and which brain regions, brain circuits, type of cells, and subcellular location express NMDA receptors.
CHAPTER 2*

In the Telencephalon, GluN2C NMDA Receptor Subunit mRNA is Predominantly Expressed in Glial Cells

* Majority of this chapter is published (Alsaad, et al., 2018).
Introduction

As described in Chapter 1, NMDA receptors have important roles in cognition, working memory, and synaptic plasticity. NMDA receptors are also involved in some neurological/psychiatric disorders such as schizophrenia, traumatic brain injury, Alzheimer disease, epilepsy, and depression (Traynelis, et al., 2010; Yamamoto, et al., 2015). These complex actions of NMDA receptors are generated by a diverse family of NMDA receptor subunits which display distinct biochemical and physiological properties. NMDA receptors are tetrameric assemblies containing two GluN1 subunits and two subunits from the GluN2 (GluN2A-GluN2D) and/or GluN3 (GluN3A, GluN3B) subunit families. Receptors with different GluN2 subunits have distinct functional properties in terms of voltage-dependency, agonist affinity, deactivation kinetics, and desensitization properties (Ishii, et al., 1993; Mishina, et al., 1993; Buller, et al., 1994; Monyer, et al., 1994; Traynelis, et al., 2010). Variations in these properties, together with the distinct distributions of the GluN2 subunits, can account for much of the functional diversity of NMDA receptors.

To date, most studies of NMDA receptor function in the telencephalon have focused upon GluN2A and GluN2B subunits because these are the predominant GluN2 subunits in the forebrain. GluN2C-containing NMDA receptors are important for cerebellar function/motor coordination (Ebralidze, et al., 1996; Kadotani, et al., 1996) and thalamic reticular nucleus modulation of delta oscillations (Zhang, et al., 2012), but relatively little is known about the role of GluN2C-containing NMDA receptors in the telencephalon. While the distribution of GluN2C subunit expression has been well characterized, an important question remains as to their localization in the telencephalon.
GluN2C subunits are highly expressed in cerebellar granular cells, moderately expressed in various lateral thalamic nuclei and in the mitral cells and glomerular layer of the olfactory bulb, and weakly expressed in the telencephalon (cortex, hippocampus, amygdala, and striatum) (Watanabe, et al., 1993a; Monyer, et al., 1994). However, the identity of the cells expressing GluN2C in the telencephalon has been unclear. In the rodent telencephalon, GluN2C has a diffuse, non-laminar distribution pattern that is consistent with both interneurons and glial cells (Watanabe, et al., 1993a; Monyer, et al., 1994). Noting that the GluN2C-labelled cells were also found in the white matter and had small nuclei that stain well for hematoxylin, Watanabe and colleagues proposed that the cortical GluN2C-labelled cells were glia (Watanabe, et al., 1993a); see also (Landwehrmeyer, et al., 1995). However, RT-PCR studies reported that parvalbumin-positive cells display significant levels of GluN2C mRNA (Xi, et al., 2009). Additionally, other recent studies have used two different transgenic animals which express β-galactosidase (β-gal) as an indicator for cells expressing the GluN2C gene. These studies reported β-gal staining in neurons of the cerebral cortex (Binshtok, et al., 2006; Karavanova, et al., 2007). This localization is also consistent with layer IV neuronal labeling by GluN2C probes in human and monkey cortex (Scherzer, et al., 1998; Munoz, et al., 1999).

The purpose of this study was to take advantage of a GluN2C knockout (GluN2C-KO) mouse as a negative control and to use highly sensitive fluorescence in situ hybridization (FISH) together with multiple cell-specific markers to further define the distribution and identity of cells that express GluN2C subunit mRNA in the telencephalon. These results suggest that in the telencephalon, GluN2C mRNA is almost
exclusively expressed by non-neuronal cells. In the CNS, proteins and mRNAs can be transferred from one cell to another by exosomal transfer. Thus, a possible explanation for the expression of β-gal in cortical neurons, which is driven by the GluN2C promotor, is the exosomal transfer of β-gal mRNA from thalamic neurons that terminate on layer IV cortical neurons (White, 1978; Bernardo and Woolsey, 1987). To test this hypothesis, we examined the effect of inhibiting exosome release on the expression of β-gal/GluN2C in layer IV of the somatosensory cortex in GluN2C-KO mice. These results suggest that expression of β-gal/GluN2C in the mouse somatosensory is not mediated through intercellular exosomal transfer.

Methods

1. Detection and Colocalization of GluN2C mRNA

Animal and Section Preparation

Experiments were in accordance with University of Nebraska Medical Center’s Institutional Animal Care and Use Committee (IACUC) guidelines. Adult C57BL/6 wild-type mice and β-galactosidase knock-in (GluN2C-KO) mice (Karavanova, et al., 2007) on the same background, were used to identify GluN2C mRNA signal and confirm its specificity. Mice were sacrificed under deep isoflurane anesthesia and brains were removed and immediately frozen on powdered dry ice. Brains were cut at a thickness of 20 µm, thaw-mounted onto Superfrost Plus™ slides, and air-dried. Sections were fixed in ice-cold 4% paraformaldehyde, washed in phosphate-buffered saline (PBS), dehydrated in 70% ethanol, and stored in 95% ethanol at 4 °C.
cRNA Probe Design

Fluorescein-labeled and digoxigenin-labeled cRNA probes were designed to detect GluN2C mRNA in wild-type mice but not in GluN2C-KO mice. A fragment of rat GluN2C cDNA (360–2402 nucleotide residues; GenBank accession number, NM_012575.3) that corresponds to the deleted fragment in the GluN2C-KO mouse genome (Karavanova, et al., 2007) was subcloned into the pSPT 19 plasmid vector. GluN2C cRNA probes were prepared by in vitro transcription (DIG RNA Labeling Kit; from Roche), and fragmented by alkaline digestion as previously described (Angerer and Angerer, 1992).

Combined Fluorescence In Situ Hybridization and Immunohistochemistry

To detect GluN2C mRNA, sections were rehydrated in 70% ethanol and TNT buffer (0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl, and 0.05% Tween 20), acetylated in fresh solution of 0.25% acetic anhydride in 0.1 M triethanolamine-HCl, pH 8.0, for 10 min, and rinsed in TNT. Sections were prehybridized in hybridization buffer (50% formamide, 50 mM Tris–HCl, pH 7.5, 1 x Denhardt’s solution, 600 mM NaCl, 200 g/mL yeast tRNA, 1 mM ethylenediaminetetraacetic acid (EDTA), and 10% dextran sulfate) for 1 h. Sections were then hybridized with GluN2C cRNA probes-containing hybridization buffer at 63–67 °C overnight and then were washed stringently at 61–65 °C with frequent gentle agitation, as follow: 5 × saline-sodium citrate (SSC) for 10 min, 4 × SSC/ 50% formamide for 20 min, 2 × SSC/ 50% formamide for 20 min, and 0.1 × SSC for 10 min. After rinsing in TNT, sections were incubated in 2% H2O2 in TNT for 10 min, washed in TNT, and blocked with 5% bovine serum and normal goat serum/TNT for 1 h. Sections were incubated with peroxidase-conjugated anti-digoxigenin or anti-fluorescein antibody
(Roche Diagnostics, 1:100–1:200) diluted in TNB for 1 h, and washed. Cy3-TSA or fluorescein-TSA reagent (tyramide signal amplification plus kit, PerkinElmer) was used to develop the signal.

To combine FISH and immunohistochemistry (IHC) techniques in frozen sections, we used an adjusted protocol based on previous protocols (Yamasaki, et al., 2010; Chaudhuri, et al., 2013). After washing non-specific binding of cRNA probe and blocking sections in bovine serum and normal goat serum as described above, sections were incubated with peroxidase-conjugated anti-digoxigenin antibody (Roche Diagnostics, 1:100–1:200), mouse anti-NeuN antibody (Millipore, 1:100), and rabbit anti-glial fibrillary acidic protein (GFAP) antibody (Abcam, 1:1000) diluted in TNB at 4 °C overnight. TNT-washed sections were then incubated with Cy3-TSA (tyramide signal amplification plus kit, PerkinElmer), and washed. Sections were then incubated with Alexa 647-conjugated goat anti-mouse antibody (Invitrogen, 1:200) and Alexa 488-conjugated goat anti-rabbit antibody (Invitrogen, 1:200) for 2 h. Sections were then rinsed in TNT and distilled water before being sealed with Fluoroshield™ with 4,6-diamidino-2-phenylindole (DAPI) medium (Sigma–Aldrich) and a coverslip.

**Quantification of Signal and Colocalization**

Confocal fluorescent images were captured using a Zeiss confocal microscope. Z-series stack images were used for quantification and determination of colocalization. For quantitative analysis, cell or puncta counting was performed using 40X magnification. GluN2C mRNA puncta, NeuN-positive cells, GFAP-positive cells, and DAPI-positive nuclei were identified and counted manually one-by-one in a single channel display mode. Colocalization of GluN2C mRNA, NeuN-positive cells, GFAP-positive cells, and
GFAP/NeuN-negative cells was determined based on coincidence of these markers in the same cell as revealed by DAPI staining. Images shown here were projected using maximum intensity projection of Z-series stacks, and image brightness was adjusted using ImageJ program. Results are expressed as mean ± SEM.

2. Inhibition of Exosome Release in GluN2C-KOMice

Animal and Section Preparation

Adult male GluN2C-KO mice (Karavanova, et al., 2007) were intraperitoneally injected with 1.25 mg/kg GW4869 (Cayman Chemical, MI) or DMSO once a day for 6 days (Xu, et al., 2016). Mice were anesthetized with isoflurane prior to brain removal and freezing on powdered dry ice. Coronal brain sections were prepared at a thickness of 30 µm, thaw-mounted onto Superfrost Plus™ slides, and stored at -20 °C until processing.

Histochemical Detection and Analysis of β-galactosidase

Frozen brain sections were air-dried for 20 min at room temperature. Sections were fixed in ice-cold 4% paraformaldehyde for 5 min and washed in PBS. Sections were then rinsed in the washing buffer (2mM MgCl₂, 0.01% deoxycholate, and 0.02 % Nonidet P-40 in 0.1M phosphate buffer, pH 7.4), and incubated at 37 °C with 0.1% 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) (Sigma–Aldrich) and 0.4 mg/ml nitroblue tetrazolium (NBT) (Promega corp., WI) in the washing buffer. After washing in PBS, sections were dried and sealed with Permount mounting medium (Fisher Scientific Inc.) and a coverslip. Stained sections were imaged and analyzed using a quantitative image analysis system (MCID system, St. Catharines, ON, Canada). The staining intensity was measured in layer IV of somatosensory cortex. Absolute values
were normalized to the average of the staining intensity of the entire corresponding cortex. Data are expressed as mean relative ± SEM and analyzed using two-tailed student t-test.

Results

Specificity of Antisense RNA Probe to GluN2C mRNA

GluN2C mRNA distribution was studied in C57BL/6 wild-type mice and GluN2C-KO mice in which most of the GluN2C coding region was replaced by β-galactosidase. Since the GluN2C-KO mice have a fragment of the GluN2C gene left in their genome, we designed the antisense RNA probe so that its sequence only complements the deleted GluN2C exons in the GluN2C-KO mice. Non-isotopic in situ hybridization was performed using fluorescein-labeled and digoxigenin-labeled cRNA probes for GluN2C mRNA to determine GluN2C mRNA distribution in the brain. The probes displayed a signal for GluN2C mRNA that matches the previously described general GluN2C distribution in the brain (Monyer, et al., 1992; Watanabe, et al., 1992; Ishii, et al., 1993; Watanabe, et al., 1993a; Buller, et al., 1994). GluN2C mRNA signal was the strongest in the granular cell layer of the cerebellum and moderately strong in mitral cell and glomerular layers of the olfactory bulb. In the lateral thalamus, GluN2C mRNA signal level was low but higher than in striatum, hippocampus, and cortex. Importantly, cRNA probe signal discussed here was not observed in GluN2C-KO mice brain sections that were processed in parallel using the same FISH/IHC procedures and the same imaging conditions that detected GluN2C signal in wild-type brain (Figure 1).
Figure 1. Overall distribution and specificity of fluorescein-tagged (a, b), and digoxigenin-tagged (c–f) antisense RNA probe binding in horizontal mouse brain sections. a, c, GluN2C mRNA signal in wild-type mouse brain showing the highest expression levels in the granular layer of the cerebellum. b, d, GluN2C-KO mouse brain sections showing very low (b) and low (d) background staining in cerebellum and olfactory bulb that was independent of the tag used. e, f, Enlarged images of the cortex, hippocampus, and thalamus which are highlighted by the box in (c). Scattered GluN2C signal is displayed in these regions of wild-type mouse (e) but not in GluN2C-KO mouse (f). T, thalamus; Ctx, cortex; H, hippocampus. Positive staining in the telencephalon is more apparent in the enlarged, high resolution images of this figure. Scale bars: c, d, 1 mm; e, f, 500 µm
GluN2C mRNA Distribution and Cell-Type Identification in Telencephalon and Thalamus

Overall, GluN2C mRNA signal showed a greater density of labeled cells in the lateral thalamic structures in contrast to the signal in the telencephalon. In the cortex, the scattered, non-laminated GluN2C mRNA distribution was consistent with the distributions of cortical interneurons and glial cells. In preliminary experiments, we evaluated GluN2C colocalization with the vesicular glutamate transporters 1 and 2 (VGluT1 and 2) and glutamate decarboxylase 67 (GAD67) as markers for glutamate- and gamma-aminobutyric acid (GABA)-using neurons, respectively. We found no GluN2C colocalization with either of these signals (Figure 2). Thus, we focused further studies using a combination of FISH and IHC to characterize GluN2C colocalization with NeuN and GFAP as markers for neurons and astrocytes, respectively. As expected, the signal for GFAP and NeuN did not colocalize with each other under our conditions. In representative brain regions, we determined the percentage of NeuN-positive cells, GFAP-positive astrocytes, and GFAP/NeuN-negative cells that expressed GluN2C mRNA.

Thalamus

As previously reported, GluN2C mRNA was expressed by many thalamic nuclei (Watanabe, et al., 1993a). The signal of GluN2C mRNA was displayed in the mediodorsal nucleus (nuc.), the ventral posteromedial nuc. (VPM), the ventral posterolateral nuc. (VPL), the posterior thalamic nuc., and the reticular thalamic nuc. (RTN). GluN2C mRNA signal was lower in the midline thalamic nuc., and there was no signal in the medial and lateral habenular nuc. In the VPM/VPL and RTN, most NeuN-positive cells expressed GluN2C mRNA. GFAP-labeling was only strong in RTN,
Figure 2. GluN2C mRNA is not expressed in cortical excitatory neurons or inhibitory interneurons. GluN2C signal does not colocalize with GAD67 mRNA, VGlut1 mRNA, or VGlut2 mRNA in the cortex (arrows).
thus GFAP/GluN2C colocalization was only determined for this thalamic nucleus. Most of the RTN GFAP-positive cells were negative for GluN2C mRNA with only 20 ± 7% of the GFAP-labeled cells expressing GluN2C mRNA. The percentage of NeuN-positive cells that were also positive for GluN2C in the VPM/VPL, RTN, and midline thalamus were 94 ± 1.6%, 87 ± 3.2%, and 21 ± 3.9%, respectively (Figure 3).

**Cerebral Cortex**

Previous studies have shown that GluN2C mRNA hybridization signal is faint in the cerebral cortex and found in all cortical layers (Watanabe, et al., 1993a). Consistent with those findings, in the present study, specific GluN2C signal was seen for scattered cells without evidence of a layered distribution. Thus, the GluN2C signal is not as would be expected for pyramidal or stellate neurons in the cerebral cortex which have a layer-specific distribution. We quantified GluN2C mRNA distribution in the retrosplenial cortex and somatosensory cortex as examples of distinct cortical areas.

GluN2C mRNA signal was distributed throughout the cortical layers of the retrosplenial cortex and was not found in NeuN-positive cells. However, GluN2C mRNA was expressed in GFAP-positive cells and NeuN/GFAP-negative cells. GFAP-labeling was most strongly expressed in layer I of the retrosplenial cortex. Within this layer, 76 ± 1.8% of the GFAP-positive astrocytes expressed GluN2C mRNA. In contrast, GFAP-labeling was weak and showed a very low number of GFAP-positive astrocytes in the other layers. Thus, GluN2C/GFAP mRNA colocalization was not analyzed in these layers. However, the few GFAP-positive cells in these layers were usually also positive for GluN2C signal. Cells that were either negative or positive for NeuN were quantified in all layers; only NeuN-negative cells colocalized with GluN2C signal (Figure 4).
Figure 3. Expression and colocalization of GluN2C mRNA in thalamus. a, GluN2C mRNA colocalizes with NeuN-positive cells in RTN, VPM/VPL, and midline thal (arrows). GluN2C mRNA is expressed by a GFAP-positive cell in the RTN (arrowhead). b, Percent of NeuN-positive, GFAP-positive, or NeuN-negative/GFAP-negative cells that were also labeled by GluN2C probe in the three thalamic regions. Scale bar = 10 µm.
Figure 4. GluN2C mRNA expression in multiple layers of retrosplenial cortex. a, GluN2C mRNA is expressed in GFAP-positive cells in layer I (arrows). GluN2C mRNA (arrows/arrowheads) does not colocalize with NeuN-positive cells in any layer of the retrosplenial cortex. GluN2C mRNA colocalizes with NeuN-negative/GFAP-negative cells in the deep layers of the retrosplenial cortex (arrowheads). b, Percent of NeuN-positive, GFAP-positive, or NeuN-negative/GFAP-negative cells that were also labeled by GluN2C probe in different layers of RSC. Scale bar = 10 µm
As found for retrosplenial cortex, expression of GluN2C mRNA in the somatosensory cortex was found in GFAP-positive cells and GFAP-negative/NeuN-negative cells but not in NeuN-positive cells. GFAP-labeling for the somatosensory cortex displayed the same pattern as found for the retrosplenial cortex, only cells in the superficial layer were labeled strongly with GFAP. We found that 86 ± 2.2% of the GFAP-positive astrocytes expressed GluN2C mRNA. GFAP-labeling was again weak in the deeper cortical layers, and thus GluN2C/GFAP colocalization was not quantified in these layers. However, when GFAP was present, GluN2C was frequently co-expressed. Most GluN2C-positive cells in the deeper layers were GFAP-negative/NeuN-negative cells (Figure 5).

**Hippocampus and Dentate Gyrus**

The GluN2C mRNA signal was distributed throughout the hippocampus and dentate gyrus. As previously reported by others, the distribution of cells labeled by the GluN2C probe is inconsistent with GluN2C presence in the pyramidal or granular cells. GluN2C-positive cells were found at low density in all hippocampal layers (stratum oriens, stratum pyramidale, stratum radiatum, and stratum lacunosum-moleculare) and the dentate gyrus (granule cell layer, molecular layer, and hilus). NeuN-positive cells did not express GluN2C mRNA. GFAP-labeling was strong in both the hippocampus and dentate gyrus which allowed for quantification of GluN2C colocalization. GFAP was co-expressed with GluN2C mRNA by 72 ± 3.6% in the stratum radiatum and by 78 ± 5.4% in the molecular layer. Also, 7 ± 1.3% and 10 ± 2% of the NeuN/GFAP-negative cells expressed GluN2C mRNA in the radiatum and the molecular layer, respectively (Figure 6).
Figure 5. GluN2C mRNA expression in somatosensory cortex. a, GluN2C mRNA is expressed by GFAP-positive cells in layer I and layer VI (arrows). GluN2C mRNA does not colocalize with NeuN-positive cells in any layers of the somatosensory cortex (arrows/arrowheads). GluN2C mRNA colocalizes with NeuN-negative/GFAP-negative cells (arrowheads). b, Percent of NeuN-positive, GFAP-positive, or NeuN-negative/GFAP-negative cells that were also labeled by GluN2C probe in each of the layers of somatosensory cortex. Scale bar = 10 µm
a

GluN2C mRNA  GFAP  NeuN  DAPI  Merge

Layer I

Layer II/III

Layer IV

Layer V

Layer VI

b

% Co-localization with GluN2C

Layer I

Layer II/III

Layer IV

Layer V

Layer VI

NeuN+  GFAP+  NeuN/GFAP−
Figure 6. GluN2C mRNA expression in hippocampus. a, GluN2C mRNA colocalizes with GFAP-positive cells in stratum oriens, the pyramidal cell layer, stratum radiatum, the molecular layer, the dentate gyrus-granular cell layer, and the hilus (arrows). GluN2C mRNA does not colocalize with NeuN-positive cells in these regions. GluN2C mRNA colocalizes with NeuN-negative/GFAP-negative cells. b, Percent of NeuN-positive, GFAP-positive, or NeuN-negative/GFAP-negative cells that were also labeled by GluN2C probe in the stratum radiatum and the molecular layer. Scale bar = 10 µm
**Striatum, Amygdala, and Globus Pallidus**

GluN2C mRNA signal was scattered throughout the striatum, amygdala, and globus pallidus at a low density. In the striatum and amygdala, NeuN-positive cells were not co-labeled with GluN2C mRNA signal. In contrast, GFAP-positive cells frequently co-expressed GluN2C mRNA. As found for the deep layers of the cerebral cortex, GFAP staining was weak in the basal ganglia, but when present was frequently co-expressed with GluN2C mRNA. GluN2C signal was also found in 20 ± 2.5 and 24 ± 1.2% of GFAP-negative/NeuN-negative cells in the striatum and amygdala, respectively. Unlike the striatum and the amygdala, GluN2C mRNA was expressed by NeuN-positive cells in the globus pallidus. 47 ± 7.8% of the NeuN-positive cells and 2 ± 0.9% of GFAP-negative/NeuN-negative cells were found to express GluN2C mRNA (Figure 7).

**Inhibition of Exosomal Release Does Not Affect X-gal Staining in Layer IV of Somatosensory Cortex in GluN2C-KO Mice**

β-galactosidase was used as an indicator for cells expressing the GluN2C gene in two different transgenic mice. These mice showed that β-gal is expressed in neurons of the cerebral cortex (Binshtok, et al., 2006; Karavanova, et al., 2007). It has also been suggested that neurons of somatosensory cortex express functional GluN2C-containing NMDA receptor (Binshtok, et al., 2006). However, our findings here were unable to confirm that GluN2C mRNA is expressed in cortical neurons. Taken together, it is possible cortical neurons may have GluN2C subunit as protein but not as mRNA.

Accumulating evidence shows that membrane proteins and RNAs in many cell types are incorporated into small vesicles called exosomes. These exosomes are secreted into the extracellular space to discard membrane proteins or transfer them to other cells
Figure 7. GluN2C mRNA expression in striatum, amygdala, and globus pallidus. a, GluN2C mRNA colocalizes with GFAP-positive cells in the striatum and the amygdala (arrows) and with NeuN-negative/GFAP negative cells in the striatum (arrowhead). GluN2C mRNA colocalizes with NeuN-positive cells in the globus pallidus (arrow). b, Percent of NeuN-positive and NeuN-negative/GFAP-negative cells that were also labeled by GluN2C probe in the striatum, amygdala, and globus pallidus. Scale bar = 10 µm
The later mechanism is thought to be an important mode of intercellular communication in the CNS. In primary cortical cultures, neurons have been reported to release exosomes which contained AMPA receptors (Faure, et al., 2006). Although these exosomes did not contain GluN1 subunits (Faure, et al., 2006), it is still possible to have GluN2C subunits in exosomes because GluN1 is ubiquitously expressed in many cell types in the CNS, suggesting that there is no necessity for the exosomal transfer of GluN1 subunits. Since it is thought that β-gal/GluN2C subunits exist in cortical neurons and the subunit mRNA was not found, the exosomal transfer may mediate the β-gal expression. Since layer IV cortical neurons are innervated by GluN2C-containing thalamic neurons (Bernardo and Woolsey, 1987; White, 1978), the β-gal expression could be driven by exosomal release from presynaptic nerve terminals that innervate cortical layer IV neurons. So, we examined the effect of inhibiting exosome-release on β-gal expression in cortical neurons of GluN2C-KO mouse. To test that, GluN2C-KO mice were injected GW4869 (1.25 mg/kg, i.p.) or DMSO once a day for 6 days. This protocol has been shown to significantly reduce exosome release (Xu, et al., 2016). The intensity of X-gal staining in layer IV of somatosensory cortex was determined using the image analyzer MCID. GW4869-treated and DMSO-treated mice showed similar X-gal staining patterns in the brain, and equal staining intensity ratios (0.99 ± 0.01) (Figure 8).

**Discussion**

Studies have consistently shown that GluN2C mRNA is found in cerebellar granule cells, lateral nuclei of the thalamus, and in the mitral cells and glomerular layer
Figure 8. Quantification of X-gal staining in layer IV of the somatosensory cortex in GluN2C-KO mice. Mice were administered GW4869 (1.25 mg/kg) or DMSO once a day for 6 days. Data are expressed as mean relative ± SEM of three mice per group and analyzed using two-tailed student t-test. Statistical analysis showed no significant differences between groups (p > 0.05).
of the olfactory bulb. However, the identity of cells expressing GluN2C in the
telencephalon is less clear, and the literature is inconsistent. In the present study, we find
that essentially all of the GluN2C signal in the telencephalon is expressed in glial cells
and colocalized well with GFAP when GFAP was present. The only evidence that we
found for a neuronal localization of GluN2C in the telencephalon was for the globus
pallidus and the olfactory bulb. These results are consistent with the original
characterizations of GluN2C mRNA distribution (Watanabe, et al., 1993a; Monyer, et al.,
1994) which reported GluN2C in cells sparsely scattered throughout each region of the
telencephalon and that these cells are likely to be glial cells based on their small size and
dark staining by hematoxylin (Watanabe, et al., 1993a). In the same tissue sections,
GluN2C mRNA was expressed in neurons of the thalamus and cerebellum, thus both the
neuronal marker and GluN2C probe were staining as expected in these sections.

Other studies have suggested that GluN2C is expressed in cortical neurons. Using
a transgenic approach to express the β-gal reporter under control of the endogenous
GluN2C promotor, neurons of layer VI of the retrosplenial cortex were found to express
β-gal (Karavanova, et al., 2007). Similarly, in a different mouse construct, putting β-gal
under control of a large portion of the GluN2C promotor resulted in β-gal-expressing
neurons in layer IV of the somatosensory cortex (Binshtok, et al., 2006). This latter study
also provided evidence for functional GluN2C-containing NMDA receptor in neurons by
showing single channel properties indicative of GluN2C-containing receptors. Using the
GluN2C-KO/ β-gal mouse (Karavanova, et al., 2007) (the GluN2C knockout control in
this study), we find β-gal expression in both layer IV of somatosensory cortex and layer
VI of the retrosplenial cortex.
Proteins and RNAs are transferred between cells in the brain by exosomes (Smalheiser, 2007). Thus, this mode of intercellular communication could potentially explain the β-gal expression in layer IV of somatosensory cortex. However, inhibiting exosome release in GluN2C-KO mice by GW4869 did not decrease X-gal staining intensity in the somatosensory cortex. These findings suggest that exosomal transfer does not contribute to β-gal expression in cortical neurons. However, more experiments are required to confirm this conclusion and confirming that exosomal release was inhibited in the brain.

The possible expression of GluN2C in cortical layer IV is consistent with studies of monkey brain in which riboprobes for GluN2C specifically labeled layer IV of somatosensory cortex area 3b (Munoz, et al., 1999) and human brain studies where riboprobes labeled the granular layer of prefrontal cortex and visual cortex (Scherzer, et al., 1998). As has been suggested, neuronal layer IV GluN2C expression may be specific to primates (Scherzer, et al., 1998). In rodents, there is no evidence of layer IV-specific staining; GluN2C probe binding is uniformly scattered throughout the cortex (Watanabe, et al., 1992; Watanabe, et al., 1993a; Akazawa, et al., 1994b; Buller, et al., 1994; Rudolf, et al., 1996). Interestingly, stellate layer IV cortical neurons in a mouse model of tuberous sclerosis complex display thalamocortical synaptic responses with GluN2C-like pharmacological properties whereas wildtype mice do not (Lozovaya, et al., 2014). Thus, GluN2C expression in rodent stellate cells may normally be suppressed but can be expressed under pathological conditions. Perhaps GluN2C promoter driven β-gal expression escapes this regulatory suppression.
In this study, we used the GluN2C knockout as a negative control and a probe targeting the RNA sequence missing in the knockout to label GluN2C mRNA in wild-type mice. By using identical hybridization/imaging conditions for parallel experiments in wild-type and knockout mice, we were able to distinguish low levels of background staining from positive staining. This approach should greatly reduce any false positives due to the probe binding to non-GluN2C mRNA. However, since our GluN2C probe does have some complementary homology to GluN2A, B, D mRNA, defining conditions that eliminated this binding could conceivably have reduced the levels of probe binding to GluN2C mRNA. Thus, we cannot rule out that in some cases the absence of staining is a false negative, especially if the cell had very low levels of GluN2C mRNA. Using these conditions, probe binding displayed punctate staining near the nucleus as has been reported for GluN2D probes (Yamasaki, et al., 2014) and thus we do not appear to be detecting mRNA in dendrites or astrocyte processes.

GluN2C mRNA colocalized with GFAP in the telencephalon when GFAP was present. For telencephalic regions that poorly stain for GFAP (e.g. deep cortical layers), GluN2C signal was found predominantly in NeuN-negative/GFAP-negative cells, which are likely to be astrocytes that are not expressing GFAP. NeuN staining was robust in these regions but did not colocalize with GluN2C. Relatively few neuronal types are not stained by NeuN, and these are predominantly expressed outside of telencephalon (Mullen, et al., 1992; Sarnat, et al., 1998; Weyer and Schilling, 2003). It is also possible that some of the GluN2C+/NeuN-/GFAP- cells include oligodendrocytes or other glial cell populations. Overall, these results are consistent with the findings of RNA-Seq transcriptomes for the cerebral cortex (Zhang, et al., 2014; Mancarci, et al., 2017). From
these studies, GluN2C mRNA from the cerebral cortex was much more highly expressed in astrocytes than in oligodendrocytes, microglia, and neurons. Immunohistochemical studies indicate the presence of GluN1 and GluN2A and/or GluN2B in astrocyte processes (Conti, et al., 1996) while evaluation of RNA from isolated astrocytes indicates the presence of mRNA for GluN1, GluN2B, and GluN2C (Schipke, et al., 2001), or find mRNA for all NMDA receptor subunits but with relatively high levels of GluN2C and low levels of GluN2D expression (Dzamba, et al., 2015).

Electrophysiological studies in cortical and hippocampal astrocytes have demonstrated functional NMDA receptors in astrocytes, and these receptors can mediate neuron-to-glia communication (Lalo, et al., 2006; Palygin, et al., 2010; Letellier, et al., 2016). The astrocytic endfeet contain NMDA receptors and AMPA receptors and enwrap the synaptic clefts. By this mechanism, astrocytes can sense the glutamate concentration in the synapse. Stimulation of NMDARs in astrocytes can increase intracellular calcium levels (Palygin, et al., 2010; Verkhratsky, et al., 2012). In turn, this increase in intracellular calcium may potentially cause or enhance the calcium-dependent release of gliotransmitters such as ATP, D-serine, and L-glutamate into the perisynaptic region. D-serine/L-glutamate released from astrocytes appears to be released at sites directly opposing neuronal extrasynaptic NMDA receptors (Bezzi, et al., 2004) and thereby causing slow inward NMDA receptor-mediated currents in neurons (Parri, et al., 2001; Angulo, et al., 2004; Fellin, et al., 2004; Perea and Araque, 2005; Lee, et al., 2007).

Pharmacological studies support a role for functional NMDA receptors containing GluN2C and/or GluN2D subunits in astrocytes (Palygin, et al., 2011). Agonist-evoked NMDA receptor currents in cortical astrocytes display low sensitivity to magnesium
blockade and are especially sensitive to the partially-selective GluN2C/D antagonist UBP141 (Morley, et al., 2005; Palygin, et al., 2011). Similarly, cortical glial cell transmembrane NMDA receptor currents activated by stimulating cortical neuronal afferents are likewise preferentially blocked by UBP141 and not by GluN2B-selective antagonists (Palygin, et al., 2011). Among NMDA receptor subunits, GluN2C, GluN1, and GluN3A display the strongest expression in cortical astrocytes as reported in RNA-sequencing/transcriptome databases (Cahoy, et al., 2008; Zhang, et al., 2014). Thus, it is possible that astrocytes express a heterotrimeric GluN1/GluN2C/GluN3A receptor, as suggested by (Palygin, et al., 2011) which would also impart low magnesium sensitivity, or GluN2C and GluN3A subunits may be in separate NMDA receptor complexes in the same or different astrocytes.

The ability of astrocyte NMDA receptors to elicit glutamate release and a subsequent extrasynaptic NMDA receptor neuronal current might account for extrasynaptic NMDAR currents that are seen upon short-burst stimulation in the hippocampus (Lozovaya, et al., 2004; Costa, et al., 2009). These long-lasting neuronal NMDA receptor currents are activated by glutamate spillover during repetitive synaptic stimulation in the CA3-CA1 hippocampal synapse. This response is especially sensitive to PPDA, UBP141, and ifenprodil, suggesting involvement of GluN2C/D and GluN2B subunits. The present study suggests the possibility that GluN2C in astrocytes could be responding to the glutamate spillover to cause glutamate release from astrocytes which in turn may activate neuronal extrasynaptic GluN2B-containing receptors (Figure. 9).

GluN2C-containing NMDA receptors have 3 properties that make them ideal as detectors of synaptic L-glutamate spillover. They display a high affinity for L-glutamate
and glycine/D-serine (Kutsuwada, et al., 1992; Ishii, et al., 1993; Monyer, et al., 1994) so they can detect low, extrasynaptic concentrations of L-glutamate. They are also weakly inhibited by Mg$^{2+}$ so their channel activity does not require coincident depolarization as do GluN2A or GluN2B-containing NMDA receptors (Kutsuwada, et al., 1992; Monyer, et al., 1992; Ishii, et al., 1993; Monyer, et al., 1994). Thirdly, GluN2C-containing NMDA receptors do not desensitize (Monyer, et al., 1994; Krupp, et al., 1996), so these receptors would stay responsive to slowly changing or tonic extracellular L-glutamate.

Taken together, these results suggest that astrocytes with GluN2C-containing NMDA receptors can modulate the excitability of neuronal NMDA receptors throughout the telencephalon. Since schizophrenia is thought to reflect NMDA receptor hypofunction, it is interesting that the expression of GluN2C mRNA in the dorsolateral prefrontal cortex was reduced in people with schizophrenia (Beneyto and Meador-Woodruff, 2008) and that GluN2C knockout mice display a phenotype resembling schizophrenia (Gupta, et al., 2016). Therefore, the modulation of GluN2C function may represent a new target to treat schizophrenia.
Figure 9. Schematic diagram showing a potential role for GluN2C-containing NMDA receptors in the tripartite synapse. (1) L-Glutamate released from presynaptic nerve endings activates AMPA receptors and GluN2A-and/or GluN2B-containing NMDA receptors (NMDAR-2A/2B) on the postsynaptic dendrite. (2) With sufficient synaptic activation, glutamate spillover activates GluN2C-containing NMDA receptors (NMDAR-2C) and metabotropic glutamate receptors (mGluR) on the astrocyte, leading to increased cytoplasmic calcium levels from extracellular and intracellular sources, respectively. (3) In response to elevated intracellular Ca\(^{2+}\) from intracellular stores, and possibly from GluN2C currents, astrocytes release glutamate (and other gliotransmitters) which acts on extrasynaptic GluN2B-containing NMDA receptors (NMDAR-2B) on the postsynaptic structure on the same neuron, and potentially additional neurons, to modulate excitability and synaptic plasticity.
CHAPTER 3*

GluN2D NMDA Receptor Subunit mRNA is Widely Expressed in GABAergic Inhibitory Interneurons in the Telencephalon

* Some data in this chapter is published (Alsaad, et al., 2018).
Introduction

As described in Chapter 1, NMDA receptors are composed of a variety of the receptor subunits (GluN1, GluN2, and GluN3 subunits). The GluN2 subunits (GluN2A-D) are known to have distinct distributions throughout the brain during development and in adulthood. These receptor subunits are also different in terms of their physiological properties (Traynelis, et al., 2010; Hansen, et al., 2017). Among the GluN2 receptor subunits, little is known about the function of GluN2C and GluN2D subunits in the brain. Knowing the regional distribution, the specific cellular localization, and the physiology of the GluN2 subunits is critical to understanding how different GluN2-containing NMDA receptors function in the brain.

The unusual physiological properties of the GluN2D subunit allow this subunit to dramatically affect NMDA receptor function. Receptors containing the GluN2D subunit show unique properties, specifically high affinity for L-glutamate and glycine, very slow deactivation kinetics, low open probability, reduced Ca\(^{2+}\) permeability, and reduced Mg\(^{2+}\) sensitivity (Ishii, et al., 1993; Mishina, et al., 1993; Buller, et al., 1994; Monyer, et al., 1994). How these properties affect CNS function depends critically on the regional and cellular location of the GluN2D subunits.

The distribution of GluN2D subunit mRNA in the brain has been reported by numerous studies, however, the existence of the GluN2D mRNA in some brain regions (e.g., cerebral cortex) is still controversial. Furthermore, the type of cells that express the GluN2D subunits is unclear. In the embryonic brain, GluN2D mRNA is expressed widely throughout the brain with highest levels in the diencephalon and brainstem. After birth, the level of GluN2D expression is markedly changed in the brain with a reduction in the
telencephalon. In adult brain, GluN2D mRNA appears largely restricted to the thalamus, 
the glomerular layer of the olfactory bulb, and the midbrain (Watanabe, et al., 1993b; 
Akazawa, et al., 1994a; Monyer, et al., 1994). Among the thalamic nuclei, the GluN2D 
subunits show higher mRNA expression in the paraventricular nucleus, the paratenial 
nucleus, the laterodorsal geniculate nucleus, the medial geniculate (medial and ventral 
divisions) nucleus, the central medial nucleus, the nucleus reuniens, and the rhomboid 
nucleus. On the other hand, GluN2D mRNA displays a low density, scattered distribution 
in different regions of the telencephalon, such as cerebral cortex, hippocampus, and 
striatum (Watanabe, et al., 1993a).

The low expression of the GluN2D mRNA in the telencephalon has made it 
difficult to distinguish the GluN2D signal from background staining levels in the brain 
sections. GluN2D mRNA has a diffuse, non-laminar distribution pattern that is consistent 
with both interneurons and glial cells in the rodent cortex, hippocampus, and striatum. 
Noticing the distribution pattern of the GluN2D-labeled cells in the internal granule cell 
layer of the hippocampal formation, it was proposed that these GluN2D-labeled cells 
were interneurons (Monyer, et al., 1994). Later, using double-label ISH, GluN2D mRNA 
was found to be expressed in a group of neurochemically identified interneurons 
(including GAD67-positive cells and parvalbumin-positive cells) in the cortex, striatum, 
and hippocampus (Standaert, et al., 1996). More recently, GluN2D was found to be 
expressed in somatosensory interneurons (Yamasaki, et al., 2014) and hippocampal 
interneurons when detected in transgenic mice that express GluN2D receptor tagged with 
EGFP (von Engelhardt, et al., 2015). However, RT-PCR studies reported that cortical
parvalbumin-positive cells (a subclass of GABAergic interneuron) have no detectable GluN2D mRNA (Xi, et al., 2009).

In this study, a highly sensitive FISH was used together with multiple cell-specific markers to further determine the distribution and the cellular expression of the GluN2D mRNA in the telencephalon. Since GluN2C subunits also display a non-laminated, scattered distribution in the telencephalon, we also examined the cellular colocalization of GluN2C and GluN2D mRNA in different brain regions. These results suggest that in the telencephalon, the GluN2D mRNA is widely expressed in GAD67-positive cells and that was consistent with the non-overlapping of GluN2D mRNA with the GluN2C mRNA signal.

Methods

Animal and Section Preparation

This was previously described on pages 32-33 in Chapter 2.

cRNA Probe Design

The complete coding sequence of rat GluN2D cDNA (Accession number: L31611.1) was used to synthesize digoxigenin-labeled and fluorescein-labeled GluN2D cRNA probes. A fragment of Mouse 67 kDa-glutamic acid decarboxylase (GAD67) cDNA (980-2017 nucleotide residues; GenBank accession number, NM_008077) was subcloned into the pSPT 19 plasmid vector to synthesize digoxigenin-labeled GAD67 cRNA probe. GluN2D and GAD67 cRNA probes were prepared by in vitro transcription (DIG RNA Labeling Kit; from Roche), and fragmented by alkaline digestion as
previously described (Angerer and Angerer, 1992). Synthesis of digoxigenin-labeled GluN2C cRNA probe was previously described on page 33 in Chapter 2.

**Combined Fluorescence In Situ Hybridization and Immunohistochemistry**

To do the combined GluN2D FISH and GFAP/NeuN immunohistochemistry (IHC) in frozen brain sections, we used the protocol described on pages 33-34 in Chapter 2.

**Dual Fluorescence In Situ Hybridization**

Brain sections were incubated with GluN2D/GAD67 or GluN2C/GluN2D cRNA probes. Hybridization and stringent washing conditions were previously described in Chapter 2. After developing the GluN2D signal, sections were treated with 100 mM sodium azide to block residual peroxidase activity (King and Newmark, 2013), and incubated with the antibody that detects GluN2C or GAD67 cRNA probes. Signal was developed using Cy3-TSA or fluorescein-TSA reagent (tyramide signal amplification plus kit, PerkinElmer).

**Cellular Colocalization of GluN2D Signal with Other Markers**

This was previously described on pages 34-35 in Chapter 2.

**Results**

**Specificity of Antisense RNA Probe to GluN2D mRNA**

GluN2D mRNA distribution was studied in C57BL/6 wild-type mouse brain. GluN2D mutant/knock-out (GluN2D-KO) mouse (Ikeda, et al., 1995) was tested for possible use as a negative control. However, the mutant mouse showed a signal for the
GluN2D cRNA probe that has a pattern similar to the previously reported GluN2D distribution. This indicates the presence of detectable GluN2D mRNA in the GluN2D-KO mouse (data not shown). The GluN2D-KO mouse was generated by gene targeting technique, in which neomycin resistance gene was inserted into the *Grin2d* gene to disrupt the synthesis of functional GluN2D subunit mRNA and protein.

The GluN2D subunit mRNA is known to be very weakly expressed in the telencephalon. Thus, we used a full-length antisense RNA probe to label GluN2D mRNA with high sensitivity. Using non-isotopic *in situ* hybridization, fluorescein-labeled and digoxigenin-labeled cRNA probes for GluN2D mRNA were used to determine GluN2D mRNA distribution in the brain. GluN2D cRNA probes displayed a signal for GluN2D mRNA that matches the previously described general GluN2D distribution in the brain (Watanabe, et al., 1993a; Buller, et al., 1994; Monyer, et al., 1994). The GluN2D mRNA signal was strong in the midline thalamus and weak in the telencephalon regions, such as cortex, and hippocampus (Figure 1).

**GluN2D mRNA Distribution and Cell-Type Identification in Telencephalon and Thalamus**

Using conditions that generate the thalamus-specific GluN2D-like distribution, which has been reported in previous studies (Watanabe, et al., 1993a; Buller, et al., 1994), we identified the localization of GluN2D mRNA in neuronal and glial cells in different regions of the forebrain. Overall, GluN2D mRNA-labeled cells in the thalamic nuclei showed a higher expression signal compared to the labeled cells in the telencephalon. GluN2D mRNA signal in the cerebral cortex and hippocampus displayed a scattered distribution which is similar to the distributions of interneurons and glial cells.
Figure 1. Overall labeling pattern of GluN2D antisense RNA probe in a coronal mouse brain section. GluN2D mRNA displays higher signal in the thalamus than in the cortex and the hippocampus. T, thalamus; Ctx, cortex; H, hippocampus. MT, midline thalamus; RT, reticular thalamic nucleus; VBM/VPL, ventral posteromedial nucleus/ventral posterolateral nucleus. Scale bar = 1 mm. Scheme of mouse brain is modified from (Franklin and Paxinos, 2001).
By combining the FISH and IHC techniques, we identified the distribution of GluN2D mRNA in neurons (NeuN-positive cells) and astrocytes (GFAP-positive cells). As expected, GFAP and NeuN markers did not colocalize with each other under the conditions used here. Furthermore, we studied the GluN2D mRNA colocalization with GAD67 mRNA as a marker for inhibitory interneurons. Since a GluN2D-KO that does not express GluN2D mRNA was not available, we were not able to define precisely background staining levels of the GluN2D probe, and so we did not attempt a quantitative colocalization analysis as we performed for GluN2C.

**Thalamus**

Signal of the GluN2D cRNA probe in the thalamic complex showed the anatomically specific pattern of GluN2D staining with highest levels in the midline thalamus (paraventricular nucleus, central medial nucleus, nucleus reuniens, and rhomboid nucleus) and lower levels in lateral thalamus as others have reported for GluN2D mRNA (Watanabe, et al., 1993a; Monyer, et al., 1994). This specific distribution also corresponds to L-[³H]-glutamate binding sites that display a GluN2D-like pharmacological profile (Buller, et al., 1994). GluN2D mRNA was highly expressed in NeuN-positive cells in the central medial nucleus and to a lesser extent in the VPM/VPL and RTN. GFAP-positive cells showed no colocalization with GluN2D mRNA (Figure 2).

**Cerebral Cortex**

As previously reported, GluN2D mRNA signal displays a scattered distribution throughout all the layers of the cerebral cortex (Watanabe, et al., 1993a). The non-layered
Figure 2. Combined FISH and IHC showing neuronal expression of GluN2D mRNA in different thalamic structures. GluN2D signal colocalizes with NeuN-labeled cells in RTN, VPM/VPL nuclei, and midline thalamus (arrows). Note that GFAP staining is weak particularly in VPM/VPL nuclei, and midline thalamus. Scale bar = 10 µm
distribution of GluN2D mRNA that was found in different cortical regions is consistent with either the cortical distribution of interneurons or glial cells. To identify the type of cells that express the GluN2D mRNA in the cerebral cortex, we examined the neuronal and glial expression of GluN2D mRNA in the retrosplenial cortex and somatosensory cortex as examples of distinct cortical areas. We found that the NeuN-positive cells were also stained with the GluN2D probe in the retrosplenial cortex and somatosensory cortex. Although a much higher number of the GFAP-positive cells was detected in the superficial layers of the cortex but not in the other layers, these GFAP-positive cells did not express the GluN2D mRNA (Figure 3). The selective expression of the sparse GluN2D mRNA signal by the cortical neurons suggests colocalization of GluN2D mRNA with interneurons.

**Hippocampus and Dentate Gyrus**

The hybridization signal of GluN2D mRNA was sparsely distributed throughout the hippocampal formation as has been reported (Watanabe, et al., 1993a; Monyer, et al., 1994). Consistent with the distribution of interneurons, the internal granule cell layer of the dentate gyrus showed a higher density of GluN2D-positive cells compared to other areas in the hippocampal formation. We studied the type of cells that express the GluN2D mRNA in different hippocampal layers (stratum oriens, stratum pyramidale, stratum radiatum, and stratum lacunosum-moleculare) and dentate gyrus (granule cell layer, molecular layer, and hilus). In those regions, we found that GluN2D mRNA was selectively expressed in NeuN-positive cells and not in GFAP-positive cells (Figure 4).
Figure 3. Expression of GluN2D mRNA in neurons of retrosplenic cortex and somatosensory cortex. GluN2D mRNA colocalizes with NeuN-positive cells (arrows) in retrosplenic cortex (RSC) and somatosensory cortex (SSC) but not with GFAP-positive cells (arrowheads). Scale bar = 10 µm
**Figure 4. Neuronal expression of GluN2D mRNA in hippocampus and dentate gyrus.** GluN2D mRNA is expressed in NeuN-labeled cells (arrows) in hippocampus (stratum oriens, and pyramidal cell layer) and dentate gyrus (granule cell layer, molecular layer, and hilus). GluN2D mRNA does not colocalize with GFAP-labeled cells in these regions (arrowheads). Scale bar = 10 µm
**Striatum, Amygdala, and Globus Pallidus**

A low density, scattered distribution of GluN2D mRNA expression was detected in the striatum, amygdala, and globus pallidus. In these brain regions, GFAP staining was weak and only showed a low number of GFAP-positive cells. Nevertheless, cells that were stained with GFAP marker were negative for GluN2D signal. On the other hand, as found in the other brain regions, GluN2D mRNA signal intensely colocalized with NeuN-positive cells in the striatum, amygdala, and globus pallidus (Figure 5).

**GABAergic Inhibitory Interneurons Express GluN2D mRNA in Various Brain Regions**

From the data shown above, the distribution of the GluN2D mRNA in the cerebral cortex, hippocampus, and dentate gyrus was consistent with the distribution of the interneurons and glial cells in these brain regions. Also, the GluN2D mRNA was expressed in the RTN and globus pallidus which are areas known to be enriched by the inhibitory interneurons. Based on the colocalization of GluN2D mRNA signal in NueN-positive cells but not in GFAP-positive cells, it is very likely that the GluN2D mRNA is expressed widely in inhibitory interneurons. To examine that, we used double-labeling FISH to stain GAD67 mRNA and GluN2D mRNA as markers for GABAergic interneurons and GluN2D-expressing cells, respectively.

In the thalamus, GAD67-expressing neurons were present in the RTN and absent in the thalamic VB nucleus. This result is consistent with previous studies (Liu, et al., 1995) and suggests that the GAD67 cRNA probe is specific. These GAD67-expressing neurons were largely found to express GluN2D mRNA. In the telencephalon, the globus pallidus displayed intense colocalization of GluN2D mRNA with GAD67 mRNA. The
Figure 5. GluN2D mRNA expression and neuronal localization in striatum, amygdala, and globus pallidus. GluN2D signal colocalizes with NeuN-positive cells (arrows) in striatum, amygdala, and globus pallidus. GluN2D signal does not colocalize with GFAP-positive cells in amygdala (arrowhead). Scale bar = 10 µm
cortex and hippocampal formation showed GluN2D mRNA colocalized with scattered GAD67-positive neurons. Some of the GluN2D signal was also expressed in GAD67-negative cells, particularly in the cortex (Figure 6).

**Comparison between GluN2C and GluN2D mRNA Distribution in the Telencephalon and Thalamus**

Both GluN2C and GluN2D subunit mRNA displayed low levels of expression and were expressed in scattered cells in the hippocampus, striatum, and cerebral cortex. Thus, in these areas, the GluN2C and GluN2D distributions appear similar, and they may be expressed in the same cells. As shown above, we found that the GluN2D-positive cells were also stained with NeuN and not with GFAP in the cerebral cortex, hippocampus, striatum, and thalamus. In contrast, GluN2C mRNA colocalized with GFAP marker but did not colocalize with the neuronal marker in the telencephalon other than in the globus pallidus. The idea that GluN2C and GluN2D are expressed in distinct cell populations in the telencephalon (except for globus pallidus) was directly tested by colocalization of GluN2C and GluN2D probes. Examination of dual-labeled tissue section revealed no double-labeled cells in the cerebral cortex, hippocampus, or striatum. The GluN2C and GluN2D probes did, however, colocalize in the thalamus (reticular nucleus and ventral posterior nucleus) and the globus pallidus, regions where GluN2C is also expressed in neurons (Figure 7).
Figure 6. Colocalization of GluN2D and GAD67 mRNA in the forebrain. GluN2D mRNA colocalizes with GAD67-labeled cells (arrows) in RTN, perirhinal cortex, dentate gyrus (hilus), and globus pallidus. Note that GAD67 mRNA signal is not expressed in VPL. Scale bar = 20 µm
Figure 7. Localization of GluN2C and GluN2D mRNA in different forebrain regions. GluN2C and GluN2D mRNA do not colocalize in the cortex or the dentate gyrus. GluN2C and GluN2D mRNA display partial colocalization in the lateral thalamus (VPM/VPL). GCL, granule cell layer; H, hilus. Scale bar = 20 µm
**Discussion**

GluN2D mRNA is well known to be highly expressed in the embryonic brain. In the adult brain, the expression of the GluN2D mRNA is consistently found in the thalamus, the mitral cells of the olfactory bulb, and the midbrain (Akazawa, et al., 1994a; Monyer, et al., 1994). However, the presence of the GluN2D subunit in the adult telencephalon is controversial. Moreover, another question that does not have a consistent, clear answer is in what type of cells the GluN2D mRNA is expressed. In this study, the GluN2D mRNA was found in neurons but not in GFAP-expressing cells in the adult telencephalon (cortex, hippocampus, striatum, amygdala, and globus pallidus). The GluN2D-labeled neurons in these brain regions were largely found to be GABAergic inhibitory interneurons as demonstrated by GAD67 colocalization. This result is consistent with demonstrations that GluN2D expression is in GABAergic cells in the cerebral cortex and hippocampus (Yamasaki, et al., 2014; von Engelhardt, et al., 2015; Perszyk, et al., 2016). From our results, however, there could be a minor subpopulation of cortical pyramidal cells that express GluN2D mRNA.

The distribution of GluN2D mRNA was in contrast to what was found for GluN2C mRNA in the telencephalon (except for globus pallidus), where the GluN2C signal was predominantly expressed in glial cells and colocalized well with GFAP when GFAP was present as reported in Chapter 2. Consistently, GluN2C and GluN2D signals did not colocalize in the telencephalic regions other than globus pallidus. Conversely, GluN2C and GluN2D mRNA colocalized largely in the thalamic neuronal cells, which is a likely finding since both probes labeled NeuN-positive cells in the thalamus. Importantly, of the mouse genome, the *Grin2c* (GluN2C) and *Grin2d* (GluN2D) genes...
are more closely related to each other than any other gene. Consequently, non-specific probe labeling of other mRNAs would most likely cause a false positive of GluN2C/GluN2D colocalization and thus would not account for the dissimilar distributions seen for the GluN2C and GluN2D probes. Thus, the lack of GluN2C/GluN2D colocalization in cortex and dentate gyrus is consistent with the probes being specific (i.e., neither probe appears to be labeling the other’s mRNA). However, as described for GluN2C FISH in Chapter 2, GluN2D cRNA probe has some complementary homology to GluN2A/B/C mRNA, so using conditions that eliminate GluN2D probe binding to other GluN2 mRNA may reduce the levels of probe binding to GluN2D mRNA. Thus, we cannot rule out that in some cases the absence of staining is a false negative, especially when a cell has a very low level of GluN2D mRNA. The pattern of probe staining seen here is as reported for FISH of NMDA receptor subunits probes (Yamasaki, et al., 2014).

The existence of the GluN2D signal in the NeuN-positive cells and their absence in GFAP-positive cells is consistent with the data from the RNA-Seq transcriptomes studies (Zhang, et al., 2014; Mancarci, et al., 2017). According to these studies, GluN2D mRNA from the cerebral cortex and hippocampus showed much higher expression in neurons than in astrocytes. Furthermore, immunohistochemical studies of cortical astrocytes and evaluation of RNA from isolated astrocytes have revealed the absence of GluN2D subunit and the presence of other NMDA receptor subunits in astrocytes (Conti, et al., 1996; Schipke, et al., 2001).

As described in Chapter 1, GABAergic interneurons are known to express functional NMDA receptors based on the reported receptor properties in physiological
studies. Different types of NMDA receptors play an important, distinct role in synaptic plasticity and fine-tuning of neuronal connections. GluN2D-containing NMDA receptors are reported to mediate synaptic transmission in the hippocampal inhibitory interneurons using a pharmacological approach in brain slices which used GluN2D-KO mice to confirm their results (von Engelhardt, et al., 2015; Perszyk, et al., 2016). Stimulation of NMDA receptors in inhibitory interneurons could potentially trigger the release of the inhibitory neurotransmitter, GABA, into the synaptic cleft. In this manner, GluN2D-containing NMDA receptors can inhibit the function of local excitatory neurons.

In conclusion, the localization of GluN2D subunit mRNA in cortical and hippocampal GABAergic inhibitory interneurons can increase our understanding about the function of the NMDA receptor subtypes and potentially lead to new therapeutic targets for several brain disorders. For example, it is hypothesized that the hypofunction of NMDA receptors in the cortical interneurons underlies the pathophysiology of schizophrenia (Sodhi, et al., 2011; Tarabeux, et al., 2011; Ayalew, et al., 2012). GluN2D-KO mouse is reported to have schizophrenia-like symptoms (Sapkota, et al., 2015). So, our results support that dysfunction of GluN2D NMDA receptor subunits may be involved in causing schizophrenia. Thus, reagents that potentiate the function of GluN2D-containing NMDA receptors in inhibitory interneurons are a potential therapeutic for schizophrenia. Another example, normal learning and memory are associated with a finely tuned neuronal circuitry, which is mediated by a balance of excitatory and inhibitory inputs. In Alzheimer’s disease (AD), it is suggested that the disruption of excitatory/inhibitory balance in the brain leads to impaired memory function (Villette and Dutar, 2017). Consistent with that, it is suggested that reducing the
tonic current in interneurons (i.e., treating excitatory/inhibitory imbalance) may explain the therapeutic effect of the NMDA receptor blocker, memantine, in treating AD.

Additionally, it is reported that the selective GluN2C/GluN2D antagonist (UBP141) inhibited the tonic current in interneurons, whereas the selective GluN2C/GluN2D potentiator (CiQ) enhanced the NMDA receptor-mediated current in the interneurons (Riebe, et al., 2016). Thus, the findings here and in Chapter 2 suggest that the effects of UBP141 and CiQ are mediated by GluN2D-containing NMDA receptors in GABAergic interneurons, which could be a potential target to treat AD.
CHAPTER 4

Role of GluN2C-Containing NMDA Receptors in the Regulation of Metabolic Brain Activity
Introduction

GluN2C NMDA receptor subunits show a distinct distribution compared to the other GluN2 subunits. In the telencephalon, as shown in previous chapters, GluN2A, B, and D subunits are widely expressed in neurons, however GluN2C subunits are expressed almost exclusively in astrocytes. This distinct distribution of GluN2C subunits implies a very distinct function for GluN2C-containing NMDA receptors in the brain. Thus, the study in this chapter was aimed to evaluate a potential astrocyte-specific function for GluN2C in the telencephalon.

As described in Chapter 1, L-glutamate is the principal excitatory neurotransmitter and activates different types of receptors in the brain (including GluN2C-containing NMDA receptors) (Traynelis, et al., 2010). L-Glutamate is suggested to have a critical role in mediating neuronal oscillations, which are critical for normal brain function. Neuronal oscillations are rhythmic and repetitive frequency patterns that are generated during the activation of a group of neurons. The oscillatory activity generated by a synchronized, large cluster of neurons can be recorded by electroencephalograms. The recorded oscillatory data can be described according to their frequency and amplitude (Buzsaki and Wang, 2012). It is thought that neuronal oscillations, especially in the gamma frequency (30-120 Hz), positively correlate with glucose uptake and utilization by cells in the brain (Roy and Sherrington, 1890; Sokoloff, et al., 1977; Nishida, et al., 2008; Galow, et al., 2014).

Glucose is almost the exclusive metabolic energy source for cells, including neurons and astrocytes, in the brain. It is widely accepted that astrocytes express glucose transporters to uptake, and then metabolize glucose. On the other hand, neurons have a
less clear role in directly metabolizing glucose (Pellerin and Magistretti, 1994; Barros, et al., 2007; Jakoby, et al., 2014). There are two main hypotheses that describe how neurons get fuel from glucose: 1) the simple hypothesis is that neurons directly uptake glucose from the interstitium and produce ATP-energy by glycolysis and oxidative metabolism, and 2) the astrocyte-to-neuron lactate shuttle hypothesis. According to the second hypothesis, astrocytes take up glucose, which can be enhanced by neuronal activity, and convert glucose to lactate. Then, lactate is transported to neurons, oxidized, and used as a source of energy. Nevertheless, neuronal activation can increase glucose uptake and utilization in the brain (Hyder, et al., 1996). Consistent with this correlation, it has been shown that ketamine, which blocks NMDA receptors, induces a parallel increase in neuronal oscillations and glucose uptake in wild-type mice. In the same manner, both neuronal oscillations and glucose uptake show no significant changes after ketamine treatment in GluN2D-KO mice (Sapkota, et al., 2015).

GluN2C-containing NMDA receptors are reported to play an important role in neuronal oscillations. Ketamine treatment in GluN2C-KO mice resulted in a two-fold greater power of gamma oscillations relative to wild-type mice (Gupta, et al., 2016). Since ketamine induced a much higher increase in neuronal oscillations in GluN2C-KO mice compared to wild-type mice, we hypothesized that ketamine would have a significantly greater effect on glucose uptake in GluN2C-KO mice compared to wild-type mice. In other words, neuronal oscillations should correlate with glucose uptake in GluN2C-KO. To examine this hypothesis, glucose uptake was measured using autoradiographic analysis of $[^{14}C]$-2-deoxy glucose ($[^{14}C]$-2DG) uptake. The effect of ketamine on radioactive glucose density in different brain regions was evaluated for wild-
type and GluN2C-KO mice. Wild-type mice showed higher $[^{14}\text{C}]-2\text{DG}$ uptake than GluN2C-KO mice after ketamine treatment in the medial prefrontal cortex and striatum. However, wild-type and GluN2C-KO mice showed similar changes in the $[^{14}\text{C}]-2\text{DG}$ uptake after ketamine treatment in the somatosensory cortex, the hippocampus, and the entorhinal cortex. These findings revealed that wild-type and GluN2C-KO mice display a different correlation pattern between neuronal oscillations and glucose uptake. The regulation of glucose uptake in astrocytes by GluN2C-containing NMDA receptors may potentially contribute to the observed results, as discussed below.

**Materials and Methods**

**Chemical Reagents**

Ketamine HCl (100 mg/mL; Hospira, Lake Forest, IL) and 2-$[^{14}\text{C(U)}]-$Deoxy-D-Glucose ($[^{14}\text{C}]-2\text{DG}$) (PerkinElmer; Boston, MA) were used in this study.

**Animals**

Male adult wild-type C57BL/6 mice and β-galactosidase knock-in (GluN2C-KO) mice (Karavanova, et al., 2007) on the same background were used in this study. Wild-type and mutant mice genotypes were confirmed by DNA genotyping. Experiments were performed in accordance with University of Nebraska Medical Center’s Institutional Animal Care and Use Committee (IACUC) guidelines.

**Autoradiographic Analysis of $[^{14}\text{C}]-2\text{DG}$ Uptake: Experimental Protocol**

Regional brain uptake of $[^{14}\text{C}]-2\text{DG}$ was measured as an indicator for the activity of different brain regions as previously described elsewhere but for some changes
Wild-type and GluN2C-KO mice were given intraperitoneal injection of either ketamine (30 mg/kg) or 0.9% saline. Two minutes later, each mouse was injected with intraperitoneal injection of $[^{14}\text{C}]-2\text{DG}$ (0.11-0.16 µCi/g). Fifteen minutes after that, the mouse was sacrificed under deep isoflurane anesthesia and brains were removed and rapidly frozen on powdered dry ice. The mice brains were stored at -80 °C until further processing. Brains were sectioned horizontally at a thickness of 20 µm, thaw-mounted onto Superfrost Plus™ glass slides, and rapidly dried. Brain sections were stored at -20 °C or placed in autoradiography cassettes against Kodak BioMax MR Film (Carestream Health, Rochester, New York) together with C-14 standards (ARC146; American Radiolabeled Chemicals, St. Louis, MO) to quantify the tissue radioactivity. Autoradiography cassettes were kept for two to four weeks at 4 °C, and then films were developed. A quantitative image analysis system (MCID system, St. Catharines, ON, Canada) was used to analyze the developed films. The radioactive density was measured in different brain regions for each mouse brain. Absolute values from each brain region were normalized to the average of the radioactive density of the whole corresponding section.

**Statistical Analysis**

Data were analyzed using two-way analysis of variance (ANOVA) with multiple comparisons test by GraphPad Software. Data are expressed as mean relative ± SEM, and statistical significance was considered when the p-value was less than 0.05.
Results

Pattern of Metabolic Brain Activity Induced by Ketamine in Wild-Type and GluN2C-KO Mice

The quantitative autoradiography of the cellular uptake of $[^{14}\text{C}]-2\text{DG}$ can provide a measurement for glucose uptake in different brain regions. In each brain region, the autoradiography measurement positively correlates to the brain activity. Overall, mice within the same treatment group showed similar, consistent relative patterns of $[^{14}\text{C}]-2\text{DG}$ uptake between brain regions. Both wild-type and GluN2C-KO mice showed altered patterns of $[^{14}\text{C}]-2\text{DG}$ uptake in some brain regions after treatment with ketamine. These results are consistent with previous reports, where the relative $[^{14}\text{C}]-2\text{DG}$ uptake increased in several brain regions and reduced in other regions after ketamine treatment in wild-type rats and mice (Duncan, et al., 1999; Miyamoto, et al., 2000). Representative autoradiograms from the treatment groups are presented in Figure 1.

Several brain regions were quantified by measuring their radioactive density following the $[^{14}\text{C}]-2\text{DG}$ uptake. Standard curves for C-14 concentration were generated by measuring film optical density over the C-14 standards with known, varied C-14 concentrations. After calibration, experimental images were immediately captured, and the standard values were subsequently confirmed. For each brain region, the differences in the radioactive density between the treatment groups were used to examine the statistical significance (Figure 2). Using two-way ANOVA with Tukey's multiple comparisons test, a comparison between brain regions and animal groups showed a significant interaction effect ($p < 0.0001$), region effect ($p < 0.0001$), and animal group effect ($p = 0.0013$).
Figure 1. Effects of ketamine on regional $[^{14}\text{C}]$-2DG uptake in autoradiograms of mouse horizontal sections. Wild-type and GluN2C-KO mice were treated with ketamine (30 mg/kg) or saline before administration of the radioactive 2DG. Values on the scale bar (in nCi/mg) indicate the amount of radioactivity. mPFC, medial prefrontal cortex; SSC, somatosensory cortex; H, hippocampus; S, striatum; Ent, entorhinal cortex
Both wild-type and GluN2C-KO mice displayed similar $[^{14}\text{C}]-2\text{DG}$ distributions in the brain after saline or ketamine (30 mg/kg) treatment (Figure 1). Also, the relative $[^{14}\text{C}]-2\text{DG}$ values in the studied brain regions showed no statistical significance between the different genotypes after saline injection (Figure 2). However, after ketamine treatment, wild-type mice showed higher levels of $[^{14}\text{C}]-2\text{DG}$ uptake than GluN2C-KO mice in the medial prefrontal cortex and striatum. On the other hand, $[^{14}\text{C}]-2\text{DG}$ uptake in wild-type and GluN2C-KO mice showed similar changes in the somatosensory cortex, the hippocampus, and the entorhinal cortex after ketamine treatment.

As previously reported, treating wild-type mice with ketamine increased relative $[^{14}\text{C}]-2\text{DG}$ uptake in the medial prefrontal cortex (27.3%, $P < 0.0001$), the striatum (20.9%, $P = 0.0002$), and the hippocampus (15.31%, $P = 0.0298$), and reduced relative $[^{14}\text{C}]-2\text{DG}$ uptake in the somatosensory cortex (15.76%, $P = 0.0028$). GluN2C-KO mice showed increase in relative $[^{14}\text{C}]-2\text{DG}$ uptake in the medial prefrontal cortex (15.52%, $P = 0.0006$), the striatum (9.18%, $P = 0.0438$), the hippocampus (11.97%, $P = 0.0374$), and the entorhinal cortex (13.47%, $P = 0.0125$), and reduced relative $[^{14}\text{C}]-2\text{DG}$ uptake in the somatosensory cortex (10.94%, $P = 0.0104$) after ketamine treatment (Figure 2). The higher $[^{14}\text{C}]-2\text{DG}$ uptake in wild-type mice than GluN2C-KO mice after ketamine treatment was statistically significant in the medial prefrontal cortex ($P = 0.0016$) and striatum ($P = 0.0246$). Using two-tailed t-test, absolute values of $[^{14}\text{C}]-2\text{DG}$ density for the whole brain section showed no statistical significance between ketamine-treated ($n = 5$) and saline-treated wild-type mice ($n = 5$) ($0.4427 \pm 0.1488$ and $0.3966 \pm 0.1484$ nCi/mg, respectively; $P = 0.8319$) or ketamine-treated ($n = 8$) and saline-treated GluN2C-
Figure 2. Relative [14C]-2DG uptake in different brain regions of wild-type and GluN2C-KO mice. Data are ratios of radioactivity amount in the indicated brain regions relative to radioactivity of the corresponding section. Data are expressed as mean ± SEM and analyzed using two-way ANOVA with Dunnett's multiple comparisons test. Statistical significance is indicated by * (p < 0.05), ** (p < 0.01), *** (p < 0.001), and **** (p < 0.0001). mPFC, medial prefrontal cortex; SSC, somatosensory cortex; H, hippocampus; Ent, entorhinal cortex.
KO mice (n = 7) (0.2610 ± 0.01813 and 0.2986 ± 0.04004 nCi/mg, respectively; p = 0.4165), as previously reported (Duncan, et al., 1999; Sapkota, et al., 2015).

**Discussion**

In GluN2C-KO mouse, it has been reported that ketamine (30 mg/Kg) robustly increased the amplitude of cortical neuronal oscillations in the low gamma range (30-70 Hz) (Gupta, et al., 2016). Because neuronal oscillations are known to positively correlate with glucose uptake, it was expected to find here that [14C]-2DG uptake in the medial prefrontal cortex would be increased by ketamine administration more in GluN2C-KO mice than in wild-type mice. However, when compared to wild-type mice, the increase in [14C]-2DG uptake in GluN2C-KO mice was less than what would be anticipated based on the amplitudes of neuronal oscillations seen in these mice. GluN2C-KO mice show a two-fold increase in the ketamine-induced neuronal oscillations relative to the wild-type mice. However, GluN2C-KO mice showed less increase in [14C]-2-DG uptake (15.52%) compared to wild-type mice (27.3%) in the medial prefrontal cortex. Taken together, wild-type and GluN2C-KO mice showed different correlation patterns between neuronal oscillations and glucose uptake in the cortex.

Previously, it has been reported that GluN2C and GluN2D subunits are expressed in interneurons of the telencephalon. Consistent with GluN2D localization in interneurons, ketamine does not cause an increase in oscillations nor in [14C]-2-DG uptake in GluN2D-KO mice. The ability of ketamine to increase [14C]-2DG uptake in GluN2C-KO mice is consistent with our finding that GluN2C is not expressed in interneurons that are responsible for ketamine-induced neuronal oscillations. The
inability of GluN2C-KO mice to display a two-fold increase in [14C]-2DG uptake compared to wild-type mice suggests the possibility that GluN2C may have a role in glucose uptake in astrocytes. Thus, GluN2C-containing NMDA receptors in astrocytes may potentially contribute to these different correlation patterns.

Astrocyte processes are in close contact with neuronal synapses; this allows astrocytes to sense and respond to changes in neuronal synaptic activity (neuro-glial communication) (Ventura and Harris, 1999; Perea, et al., 2009). Numerous studies have reported that glutamate stimulates glucose transporters and glucose uptake in astrocytes. This relationship is suggested to underlie the coupling of neuronal activity and metabolic responses (glucose utilization) seen in functional brain mapping studies (Loaiza, et al., 2003; Magistretti, 2009). In such brain regions, the more active the neurons, the higher glutamate release into the synaptic cleft that could stimulate glucose transport into astrocytes, which in turn enhances glucose metabolism to support active neurons with lactate (Pellerin and Magistretti, 1994; Porras, et al., 2008).

Studies have investigated the mechanism by which glutamate triggers glucose uptake in astrocytes. It is thought to be dependent on the Na+-glutamate co-transporter. According to this mechanism, the electrochemical gradient of sodium is the driving force for the glutamate co-transporter. To transport one glutamate molecule, three Na+ ions enter the astrocyte. This results in transient sodium currents and a change in the intracellular sodium concentration, which is suggested to be a signaling mechanism to couple synaptic activity to metabolic process in astrocyte. By this proposed mechanism, the activation of Na+-K+ ATPase stimulates glucose uptake and glycolysis (Magistretti, 2009). Additionally, other studies reported an essential role of Ca2+ signaling, along with
glutamate, in the stimulation of glucose transport in astrocytes following synaptic activity (Porras, et al., 2008). Taken together, triggering glucose uptake in astrocytes requires the presence of glutamate, sodium, and calcium. These elements are known to be a function of glutamate receptors, and NMDA receptors in particular.

NMDA receptors are a likely target for glutamate to couple neuronal activity to glucose utilization by astrocytes. Particularly, GluN2C- and GluN2D-containing NMDA receptors have the characteristics that make them ideal detectors for synaptic activity (i.e., L-glutamate spillover). As described in Chapter 2, GluN2C- and GluN2D-containing NMDA receptors have a high affinity for L-glutamate, weakly inhibited by Mg\(^{2+}\), and do not desensitize. So, these NMDA receptor subtypes can be responsive to slowly changing L-glutamate concentrations and do not require coincident depolarization (Ikeda, et al., 1992; Kutsuwada, et al., 1992; Monyer, et al., 1992; Ishii, et al., 1993; Monyer, et al., 1994; Krupp, et al., 1996). These properties allow GluN2C-containing NMDA receptors in astrocytes to precisely sense the changes in the surrounding neuronal network activity and support neurons with lactate/energy. Using pharmacological reagents, a study has reported that NMDA receptors did not contribute to the glutamate-stimulated glucose transport in primary mouse astrocytes, which were cultured in vitro for 19-22 days, (Pellerin and Magistretti, 1994). However, the expression of NMDA receptors could be affected in the cultured cells. For example, expression of GluN1 mRNA has been reported to decrease in primary mouse astrocytes after 2 weeks in culture, while expression of GluN2A and GluN2B mRNA increased with time in culture (Zhou, et al., 2010). Consistent with this observation, we found that different batches of astrocyte cultures showed variable responses to NMDA receptor agonists, which were
recorded using Ca$^{2+}$ dyes (Figure 3). Therefore, NMDA receptors are still a potential mechanism for inducing glucose uptake in astrocytes; particularly, GluN2C-containing NMDA receptors because of their physiological properties and existence of GluN2C mRNA in astrocytes, as described in Chapter 2.

The results in this chapter also support the potential role of GluN2C-containing NMDA receptors in astrocytes in the regulation of glucose uptake. Although ketamine-treated GluN2C-KO mouse has higher neuronal oscillations than wild-type mouse, it showed a lower level of $[^{14}\text{C}]$-2DG uptake. In other words, there is a reduced capability for synaptic stimulation of glucose uptake in GluN2C-KO mice compared to wild-type mice. Therefore, lacking GluN2C subunit disrupted the correlation between neuronal activity and glucose utilization. So, these findings are consistent with a potential contribution of GluN2C-containing NMDA receptors in regulating glucose uptake in astrocytes in response to synaptic activity.

Knocking-out GluN2C subunit did not completely eliminate the ketamine-induced glucose uptake in the brain. So, other factors (e.g. transporters and mGluRs) may also play roles in regulating glucose uptake and contribute to the limited/ non-correlated increase in the ketamine-induced glucose uptake (Gutierrez Aguilar, et al., 2017).

Ketamine preferentially blocks GluN2C- and GluN2D-containing NMDA receptors (Kotermanski and Johnson, 2009), so if GluN2C does contribute to astrocyte glucose transporter activity, then ketamine may be limiting the increase in glucose uptake in wild-type mice. In such a condition, inducing neuronal oscillations by a reagent that does not block GluN2C in wild-type mice would result in higher levels of glucose uptake.
Figure 3. Calcium response in primary mouse astrocytes measured with Fluo-4 NW Calcium Assay Kit. Astrocytes were seeded in a 96-well black wall/clear bottom plate. A, B, Variable calcium response in different batches of primary mouse astrocytes.
relative to ketamine-treated wild-type mice and GluN2C-KO mice. This would also show
a more dramatic effect of GluN2C-containing NMDA receptors in regulating glucose
uptake in the brain.

In conclusion, findings in this study show that GluN2C- and GluN2D-containing
NMDA receptors are functionally distinct in the cerebral cortex. GluN2C-KO mouse can
support both enhanced neuronal oscillations and increased $[^{14}C]$-2DG uptake in response
to ketamine administration, whereas GluN2D-KO mouse eliminates both responses to
ketamine. However, ketamine does not cause as large an increase in $[^{14}C]$-2DG uptake as
predicted by the greater increase in gamma oscillations evoked by ketamine in the
GluN2C-KO than in the wild-type mouse. It is possible that this disparity is due to the
contribution of GluN2C-containing NMDA receptors to the regulation of glucose uptake
in astrocytes.
CHAPTER 5

Summary and Discussion
In the brain, neurons largely connect with each other through synaptic transmission. The vast majority of excitatory synaptic transmission is mediated by glutamate receptors, which are widely expressed in the brain. These receptors are primarily activated by L-glutamate. Glutamate receptors are classified into 1) iGluRs which act as ligand-gated ion channels and 2) mGluRs which act as G-protein coupled receptors (Niswender and Conn, 2010; Traynelis, et al., 2010; Willard and Koochekpour, 2013).

NMDA receptors are a subtype of iGluRs that mediate a major component of excitatory neurotransmission in the brain. NMDA receptors are tetraheteromeric complexes formed by subunits from three families (GluN1, GluN2A-D, and GluN3A, B subunits). Under physiological conditions, NMDA receptors play central roles in synaptic plasticity, learning, and memory. On the other hand, dysfunction of NMDA receptors is implicated in neurological diseases and psychiatric disorders such as Alzheimer’s disease, epilepsy, ischemic stroke, schizophrenia and depression (Zhou and Sheng, 2013; Yamamoto, et al., 2015; Hansen, et al., 2017).

Among NMDA receptor subunits, GluN2 subunits are thought to have the most impact on the heterogeneity of NMDA receptor function. The four GluN2 subunits have distinct physiological properties and anatomical distributions in the brain. GluN2 subunits are largely regulated by the same endogenous and exogenous compounds (e.g. L-glutamate, and ketamine), yet each subunit differently impacts how NMDA receptors response. These NMDA receptor subunits show different affinities to L-glutamate, sensitivities to Mg\(^{2+}\) block, deactivation kinetics, and desensitization properties. Anatomically, GluN2 subunits display distinct regional, cellular, and subcellular
distributions in the brain during development and adulthood (Ishii, et al., 1993; Mishina, et al., 1993; Monyer, et al., 1994; Buller, et al., 1994). GluN2A and GluN2B subunits, the predominant GluN2 subtypes in the forebrain, have been extensively studied in the past decades and well-known.

Unlike GluN2A and GluN2B, the distribution of GluN2C and GluN2D subunits in the telencephalon is still unclear and controversial. Particularly, the type of cells that express GluN2C and GluN2D subunits is inconsistent between studies that have been done so far. In the telencephalon, GluN2C subunits were suggested to be expressed in glial cells (Watanabe, et al., 1993a). However, later studies using newer techniques have suggested that GluN2C subunits are expressed in cortical neurons (Binshtok, et al., 2006; Karavanova, et al., 2007). Similarly, GluN2D subunits were thought to be expressed in interneurons of the telencephalon. However, other studies suggested that GluN2D subunits are not expressed in neuronal cells (Xi, et al., 2009).

To better understand the possible role of GluN2C and GluN2D subunits in the telencephalon, it is critical to identify their location within the neuronal circuitry. Therefore, FISH, together with multiple cell markers, was used to define the distribution and type of cells expressing GluN2C and GluN2D mRNA. GluN2C-KO mice were used as a negative control to confirm GluN2C mRNA signal and set levels of background staining in tissues. Also, to test a possible function of GluN2C-containing NMDA receptors in the cerebral cortex, we studied the effect of GluN2C subunits on the cellular glucose uptake in different brain regions.
In Chapter 2, we found that the GluN2C signal in the telencephalon was predominantly expressed in non-neuronal cells and colocalized well with GFAP staining when GFAP was present. The only evidence that we found for a neuronal localization of GluN2C in the telencephalon was for the globus pallidus and the olfactory bulb. These results are consistent with the original characterizations of GluN2C mRNA distribution (Watanabe, et al., 1993a) which reported GluN2C in cells sparsely scattered in the telencephalon and that these cells are likely to be glial cells based upon their small size (Watanabe, et al., 1993a).

GluN2C transgenic mice, which use β-gal under a GluN2C promotor, express β-gal in cortical neurons and thus these neurons were expected to express GluN2C subunit in wild-type mice (Karavanova, et al., 2007). These β-gal-positive neurons are in layer IV of the somatosensory cortex and are likely to be innervated by GluN2C-expressing thalamic neurons (White, 1978; Bernardo and Woolsey, 1987). It was possible that β-gal is transferred by exosome release from thalamic neurons to layer IV somatosensory cortical neurons (Smalheiser, 2007). This possibility was examined by treating GluN2C-KO mice with the exosome release inhibitor, GW4869. However, the intensity of X-gal staining in the somatosensory cortex was similar in DMSO-treated and GW486-treated GluN2C-KO mice. These findings suggest that exosomal transfer does not contribute to the β-gal expression in layer IV neurons of the somatosensory cortex. Alternatively, as we previously proposed, GluN2C can be expressed in these cells in pathological conditions but appear regulated in normal conditions (Scherzer, et al., 1998; Munoz, et al., 1999). Thus, β-gal under the GluN2C promotor may be escaping this regulatory inhibition.
In Chapter 3, we found that GluN2D mRNA was expressed in NeuN-positive cells but not in GFAP-positive cells in different regions of the adult telencephalon. The GluN2D-stained neurons in the telencephalon were largely found to be GABAergic inhibitory interneurons as demonstrated by GAD67 mRNA colocalization. This result is consistent with demonstrations that GluN2D is expressed in GABAergic cells in the cerebral cortex and hippocampus (Yamasaki, et al., 2014; von Engelhardt, et al., 2015; Perszyk, et al., 2016). Additionally, the signal of GluN2D mRNA was evaluated for colocalization with the signal of GluN2C mRNA. This comparison revealed no double-labeled cells in the cerebral cortex, hippocampus, or striatum. However, signals of GluN2C and GluN2D mRNA colocalized in the thalamus (reticular nucleus and ventral posterior nucleus) and the globus pallidus, regions where GluN2C is also expressed in neurons. Since Grin2c is the most homologous gene to Grin2d, the dissimilar distributions seen for the GluN2C and GluN2D cRNA probes in the telencephalon in the same section suggest that there is minimal false positive signal due to the GluN2D probe binding to non-GluN2D mRNA (or GluN2C probe binding to non-GluN2C mRNA). Thus, the lack of GluN2C/GluN2D colocalization in the telencephalon further confirms the specificity of probes.

In Chapter 4, because neuronal oscillations have been shown to correlate with glucose uptake, and ketamine induced a much higher increase in neuronal oscillations in GluN2C-KO mice compared to wild-type mice, we hypothesized that ketamine would have a significantly greater effect on glucose uptake in GluN2C-KO mice compared to wild-type mice (Roy and Sherrington, 1890; Sokoloff, et al., 1977; Nishida, et al., 2008; Galow, et al., 2014; Gupta, et al., 2016). Using autoradiographic analysis, the uptake of
[14C]-2DG in different brain regions was measured to evaluate the effect of ketamine on wild-type and GluN2C-KO mice. In the medial prefrontal cortex, we found that ketamine induced higher glucose uptake in wild-type mice compared to GluN2C-KO mice. These findings showed that wild-type and GluN2C-KO mice have different correlation patterns between neuronal oscillations and glucose uptake. GluN2C-containing NMDA receptors in astrocytes may potentially contribute to the regulation of glucose uptake.

In conclusion, GluN2C and GluN2D NMDA receptor subunit mRNA show scattered distributions and cell-type specific expression in the telencephalon. Expression of GluN2C mRNA in astrocytes and GluN2D mRNA in interneurons is very likely to underlie distinct functions of these NMDA receptor subunits in the brain.

Astrocytes and interneurons are suggested to regulate neuronal function by synchronizing the firing of excitatory neuronal networks in the brain. These two cell-types have features that enable precise synchrony of neuronal firing. Astrocytes show a scattered distribution in the brain, where they are organized in a non-overlapping tile-like manner (Bushong, et al., 2002). This pattern of cellular arrangement allows every astrocyte to connect with a different group of neurons. Also, it has been reported that a single astrocyte can enwrap a group of neuronal cell bodies, and contact thousands of synapses (Halassa, et al., 2007b). Physiologically, activation of a single astrocyte is reported to simultaneously induce responses in a few neighboring neurons (Angulo, et al., 2004; Fellin, et al., 2004). Furthermore, astrocytes are connected with each other by gap junctions (Nimmerjahn, et al., 2004), allowing communication between neighboring astrocytes to coordinate their activity. Similarly, interneurons display a scattered distribution in the telencephalon and connect with a local cluster of neurons. Also,
interneurons activity can be coupled by chemical synaptic transmission, gap junctions, or both (Swadlow, et al., 1998; Galarreta and Hestrin, 1999; Tamas, et al., 2000; Bruno, 2011). *In vivo*, activity of astrocytes and interneurons is reported to affect gamma oscillations (i.e. neuronal activity), where inhibiting glutamate release from astrocytes reduces gamma oscillations while astrocyte-released ATP can promote gamma oscillations (Fries, et al., 2007; Buzsaki and Wang, 2012; Lee, et al., 2014b; Tan, et al., 2017). Thus, astrocytes and interneurons have the potential to synchronize the neuronal firing of a cluster of neurons or spatially dispersed neuronal clusters.

To synchronize activity of neuronal firing, astrocytes and interneurons provide feedback to neighboring excitatory neurons. Bursts of excitatory synaptic transmission result in spill-over of L-glutamate from the synaptic cleft and stimulation of astrocytes processes, which surround excitatory synapses (Ventura and Harris, 1999; Lozovaya, et al., 2004; Halassa, et al., 2007a; Costa, et al., 2009). In turn, astrocyte depolarization can cause the release of gliotransmitters onto a local population of excitatory neurons, thereby providing a synchronizing signal (Parpura, et al., 1994; Perea, et al., 2009; Lee, et al., 2014b). Likewise, interneurons are stimulated by excitatory neurons and in turn provide a coordinated inhibitory signal to a local cluster of excitatory neurons, thus providing a synchronizing inhibitory signal (Cobb, et al., 1995; Buzsaki and Wang, 2012; Otsuka and Kawaguchi, 2013; Kann, et al., 2014). Thus, these astrocytes and interneurons can provide feedback and coordinate the firing of neuronal clusters.

As described in Chapter 2, GluN2C- and GluN2D-containing NMDA receptors are ideal detectors of synaptic L-glutamate spillover and generators of tonic currents. GluN2C- and GluN2D-containing NMDA receptors display a high affinity for L-
glutamate (Ikeda, et al., 1992; Kutsuwada, et al., 1992; Ishii, et al., 1993; Monyer, et al., 1994), which enable them to detect low concentrations of L-glutamate. Also, these receptor subtypes are less sensitive to Mg$^{2+}$ inhibition, so their channel activity does not require coincident depolarization (Ikeda, et al., 1992; Kutsuwada, et al., 1992; Monyer, et al., 1992; Ishii, et al., 1993; Monyer, et al., 1994). Furthermore, GluN2C- and GluN2D-containing NMDA receptors do not desensitize (Monyer, et al., 1994; Krupp, et al., 1996), so these receptors would stay responsive to slowly changing or tonic extracellular L-glutamate. Therefore, GluN2C- and GluN2D-containing NMDA receptors can allow astrocytes and interneurons to effectively sense and respond to surrounding neuronal activity. Accordingly, GluN2C-containing NMDA receptors could lead to glutamate release from astrocytes and subsequent neuronal activation, whereas GluN2D-containing NMDA receptors could result in activation of inhibitory interneurons to release GABA and subsequently inhibit excitatory neurons. In this manner, GluN2C- and GluN2D-containing NMDA receptors can have opposing modulating effects on neuronal oscillations.

NMDA receptors are reported to mediate persistent currents in several neuronal types at ambient glutamate concentration (Sah, et al., 1989; Cavelier and Attwell, 2005). Moreover, tonically active GluN2D-containing NMDA receptors are suggested to mediate persistent currents in hippocampal interneurons (Riebe, et al., 2016). Thus, GluN2C- and GluN2D-containing NMDA receptors can generate a long-lasting tonic current that changes the excitatory threshold for that cell (i.e., synchronizes neuronal firing) and maintain excitatory/inhibitory balance in the brain.
Taken together, the finding that GluN2C mRNA is expressed in astrocytes and GluN2D mRNA is expressed in interneurons, highlight a potential mechanism by which astrocytes and interneurons could be regulating the activity of neuronal networks. Since abnormal neuronal firing is a hallmark of neurological and psychiatric disorders, GluN2C- and GluN2D-containing NMDA receptors could be potential therapeutic targets for these disorders.
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