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Synaptic and Cellular Evaluation of NMDA Receptors in Health and Disease

By

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Graduate Division of Biological and Biomedical Science
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# Synaptic and Cellular Evaluation of NMDA Receptors in Health and Disease

By

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M.P.H., Environmental Health, Rollins School of Public Health, Emory University, 2016
B.S. Biology, The University of Georgia, 2013

Advisor: Stephen F. Traynelis, Ph.D.

An abstract of
A dissertation submitted to the Faculty of the
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Abstract

Synaptic and Cellular Evaluation of NMDA Receptors in Health and Disease

By Chad R. Camp

N-methyl-D-aspartate receptors are glutamate-gated, calcium-permeable ion channels involved in a host of normal brain functions, including neuronal development. To non-specialists, NMDARs are important neurotransmitter receptors involved in synaptic plasticity, mainly long-term potentiation. In this dissertation, however, expanded roles of NMDARs are described that have come to light thanks to recent technological advances in genetic sequencing, transgenic mouse model production, and small molecule drug design. The overarching goals of the work presented in this thesis are two-fold: 1) to provide cellular and synaptic evaluation of mouse models based on NMDAR human patient-derived variants and 2) show efficacy of subunit-selective NMDAR modulation in altering circuit function for potential therapeutic gain. In Chapter 1, various roles of NMDARs are reviewed and intended to highlight the true omnipresent nature of these receptors within the central nervous system. In Chapters 2 and 3, mouse models of human patient-derived NMDAR variants are used to explore the pathophysiology of disease at the cellular and circuit levels using patch-clamp electrophysiology and immunohistochemical techniques. Chapter 3 also highlights the importance of viewing animal model age as a potent experimental variable while providing novel supporting data for GluN2A's role in neurodevelopment and circuit maturation. In Chapter 4, a novel positive allosteric modulator is used to provide functional data on synaptic NMDAR subunit expression in groups of relatively understudied GABAergic interneurons. Overall, the work presented in this thesis is aimed at pushing the field of precision medicine forward via detailed cellular and synaptic analyses of mouse models of human disease, combined with novel subunit-selective NMDAR modulators.

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This thesis would not have been possible without the undying support from family, friends, mentors, and colleagues throughout my first 32 years.

First and foremost, I would like to thank Steve. I completed my lab rotations in Spring 2017, but knew I wanted to join Steve's lab in July 2016. I remember sitting in lab meeting early during my rotation and being completely captivated by the discussion amongst the lab during journal club. I had never been in a room with such intelligent people capable of entertaining multiple perspectives all at once. I was completely lost and that's exactly why I knew that I had to join his lab. I'd been interested in neuroscience research for some time, but my rotation was my first real exposure to electrophysiology. The ability to 'listen' to neuronal networks communicate in real time and then change their responses by applying a drug felt, and still feels, so visceral. He truly cares about his students, and his mentor style is perfectly suited for someone like me that enjoys pretending to be independent. Steve is an endless pool of scientific ideas and I have grown to thoroughly enjoy our caffeine-fueled chats, even when we disagree. We have cultivated a productive and professional relationship where I can feel free to share my ideas, regardless of their feasibility. Instead of immediately shutting down some of my more ludicrous ideas, he takes the time to ponder them before responding with very insightful suggestions about how we may be able bring them to fruition. He is (usually) always the first person to say, "Okay, just do it" and for that I am eternally grateful.

I would also like to thank my committee members: Drs. Ray Dingledine, Chris McBain, Shannon Gourley, and Gary Bassell for their mentorship, guidance, and encouragement. I have had the pleasure to interact with each of them individually and this thesis would not be presented how it is today without each of their respective talents and contributions to my overall training.

I also want to recognize that I would not be in the Emory Molecular and Systems Pharmacology PhD program if it were not for the helping hands of Dr. Mike Caudle for taking a chance on a Masters student with absolutely zero laboratory skills, Dr. Eddie Morgan for the initial unending support and letter of recommendation to joint our PhD program, and Dr. TJ Murphy for the many late night email conversations of support and blunt advice.

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Agonist binding domain (ABD);

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA);

(2)-amino-5-phosphonovaleric acid (APV);

Amino-terminal domain (ATD);

Artificial cerebrospinal fluid (aCSF);

Attention deficit/hyperactivity disorder (ADHD);

Autism spectrum disorder (ASD);

C-terminal domain (CTD);

Caudal ganglionic eminence (CGE);

Calcium/calmodulin-dependent protein kinase II (CamKII)

(3-Chlorophenyl)[3,4-dihydro-6,7-dimethoxy-1-[(4-methoxyphenoxy)methyl]-2(1H)-isoquinolinyl]methanone (CIQ);

Center for Functional Evaluation of Rare Variants (CFERV);

Central nervous system (CNS);

Cerebrospinal fluid (CSF);

Cholecystokinin (CCK);

Copy number variants (CNV);

Developmental and epileptic encephalopathy (DEE);

Effective concentration (EC);

Electroconvulsive therapy (ECT);

Epilepsy (EPI);

excitatory postsynaptic current (EPSC);

Exon 5 *GRIN1* gene splice variant (exon5);

Field excitatory postsynaptic potentials (fEPSPs);

γ-Aminobutyric acid (GABA);

G protein-coupled receptors (GPCRs);

Gain-of-function (GoF);

Intellectual disability (ID);

Inhibitory postsynaptic current (IPSC);

Interneuron-selective interneurons (ISIs);

Ligand binding domain (LBD);

Long-term depression (LTD);

Long-term potentiation (LTP);

Loss of Function (LoF);

Medial ganglionic eminence (MGE);

Messenger RNA (mRNA);

Metabotropic glutamate receptors (mGluRs)

Movement disorders (MD);

N-methyl-D-aspartate (NMDA);

N-terminal domain (NTD);

negative allosteric modulators (NAMs);

Neuronal nitric oxide synthase (nNOS);

Neuropeptide Y (NPY);

Parvalbumin (PV);

Postnatal (P);

Postsynaptic density (PSD);

Protein kinase A (PKA);

Protein kinase C (PKC);

Protein kinase G (PKG);

Residual Variation Intolerance Score (RVIS);

Reverse transcription PCR (rtPCR);

Schizophrenia (SCZ);

Single nucleotide polymorphism (SNP);

Small conductance calcium-activated potassium channels (SK channels);

Spontaneous inhibitory postsynaptic current (sIPSC);

Transmembrane domain (TMD);

Vasoactive intestinal peptide (VIP);

Whole exome sequencing (WES)

# CHAPTER 1: NMDA Receptors 101 – Structure, Function, Kinetics, Pharmacology, Expression, Roles in Normal Brain Function, and Genetic Variation

Sections of this introductory chapter are adapted from:

Hansen KB, Wollmuth LP, Bowie D, Furukawa H, Menniti FS, Sobolevsky AI, Swanson GT, Swanger SA, Greger IH, Nakagawa T, McBain CJ, Jayaraman V, Low CM, Dell'Acqua ML, Diamond JS, **Camp CR**, Perszyk RE, Yuan H and Traynelis SF (2021) Structure, Function, and Pharmacology of Glutamate Receptor Ion Channels. *Pharmacol Rev* **73**(4): 298-487.

and

Benke TA, Park K, Krey I, **Camp CR**, Song R, Ramsey AJ, Yuan H, Traynelis SF and Lemke J (2021) Clinical and therapeutic significance of genetic variation in the GRIN gene family encoding NMDARs. *Neuropharmacology* **199**: 108805.

*1.1 Glutamatergic Neurotransmitter Receptors*

The hallmark feature of the central nervous system (CNS) is the chemical synapse, a narrow cleft (~50 nm) in which two adjacent neurons communicate via released chemicals called neurotransmitters. These neurotransmitters bind to largely postsynaptic receptors, facilitating signal transduction. The most common neurotransmitter in the CNS is glutamate, which binds to both metabotropic and ionotropic receptor classes. Of the roughly 100 trillion-plus synapses in the adult human brain, upwards of 90% are thought to be glutamatergic (Abeles, 1991). Metabotropic glutamate receptors (mGluRs) are a family of ligand-gated G-protein coupled receptors (GPCRs), which can be further subdivided into two major categories: excitatory, Gq-coupled receptors from Group I ($mGluR_1$ and $mGluR_5$) and inhibitory, Gi/o-coupled receptors from Groups II and III ($mGluR_2$, $mGluR_3$, $mGluR_4$, $mGluR_6$, $mGluR_7$, and $mGluR_8$). Ionotropic glutamate receptors (iGluRs) are a family of ligand-gated cation-permeant ionotropic receptors which can be further subdivided into four different functional classes: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, kainate receptors, N-methyl-D-aspartate (NMDA) receptors, and GluD receptors (also known as δ (delta) receptors).

mGluRs and iGluRs are typically found within the same synapse yet produce distinct cellular responses on vastly different time scales. iGluR activation generally produces a rapid and measurable current response in the postsynaptic neuron, however, there is a growing appreciation of the metabotropic signaling ability of iGluRs. Given their ability to directly depolarize the postsynaptic cell, which is vital for further signal transduction, and rapid signal generation, iGluRs have been an important field of study for neuroscientists and cellular physiologists for decades. More specifically, NMDA receptors (NMDARs) are a particularly intriguing subtype of iGluR because of their unique activation requirements, calcium permeability, and roles in learning and

memory. The discovery of an NMDAR-dependent form of long-term potentiation – the cellular correlate to learning – has been a topic of intense investigation ever since the seminal findings by Graham Collingridge and colleagues in 1983 (Collingridge et al., 1983). In this dissertation expanded roles of NMDARs are described that have come to light thanks to recent technological advances in genetic sequencing, transgenic mouse model production, and small molecule drug design.

*1.2 NMDAR Subunit Stoichiometry and Structure*

The iGluR subunits share a common modular design and membrane topology despite having modest primary sequence identity (Paas, 1998; Wo and Oswald, 1995) (**Figure 1.1**). Each subunit includes a large extracellular region comprising the N-terminal domain (NTD, also referred to as amino-terminal domain, ATD), which mediates receptor assembly, trafficking, and functional regulation. In NMDARs, the *GRIN1* gene has eight different splice variants, with the most studied being the GluN1-1a splice isoform which includes residues encoded by exons 21 and 22, but lacks 21 amino acids in the NTD, encoded by exon 5 of the *GRIN1* gene (hereafter exon5). The exon5 variant lowers sensitivity to proton inhibition (Traynelis et al., 1995), promotes faster deactivation kinetics compared to receptors lacking exon5 (Regan et al., 2018; Rumbaugh et al., 2000; Vance et al., 2012; Yi et al., 2019a; Yi et al., 2018), and has a unique spatial expression pattern in the CNS gyrus (Laurie and Seeburg, 1994; Luque et al., 1994; Paupard et al., 1997). The agonist binding domain (ABD, also referred to as ligand binding domain, LBD), contains binding sites for agonists, competitive antagonists, and some allosteric modulators. Each ABD is composed of two segments of the polypeptide chain, S1 and S2, separated by two transmembrane helices (M1 and M3) and a membrane reentrant loop (M2). The M1-M2-M3 regions, together with a fourth transmembrane helix (M4) compose the transmembrane domain (TMD). The cytoplasmic C-

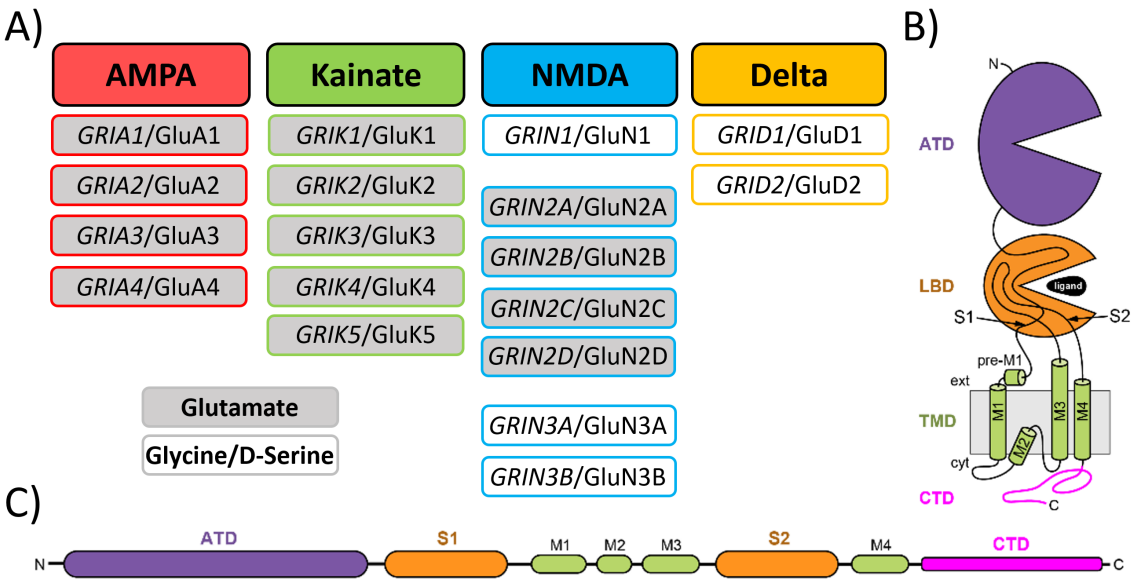


**Figure 1.1.** Ionotropic glutamate receptor functional classes and domain structure. A) All four ionotropic glutamate receptor classes, with each gene (italic font) and the protein encoded by that gene (standard font) listed. B) Cartoon showing the different domains of all ionotropic glutamate receptors, with C) each domain represented along the linear structure of the polypeptide chain. Adapted from (Hansen et al., 2021).

terminal domain (CTD) directs receptor localization and regulation, is the locus of post-translational modifications (*e.g.*, phosphorylation and palmitoylation), and varies in length and sequence among subunits.

All iGluRs are integral membrane proteins assembled from four large (>850 residues) multi-domain subunits to form an ion channel, that is, a central pore spanning the membrane through which ions can pass. NMDARs are heterotetrameric complexes, comprising two obligate GluN1 subunits (encoded by the *GRIN1* gene) and two GluN2A-D and/or GluN3A-B subunits (encoded by the *GRIN2A-D* and *GRIN3A-B* genes). Glutamate binding is required to activate most iGluRs, however, NMDARs require both glutamate (GluN2 subunits) and glycine/D-serine (GluN1 and GluN3A-B subunits), combined with membrane depolarization, for receptor activation. NMDARs can be classified as diheteromeric (comprised of two GluN1 subunits and two identical GluN2 or GluN3 subunits) and triheteromeric (comprised of two GluN1 subunits and two different GluN2 subunits, two GluN1 subunits and two different GluN3 subunits, or two GluN1 subunits combined with one GluN2 and one GluN3 subunits) assemblies (**Figure 1.2**). In native tissue, triheteromeric NMDARs comprising two GluN1 and two different GluN2 subunits are thought to be in the majority (Hansen et al., 2014; Karakas and Furukawa, 2014; Lee et al., 2014; Lu et al., 2017; Monyer et al., 1992; Ulbrich and Isacoff, 2007; Ulbrich and Isacoff, 2008) However, other variations of diheteromeric (Grand et al., 2018; Otsu et al., 2019; Zhu et al., 2020) and triheteromeric complexes (Perez-Otano et al., 2016) have been reported. Although the existence of GluN3-containing NMDARs in native tissue is unequivocal, the presence of GluN1/GluN2/GluN3 triheteromeric assemblies is controversial (see (Ulbrich and Isacoff, 2008) and (Perez-Otano et al., 2001). Moreover, given our tenuous understanding of how these seemingly constitutively desensitized glycine-binding receptors may be activated in neurons, they will be

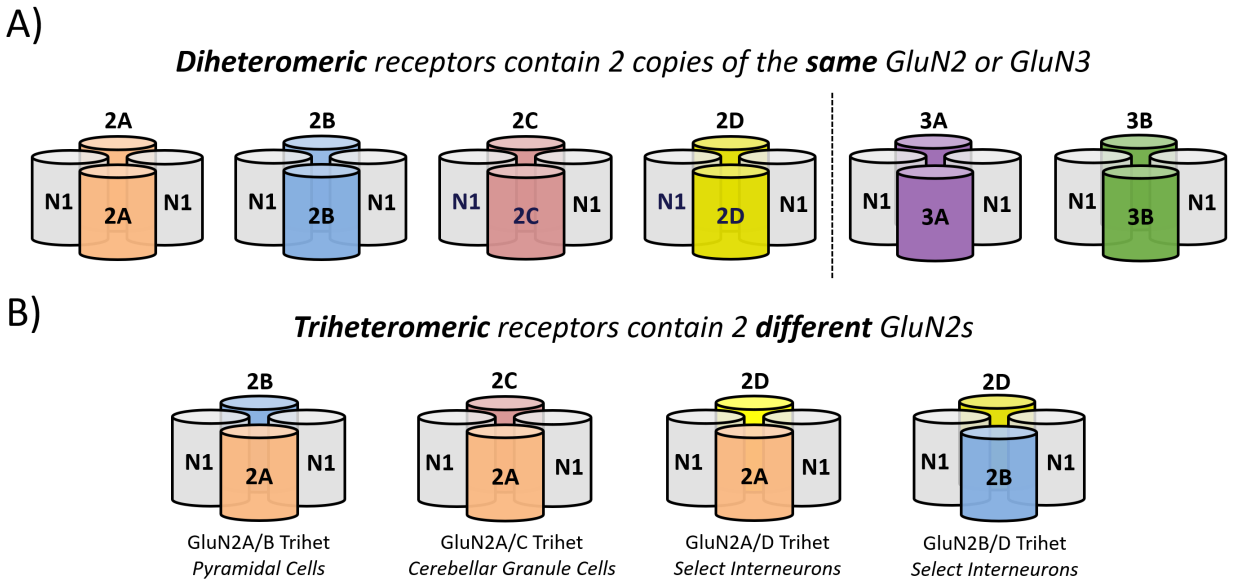


**Figure 1.2.** NMDA receptor subunit stoichiometry. NMDARs are heterotetrameric protein assemblies of two obligate GluN1 subunits, and GluN2 or GluN3 subunits. The construction of NMDARs can be split into two groups: A) diheteromeric assemblies containing two copies of the same GluN2 or GluN3 subunit, or B) triheteromeric assemblies containing two different GluN2 subunits. The possibility of triheteromeric receptors containing GluN3 subunits has yet to be confirmed in native tissue and has thus been omitted from this figure.

excluded from the discussion hereafter.

*1.3 NMDAR Activation and Deactivation in Heterologous Expression Systems*

Glutamate receptors are best understood for their role in synaptic transmission, in which they are activated by rapidly released glutamate that reaches high concentration (peak ~1.2 mM) and endures in the synaptic cleft for a few milliseconds before diffusion and uptake reduce the concentration (Budisantoso et al., 2013; Clements et al., 1992; Diamond, 2005). Since the mechanisms that control NMDAR channel opening and closing dictate their postsynaptic response to brief synaptic pulses of glutamate, the rate at which glutamate dissociates from the receptor is the strongest determinant of the time course of their synaptic currents (Jonas, 2000; Lester et al., 1990; Paoletti et al., 2013; Silver et al., 1996a; Traynelis et al., 2010). Additionally, since NMDARs are gated by glycine/D-serine, the steady-state concentration of extracellular glycine (~6 μM in cerebrospinal fluid (CSF) (D'Souza et al., 2000)) and D-serine (~2 μM in CSF (Madeira et al., 2015)) will also impact receptor function. The glycine site is usually not saturated (Berger et al., 1998; Bergeron et al., 1998), and phasic changes in D-serine release from astrocytes, or glycine release from nearby glycinergic terminals offer another opportunity for the modulation of NMDAR activation and deactivation. For extrasynaptic or perisynaptic NMDARs, their affinity for glutamate will control their response to extrasynaptic glutamate following synaptic or glial release (Kessler, 2013), as well as the response to steady state levels of glutamate in the extracellular space at concentrations near 80 nM (Moldavski et al., 2020).

Much of our understanding of NMDAR activation and deactivation comes from electrophysiological recordings performed in heterologous expression systems or from cultured neurons. After the NMDAR subunits were cloned in the late 1980's, a wealth of functional data was obtained describing ion channel properties including the time course and kinetics of activation

and deactivation. There are now strong and well-supported hypotheses to explain the biophysical properties possessed by this class of ion channels, which are controlled by the GluN2/GluN3 subunits. Outside of alternative splicing of the GluN1 subunit (*e.g.*, exon5 mentioned previously), the different GluN2/GluN3 subunits give each receptor assembly a unique agonist potency, deactivation time course, level of magnesium block, open channel probability, level of desensitization, and vulnerability to endogenous modulation.

Most NMDAR complexes in native tissue are triheteromeric assemblies comprised of two different GluN2 subunits (hereafter triheteromeric receptors), further expanding the repertoire of different NMDARs that can be utilized in native circuits. We can selectively express triheteromeric receptors in heterologous systems utilizing chimeric receptors that include intracellular domains from $GABA_B$ receptors that can be arranged to control NMDAR trafficking (Hansen et al., 2014), allowing for the characterization of physiologically relevant receptors with different GluN2 subunits or different GluN1 splice variants. Triheteromeric receptors show a complex blending of individual diheteromeric subunit properties, rather than an average of properties of the two GluN2 subunits (Bhattacharya et al., 2018; Brickley et al., 2003; Cheffings and Colquhoun, 2000; Jones and Gibb, 2005). Some features of receptor function, such as deactivation time course, can be dominated by one subunit (Hansen et al., 2014; Sun et al., 2017), whereas other functional and pharmacological features of the receptor draw on unique features of each subunit (Bhattacharya et al., 2018; Cheriyan et al., 2016; Hansen et al., 2014; Stroebel et al., 2014; Yi et al., 2019a; Yi et al., 2018). **Table 1.1** summarizes current knowledge on various pharmacological and kinetic properties of diheteromeric and triheteromeric NMDARs expressed in heterologous systems.

### *1.4 Synaptic NMDARs: Activation and Deactivation*

Synaptic NMDARs respond to rapid and brief elevation of glutamate concentration within

| Construct | Glutamate $EC_{50}$ (μM) | Glycine $EC_{50}$ (μM) | D-Serine $EC_{50}$ (μM) | $\tau$-Deactivation (ms) | $\tau$-Desensitization (ms) | $P_{OPEN}$ |
|---|---|---|---|---|---|---|
| **GluN1/N2A** | 4.5 | 0.9 | 1.3 | 85 | 182 | 0.43 |
| **GluN1-1b/N2A** | 3.4 | 1.3 | | 44 | -- | |
| **GluN1/N2B** | 2.5 | 0.3 | 0.7 | 394 | 349 | 0.12 |
| **GluN1-1b/N2B** | 2.9 | 0.3 | | 150 | -- | |
| **GluN1/N2C** | 1.0 | 0.1 | 0.3 | 321 | 389 | 0.01 |
| **GluN1/N2D** | 0.4 | 0.1 | 0.2 | 3050 | N/A | 0.01 |
| **GluN1-1b/N2D** | 0.9 | 0.2 | | 846 | -- | |
| **GluN1/N2A/N2B** | 2.5 | 0.6 | | 100 | -- | |
| **GluN1/N2A/N2C** | 1.1 | 0.5 | | 301 | -- | 0.02 |
| **GluN1/N2A/N2D** | -- | -- | -- | -- | -- | -- |
| **GluN1/N2B/N2B** | 0.5 | 0.3 | | 1770 | -- | 0.02 |

**Table 1.1** Pharmacological and kinetic properties of diheteromeric and triheteromeric NMDARs expressed in heterologous systems**.** These data are adapted from Hansen et. al. 2021 and have been simplified to only be a single value. Measures that were displayed as a range in Hansen et. al. 2021 have been represented as the average of the lowest and highest values given for that measure. Thus, this table is *only* designed to give readers a quick snapshot of pharmacological and kinetic data.

the synaptic cleft with a relatively slow time course, activating in several milliseconds and deactivating with a time course (10-100's of ms) that can be orders of magnitude slower than that for AMPARs, and moderately slower than kainate receptors (Monyer et al., 1992; Vicini et al., 1998; Wyllie et al., 1998) (**Figure 1.3**). Unlike synaptic AMPARs, synaptic NMDARs show little to no desensitization, with their synaptic time course of deactivation being directly related to open-channel probability, receptor gating, and agonist potency. Simulation studies found that diheteromeric GluN2A and GluN2B NMDARs will open at least once (open-channel probability > 0.5) in response to a single synaptic event (brief pulse of high concentration of glutamate) (Erreger et al., 2005). Moreover, in response in a single synaptic event, diheteromeric GluN2A NMDARs will exhibit a higher peak amplitude (higher open-probability), however, their overall charge transfer is only half as large as diheteromeric GluN2B NMDARs (Sheng et al., 1994). This is because GluN2B-containing NMDARs have a higher potency for glutamate, and exhibit a slower conformational change (*i.e.*, gating steps) in response to agonist binding (Erreger et al., 2005). In total, this gives rise to synaptic GluN2A-containing receptors displaying a faster synaptic deactivation time course than GluN2B-containing receptors. Given that most NMDARs in native tissue are GluN2A/GluN2B triheteromeric receptors, there will be synaptic NMDARs with intermediate charge transfer. This may explain why the typical weighted deactivation time course of an evoked NMDAR-mediated excitatory postsynaptic current (EPSC) onto a neuron which expresses both GluN2A and GluN2B receptors is 70-110 ms compared to the deactivation time course of ~35 ms for diheteromeric GluN2A NMDARs and 200-500 ms for diheteromeric GluN2B NMDARs in heterologous systems.

Synaptic activation properties of GluN2C- and GluN2D-containing NMDARs have been studied less than GluN2A and GluN2B, and thus are not as well understood. Both diheteromeric

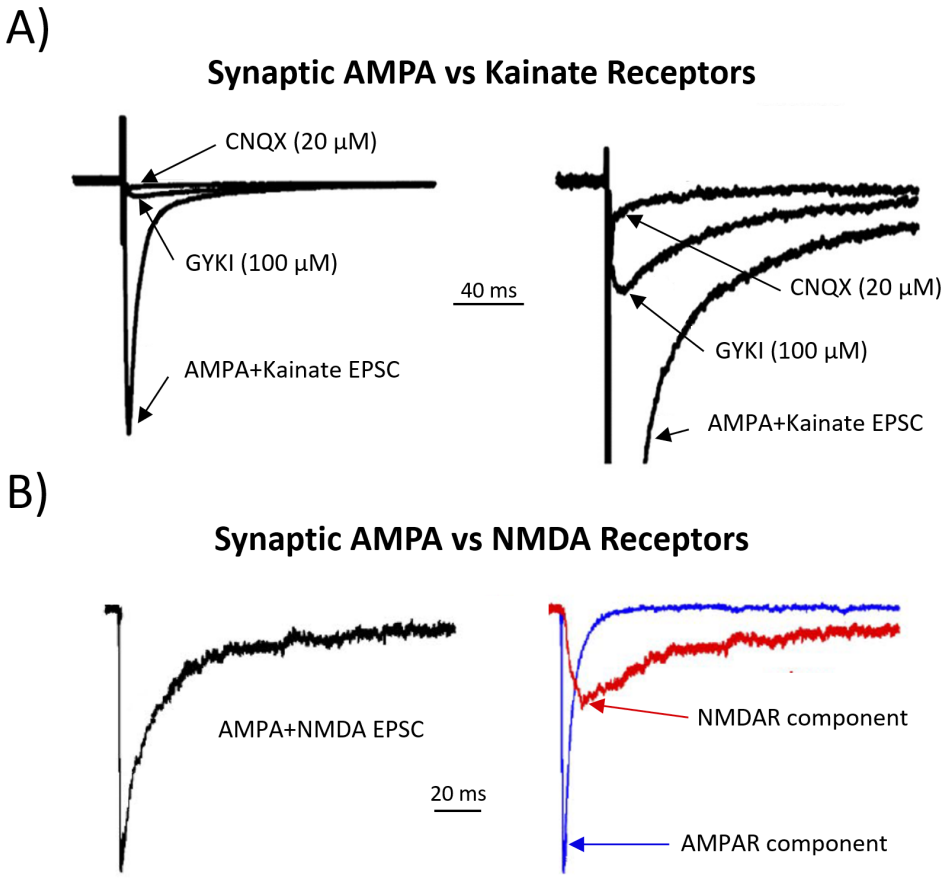


**Figure 1.3.** Time course of synaptic AMPA, kainate, and NMDA receptors. A) Synaptic time course comparison between evoked AMPA and kainate receptors in the anterior cingulate cortex of adult mice. Compound AMPA and kainate receptor evoked EPSC recorded at -65 mV in presence of 2.0 mM $Mg^{2+}$, 100 µM picrotoxin, and 50 µM D-APV. Kainate-only component revealed with the AMPAR-selective noncompetitive antagonist GYK1 53655 (100 µM). Kainate component was nearly abolished after application of CNQX (20 µM) in presence of 100 µM GYKI 53655. B) AMPA and NMDA receptor-mediated EPSC at the pyramidal to multipolar interneuron synapse in the visual cortex. Recordings made at -60 mV in the absence of $Mg^{2+}$ and 10 µM gabazine. The NMDAR component is isolated via 20 µM CNQX. There is no significant kainate receptor component at this synapse. Figures in panel A adapted from (Wu et al., 2005); Figures in panel B contains unpublished data from Lonnie P. Wollmuth and is adapted from (Hansen et al., 2017).

GluN2C and GluN2D NMDARs have low open-channel probabilities (~0.01) and low potency for $Mg^{2+}$ block ($IC_{50}$ ~ 11 μM) (Kuner and Schoepfer, 1996). GluN2C diheteromeric receptors have a deactivation time course of ~300 ms in heterologous expression systems, compared to over 1 second for diheteromeric GluN2D receptors. These differences are likely due to GluN2C-containing receptors having a lower potency for glutamate ($EC_{50}$ of 1 μM for GluN2C vs 0.4 μM for GluN2D) and possibly faster gating steps (Dravid et al., 2008; Kuner and Schoepfer, 1996; Vance et al., 2013). GluN2D-containing receptors additionally have the unusual property of entering an inactive state even without glutamate bound, impacting their overall time course for deactivation (Vance et al., 2013). Regardless of their increased glutamate potency of GluN2C/GluN2D compared to GluN2A/GluN2B and minimal $Mg^{2+}$ block, their low open-channel probabilities suggest, unlike synaptic GluN2A and GluN2B NMDARs, GluN2C and GluN2D NMDARs may not always open during a single synaptic event.

At native synapses, GluN2C is highly expressed in cerebellar tissue and recent data suggest that these GluN2C-containing NMDARs exist primarily as triheteromeric GluN2A/GluN2C NMDARs (Bhattacharya et al., 2018). The weighted deactivation time course of an evoked NMDAR-mediated excitatory postsynaptic current (EPSC) onto cerebellar granule cells is ~160 ms in post-adolescent tissue (Cathala et al., 2000). Thus, synaptic triheteromeric GluN2A/GluN2C NMDARs exhibit a blending of their diheteromeric constructs in terms of recorded deactivation time constant, a property also observed with synaptic GluN2A/GluN2B triheteromeric receptors (mentioned above).

Synaptic GluN2D NMDARs also likely exist as triheteromeric receptors, namely GluN2A/GluN2D and GluN2B/GluN2D complexes. Evidence against the concept of diheteromeric GluN2D NMDARs being trafficking to the postsynaptic density has not been

presented, however, based on observed weighted deactivation time course data of evoked NMDAR-mediated EPSCs in cells known to express GluN2D, this seems unlikely. The only known report of a deactivation time course consistent with diheteromeric GluN2D-containing NMDARs was in cerebellar Purkinje cells prior to climbing fiber innervation (Brickley et al., 2003). Heterologous expression system data on the deactivation time course of triheteromeric GluN2B/GluN2D NMDARs is ~1700 ms in the absence of GluN1 exon5 and is ~300 ms in the presence of GluN1 exon5 (Yi et al., 2019b). Analogous heterologous expression system data for triheteromeric GluN2A/GluN2D NMDARs has not been reported. The synaptic weighted deactivation time course data of evoked NMDAR-mediated EPSCs in cells known to express GluN2D is 120-150 ms (Swanger et al., 2018; Swanger et al., 2015; Yi et al., 2019a). This discrepancy in heterologous versus synaptic deactivation time of GluN2D-containing NMDARs could be explained by: 1) the presence of synaptic GluN2A/GluN2B within the same synapse as GluN2D triheteromeric receptors decreasing deactivation time, 2) GluN2A/GluN2D triheteromeric NMDARs influencing deactivation time over GluN2B/GluN2D triheteromeric receptors given the assumed higher open-channel probability endowed by GluN2A-expressing NMDARs, 3) GluN2D-expressing neurons exclusively expressing GluN1 exon5 (see (Li et al., 2021) for support), and 4) GluN2D triheteromeric NMDARs does not display a blending of diheteromeric NMDAR, even in heterologous expression systems (Yi et al., 2019a).

As with all NMDARs in heterologous systems, the ion permeation pore of synaptic NMDARs is blocked by extracellular $Mg^{2+}$ at the resting membrane potential most neurons in the CNS. As such, the typical glutamatergic synapse contains both AMPARs and NMDARs, with AMPARs endowing rapid signaling properties and membrane depolarization necessary for relief of magnesium block. Although GluN2C- and GluN2D-containing NMDARs have a weaker

magnesium block at -60 mV compared to GluN2A- and GluN2B-containing NMDARs, neurons that express GluN2C or GluN2D NMDARs still display IV-relationships suggesting strong magnesium block in the presence of physiological concentrations of $Mg^{2+}$ when the neuron is not depolarized (Huang and Gibb, 2014). NMDAR-containing glutamatergic synapses without AMPARs, so-called silent synapses, play important roles in both the developing (Rumpel et al., 1998) and adult brain (Vardalaki et al., 2022), with activation being largely driven by depolarization from adjacent dendritic spines.

Another wrinkle in the activation of synaptic NMDARs is the ability of the GluN1 to initiate gating after binding glycine or D-serine. The contributions of glycine and D-serine to the activation of NMDARs appear to overlap, albeit there are differences between synapses in the preference for glycine or D-serine (reviewed in (Mothet et al., 2015). In the mature hippocampus for example, D-serine is the main co-agonist in Schaffer collateral to CA1 synapses, where there is a switch from glycine to D-serine as the co-agonist during postnatal development (Le Bail et al., 2015) that parallels the timing of GluN2A expression in these neurons (Gray et al., 2011; Monyer et al., 1994; Rauner and Kohr, 2011; Rodenas-Ruano et al., 2012). By contrast, glycine is predominant in medial perforant path to dentate gyrus synapses (Le Bail et al., 2015).

### *1.5 Synaptic NMDARs: Spatiotemporal Expression Patterns*

Distinct expression patterns for GluN1 splice isoforms and GluN2 subtypes were established three decades ago using the then new technique of *in situ* hybridization (*e.g.*, (Akazawa et al., 1994; Monyer et al., 1994)) (**Figure 1.4**). Recent advances in the sensitivity of single-cell sequencing and new imaging methods have enriched our understanding of cell-type-specific gene expression. Consistent with its role as an obligate NMDAR subunit, GluN1 is expressed throughout the nervous system as early as E13 in rodents and gestational week 8 in humans

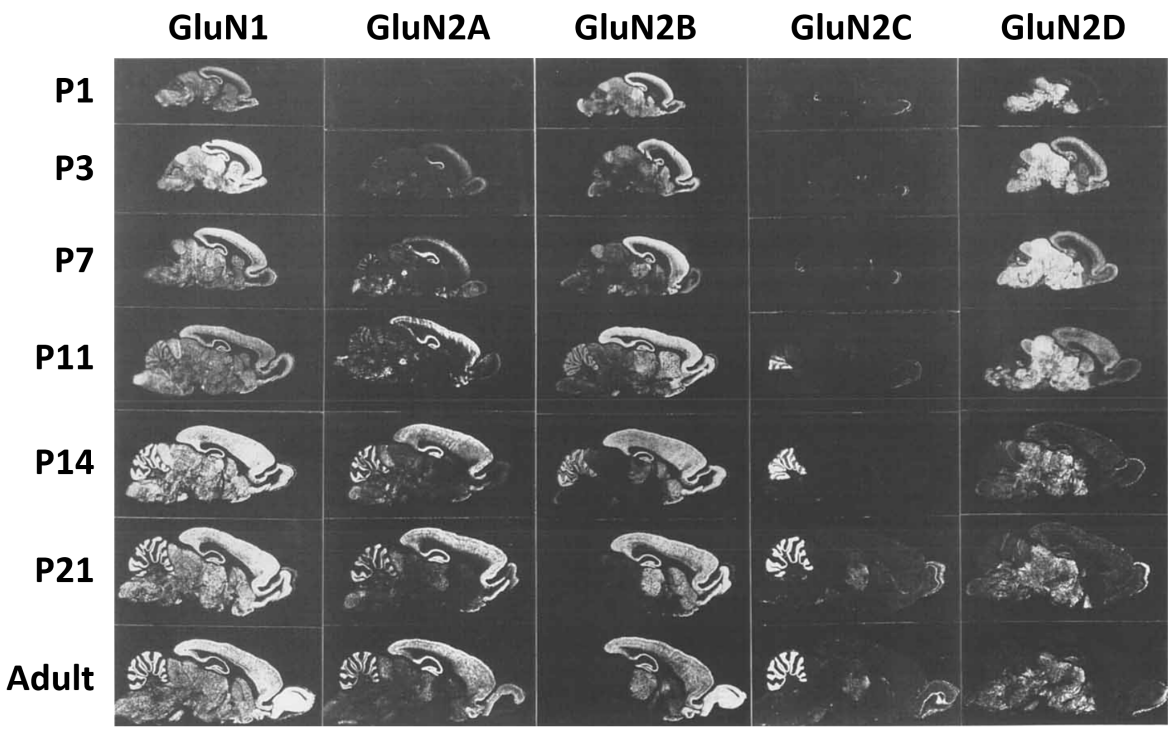


**Figure 1.4.** NMDAR subunit spatiotemporal expression patterns in developing rat brain. Data represented are negative film images for *in-situ* hybridization experiments for the mRNA of each NMDAR subunit. Figure is adapted from (Akazawa et al., 1994).

(Moriyoshi et al., 1991; Ritter et al., 2001; Ritter et al., 2002; Watanabe et al., 1992). This high level of GluN1 expression is maintained in adults. Splice variants of GluN1 are tightly regulated in a regional pattern that is established at birth (Laurie and Seeburg, 1994; Nakanishi et al., 1992; Paupard et al., 1997; Zhong et al., 1995). The most prominent splice variant, GluN1 exon5 as discussed earlier, is abundant in the thalamus, midbrain, hindbrain, and spinal cord, but restricted to only a few cortical areas, hippocampal CA3, and the dentate gyrus, (Laurie and Seeburg, 1994; Luque et al., 1994; Paupard et al., 1997). The GluN1-1a splice variant shows high expression in the telencephalon, with spare expression in the midbrain, playing a complementary role to GluN1 exon5 expression. In the developing cerebellum, 20% of GluN1 contains residues encoded by exon5, compared to 80% of GluN1 in adult (Prybylowski et al., 2000), suggesting alternative splicing may be an important developmental factor (Paupard et al., 1997).

The different spatial gene expression patterns of GluN2 subunits are major determinants of NMDAR functional diversity across the CNS. The GluN2B and GluN2D subunits are highly expressed during embryonic development, whereas GluN2A and GluN2C expression increases postnatally (Akazawa et al., 1994; Monyer et al., 1994; Watanabe et al., 1992; Watanabe et al., 1993; Wenzel et al., 1997; Wenzel et al., 1996). GluN2A mRNA expression in rodents begins a few days after birth and becomes highly expressed in nearly all CNS regions. GluN2B expression remains high in most brain regions through early postnatal development and then becomes restricted primarily to the forebrain. The increase in synaptic GluN2A expression and coordinated reduction in synaptic GluN2B expression is regulated during development in the cortex, hippocampus, amygdala, thalamus, and cerebellum (Barria and Malinow, 2002; Gray et al., 2011; Lopez de Armentia and Sah, 2003; Philpot et al., 2001; Tovar and Westbrook, 1999). This developmental GluN2B-GluN2A switch occurs over timelines that differ across cortical regions

and between GABAergic interneurons and principal glutamatergic cells within the forebrain (Dumas, 2005; Mierau et al., 2016; Wang and Gao, 2009). Furthermore, some cell types, such as neurons in the dorsal horn, do not undergo a developmental loss of synaptic GluN2B (Hildebrand et al., 2014; Mahmoud et al., 2020). The relative levels of synaptic GluN2A and GluN2B also differ between anatomically distinct inputs to a single neuron type in the cortex, hippocampus, striatum, thalamus, and retina (Carta et al., 2018; Fritschy et al., 1998; Kalbaugh et al., 2009; Kumar and Huguenard, 2003; Li and Pozzo-Miller, 2019; Miyata and Imoto, 2006; Shinohara et al., 2008).

GluN2C expression begins at approximately postnatal day 10 in the rodent, and it is abundantly expressed in cerebellar granule cells, lateral nuclei of the dorsal thalamus, and the glomerular layer and mitral cells of the olfactory bulb (Akazawa et al., 1994; Monyer et al., 1994; Watanabe et al., 1993). GluN2C is weakly expressed in the cortex, hippocampus, striatum, and amygdala, and recent evidence suggests that GluN2C is expressed primarily in astrocytes, not neurons, in these regions (Alsaad et al., 2019; Karavanova et al., 2007; Ravikrishnan et al., 2018). Functional studies utilizing GluN2C-selective pharmacology support neuronal expression in the cerebellum, thalamus, and globus pallidus (Bhattacharya et al., 2018; Fernandez et al., 2017; Liu et al., 2021; Liu et al., 2019).

GluN2D is widely expressed in the CNS during embryonic and early postnatal development, but after the first postnatal week GluN2D expression becomes restricted to GABAergic interneurons in the cortex and hippocampus, cholinergic interneurons in the striatum, and select neurons within the thalamus, basal ganglia, bed nucleus of the stria terminalis, substantia gelatinosa of the spinal cord, and cerebellum (Akazawa et al., 1994; Dubois et al., 2016; Monyer et al., 1994; Salimando et al., 2020; Standaert et al., 1996; Standaert et al., 1994; Tolle et al., 1993;

Wenzel et al., 1996). Currently, the evidence does not support restriction of GluN2D to particular interneuron classes in the cortex and hippocampus, and it appears to be widely expressed in parvalbumin- and somatostatin-positive cells (Perszyk et al., 2016; von Engelhardt et al., 2015). Recent functional studies utilizing subtype-selective pharmacology support GluN2D expression in cortical and hippocampal interneurons (Garst-Orozco et al., 2020; Perszyk et al., 2016; Swanger et al., 2018; von Engelhardt et al., 2015; Yi et al., 2019a; Yi et al., 2020), striatum (Feng et al., 2014; Nouhi et al., 2018; Zhang et al., 2014a; Zhang et al., 2014b), subthalamic nucleus (Swanger et al., 2018; Swanger et al., 2015; Yi et al., 2020), substantia nigra (Morris et al., 2018; Pearlstein et al., 2015; Sitzia et al., 2020; Wu and Johnson, 2015), and spinal cord (Hildebrand et al., 2014; Mahmoud et al., 2020).

### *1.6 Synaptic NMDARs: Roles in Neurogenesis and Neuronal Migration*

NMDA receptors are expressed early in CNS development to impact the generation of neurons as well as their migration. NMDAR activity supports mammalian neurogenesis in the fetal brain by promoting neural progenitor cell proliferation and neural migration (Li et al., 2011; Luk et al., 2003; Mochizuki et al., 2007; Sadikot et al., 1998; Suzuki et al., 2006). *In vitro,* NMDAR signaling can promote the migration of cerebellar granule cells and glutamatergic cortical neurons (Behar et al., 1999; Hirai et al., 1999; Hirasawa et al., 2003; Komuro and Rakic, 1993; Mancini and Atchison, 2007; Tarnok et al., 2008). *In vivo*, NMDAR agonist and antagonist administration causes abnormal cortical architecture, indicative of altered radial neuronal migration (Marret et al., 1996; Reiprich et al., 2005). However, GluN1 deletion does not have significant effects on cortical architecture (Iwasato et al., 2000; Messersmith et al., 1997), although *GRIN1 de novo* variants have been associated with cortical abnormalities (Fry et al., 2018). Interestingly, AMPARs, but not NMDARs, regulate hippocampal interneuron migration (Bortone and Polleux, 2009; Manent

et al., 2006). In contrast to early development, NMDAR activity can restrict neurogenesis as the brain matures. In the postnatal and adult dentate gyrus, NMDAR blockade increases neural progenitor cell proliferation (Bernabeu and Sharp, 2000; Bunk et al., 2014; Bursztajn et al., 2007; Cameron et al., 1995; Gould et al., 1994; Kitayama et al., 2003; Nacher et al., 2003; Nacher et al., 2001; Okuyama et al., 2004). This blockade can cause a long-lasting increase in adult-born neurons, while also depleting the precursor cell population, potentially restricting behaviorally-relevant neurogenesis that may be required at a later time (Joo et al., 2007; Petrus et al., 2009).

*1.7 Synaptic NMDARs: Roles in Neurite Development*

Subunit-specific NMDAR signaling has a complex role in the initial stages of neuronal differentiation, both promoting and inhibiting growth of axons and dendrites. Investigations of topographic sensory map formation revealed that NMDARs regulate axonal arborization, and disrupting NMDAR function causes axons to overlap erroneously in the visual and somatosensory cortices (Cline and Constantine-Paton, 1989; Cline et al., 1987; Iwasato et al., 1997; Lee et al., 2005; Rajan et al., 1999; Ramoa et al., 2001; Ruthazer et al., 2003; Simon et al., 1992; Van Horn et al., 2017; Yamasaki et al., 2014). Initially, dendritic NMDARs were thought to orchestrate axonal arborization through retrograde signaling (Schmidt, 2004), but more recent studies indicate axonal NMDARs regulate axon growth and pathfinding by controlling axon dynamics. In the presynaptic bouton, NMDAR regulate maturation by promoting assembly of synaptic vesicles and active zone proteins (Corlew et al., 2008; Corlew et al., 2007; Gao et al., 2018; Gill et al., 2015; Sceniak et al., 2012).

During embryonic and early postnatal development, NMDARs promote dendrite dynamics, growth, and branching (Kalb, 1994; Rajan and Cline, 1998; Rajan et al., 1999). NMDAR activation is triggered by spontaneous glutamate release from developing axons to

promote dendrite growth prior to axodendritic contact (Andreae and Burrone, 2015). GluN2B signaling promotes dendrite growth and GluN2A signaling facilitates dendrite refinement and stability in glutamatergic neurons (Bustos et al., 2014; Espinosa et al., 2009; Sceniak et al., 2019; Sepulveda et al., 2010). However, such effects are context specific, as indicated by the finding that GluN2B-CaMKII signaling limited dendrite growth in immature cultured neurons (5 days in vitro), but enhanced growth in older neurons (12 days in vitro). Furthermore, artificially enhancing an interaction between GluN2A and CaMKII re-activated dendritogenesis *in vivo* (Bustos et al., 2017). NMDARs also regulate dendrite development in GABAergic interneurons (Chittajallu et al., 2017; Hanson et al., 2019; Pla et al., 2018), with GluN2D activation being required during a critical developmental window for proper cortical interneuron dendrite arborization (Hanson et al., 2019).

### *1.8 Synaptic NMDARs: Roles in Synapse Formation*

Many studies suggest that synaptogenesis triggers NMDAR clustering at nascent glutamatergic synapses (Cottrell et al., 2000; Washbourne et al., 2002), allowing NMDARs to regulate dendritic spine formation, maturation, and stabilization in developing and mature neurons (Engert and Bonhoeffer, 1999; Kwon and Sabatini, 2011; Maletic-Savatic et al., 1999; McKinney, 2010; Yasuda et al., 2011). Indeed, NMDAR activity has a well-established role in synaptic refinement by controlling the pruning and strengthening of immature synapses during development (Colonnese and Constantine-Paton, 2006; Luthi et al., 2001; Personius et al., 2016; Rabacchi et al., 1992; Zhang et al., 2000; Zhang et al., 2013). Several lines of evidence suggest GluN2B-mediated signaling supports dendritic spine dynamics and synapse refinement to exert a brake on synapse maturation, while GluN2A-mediated signaling hinders spine dynamics and promotes synapse maturation and stabilization (Gray et al., 2011; Henle et al., 2012; Kelsch et al., 2014;

Ohno et al., 2010; Wang et al., 2011; Wang et al., 2021). Subunit-specific regulation of synapse maturation depends upon distinct CTD interactions, with GluN2B-CaMKII signaling playing an important role (Alvarez et al., 2007; Dupuis et al., 2014; El Gaamouch et al., 2012; Gambrill and Barria, 2011); but see (McKay et al., 2018).

NMDARs are also critical in inhibitory synapse development. Chronic NMDAR blockade alters GABAergic synapse number (Aamodt et al., 2000; Henneberger et al., 2005; Rosato-Siri et al., 2002). GluN1 deletion from hippocampal pyramidal neurons impaired inhibitory synapse formation via a mechanism requiring a calmodulin binding motif in the GluN1 CTD (Gu et al., 2016; Lu et al., 2013). GluN2D-mediated signaling may be particularly involved, since GluN2D-selective inhibition reduces GABAergic synapse density in the adult cortex (Hanson et al., 2019). Interestingly, GluN2D is highly expressed in early postnatal development, but the role of GluN2D in the effects of GluN1 deletion or non-selective NMDAR blockers is unknown. In addition, NMDARs are embedded postsynaptically with GABA receptors at some developing GABAergic synapses, suggesting a unique developmental mechanism in formation of these synapses (Cserep et al., 2012; Gundersen et al., 2004; Szabadits et al., 2011).

### *1.9 Roles of NMDARs in Presynaptic Signaling*

Presynaptic NMDARs control synaptic transmission by influencing neurotransmitter release (Aoki et al., 1994; Charton et al., 1999; Corlew et al., 2008). NMDARs enhance both spontaneous and evoked glutamate release at synapses in the cortex (Berretta and Jones, 1996; Corlew et al., 2007; Larsen et al., 2011; Sjostrom et al., 2003; Woodhall et al., 2001), hippocampus (McGuinness et al., 2010; Prius-Mengual et al., 2019), cerebellum (Bidoret et al., 2009; Glitsch and Marty, 1999), and spinal cord (Bardoni et al., 2004; Robert et al., 1998). Presynaptic NMDARs also regulate neurotransmitter release at GABAergic synapses in the reticular nucleus of the

thalamus (Crabtree et al., 2013), cortex (Pafundo et al., 2018), and cerebellum (Duguid and Smart, 2004; Rossi et al., 2012). Ambient glutamate likely activates NMDARs regulating spontaneous release, whereas action potential driven release is likely mediated by autoreceptors or by glutamate spillover from adjacent synapses (Duguid and Smart, 2004; Duguid and Smart, 2009). GluN2B-containing receptors make up the majority of presynaptic NMDA receptors in the cortex, but GluN2D may also regulate presynaptic release in the hippocampus and cerebellum (Berg et al., 2013; Dubois et al., 2016; Mameli et al., 2005; Prius-Mengual et al., 2019). In addition, several studies implicate GluN2A in presynaptic regulation of neurotransmitter release in the spinal cord (Xie et al., 2016), and the parallel fiber-Purkinje cell synapse in the cerebellum (Bidoret et al., 2009).

*1.10 Roles of NMDARs in Postsynaptic Signaling*

The primary function of postsynaptic NMDARs is to transduce synaptic signals by mediating excitatory synaptic currents and initiating $Ca^{2+}$-dependent intracellular signaling. Since depolarization is required to relieve channel block by extracellular $Mg^{2+}$, NMDAR activity can detect coincident activity of presynaptic and postsynaptic neurons – a mechanism critical for some types of synaptic plasticity. A classical role of postsynaptic NMDA receptors is to modify the function, structure, and molecular composition of synapses via $Ca^{2+}$-dependent activation of local intracellular signaling pathways, cytoskeleton dynamics, and synapse-to-nucleus signaling plasticity.

The impact of NMDAR activation on neuronal function depends upon receptor composition, as the properties of the NMDAR will control the amount of $Ca^{2+}$ entering the postsynaptic spine. As discussed previously, the overall charge transfer through NMDARs is impacted by open probability, deactivation time course, $Mg^{2+}$ sensitivity, and $Ca^{2+}$ permeability –

all properties that vary with the GluN2 subunits and to some degree GluN1 alternative splicing. The requirement for depolarization to relieve $Mg^{2+}$ block and allow current flow, in combination with the longer deactivation time of NMDARs, allows their responses to summate during trains of synaptic input, which is necessary for synaptic activity to drive action potential firing (Iacobucci and Popescu, 2017; Stuart and Spruston, 2015). Thus, NMDARs are critical to the primary function of neurons, which is to integrate synaptic signals and transduce them into patterns of neuronal activity.

One form of dendritic integration is the nonlinear summation of NMDAR-mediated synaptic potentials that generate large, sometimes regenerative, dendritic potentials referred to as "NMDA spikes" (Schiller et al., 2000). In the cortex and thalamus, these depolarizing potentials enhance neuronal excitability and drive action potential generation directly (Farinella et al., 2014; Larkum et al., 2009; Lavzin et al., 2012; Losonczy and Magee, 2006; Major et al., 2008; Manita et al., 2011; Smith et al., 2013). NMDARs may generate depolarizing potentials indirectly via activating voltage-gated $Ca^{2+}$ channels that facilitate dendritic calcium spikes capable of inducing action potential bursts (Calton et al., 2000; Milojkovic et al., 2004; Palmer et al., 2014; Polsky et al., 2009; Wei et al., 2001). NMDAR-generated dendritic calcium spikes can also shape dendritic spine structural plasticity (Dittmer et al., 2019) and rapid signaling to the nucleus that can regulate gene transcription (Wild et al., 2019).

### *1.11 Roles of NMDARs in Extrasynaptic Signaling*

Extrasynaptic NMDARs include receptors expressed in somas, dendritic shafts, and perisynaptic regions. Morphologically, extrasynaptic receptors lie more than 100 nm away from the postsynaptic density, with perisynaptic receptors generally being 100-300 nm from the postsynaptic density (Groc et al., 2009; Hardingham and Bading, 2010; Kohr, 2006; Petralia et al.,

2010). However, many physiologists define synaptic NMDARs as those activated by low frequency synaptic activity, such as spontaneous synaptic release, and extrasynaptic receptors are not typically activated under these conditions (Chen and Diamond, 2002; Harris and Pettit, 2007; Tovar and Westbrook, 1999; Zhang and Diamond, 2009). Perisynaptic receptors are activated by spillover of synaptic glutamate during high frequency activity (Asztely et al., 1997; Clark and Cull-Candy, 2002; Harris and Pettit, 2008; Herman et al., 2011; Wild et al., 2015), whereas NMDARs farther from synapses on dendritic shafts and somas are likely activated by ambient glutamate (Cavelier and Attwell, 2005; Herman and Jahr, 2007; Sah et al., 1989) or glutamate released from astrocytes (Angulo et al., 2004; Bardoni et al., 2010; Bezzi et al., 2004; Fellin et al., 2004; Fleming et al., 2011; Hamilton and Attwell, 2010; Jourdain et al., 2007; Lee et al., 2007; Nie and Weng, 2010; Oh et al., 2012; Park et al., 2015; Shigetomi et al., 2008). It is also possible that synaptic NMDARs in quiescent synapses may be activated by glutamate spillover ("crosstalk") from neighboring, active synapses (Arnth-Jensen et al., 2002; Henneberger et al., 2020; Scimemi et al., 2004), although the extent to which this occurs is debated (Barbour, 2001).

Extrasynaptic NMDARs were detected widely in the CNS more than two decades ago (Aoki et al., 1997; Clark et al., 1997; Kharazia and Weinberg, 1999; Momiyama, 2000; Rao and Craig, 1997; Valtschanoff et al., 1999), but we still lack a clear picture of how synaptic versus extrasynaptic receptors are organized, the degree to which receptors move between synaptic and extrasynaptic domains, and the physiological and pathophysiological roles for these two receptor populations (Papouin and Oliet, 2014; Parsons and Raymond, 2014; Petralia, 2012; Zhou et al., 2015). Perisynaptic NMDARs may play a role in long-term synaptic plasticity, but also can diffuse laterally into the synapse in an activity-dependent manner, which blurs the distinction between synaptic and extrasynaptic populations. Extrasynaptic receptors are also well-positioned to detect

glial release of glutamate, which can be triggered by G protein-coupled receptor activation as well as other signals (Haroon et al., 2017; Kofuji and Araque, 2020; Mannaioni et al., 2001). Furthermore, NMDARs in perisynaptic regions may have unique roles in dendritic excitability via activation of SK channels (Isaacson and Murphy, 2001) and "NMDA spikes" (Jourdain et al., 2007; Oikonomou et al., 2012) due to their localization near $Ca^{2+}$- and voltage-activated channels.

Extrasynaptic NMDARs, particularly those expressed on dendritic shafts and somas, mediate tonic currents or slow inward currents that enhance neuronal excitability in response to ambient glutamate when the membrane potential is sufficiently depolarized (Herman and Jahr, 2007; Le Meur et al., 2007; Povysheva and Johnson, 2012; Sah et al., 1989; Wu et al., 2012). Tonic NMDAR currents may facilitate coupling between dendritic and somatic compartments, enhancing action potential firing in pyramidal neurons (Riebe et al., 2016; Sah et al., 1989; Wu et al., 2012). Furthermore, tonic NMDAR currents in interneurons regulate gamma oscillatory activity (Mann and Mody, 2010) and slow inward currents activated by glial glutamate release contribute to the synchronization of neuronal firing (Angulo et al., 2004; D'Ascenzo et al., 2007; Fellin et al., 2004; Fellin et al., 2006; Nie and Weng, 2010). The hypothesis that NMDARs containing GluN2C or GluN2D, which have reduced $Mg^{2+}$ sensitivity and higher glutamate potency relative to GluN2A and GluN2B, mediate tonic currents is supported by studies in the substantia nigra (Morris et al., 2018), thalamus (Zhang et al., 2012), and cortex (Hanson et al., 2019).

Extrasynaptic NMDARs have been proposed to be an important mediator of excitotoxicity in neurons in response to acute injury (Choi et al., 1988; Choi et al., 1987). Multiple studies suggest that extrasynaptic NMDAR signaling activates cell death pathways, whereas synaptic NMDARs can support cell survival (Hardingham and Bading, 2010; Kaufman et al., 2012; Sattler et al., 2000;

Vanhoutte and Bading, 2003; Xu et al., 2009). Recent work contradicts these studies, however, by suggesting that synaptic receptors can contribute to excitotoxic cell death, and it seems likely that activation of both synaptic and extrasynaptic NMDARs can participate in the induction of cell death (Papouin et al., 2012; Wroge et al., 2012; Zhou et al., 2013a; Zhou et al., 2013b). Thus, current evidence does not unequivocally support a rule for exclusively linking synaptic or extrasynaptic NMDARs to cell survival or death.

### *1.12 Roles of NMDARs in Glial Cells*

Glial cells – astrocytes, microglia, and oligodendrocytes – are estimated to be in a 1:1 ratio with neurons in the CNS. NMDAR expression has been reported on all types of glia, with GluN2A and GluN2C being the most common. In astrocytes, GluN2C expression has been confirmed in hippocampus (Alsaad et al., 2019; Chipman et al., 2021; Ravikrishnan et al., 2018), cortex (Alsaad et al., 2019; Ravikrishnan et al., 2018), striatum (Alsaad et al., 2019; Ravikrishnan et al., 2018; Shelkar et al., 2019), and amygdala (Alsaad et al., 2019; Ravikrishnan et al., 2018). In hippocampus, activation of GluN2C-containing NMDARs on astrocytes modulates presynaptic glutamate release (Chipman et al., 2021), whereas activation of GluN2C astrocytic NMDARs in the nucleus accumbens can modulate cocaine-conditioned place preference (Shelkar et al., 2022). As previously shown, GluN2C does not form diheteromeric receptors at native synapses in cerebellar granule cells (Bhattacharya et al., 2018) which has been confirmed for astrocytes (Shelkar et al., 2022). However, in the absence of GluN2A, it appears GluN2C diheteromeric receptors can reach the neuronal surface, as implied by their single channel properties (Takahashi et al., 1996). Indeed, as with cerebellar granule cells, NMDARs on astrocytes are GluN2A/GluN2C triheteromeric assemblies (Bhattacharya et al., 2018; Shelkar et al., 2022).

Microglia – the resident immune cell in the CNS – also likely express NMDARs, however, their subunit composition is largely unknown. Cultured microglia show robust motility responses to NMDA (Raghunatha et al., 2020) and mice lacking GluN1 driven by a myeloid precursor cell promoter show protection from excitotoxicity-mediated neuronal cell death (Kaindl et al., 2012). Microglia are essential for proper synaptic pruning during development (Paolicelli et al., 2011), where microglial NMDARs may play a modulatory role in this process (Eyo et al., 2018). In all, however, data for functional NMDARs on microglia are limited. Several reports suggest GluN2C is also expressed in oligodendrocytes in the white matter of the cerebellum, corpus callosum, and optic nerve (Burzomato et al., 2010; Doyle et al., 2018; Karadottir et al., 2005; Micu et al., 2006; Salter and Fern, 2005). In the spinal cord, GluN2C was weakly detected in non-neuronal cells in white matter (Akesson et al., 2000; Shibata et al., 1999; Sundstrom et al., 1997; Tolle et al., 1993), however, the exact role of oligodendrocyte GluN2C-containing NMDARs is not well understood.

### *1.13 Roles of NMDARs in Synaptic Plasticity*

Synaptic plasticity, or the ability to bidirectionally modulate intercellular signaling strength, is a feature of the CNS. Synaptic plasticity takes many different forms, producing changes in synaptic efficacy ranging from seconds to days that are thought to encode complex behaviors such as learning, memory, sensory processing, emotional responses, addiction, and general cognition. NMDARs play essential roles in several key forms of synaptic plasticity.

The postsynaptic density (PSD) at the Schaffer collateral-CA1 synapse, or the portion of the dendritic spine with a cluster of neurotransmitters receptors and signaling proteins, is directly opposed to presynaptic release sites and is organized into several nanoscale domains. This organization holds important implications for participation of glutamate receptors in synaptic plasticity. The center of the PSD contains several NMDARs, with AMPAR clusters adjacent to,

but not overlapping with, NMDAR clusters (Fukata et al., 2013; Goncalves et al., 2020; Kellermayer et al., 2018; MacGillavry et al., 2013; Nair et al., 2013; Shinohara, 2012; Tang et al., 2016). AMPAR clusters may position directly underneath presynaptic neurotransmitter vesicular release sites, forming a nanoscale column of presynaptic release machinery and postsynaptic AMPARs (Haas et al., 2018; Nair et al., 2013; Tang et al., 2016) (**Figure 1.5**). Each nanodomain is roughly 70 nm in diameter (MacGillavry et al., 2013; Nair et al., 2013), with adjacent nanodomains within the same synapse being approximately 100 nm apart (Choquet and Hosy, 2020; Haas et al., 2018). The glutamate concentration within the synapse rapidly diminishes to 500 μM or less about 100 nm outside of the activated nanodomain per given *quanta*, and this may be even faster in some regions because of the segregated architecture of the nanodomains (Choquet and Hosy, 2020; Haas et al., 2018; Nair et al., 2013) (**Figure 1.5**). Since NMDARs are at the center of the PSD, roughly 80-100 nm from each AMPAR nanodomain, and require co-agonists glutamate and glycine to bind as well as membrane depolarization to relieve $Mg^{2+}$ block to generate current flow, their activation might require multiple presynaptic events at some synapses (Helassa et al., 2018; Silver et al., 1996b); but see (Bekkers and Stevens, 1989). This creates opportunities for plasticity to proceed through modulation of both postsynaptic receptors and presynaptic release.

Short-term plasticity refers to the ability of a synapse to increase or decrease postsynaptic signaling output and spine depolarization when pairs of presynaptic stimuli arrive within the same presynaptic bouton on a millisecond timescale. Short-term plasticity at excitatory synapses is important for several forms of memory (Ferguson et al., 2004; Hansel and Mato, 2013; Jaaskelainen et al., 2011), can serve to counterbalance frequency-dependent depression (Turecek et al., 2016), can exert frequency-dependent filtering (Jackman and Regehr, 2017), and can

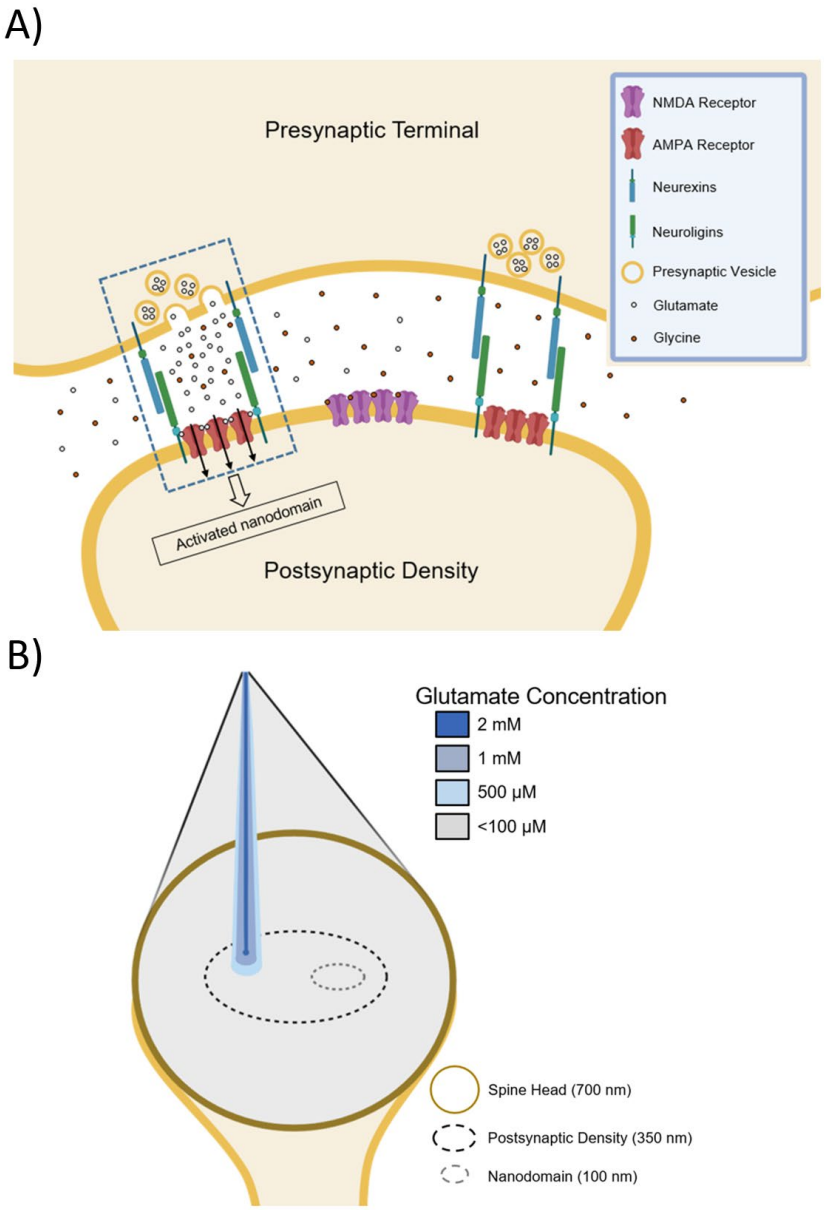


**Figure 1.5.** Organization of a typical glutamatergic synapse. A) The center of the postsynaptic density contains several NMDARs, with adjacent AMPARs arranged into nanocolumns where AMPAR clusters are positioned directly below vesicular release machinery. B) Simulations of glutamate concentration and diffusion within the synaptic cleft after a single quantum of glutamate is released. Figure adapted from (Hansen et al., 2021).

influence sensory processing (Fortune and Rose, 2002). Short-term plasticity only lasts for a matter of seconds to minutes and both pre- and postsynaptic mechanisms are involved. Short-term plasticity is classically divided into paired-pulse facilitation and paired-pulse depression, although other forms exist (Zucker and Regehr, 2002). Both forms of short-term plasticity largely center around AMPARs and thus will not be discussed. However, short-term plasticity is also driven by presynaptic calcium load where presynaptic NMDARs may be important (Chamberlain et al., 2008).

Long-term potentiation (LTP) and long-term depression (LTD) refer to forms of synaptic plasticity whereby specific stimulation of afferent glutamatergic fibers can induce long-lasting (sustained for hours and days) increases or decreases in synaptic signaling, respectively. Both LTP and LTD are thought to be the molecular correlates for encoding and storing long-term memories throughout the brain (Lisman, 1989; Nabavi et al., 2014). While the exact molecular mechanisms involved in LTP and LTD are still under investigation, there is a consensus that NMDARs participate in the expression phase of long-term plasticity. A vast number of LTP studies have explored the Schaffer collateral-CA1 synapse as this laminar pyramidal cell structure simplifies experimentation and allows recording of robust field excitatory postsynaptic potentials (fEPSPs). LTP induction at the Schaffer collateral-CA1 synapse requires sufficient postsynaptic depolarization to relieve $Mg^{2+}$ block of NMDARs, co-agonists glutamate and glycine binding to the NMDAR, and $Ca^{2+}$ influx into the postsynaptic spine (Luscher and Malenka, 2012). In the classical paradigm, this is accomplished by a high-frequency burst or tetanic stimulation, which produces more persistent depolarization than a single synaptic event (Schwartzkroin and Wester, 1975). Pharmacological inhibition of NMDARs with competitive antagonists like AP5 (Collingridge et al., 1983), or channel blockers like MK-801 (Gilbert and Mack, 1990), or

chelation of postsynaptic $Ca^{2+}$ with EGTA (Lynch et al., 1983) prevent induction of LTP following tetanic stimulation. Multiple biochemical experiments have shown that a rise in postsynaptic $Ca^{2+}$ via NMDARs activates intracellular signaling cascades, including PKA (Otmakhova et al., 2000), PKC (Malinow et al., 1989), PKG (Serulle et al., 2007), and CaMKII (Lisman et al., 2012), all of which help traffic, anchor, and sustain AMPARs within the PSD to produce a potentiated synaptic response.

Several lines of early evidence suggested that the GluN2B subunit was the key driver for postsynaptic LTP induction, including the observation that RNA knock-down of GluN2B in hippocampus abolishes LTP at the Schaffer collateral-CA1 synapse (Clayton et al., 2002; Kutsuwada et al., 1996a), and overexpression of GluN2B in hippocampus enhanced spatial learning (Tang et al., 2001). In addition, the GluN2B subunit harbors a CaMKII binding site on its intracellular CTD (Barria and Malinow, 2005; Leonard et al., 1999; Strack and Colbran, 1998; Strack et al., 2000), along with several phosphorylation sites critical for LTP (Nakazawa et al., 2006; Rostas et al., 1996). However, experiments using GluN2A knockout mice indicate the involvement of the GluN2A in LTP induction, as noted by their lower level of potentiation compared to wild type mice (Ito et al., 1997; Sakimura et al., 1995b). GluN2A knockout mice show deficits in spatial learning and memory, providing behavioral evidence to support the importance of GluN2A in LTP induction (Bannerman et al., 2008a; Brigman et al., 2008).

Despite these observations, there is no clear evidence suggesting only one GluN2 subunit controls LTP induction and/or facilitates expression. Genetic deletion experiments carry the caveat of compensation, and pharmacological experiments also yield complex data. Furthermore, the majority of postsynaptic NMDARs at Schaffer collateral to CA1 synapses are triheteromeric assemblies containing two GluN1 subunits, one GluN2B subunit, and one GluN2A subunit (Gray

et al., 2011; Rauner and Kohr, 2011; Sheng et al., 1994; Tovar et al., 2013; Yi et al., 2019a). For example, the GluN2B-selective inhibitor ifenprodil can act on triheteromeric GluN2A/GluN2B receptor complexes, and the competitive antagonist NVP-AAM007, which in error was described as GluN2A-selective (Frizelle et al., 2006; Lind et al., 2017), lacks sufficient selectivity from GluN2B-containing NMDARs to yield interpretable results. Thus, consistent with the triheteromeric view of NMDARs at hippocampal synapses, it is likely that both GluN2A and GluN2B subunits are important for LTP induction (*e.g.*, (Volianskis et al., 2013; Volianskis et al., 2015).

A feature of GluN2 subunits is their distinct CTDs that harbor multiple binding sites for scaffolding proteins, kinases, and other binding partners. There may well be some GluN2-specific aspects to types and strength of LTP depending on, for example, CaMKII binding (Barria and Malinow, 2005; Halt et al., 2012; Incontro et al., 2018; Zhou et al., 2007). Of note, deletion of the CaMKII binding site on GluN2B reduces LTP and transfer of this binding site to GluN2A increases LTP (Barria and Malinow, 2005; Halt et al., 2012). Whereas NMDARs and CaMKII are also required for induction of LTD, activation of a calcineurin-DAPK1 signaling pathway that prevents CaMKII from binding to GluN2B appears to be crucial for promoting LTD and preventing inappropriate synaptic potentiation (Coultrap et al., 2014; Goodell et al., 2017).

Since the initial description of LTP, many additional stimulus paradigms have been shown to elicit long-lasting potentiation, leading to the idea that multiple mechanisms exist by which synaptic strength can be enhanced. High frequency stimulation applied in a series of bursts at a frequency within the theta range may be a more physiological stimulus that mimics the endogenous theta rhythms of pyramidal cells. While both tetanic and theta burst stimulus protocols can induce LTP, there may be distinctions between the underlying mechanisms. Application of several theta

bursts produces a robust LTP signal, as these bursts can relieve $Mg^{2+}$ block to allow increased $Ca^{2+}$ flux through NMDARs (Larson and Munkacsy, 2015). LTP at CA1 synapses and in cultured hippocampal neurons can also be induced chemically by a variety of means, including through the application of forskolin (Otmakhov et al., 2004) to activate adenylyl cyclase and rolipram to inhibit phosphodiesterases, which is sufficient to promote LTP induction, expression, and maintenance via activation of the PKA pathway along with spontaneous burst firing of CA3 neurons (Otmakhova et al., 2000). Likewise, some paradigms that utilize glycine application (to ensure saturation of GluN1) in low extracellular $Mg^{2+}$ (to promote activation of synaptic NMDARs by glutamate released in response to spontaneous activity) also induce LTP (*e.g.*, (Lu et al., 2001; Musleh et al., 1997). These forms of chemical LTP have allowed biochemical, imaging, and genetic approaches to better explore signaling systems in LTP, since chemical LTP can produce plastic changes at a much larger number of synapses than local electrical stimulation, although mechanisms may be somewhat different from LTP that is induced by electrical stimulation. While there is enormous volume of work completed at the Schaffer collateral-CA1 synapse, there is ample precedent for both similar and different mechanisms at other central synapses. For example, the mossy fiber-CA3 synapse utilizes an NMDAR-independent LTP induction mechanism (Johnston et al., 1992; Nicoll and Schmitz, 2005).

The induction of LTD has typically involved a low-frequency stimulus paradigm (Kemp et al., 2000), but can also be evoked by postsynaptic action potential generation before a presynaptic stimulation (*i.e.*, spike-timing protocols) (Feldman, 2012) or chemically by low concentrations of NMDA (Lee et al., 1998). These LTD induction paradigms suggest a need for low levels of intracellular $Ca^{2+}$, as opposed to high levels needed to induce LTP. For NMDAR-dependent LTD, $Ca^{2+}$ influx into the PSD activates several phosphatases, including calcineurin and

protein phosphatase 1 (Mulkey et al., 1994) (Figure 5.8). Low levels of $Ca^{2+}$ promote phosphatase activity, which can promote the removal of phosphate from GluA1 Ser831 and Ser845 (Lee et al., 2003) and AMPAR auxiliary proteins (Tomita et al., 2005), which can cause destabilization of AMPAR anchoring and removal from the PSD (Diering and Huganir, 2018). NMDAR-independent LTD is thought to rely on the metabotropic actions of mGluRs, mainly mGluR5, which activates PLC-PKC and tyrosine phosphatase signaling pathways (Gladding et al., 2009; Oliet et al., 1997). It remains a matter of investigation as to how NMDAR-dependent versus NMDAR-independent LTD induction mechanisms are coordinated to depress synaptic strength. Moreover, pharmacological studies initially indicated a primary role for GluN2B-containing receptors in LTD induction (Massey et al., 2004); but see (Morishita et al., 2007), LTD induction can proceed with either GluN2A or GluN2B alone, since genetic deletion of either subunit is insufficient to block LTD (Wong and Gray, 2018).

The CTD of NMDARs respond to conformational changes within the ABD, initiating mechanisms for synaptic plasticity via intracellular signaling that are independent of current flow. NMDAR-dependent LTD can be induced by glutamate binding to the GluN2 subunit in the absence of ion flux (Nabavi et al., 2013; Stein et al., 2015; Stein et al., 2020); but see (Babiec et al., 2014; Sanderson et al., 2016). Here, exposing synaptic receptors to glutamate but blocking the glycine site on GluN1 to block channel gating is sufficient for LTD induction and associated dendritic spine shrinkage. This data indicate that conformational changes within NMDARs enable metabotropic intracellular signaling that leads to LTD induction. Agonist binding to NMDARs, independent of channel gating, generates a conformational rearrangement of the CTD that promotes protein phosphatase 1 disengagement from the receptor complex (Aow et al., 2015; Dore et al., 2015). Sustained, non-ionotropic signaling can lead to dephosphorylation of CaMKII,

potentially via newly-released protein phosphatase 1, culminating in CaMKII removal from the NMDAR CTD (Dore et al., 2015). The diminished catalytic activity of CaMKII and its removal from NMDARs via dephosphorylation may be a priming factor in generating non-ionotropic NMDAR-dependent LTD and associated dendritic spine shrinkage (Nabavi et al., 2013; Stein et al., 2015; Stein et al., 2020), and could also represent a mechanism for synaptic scaling in response to low presynaptic activity.

Glutamate binding to NMDARs can also activate p38 MAP kinase independent of glycine occupancy. Blockade of the GluN1 glycine binding site with antagonist when GluN2 is bound to glutamate reduces dendritic spine diameter in a manner dependent on p38 MAPK kinase (Stein et al., 2015). This non-ionotropic signaling is activated via neuronal nitric oxide synthase and cofilin, which promote cytoskeletal remodeling (Stein et al., 2020). Considering that some extracellular glycine is always present, even if phasically regulated, it is difficult to imagine a situation in which the GluN1 ABD would be unoccupied. However, given the higher potency of glutamate for NMDARs compared to AMPARs, glutamate spillover might preferentially bind to NMDARs without mediating ionotropic signaling due to $Mg^{2+}$ block if the membrane is not adequately depolarized. Thus, non-ionotropic spine shrinkage may influence synaptic strength via unconventional mechanisms (reviewed in (Dore et al., 2017; Rajani et al., 2020).

### *1.14 NMDARs, Genetic Variation, and Disease*

Rapid advances in DNA sequencing technologies have enabled genome-wide sequencing as a means of diagnosis and have yielded an unprecedented amount of genetic data from both patients and healthy individuals. Informatics tools such as the residual variation intolerance score (RVIS) (Petrovski et al., 2013) can be used to analyze genetic variation in the healthy population. These scores indicate that the *GRIN* genes encoding NMDARs show fewer single nucleotide

polymorphisms than most genes, making them among the genes least tolerant to missense mutations (Petrovski et al., 2013) (**Table 1.2** and **1.3**). The intolerance to variation raises the likelihood that variants in these genes will result in disease. Comparison of the observed and expected frequency of genetic missense variations at specific amino acid positions in the healthy population, with the observed/expected ratio referred to as the missense tolerance ratio, can identify regions of a gene that are under purifying selection, also known as negative selection or the selective removal of alleles that are deleterious (Traynelis et al., 2017). Moreover, there are also distinct intolerant regions for each gene product, consistent with the fact that the different NMDAR subunits play unique roles in brain function despite high homology and similar functional properties within a family.

Whole exome sequencing (WES) is widely considered to be the first stage of diagnostic testing when neurodevelopmental disorders are suspected (Srivastava et al., 2019), which includes individuals who show concerns for developmental delay, intellectual disability, and/or seizures. While gene panel testing typically utilizes the same technology as next generation sequencing, it still has limitations in terms of analysis compared to WES, such as a reduced capability to detect genomic copy number variants (CNVs, including deletion or duplication). Variation in genomic copy number will usually affect multiple genes and is detected using chromosomal or SNP microarrays. Genetic diagnosis via WES has changed therapeutic treatments, can produce improved outcomes (Berg et al., 2014; Kuperberg et al., 2016), and when applied early, is cost-effective (Howell et al., 2018).

Patients with *GRIN1*-related neurodevelopmental disorder show multiple deficits, including ID, epilepsy, hypotonia, and for some individuals, movement disorders. All affected individuals evaluated to date show variable levels of intellectual disability (ID): 5% mild, 7% moderate, 71% severe, or 17% profound (Platzer and Lemke, 1993). 65% of *GRIN1* variant

| | RVIS (%) | Total | Missense | Nonsense | Frameshift | Splice | Other |
|---|---|---|---|---|---|---|---|
| ***GRIN1*, GluN1** | **6.92** | **85** | **82** | **2** | **1** | **0** | **0** |
| ***GRIN2A*, GluN2A** | **1.96** | **297** | **199** | **22** | **34** | **13** | **29** |
| ***GRIN2B*, GluN2B** | **1.28** | **252** | **190** | **21** | **21** | **6** | **14** |
| ***GRIN2C*, GluN2C** | **62** | **19** | **13** | **1** | **5** | **0** | **0** |
| ***GRIN2D*, GluN2D** | **11.7** | **26** | **25** | **0** | **0** | **1** | **0** |
| **Total** | **--** | **679** | **509** | **46** | **61** | **20** | **43** |

**Table 1.2.** NMDAR variants and intolerance scores**.** Residual Variation Intolerance Score (RVIS) is given as a percentile rank among all genes. A RVIS of 2.55 means that 97.45% of all genes have more variation in the healthy population than the gene of interest. All variants accounted for in this table were not present in the healthy population (gnomAD database). Other includes large-scale chromosomal deletions, translocations, inversions, or duplications. Table adapted from (Hansen et al., 2021).

| | EPI | ID | ASD | ADHD | MD | SCZ |
|---|---|---|---|---|---|---|
| ***GRIN1*, GluN1** | **27** | **38** | **4** | **0** | **21** | **3** |
| ***GRIN2A*, GluN2A** | **203** | **190** | **19** | **5** | **28** | **5** |
| ***GRIN2B*, GluN2B** | **99** | **187** | **35** | **2** | **10** | **5** |
| ***GRIN2C*, GluN2C** | **1** | **4** | **8** | **0** | **0** | **7** |
| ***GRIN2D*, GluN2D** | **13** | **13** | **5** | **0** | **0** | **9** |
| **Total** | **343** | **432** | **71** | **7** | **59** | **29** |

**Table 1.3.** NMDAR variants and associated disease. All variants accounted for in this table were not present in the healthy population (gnomAD database). EPI means epilepsy; ID means intellectual disability; ASD means autism-spectrum disorder; ADHD means attention deficit/hyperactivity disorder; MD means movement disorders; SCZ means schizophrenia. Table adapted from (Hansen et al., 2021).

individuals present with epilepsy. Onset ranges from birth to 11 years of age, and two thirds of demonstrated resistance to conventional antiseizure treatment. Some individuals show features of autism spectrum disorders, or exhibit other behavior problems such as stereotypic movement disorder (32%), sleep problem (15%), and self-harm behavior (7%) (Platzer and Lemke, 1993). A subset of individuals show an unusual type of cortical malformation that consisted of extensive bilateral polymicrogyria together with lateral ventriculomegaly, enlarged extra-axial spaces, reduced thickness of the corpus callosum, basal ganglia dysplasia, and decreased white matter volume (Fry et al., 2018).

Neurodevelopmental disorders in *GRIN2A* individuals are associated predominantly with epilepsy and ID. However, as many as 37% of the individuals demonstrate normal intelligence, 63% have (often mild) ID (46%), and others with moderate (22%), severe (11%) or profound (21%) ID (Strehlow et al., 2019). Brain imaging is usually normal and only a minority (14%) reveal nonspecific changes (Strehlow et al., 2019). Epilepsy is present in almost all *GRIN2A* individuals with onset from birth to 8 years of age. Interestingly, seizures may resolve between 8 and 20 years of age (see Chapter 3). Additional challenges include hypotonia (29%), movement disorders (27%), autism spectrum disorders (9%), and/or psychiatric disorders, such as schizophrenia (3%) (Strehlow et al., 2019). A unique feature associated within the *GRIN*-associated disorders is the breadth of language/speech problems observed in *GRIN2A*-related developmental disorders, which include dysarthria, dysphasia, speech dyspraxia, speech regression with residual impairments in more than a third of all cases (Strehlow et al., 2019; Turner et al., 2015); 19% of *GRIN2A* individuals have aphasia.

Individuals affected with *GRIN2B*-associated disorders exhibit ID, hypotonia, epilepsy, and movement disorders. All affected (so far) have developmental delay preceding certain degrees

of ID. Some form of epilepsy is present in half of the affected individuals and shows an onset between birth to 9 years of age. Seizures are medically refractory for half. Additional clinical characteristics are perhaps less frequent and/or are somewhat milder than *GRIN1*-associated neurodevelopmental disorders. However, the spectrum of clinical characteristics is similar to *GRIN1* with hypotonia (56%) and spasticity (23%), autism spectrum disorder (26%), movement disorders (10%), and cortical visual impairment (8%) (Platzer and Lemke, 1993) (Table 2). Cortical malformations with polymicrogyria and basal ganglia dysplasia in *GRIN1* is mirrored in a subset of individuals with *GRIN2B* disorders (Platzer et al., 2017).

Variation in *GRIN2D* appears far less frequent than that in *GRIN1*, *GRIN2A*, or *GRIN2B* (Camp and Yuan, 2020; XiangWei et al., 2018). One population-based study reported no truncated *GRIN2D* variants, suggesting a crucial role in early development and survival (Tarabeux et al., 2011). However, a different conclusion was reached by other investigators (Santos-Gomez et al., 2021), who raised the idea that intronic variations (i.e. missense) might be related to the risk for schizophrenia (Yu et al., 2018). *GRIN2D* missense variants have been observed in individuals with severe, drug-resistant epileptic encephalopathy with an early onset (Camp and Yuan, 2020; Jiao et al., 2021; Li et al., 2016; Tsuchida et al., 2018; XiangWei et al., 2019a). Functional analysis of variants introduced into *GRIN2D* cDNA have shown gain of function characteristics (Camp and Yuan, 2020; Li et al., 2016), possibly with a compensatory reduced expression (XiangWei et al., 2019a). Only 28 *GRIN2D* variants are currently documented in the literature (Hansen et al., 2021).

An understanding of the mechanisms by which the disease-associated variants produce a clinical phenotype requires functional evaluation of the variant receptors. This functional understanding is essential to classify variants of unknown significance, develop therapeutic options, and offers a logical way to stratify patients. Among the ~700 known *GRIN* variants,

functional evaluation has been published in peer-reviewed literature for well over 200 variants (see Center for Functional Evaluation of Rare Variants (CFERV), http://functionalvariants.emory.edu/). The published functional evaluations of disease-associated variants range from evaluation of a single parameter (*i.e.*, current amplitudes or agonist potency) to comprehensive evaluation of multiple parameters *in vitro* and *in vivo*. Multiple parameters can be assessed *in vitro* using recombinant receptors and native cells/tissues from transgenic animals to determine current amplitude, agonist (glutamate and glycine) potency, voltage-dependent $Mg^{2+}$ block, sensitivity to endogenous negative modulators ($Zn^{2+}$ and protons), channel open probability, channel activation time course, glutamate deactivation time course (which can be predictive of synaptic response time course), desensitization, long-term potentiation/depression, receptor trafficking, and neuronal excitotoxicity (Addis et al., 2017; Amador et al., 2020; Amin et al., 2018; Fedele et al., 2018; Li et al., 2019; Ogden et al., 2017; Shin et al., 2020; Strehlow et al., 2019; Swanger et al., 2016; Vyklicky et al., 2018; XiangWei et al., 2018; XiangWei et al., 2019b). These *in vitro* evaluations can be combined for many variants to provide a quantitative prediction of the overall effects that variants have on NMDAR pharmacology, function, and receptor localization. While this approach has the capacity to approximate the contribution of the *GRIN* variants to NMDAR-mediated synaptic and non-synaptic signaling (Swanger et al., 2016); see also (XiangWei et al., 2018), it will be critical to evaluate function, surface expression, and subcellular localization of variants in neurons. If the variants are not trafficked to the neuronal surface or incorporated into the synapse, then a functionally null phenotype would be seen in transgenic animals or transfected neurons.

The term gain-of-function is defined as any variant-related increase in NMDAR-mediated signaling, and could arise from enhanced agonist potency (*e.g.*, glutamate and/or glycine), reduced

sensitivity to voltage-dependent $Mg^{2+}$ block, reduced sensitivity to endogenous negative modulators, prolonged synaptic response time course, enhanced gating or open probability, reduced receptor desensitization, and/or enhanced receptor cell surface trafficking. Each of these effects could differentially impact synaptic and perisynaptic receptors, which are exposed to different concentrations of glutamate (*e.g.*, (Moldavski et al., 2020; Swanger et al., 2016). Ultimately, these variants will likely have different clinical manifestations, since they would be expected to alter circuit function in different ways. Similarly, loss-of-function variants, defined as producing a reduction in NMDAR-mediated signaling, may arise from reduced agonist potency, enhanced sensitivity to voltage-dependent magnesium block or other endogenous negative modulators, shortened deactivation time course, reduced response amplitude, and/or decreased receptor cell surface trafficking. While these parameters can be easily measured *in vitro*, compensatory and developmental changes could produce equally (or more) important changes in the expression profile of other genes and connectivity of neural circuits. Furthermore, it is unclear if loss-of-function variants caused by nonsense mutations or deletions will behave the same as missense loss-of-function variants, which may assemble with other subunits and could exert a dominant negative effect. While global designations of variant function are helpful, clinical phenotypes are likely driven by variant-induced changes in specific receptor properties, rather than the overall net effect captured by the gain- and loss-of-function categorization.

The complexity of the effects of *GRIN* variants on clinical phenotypes is illustrated by the finding that both gain- and loss-of-function variants in the same gene can result in similar neurologic symptoms, such as seizure disorders. For instance, *GRIN2A* gain-of-function human variants L812M (Yuan et al., 2014) and P552R (Ogden et al., 2017), and loss-of-function *GRIN2A* variants D731N (Gao et al., 2017) and V685G (Swanger et al., 2016) are observed in patients with

infantile onset epilepsy and cognitive impairment. This is intriguing as one might hypothesize that the loss of excitatory synaptic GluN2A subunits would decrease excitability, rather than promote an epileptic phenotype. This paradoxical observation is also seen for loss-of-function *GRIN2A* truncation variants, which display seizure phenotypes (Carvill et al., 2013; Lemke et al., 2013; Lesca et al., 2013), and *Grin2a* knockout mice that show hyperexcitability (Salmi et al., 2018; Salmi et al., 2019). These observations suggest that the loss of signaling from GluN2A-containing NMDAR must be placed into a developmental context, whereby change in NMDAR function in particular cells (*i.e.*, interneuron vs principal cell) impacts a critical developmental window and likely changes the overall balance of excitation and inhibition (see Chapter 3). Moreover, functional evaluation of variants can result in conflicting results from multiple parameters. For example, the *GRIN2A* variant A643D showed enhanced glutamate potency, but decreased current response and reduced cell surface expression (Fernandez-Marmiesse et al., 2018). Therefore, evaluation of only one or two aspects of the functional effects of variants is insufficient for reaching a conclusion on the physiological consequence of a given variant and could lead to ineffective therapeutic strategies.

### *1.15 NMDAR Variants and Models of Disease*

To move a step closer to human physiology, the generation of mouse models containing human-specific variants allows for the detailed exploration of *GRIN* variants. More specifically, genetically-modified mice are essential for the elucidation of how these variants impact the brain on a developmental, circuit, cellular, and molecular level, as well as provide investigators with a mammalian platform for testing therapeutic approaches. At the gross anatomical level, mice and man have remarkable similarities (Hodge et al., 2019; Semple et al., 2013), however, recent single cell sequencing studies have confirmed at the level of neurotransmitter receptors, mice and man

are unsurprisingly disparate (Hodge et al., 2019; Sjostedt et al., 2020). Still, the use of mouse models prevails in modern bench research thanks to ease with which their genome can be manipulated, monetary efficiency, and wealth of information in the literature about murine-derived neurons. Although limitations exist, the use of mouse models has progressed scientific discovery in countless ways and is considered the gold standard starting point for exploration of human disease pathophysiology.

Homozygous null (knockout) mutations for each of the seven *GRIN* genes have been generated, however, information gleaned from the study of these mice should be viewed cautiously, as the majority with *GRIN* variants only have one impacted allele. Gene deletions can affect individuals differently than LoF missense and nonsense variants, which suggests that LoF missense and nonsense variants have the capacity to act as dominant negatives. Thus, heterozygous null (knockout) or variant knock-in mouse models – whether nonsense, missense, or deletion – are highly relevant to the human condition and should thus be given precedent when making comparisons to affected individuals.

The first targeted mutation in the *GRIN* gene family was the homozygous null mutation in the *Grin1* gene, which resulted in perinatal lethality (Forrest et al., 1994; Li et al., 1994). *Grin1* null mice die due to respiratory failure as well as failure to suckle, highlighting the omnipresent roles of NMDARs in all facets of early brain function, including those in the brainstem. Like homozygous *Grin1* null mice, homozygous *Grin2b* null mice also suffer perinatal lethality (Kutsuwada et al., 1996b). Although *Grin2b* knockout mice do breathe, they do not suckle and thus do not survive the neonatal period. The homozygous null mutations of *Grin2a* (Kadotani et al., 1996; Sakimura et al., 1995a), *Grin2c* (Ebralidze et al., 1996), and *Grin2d* (Ikeda et al., 1995a) appear less severe than those homozygous null *Grin1* and *Grin2b* mice, with each being fertile and

viable, albeit with aberrations in a variety of behavioral and cognitive tasks (Bannerman et al., 2008b; Boyce-Rustay and Holmes, 2006; Brigman et al., 2008; Camp and Yuan, 2020; Hillman et al., 2011; Salimando et al., 2020; Shelkar et al., 2019; Yamamoto et al., 2017). See **Table 1.4** for full details on all null NMDAR mouse models and their associated neurobehavioral outcomes.

### *1.16 Conclusions*

NMDARs are a multifaceted group of ionotropic glutamate receptors whose roles in neurobiology have extended far beyond an ion channel that helps induce long-term potentiation in recent decades. The overarching goals of the work presented in this thesis are two-fold: 1) to provide cellular and synaptic characterization of mouse models based on NMDAR human patient-derived variants and 2) show efficacy of subunit-selective NMDAR modulation in altering circuit function for potential therapeutic gain. The totality of these data adds significantly to the field of NMDAR biology and neuroscience as whole, providing 1) groundwork characterization of several novel mouse models of NMDAR human patient-derived variants, 2) supportive data in the emerging roles of NMDARs in neurodevelopment, and 3) data that refines the ideal that all GABAergic interneurons show functional synaptic GluN2D-containing receptors.

| Protein Subunit Name | Heterozygous Null Phenotype | Homozygous Null Phenotype | Individuals with Large-Scale Disruptions (LSD) or Premature Stops |
|---|---|---|---|
| | ***Severe Phenotypes in Mice*** | | |
| GluN1 | Viable and fertile (Elberger and Deng, 2003; Forrest et al., 1994); accelerated cortical neuron development (Elberger and Deng, 2003) | Lethal – No suckling response and poor lung development (Forrest et al., 1994); altered brainstem nuclei development (Li et al., 1994) | 4 total (4/91; 4.4%):<br>4 preSTOP |
| GluN2B | Viable and fertile (Kutsuwada et al., 1996b) | Lethal – No suckling response with altered brainstem nuclei development (Kutsuwada et al., 1996b) | 36 total (36/258; 14%):<br>14 LSD and 22 preSTOP |
| | ***Viable, but with deficits*** | | |
| GluN2A | Viable and fertile (Sakimura et al., 1995a); transient changes in brain microstructure assessed via diffusion tensor imaging (Salmi et al., 2018) | Viable and fertile (Sakimura et al., 1995a); diminished spatial learning (Sakimura et al., 1995a) with largely transient epileptiform activity (Salmi et al., 2018; Salmi et al., 2019); | 53 total (53/311; 17%):<br>29 LSD and 24 preSTOP |
| GluN2C | Viable and fertile (Ebralidze et al., 1996); alterations in sensorimotor gating (Gupta et al., 2016) | Viable and fertile (Ebralidze et al., 1996); some alterations in working memory (Hillman et al., 2011) and sensorimotor gating (Gupta et al., 2016) | 1 total (1/19; 5.3%):<br>1 preSTOP |
| GluN2D | Viable and fertile (Ikeda et al., 1995b) | Viable and fertile; hypolocomotion and increased anxiety-like behaviors (Ikeda et al., 1995b; Salimando et al., 2020; Yamamoto et al., 2017) | Yet to be reported |
| GluN3A | Viable and fertile (Das et al., 1998) | Viable and fertile (Das et al., 1998); impaired locomotor activity (Mohamad et al., 2013); changes to spatial and aversion learning (Mohamad et al., 2013; Otsu et al., 2019) | 2 total (2/13; 15%):<br>2 preSTOP |
| GluN3B | Viable and fertile (Niemann et al., 2007) | Viable and fertile (Niemann et al., 2007); impaired motor learning and coordination (Niemann et al., 2007) | Yet to be reported |

**Table 1.4.** NMDAR subunit null mouse models and prevalence of null mutations in affected individuals**.** Heterozygous and homozygous null (knockout) mouse models have been generated for each of the seven *GRIN* genes. Mice containing homozygous null mutations for GluN1 and GluN2B are postnatal lethal, while heterozygous null offspring survive normally. In general, homozygous null mice, regardless of which gene has been disrupted, display some sort of aberrant phenotype that could be extrapolated to patients. It should be noted, however, only one large-scale chromosomal disruption has been reported to be homozygous in human, an inherited *GRIN2A* deletion affecting both alleles, with all others reported being heterozygous. Given the overall lack of characterization of heterozygous null *GRIN* mouse models, it is difficult to determine whether they can truly mimic features of the human condition. Large-scale disruptions (LSD) refers to chromosomal deletions, duplications, inversions, insertions, or translocations; premature stops (preSTOP) refers to a nonsense mutation resulting in a premature stop codon in the mRNA. Table adapted from (Benke et al., 2021).

*1.17 References for Chapter 1*

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# CHAPTER 2: Mouse Models of Gain-of-Function NMDAR Variants

*2.1 Introduction*

Functional information gathered from heterologous systems expressing human patient-derived NMDAR variants, can advance our understanding of which regions of the receptor are most critical for its function. Unsurprisingly, variants within the agonist-binding domain (ABD) and around the transmembrane domain (TMD) helices, regardless of NMDAR subunit, are usually the most deleterious (Strehlow et al., 2019). For example, variants in the ABD and TMD regions show the most change from wildtype NMDARs in terms of a variety of measurable factors such as: agonist potency, magnesium block, deactivation time course, rise time, and/or open-channel probability (Benke et al., 2021). Functional analysis is a necessary step that provides a wealth of opportunities to advance understanding of each variant as well as overall receptor biology. It allows stratification of individuals for future approaches to precision therapies, as individuals with variants that produce similar changes in receptor function are more likely to identify a signal in clinical trials than variants that simply happen to be close to each other on the polypeptide chain. This is because similar changes in receptor function are likely to have similar action on circuit function.

Given the expense and time investment associated with generating and investigating human variants using knock-in mouse models, a prudent selection strategy would be to pick variants that show the most drastic changes from wildtype receptors *in vitro* and/or those that produce the most severe phenotypes in humans. An agnostic evaluation of gain-of-function (receptor performs better than wildtype receptor, *e.g.*, a prolongation of deactivation time) versus loss-of-function (receptor performs worse than wildtype receptor, *e.g.*, a shortening of deactivation time) *GRIN2A* variants showed gain-of-function *GRIN2A* variants were more likely to be associated with more severe phenotypes, such as epileptic spasms and detrimental levels of intellectual disability (Strehlow et

al., 2019). Moreover, this study revealed that severe gain-of-function *GRIN2A* variants were centered around the TMD and its associated linker regions which are responsible for transducing motion in the agonist binding into channel opening (Strehlow et al., 2019).

Using variant severity as a selection strategy, one of the first knock-in mouse models generated by Dr. Wayne Frankel and Jackson Laboratories in Bar Harbor, was the gain-of-function (GoF) GluN2A-P552R mouse. In humans, this missense variant is associated with severe intellectual disability (de Ligt et al., 2012) and in transfected HEK cells, this variant produces a unique prolongation of the activation rise time not seen in many other GoF *GRIN2A* variants (Ogden et al., 2017). Collaborations with over a hundred clinicians developed by the Center for Functional Evaluation of Rare Variants at Emory allowed us to sample a large number of human variants, from which we selected an additional variant to study from a patient with intractable seizures. This new *GRIN2A* variant, GluN2A-S644G, produced a strong GoF when studied *in vitro*. Dr. Frankel generated a second knock-in mouse model containing this GoF *GRIN2A* variant, GluN2A-S644G, and in collaboration we were able to show efficacy of FDA-approved NMDAR modulators in reducing seizure burden (Amador et al., 2020). Given our relative success with these first two mouse models of human NMDAR variants, we generated a third mouse model based on a GoF *GRIN2D* variant, GluN2D-V667I. We picked a variant in the GluN2D gene as *GRIN2D* variants also show severe epileptic phenotypes. Additionally, given GluN2D's sparse expression pattern which includes cortical GABAergic interneurons, investigation of a GoF *GRIN2D* variant provides us with a unique opportunity to learn more about GluN2D's role in neurobiology.

Here, I describe experiments with three different gain-of-function NMDAR mouse models that were generated to serve as mammalian model systems to understand human disease. Results from this modelling approach provide some of the first insights into the nature of NMDAR variant-

mediated disease at the synaptic and circuit level. Moreover, mouse models of human disease provide a starting point to test new therapeutic approaches such as gene replacement and/or silencing therapies.

## *2.2 Methods*

### 2.2.1 Mouse Model Generation

All mouse procedures were conducted at The Jackson Laboratory, Columbia University Irving Medical Center, or Emory University, each fully accredited by AAALAC, approved by respective IACUC and performed in accordance with state and federal Animal Welfare Acts and Public Health Service policies. *Grin2a*$^{P552R}$ and *Grin2a*$^{S644G}$ mice were generated in the C57BL/6NJ (B6NJ) mouse strain using CRISPR/Cas9 and an oligonucleotide donor sequence as part of the JAX Center for Precision Genetics (JCPG) and maintained by backcrossing heterozygous males to wildtype B6NJ females. For GluN2D-V667I, the conditional knock-in mice were generated using a knockout-first strategy. Briefly, a DNA cassette containing LoxP-Stop-LoxP V664I knockin cassette was inserted between exon 7 and 8 and injected into single-cell mouse embryos of founder mice. Genotypes were confirmed by long- and short-range Sanger sequencing and heterozygotes crossed with wildtype C57BL/6J for maintenance. Experimental mice that remove the knock-in cassette (allowing expression of the mutated exon) were generated following crosses with suitable cre driver strains, such as the Sox2-Cre line used induced recombination in all neuronal cells. Mice were housed in ventilated cages at controlled temperature (22–23ºC), humidity ~60%, and 12h:12h light:dark cycles. Mice had *ad libitum* to regular chow and water.

2.2.2 Electroconvulsive Threshold

Tests on adult mice between 8 and 13 weeks of age were performed using transcorneal electrodes with the Ugo Basile Model 7801 electroconvulsive device as described previously (Asinof et al., 2015). Settings for minimal or maximal generalized seizure endpoints were 299 Hz, 1.6 ms pulse width, 0.2 s duration, variable current; those for 6 Hz partial or psychomotor seizure endpoints were 6 Hz, 0.2 ms pulse width, 3.0 s duration, variable current. Data were analyzed as the integrated root mean square (iRMS, area under the curve) as (square root of Hz) × current × pulse width × duration.

2.2.3 Acute Hippocampal Slice Preparation and Electrophysiological Recordings

P14-20 (GluN2A-P552R and GluN2A-S644G) or P21-26 (GluN2D-V667I) mice from both sexes were overdosed with inhaled isoflurane, brains were rapidly removed and immediately placed in an ice-cold, sucrose-based artificial cerebrospinal fluid (aCSF) containing the following (in mM): 88 sucrose, 80 NaCl, 2.5 KCl, 1.25 $HNa_2PO_4$, 26 $HNaCO_3$, 10 glucose, 2 thiourea, 3 sodium pyruvate, 5 sodium ascorbate, 12 N-acetylcysteine, 10 $MgSO_4$, and 0.5 $CaCl_2$ bubbled in 95% $O_2$/5% $CO_2$. 300-µm thick horizontal hippocampal sections were obtained using a vibratome (Lecia, VT1200S). After sectioning, slices were incubated in a sucrose-based aCSF as described above but with 4 mM $MgSO_4$ at 32°C for 30 minutes then returned to room temperature for at least an hour before use. Cells were visualized using an upright Olympus BX50W microscope with IR-DIC optics coupled to a Dage IR-2000 camera. Whole-cell patch clamp recordings were obtained using an Axopatch 200B (Molecular Devices) or a Multiclamp 700B (Molecular Devices, digitized at 20 kHz using a Digidata 1440a (Molecular Devices) controlled by pClamp 10.6 software

(Molecular Devices). All signals were low-pass filtered at 2 kHz using a Bessel 8-pole filter (Warner, LPF-8).

For NMDAR-mediated EPSCs, the following intracellular solution was used (in mM): 100 Cs-gluconate, 5 CsCl, 0.6 EGTA, 5 BAPTA, 5 $MgCl_2$, 8 NaCl, 2 Na-ATP, 0.3 Na-GTP, 40 HEPES, 5 Na-phosphocreatine, and 3 QX-314. All recordings were made in the following aCSF extracellular solution (in mM): 126 NaCl, 2.5 KCl, 1.25 $HNa_2PO_4$, 26 $HNaCO_3$, 20 glucose, 0.2 $MgSO_4$, and 1.5 $CaCl_2$ bubbled with 95% $O_2$/5% $CO_2$ and held at 30-32°C using an inline heater (Warner, SH-27B). A monopolar iridium-platinum stimulating electrode (FHC, Inc.) was placed in the upper 1/3$^{rd}$ of the Schaffer collaterals to elicit a single 50 μs stimulation at a frequency of 0.03 Hz and the NMDAR-mediated EPSC was pharmacologically isolated with 10 μM NBQX and 10 μM gabazine. For GluN2A-P552R and GluN2A-S644G mouse recordings, cells were held at -30 mV while cells from GluN2D-V667I mice were recorded at +40 mV. For all cells, stimulation intensity was chosen to be near 50% of the maximum peak amplitude of the EPSC. A total of 8-12 epochs were recorded and averaged together. At the conclusion of recording, 200 μM DL-APV was applied to ensure responses were mediated via NMDARs. The APV-sensitive leak was calculated as a leak current after 5 minutes of 200 μM DL-APV application subtracted from the leak current at the beginning of the NMDAR-mediated EPSC experiment. A weighted time constant was calculated using the following formula by fitting a dual-exponential function to each composite EPSC trace in ChanneLab (Synaptosoft):

(1) $\tau_W = (\tau_{FAST}\,\text{amplitude}_{FAST} + \tau_{SLOW}\,\text{amplitude}_{SLOW})/(\text{amplitude}_{FAST} + \text{amplitude}_{SLOW})$ .

For sIPSCs and mIPSCs, cells were held at -60 mV to minimize depolarization-induced suppression of inhibition (Diana and Marty, 2003) in normal aCSF (1.5 mM $Mg^{2+}$) with NBQX (10 μM) present at all times. A high chloride internal solution was used, containing the following

(in mM): 135 cesium chloride, 3.5 NaCl, 1 $MgCl_2$, 10 HEPES, 0.6 EGTA, 2 $Na_2ATP$, 0.3 $Na_2GTP$, 5 sodium phosphocreatine, and 3 QX-314. sIPSCs recordings were made for a total of five minutes (with the last two minutes being used for analysis). 1 μM TTx was washed onto the slice for ten minutes and then mIPSC recordings were made for a total of five minutes (with the last two minutes being used for analysis). All experiments ended by washing on 10 μM gabazine to ensure all responses were mediated by $GABA_A$ receptors. Data were analyzed offline using MiniAnalysis (Synaptosoft) with an 8-pA amplitude threshold for event detection.

For all electrophysiological recordings, series resistance was monitored throughout all experiments and was typically 8−20 MΩ. For voltage clamp recordings, a 50 ms, 5-mV square wave was included in the stimulation paradigm or inserted once every 30 seconds in gap-free mode for sIPSCs and mIPSCs. Series resistance was monitored throughout the entire recording. All series resistances were measured offline by analyzing the peak of the capacitive charging spike and applying Ohm's law. If the series resistance changed >25% during the experiment, or ever exceeded 30 MΩ, then the cell was excluded.

### 2.2.4 Statistical Analysis and Figure Preparation

Two-tailed, unpaired Student's t-test, one-way ANOVA with Bonferroni's correction method for multiple comparisons, and the Kolomorgov-Smirnov tests were used were appropriate. For experiments where multiple statistical analyses were performed on the same dataset, our significance threshold was lowered to correct for family-wise error rate (FWER) using the Bonferroni post-hoc correction method. All studies were designed so that an effect size of at least 1 was detected at 80% or greater power. All statistical analyses were performed in Prism's GraphPad software all figures were generated in Microsoft's PowerPoint.

*2.3 Results*

All three variants – GluN2A-P552R, GluN2A-S644G, and GluN2D-V667I – are in the regions determined to be intolerant to variation based on lack of variants in these regions in the healthy population (Ogden et al., 2017). The P552 residue is in the pre-M1 helix of the TMD (**Figure 2.1**). This portion of the receptor is critical for gating, helping to transduce molecular motion in the ABD from ligand binding into motion within the TMD sufficient for pore opening (McDaniel et al., 2020; Ogden et al., 2017). Both the S644 and V667 residues are part of the M3 helix within the TMD, with the GluN2A-S644G variant being the start of the highly conserved SYTANLAAF motif (Jones et al., 2002) (**Figure 2.1**). This SYTANLAAF motif is found in all iGluRs and is also one of the most intolerant regions of the entire receptor. Moreover, SYTANLAAF and the M3 helix, in cooperation with the pre-M1 helix and M4 helix of a neighboring subunit within the NMDAR complex form the gating 'triad' as these key regions lie within 5 Å of each other (Gibb et al., 2018; McDaniel et al., 2020; Ogden et al., 2017). In essence, these three regions of the receptor work together to initiate pore opening facilitating ion flux. Thus, each of these variants are in highly conserved and functionally important portions of the receptor, explaining why these were some of the first mouse models we generated. Moreover, as evident from **Table 2.1**, each of these variants show severe human phenotypes.

The GluN2A-P552R mutant mouse model did not display spontaneous seizures. Heterozygous knock-in mice showed no premature lethality, however, homozygous GluN2A-P552R/GluN2A-P552R mice died of lethal seizures around 30 days postnatally (P) (data not shown). Maximum (tonic-clonic) electroconvulsive threshold (ECT) did not appear to be unchanged between wildtype (+/+) and heterozygous GluN2A-P552R mice, regardless of sex (**Figure 2.2A**). Data were not tested for significance as current data are underpowered. Unlike the

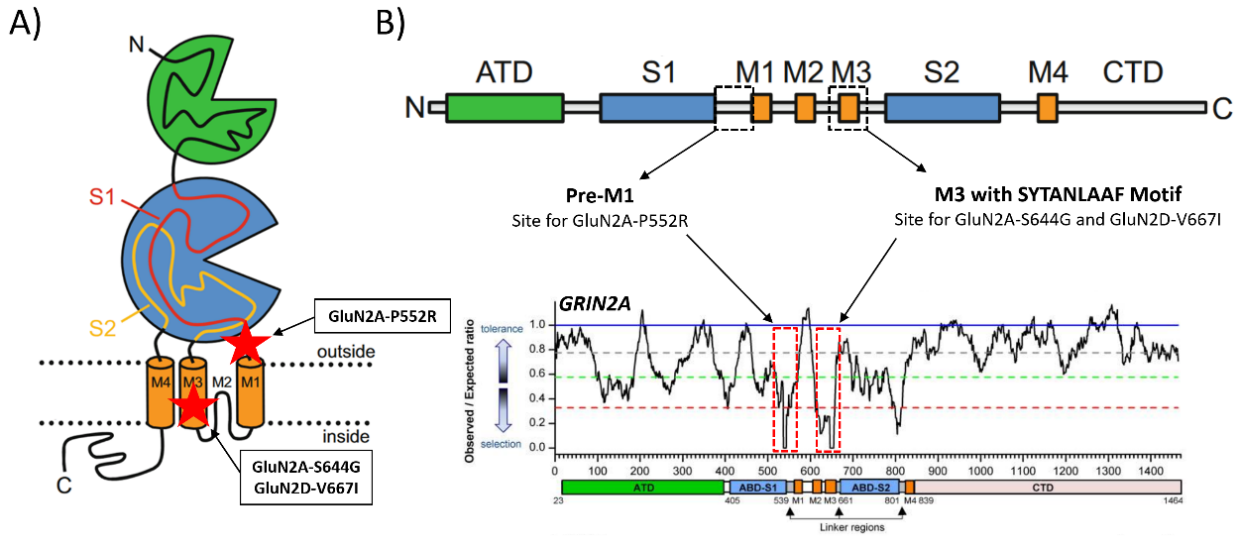


**Figure 2.1.** Topographical location of variants used in mouse model experiments. A) Cartoon structure of a typical NMDAR subunit. The location of each variant is denoted with a red star. Figure adapted from (Hansen et al., 2017) B) *Top*: cartoon structure of the polypeptide chain showing each domain of a typical subunit. The pre-M1 and M3 regions are highlighted to demonstrate where each variant lies along the protein. *Bottom*: *GRIN2A* genetic intolerance plot showing sliding window of observed versus expected (OE) variant estimates (black full line), neutrality expected OE-ratio estimate (blue full line), median OE-ratio for the gene (dark grey dashed line), 25$^{th}$ percentile of OE-ratio (green dashed line), and 5$^{th}$ percentile of OE-ratio (red dashed line). Red dashed boxes are around pre-M1 and M3 to highlight absolute intolerance to genetic mutation. Data represented are for the *GRIN2A* gene, however, the OE-ratio sliding window plot for *GRIN2D* also shows absolute intolerance in pre-M1 and M3. Figure adapted from (Hansen et al., 2017) and (Ogden et al., 2017).

| | Variant Origin | Epilepsy? | Other clinical manifestations? | Impacted Individuals | Response to NMDAR modulators |
|---|---|---|---|---|---|
| **GluN2A-P552R** | *De novo* | Yes – refractory; since 9 months of age | Yes – severe developmental and intellectual delay; no speech, aphasia | One to date | Currently unknown |
| **GluN2A-S644G** | *De novo* | Yes – refractory; over 200 seizures/month | Yes – severe developmental and intellectual delay, no independent head control | One to date | Limited: Memantine and dextromethorphan limited seizure burden, but daily seizures still exist; no recovery from other clinical symptoms; magnesium treatment not given at time of report |
| **GluN2D-V667I** | *De novo* | Yes – refractory; multiple seizures a day | Yes – severe developmental and intellectual delay | Two to date | Depends on age at administration: patient 1 given memantine at 6.5 years which worsened seizures, but patient did respond to magnesium and ketamine; patient 2 was given memantine at 2.5 years which improved seizure burden |

**Table 2.1.** Human patient data of gain-of-function missense NMDAR variants.

Data for GluN2A-P552R patient from (de Ligt et al., 2012; Ogden et al., 2017)

Data for GluN2A-S644G patient from (Amador et al., 2020)

Data for GluN2D-V667I patient from (Camp and Yuan, 2020; Li et al., 2016)

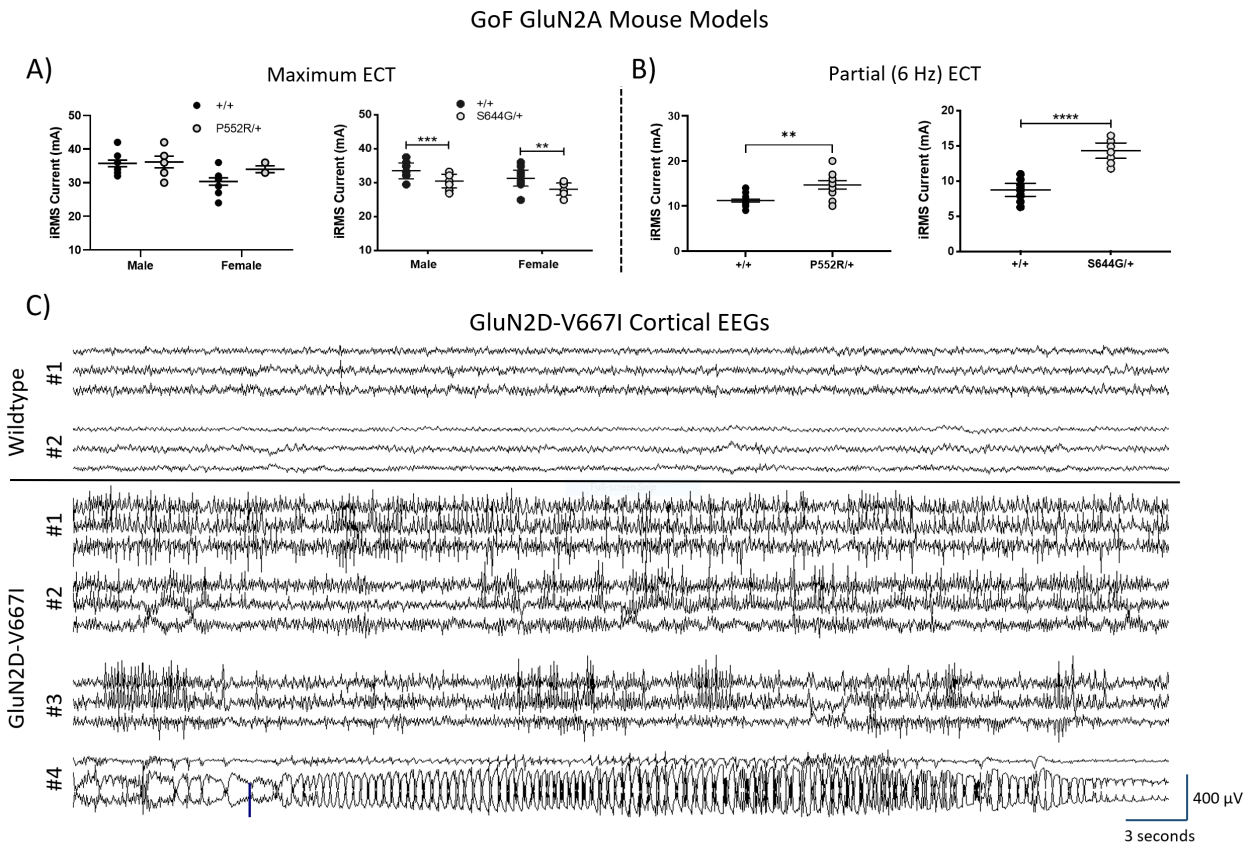


**Figure 2.2.** Seizure phenotypes of mouse models of human NMDAR variants. A) Maximum (generalized tonic-clonic) electroconvulsive current threshold (ECT) in both GluN2A-P552R (left) and GluN2A-S644G (right) mouse models. Data from GluN2A-P552R are underpowered and thus could not be subjected to statistical testing. Maximal ECT is significantly lower compared in GluN2A-S644G mice compared to age-matched +/+ littermates, regardless of sex (female: n=7 for +/+ and n=6 for GluN2A-S644G/+, p = 0.0434, Mann-Whitney rank-sum test; male: n=9 for +/+ and n=15 for GluN2A-S644G/+, p = 0.0007, Mann-Whitney rank-sum test). B) Partial (6 Hz) ECT in both GluN2A-P552R (left) and GluN2A-S644G (right) mouse models. Partial ECT is significantly higher in both GluN2A-P552R mice (n=21 for +/+ and n=10 for GluN2A-S644G; p=0.003, Mann-Whitney rank-sum test) and GluN2A-S644G mice (n=31 for +/+ and n=33 for GluN2A-S644G; p=4.1e-12, Mann-Whitney rank-sum test). C) Cortical electroencephalographic (EEG) recordings from preadolescent (postnatal 16-22) +/+ (n=2 mice) and GluN2D-V667I (n=4 mice).

Data collection is on-going. Data are mean±SEM. ** = $p<0.01$; *** = $p<0.001$; **** = $p<0.0001$. GluN2A-P552R and GluN2D-V667I data are unpublished. GluN2A-S644G data adapted from (Amador et al., 2020).

GluN2A-P552R mice, heterozygous GluN2A-S644G mice have a significantly lower current on the maximum ECT assay compared to wildtype (+/+) mice, in both males and females (**Figure 2.2A**). Surprisingly, both heterozygous GluN2A-P552R mice (**Figure 2.2B**) and heterozygous GluN2A-S644G mice (**Figure 2.2B**) showed a statistically significant decreased current threshold for partial (6 Hz) corneal seizures compared to their respective wildtype littermates. In all, these data suggest that both GoF GluN2A mouse models show signs of altered circuit excitability.

Heterozygous GluN2D-V667I mice appear to have the most robust epileptic phenotype as these mice died from spontaneous lethal seizures before P35 (data not shown). Obtaining homozygous GluN2D-V667I mice has not been attempted. Cortical EEG recordings from preadolescent mice show epileptiform activity with large spike-and-wave discharges not observed in wildtype littermates (**Figure 2.2C**). ECT assays in these mice are currently on-going. Given the lethal seizure phenotype observed in adolescent heterozygous GluN2D-V667I mice, an alternative strategy for generating F1 and F2 generations had to be employed since mice never reach sexual maturity (see methods for details).

After mouse model generation, we wanted to compare NMDAR-mediated EPSC kinetic properties to responses obtained in heterologous expression systems. This would allow us to see how well our heterologous data translates to native synapses, as well as verify that the mutant receptor is made by the cell, is trafficked to the postsynaptic density, and can participate in synaptic neurotransmission. HEK cell recordings can be designed to mimic a single synaptic event in that a transfected cell can be rapidly exposed (1-5 ms) to saturating concentrations of glutamate (1 mM) in presence of glycine to generate a synaptic-like activation and deactivation time course. These data can then be compared to synaptic data as a means of mouse model validation and to

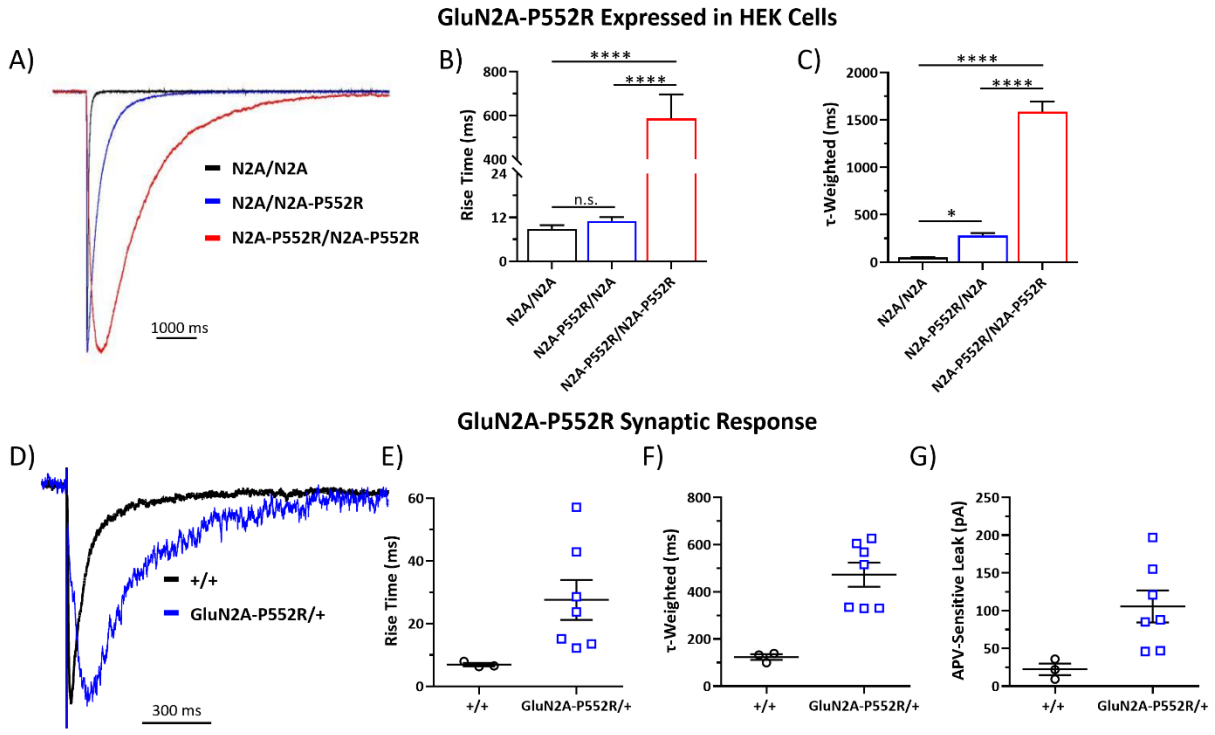


**Figure 2.3.** Comparison of GluN2A-P552R kinetic properties in HEK cells and at native synapses. A) Representative, normalized whole-cell current time course from HEK cells in response to brief (1–5 ms) application of maximally effective concentrations of glutamate (100 μM) and glycine (30 μM) in wildtype (N2A/N2A; black), triheteromeric GluN2A-P552R (N2A-P552R/N2A; blue), and diheteromeric GluN2A-P552R (N2A-P552R/N2A- P552R; red) NMDARs. B) Rise time of the activation phase of the response to a brief pulse saturating glutamate and glycine shows that only diheteromeric GluN2A-P552R constructs have a significant prolongation of the rise time ($p<0.0001$, one-way ANOVA with multiple comparisons) while triheteromeric GluN2A-P552R receptors show no difference from wildtype. C) Tau weighted describing the deactivation show significant prolongations of deactivation in both triheteromeric GluN2A-P552R and diheteromeric GluN2A-P552R constructs (one-way ANOVA with multiple comparisons). D) Representative, normalized NMDAR-mediated EPSC onto CA1 pyramidal cells in +/+ mice (black) and heterozygous GluN2A-P552R mice (blue). E) The rise time of the activation phase of the EPSC, with F) the tau weighted of the decaying phase of the EPSC. G) APV-sensitive leak current recorded at the end of

the EPSC experiments. All data are underpowered and thus cannot be subjected to statistical testing. See methods for more details. Data are mean±SEM. HEK cell data are adapted from (Ogden et al., 2017) and synaptic data are unpublished. * = $p<0.05$; **** = $p<0.0001$; n.s. = not significant.

| | **Rise Time** (ms) | **Peak Amplitude** (pA) | **Tau Weighted** (ms) | **APV-Sensitive Leak** (pA) | **Charge Transfer** (pC) |
|---|---|---|---|---|---|
| **GluN2A-P552R/+** (n=7 cells/4 mice) | 28 ± 6.4 | -136 ± 34 | 473 ± 52 | 106 ± 21 | -53 ± 13 |
| **WT Littermate** (n=3 cells/2 mice) | 6.9 ± 0.5 | -152 ± 26 | 124 ± 12 | 22 ± 7.4 | -19 ± 4.8 |
| | | | | | |
| **GluN2A-S644G/+** (n=9 cells/4 mice) | 7.6 ± 0.6 | -178 ± 24 | 150 ± 9.6 | 29 ± 3.8 | -18 ± 4.0 |
| **WT Littermate** (n=15 cells/5 mice) | 7.2 ± 0.4 | -158 ± 39 | 104 ± 4.1 | 15 ± 3.2 | -29 ± 4.3 |

**Table 2.2.** Kinetic properties of NMDAR-mediated EPSCs onto CA1 pyramidal cells from GoF GluN2A missense mouse models. All data represented are mean ± SEM. GluN2A-P552R data are unpublished and GluN2A-S644G data are adapted from (Amador et al., 2020).

learn how the mutant receptor may impact the kinetic profile of an NMDAR-mediated EPSC. Moreover, thanks to clever molecular engineering, we can selectively express one or two copies of our mutant subunit in a single receptor complex as described in (Hansen et al., 2014). These data are clinically relevant in that the majority of human patients have only heterozygous missense mutations and most native NMDARs are triheteromeric.

Heterologous data from GluN2A-P552R containing receptors show a prolongation of both the activation and deactivation time course in response to a synaptic-like pulse of saturating glutamate (**Figure 2.3A-C**) (**Table 2.2**). There was a subunit dependent prolongation of the deactivation time course, with only one copy of the mutant GluN2A-P552R subunit showing an intermediate deactivation time course compared to wildtype and NMDARs containing two copies of GluN2A-P552R (**Figure 2.3A** and **Figure 2.3C**). Surprisingly, NMDARs containing two copies of GluN2A-P552R also showed a significant prolongation of the activation time course not seen in constructs with only one copy of the GluN2A-P552R subunit (**Figure 2.3A-B**). Here, the current response continued to rise after glutamate application had concluded and the cell had been returned to glutamate-free wash solution. Thus, glutamate was most likely able to bind during the 5 ms application, which suggests that the prolonged activation time course is due to a slowing of the conformational changes that precede channel opening. In fact, this change to the activation time course helped to establish the pre-M1 helix as crucial for helping translate molecular motion within the agonist binding domain into channel opening.

Given the drastic slowing of channel opening, we were particularly interested to study the knock-in GluN2A-P552R mouse as changes in the temporal resolution of NMDAR activation *in vivo* would likely have a host of unexplored outcomes on circuit function and behavior. Unfortunately, however, we later discovered that during the generation of our knock-in mouse, we

inadvertently introduced an alternative splice site that could be utilized by the cell to synthesize a transcript where glutamine was inserted before (551E) the desired P552R mutation. Our best estimates suggest that this alterative splice site would be used 1/4$^{th}$ of the time, ultimately limiting any real conclusions that could be gleaned from this mouse. This also explains why synaptic data shown for this mouse in **Figure 2.3** are incomplete. We did, however, obtain promising NMDAR-mediated EPSCs that contained both a prolongation of the activation and deactivation time course at native synapses onto CA1 pyramidal cells from juvenile (P14-20) mice. (**Figure 2.3D-G**). These data suggest that, despite some NMDARs containing an extra glutamine at residue 551 of GluN2A, we were able to successfully show our GluN2A-P552R subunit trafficked to the synapse and could participate in synaptic neurotransmission. Our preliminary synaptic data show robust APV-mediated changes in the basal leak current, suggesting an underlying tonic activation of NMDARs (**Figure 2.3G**).

Heterologous data from GluN2A-S644G containing receptors show a prolongation of the deactivation time course in response to a synaptic-like pulse of saturating glutamate (**Figure 2.4A and Figure 2.4C**) (**Table 2.2**). There was a subunit dependent prolongation of the deactivation time course, with only one copy of the mutant GluN2A-S644G subunit showing an intermediate deactivation time course compared to wildtype and NMDARs containing two copies of GluN2A-S644G (**Figure 2.3A** and **Figure 2.3C**). Unlike the pre-M1 P552R mutant, GluN2A-S644G-containing NMDARs show no change in their activation time course or the rise time of the EPSC (**Figure 2.4B**). Synaptic data from juvenile (P14-20) heterozygous GluN2A-S644G mice confirm no changes in the activation time course (**Figure 2.4D-E**) but do show a significant prolongation of the deactivation time course, shown as a change in the weighted tau (**Figure 2.4D** and **Figure 2.4F**). There were, however, no detectable changes in the APV-sensitive leak current, a measure

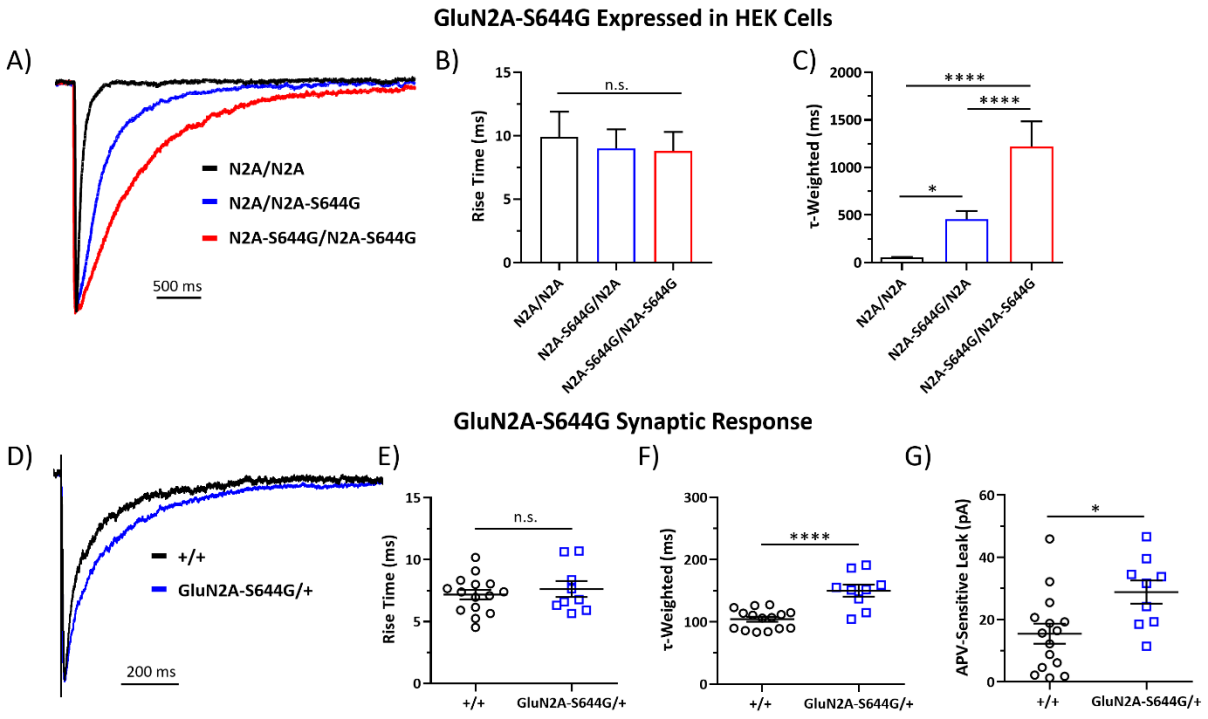


**Figure 2.4.** Comparison of GluN2A-S644G kinetic properties in HEK cells and native synapses. A) Representative, normalized whole-cell current time course from HEK cells in response to brief (1–5 ms) application of maximally effective concentrations of glutamate (100 μM) and glycine (30 μM) in wildtype (N2A/N2A; black), triheteromeric GluN2A-S644G (N2A-S644G/N2A; blue), and diheteromeric GluN2A-S644G (N2A-S644G/N2A-S644G; red) NMDARs. B) No significant change in the rise time of the activation phase of the response to a brief pulse saturating glutamate and glycine. C) Tau weighted describing the deactivation show significant prolongations of deactivation in both triheteromeric GluN2A-S644G and diheteromeric GluN2A-S644G constructs (one-way ANOVA with multiple comparisons). D) Representative, normalized NMDAR-mediated EPSC onto CA1 pyramidal cells from +/+ mice (black) and heterozygous GluN2A-P552R mice (blue). E) No significant difference observed in the rise time of the EPSC. F) The weighted tau describing the decaying phase of the EPSC shows a significant prolongation in GluN2A-S644G/+ mice (Student's two-sample *t*-test, $p = 0.0000053$). G) Significant increase in the APV-sensitive leak current recorded at the end of the EPSC experiments in GluN2A-S644G/+ mice (Student's

two-sample *t*-test, $p = 0.015$). See methods for more details. Data are mean±SEM. **** = $p<0.0001$. All data represented in this figure are adapted from (Amador et al., 2020).

of potential tonic activation of NMDARs (**Figure 2.4G**).

Heterologous data from diheteromeric GluN2D-V667I receptors show a prolongation of the deactivation time course in response to a synaptic-like pulse of saturating glutamate (**Figure 2.5A-B**) (**Table 2.3**). Data on GluN2D/GluN2D-V667I receptors has not yet been collected. Diheteromeric GluN2D-V667I channels have an open channel probability 10-fold higher than wildtype GluN2D receptors (**Figure 2.5C-E**). An increase in $P_{OPEN}$ helps explain the prolongation of the deactivation time course given the modest, but significant, increased glutamate potency caused by this variant (0.4 μM for wildtype vs 0.3 μM for diheteromeric GluN2D-V667I) (Li et al., 2016).

Unlike GluN2A-containing NMDARs which is expressed in both principal cells and GABAergic interneurons, GluN2D-containing NMDARs are primarily expressed in interneurons only. Thus, we measured NMDAR-mediated EPSCs onto both CA1 pyramidal cells and CA1 *stratum radiation* interneurons. As expected, we observed no change in tau weighted describing the deactivation time course of the NMDAR-mediated EPSCs onto CA1 pyramidal cells (**Figure 2.5F** and **Figure 2.5H**), but we did see a significant prolongation of tau weighted describing the deactivation time course of the NMDAR-mediated EPSCs onto CA1 radiatum interneurons (**Figure 2.5G-H**). We also observed a significant change in the APV-sensitive basal leak current in interneurons (**Figure 2.5I**), but not in CA1 pyramidal cells (**Figure 2.5I**). This suggests that the V667I variant may increase excitatory drive of GABAergic interneurons, potentially via tonically active GluN2D-containing NMDARs activated by ambient glutamte present in the extracellular space. These data also suggest that, like both the GluN2A-P552R and GluN2A-S644G mouse models, NMDARs containing the GluN2D-V667I mutant subunit traffic to the postsynaptic density and can participate in synaptic neurotransmission. Moreover, we do not see ectopic

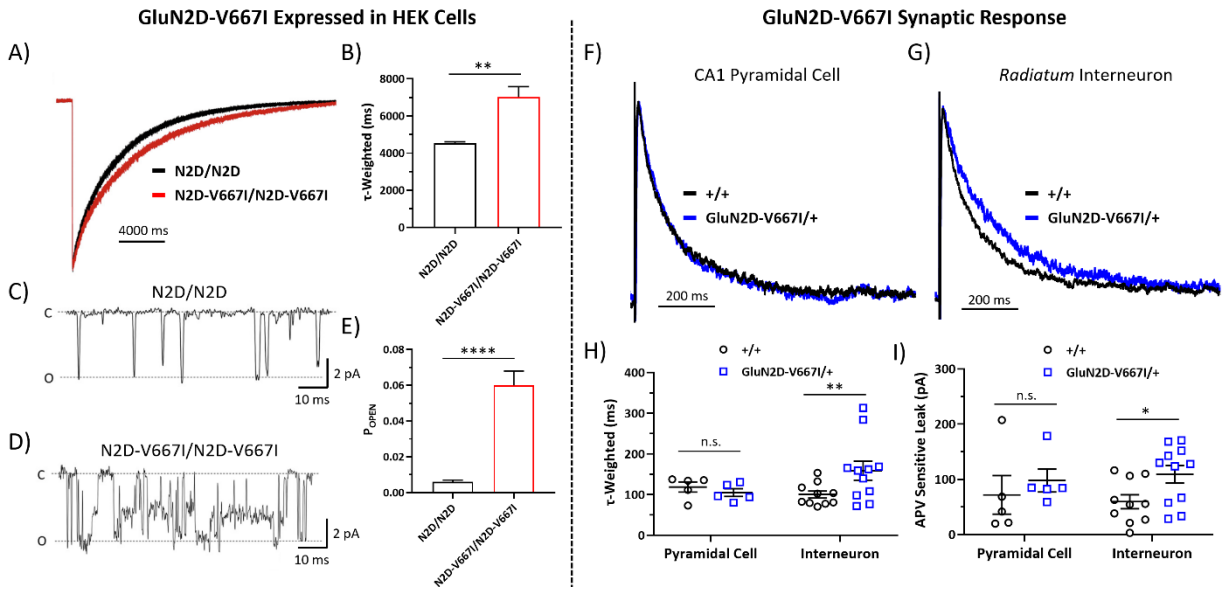


**Figure 2.5**. Comparison of GluN2D-V667I kinetic properties in HEK cells and native synapses. A) Representative, normalized whole-cell current time course from HEK cells in response to brief (1–5 ms) application of maximally effective concentrations of glutamate (100 μM) and glycine (30 μM) in wildtype (N2D/N2D; black) and diheteromeric GluN2D-V667I (N2D-V667I/N2D-V667I; red) NMDARs. B) Tau weighted describing the deactivation time course is significantly prolonged in the GluN2D-V667I receptors (Student's two-sample t-test, $p = 0.0014$). Representative single-channel recordings of C) wildtype (+/+) and D) diheteromeric GluN2D-V667I (N2D-V667I/N2D-V667I) NMDARs. E) POPEN is significantly increased in the GluN2D-V667I receptors (Student's two-sample t-test, $p < 0.000001$). Normalized NMDAR-mediated EPSCs onto F) CA1 pyramidal cells and G) stratum radiatum GABAergic interneurons from preadolescent (postnatal 20-26) +/+ mice (black) and heterozygous GluN2D-V667I mice (blue). H) The weighted tau describing the decaying phase of the EPSC is unchanged in CA1 pyramidal cells but is significantly prolonged in radiatum interneurons from heterozygous GluN2D-V667I mice (Student's two-sample t-test, $p = 0.036$). I) APV-sensitive leak current recorded at the end of the EPSC experiments in unchanged in CA1 pyramidal cells but is significantly increased in heterozygous GluN2D-V667I mice (Student's two-sample t-test, $p = 0.038$). See methods for more details. Data are mean±SEM. **** =

$p<0.0001$. Heterologous expression data adapted from (Li et al., 2016) and all synaptic data are unpublished.

| | **Rise Time** (ms) | **Peak Amplitude** (pA) | **Tau Weighted** (ms) | **APV-Sensitive Leak** (pA) | **Charge Transfer** (pC) |
|---|---|---|---|---|---|
| *CA1 Pyramidal Cells* | | | | | |
| **GluN2D-V667I/+** (n=5 cells/3 mice) | 11 ± 1.6 | 199 ± 77 | 105 ± 9.6 | 98 ± 21 | 24 ± 11 |
| **WT Littermate** (n=5 cells/3 mice) | 9.5 ± 1.9 | 126 ± 33 | 118 ± 12 | 72 ± 35 | 17 ± 4.7 |
| *CA1 Radiatum Interneurons* | | | | | |
| **GluN2D-V667I/+** (n=11 cells/4 mice) | 10 ± 1.5 | 179 ± 30 | 159 ± 23 | 109 ± 16 | 23 ± 3.9 |
| **WT Littermate** (n=10 cells/4 mice) | 11 ± 1.8 | 149 ± 19 | 100 ± 8.6 | 60 ± 13 | 13 ± 1.4 |

**Table 2.3.** Kinetic properties of NMDAR-mediated EPSCs onto CA1 pyramidal cells and CA1 *stratum radiatum* from GluN2D-V667I mice. All data represented are mean ± SEM. All data are unpublished.

receptor expression in CA1 pyramidal cells.

Next, we wanted to probe spontaneous and miniature inhibitory tone onto pyramidal cells given a probable dysfunction in the interneuron network. The spontaneous postsynaptic inhibitory current (sIPSC) interevent interval and sIPSC amplitude cumulative histogram plots are both significantly shifted to the left (Kolmogorov-Smirnov test; $p<0.000001$, Kolmogorov-Smirnov $D=0.36$ for interevent interval; $p<0.000001$, Kolmogorov-Smirnov $D=0.25$ for amplitude; $N=14$ cells/4 animals for each genotype; **Figure 2.6A-D**) (**Table 2.4**). These data suggest that the gain-of-function GluN2D-V667I variant produces an increase in spontaneous inhibitory tone, while generating a decrease in overall amplitude. Additionally, we wanted to probe the miniature inhibitory postsynaptic tone (mIPSC) to gain information on the phasic inhibitory tone. The mIPSC interevent interval is significantly shifted to the left (Kolmogorov-Smirnov test; $p<0.000001$, Kolmogorov-Smirnov $D=0.27$; $N=14$ cells/4 animals for each genotype), while mIPSC amplitude is unchanged (**Figure 2.6E-H**). Given that mIPSCs represent single quantal events (Nusser et al., 1997), a leftward shift in interevent interval suggests that heterozygous GluN2D-V667I mice may have a decreased presynaptic release probability and/or changes in the number of inhibitory synaptic connections made onto CA1 pyramidal cells. Moreover, the lack of impact on mIPSC amplitude and decay time (**Figure 2.7A-B**) (**Table 2.4**). suggests that the number of postsynaptic $GABA_A$ receptors per inhibitory synapse and the subunit composition of postsynaptic $GABA_A$ receptors, respectively, is unlikely unchanged (Nusser et al., 1997).

*2.4 Discussion*

Data presented in this chapter are from neurons in acute brain slices prepared from mice harboring each of three different gain-of-function missense NMDAR variants with severe

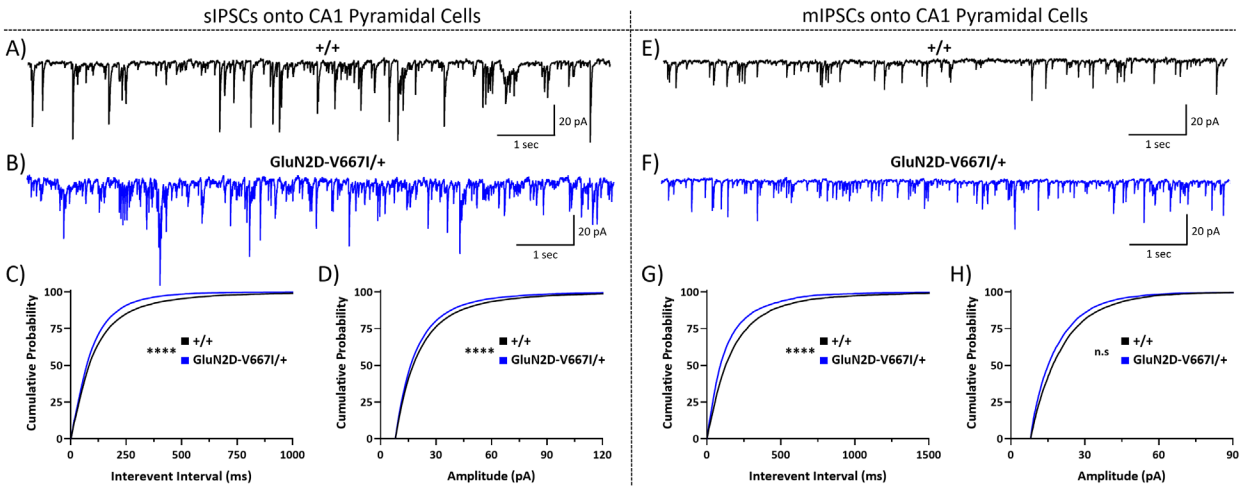


**Figure 2.6.** Spontaneous and miniature inhibitory postsynaptic currents (IPSCs) onto CA1 pyramidal cells. All recordings were made using preadolescent mice (P20-26) at -60 mV in presense of normal aCSF (1.5 mM $Mg^{2+}$) with 10 µM NBQX. Miniature IPSCs (mIPSCs) were isolated via application of 1 µM TTx. Representative traces of spontaneous IPSCs (sIPSCs) from A) wildtype (+/+) and B) heterozygous GluN2D-V667I (GluN2D-V667I/+) mice. C) The sIPSC interevent interval ($p<0.00001$; Kolmogorov-Smirnov D = 0.36) and D) amplitude ($p<0.00001$; Kolmogorov-Smirnov D = 0.25) cumulative histogram plots were both significantly shifted to the left in heterozygous GluN2D-V667I mice. Representative traces of mIPSCs from A) wildtype (+/+) and B) heterozygous GluN2D-V667I (GluN2D-V667I/+) mice. G) The mIPSC interevent interval cumulative histogram plot was significantly shifted to the left in heterozygous GluN2D-V667I mice ($p<0.00001$; Kolmogorov-Smirnov D = 0.26) while the H) amplitude cumulative histogram plot was unchanged. **** = $p<0.0001$. Data represented are from n=14 cells/4 mice per genotype. All data are unpublished.

| | **sIPSC Freq.** (Hz) | **sIPSC Amplitude** (pA) | **mIPSC Freq.** (Hz) | **mIPSC Amplitude** (pA) | **mIPSC Decay** (ms) |
|---|---|---|---|---|---|
| **GluN2D-V667I/+** (n=14 cells/4 mice) | 14 ± 2.7 | -24 ± 1.6 | 8.4 ± 1.2 | -19 ± 1.0 | 13 ± 0.9 |
| **WT Littermate** (n=14 cells/4 mice) | 9.2 ± 1.1 | -25 ± 2.1 | 5.9 ± 0.7 | -21 ± 1.0 | 14 ± 1.0 |

**Table 2.4.** Properties of spontaneous inhibitory postsynaptic currents (sIPSCs) and miniature inhibitory postsynaptic currents (mIPSCs) onto CA1 pyramidal cells from GluN2D-V667I mice. Freq. = frequency. All data represented are mean ± SEM. All data are unpublished.

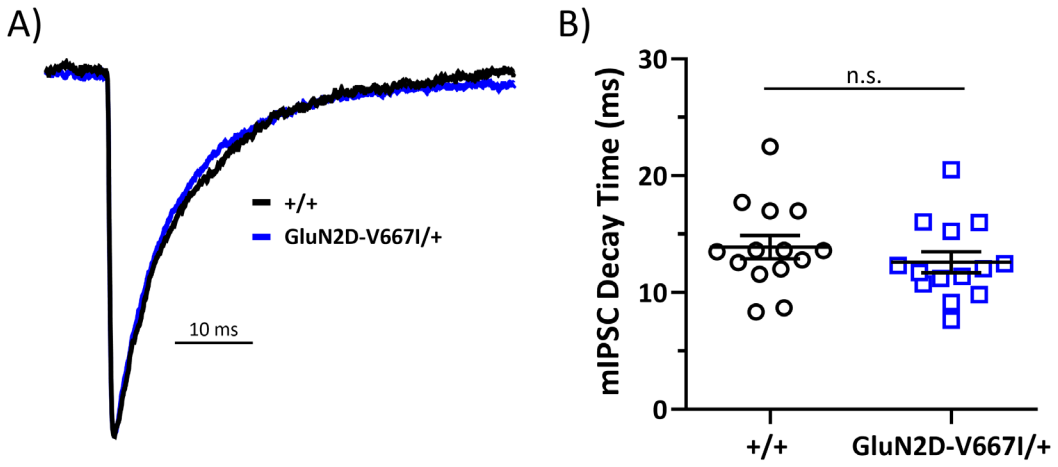


**Figure 2.7.** The decay time of mIPSCs onto CA1 pyramidal cells is unchanged in GluN2D-V667I/+ mice. A) Representative, normalized mIPSC traces from +/+ (black) and GluN2D-V667I/+ (blue) mice. B) No significant change in the decay time of mIPSCs onto CA1 pyramidal cells in GluN2D-V667I/+ mice. Data represented are from n=14 cells/4 mice per genotype. n.s. = not significant. All data are unpublished.

developmental and epileptic encephalopathy (DEE). The severity of the human phenotypes associated with these variants are likely to due each variant being found within the highly intolerant TMD. This region of the NMDAR is cruical for gating – the act of transducing molecular motion from ligand binding within the ABD into pore opening for ion flux and signal propagation. Given its important role in channel opening it seems logical that there is abolute selection against mutations within this region. To our surprise, however, only one our mouse models displayed spontaneous seizures – the heterozygous GluN2D-V667I mouse. Heterozygous gain-of-function GluN2A-P552R and GluN2A-S644G mice showed no spontaneous seizures, despite those same heterozygous variants causing multiple seizures a day in humans. Interpretation of data obtained from the GluN2A-P552R mouse model was complicated by the introduction of an unplanned additional amino acid which may impact any oberseved epileptic phenotypes. This information was not obtained until after experimentation was started, and led to early termination of that project. While the GluN2A-S644G mice do not have spontaneous seizures, they do display some signs of DEE including seizure susceptibility and distinct neurobehavioral features described in (Amador et al., 2020).

Whereas we observed some features of the expected phenotypes observed in patients in the GluN2A-S644G mice, the etiology of DEE resulting from the GluN2A-S644G variant is biologically complex. Depending on mutant gene dosage and seizure assessment, GluN2A-S644G mice are both seizure-susceptible (spontaneous lethal seizures in homozygous GluN2A-S644G mice, not shown; lower threshold to electroconvulsive minimal and maximal seizure endpoints in heterozygous GluN2A-S644G) and seizure-resistance (elevated threshold to electroconvulsive partial seizures; relative resistance to PTZ-induced tonic-clonic seizures, not shown). Interestingly, mice lacking the excitatory postsynapse scaffold protein IQSEC2, which also exhibit lethal

spontaneous seizures and seizure resistance in 6 Hz ECT and PTZ tests, have increased excitatory drive onto hippocampal interneurons and altered intrinsic hippocampal interneuron properties (Sah et al., 2020). The two models compared suggest possible convergence of mechanisms of genetically diverse DEE's, which might help in identifying common therapies - invaluable given the extensive genetic heterogeneity of DEE.

Functional characterization of mutant channel activity shows a strong gain-of-function effect for all three variants. There does, however, appear to be a quantitative difference between the level of gain-of-function properties (i.e. deactivation/decay times) between heterologous data and data obtained from native synapses. Although all three mutant mouse models show that, at the synaptic level, gain-of-function properties such as a prolongation of the synaptic decay are present, they appear less striking than those observed in HEK recordings. The reasons for this are numerous and these discrepancies have been observed with other mouse models of NMDAR dysfunction (Benke et al., 2021). The most likely explanation is the binomial probability of receptors having 0, 1, or 2 copies of the mutant, with the inclusion of any wildtype subunits conferring faster synaptic decay times. Moreover, even within the same brain region (CA1 hippocampus) synaptic biology is heterogeneous, with synaptic receptor turnover being activity-dependent. Each of these heterogeneous synapses will be activated during Schaffer collateral stimulation and summed together with the confound of electrotonic filtering through a large dendritic tree, to generate a single macroscopic current response.

In addition to uncertainty in terms of true synaptic expression of each mutant subunit, several other factors – such as age, cell type selection, and human bias – will also impact observed synaptic decay times. All recordings were made in young mice (P14-26), mainly out of expediency and efficiency, which will not capture the true breadth of synaptic decay times possible. At this

age, pyramidal cells still express GluN2B-containing NMDARs in the synapse (Hansen et al., 2014). For GluN2A variants, this means the number of possible subunit combinations – both diheteromeric and triheteromeric – is large. For the GluN2D-V667I mouse, random *stratum radiatum* interneurons were picked, with no regard to interneuron subtype. Although the general consensus is that GABAergic interneurons express GluN2D (Perszyk et al., 2016; von Engelhardt et al., 2015), this canonical view has not been confirmed for all subtypes of interneuron (see Chapter 4, for example). Thus, some of the cells recorded may not even contain functional GluN2D-containing receptors at the postsynaptic density, let alone the mutant GluN2D-V667I subunit. There also may be systematic bias towards healthy neurons that allowed stable patch clamp recording, which may have been selected against cells with strong expression of our mutants across all three models.

Overall, data from these mutant mouse lines confirms many features of patient-derived variants studied in heterologous expression systems. Furthermore, these data support the initial goal of creating new models of neurological disease based on existing mutations observed in patients. Such models are not only relevant for individuals with *GRIN* variants, but also useful as models of synaptic dysfunction. Thus, they could allow evaluation of ideas and therapeutic strategies to address synaptic pathology arising from a broad range of factors, both genetic and independent of genetic variation.

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# CHAPTER 3: *GRIN2A* Null Variants and Parvalbumin-Positive Interneuron Maturation

Adapted from:

**Camp CR**, Vlachos A, Klöckner C, Banke TG, Shariatzadeh N, Ruggiero SM, Galer P, Helbig I, Yuan H, McBain CJ, Benke TA, Lemke JR, Pelkey KA, Traynelis SF (2022) Loss of *Grin2a* Causes a Transient Delay in the Electrophysiological Maturation of Hippocampal Parvalbumin Interneurons: A Possible Mechanism for Transient Seizure Burden in Patients with Null *GRIN2A* Variants.

*3.1 Introduction*

N-methyl-D-aspartate receptors (NMDARs) comprise a family of ligand-gated ionotropic glutamate receptors that mediate a calcium-permeable component to fast excitatory neurotransmission (Hansen et al., 2021). NMDARs are heterotetrameric assemblies of two obligate GluN1 subunits (encoded by the *GRIN1* gene) and two GluN2 subunits (encoded by the *GRIN2A*-*GRIN2D* genes) (Hansen et al., 2021). Given their ubiquitous expression, participation in glutamatergic neurotransmission, and facilitation of calcium entry into cells, NMDARs have been implicated in a host of physiological and developmental roles including learning, memory, spatial navigation, coordinated movement, decision making, neuronal migration, morphological development, and synaptic connectivity (Adesnik et al., 2008; Collingridge, 1987; Hansen et al., 2021; Komuro and Rakic, 1993; Nakazawa et al., 2004; Nash and Brotchie, 2000; Ultanir et al., 2007; Wang, 2002; Watanabe et al., 1998).

A growing volume of sequencing data implicates genetic variation within NMDARs as a contributing factor to neuropathological conditions including epilepsy, schizophrenia, autism, intellectual disability, and developmental delay (Benke et al., 2021; Yuan et al., 2009). These genetic variants, which are absent from the healthy population, illustrate a critical role for NMDARs in basic and higher-level cognitive function (Amin et al., 2021; Perszyk et al., 2020). Moreover, when stratified by subunit, 43% (297/679) of all currently known NMDAR genetic variants are within the *GRIN2A* gene, which encodes the GluN2A subunit (Benke et al., 2021; Hansen et al., 2021). Neurological conditions associated with *GRIN2A* variation present with a range of symptoms, with the most common being epilepsy and intellectual disability, coupled with some form of aphasia (Benke et al., 2021; Carvill et al., 2013; Hansen et al., 2021). Additionally, mutations in the *GRIN2A* gene have been identified as a high-risk factor for schizophrenia in

genome-wide association studies (Schizophrenia Working Group of the Psychiatric Genomics, 2014; Singh et al., 2022). Nearly one-third (98/297) of *GRIN2A* variants can be classified as null variants (Hansen et al., 2021), mainly nonsense mediated single nucleotide polymorphisms and chromosomal deletions, in which no functional GluN2A protein would be made. Here, we show that unlike those with loss-of-function or gain-of-function missense *GRIN2A* variants, the majority of those affected with a null *GRIN2A* variant demonstrate a transient period of seizure susceptibility that begins during infancy and diminishes near adolescence.

To investigate the cellular mechanisms for this transient seizure burden, we used global *Grin2a*$^{+/-}$ and *Grin2a*$^{-/-}$ mice as models for null *GRIN2A* variants. While individuals with null *GRIN2A* variants are only haploinsufficient, current data on *Grin2a*$^{+/-}$ mice is limited. *Grin2a*$^{-/-}$ mice, however, display neurological characteristics similar to those affected with null *GRIN2A* variants, such as transient cortical epileptiform discharges and deficits in spatial learning (Sakimura et al., 1995b; Salmi et al., 2019). Given that this transient seizure burden manifests early in life, we hypothesized that this may be a neurodevelopmental disease, in which aberrant circuit activity during the critical plasticity period of development disrupts the excitatory-to-inhibitory balance. Loss of early GluN2A signaling would promote profound network disruptions as the GluN2B-to-GluN2A switch, a period in which the relative ratio of GluN2B:GluN2A transcript tilts in favor of GluN2A being in the majority, confers cells with faster NMDAR-mediated excitatory postsynaptic currents and less overall calcium transfer per synaptic event (Carmignoto and Vicini, 1992; Erreger et al., 2005; Kirson and Yaari, 1996; Sans et al., 2000; Schneggenburger, 1996; Williams et al., 1993). This change in postsynaptic calcium signaling coincides with immense periods of neurodevelopment in which cells display morphological changes, synaptic

connections are established and pruned, and various ion channels regulating cellular excitability are upregulated (Oswald and Reyes, 2008; Piatti et al., 2011; Zhang, 2004).

The GluN2A subunit is expressed in excitatory glutamatergic pyramidal cells (Hansen et al., 2021; Perszyk et al., 2016) and multiple interneuron subtypes (Perszyk et al., 2016). Inhibition or targeted knockdown of the GluN1, GluN2B, or GluN2D subunits impede interneuron development, suggesting an active role for NMDARs in interneuron function and maturation (Chittajallu et al., 2017; Hanson et al., 2019; Hanson et al., 2013; Kelsch et al., 2014). Given the transient nature of seizure susceptibility observed in null *GRIN2A* patients, the temporal expression pattern of GluN2A, and potential roles of NMDARs in circuit refinement and interneuron maturation, we hypothesized that the reduced GluN2A signaling may impact interneuron function and thereby contribute to the formation of a transiently hyperexcitable network. Our data suggest that *Grin2a*$^{+/-}$ and *Grin2a*$^{-/-}$ mice show a delay in parvalbumin-positive (PV) interneuron maturation, with resolution of aberrant interneuron function occurring at a time – post adolescence – roughly corresponding to the time null *GRIN2A* variant patients show seizure offset. These data suggest a molecular mechanism for the transient seizure burden observed in null *GRIN2A* patients and provide further evidence for GluN2A's role in circuit maturation.

## *3.2 Methods*

### 3.2.1 Animals and Breeding

All procedures involving the use of animals performed at Emory University were reviewed and approved by the Emory University IACUC, and were performed in full accordance with state and federal Animal Welfare Acts and Public Health Service policies. *Grin2a*$^{-/-}$ mice were obtained from the laboratory of Masayoshi Mishina (University of Tokyo, Japan) and were generated via insertion of a neomycin resistance gene and Pau sequence (mRNA destabilizing and transcription-

pausing signals) into the coding region of the transmembrane domain of the *Grin2a* gene as previously described (Sakimura et al., 1995b). These mice were then backcrossed more than 15 times to a C57BL/6J (Jax stock number: 00664) background at Emory before use.

Mice were genotyped by performing PCRs for the neomycin cassette (forward: GGGCGCCCGGTTCTT; reverse: CCTCGTCCTGCAGTTCATTCA) and the WT *Grin2a* gene (forward: GCCCGTCCAGAATCCTAAAGG; reverse: GCAAAGAAGGCCCACACTGATA). Heterozygous *Grin2a*$^{+/-}$ mice were identified as being positive for both probes. In order to visualize PV cells for use in electrophysiological experiments, *Grin2a*$^{-/-}$ mice were crossed with *Pvalb*-TdTomato mice (Jax stock number: 027395 – Tg(*Pvalb*-tdTomato15Gfng)) to generate *Grin2a*$^{+/+}$:*Pvalb*-TdTom, *Grin2a*$^{+/-}$:*Pvalb*-TdTom, and *Grin2a*$^{-/-}$:*Pvalb*-TdTom mice. This reporter line has already been backcrossed to a C57BL/6J background and has been previously validated to be selective and specific for PV$^{+}$-GABAergic interneurons, including those within the CA1 subfield (Ekins et al., 2020; Kaiser et al., 2016). For identification of neonatal PV cells in acutely prepared hippocampal tissue, *Tac1*-Cre (Jax stock number: 021877 – B6;129S-*Tac1*$^{tm1.1(cre)Hze}$/J) driver mice were crossed with eGFP-Floxed (Jax stock number: 004077 – B6;129-Gt(ROSA)26Sor$^{tm2Sho}$/J) mice to produce *Tac1*-Cre:eGFP mice. *Tac1* has previously been described to be specifically expressed in PV cells, with no expression in MGE-derived somatostatin-positive cells (Que et al., 2021).

The following definitions are used for mice of various ages: neonatal (P6-8), juvenile (P13-15), preadolescent (P20-26), and adult (P70-125) as described previously (Spear, 2000). All mice were maintained in a conventional vivarium, given standard chow and water *ad libitum*, with a 12-hour light cycle. Both male and female mice were used in all experiments. Both male and female mice were used in all experiments.

3.2.2 Human Patient Data

Deidentified data on seizure onset and offset were obtained from consented patients under protocols approved by the University of Colorado IRB, the University of Pennsylvania IRB, or University of Leizig IRB. Freedom from seizures was defined as no seizures for at least 365 consecutive days.

3.2.3 Acute Hippocampal Slice Preparation and Electrophysiological Recordings

After mice were overdosed with inhaled isoflurane, brains were rapidly removed and immediately placed in ice-cold ACSF (see below), and 300-μm thick horizontal hippocampal slices were made using a vibratome (Lecia, VT-1200S) in an ice-cold, sucrose-based artificial cerebrospinal fluid (aCSF) containing the following (in mM): 88 sucrose, 80 NaCl, 2.5 KCl, 1.25 $HNa_2PO_4$, 26 $HNaCO_3$, 10 glucose, 2 thiourea, 3 sodium pyruvate, 5 sodium ascorbate, 12 N-acetylcysteine, 10 $MgSO_4$, and 0.5 $CaCl_2$ bubbled in 95% $O_2$/5% $CO_2$. After sectioning, slices were incubated in a sucrose-based aCSF as described above but with 4 mM $MgSO_4$ at 32°C for 30 minutes then returned to room temperature for at least an hour before use. All recordings were made in the following aCSF extracellular solution (in mM): 126 NaCl, 2.5 KCl, 1.25 $HNa_2PO_4$, 26 $HNaCO_3$, 20 glucose, 1.5 $MgSO_4$, and 1.5 $CaCl_2$ bubbled with 95% $O_2$/5% $CO_2$ and held at 30-32°C using an inline heater (Warner, SH-27B). Cells were visualized using an upright Olympus BX50W microscope with IR-DIC optics coupled to a Dage IR-2000 camera. Whole-cell patch clamp recordings were obtained using an Axopatch 200B (Molecular Devices) or a Multiclamp 700B (Molecular Devices, digitized at 20 kHz using a Digidata 1440a (Molecular Devices) controlled by pClamp 10.6 software (Molecular Devices). All signals were low-pass filtered at 2 kHz using a Bessel 8-pole filter (Warner, LPF-8). Patch clamp electrodes were pulled using a

Sutter P1000 horizontal puller from thin-walled borosilicate capillary tubes (WPI), with a typical resistance of 4-8 MΩ.

For current clamp recordings (action-potential spiking probability, intrinsic excitability, and action potential firing properties), the following intracellular solution was used (in mM): 115 potassium gluconate, 0.6 EGTA, 2 $MgCl_2$, 2 $Na_2ATP$, 0.3 $Na_2GTP$, 10 HEPES, 5 sodium phosphocreatine, 8 KCl, and 0.3-0.5% biocytin. After obtaining a whole-cell configuration, all cells were allowed to dialyze for 5 minutes in current-clamp mode at I=0. The liquid junction potential was not corrected and all current-clamp responses were automatically bridge-balanced using the Multiclamp 700B clamp commander software. For action-potential spiking probability experiments, a monopolar iridium-platinum stimulating electrode (FHC, Inc.) was placed in the upper 1/3$^{rd}$ of the Schaffer collaterals to elicit a single 50 μs stimulation at a frequency of 0.03 Hz. This stimulation paradigm was used to find a stimulation intensity that would be just below a threshold that would produce a single action-potential spike of the patched CA1 pyramidal cell held at -60 mV in current-clamp mode (typically 40-70 μA). Once this stimulation intensity was established, the stimulation paradigm was shifted to deliver five 50 μs bursts at a frequency of 100 Hz every 30 seconds as previously described (Jami et al., 2021). A total of five epochs were recorded per cell with action-potential spiking probability calculated per stimulation number as number of spikes/5. Input resistance was calculated using the slope of voltage deflections in response to 500 ms current injections of -200, -150, -100, -50, 0, and 50 pA, with an inter-sweep interval of 2 seconds. The membrane time constant was calculated in response 20-30 sweeps of a 500 ms, -50 pA current injection, with an inter-sweep interval of 2 seconds. Composite responses were made by averaging 20-25 traces together using Clampfit (Molecular Devices). A weighted time constant was calculated using formula (1) by fitting a dual-exponential to each response in

ChanneLab (Synaptosoft). Rheobase was calculated in response to 500 ms current injections starting at 0 pA and increasing by 2 pA every 2 seconds. Rheobase was defined as the minimal current injection required to elicit an action potential during the current injection period. Action potential firing frequency was calculated in response a 500 ms current injection every 2 seconds starting at -100 pA, and increasing by 20 pA for neonate and juvenile mice and 50 pA for preadolescent and adult mice, until the cell displayed depolarization-induced block of firing. Number of action potentials per current injection were calculated using pClamp (Molecular Devices). Action potential half-width, action potential amplitude, action potential threshold, and afterhyperpolarization amplitude were calculated using pClamp (Molecular Devices), from action potentials obtained during rheobase recordings.

For voltage clamp experiments (NMDAR-mediate EPSCs), the following intracellular solution was used (in mM): 100 Cs-gluconate, 5 CsCl, 0.6 EGTA, 5 BAPTA, 5 $MgCl_2$, 8 NaCl, 2 Na-ATP, 0.3 Na-GTP, 40 HEPES, 5 Na-phosphocreatine, and 3 QX-314. A monopolar iridium-platinum stimulating electrode (FHC, Inc.) was placed in the upper $1/3^{rd}$ of the Schaffer collaterals to elicit a single 50 μs stimulation at a frequency of 0.03 Hz and the NMDAR-mediated EPSC was pharmacologically isolated with 10 μM NBQX and 10 μM gabazine. Cells were held at +40 mV and stimulation intensity was chosen to be near 50% of the maximum peak amplitude of the EPSC. A total of 8-12 epochs were recorded and averaged together. At the conclusion of recording, 200 μM DL-APV was applied to ensure responses were mediated via NMDARs. A weighted time constant was calculated using the following formula by fitting a dual-exponential function to each composite mIPSC trace in ChanneLab (Synaptosoft):

(2) $\tau_W = (\tau_{FAST}\,\text{amplitude}_{FAST} + \tau_{SLOW}\,\text{amplitude}_{SLOW})/ (\text{amplitude}_{FAST} + \text{amplitude}_{SLOW})$

For all electrophysiological recordings, series resistance was monitored throughout all experiments and was typically 8−20 MΩ. For current clamp recordings, cells were briefly switched to voltage clamp and held at -60 mV while a series of 50 ms, 5-mV square waveforms were applied to the cell. For voltage clamp recordings, this 50 ms, 5-mV square wave was included in the stimulation paradigm. Series resistance was monitored throughout the entire recording, while current clamp recordings had series resistance measurements made at the beginning and at the end of each experiment, which usually lasted 5-7 minutes total. All series resistances were measured offline by analyzing the peak of the capacitive charging spike and applying Ohm's law. If the series resistance changed >25% during the experiment, or ever exceeded 30 MΩ, then the cell was excluded.

### 3.2.4 Interneuron Anatomical Reconstructions

After biocytin filling during whole-cell recordings, slices were fixed with 4% PFA in 1x PBS overnight, then permeabilized with 0.3% Triton-X in 1x PBS and incubated with streptavidin Alexa546 (Invitrogen, S11225; 1:500). Resectioned slices (75-µm) were mounted on gelatin-coated slides using Mowiol mounting medium. We also found similar axonal recovery success by filling cells with 0.5% biocytin and permeabilizing slices with 1.2% Triton-X in 1x PBS for 10 minutes prior to incubation in streptavidin AlexaFluor546 without resectioning. Cells were visualized using epifluorescence microscopy and images for representative examples were obtained with confocal microscopy. Cells were reconstructed and analyzed with Sholl analysis using Neurolucida software (MBF Bioscience). Polar histograms of dendrites and axons were created using the Neurolucida function (10 degree bins). Polarity preference was determined by

calculating the percentage of horizontally (150–210, 330–30 degrees) or vertically (60–120, 240–300 degrees) oriented axons for each genotype.

### 3.2.5 Immunohistochemistry for GABAergic Interneuron Markers

After mice were overdosed with inhaled isoflurane, they were transcardially perfused with cold 1x phosphate buffered saline (PBS; pH 7.35), and subsequently perfused with cold 4% paraformaldehyde (PFA) in 1x PBS. Brains were removed and fixed for 24 hours in 4% PFA in 1X PBS before being transferred to a 30% sucrose solution dissolved in 1x PBS until the brains sank. After cryoprotection, brains were frozen in optimal cutting temperature solution (OCT, Fisher) and serial 50-µm coronal hippocampal sections were obtained, with a total of five sections per animal, spaced roughly 250-µm apart. Slices were then transferred to a permeabilization solution containing 1.2% Triton-X in 1x PBS for 10 minutes, as previously described (Kaiser et al., 2016), before they were incubated in blocking solution containing 15% normal goat serum, 1% bovine serum albumin, and 0.5% Triton-X for 2-4 hours at room temperature. Primary antibodies were diluted in this same blocking solution at the following concentrations and incubated at 4°C for 72 hours: rabbit anti-parvalbumin (Swant, PV27; 1:5000) and rabbit anti-prepro-cholecystokinin (Frontier Institute Co., Ab-Rf350; 1:1000). After primary incubation, slices were washed 3 times in 1x PBS for 10 minutes then incubated in blocking solution containing AlexaFlour488 conjugated secondary antibodies (Abcam, ab150077; 1:1000) for 2-4 hours at room temperature. Slices were then washed 3 times in 1x PBS for 10 minutes then incubated in DAPI counterstain (Abcam, ab228549; 2 µM) for 30 minutes before being washed 3 more times in 1x PBS for 10 minutes. Slices were mounted on Superfrost Plus slides (Fisher Scientific, 12-550-15) and coverslipped with #1.5 coverslips (Thomas Scientific, 64-0717) using ProLong Gold

Antifade mounting media (ThermoFisher, P36930). After mounting media had cured, slides were sealed with CoverGrip (Biotium, 23005).

### 3.2.6 Image Acquisition and Analysis

All GABAergic interneuron marker images were acquired using a Nikon A1R HD25 line-scanning confocal microscope using NIS Elements software. The following argon laser lines were used (in nm): 405 and 488 collected using GaAsP PMTs. All images were a series of z-stacks captured using a piezo motor z-controller, with software set to acquire data in 1024 x 1024 pixel format at a $1/8^{th}$ frame rate dwell time. One experimenter performed all analyses and was blinded to genotype during acquisition and counting of confocal microscopy data. All GABAergic interneuron marker images were captured using Plan Apo 10x 0.45 NA objective, with some representative images captured using a Plan Apo 20x 0.75 NA objective, with images consisting of 11-14 stacks with a z-stack distance of 1 μm and a pinhole size of 19.4 μm. 4-5 hippocampi from each animal were imaged across 4 animals per genotype. All images were analyzed in Imaris 9.5 (Bitplane) by manually drawing hippocampal subregion boundaries and hand-counted.

### 3.2.7 Statistical Analysis and Figure Preparation

One-way or two-way ANOVA statistical tests were performed where appropriate. For experiments where multiple statistical analyses were performed on the same dataset, our significance threshold was lowered to correct for family-wise error rate (FWER) using the Bonferroni post-hoc correction method. All studies were designed so that an effect size of at least 1 was detected at 80% or greater power. All statistical analyses were performed in Prism's GraphPad software all figures were generated in Adobe's Illustrator software.

*3.3 Results*

3.3.1 Null GRIN2A Variants May Have a Transient Seizure Burden

Despite strong selective pressure against mutation in various *GRIN* genes, hundreds of human variants have been reported (Hansen et al., 2021). Summarizing variant data from Hansen et. al. 2021, **Figure 3.1A** highlights that the *GRIN2A* gene contains 44% (297/679) of all known *GRIN* variants. **Figure 3.1B** shows that only 67% (199/297) of *GRIN2A* variants are missense, while the remaining 33% (98/297) are null variants. Given this striking number of null *GRIN2A* variants, we utilized publicly available patient data (https://grin-portal.broadinstitute.org/) and unpublished clinical data (**Supplemental Table S3.1**), to evaluate seizure burden. **Figure 3.1C** reports a total of 92 null *GRIN2A* variants, with a mean age of seizure onset of 4.5 ± 0.2 years. We also report that 22 null *GRIN2A* patients with previous history of seizures have been seizure-free for at least two years, with a mean seizure offset of 10.2 ± 0.8 years. These data are in stark contrast to missense *GRIN2A* variants with available seizure onset/offset data. **Figure 3.1D** reports a total of 47 missense *GRIN2A* variants, with a mean age of seizure onset of 4.9 ± 0.7 years (**Supplemental Table S3.2**). To date, only one missense *GRIN2A* patient has been deemed seizure-free at the age of 1.7 years. These data suggest seizure susceptibility in null *GRIN2A* patients is transient, while seizure burden in missense *GRIN2A* patients may be more permanent. More data is needed to make concrete conclusions from these findings; however, they do raise the possibility that loss of *GRIN2A* produces a transient increase in circuit excitability.

3.3.2 Developing Hippocampus Shows Hyperexcitability in *Grin2a*$^{+/-}$ and *Grin2a*$^{-/-}$ Mice

Previous data using *Grin2a*$^{-/-}$ mice has shown cortical epileptiform activity in preadolescent mice (Salmi et al., 2019), as well as a prolongation of the NMDA receptor-mediated

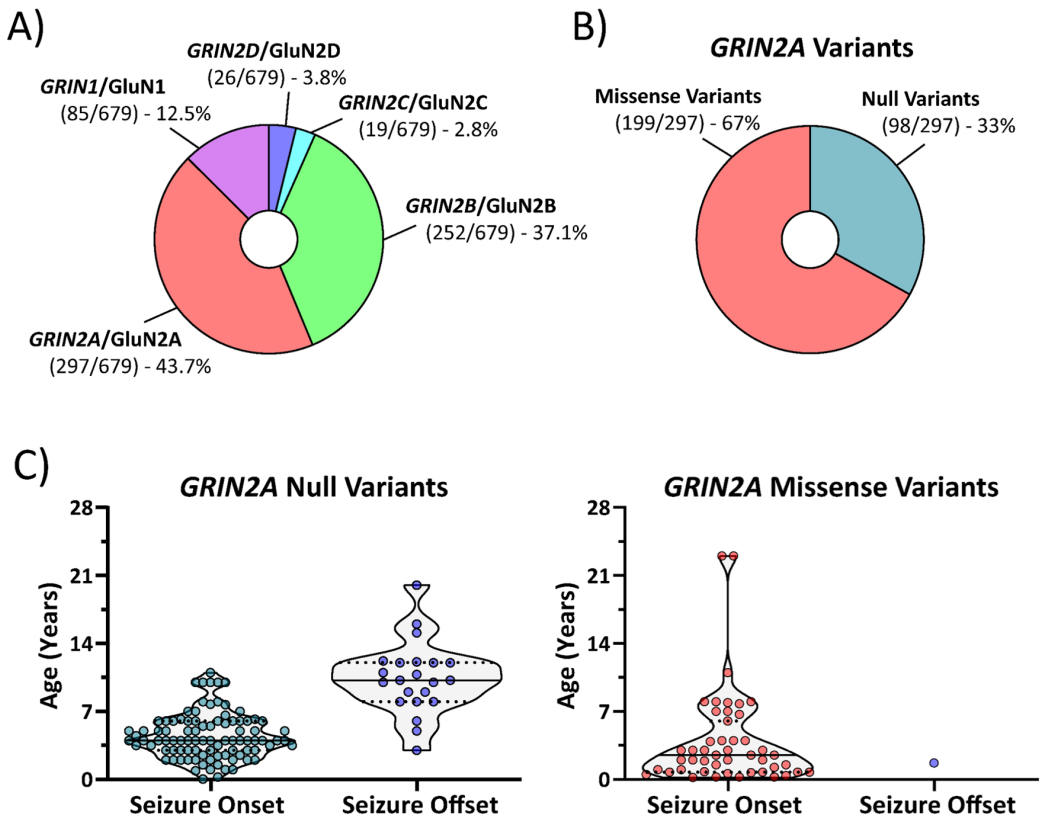


**Figure 3.1**. Null *GRIN2A* null patients display a transient seizure burden not seen in missense *GRIN2A* patients. **A)** Summary data adapted from Hansen et. al. 2021 showing that most *GRIN* variants are found in the *GRIN2A* (43.7%; 297/679) gene. **B)** Summary data adapted from Hansen et. al. 2021 highlighting that 1/3$^{rd}$ (98/297) of all *GRIN2A* variants are null variants, which include nonsense variants, as well as chromosomal insertions, deletions, inversions, and translocations. **C)** Null *GRIN2A* patient data shows both a seizure onset (4.5 ± 0.2 years; n=92) as well as a seizure offset (10.4 ± 0.8 years; n=22). Seizure offset is defined as being seizure-free for two consecutive years in a patient that had a previous history of seizures. **D)** Missense *GRIN2A* patient data shows seizure onset (4.9 ± 0.7 years; n=47), with only one patient being described as seizure-free at the time of reporting. For violin plots, solid middle line represents the median, with the dashed lines representing the 25$^{th}$ and 75$^{th}$ quantile.

excitatory postsynaptic current (EPSC) onto CA1 pyramidal cells (Booker et al., 2021) and dentate gyrus granule cells (Kannangara et al., 2015). The prolongation of the NMDA receptor-mediated EPSC is expected given the mixed expression of rapidly deactivating GluN2A- and slowly deactivating GluN2B-containing NMDA receptors on excitatory cells and the decay kinetics of a pure GluN2B-containing NMDA receptor population. Moreover, these data suggest that the loss of GluN2A-mediated signaling generates an increased excitability of cortical and hippocampal circuits given the longer time course of the NMDA receptor-mediated EPSC, however, the exact age range in when this excitability change occurs has not been explored. Since GluN2A expression shows a strong upregulation of expression during early postnatal development (Monyer et al., 1994), we explored the impact of developmental age on the prolongation of the NMDA receptor-mediated EPSC (data not shown).

Since juvenile mice have the most divergent NMDA receptor-mediated EPSC decay time across the three genotypes, this age was our benchmark to explore what other changes to hippocampal circuit function, if any, occur in *Grin2a*$^{+/-}$ and *Grin2a*$^{-/-}$ mice. Thus, we recorded action-potential generation probability in juvenile CA1 pyramidal cells during five Schaffer collateral stimulations at 100 Hz (e.g. a 50 ms burst of 5 pulses) to assess circuit output after excitatory afferent signaling. Two-way ANOVA shows a statistically significant main effect for both stimulation number and genotype (**Figure 3.2B-2C; Supplemental Table S3.3**), however, there are no significant interactions nor differences in the total number of action-potentials generated (**Figure 3.2D**; **Supplemental Table S3.3**). These data suggest that *Grin2a*$^{+/-}$ and *Grin2a*$^{-/-}$ mice are more likely to fire action-potentials when subjected to similar excitatory afferent activity compared to *Grin2a*$^{+/+}$ mice. One possible interpretation of these data is that the loss of *Grin2a* impacts the intrinsic excitability of the CA1 pyramidal cells themselves. We show no

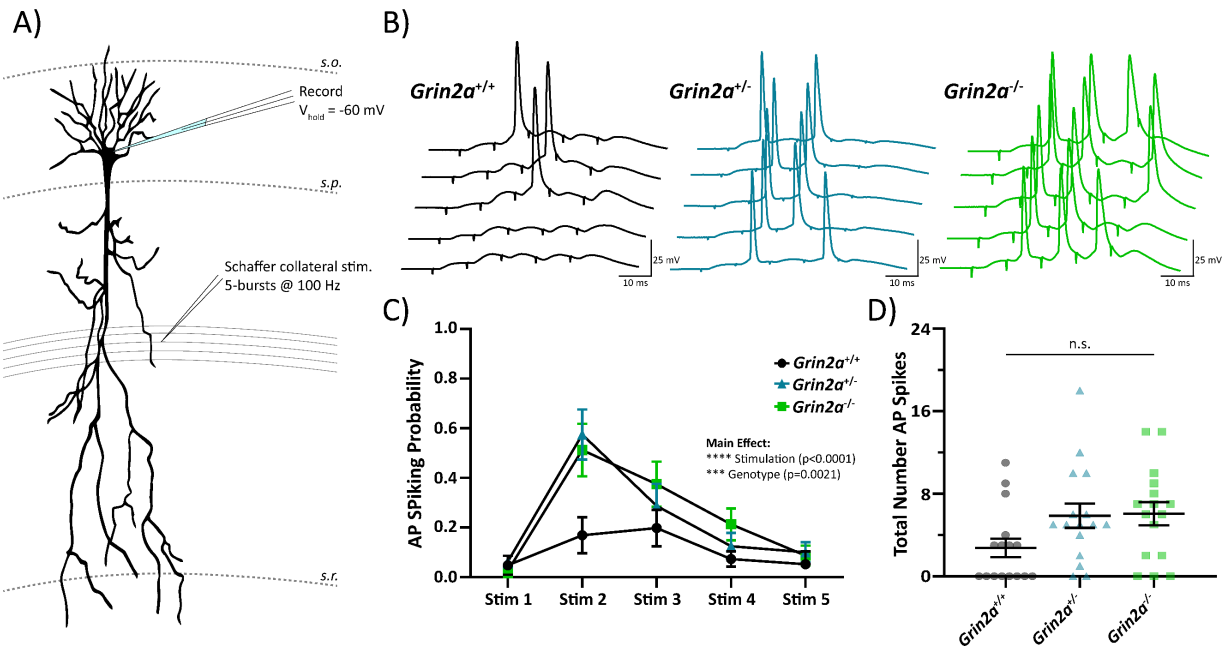


**Figure 3.2.** Juvenile CA1 circuit shows hyperexcitability in *Grin2a*$^{+/-}$ and *Grin2a*$^{-/-}$ mice. **A)** CA1 pyramidal cells from juvenile (P14-16) mice were current clamped at -60 mV and Schaffer collateral afferents were stimulated five times at 100 Hz for a total of 5 epochs. Stimulation intensity was set just below threshold to produce an action-potential spike after a single Schaffer collateral stimulation. **B)** Representative traces showing action-potential spiking in response to successive Schaffer collateral stimulations. **C)** Action-potential spiking probability for each stimulus averaged over 5 epochs across all genotypes. Two-way ANOVA showed significant main effects for both genotype ($F_{2, 225} = 6.351$; $p = 0.0021$) and stimulation number ($F_{4, 225} = 16.82$; $p < 0.0001$), however, there was no interaction. **D)** Total number of action potentials elicited over 5 epochs of 5-burst Schaffer collateral stimulation. One-way ANOVA showed there was no significant difference across the three genotypes ($F = 2.997$; $p = 0.06$). Data represented show mean ± SEM. AP = action-potential; *s.o.* = *stratum oriens*; *s.p.* = *stratum pyramidale*; *s.r.* = *stratum radiatum*; n.s. = not significant

difference in any measurable electrophysiological passive property such as resting membrane potential or input resistance, as well as no changes in action-potential firing (**Supplemental Figure S3.1**; **Supplemental Table S3.4**). Thus, changes in action-potential firing probability are not due to alterations in pyramidal cell intrinsic excitability. In addition to providing direct excitatory afferent signaling onto pyramidal cells, Schaffer collateral stimulation will also provide excitatory tone onto feedforward GABAergic interneurons. Alternatively, the loss of *Grin2a* may cause compensatory alterations in other glutamatergic neurotransmitter receptors which could impact action-potential spiking probability.

### 3.3.3 Alterations in Hippocampal PV Cell Density

Alterations in synaptic excitability as described Figure 2 could be due to changes in several features of the hippocampal circuit, including GABAergic inhibition which is mediated by a wide range of different interneurons. Inhibitory GABAergic basket cells primarily make somatic inhibitory synaptic connections where they exert profound control on pyramidal cell firing, and in CA1, are heavily innervated by Schaffer collateral afferents. CA1 basket cells can be split into two main subtypes: parvalbumin (PV)-expressing cells and cholecystokinin (CCK)-expressing cells. PV and CCK cells have opposing transcriptomic profiles and each represents a major subtype of interneuron arising from the medial ganglionic eminence (MGE) and caudal ganglionic eminence (CGE), respectively (Pelkey et al., 2017). Since early pyramidal cell activity has been shown to control interneuron apoptosis (Wong et al., 2018), and we report alterations to the NMDA receptor-mediated EPSC onto young pyramidal cells, we hypothesized that the loss of *Grin2a* may impact basket cell density in CA1. We therefore determined cell density of PV and CCK interneurons in *Grin2a*$^{+/+}$, *Grin2a*$^{+/-}$, and *Grin2a*$^{-/-}$ mice via immunohistochemical staining.

The total loss of *Grin2a* promotes an upregulation of PV cells in CA1 (**Figure 3.3A-B; Supplemental Table S3.5**) compared to both *Grin2a*$^{+/+}$ and *Grin2a*$^{+/-}$mice. Although the overall cell density of PV cells is altered in *Grin2a*$^{-/-}$ mice, there is no difference in PV cellular lamination (**Figure 3.3A** and **3.3C; Supplemental Table S3.5**) regardless of genotype, with most PV cells residing in *stratum oriens* and *stratum pyramidale* as previously reported (Pelkey et al., 2017). Unlike PV cells, we show that the loss of *Grin2a* does not impact CCK cell density (**Supplemental Figure S3.2A-S2B; Supplemental Table S3.6**) or CCK cellular lamination (**Supplemental Figure S3.2A** and **S3.2C; Supplemental Table S3.6**). Thus, these data indicate that the loss of *Grin2a* may preferentially impact PV interneuron survival/apoptosis in CA1. Moreover, the effect of increased cell density in only *Grin2a*$^{-/-}$ mice suggests that a threshold of aberrant pyramidal cell activity must be reached before PV cell survival/apoptosis is impacted. Pyramidal cell activity thus far has only been shown to impact MGE-derived interneurons, with no data on survival/apoptosis of CGE-derived interneurons (Wong et al., 2018). Alternatively, CCK-expressing cells may not be affected since previous reports have shown that these cells have little to no GluN2A-mediated synaptic signaling (Booker et al., 2021; Matta et al., 2013).

### 3.3.4 Age-Dependent Changes in Passive and Action-Potential Firing Properties of CA1 PV Cells

In addition to controlling interneuron survival/apoptosis, local pyramidal cell activity has also been implicated in controlling the maturation of GABAergic interneurons (Lim et al., 2018; Wong et al., 2018). Given that our transcriptomic data highlights differences in several voltage-gated sodium and potassium channels and the lack of intrinsic excitability perturbations in pyramidal cells, we hypothesized that the loss of *Grin2a* may be impacting PV cell intrinsic

excitability. Previous work has shown that PV cells undergo an electrophysiological maturation of both their

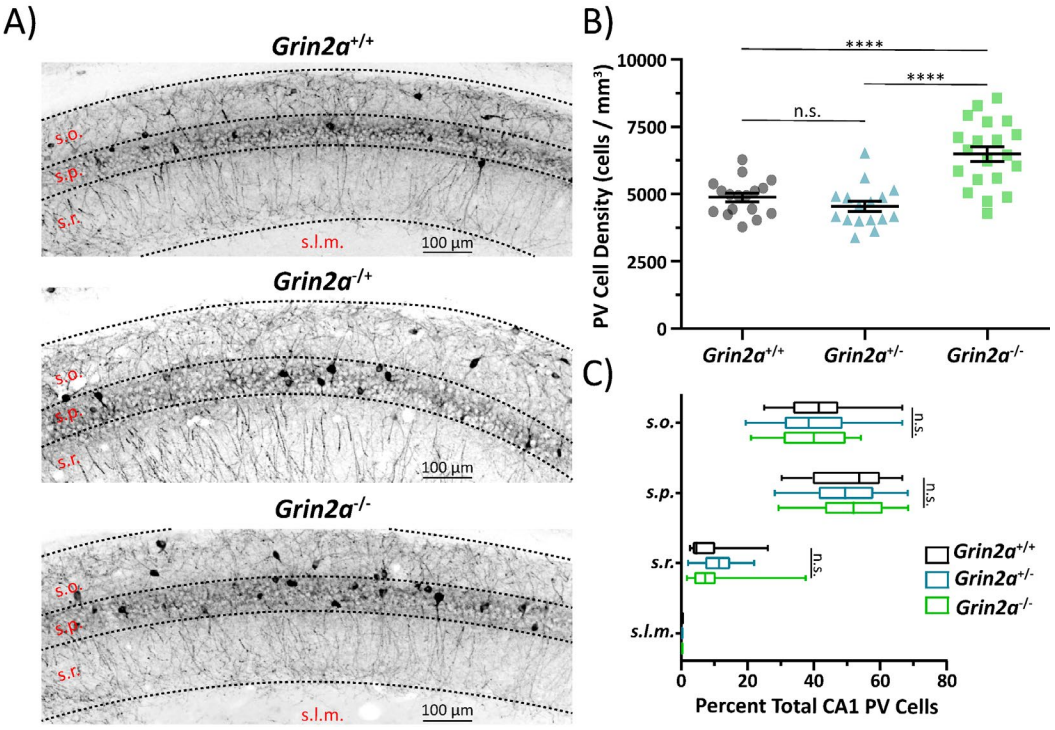


**Figure 3.3.** Loss of *Grin2a* causes an increase in parvalbumin (PV) cell density in CA1. **A)** Representative images of CA1 hippocampal sections stained for PV in preadolescent mice. **B)** CA1 PV cell density is significantly increased in *Grin2a*$^{-/-}$ mice compared to *Grin2a*$^{+/+}$ mice (6,488 ± 276 cells per mm$^3$ in *Grin2a*$^{-/-}$ vs 4,875 ± 162 cells per mm$^3$ in *Grin2a*$^{+/+}$; one-way ANOVA, $p<0.0001$) and *Grin2a*$^{+/-}$ mice (6,488 ± 276 cells per mm$^3$ in *Grin2a*$^{-/-}$ vs 4,542 ± 198 cells per mm$^3$ in *Grin2a*$^{+/-}$; one-way ANOVA, $p<0.0001$). There is no difference in CA1 PV cell density between *Grin2a*$^{+/+}$ and *Grin2a*$^{+/-}$ mice (one-way ANOVA, $p=0.58$). **C)** Despite an increase in cell density in *Grin2a*$^{-/-}$ mice, there is no difference in PV CA1 cellular lamination across all three genotypes. Data represented show mean ± SEM. *s.o.* = *stratum oriens*; *s.p.* = *stratum pyramidale*; *s.r.* = *stratum radiatum*; *s.l.m.* = *stratum lacunosum moleculare*; PV = parvalbumin; **** = $p<0.0001$; n.s. = not significant.

passive and action-potential firing properties, however, many of these studies have been performed in cortical PV cells (Goldberg et al., 2011; Miyamae et al., 2017; Okaty et al., 2009) and dentate gyrus PV cells (Doischer et al., 2008). Since clear data highlighting an electrophysiological maturation in CA1 is limited (however, see (Que et al., 2021)), we first wanted to demonstrate that hippocampal PV cells also show an electrophysiological maturation pattern like those seen in cortex.

Detailed analysis of the electrophysiological maturation pattern in young PV cells has been hampered by the age-dependent expression of the PV gene itself, which begins around P14 (de Lecea et al., 1995). To bypass this limitation, we used another driver mouse line, *Tac1*-Cre (Jax #:021877), to obtain neonatal (P6-8) PV cells recordings in developing hippocampus. Tac1 is exclusively expressed in PV cells, with no expression reported in MGE-derived somatostatin cells (Que et al., 2021). Additionally, Tac1 expression begins early in embryogenesis and is sustained well into the third week of development (Allen Developing Mouse Brain Atlas). To confirm that *Tac1*-Cre mice are a viable tool for studying neonatal PV cells, we compared several passive and action-potential firing properties in juvenile (P13-15) *Tac1*-Cre positive cells against a traditional *Pvalb*-TdTomato driver line (Jax #:027395) and found no differences (See **Supplemental Figure S3.3A-H**). Each *Tac1*-Cre cell was also filled with biocytin, with all visually confirmed to be of the basket cell subtype (See **Supplemental Figure S3.3I-J**), as previously reported (Que et al., 2021).

Using *Tac1*-Cre mice for neonatal recordings and *Pvalb*-TdTomato mice for juvenile, preadolescent, and adult recordings we recorded various passive and action-potential firing properties of CA1 PV cells at four stages during neurodevelopment. We report that neonatal CA1 PV cells show a transient prolongation of their membrane time constant, which is significantly

increased compared to juvenile, preadolescent, and adult CA1 PV cells (**Figure 3.4B-C; Supplemental Table S3.7**). A similar trend is observed for input resistance in neonatal CA1 PV Cells (See **Supplemental Table S3.7**). The action-potential half-width of neonatal CA1 PV cells is also transiently prolonged and is significantly increased compared to juvenile, preadolescent, and adult cells (**Figure 3.4D-E; Supplemental Table S3.7**). A transient prolongation of the membrane time constant and action-potential half-width observed here matches previously reported data for developing cortical PV cells. Additionally, it suggests that the excitability of neonatal PV cells is increased, but that their signaling is not temporally precise, typical of a fledging network.

We also report dampening of the maximum action-potential firing frequency in developing CA1 PV cells. Neonatal CA1 PV cells maximum action-potential firing capacity is significantly decreased compared to juvenile, preadolescent, and adult CA1 PV cells (**Figure 3.4F-G; Supplemental Table S3.7**). The maximum action-potential firing frequency in juvenile CA1 PV cells is also significantly decreased compared to adult cells (**Figure 3.4F-G; Supplemental Table S3.7**). An age-dependent increase in the fast-spiking nature of PV cells has been well characterized and is thought to be driven by a delay in the upregulation of the rapid Kv3-family of voltage-gated potassium channels (Goldberg et al., 2011). The current required for depolarization-induced block of action-potential firing in neonatal CA1 PV cells is significantly decreased compared to juvenile, preadolescent, and adult cells (**Figure 3.4F and 3.4H; Supplemental Table S3.7**). Here, the decreased current for depolarization-induced block is likely caused by an increased input resistance measured in neonatal mice. In all, we show that CA1 PV cells do undergo significant electrophysiological maturation programs, which helps transform them from simple signal propagators to precise signaling machines.

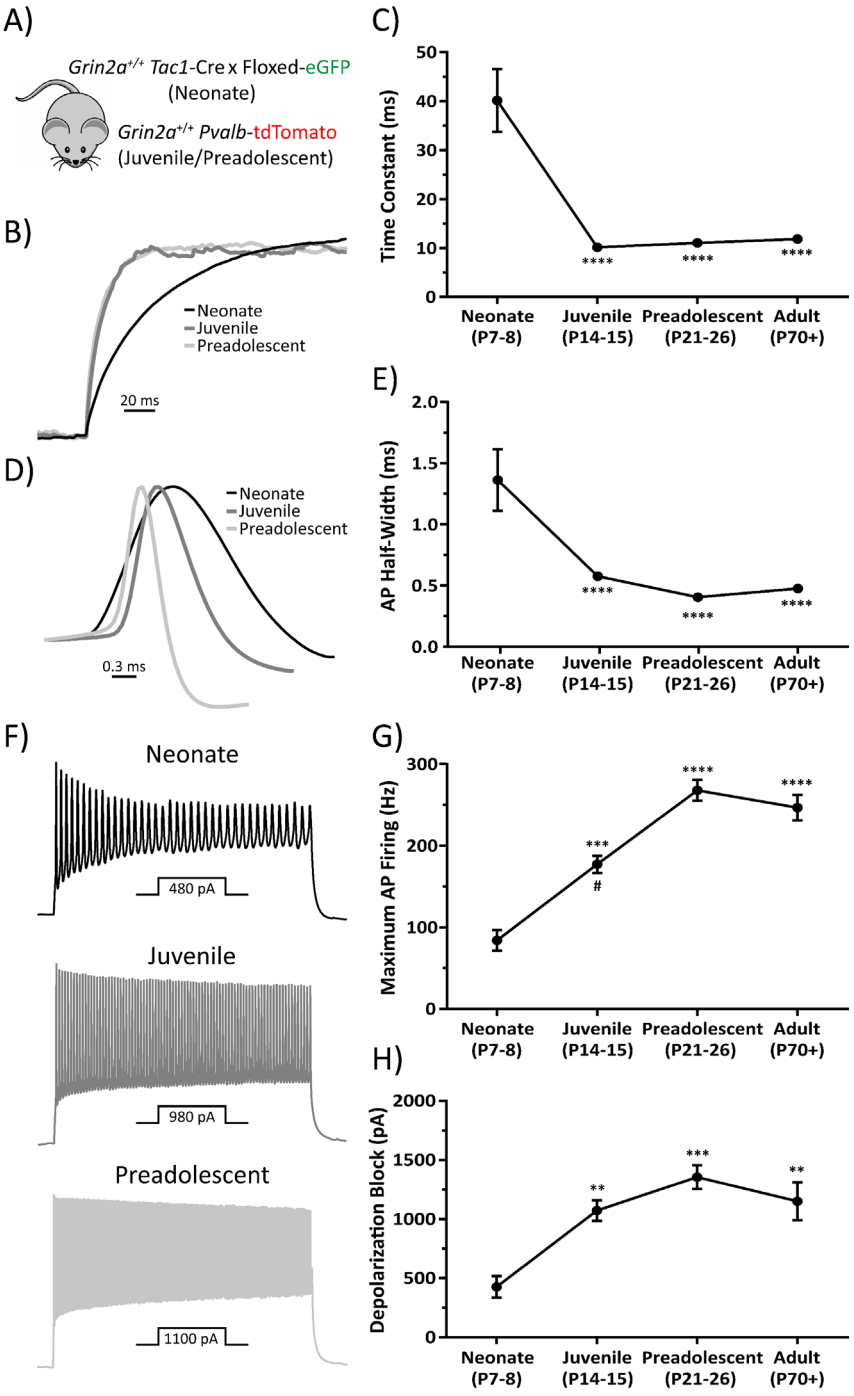


**Figure 3.4.** CA1 PV cells undergo electrophysiological maturation of passive and action-potential firing properties. **A)** PV cells were visualized using either PV-tdTomato or Tac1-CREx floxed GFP (See Supplemental Figure S6). T**B)** Representative, amplitude-normalized repolarization traces to highlight differences in membrane time constant following a -50 pA current injection at different developmental timepoints. **C)** Membrane time constant is significantly prolonged in neonatal mice (F = 40.68, one-way

ANOVA; $p<0.0001$ for post-hoc multiple comparisons with every other developmental timepoint). **D)** Action-potential half-width is significantly prolonged in neonatal mice ($F = 28$, one-way ANOVA; $p<0.0001$ for post-hoc multiple comparisons with every other developmental timepoint). **E)** Representative, amplitude-normalized single action-potential traces to highlight differences in half-width at different developmental timepoints. **F)** Representative action-potential trains elicited by various current injections depicted below each train to illustrate change in maximum action-potential firing frequency during development. Traces shown are those just below threshold for depolarization-induced block of action-potential firing. **G)** Maximum action-potential firing frequency is significantly decreased in neonatal mice ($F = 28.3$, one-way ANOVA; $p<0.001$ for post-hoc multiple comparison test with juvenile mice, and $p<0.0001$ for post-hoc multiple comparison test with preadolescent and adult mice). Juvenile mice also show a significantly decreased maximum action-potential firing frequency compared to preadolescent mice (one-way ANOVA post-hoc multiple comparison test; $p=0.0039$). **F)** Current required for depolarization-induced block of action-potential firing is significantly decreased in neonatal mice ($F = 8.36$, one-way ANOVA; $p<0.01$ for post-hoc multiple comparison test with juvenile and adult mice, and $p<0.0001$ for post-hoc multiple comparison test with preadolescent mice). Symbols are mean ± SEM. AP = action-potential; depolarization block = Current required for depolarization-induced block of action-potential firing. ** = $p<0.01$; ***$p<0.001$; **** = $p<0.0001$; # = juvenile mice significantly different than adult mice in one-way ANOVA post-hoc multiple comparison

3.3.5 Electrophysiological Maturation of PV Cells is Delayed in *Grin2a*$^{+/-}$ and *Grin2a*$^{-/-}$ Mice

After showing that WT CA1 PV cells undergo electrophysiological maturation of their passive and action-potential firing properties, we tested our hypothesis that altered network activity may impact the rate of PV cell maturation in *Grin2a*$^{+/-}$ and *Grin2a*$^{-/-}$ mice. We generated *Grin2a*$^{+/-}$ x *Pvalb*-TdTomato and *Grin2a*$^{-/-}$ x *Pvalb*-TdTomato mice via selective breeding (see methods) to visualize PV cells in CA1 across our three genotypes. We found no difference in the resting membrane potential of juvenile, preadolescent, or adult CA1 PV cells in *Grin2a*$^{+/+}$, *Grin2a*$^{+/-}$, and *Grin2a*$^{-/-}$ mice (**Figure 3.5B; Supplemental Table S8**). While we did not find any statistically significant main effects of age or genotype for PV cellular capacitance, we did find a statistically significant interaction such that juvenile PV cells from *Grin2a*$^{+/-}$ mice displayed a higher capacitance than juvenile PV cells from both *Grin2a*$^{+/+}$ and *Grin2a*$^{-/-}$ mice (**Figure 3.5C**; **Supplemental Table S8**). The membrane time constant and input resistance both had statistically significant main effects for age and genotype, indicating a transient increase in both measures of passive membrane excitability (**Figure 3.5E-F**; **Supplemental Table S8**).

Statistically significant interactions for membrane time constant values indicate that juvenile CA1 PV cells from *Grin2a*$^{+/+}$ mice are significantly lower compared to juvenile CA1 PV cells from both *Grin2a*$^{+/-}$ and *Grin2a*$^{-/-}$ mice (**Figure 3.5D-E**; **Supplemental Table S8**). In preadolescent mice, the membrane constant of CA1 PV cells from *Grin2a*$^{+/+}$ mice are significantly lower than CA1 PV cells from *Grin2a*$^{-/-}$ mice, while there is no difference in membrane time constant of CA1 PV cells from preadolescent *Grin2a*$^{+/+}$ and *Grin2a*$^{+/-}$ mice (**Figure 3.5D-E**; **Supplemental Table S8**). All membrane time constant values were not significantly different in adult mice, regardless of genotype (**Figure 3.5D-E**; **Supplemental Table S8**). Statistically significant interactions for input resistance values indicate that juvenile and preadolescent CA1

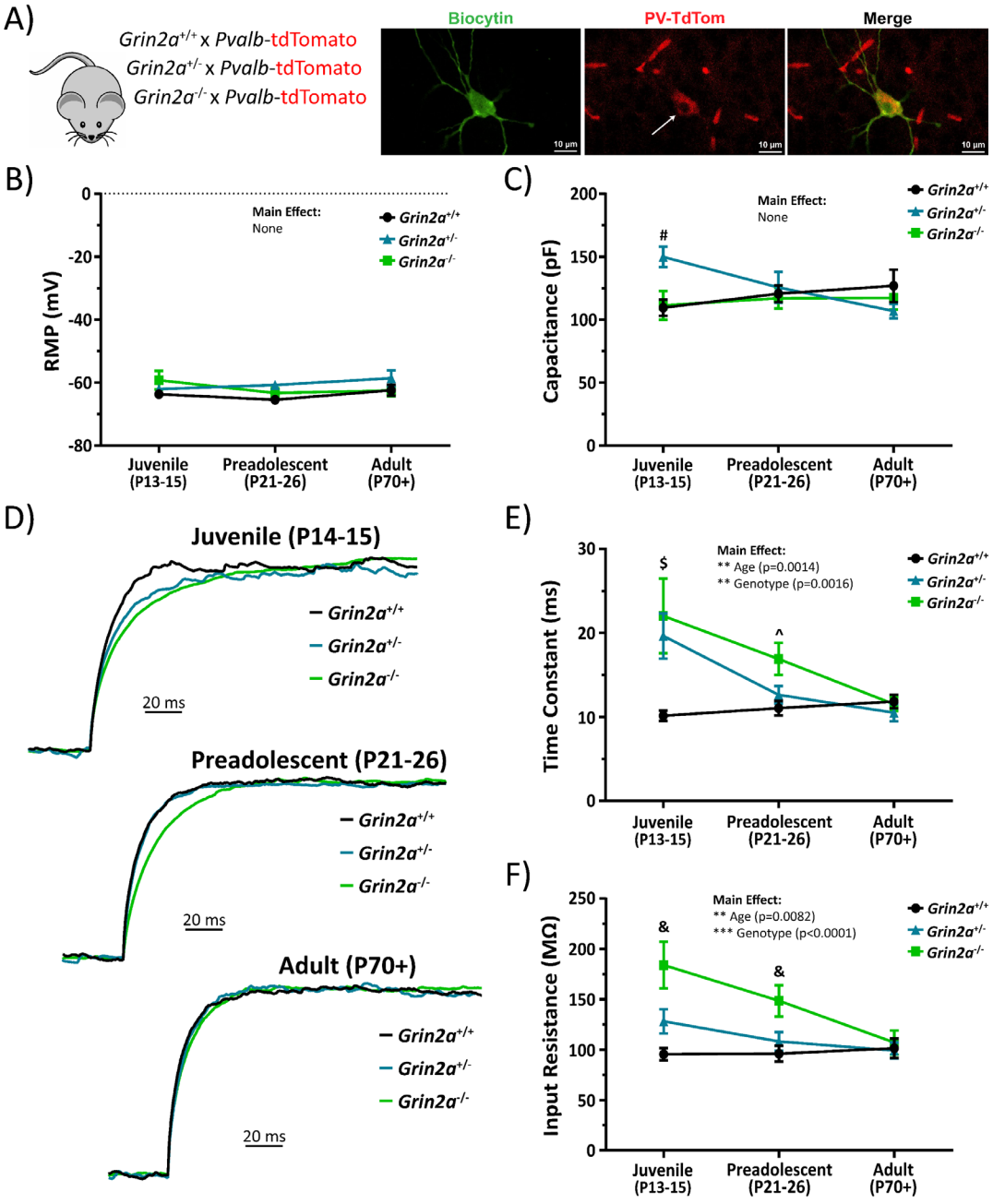


**Figure 3.5.** The loss of *Grin2a* causes a transient change in passive electrophysiological properties in CA1 PV cells. **A)** Mouse model used to visualize PV cells in CA1 next to examples of a biocytin backfilled CA1 PV cell that was stained for TdTomato to indicate successful cellular identification. **B)** No change in resting membrane potential across all three genotypes at different developmental time points (two-way ANOVA). **C)** No main effect of cellular capacitance across genotype ($F_{2, 136}$

= 1.3; p=0.28; two-way ANOVA) or age ($F_{2, 136}$ = 0.32; p=0.72; two-way ANOVA), however, there is a statistically significant interaction such that juvenile *Grin2a*$^{+/-}$ mice have a higher cellular capacitance than both *Grin2a*$^{+/+}$ mice (150 ± 8.0 pF for *Grin2a*$^{+/-}$ vs 110 ± 6.5 pF for *Grin2a*$^{+/+}$; p=0.0061) and *Grin2a*$^{-/-}$ mice (150 ± 8.0 pF for *Grin2a*$^{+/-}$ vs 111 ± 11 pF for *Grin2a*$^{-/-}$; p=0.014). **D)** Representative, amplitude-normalized repolarization traces to highlight differences in membrane time constant following a -50 pA current injection at different developmental timepoints. **E)** Membrane time constant measurements show statistically significant main effects for both age ($F_{2, 136}$ = 6.9; p=0.0014; two-way ANOVA) and genotype ($F_{2, 136}$ = 6.8; p=0.0016; two-way ANOVA). There are also several statistically significant interactions such that both juvenile *Grin2a*$^{+/-}$ mice (20 ± 2.7 ms for *Grin2a*$^{+/-}$ vs 10 ± 0.6 ms for *Grin2a*$^{+/+}$; p=0.0011) and *Grin2a*$^{-/-}$ mice (22 ± 4.4 ms for *Grin2a*$^{-/-}$ vs 10 ± 0.6 ms for *Grin2a*$^{+/+}$; p<0.0001) displayed higher membrane time constants than *Grin2a*$^{+/+}$ mice. Additionally, preadolescent *Grin2a*$^{-/-}$ mice showed a higher membrane time constant than *Grin2a*$^{+/+}$ mice (17 ± 1.9 ms for *Grin2a*$^{-/-}$ vs 11 ± 0.9 ms for *Grin2a*$^{+/+}$; p=0.048). **F)** Input resistance measurements show statistically significant main effects for both age ($F_{2, 133}$ = 5.0; p=0.0082; two-way ANOVA) and genotype ($F_{2, 133}$ = 12.2; p<0.0001; two-way ANOVA). There are also several statistically significant interactions such that juvenile *Grin2a*$^{-/-}$ mice displayed higher input resistances than *Grin2a*$^{+/+}$ mice (184 ± 23 MΩ for *Grin2a*$^{-/-}$ vs 96 ± 6.1 MΩ for *Grin2a*$^{+/+}$; p<0.0001) and *Grin2a*$^{+/-}$ mice (184 ± 23 MΩ for *Grin2a*$^{-/-}$ vs 128 ± 12 MΩ for *Grin2a*$^{+/+}$; p=0.0054). Additionally, preadolescent *Grin2a*$^{-/-}$ mice showed a higher input resistance than *Grin2a*$^{+/+}$ mice (149 ± 15 MΩ for *Grin2a*$^{-/-}$ vs 96 ± 7.9 MΩ for *Grin2a*$^{+/+}$; p=0.0031) and *Grin2a*$^{+/-}$ mice (149 ± 15 MΩ for *Grin2a*$^{-/-}$ vs 108 ± 9.3 MΩ for *Grin2a*$^{+/+}$; p=0.043). The sum of these data indicates an age- and gene-dependent transient delay in various passive electrical properties of CA1 PV cells. Symbols are mean ± SEM. RMP = resting

membrane potential; # = *Grin2a*$^{+/-}$ significantly different than both *Grin2a*$^{+/+}$ and *Grin2a*$^{-/-}$ at that age; $ = *Grin2a*$^{+/+}$ significantly different than both *Grin2a*$^{+/-}$ and *Grin2a*$^{-/-}$ at that age; ^ = *Grin2a*$^{-/-}$ significantly different than *Grin2a*$^{+/+}$ at that age; & = *Grin2a*$^{-/-}$ significantly different than both *Grin2a*$^{+/+}$ and *Grin2a*$^{+/-}$ at that age; ** = $p<0.01$; ***$p<0.001$.

PV cells from *Grin2a*$^{-/-}$ mice are significantly higher than juvenile and preadolescent CA1 PV cells from both *Grin2a*$^{+/+}$ and *Grin2a*$^{+/-}$ mice (**Figure 3.5F**; **Supplemental Table S8**). All input resistance values were not significantly different in adult mice, regardless of genotype (**Figure 3.5F**; **Supplemental Table S8**). The sum of these data illustrates that there is an age- and genotype-dependent transient delay in the passive membrane excitability of CA1 PV cells.

We also examined the action-potential waveform and firing properties of CA1 PV cells in *Grin2a*$^{+/-}$ and *Grin2a*$^{-/-}$ mice during development. We show no statistically significant effects of age or genotype on rheobase or action-potential amplitude, as well as no significant interactions for each measure (**Figure 3.6A-B**; **Supplemental Table S9**). Action-potential half-width shows statistically significant effects for both age and genotype (**Figure 3.6C-D**; **Supplemental Table S9**). Statistically significant interactions for the action-potential half-width indicate that juvenile CA1 PV cells from *Grin2a*$^{-/-}$ mice have significantly broader action potentials compared to juvenile CA1 PV cells from both *Grin2a*$^{+/+}$ and *Grin2a*$^{+/-}$ mice (**Figure 3.6C-D**; **Supplemental Table S9**). In preadolescent mice, the action-potential half-width of CA1 cells from *Grin2a*$^{-/-}$ mice is significantly higher than CA1 PV cells from *Grin2a*$^{-/-}$ mice, while there is no difference in the action-potential half-width of CA1 PV cells from preadolescent *Grin2a*$^{+/+}$ and *Grin2a*$^{+/-}$ mice (**Figure 3.6C-D**; **Supplemental Table S9**). All action-potential half-width values were not significantly different in adult mice, regardless of genotype (**Figure 3.6C-D**; **Supplemental Table S3.9**). These data indicate a significant but transient prolongation of the action-potential half-width that is dependent on both age and genotype. The afterhyperpolarization amplitude shows a statistically significant main effect for age, but not for genotype, which suggests that the channels responsible for afterhyperpolarization amplitude are not subjected to the same delay observed for other measures like membrane time constant, input resistance, and action-potential half-width

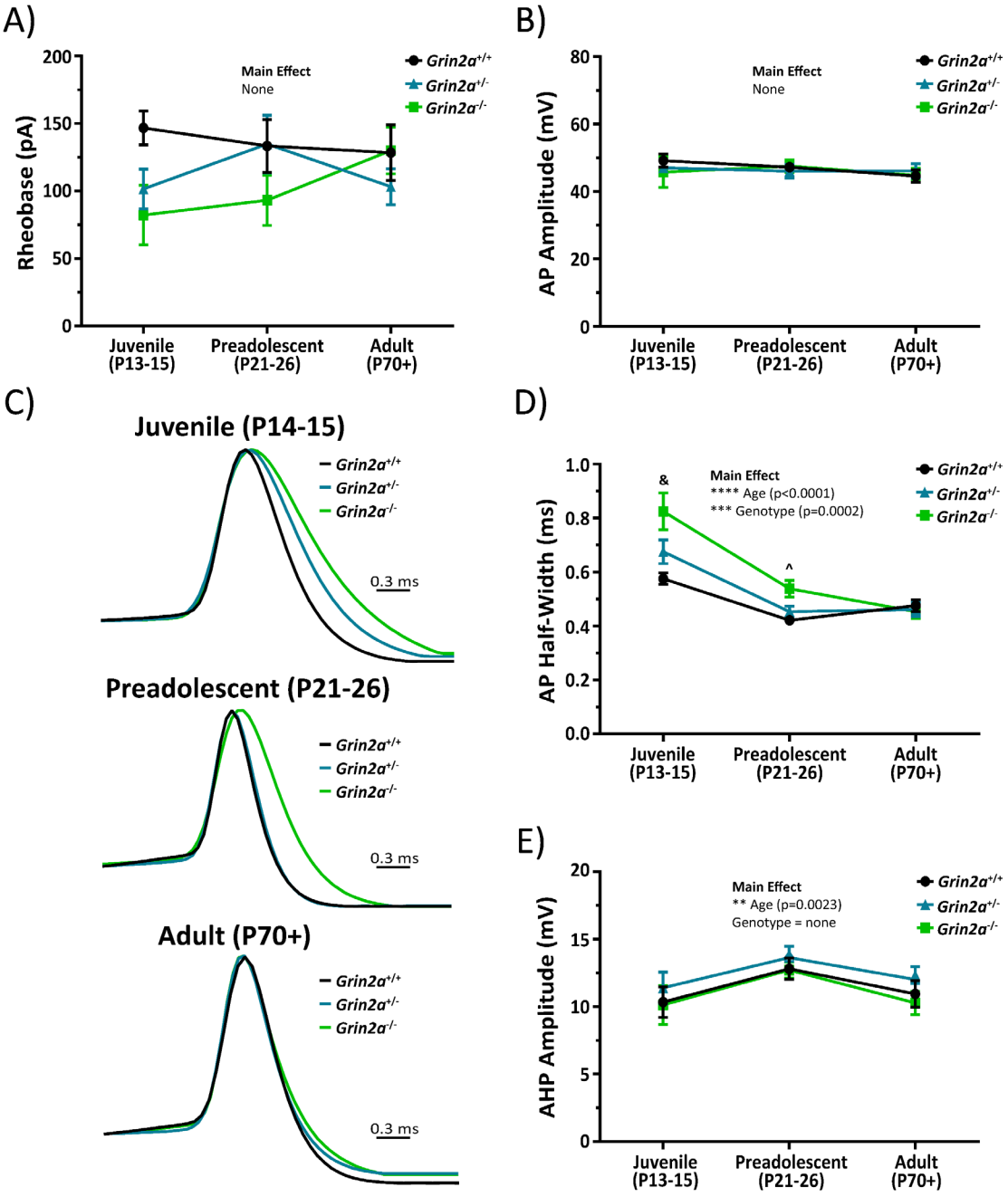


**Figure 3.6.** The loss of *Grin2a* causes a transient change in action-potential waveform properties of CA1 PV cells. There are no differences in **A)** rheobase or **B)** action-potential amplitude regardless of age or genotype in CA1 PV cells. **C)** Representative, amplitude-normalized single action-potential traces to highlight differences in half-width at different developmental timepoints. **D)** Action-potential half-width measurements show statistically significant main effects for both

age ($F_{2, 134}$ = 47; p<0.0001; two-way ANOVA) and genotype ($F_{2, 134}$ = 9.3; p=0.0002; two-way ANOVA). There are also several statistically significant interactions such that both juvenile *Grin2a*$^{+/+}$ mice (0.58 ± 0.02 ms for *Grin2a*$^{+/+}$ vs 0.82 ± 0.07 ms for *Grin2a*$^{-/-}$; p<0.0001) and *Grin2a*$^{+/-}$ mice (0.68 ± 0.04 ms for *Grin2a*$^{+/-}$ vs 0.82 ± 0.07 for *Grin2a*$^{-/-}$; p<0.005) displayed longer action-potential half-widths than *Grin2a*$^{-/-}$ mice. Additionally, preadolescent *Grin2a*$^{-/-}$ mice showed longer action-potential half-widths than *Grin2a*$^{+/+}$ mice (0.54 ± 0.03 ms for *Grin2a*$^{-/-}$ vs 0.42 ± 0.01 ms for *Grin2a*$^{+/+}$; p=0.0152). **E)** Afterhyperpolarization amplitude of the action-potential waveform showed a significant main effect for age ($F_{2, 127}$ = 6.36; p=0.0023; two-way ANOVA), but no main effect for genotype ($F_{2, 127}$ = 2; p=0.14; two-way ANOVA). Symbols are mean ± SEM. AP = action-potential; AHP = afterhyperpolarization; ^ = *Grin2a*$^{-/-}$ significantly different than *Grin2a*$^{+/+}$ at that age; & = *Grin2a*$^{-/-}$ significantly different than both *Grin2a*$^{+/+}$ and *Grin2a*$^{+/-}$ at that age; ** = p<0.01; ***p<0.001; **** = p<0.0001.

(**Figure 3.6E**; **Supplemental Table S3.9**).

We also found that both the maximum action-potential firing frequency before depolarization-induced block and the current required to reach depolarization-induced block of action potential firing show statistically significant main effects for age and genotype (**Figure 3.7A-E**; **Supplemental Table S9**). The transient delay in the maximum action-potential firing frequency suggests a delay in the upregulation of Kv3-family of voltage-gated potassium channels, while the transient decrease in the current required for depolarization-induced block is likely caused by the transient increase in input resistance described above. The sum of these data suggests that although PV cells from preadolescent *Grin2a*$^{-/-}$ mice may be fast to fire given their increased input resistance, they are also fast to retire given their alterations in depolarization-induced block and action-potential firing frequency. That is, inhibitory firing will fade in response high frequency firing, as might occur during initial runup toward seizure initiation. This diminished action potential generation could have profound consequences on circuit excitability and network wiring during the critical plasticity period during preadolescent development, which may manifest as epileptiform activity, maladaptive plasticity, or cognitive impairment. Moreover, if the transient nature of these reported alterations in PV cell function for *Grin2a*$^{-/-}$ interneurons also occur in PV interneurons from *GRIN2A* null variant patients, this could provide a molecular mechanism driving hyperexcitability and hypersynchronous epileptiform activity in these patients.

### *3.4 Discussion*

The most important finding of this study is that *GRIN2A* null human patients display a largely transient seizure burden, that can resolve with age. We report that *Grin2a*$^{-/-}$ mice, a model for *GRIN2A* null variants, also display transient excitability changes in hippocampal parvalbumin interneuron excitability that correlates developmentally with the period of seizure susceptibility in

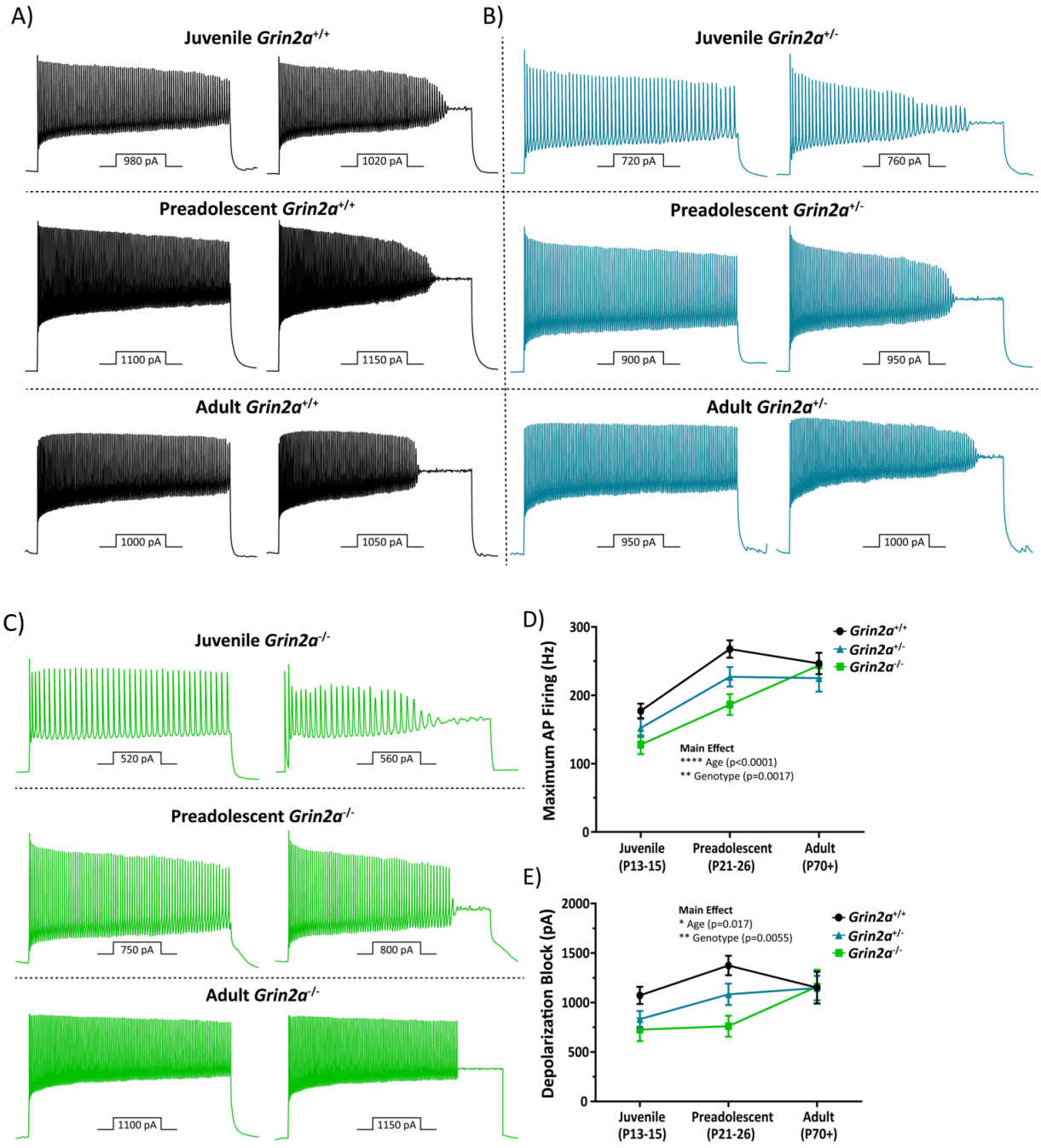


**Figure 3.7.** The loss of *Grin2a* causes a transient change in action-potential firing properties of CA1 PV cells. Representative action-potential trains elicited by current injections depicted below each train to illustrate the maximum action-potential firing frequency and the current required for depolarization-induced block of action-potential firing for **A)** *Grin2a*$^{+/+}$, **B)** *Grin2a*$^{+/-}$, and **C)**

*Grin2a*$^{-/-}$ mice during development. **D)** Maximum action potential firing frequencies show a significant main effect for both age ($F_{2, 135} = 29.8$; $p<0.0001$; two-way ANOVA) and genotype ($F_{2, 135} = 6.7$; $p=0.0017$; two-way ANOVA). **E)** Currents required to reach depolarization-induced block of action potential firing show a significant main effect for both age ($F_{2, 135} = 4.2$; $p=0.017$; two-way ANOVA) and genotype ($F_{2, 135} = 5.4$; $p=0.006$; two-way ANOVA). Symbols are mean ± SEM. AP = action-potential; depolarization block = current required to reach depolarization-induced block of action-potential firing; * = $p<0.05$; ** = $p<0.01$; ***$p<0.001$; **** = $p<0.0001$.

human patients with null *GRIN2A* variants. This appears to primarily reflect a change in PV cell spike firing propensity, and thus we suggest that this transient change in PV cell function as a plausible mechanism that may contribute to the transient seizure susceptibility in *GRIN2A* null variant patients. These deficits in PV cell function largely mirror a delay in the electrophysiological maturation and likely shift network excitability towards excessive pyramidal cell firing, allowing synchronized epileptiform discharge formation. This perturbation resolves with further development, as adult PV cells show similar electrophysiological properties as age-matched WT cells, corresponding developmentally to the time patients show reduced seizure burden. In addition to the perturbations in PV interneuron function, these data highlight a previously unrecognized role for GluN2A signaling in the cellular maturation and preservation of the PV interneuron population in hippocampal CA1. Alterations in circuit excitability, the changes in the number of PV cells, which are the most common interneuron in the CNS, could have far reaching impact on other aspects of brain function and development.

The data we report here suggests multiple potential roles for the GluN2A subunit of NMDARs in the regulation of function, development, and maturation of GABAergic interneurons in the mouse hippocampus. We show that CA1 PV cells from *Grin2a*$^{-/-}$ mice display genotype- and age-dependent alterations in passive electrical and action-potential firing properties. By examining both passive intrinsic properties and action potential firing characteristics, we can estimate how these cells may respond to excitatory afferent activity and how efficiently they can transduce somatic depolarizations into signal-carrying action potentials. The exact mechanism for this transcriptional shift from immature to mature PV electrophysiological properties is not fully understood but has been shown to be activity-dependent (Dehorter et al., 2015; Miller et al., 2011). Thus, the loss of *Grin2a* in developing PV interneurons appears to slow cellular maturation,

potentially stemming from a loss of GluN2A signaling on PV cell dendrites or changes in non-cell autonomous signaling. All these properties ultimately reach wild type levels in adult mice, suggesting that although GluN2A signaling may be sufficient to initiate these maturation transcriptional programs, it is not absolutely required. While the overall effects of a prolonged immature electrophysiological profile in preadolescent PV cells are not known, this period coincides with critical period plasticity in various brain regions (Hensch, 2005; Takesian and Hensch, 2013), and thus, could critically alter circuit formation in the hippocampus, and other regions. Specifically, larger input resistances and lower peak action potential firing frequencies will likely dampen the temporal resolution of inhibitory tone in a developing network, which may promote maladaptive plasticity and incorrect circuit wiring. Additionally, changes in depolarization-induced block of action potentials correlates with neural excitability and propagation of epileptiform activity (Calin et al., 2021). Thus, disruption of PV cell function during development could have profound consequences for eventual mature networks that arise.

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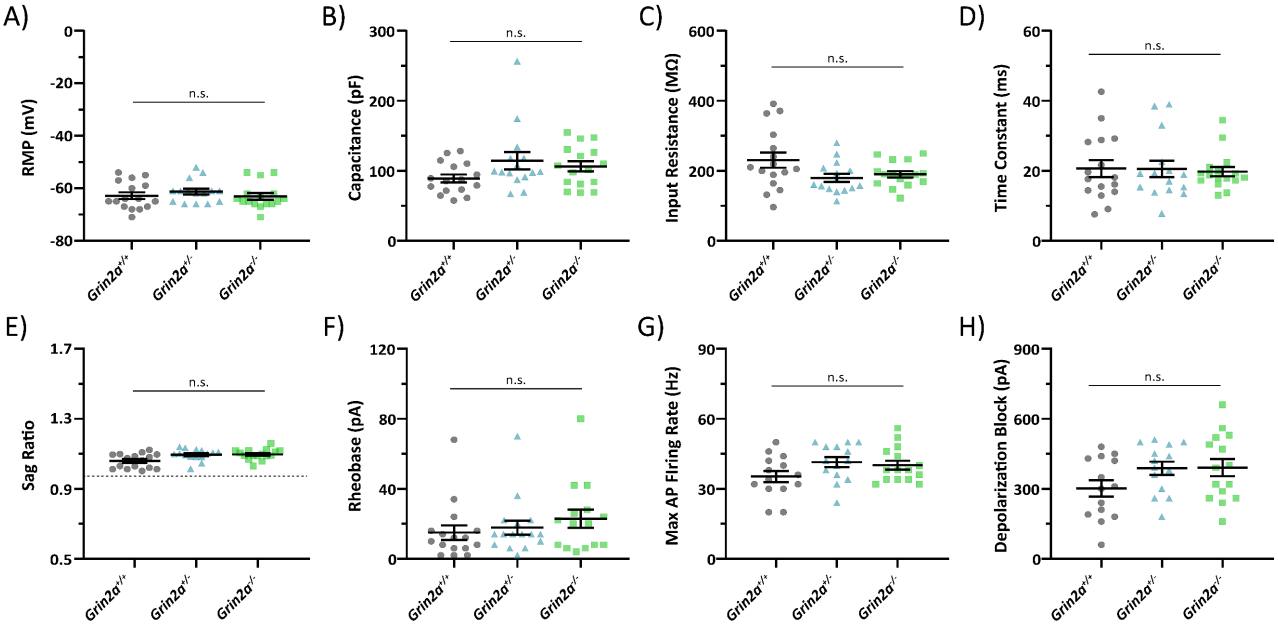
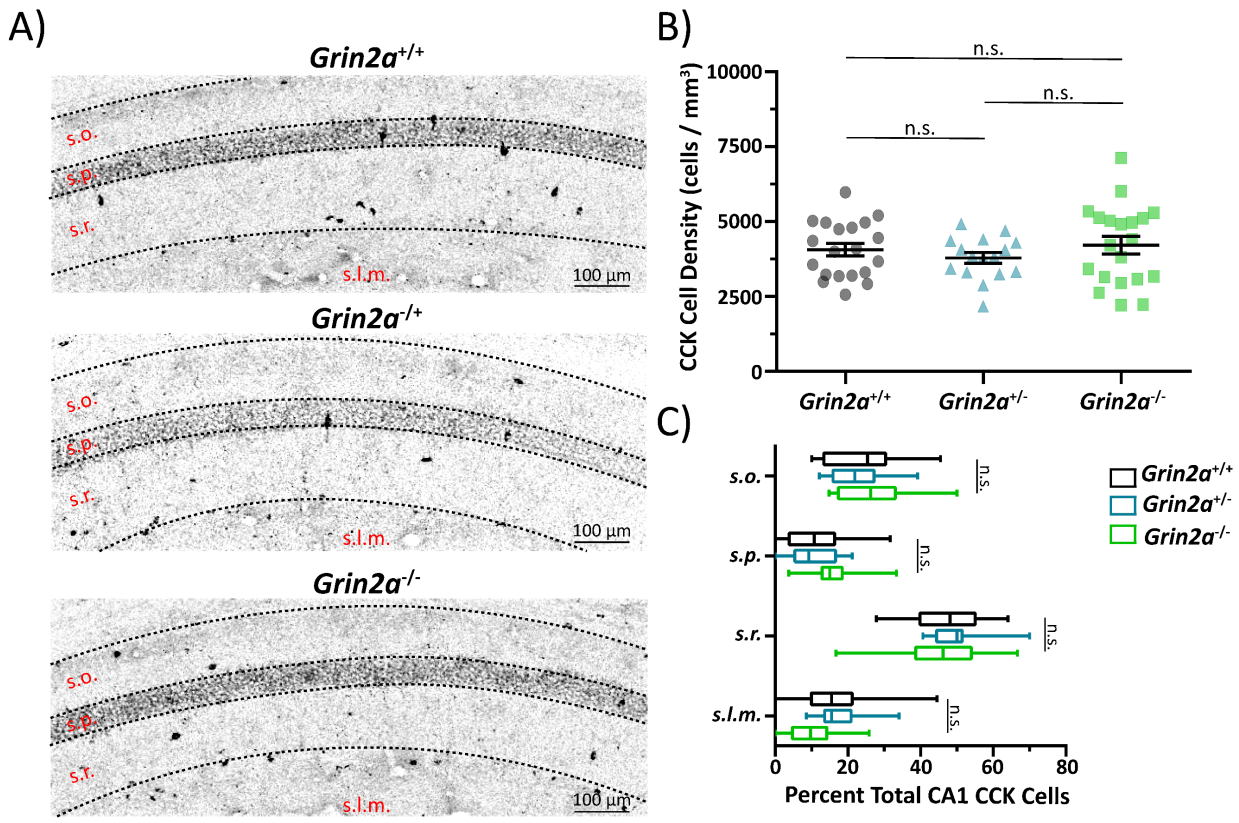
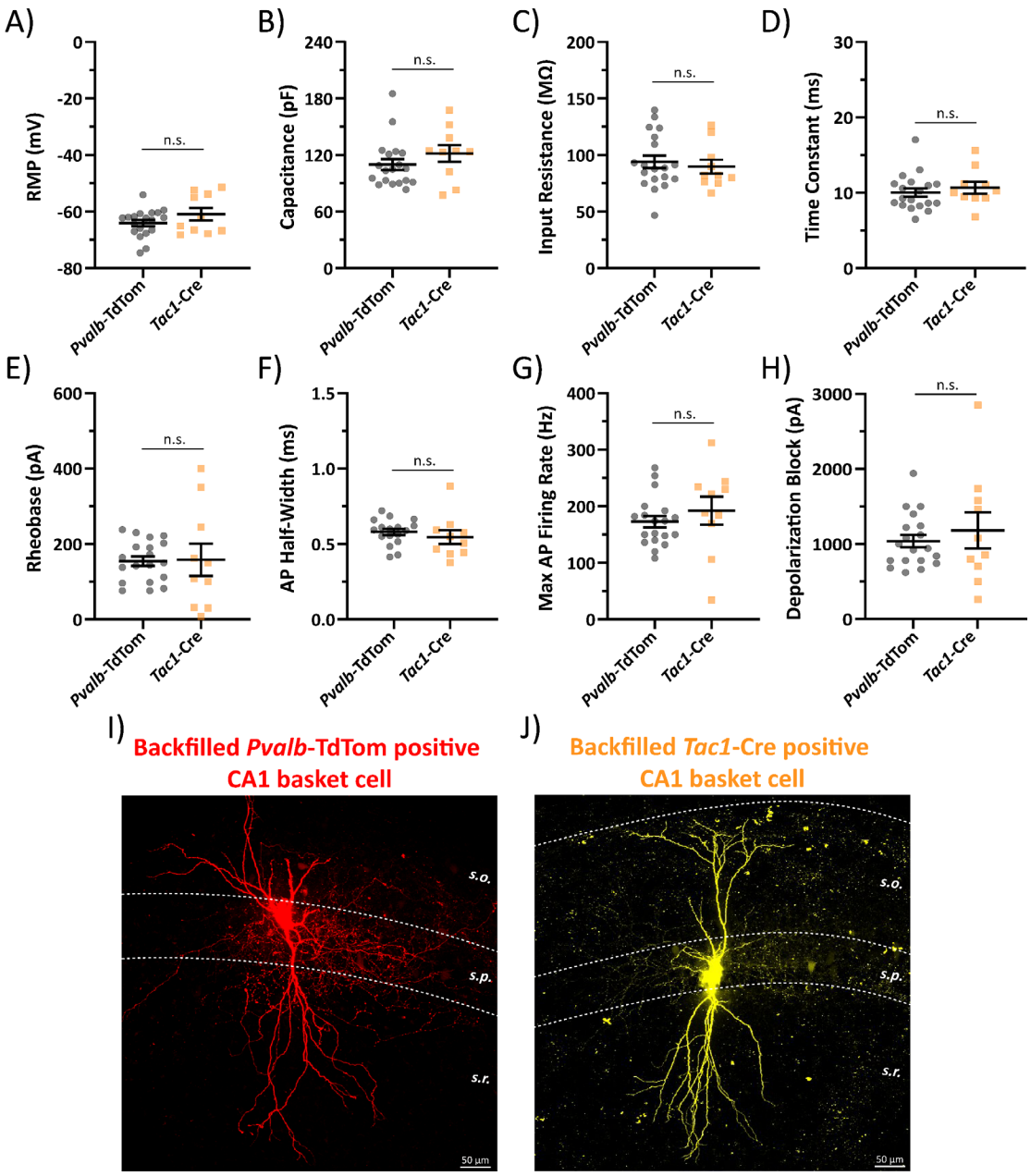



**Supplemental Figure S3.1.** Juvenile CA1 circuit hyperexcitability is not due to changes in CA1 pyramidal cell intrinsic or action-potential firing properties. Data from juvenile (P14-16) CA1 pyramidal cells show no significant differences in **A)** resting membrane potential, **B)** cell capacitance, **C)** input resistance, **D)** membrane time constant, **E)** sag ratio, **F)** rheobase, **G)** maximum action-potential firing rate, or **H)** current required to reach depolarization-induced blockade of action-potential firing when assayed via one-way ANOVA. Data represented show mean ± SEM. RMP = resting membrane potential; AP = action-potential; depolarization block = current required to reach depolarization-induced blockade of action-potential firing; n.s. = not significant.




**Supplemental Figure S3.2.** Loss of *Grin2a* does not alter cholecystokinin (CCK) cell density in CA1. **A)** Representative images of CA1 hippocampal sections stained for CCK in preadolescent mice. **B)** CA1 CCK cell density is unchanged across all three genotypes (one-way ANOVA, p=0.49). **C)** There is no difference in CCK CA1 cellular lamination across all three genotypes. Data represented show mean ± SEM. *s.o.* = *stratum oriens*; *s.p.* = *stratum pyramidale*; *s.r.* = *stratum radiatum*; *s.l.m.* = *stratum lacunosum moleculare*; CCK = cholecystokinin; n.s. = not significant.




**Supplemental Figure S3.3.** CA1 PV cells undergo electrophysiological maturation of passive and action-potential firing properties. **A)** Strategy to visualize PV cells in acute hippocampal slices. Since the parvalbumin gene is developmentally regulated, Tac1-Cre mice were used in neonate (P6-8) mice to patch immature, fast-spiking interneurons.

| Variant Type | Nucleotide Information | Protein Information | Amino Acid Number | Seizure Onset (Years) | Seizure Offset (Years) |
|---|---|---|---|---|---|
| Deletion | N/A | del exon 1-3 | N/A | 10 | -- |
| Deletion | chr16:10,227,326-10,300,839x1 | del exon 1-3 | N/A | 7 | -- |
| Deletion | chr16:10,227,326-10,300,839x1 | del exon 1-3 | N/A | 3 | -- |
| Deletion | chr16:10,227,326-10,300,839x1 | del exon 1-3 | N/A | 10 | -- |
| Deletion | chr16:10,227,326-10,300,839x1 | del exon 1-3 | N/A | 2.5 | -- |
| Deletion | chr16:10,241,998-10,300,800x1 | del exon 1-3 | N/A | 8 | -- |
| Deletion | chr16:10,241,998-10,300,800x1 | del exon 1-3 | N/A | 6 | -- |
| Deletion | chr16:10,246,239<U+0096>10,321,593 | del exon 1-3 | N/A | 6 | -- |
| Deletion | chr16:10,246,239<U+0096>10,321,593 | del exon 1-3 | N/A | 6 | -- |
| Deletion | chr16:10,246,239-10,354,862x1 | del exon 1-3 | N/A | 4 | -- |
| Deletion | possible c.1-? | Deletion exon 3 | possible 1-? | 6 | 6 |
| Deletion | | Deletion exon 3 | | 6.6 | -- |
| Deletion | c.280_283delCGCA | p.(Arg94Serfs*15) | 94 | 6.2 | 12.1 |
| Deletion | c.280_283delCGCA | p.(Arg94Serfs*15) | 94 | 6.2 | 12.2 |
| Deletion | chr16:10,000,670-10,197,654x1 | del exon 4 | possible 139-? | 2 | -- |
| Deletion | 16p13 deletion | del exon-? | possible 139-? | 2 | -- |
| Deletion | 16p13.2 292,09 kb | del exon 4-14 | possible 139-? | 2 | -- |
| Deletion | 16p13.2 deletion (6907020-10415739) | | | 2.4 | -- |
| Deletion | c.(414+1_415-1)_(1007+1_1008-1) | del exon 4 | possible 139-? | 4 | -- |
| Deletion | chr16:9,825,755-10,069,792x1 | del exon 4-14 | possible 139-? | 7.7 | -- |
| Deletion | possible c.415-?; del 292,09kb | del exon 4-14 | possible 139-? | 4 | -- |
| Deletion | possible c.415-? | Deletion exon 4 | possible 139-? | 7 | 12 |
| Deletion | possible c.415-? | Deletion exon 4 | possible 139-? | 5 | 5 |
| Deletion | possible c.415-? | del exon 4 | possible 139-? | 3 | -- |
| Deletion | possible c.415-1122 | dup exon 4-5 | possible 139-? | 0.25 | -- |
| Deletion | possible c.1009-? | Deletion exon 5 | possible 337-? | 1.5 | -- |
| Deletion | possible c.1123-? | Deletion exon 6 | possible 375-? | 3 | -- |
| Deletion | possible c.1123-? | del exon 6-11 | possible 375-? | 3 | -- |
| Deletion | possible c.1123-? | del exon 6-7 | possible 375-? | 3 | -- |
| Deletion | possible c.1123-? | del exon 6-7 | possible 375-? | 1 | -- |
| Deletion | possible c.1123-? | del exon 6-7 | possible 375-? | 8 | -- |
| Deletion | c.1585delG | p.(Val529Trpfs*22) | 529 | 2 | -- |
| Deletion | c.1586delT | p.(Val529Glyfs*22) | 529 | 5.75 | -- |
| Deletion | c.1650_1651delAGGTGTGT | p.(Leu-?) | 550 | 4 | 8 |
| Deletion | c.1650_1651delAGGTGTGT | | | -- | 11 |
| Deletion | c.1651+1del | p.(Glu-?) | 551 | 7 | -- |
| Deletion | c.1686del | p.(Phe562Leufs*2) | 562 | 2 | -- |
| Deletion | 9,915,756<U+0096>9,915,815<d7>1 | del exon 11 | possible 670-? | 5 | -- |
| Deletion | 9,915,756<U+0096>9,915,815<d7>1 | del exon 11 | possible 670-? | 5 | -- |
| Deletion | 9,915,756<U+0096>9,915,815<d7>1 | del exon 11 | possible 670-? | 4.5 | -- |
| Deletion | possible c.2170-? | del exon 12-14 | possible 724-? | | 12 |
| Deletion | c.2334_2338delCTTGC | p.(Leu779Serfs*5) | 779 | 4 | |
| Deletion | c.2408del | p.(Glu803Glyfs*5) | 803 | 2 | |
| Deletion | c.3596delC | p.(Pro1199Arg*32) | 1199 | | 10.2 |
| Deletion | c.3596delC | p.(Pro1199Arg*32) | 1199 | | 10.2 |
| Del/Insert | c.2341_2343delinsAT | p.(Gln781Ilefs*27) | 781 | 6 | -- |
| Duplication | c.90dupT | p.(Pro31Serfs*107) | 31 | 4 | -- |
| Duplication | c.176_179dupAGGC | p.(Ala61Glyfs*78) | 61 | 6 | -- |
| Duplication | possible c.415-1122 | dup exon 4-5 | possible 139-? | 0.25 | -- |
| Duplication | c.2007+2dup | p.(Lys-?) | 669 | 10 | -- |
| Duplication | c.2253dupG | p.(Ser752Glufs*34) | 752 | 5 | -- |

| | | | | | |
|---|---|---|---|---|---|
| Nonsense | c.2T>C | p.(Met1?) | 1 | 3.5 | -- |
| Nonsense | c.2T>C | p.(Met1?) | 1 | 2 | -- |
| Nonsense | ? | p.(Ala27Glyfs*112) | 27 | -- | 16 |
| Nonsense | c.172G>T | p.(Glu58*) | 58 | 5 | -- |
| Nonsense | ? | p.(Ala61Glyfs*78) | 61 | -- | 8 |
| Nonsense | c.500G>A | p.(Trp167*) | 167 | 4 | -- |
| Nonsense | c.594G>A | p.(Trp198*) | 198 | 5 | -- |
| Nonsense | c.594G>A | p.(Trp198*) | 198 | 4 | -- |
| Nonsense | c.594G>A | p.(Trp198*) | 198 | 3 | -- |
| Nonsense | ? | p.(Trp198*) | 198 | -- | 12 |
| Nonsense | c.627delC | p.Phe210Leufsx10 | 210 | 7.75 | 15.1 |
| Nonsense | c.652C>T | p.(Gln218*) | 218 | 1 | -- |
| Nonsense | c.652C>T | p.(Gln218*) | 218 | 0.9 | -- |
| Nonsense | c.703G>T | p.Glu235X | 235 | 3 | 9 |
| Nonsense | c.1001T>A | p.(Leu334*) | 334 | 3 | -- |
| Nonsense | c.1001T>A | p.(Leu334*) | 334 | 1 | -- |
| Nonsense | c.1001T>A | p.(Leu334*) | 334 | 4 | -- |
| Nonsense | c.1036A>T | p.Lys346Ter | 346 | 11 | -- |
| Nonsense | C.1115G>A | p.Trp372 | 372 | 0.04 | 3 |
| Nonsense | ? | p.(Pro415Hisfs*8) | 415 | -- | 10 |
| Nonsense | ? | p.(Pro415Hisfs*8) | 415 | -- | 20 |
| Nonsense | c.1613C>G | p.(Ser538*) | 538 | 4 | -- |
| Nonsense | c.1818G>A | p.(Trp606*) | 606 | 3.5 | -- |
| Nonsense | c.2041C>T | p.(Arg681*) | 681 | 4 | -- |
| Nonsense | c.2041C>T | p.(Arg681*) | 681 | 2.5 | -- |
| Nonsense | c.2407G>T | p.(Glu803*) | 803 | 3.5 | -- |
| Nonsense | ? | p.(Arg847*) | 847 | -- | 10.8 |
| Unknown | c.415-2A>G | p.(Asp-?) | 139 | 10 | -- |
| Unknown | c.1007+1G>A | p.(?) | 336 | 6 | -- |
| Unknown | c.1007+1G>A | p.(?) | 336 | 3 | -- |
| Unknown | c.1007+1G>A | p.(?) | 336 | 4 | -- |
| Unknown | c.1007+1G>A | p.(?) | 336 | 6 | -- |
| Unknown | c.1007+1G>A | p.(?) | 336 | 1.5 | -- |
| Unknown | c.1007+1G>A | p.(?) | 336 | 2 | -- |
| Unknown | c.1007+1G>A | p.(?) | 336 | 4 | -- |
| Unknown | c.1007+1G>A | p.(?) | 336 | 4 | -- |
| Unknown | c.1007+1G>A | p.(?) | 336 | 5.5 | -- |
| Unknown | c.1007+1G>A | p.(?) | 336 | 2.5 | -- |
| Unknown | c.1007+1G>A | p.(?) | 336 | 6 | -- |
| Unknown | c.1007+1G>A | p.(?) | 336 | 10 | -- |
| Unknown | c.1007+1G>A | p.(?) | 336 | -- | 9 |
| Unknown | c.1007+1G>T | p.(?) | 336 | 4 | -- |
| Unknown | c.1123-1G>T | p.(Val-?) | 375 | 4 | -- |
| Unknown | c.1123-1G>T | p.(Val-?) | 375 | 3.5 | -- |
| Unknown | c.1123-2A>G | p.(Val-?) | 375 | 2 | -- |
| Unknown | c.1123-2A>G | p.(Val-?) | 375 | 4 | -- |
| Unknown | c.1123-2A>G | p.(Val-?) | 375 | 6 | -- |
| Unknown | c.1123-2A>G | p.(Val-?) | 375 | 5 | -- |
| Unknown | c.1123-2A>G | p.(Val-?) | 375 | 4 | -- |
| Unknown | c.1123-2A>G | p.(Val-?) | 375 | 5 | -- |
| Unknown | c.1123-2A>G | p.(Val-?) | 375 | 5 | -- |
| Unknown | c.2007+1G>A | p.(Lys-?) | 669 | 3.5 | -- |

**Supplemental Table S3.1.** Null *GRIN2A* variant patient data with time when seizures began and/or ended. Data represented are gathered from the *GRIN* portal (https://grin-

portal.broadinstitute.org/), University of Leipzig, Children's Hospital of Philadelphia, or University of Colorado Anschutz Medical Center. Seizure offset is defined as freedom from seizures for two consecutive years. N/A = not applicable. '--' = data not available at time of publication. Some patients had not reached an age where offset was expected.

| Gene | Variant Type | Nucleotide Information | Protein Information | Amino Acid Number | Seizure Onset (Years) | Seizure Offset (Years) |
|---|---|---|---|---|---|---|
| *GRIN2A* | Missense | c.583T>C | p.(Phe195Leu) | 195 | 23 | -- |
| *GRIN2A* | Missense | c.583T>C | p.(Phe195Leu) | 195 | 23 | -- |
| *GRIN2A* | Missense | c.1667C>T | p.(Arg217Trp) | 217 | 0.25 | 1.7 |
| *GRIN2A* | Missense | c.692G>A | p.(Cys231Tyr) | 231 | 3 | -- |
| *GRIN2A* | Missense | c.1232T>A | p.(Leu411Gln) | 411 | 3 | -- |
| *GRIN2A* | Missense | c.1251C>T | p.(Val417Val) | 417 | 0.8 | -- |
| *GRIN2A* | Missense | c.1306T>C | p.(Cys436Arg) | 436 | 4 | -- |
| *GRIN2A* | Missense | c.1447G>A | p.(Gly483Arg) | 483 | 4 | -- |
| *GRIN2A* | Missense | c.1492G>A | p.(Gly498Ser) | 498 | 8 | -- |
| *GRIN2A* | Missense | c.1532C>T | p.(Ser511Leu) | 511 | 2.5 | -- |
| *GRIN2A* | Missense | c.1552C>T | p.(Arg518Cys) | 518 | 3 | -- |
| *GRIN2A* | Missense | c.1553G>A | p.(Arg518His) | 518 | 3 | -- |
| *GRIN2A* | Missense | c.1553G>A | p.(Arg518His) | 518 | 2 | -- |
| *GRIN2A* | Missense | c.1592C>T | p.(Thr531Met) | 531 | 6.7 | -- |
| *GRIN2A* | Missense | c.1592C>T | p.(Thr531Met) | 531 | 7 | -- |
| *GRIN2A* | Missense | c.1592C>T | p.(Thr531Met) | 531 | 11 | -- |
| *GRIN2A* | Missense | c.1642G>A | p.(Ala548Thr) | 548 | 6 | -- |
| *GRIN2A* | Missense | c.1655C>G | p.(Pro552Arg) | 552 | 0.75 | -- |
| *GRIN2A* | Missense | c.1655C>G | p.(Pro552Arg) | 552 | 0.75 | -- |
| *GRIN2A* | Missense | c.1841A>G | p.(Asn614Ser) | 614 | 0.4 | -- |
| *GRIN2A* | Missense | c.1845C>A | p.(Asn615Lys) | 615 | 0.75 | -- |
| *GRIN2A* | Missense | c.1845C>A | p.(Asn615Lys) | 615 | 0.25 | -- |
| *GRIN2A* | Missense | c.1903G>A | p.(Ala635Thr) | 635 | 0.6 | -- |
| *GRIN2A* | Missense | c.1930A>G | p.(Ser644Gly) | 644 | 0.5 | -- |
| *GRIN2A* | Missense | c.1936A>G | p.(Thr646Ala) | 646 | 0.7 | -- |
| *GRIN2A* | Missense | c.1936A>G | p.(Thr646Ala) | 646 | 0.25 | -- |
| *GRIN2A* | Missense | c.1945C>G | p.(Leu649Val) | 649 | 0.75 | -- |
| *GRIN2A* | Missense | c.1946_1947delin | p.(Leu649Pro) | 649 | 1 | -- |
| *GRIN2A* | Missense | c.1948G>T | p.(Ala650Ser) | 650 | 1.5 | -- |
| *GRIN2A* | Missense | c.1954T>G | p.(Phe652Val) | 652 | 2 | -- |
| *GRIN2A* | Missense | c.1961T>C | p.(Ile654Thr) | 654 | 0.2 | -- |
| *GRIN2A* | Missense | c.2081T>C | p.(Ile694Thr) | 694 | 2.5 | -- |
| *GRIN2A* | Missense | c.2095C>T | p.(Pro699Ser) | 699 | 8 | -- |
| *GRIN2A* | Missense | c.2113A>G | p.(Met705Val) | 705 | 8 | -- |
| *GRIN2A* | Missense | c.2191 G>A | p.Asp731Asn | 731 | 1.9 | -- |
| *GRIN2A* | Missense | c.2191G>A | p.(Asp731Asn) | 731 | 4 | -- |
| *GRIN2A* | Missense | c.2191G>A | p.Asp731Asn | 731 | 3.9 | -- |
| *GRIN2A* | Missense | c.2326G>T | p.(Asp776Tyr) | 776 | 7 | -- |
| *GRIN2A* | Missense | c.2434C>A | p.(Leu812Met) | 812 | 1.5 | -- |
| *GRIN2A* | Missense | c.2449A>G | p.(Met817Val) | 817 | 2 | -- |
| *GRIN2A* | Missense | c.2450T>C | p.(Met817Thr) | 817 | 2 | -- |
| *GRIN2A* | Missense | c.2450T>C | p.(Met817Thr) | 817 | 1.25 | -- |
| *GRIN2A* | Missense | c.2452G>A | p.(Ala818Thr) | 818 | 3 | -- |
| *GRIN2A* | Missense | c.2453C>A | p.(Ala818Glu) | 818 | 1 | -- |
| *GRIN2A* | Missense | c.2907C>G | p.(Asn969Lys) | 969 | 2.5 | -- |
| *GRIN2A* | Missense | c.3664C>G | p.(Pro1222Ala) | 1222 | 7.9 | -- |
| *GRIN2A* | Missense | c.3664C>G | p.(Pro1222Ala) | 1222 | 7.8 | -- |

**Supplemental Table S3.2.** Missense *GRIN2A* variant patient data with information on when seizure burden began and/or ended. Data represented are gathered from the *GRIN* portal (https://grin-portal.broadinstitute.org/), University of Leipzig, Children's Hospital of Philadelphia,

or University of Colorado Anschutz Medical Center. Seizure offset is defined as freedom from seizures for two consecutive years. ‘--' = data not available at time of publication.

| | AP Probability 1st Stimulation | AP Probability 2nd Stimulation | AP Probability 3rd Stimulation | AP Probability 4th Stimulation | AP Probability 5th Stimulation | Total Number of APs Generated |
|---|---|---|---|---|---|---|
| ***Grin2a*$^{+/+}$ mice** N=16 cells/5 mice | 0.05 ± 0.04 | 0.17 ± 0.07 | 0.2 ± 0.07 | 0.08 ± 0.03 | 0.05 ± 0.05 | 2.75 ± 0.91 |
| ***Grin2a*$^{+/-}$ mice** N=16 cells/4 mice | 0.06 ± 0.02 | 0.58 ± 0.1 | 0.29 ± 0.09 | 0.13 ± 0.05 | 0.1 ± 0.04 | 5.88 ± 1.18 |
| ***Grin2a*$^{-/-}$ mice** N=16 cells/4 mice | 0.03 ± 0.04 | 0.51 ± 0.1 | 0.38 ± 0.09 | 0.21 ± 0.06 | 0.09 ± 0.04 | 6.06 ± 1.12 |

**Supplemental Table S3.3.** Action-potential spiking probability in *Grin2a*$^{+/+}$, *Grin2a*$^{+/-}$, and *Grin2a*$^{-/-}$ juvenile (P14-16) mice. Data are mean ± SEM. AP = action potential.

| | **RMP** (mV) | **Capacitance** (pF) | **Input Resistance** (MΩ) | **Time Constant** (ms) | **Rheobase** (pA) | **Sag Ratio** | **Max AP Firing** (Hz) | **Depolarization Block** (pA) |
|---|---|---|---|---|---|---|---|---|
| ***Grin2a*$^{+/+}$ mice** N=16 cells/5 mice | -62 ± 1.3 | 89 ± 5.8 | 231 ± 22 | 21 ± 2.4 | 20 ± 4.4 | 1.06 ± 0.01 | 35 ± 2.4 | 301 ± 35 |
| ***Grin2a*$^{+/-}$ mice** N=15 cells/4 mice | -61 ± 1.1 | 114 ± 12 | 179 ± 12 | 21 ± 2.4 | 23 ± 5.2 | 1.1 ± 0.01 | 41 ± 2 | 389 ± 27 |
| ***Grin2a*$^{-/-}$ mice** N=16 cells/4 mice | -63 ± 1.2 | 106 ± 7.3 | 190 ± 8.9 | 20 ± 1.4 | 19 ± 4 | 1.1 ± 0.01 | 40 ± 1.9 | 391 ± 36 |

**Supplemental Table S3.4.** Passive and action-potential firing properties of CA1 pyramidal cells in *Grin2a*$^{+/+}$, *Grin2a*$^{+/-}$, and *Grin2a*$^{-/-}$ juvenile (P14-16) mice. Data are mean ± SEM. RMP = resting membrane potential; AP = action potential; depolarization block = current required for depolarization-induced block of AP firing.

| | **CA1 Cell Density** (# / $mm^3$) | ***stratum oriens*** (% total CA1 PV cells) | ***stratum pyramidale*** (% total CA1 PV cells) | ***stratum radiatum*** (% total CA1 PV cells) | ***stratum lacuosum moleculare*** (% total CA1 PV cells) |
|---|---|---|---|---|---|
| ***Grin2a*$^{+/+}$ mice** N=17 slices/4 mice | 4875 ± 162 | 42 ± 2.8 | 51 ± 2.8 | 7.5 ± 1.5 | 0 |
| ***Grin2a*$^{+/-}$ mice** N=16 slices/4 mice | 4542 ± 198 | 40 ± 2.7 | 49 ± 2.4 | 11 ± 8.4 | 0 |
| ***Grin2a*$^{-/-}$ mice** N=20 slices/4 mice | 6488 ± 276 | 40 ± 2.3 | 52 ± 2.3 | 9.4 ± 5.4 | 0 |

**Supplemental Table 3.5.** Parvalbumin-positive CA1 density and cellular lamination in *Grin2a*$^{+/+}$, *Grin2a*$^{+/-}$, and *Grin2a*$^{-/-}$ preadolescent (P21-26) mice. Data are mean ± SEM. 4-5 hippocampi from each animal were imaged across 4 animals per genotype for all staining and cell counts.

| | **CA1 Cell Density** (# / $mm^3$) | ***stratum oriens*** (% total CA1 CCK cells) | ***stratum pyramidale*** (% total CA1 CCK cells) | ***stratum radiatum*** (% total CA1 CCK cells) | ***stratum lacuosum moleculare*** (% total CA1 CCK cells) |
|---|---|---|---|---|---|
| ***Grin2a*$^{+/+}$ mice** N=20 slices/4 mice | 4060 ± 210 | 24 ± 2.4 | 11 ± 2.1 | 47 ± 2.2 | 17 ± 2.4 |
| ***Grin2a*$^{+/-}$ mice** N=16 slices/4 mice | 3790 ± 177 | 23 ± 1.9 | 10 ± 1.7 | 50 ± 1.9 | 17 ± 1.6 |
| ***Grin2a*$^{-/-}$ mice** N=20 slices/4 mice | 4209 ± 299 | 27 ± 2.3 | 16 ± 1.7 | 45 ± 2.8 | 12 ± 9 |

**Supplemental Table S3.6.** Cholecystokinin-positive CA1 density and cellular lamination in *Grin2a*$^{+/+}$, *Grin2a*$^{+/-}$, and *Grin2a*$^{-/-}$ preadolescent (P21-26) mice. Data are mean ± SEM.

| | RMP (mV) | Capacitance (pF) | Input Resistance (MΩ) | Time Constant (ms) | Rheobase (pA) | Sag Ratio |
|---|---|---|---|---|---|---|
| **Neonate (P6-7)** N=7 cells/3 mice | -57 ± 4.5 | 193 ± 27 | 211 ± 28 | 40 ± 6.4 | 33 ± 12 | 1.11 ± 0.02 |
| **Juvenile (P13-15)** N=19 cells/6 mice | -64 ± 1.1 | 110 ± 6.5 | 96 ± 6.1 | 10 ± 0.6 | 147 ± 12 | 1.01 ± 0.01 |
| **Preadolescent (P21-26)** N=21 cells/6 mice | -65 ± 1.3 | 121 ± 7 | 96 ± 8.3 | 11 ± 0.9 | 134 ± 21 | 0.96 ± 0.05 |
| **Adult (P70+)** N=12 cells/6 mice | -62 ± 1.7 | 127 ± 13 | 101 ± 9.7 | 12 ± 0.8 | 128 ± 21 | 0.99 ± 0.01 |
| -- | **AP Half-Width** (ms) | **AP Amplitude** (mV) | **AHP Amplitude** (mV) | **AP Threshold** (mV) | **Max AP Firing** (Hz) | **Depolarization Block** (pA) |
| **Neonate (P6-7)** N=7 cells/3 mice | 1.4 ± 0.25 | 45 ± 4.0 | 10 ± 2.7 | 41 ± 2.7 | 83.9 ± 13 | 425.7 ± 91 |
| **Juvenile (P13-15)** N=19 cells/6 mice | 0.58 ± 0.02 | 49 ± 2.0 | 10 ± 1.1 | 56 ± 3.2 | 177 ± 11 | 1072 ± 87 |
| **Preadolescent (P21-26)** N=21 cells/6 mice | 0.40 ± 0.01 | 47 ± 1.0 | 13 ± 0.8 | 54 ± 4.9 | 268 ± 13 | 1355 ± 101 |
| **Adult (P70+)** N=12 cells/6 mice | 0.48 ± 0.02 | 39 ± 2.5 | 11 ± 1.0 | 58 ± 3.4 | 247 ± 16 | 1150 ± 161 |

**Supplemental Table S3.7.** Electrophysiological properties of wildtype CA1 PV cells during development. Data represented are mean ± SEM. RMP = resting membrane potential; AP = action potential; AHP = afterhyperpolarization of the AP; depolarization block = current required for depolarization-induced block of AP firing.

| | RMP (mV) | Capacitance (pF) | Input Resistance (MΩ) | Time Constant (ms) | Sag Ratio |
|---|---|---|---|---|---|
| **Juvenile *Grin2a*$^{+/+}$** N=19 cells/6 mice | -64 ± 1.1 | 110 ± 6.5 | 96 ± 6.1 | 10 ± 0.6 | 1.01 ± 0.01 |
| **Juvenile *Grin2a*$^{+/-}$** N=19 cells/6 mice | -63 ± 1.6 | 145 ± 8.1 | 133 ± 13 | 19 ± 2.5 | 1.02 ± 0.01 |
| **Juvenile *Grin2a*$^{-/-}$** N=14 cells/4 mice | -59 ± 3 | 111 ± 11 | 184 ± 23 | 22 ± 4.4 | 1.04 ± 0.02 |
| | | | | | |
| **Preadolescent *Grin2a*$^{+/+}$** N=21 cells/6 mice | -65 ± 1.3 | 121 ± 7 | 96 ± 8.3 | 11 ± 0.9 | 0.96 ± 0.05 |
| **Preadolescent *Grin2a*$^{+/-}$** N=19 cells/5 mice | -61 ± 1.1 | 126 ± 12 | 113 ± 9.4 | 13 ± 1.1 | 1.01 ± 0.004 |
| **Preadolescent *Grin2a*$^{-/-}$** N=19 cells/5 mice | -63 ± 3.8 | 117 ± 10 | 149 ± 17 | 17 ± 2 | 1.01 ± 0.05 |
| | | | | | |
| **Adult *Grin2a*$^{+/+}$** N=12 cells/6 mice | -62 ± 1.7 | 127 ± 13 | 101 ± 9.7 | 12 ± 0.8 | 0.99 ± 0.01 |
| **Adult *Grin2a*$^{+/-}$** N=12 cells/4 mice | -59 ± 2.5 | 107 ± 5.8 | 99 ± 7.9 | 11 ± 1.0 | 1.01 ± 0.003 |
| **Adult *Grin2a*$^{-/-}$** N=14 cells/5 mice | -62 ± 1.8 | 117 ± 9.3 | 107 ± 12 | 12 ± 0.8 | 1.00 ± 0.002 |

**Supplemental Table S3.8.** Passive electrophysiological properties during development in *Grin2a*$^{+/+}$, *Grin2a*$^{+/-}$, and *Grin2a*$^{-/-}$ CA1 PV cells. RMP = resting membrane potential.

| | **Rheobase** (pA) | **AP Half-Width** (mV) | **AP Amplitude** (mV) | **AHP Amplitude** (mV) | **Max AP Firing** (Hz) | **Depolarization Block** (pA) |
|---|---|---|---|---|---|---|
| **Juvenile *Grin2a*$^{+/+}$** N=19 cells/6 mice | 147 ± 12 | 0.58 ± 0.02 | 49 ± 2.0 | 10 ± 1.1 | 177 ± 11 | 1072 ± 87 |
| **Juvenile *Grin2a*$^{+/-}$** N=19 cells/6 mice | 100 ± 14 | 0.69 ± 0.04 | 47 ± 1.1 | 11 ± 1.1 | 153 ± 12 | 861 ± 78 |
| **Juvenile *Grin2a*$^{-/-}$** N=14 cells/4 mice | 82 ± 22 | 0.82 ± 0.07 | 46 ± 4.5 | 10 ± 1.3 | 135 ± 15 | 725 ± 116 |
| | | | | | | |
| **Preadolescent *Grin2a*$^{+/+}$** N=21 cells/6 mice | 128 ± 21 | 0.40 ± 0.01 | 47 ± 1.0 | 13 ± 0.8 | 268 ± 13 | 1355 ± 101 |
| **Preadolescent *Grin2a*$^{+/-}$** N=18 cells/5 mice | 135 ± 21 | 0.45 ± 0.05 | 46 ± 2 | 14 ± 0.6 | 227 ± 14 | 1083 ± 108 |
| **Preadolescent *Grin2a*$^{-/-}$** N=18 cells/5 mice | 93 ± 19 | 0.54 ± 0.04 | 45 ± 3 | 11 ± 1.6 | 186 ± 18 | 761 ± 111 |
| | | | | | | |
| **Adult *Grin2a*$^{+/+}$** N=12 cells/6 mice | 128 ± 21 | 0.48 ± 0.02 | 39 ± 2.5 | 11 ± 1.0 | 247 ± 16 | 1150 ± 161 |
| **Adult *Grin2a*$^{+/-}$** N=12 cells/4 mice | 103 ± 13 | 0.46 ± 0.03 | 46 ± 2.1 | 12 ± 0.6 | 225 ± 20 | 1145 ± 124 |
| **Adult *Grin2a*$^{-/-}$** N=14 cells/5 mice | 130 ± 19 | 0.45 ± 0.02 | 38 ± 3.3 | 10 ± 0.9 | 244 ± 18 | 1161 ± 170 |

**Supplemental Table S3.9.** Action-potential waveform and firing properties during development in *Grin2a*$^{+/+}$, *Grin2a*$^{+/-}$, and *Grin2a*$^{-/-}$ CA1 PV cells. AP = action potential; AHP = afterhyperpolarization of the AP; depolarization block = current required for depolarization-induced block of AP firing.

# CHAPTER 4: Subunit-Specific Modulation of NMDARs for Therapeutic Gain

*4.1 Introduction*

N-methyl-D-aspartate receptors (NMDARs) comprise a family of ligand-gated ionotropic glutamate receptors that mediate a calcium-permeable component to fast excitatory neurotransmission (Hansen et al., 2021). NMDARs are heterotetrameric assemblies of two obligate GluN1 subunits (encoded by the *GRIN1* gene) and two GluN2 subunits (encoded by the *GRIN2A*-*GRIN2D* genes) (Hansen et al., 2021). Given their ubiquitous expression, participation in glutamatergic neurotransmission, and facilitation of calcium entry into cells, NMDARs have been implicated in a host of physiological and developmental roles including learning, memory, spatial navigation, coordinated movement, decision making, neuronal migration, morphological development, and synaptic connectivity (Adesnik et al., 2008; Collingridge, 1987; Hansen et al., 2021; Komuro and Rakic, 1993; Nakazawa et al., 2004; Nash and Brotchie, 2000; Ultanir et al., 2007; Wang, 2002; Watanabe et al., 1998).

Modulation of NMDARs for therapeutic gain caught early traction in the ‘90s with the emergence of the NMDAR-mediated excitotoxicity hypothesis for neuropathological diseases like Alzheimer’s, Huntington’s, and Parkinson’s as well as reperfusion injury after acute injury such as ischemic stroke or traumatic brain injury (Hoyte et al., 2004; Kemp and McKernan, 2002; Lipton, 2004; Lipton and Nicotera, 1998; Lipton and Rosenberg, 1994). Although these non-selective NMDAR inhibitors were protective against excitotoxicity in preclinical models, they also inhibited the channel’s ability to operate normally, which led to many undesirable side effects such as drowsiness, hallucinations, motor dysfunction, sedation, and even coma (Hoyte et al., 2004; Lipton, 2004). The failure of these compounds in the clinic had multiple underlying causes to their mechanism of action, off target liabilities, and lack of subunit-specific action. Early NMDAR inhibitors were either competitive antagonists (*e.g.*, Selfotel and analogs) or pore blockers such as

memantine, dextromethorphan, and its metabolite dextrorphan. These inhibitors can produce complete block of all NMDARs, compromising the endogenous signaling ability of NMDARs within the circuit and blunting of basal NMDAR activity required for normal brain function. Moreover, these classes of inhibitors lacked NMDAR subunit specificity in that they were efficacious on GluN2A, GluN2B, GluN2C, and GluN2D-containing NMDARs. Thus, all NMDARs within the CNS were targeted, including those with regulatory functions such as those involved in cardiovascular function and motor control (Lipton, 2004).

Allosteric modulators of NMDARs can diminish some of the problems associated with traditional pore blockers and competitive antagonists by fine-tuning channel activity instead of acting as a binominal on/off switch. Ifenprodil and its derivatives were the first subunit-selective allosteric modulators (NAMs) developed for NMDARs, and showed promising pre-clinical efficacy in combating treatment-resistance depression (Preskorn et al., 2008) and excitotoxicity-mediated injury after ischemic stroke (Yuan et al., 2015). However, early stage GluN2B NAMs such as eliprodil failed clinical trials, partly due to poor study design, as well as undesirable off target side-effects. These early stage GluN2B-selective NAMs had multiple off target liabilities that newer generations of GluN2B NAMs lack (Perszyk et al., 2020). Thus, whereas the prospects for clinically relevant GluN2B modulation initially seemed limited, newer GluN2B inhibitors are well tolerated and have shown promise in preclinical and clinical studies. GluN2A-selective inhibitors based on the TCN-210 scaffold (Bettini et al., 2010) have also been developed and shown promise in preclinical studies. These NAMs bind at the dimer interface (Hackos et al., 2016; Hanson et al., 2020) act by reducing the glycine affinity (Hansen et al., 2012). While this class of NAMs has yet to be tested in humans, there is strong interest and therapeutic potential.

Modulation of GluN2D-containing NMDARs has recently become appreciated as an attractive target due to their limited expression profile in the adult CNS. Although highly expressed during embryogenesis and early neonatal development, GluN2D expression in the mature brain is limited to cortical GABAergic interneurons (Perszyk et al., 2016; von Engelhardt et al., 2015), as well as select neurons the spinal cord (Tolle et al., 1993), thalamus (O'Hara et al., 1995), olfactory bulb (Akazawa et al., 1994), basal ganglia (Swanger et al., 2015), and cerebellum (Akazawa et al., 1994). GluN2D hypofunction hypotheses have been suggested for both schizophrenia (Lisman et al., 2008; Schmitt et al., 2010) and Parkinson's disease (Tozzi et al., 2016), with pre-clinical models suggesting that GluN2D PAMs can modulate(Suryavanshi et al., 2014) prepulse inhibition and dopamine release from basal ganglia neurons (Nouhi et al., 2018), respectively. Moreover, since GluN2D-containing NMDARs are expressed on GABAergic interneurons, GluN2D PAMs could potentially alter overall network excitability proving advantageous for a host of neurological disorders.

CIQ or (3-chlorophenyl)(6,7-dimethoxy-1-((4-methoxyphenoxy)-methyl)-3,4-dihydroisoquinolin-2(1*H*)-yl)methanone), was the first-in-class GluN2C/GluN2D-selective positive allosteric modulator (PAM). This compound shows strong selectivity over GluN2A- and GluN2B-containing NMDARs (no significant potentiation at 30 μM) and has enabled significant progress towards understanding the effects of GluN2C/GluN2D modulation *in vivo* (Nouhi et al., 2018; Suryavanshi et al., 2014). CIQ's potency and solubility (see results), however, are poor and need to be improved. Recently, a new derivative of CIQ – (+)-EU1180-453 (hereafter referred to as **EU1180-465**) – has been synthesized with more than 15-fold improvements in doubling concentration (*i.e.*, the concentration needed to double a current response) and more than 7-fold improvements in aqueous solubility (Epplin et al., 2020). Here, the effects of EU1180-465 on

circuit excitability and ability to potentiate multiple classes of GABAergic interneuron are examined, with the aim of providing useful data to inform future pre-clinical studies designed to investigate GluN2D-selective PAMs as viable therapeutic agents.

## *4.2 Methods*

### 4.2.1 Animals and Breeding

All mouse procedures were conducted at Emory University, which is fully accredited by AAALAC and IACUC and performed in accordance with state and federal Animal Welfare Acts and Public Health Service policies. C57BL/6J mice from the Jackson Laboratory (catalog number: 000664) were used as wild type mice. NPY-hrGFP mice from the Jackson Laboratory (catalog number: 006417) were used to identify NPY-positive neurogliaform cells. VIP-IRES-Cre mice from the Jackson Laboratory (catalog number: 010908) were crossed with TdTomato-floxed (Ai9) mice (catalog number: 007909) to identify VIP-positive cells. All transgenic mice were backcrossed to a C57BL/6J background.

Preadolescent (P20-26) mice of both sexes were used in all experiments. Mice were housed in ventilated cages at controlled temperature (22–23ºC), humidity ~60%, and 12h light:dark cycles. Mice had *ad libitum* to regular chow and water.

### 4.2.2 Acute Hippocampal Slice Preparation and Electrophysiological Recordings

Mice were overdosed with inhaled isoflurane, brains were rapidly removed and immediately placed in an ice-cold, sucrose-based artificial cerebrospinal fluid (aCSF) containing the following (in mM): 88 sucrose, 80 NaCl, 2.5 KCl, 1.25 $HNa_2PO_4$, 26 $HNaCO_3$, 10 glucose, 2 thiourea, 3 sodium pyruvate, 5 sodium ascorbate, 12 N-acetylcysteine, 10 $MgSO_4$, and 0.5 $CaCl_2$ bubbled in 95% $O_2$/5% $CO_2$. 300-µm thick horizontal hippocampal sections were obtained using

a vibratome (Lecia, VT1200S). After sectioning, slices were incubated in the sucrose-based aCSF as described above but with 4 mM $MgSO_4$ at 32°C for 30 minutes then returned to room temperature for at least an hour before use. All cells were visualized using an upright Olympus BX50W microscope with IR-DIC optics coupled to a Dage IR-2000 camera. Whole-cell patch clamp recordings were obtained using a Multiclamp 700B (Molecular Devices, digitized at 20 kHz using a Digidata 1440a (Molecular Devices) controlled by pClamp 10.6 software (Molecular Devices). All signals were low-pass filtered at 2 kHz using a Bessel 8-pole filter (Warner, LPF-8).

For all patch-clamp experiments, the following intracellular solution was used (in mM): 100 Cs-gluconate, 5 CsCl, 0.6 EGTA, 5 BAPTA, 5 $MgCl_2$, 8 NaCl, 2 Na-ATP, 0.3 Na-GTP, 40 HEPES, 5 Na-phosphocreatine, and 3 QX-314 supplemented with 0.5% biocytin. Evoked NMDAR-mediated EPSC recordings were made in the following aCSF extracellular solution (in mM): 126 NaCl, 2.5 KCl, 1.25 $HNa_2PO_4$, 26 $HNaCO_3$, 20 glucose, 0.2 $MgSO_4$, and 1.5 $CaCl_2$ bubbled with 95% $O_2$/5% $CO_2$ and held at 30-32°C using an inline heater (Warner, SH-27B). sIPSC data were obtained with the same extracellular solution, except with 1.5 mM $Mg^{2+}$.

For sIPSCs, cells were held at +10 mV sIPSCs, which is the reversal potential for iGluRs. Baseline recordings were made for a total of five minutes. Then DMSO or 10 μM EU1180-465 was washed onto the slice for ten minutes and response recordings were made for a total of five minutes. All experiments ended by washing on 10 μM gabazine to ensure all responses were mediated by $GABA_A$ receptors. Data were analyzed offline using MiniAnalysis (Synaptosoft) with an 8-pA amplitude threshold for event detection.

For evoked NMDAR-mediated EPSCs, a monopolar iridium-platinum stimulating electrode (FHC, Inc.) was placed in the upper 1/3$^{rd}$ of the Schaffer collaterals to elicit a single 50 μs stimulation at a frequency of 0.03 Hz and the NMDAR-mediated EPSC was pharmacologically

isolated with 10 μM NBQX and 10 μM gabazine. Cells were held at -30 mV and stimulation intensity was chosen to be near 50% of the maximum peak amplitude of the NMDAR-mediated EPSC. Baseline recordings were made for a total of five minutes. Then DMSO or 10 μM EU1180-465 was washed onto the slice for ten minutes and response recordings were made for a total of five minutes. A total of 8-12 epochs were obtained and averaged together for each baseline and response period. At the conclusion of recording, 400 μM DL-APV was applied to ensure responses were mediated via NMDARs. A weighted time constant was calculated using the following formula by fitting a dual-exponential function to each composite EPSC trace in ChanneLab (Synaptosoft):

(3) $\tau_W = (\tau_{FAST}\,\text{amplitude}_{FAST} + \tau_{SLOW}\,\text{amplitude}_{SLOW})/(\text{amplitude}_{FAST} + \text{amplitude}_{SLOW})$ .

For all electrophysiological recordings, a wash-in period of 10 minutes was used with EU1180-465 application as previous experiments showed this period of time was sufficient to achieve maximal and steady-state levels of drug action. Aliquots of 10 mM EU1180-465 in DMSO were made from fresh powder daily and never frozen and re-used. After each experiment, lines were washed with 70% ethanol and then diH20 to remove any remaining compound. Series resistance was monitored throughout all experiments and was typically 8−20 MΩ. For voltage clamp recordings, a 50 ms, 5-mV square wave was included in the stimulation paradigm or inserted once every 30 seconds in gap-free mode for sIPSCs. Series resistance was monitored throughout the entire recording. All series resistances were measured offline by analyzing the peak of the capacitive charging spike and applying Ohm's law. If the series resistance changed >25% during the experiment, or ever exceeded 30 MΩ, then the cell was excluded.

### 4.2.3 Cell Selection and Post-Hoc Slice Processing

All fluorescently labeled cells were visualized with appropriate ThorLabs LEDs and ThorLabs or Chroma filter cube sets. Only NPY-expressing cells near the *stratum radiautm*/*stratum lacunosum-moleculare* border were chosen as this is where all neurogliaform cells reside. For VIP-expressing TdTomato cells, only cells within the *stratum radiatum* were selected. All cells from fluorescently labeled transgenic mice were backfilled with 0.5% biocytin, and the electrode tip was removed slowly to close the soma's cell membrane. Slices were then immediately placed into 4% paraformaldehyde in 1x PBS overnight. After fixation, slices were washed 3x for 10 minutes each in 1x PBS, permeabilized with 1.2% Triton-X in 1x PBS for 10 minutes, then incubated overnight in 1x PBS with 10% normal goat serum, 1% bovine serum albumin, 0.5% Triton-X, and streptavidin Alexa Flour 555 (1:500, Invitrogen). Slices were washed 3x for 10 minutes each in 1x PBS. Slices from NPY-hrGFP mice were mounted and coverslipped with ProLong gold mounting media. Neurogliaform morphology was confirmed on a spinning-disc microscope (Olympus).

Slices from VIP-Cre x Floxed-TdTomato mice were then incubated in in 1x PBS with 10% normal goat serum, 1% bovine serum albumin, 0.5% Triton-X, and rabbit anti-cholecystokinin (1:500; Frontier Institute) for 1-2 days at 4C. Slices were washed 3x for 10 minutes each in 1x PBS then incubated in 1x PBS with 10% normal goat serum, 1% bovine serum albumin, 0.5% Triton-X with anti-rabbit Alexa Flour 488 (1:500, Jackson Immuno) for 3 hours. Slices were washed 3x for 10 minutes each in 1x PBS. Slices were then mounted and coverslipped with ProLong gold mounting media.

A line-scanning confocal microscope (Nikon A1R) was used to image biocytin and CCK overlap. This microscope allowed custom detector thresholds to ensure that no false positives were identified given how close Alexa Flour 488 and Alexa Flour 555 are on the spectral wavelength

scale. This technique was verified via inclusion of several biocytin-backfilled parvalbumin-positive cells which do not express CCK (**see Figure 4.10**). In addition to classification of VIP-expressing interneuron-selective interneurons or VIP/CCK co-expressing basket cells based on CCK post-hoc staining, successful recovery of axonal morphology was also required for inclusion. VIP-expressing interneuron-selective interneurons had to have axons in either *stratum oriens* (type II) or be multiple polar with some axonal projections in *stratum lacunosum-moleculare* (type III). VIP/CCK co-expressing basket cells had to have axonal projections within *stratum pyramidale*

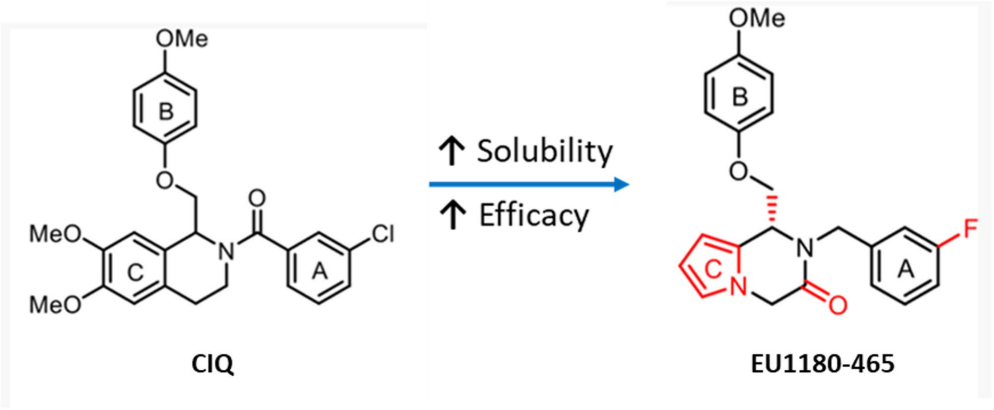
4.2.4 Statistical Analysis and Figure Preparation

Two-tailed, unpaired Student's t-test and the Kolomorgov-Smirnov tests were used were appropriate. For experiments where multiple statistical analyses were performed on the same dataset, our significance threshold was lowered to correct for family-wise error rate (FWER) using the Bonferroni post-hoc correction method. All studies were designed so that an effect size of at least 1 was detected at 80% or greater power. All statistical analyses were performed in Prism's GraphPad software all figures were generated in Adobe's Illustrator software.

*4.3 Results*

CIQ was the first-in-class GluN2C/GluN2D selective PAM of NMDARs. Its chemical structure involves a tetrahydroisoquinoline scaffold that was recently modified to generate EU1180-465 (**Figure 4.1A**) (Epplin et al., 2020). These modifications resulted in a significant increase in potency ($EC_{50}$ 5.0 μM for CIQ vs $EC_{50}$ 3.2 μM for EU1180-465 at GluN2D) and maximal achievable potentiation at saturating concentrations (350% over baseline for both GluN2C and GluN2C). Together these two enhancements produce robust improvement in

A)




B)

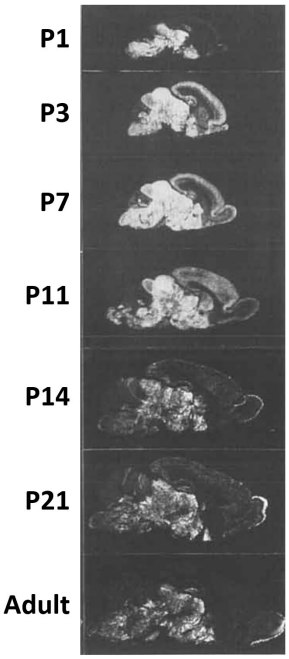
| | $EC_{50}$ (μM) (% Max) | | Doubling Concentration (μM) | | Solubility (μM) | [Brain]: [Plasma] | Half-Life (h) |
|---|---|---|---|---|---|---|---|
| | GluN2C | GluN2D | GluN2C | GluN2D | -- | -- | -- |
| **(+)-CIQ** | 5.0 (230%) | 5.0 (220%) | 12.6 | 31.6 | 8 | 6.7 ± 2.2 | 1.4 ± 0.1 |
| **EU1180-465** | 3.2 (350%) | 3.2 (350%) | 2.0 | 2.0 | 74 | 1.4 ± 0.5 | 1.1 ± 0.04 |

**Figure 4.1.** Distinctions between the exemplar GluN2C/GluN2D positive allosteric modulator, CIQ, and its predecessor, EU1180-465. A) Compound structure of CIQ and EU1180-465, with differences made to the tetrahydroisoquinoline scaffold highlighted in red. B) Various pharmacological and pharmacokinetic properties of EU1180-465 compared to CIQ. $EC_{50}$ and doubling concentration data were obtained in *Xenopus* oocytes after rat mRNA injection of each construct in presence of saturating concentrations of glutamate (100 μM) and glycine (30 μM). Doubling concentration was defined as the concentration required to increase basal receptor response to agonist only by 2-fold. For pharmacokinetic experiments, compounds were dosed i.p. in either 1:4:5 dimethylacetamide:PEG400:(5% dextrose in water) (**CIQ at 20 μM**, $n$ = 3/group) or 1:1 PEG400:water (**EU-1180–453 at 10** μM, $n$ = 3/group). Brain and plasma concentrations in ng/g and ng/mL, respectively, at $C_{max}$. See methods for more details. All data adapted from (Epplin et al., 2020).

doubling concentration (31.6 μM for CIQ vs 2.0 μM for EU1180-465 at GluN2D), which is the concentration required to increase the basal response without the modulator to saturating concentrations of agonist (100 μM glutamate/30 μM glycine) by 2-fold (**Figure 4.1B**). The aqueous solubility has also been improved by over 7-fold, from 8 μM for CIQ to 74 μM for EU1180-465, while still possessing favorable brain penetration and half-life suitable for acute *in vivo* preclinical experiments (**Figure 4.1B**). Although further refinement of the core scaffold is needed before this compound series is introduced into clinical trials, these advances represent an important step forward in terms of GluN2C/GluN2D PAM drug discovery.

Since GluN2D-containing NMDARs are found in cortical GABAergic interneurons (**Figure 4.2B-C**), we assessed the ability of EU1180-465 to potentiate spontaneous inhibitory postsynaptic currents (IPSCs) onto principal cells. The mouse CA1 region of the hippocampus was chosen as a model circuit given its well-defined cellular architecture and robust durability in acute slice preparations. CA1 pyramidal cells from preadolescent (postnatal day (P) 20-26) were recorded under whole cell voltage clamp at +10 mV, which is the reversal potential for ionotropic glutamate receptors. This allows spontaneous IPSCs to be recorded without the need to block spontaneous AMPAR-mediated synaptic events, which are critical for interneuron excitation. Unfortunately, depolarizing pyramidal cells will generate depolarization-induced suppression of inhibition (DSI) via endocannabinoid release (Pitler and Alger, 1992; Wilson and Nicoll, 2001) which alters the overall content of IPSCs recorded from various interneuron populations. Nevertheless, we were able to show a proof-of-concept enhancement of inhibitory tone as the application of 10 μM EU1180-465 caused a significant increase in the sIPSC frequency and significantly shifted the interevent interval (**Figure 4.3A-F** and **Table 4.1**). EU1180-465 did not increase the overall amplitude sIPSCs (**Figure 4.4A** and **Table 4.1**), but the cumulative probability

A)




B)

**Percent Cells Positive for NMDAR mRNA**

| | *Grin2a* | *Grin2b* | *Grin2c* | *Grin2d* |
|---|---|---|---|---|
| **PV** | 78% | 78% | 11% | 67% |
| **CCK** | 57% | 86% | 0% | 100% |
| **SOM** | 81% | 86 | 0% | 81% |
| **NPY** | 75% | 86 | 4% | 79% |

C)

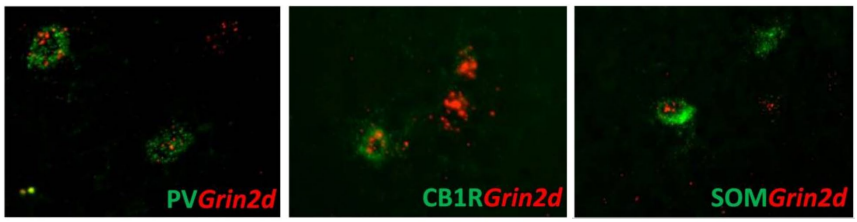



**Figure 4.2.** *Grin2d* mRNA is found in GABAergic interneurons in mouse hippocampus. A) Spatiotemporal expression profile of *Grin2d* from in-situ hybridization experiments in rat. B) Results from single-cell rtPCR experiments in patched CA1 GABAergic interneurons in juvenile (P14-20) mice. Data are reported as percentage of cells that express said NMDAR subunit mRNA, stratified by different neurochemical markers for GABAergic interneurons. C) RNAScope data showing overlay of *Grin2d* positive puncta being co-expressed by cells that express various neurochemical markers for GABAergic interneurons. PV = parvalbumin; CCK = cholecystokinin; SOM = somatostatin; NPY = neuropeptide-Y; CB1R = endocannabinoid receptor 1. Data in panel A are adapted from (Akazawa et al., 1994); Data in panels B and C are adapted from (Perszyk et al., 2016).

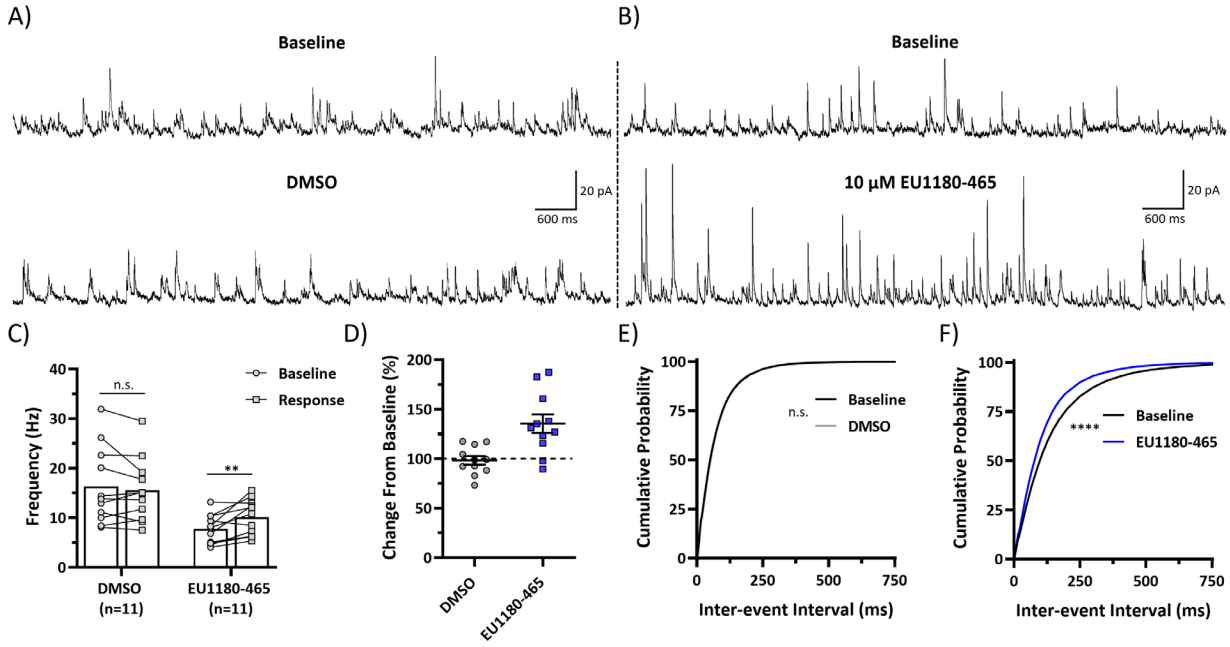



**Figure 4.3.** Application of EU1180-465 increases inhibitory tone in CA1. All data shown are spontaneous inhibitory postsynaptic currents (sIPSCs) recorded at +10 mV onto CA1 pyramidal cells from preadolescent mice (P20-26) mice. Representative traces of sIPSCs A) before and after vehicle (DMSO) application and B) before and after 10 µM EU1180-465. C) sIPSC frequency is significantly increased after 10 µM EU1180-465 application (7.7 ± 0.9 Hz for baseline vs 10 ± 1.0 Hz for 10 µM EU1180-465; paired *t*-test, p=0.008), but was unchanged in vehicle. D) sIPSC frequency normalized to baseline show a mean increase of 40% in sIPSC frequency after 10 µM EU1180-465. E) Cumulative probability plots of interevent interval are E) unchanged with vehicle but are F) significantly shifted to the left after 10 µM EU1180-465 (Kolmogorov-Smirnov test, p<0.0001). Data are mean±SEM; n=11 cells/4 mice per group. ** = p<0.01; **** = p<0.0001; n.s. = not significant.

| | Baseline | Vehicle (DMSO) (n=11) | Fold Change | | Baseline | 10 µM EU1180-465 (n=11) | Fold Change |
|---|---|---|---|---|---|---|---|
| **Frequency** (Hz) | 16 ± 2.4 | 16 ± 2.0 | **0.98** | | 7.7 ± 1 | 10 ± 1.1 | **1.35** |
| **Amplitude** (pA) | 20 ± 1.0 | 19 ± 1.1 | **0.98** | | 23 ± 1.8 | 24 ± 2.5 | **1.02** |
| **Weighted Decay** (ms) | 14 ± 1.0 | 15 ± 0.9 | **1.08** | | 11 ± 0.5 | 18 ± 1.0 | **1.02** |
| **Access Resistance** (MΩ) | 15 ± 0.7 | 14 ± 0.7 | **0.97** | | 15 ± 1.2 | 16 ± 0.9 | **1.01** |

**Table 4.1.** Summary of sIPSCs onto hippocampal CA1 pyramidal cells with EU1180-465. All data are mean ± SEM determined using MiniAnalysis; n is the number of cells. Each cell was recorded from a different slice. Slices were prepared from 4 mice for each condition. Slices from each animal were prepared on a separate day along with new solutions for that day.

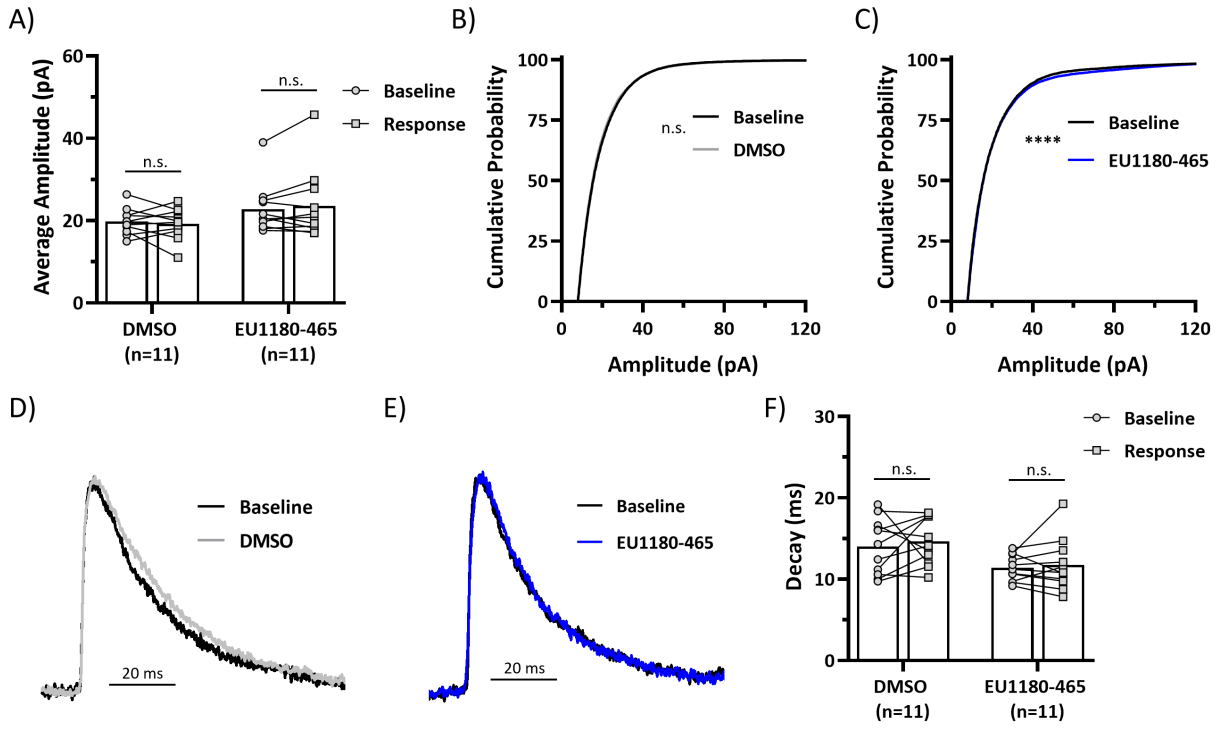



**Figure 4.4.** Application of EU1180-465 increases amplitude, but not decay time, of sIPSCs onto CA1 pyramidal cells. All data shown are spontaneous inhibitory postsynaptic currents (sIPSCs) recorded at +10 mV onto CA1 pyramidal cells from preadolescent mice (P20-26) mice. A) sIPSC amplitude is not significantly increased after vehicle or 10 µM EU1180-465 application. Cumulative probability plots of sIPSC amplitude show B) no significant shifts for vehicle, but C) a statistically significant to the right after 10 µM EU1180-465 application (Kolmogorov-Smirnov test, $p<0.0001$). Representative normalized sIPSC traces after D) vehicle or E) 10 µM EU1180-465 application. F) sIPSC decay times are unchanged in response to vehicle or 10 µM EU1180-465. Data are mean±SEM; n=11 cells/4 mice per group. **** = $p<0.0001$; n.s. = not significant.

plot of sIPSC amplitude was significantly shifted to the right **(Figure 4.4C).** Application of vehicle for EU1180-465 (DMSO) did not produce any changes in sIPSC frequency (**Figure 4.3C**), sIPSC amplitude (**Figure 4.4A**) nor the cumulative probability plots of sIPSC interevent interval (**Figure 4.3E**) or amplitude (**Figure 4.4B**). Application of 10 μM EU1180-465 or vehicle did not impact the decay time of sIPSCs (**Figure 4.4D-F**). In all, EU1180-465 increased spontaneous inhibitory tone onto excitatory principle cells by increasing frequency of inhibitory events and by increasing amplitude distribution of each event. This is likely accomplished by EU1180-465 increasing the excitatory tone onto GABAergic interneurons.

To provide direct evidence for an increased excitatory tone onto GABAergic interneurons, NMDAR-mediated EPSCs were recorded onto interneurons in *stratum radiatum* before and after EU1180-465. CA1 *stratum radiatum* has been previously shown to exclusively contain interneurons (and glia), and many of which receive Schaffer collateral excitation. Random CA1 *radiatum* interneurons were patch-clamped in 0.2 $Mg^{2+}$ and held at -30 mV as these conditions increased success of obtaining quality recordings during extended drug applications. Application of 10 μM EU1180-465 significantly increased the peak amplitude (**Figure 4.5C** and **Table 4.2**) and charge transfer (**Figure 4.5E**) of NMDAR-mediated EPSCs. The weighted decay time of the EPSC appears to be increased (**Figure 4.5D**), however, it was not statistically significant. As expected, EU1180-465 did not alter the rise time of the EPSC (**Figure 4.5F**).

Upon closer examination of the NMDAR-mediated EPSC data, there appeared to be two different groups of cells: those with clear potentiation from EU1180-465 and those which did not respond to the drug. Data were parsed using a 25% increase of charge transfer over baseline as our threshold for considering a cell 'potentiated'. Charge transfer was chosen as this measure combines both peak amplitude and decay time into one value. After stratification, seven cells met the 25%

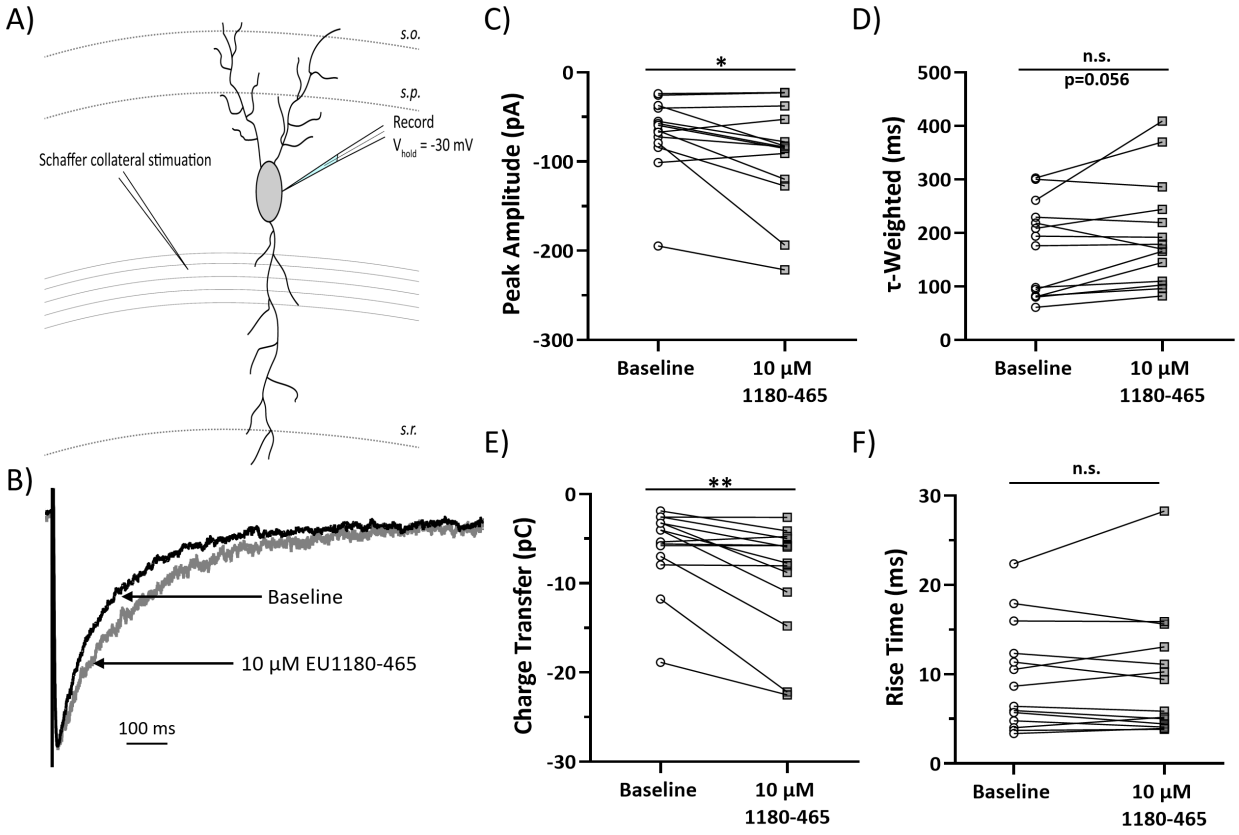



**Figure 4.5.** EU1180-465 potentiates CA1 *stratum radiatum* GABAergic interneurons. A) Diagram of CA1 *stratum radiatum* where GABAergic interneurons reside. B) Representative, normalized NMDAR-mediated EPSC onto CA1 *stratum radiatum* interneurons at baseline (black) and after application of 10 µM EU1180-465 (gray). C) Peak amplitude is significantly increased after 10 µM EU1180-465 (-69 ± 11 pA baseline vs -93 ± 15 pA for 10 µM EU1180-465; paired *t*-test, p = 0.02). D) No significant potentiation of synaptic decay time, however, E) overall charge transfer is significantly increased (-6.0 ± 4.5 pC baseline vs -9.2 ± 6.4 pC for 10 µM EU1180-465; paired *t*-test, p = 0.003). F) Application of 10 µM EU1180-465 does not alteration activation time course. All data are from wildtype preadolescent mice (P20-26). N=14 cells/4 mice for all recordings. * = p<0.05; ** = p<0.01; n.s. = not significant. s.r. = *stratum radiatum;* s.p. = *stratum pyramidale;* s.o. = *stratum oriens.*

| | **Baseline** | **10 µM EU1180-465** (n=14) | **Fold Change** |
|---|---|---|---|
| **Rise Time** (ms) | 9.5 ± 1.6 | 9.7 ± 1.8 | 1.02 |
| **Peak Amplitude** (pA) | -69 ± 11 | -93 ± 15 | 1.35 |
| **Tau Weighted** (ms) | 171 ± 23 | 198 ± 27 | 1.16 |
| **Charge Transfer** (pC) | -6.0 ± 1.2 | -9.2 ± 1.7 | 1.53 |
| **Access Resistance** (MΩ) | 15.9 ± 2.3 | 16.3 ± 1.8 | 1.03 |

**Table 4.2.** Summary data from all evoked NMDAR-mediated EPSCs onto *stratum radiatum* interneurons. All data are mean ± SEM.

threshold and were classified as 'responders' (**Figure 4.6A**), while the remaining seven cells which fell below the 25% threshold were classified as 'non-responders' (**Figure 4.6B**). Considering that the CA1 *stratum radiatum* contains many different subtypes of GABAergic interneurons (**Figure 4.6C**), we hypothesize that this binominal distribution of responses to EU1180-465 may be due to certain interneuron subtypes not expressing GluN2D-containing NMDARs. These cells may also exist as a continuum, with some cells showing an intermediate expression of GluN2D, or even use GluN2D under certain activity-dependent conditions.

To understand the functional expression of GluN2D in various interneuron subtypes further, we utilized several neurochemical driver Cre lines to identify select interneuron groups in acute hippocampal slices. CA1 *stratum radiatum* contains four major groups of GABAergic interneurons: neurogliaform cells, interneuron-selective interneurons (ISIs), ivy cells, cholecystokinin (CCK)-expressing cells (Pelkey et al., 2017). First, neurogliaform cells were targeted using the NPY-hrGFP mouse line from Jackson labs. Nearly all neurogliaform cells express neuropeptide-y (NPY), however, NPY is also expressed by some ivy cells and some O-LM somatostatin (SST) cells (**Figure 4.7A**) (Pelkey et al., 2017). Ivy cells traditionally reside at the *stratum pyramidale*/*stratum radiatum* border and SST cells are exclusively expressed in *stratum oriens* so both can be easily avoided. Thus, only NPY-positive cells at the *stratum radiatum*/*stratum lacunosum-moleculare* border were chosen and typified neurogliaform cellular morphology was confirmed via biocytin backfilling. Application of 10 μM EU1180-465 significantly increased the charge transfer of NMDAR-mediated EPSCs onto NPY-expressing neurogliaform cells (**Figure 4.7D** and **Table 4.3**), indicating that this class of interneuron likely expresses GluN2D-containing NMDARs.

Interneuron-selective interneurons (ISIs) were targeted using the vasoactive intestinal

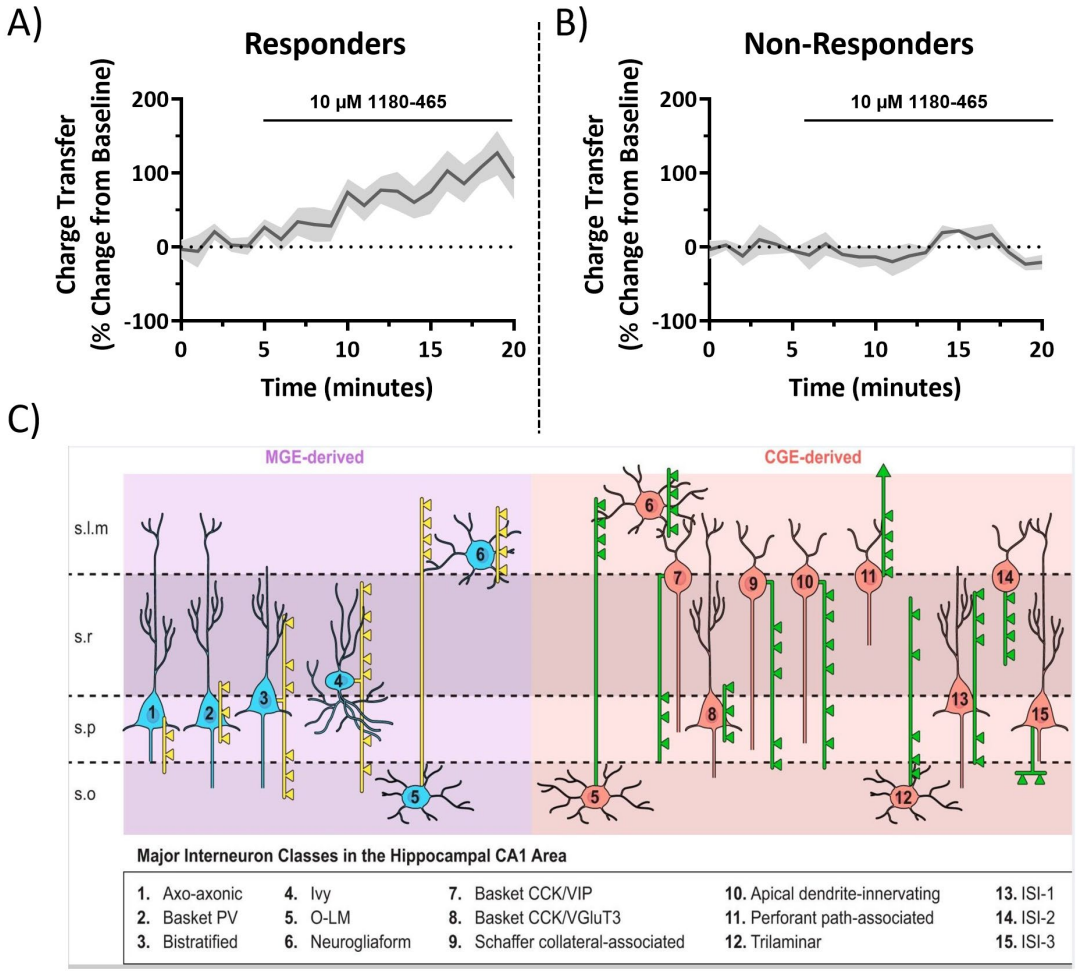



**Figure 4.6.** EU1180-465 response on *stratum radiatum* interneurons can be stratified into responders and non-responders. A) Time versus charge transfer plots of cells classified as responders (> 25% potentiation of charge transfer compared to baseline; n=7 cells) and B) time versus charge transfer plots of cells classified as non-responders (< 25% potentiation of charge transfer compared to baseline; n=7 cells). Data show a clear dichotomy of responses, which was hypothesized to be due to interneuron subtype. C) Diagram of all GABAergic interneuron subtypes found in mouse CA1. Panel C adapted from (Pelkey et al., 2017). MGE = medial ganglionic eminence; CGE = caudal ganglionic eminence; PV = parvalbumin; O-LM = *oriens-lacunosum moleculare*; CCK = cholecystokinin; VIP = vasoactive intestinal peptide; ISI = interneuron-specific interneuron; s.l.m. = *stratum lacunosum-moleculare;* s.r. = *stratum radiatum;* s.p. = *stratum pyramidale;* s.o. = *stratum oriens.*

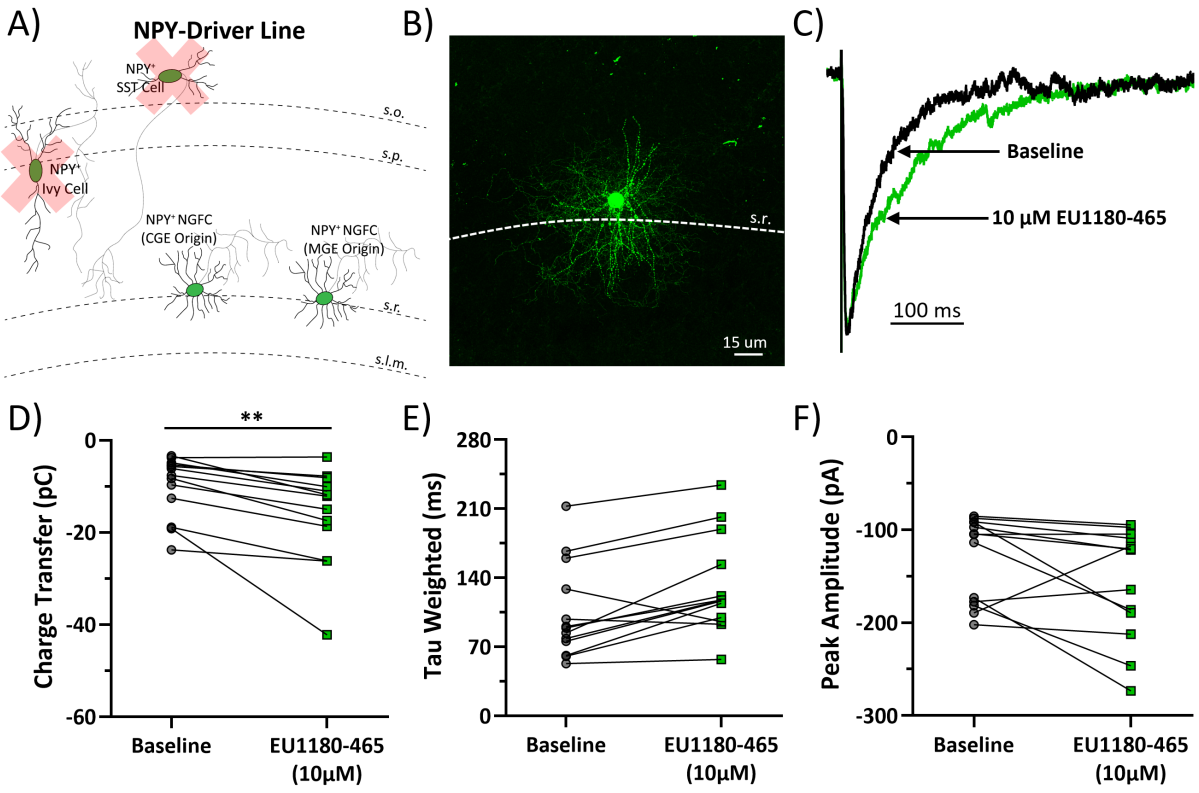



**Figure 4.7**. NPY-expressing neurogliaform cells are potentiated by EU1180-465. A) Diagram of all interneuron subtypes labeled by a neuropeptide-y (NPY) driver mouse line. In order to avoid NPY-positive somatostatin and NPY-positive Ivy cells, only cells at the *stratum radiatum*/*stratum lacunosum-moleculare* border were selected. B) Biocytin backfilled NPY-positive neurogliaform cell. C) Representative, normalized NMDAR-mediated EPSC onto NPY-positive neurogliaform cells at baseline (black) and after application of 10 µM EU1180-465 (green). D) Overall charge transfer was significantly potentiated after application of 10 µM EU1180-465 (-10 ± 1.8 pC baseline vs -16 ± 2.9 pC for 10 µM EU1180-465; paired *t*-test, p = 0.002). E) Tau weighted and F) peak amplitude also appear to be potentiated but were not tested for statistical significance. All data are from N=13 cells/4 mice. NPY = neuropeptide-y; SST = somatostatin; NGFC = neurogliaform cell; MGE = medial ganglionic eminence; CGE = caudal ganglionic eminence; s.l.m. = *stratum lacunosum-moleculare;* s.r. = *stratum radiatum;* s.p. = *stratum pyramidale;* s.o. = *stratum oriens.*

| | **Baseline** | **10 µM EU1180-465** (n=12) | **Fold Change** |
|---|---|---|---|
| **Rise Time** (ms) | 7.3 ± 2.6 | 7.9 ± 1.3 | 1.08 |
| **Peak Amplitude** (pA) | -131 ± 13 | -157 ± 17 | 1.20 |
| **Tau Weighted** (ms) | 105 ± 13 | 132 ± 14 | 1.26 |
| **Charge Transfer** (pC) | -10 ± 1.8 | -16 ± 2.9 | 1.60 |
| **Access Resistance** (MΩ) | 14.6 ± 1.9 | 15.4 ± 1.1 | 1.05 |

**Table 4.3.** Summary data from all evoked NMDAR-mediated EPSCs onto NPY-expressing neurogliaform cells. All data are mean ± SEM.

peptide (VIP)-Cre mouse line from Jackson labs. These mice were crossed with an Ai9 TdTomato-floxed mouse to visualize VIP-positive cells in acute hippocampal slices. The VIP-cre line will label type II and type III ISIs as desired, however, it will also label VIP/CCK co-expressing basket cells (**Figure 4.8A**). Since both VIP-expressing ISIs and VIP/CCK-expressing basket cells both reside in *stratum radiatum*, all cells from the VIP-Cre line were backfilled with biocytin and all slices were post-hoc stained for streptavidin and CCK. Validation of staining strategy and CCK antibody selectivity are shown in **Figure 4.10**. Only cells from the VIP-Cre line that stained negative for CCK *and* had clearly discernable axons were included in the ISI dataset. This selection strategy led to many (>7) cells being excluded from analysis. Application of 10 μM EU1180-465 did not change the charge transfer of NMDAR-mediated EPSCs onto VIP-expressing ISIs (**Figure 4.8D** and **Table 4.4**), indicating that this class of interneuron likely does not express functional GluN2D-containing synaptic NMDARs. Type I ISI's do not express VIP and thus will have to be targeted using another driver line, likely via Calretinin-Cre mice.

Cells from the VIP-Cre line that stained positive for CCK *and* had clearly discernable axons were included in the VIP/CCK dataset. Again, this selection strategy led to many (>7) cells being excluded from analysis. Application of 10 μM EU1180-465 significantly increased the charge transfer of NMDAR-mediated EPSCs onto VIP/CCK-expressing basket cells (**Figure 4.9D** and **Table 4.5**), indicating that VIP/CCK-positive basket cells likely express GluN2D-containing synaptic NMDARs. Other subtypes of CCK-expressing cells including CCK-positive/VIP-negative basket cells, CCK-expressing Schaffer collateral associated cells, CCK-expressing dendrite targeting cells, and CCK-expressing perforant pathway associated cells still need to be investigated. All these subtypes can be effectively targeted using the CCK-Cre mouse line combined with post-hoc morphological analysis. Moreover, Ivy cells can be effectively targeted

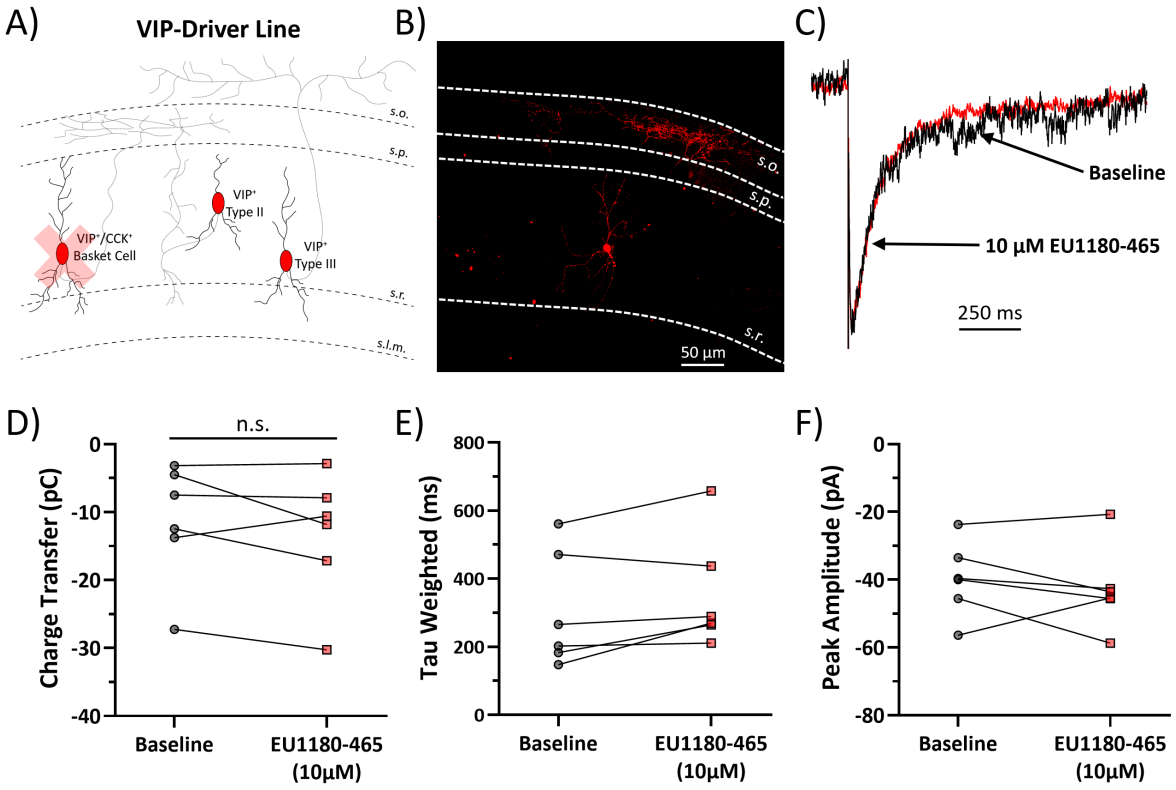



**Figure 4.8**. VIP-expressing interneuron-selective interneurons are not potentiated by EU1180-465. A) Diagram of all interneuron subtypes labeled by a vasoactive intestinal peptide (VIP) driver mouse line. In order to avoid VIP/CCK-positive basket cells, all cells were stained for CCK post-hoc and evaluated for overlap with biocytin (see methods for more details). B) Biocytin backfilled VIP-positive interneuron-selective interneuron (ISI). C) Representative, normalized NMDAR-mediated EPSC onto VIP-positive ISIs at baseline (black) and after application of 10 µM EU1180-465 (red). D) Overall charge transfer was not significantly potentiated after application of 10 µM EU1180-465 (-11 ± 3.6 pC baseline vs -13 ± 3.9 pC for 10 µM EU1180-465; paired *t*-test, p = 0.25). E) Tau weighted and F) peak amplitude also appear to be potentiated but were not tested for statistical significance. All data are from N=6 cells/5 mice. VIP = vasoactive intestinal peptide; CCK = cholecystokinin; s.r. = *stratum radiatum;* s.p. = *stratum pyramidale;* s.o. = *stratum oriens.*

| | **Baseline** | **10 µM EU1180-465** (n=6) | **Fold Change** |
|---|---|---|---|
| **Rise Time** (ms) | 8.3 ± 1.2 | 8.7 ± 1.8 | 1.05 |
| **Peak Amplitude** (pA) | -40 ± 4.5 | -43 ± 5.0 | 1.08 |
| **Tau Weighted** (ms) | 305 ± 69 | 355 ± 68 | 1.16 |
| **Charge Transfer** (nC) | -11 ± 3.6 | -13 ± 3.9 | 1.18 |
| **Access Resistance** (MΩ) | 21.6 ± 1.6 | 22.4 ± 1.1 | 1.04 |

**Table 4.4.** Summary data from all evoked NMDAR-mediated EPSCs onto VIP-expressing interneuron-selective interneurons. All data are mean ± SEM.

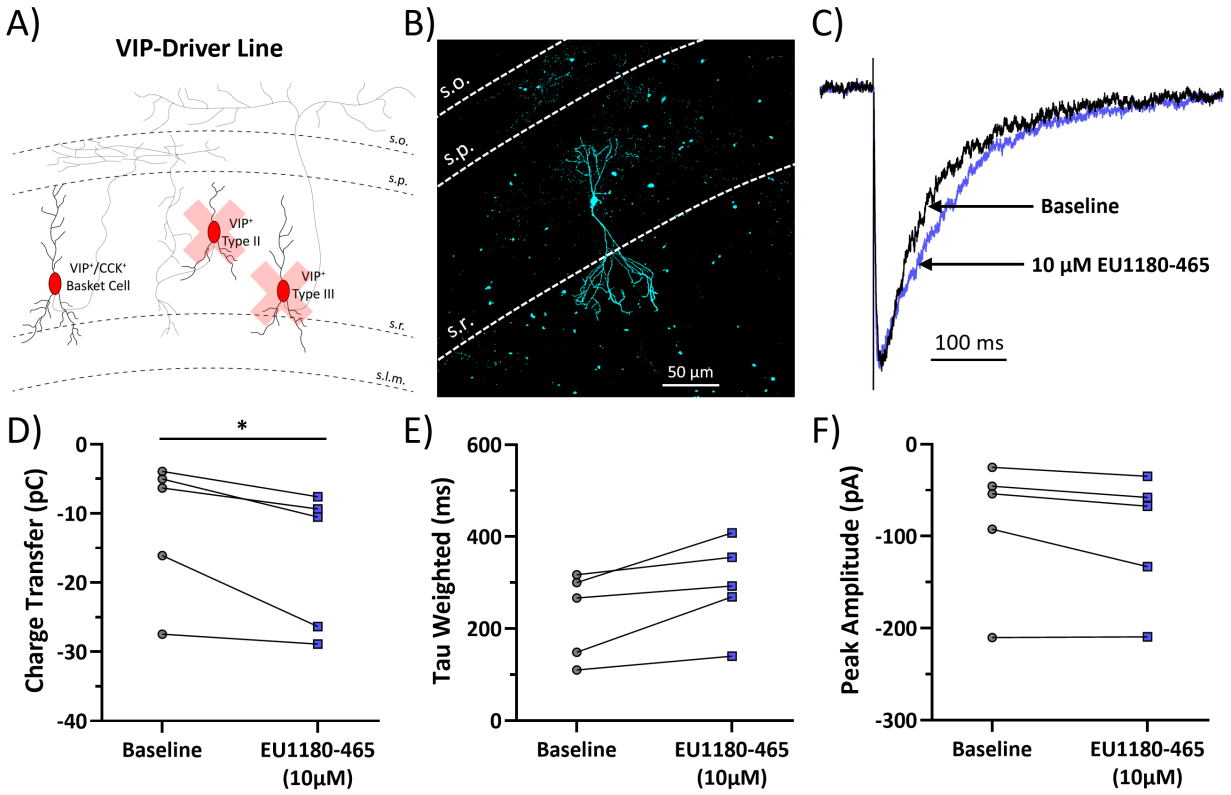



**Figure 4.9**. VIP/CCK-expressing basket cells are potentiated by EU1180-465. A) Diagram of all interneuron subtypes labeled by a vasoactive intestinal peptide (VIP) driver mouse line. To avoid VIP-expressing interneuron-selective interneurons, all cells were stained for CCK post-hoc and evaluated for overlap with biocytin (see methods for more details). B) Biocytin backfilled VIP/CCK-positive basket cell. C) Representative, normalized NMDAR-mediated EPSC onto CCK/VIP-positive basket cells at baseline (black) and after application of 10 µM EU1180-465 (blue). D) Overall charge transfer was significantly potentiated after application of 10 µM EU1180-465 (-12 ± 4.65 pC baseline vs -17 ± 1.5 pC for 10 µM EU1180-465; paired *t*-test, p = 0.035). E) Tau weighted and F) peak amplitude also appear to be potentiated but were not tested for statistical significance. All data are from N=5 cells/3 mice. VIP = vasoactive intestinal peptide; CCK = cholecystokinin*;* s.r. = *stratum radiatum;* s.p. = *stratum pyramidale;* s.o. = *stratum oriens.*

| | **Baseline** | **10 µM EU1180-465** (n=5) | **Fold Change** |
|---|---|---|---|
| **Rise Time** (ms) | 8.1 ± 0.9 | 7.8 ± 1.4 | 0.96 |
| **Peak Amplitude** (pA) | -85 ± 33 | -101 ± 32 | 1.19 |
| **Tau Weighted** (ms) | 229 ± 42 | 293 ± 45 | 1.28 |
| **Charge Transfer** (pC) | -12 ± 4.5 | -17 ± 4.6 | 1.42 |
| **Access Resistance** (MΩ) | 18.6 ± 1.8 | 19.3 ± 1.1 | 1.04 |

**Table 4.5.** Summary data from all evoked NMDAR-mediated EPSCs onto VIP/CCK co-expressing basket cells. All data are mean ± SEM.

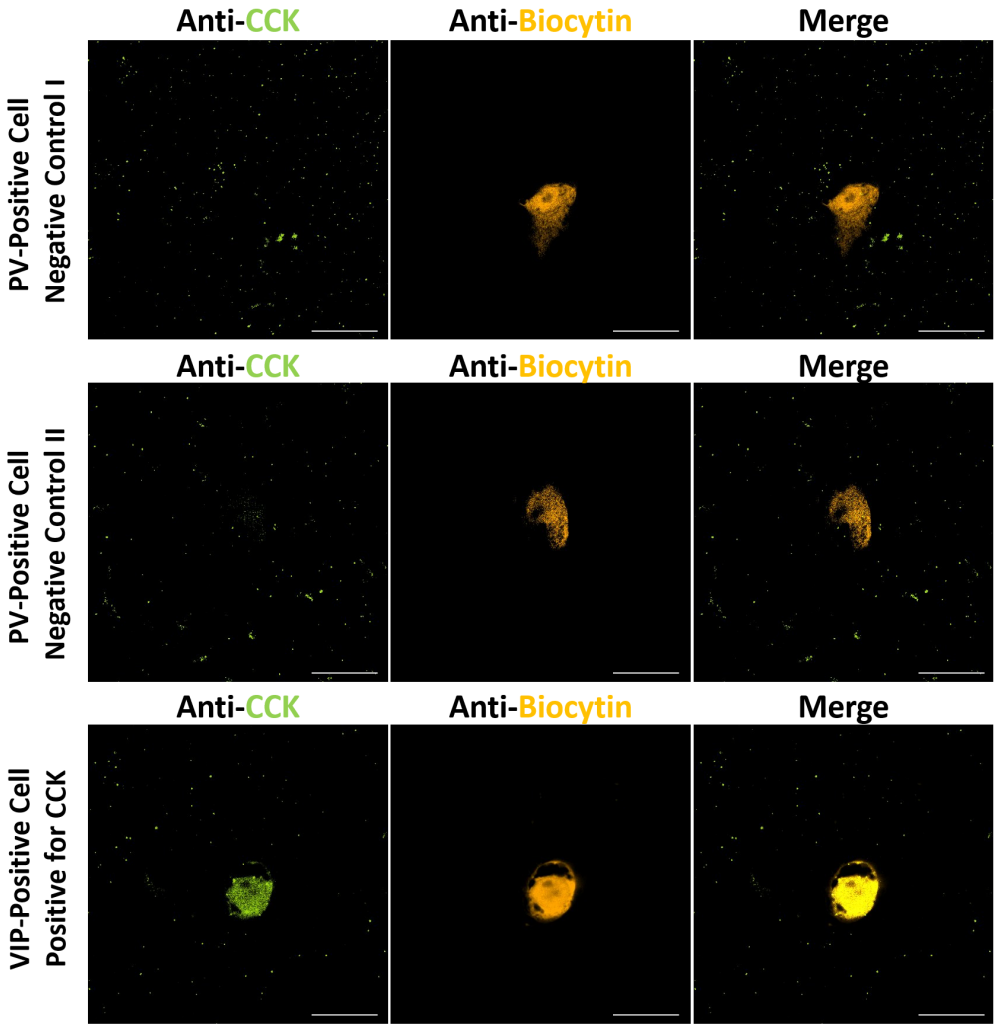



**Figure 4.10.** Validation of CCK antibody and post-hoc staining technique. Data shown are from *Pvalb* -TdTomato (top two rows) and VIP-Cre x Ai9 Td-Tomato (bottom row) mouse reporter lines. Two different cells from the *Pvalb* -TdTomato mouse were patched, backfilled with biocytin, fixed, post-hoc stained for streptavidin and CCK, and then imaged. These top two rows indicate that PV cells, which do not express CCK, do not stain positive for CCK. The bottom row is an example of a positive overlap between a VIP-positive identified cell (biocytin) that also expresses CCK. Images were acquired on a line-scanning microscope, where detector limits were set to avoid any fluorescent overlap between the two channels. PV = parvalbumin; CCK = cholecystokinin; VIP = vasoactive intestinal protein. Scale bar is 20 µm.

using the neuronal nitric oxide synthase (nNOS)-Cre mouse line (Pelkey et al., 2017).

*4.4 Discussion*

NMDAR modulation has tremendous therapeutic benefit, however, past efforts to develop clinically useful agents have been unsuccessful, due in part to a lack of subunit-selective tools. Here, we describe a novel GluN2C/GluN2D PAM – EU1180-465 – with improved drug-like properties such as increased aqueous solubility and increased doubling concentration compared to the exemplar GluN2C/GluN2D PAM (+)-CIQ. Moreover, EU1180-465 application increases spontaneous inhibitory tone in acute hippocampal slices via potentiation of GluN2D-containing NMDARs on GABAergic interneurons. By increasing the overall excitatory charge transfer onto GABAergic interneurons, EU1180-465 shows the ability to enhance overall circuit inhibition which could prove advantageous for numerous neurological diseases such as depression, movement disorders, epilepsy, and schizophrenia.

More importantly, we present data to challenge the ideal that all GABAergic interneurons express synaptic GluN2D-containing NMDARs. Although we present data showing that some interneurons subtypes have no synaptic GluN2D-expression, this does not mean they do not express GluN2D-containing NMDAR entirely. Thus far, the majority of data suggesting that GABAergic interneurons express GluN2D have been via single-cell rtPCR (Perszyk et al., 2016; Porter et al., 1998) and/or in situ hybridization studies (Alsaad et al., 2019; Monyer et al., 1994). A transgenic mouse model expressing eGFP under the GluN2D promoter has also generated some supporting data, however, it provided little evidence of functional GluN2D-containing NMDARs at the synapse (von Engelhardt et al., 2015). Functional studies with GluN2D knockout mice indicate GluN2D's involvement in interneuron function (Dubois and Liu, 2021; Hanson et al., 2019). However, given the ubiquitous expression of GluN2D during early neurodevelopment

(Monyer et al., 1994), it's difficult to interpret these data as they could just be artifacts of aberrant development instead of the acute absence of GluN2D. Pharmacological evidence using GluN2C/GluN2D selective potentiators and inhibitors provide functional data of GluN2D's involvement in synaptic neurotransmission in hippocampal GABAergic interneurons (Perszyk et al., 2016; Swanger et al., 2018; Yi et al., 2020). These studies, however, do not indicate which interneuron subtype is studied, or if they do, it is only in a select few, such as parvalbumin-positive interneurons (Garst-Orozco et al., 2020). Thus, a detailed functional investigation of GluN2D's role in synaptic neurotransmission, stratified by interneuron subtype, is needed to help guide future studies using GluN2D modulation of interneurons as a potential therapeutic target.

Agnostic selection of GABAergic interneurons in CA1 *stratum radiatum* led to a binominal distribution of EU1180-465's ability to potentiate NMDAR-mediated EPSCs. Stratification of charge transfer responses following EU1180-465 application showed that only half (n=7) of randomly selected *stratum radiatum* interneurons were potentiated more than 25% over baseline. Given that the *stratum radiatum* interneuron population is heterogeneous, a plausible explanation for these data is that not all GABAergic interneurons utilize GluN2D-containing NMDARs for synaptic neurotransmission. Through the use of transgenic mouse lines combined with post-hoc antibody staining and morphological analysis, we have shown that Type II and Type III interneuron-selective interneurons (ISIs) (Pelkey et al., 2017) are not responsive to EU1180-465. Thus, these data suggest that Type II and Type III ISIs may not rely on synaptic GluN2D-containing NMDARS. We have also shown that NPY-expressing neurogliaform cells show robust potentiation from EU1180-465. VIP/CCK co-expressing cells also show EU1180-465 potentiation, although the small size of our current dataset warrants limited conclusions at this time.

These data also highlight the relatively large NMDAR-mediated EPSCs onto NPY-expressing neurogliaform cells, which has been shown previously (Chittajallu et al., 2017). Neurogliaform cells are unique in that they can signal through 'volume transmission' in that they release bolus amounts of GABA (Karayannis et al., 2010; Price et al., 2005), with many making 'en passant' synaptic connections (Naegele and Katz, 1990; Vida et al., 1998). Neurogliaform-mediated volume transmission helps control the tonic GABA current (Bryson et al., 2020; Tamas et al., 2003), suggesting that potentiation of excitatory drive onto neurogliaform cells via EU1180-465 will likely impact circuit function outside of the canonical increased IPSC onto principle cells. Moreover, the lack of EU1180-465's effect on ISIs appears at odds with single-cell rtPCR from cortical ISIs which were shown to express GluN2D mRNA (Porter et al., 1998). However, other studies have shown little GluN2D expression in calretinin-positive cells which is another neurochemical marker for ISIs (von Engelhardt et al., 2015). Future studies on Type I ISIs are needed to flesh out the trio of ISI subtypes, however, current data suggests that any effect of EU1180-465 on circuit excitability is not due to disinhibition.

Future studies will be aimed at elucidating EU1180-465's potentiation ability of CCK expressing cells, which is one of the largest subsets of GABAergic interneuron (Pelkey et al., 2017). Within *stratum radiatum*, CCK-expressing interneurons make up the majority of all GABAergic interneurons, and given their relative size compared to ISIs and neurogliaform cells, are the most likely to be picked for patch-clamp experiments. Thus, it seems likely that some CCK cells will also not respond to EU1180-465. Although there are multiple subsets of CCK-expressing interneurons, such as Schaffer collateral and perforant-pathway associated, it is unlikely that if some CCK cells are unresponsive to EU1180-465, they will neatly be stratified into one of these

many subsets. The more probable finding is that CCK-expressing cells utilize GluN2D-containing NMDARs in a synapse- and activity-dependent manner.

*4.5 References for Chapter 4*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# CHAPTER 5: Conclusions, Discussion, and Future Directions

The overarching goals of the work presented in this thesis are two-fold: 1) to evaluate cellular and synaptic features of mouse models based on N-methyl-D-aspartate receptor (NMDAR) human patient-derived variants and 2) explore the actions of subunit-selective NMDAR modulation on circuit function for potential therapeutic gain. In Chapter 1, various roles of NMDARs were reviewed, and emphasized the omnipresent nature of these receptors within the central nervous system (CNS). In Chapters 2 and 3, mouse models of human patient-derived NMDAR variants are used to explore the pathophysiology and mechanistic underpinnings of disease at the cellular and circuit levels. Although much progress has been made in terms of variant classification (*i.e.,* in heterologous systems), the field of NMDAR variant biology is still in its infancy. Chapter 3 also highlights the importance of viewing animal model age as a potent experimental variable while providing novel supporting data for GluN2A's role in neurodevelopment and circuit maturation. In Chapter 4, a novel positive allosteric modulator is used to provide functional data on synaptic NMDAR subunit expression in groups of relatively understudied GABAergic interneurons.

### *5.1 Initial Behavioral Characterization of Gain-of-Function Mouse Models*

Chapter 2 presents three different gain-of-function mouse models based on NMDAR human patient-derived variants in the least tolerant regions of the receptor. In humans, each of these variants present as devasting developmental and epileptic encephalopathy. We show that both the GluN2A-P552R and GluN2A-S664G mouse models have altered current thresholds for partial (6-Hz) seizure induction. Although both mouse models require higher current for 6-Hz seizure induction, which is the opposite of observations in the patients who undergo spontaneous seizures, these data suggest altered levels of circuit excitability. This trend is also seen in other mouse models of epilepsy, for example the *IQSEC2* mouse (Sah et al., 2020). Heterozygous

*IQSEC2* null mice die from lethal spontaneous seizures (median survival age = P95) yet require higher current for 6-Hz induced electrical seizures (Sah et al., 2020). This paradoxical example highlights the crude nature of these electrical seizure induction experiments and suggests that any change from wildtype controls could be viewed as troubling. They also raise the possibility that altered NMDA receptor composition within the circuit required for generalization of the seizure (e.g. the lateral geniculate for corneal stimulation) may influence seizure threshold. Moreover, developmental and epileptic encephalopathy is a complex disease which can likely never be fully modeled in mice. Altered levels of circuit excitability are but one of many associated phenotypes, with comorbidities such as delayed developmental milestones and diminished levels of intellectual processing yet to be assayed in our mice.

Another factor that may contribute to our paradoxical results is animal age. Patients harboring the GluN2A-P552R and GluN2A-S644G variants began showing signs of developmental and epileptic encephalopathy often before the age of 3. However, much of our mouse model testing was completed in adult mice. Therefore, it is possible that if we could have conducted our 6-Hz seizure experiments in younger mice, we would observe our predicted trend of a lower current required for induction. Thus, the adult data could be due to maladaptive circuit compensation that over-corrected for early hyperexcitability. Although we don't have 6-Hz data for our GluN2D-V667I mice, our electroencephalographic data shows severe spontaneous epileptiform activity. These data were, however, collected in young (<P35) mice. Future experiments should consider completing seizure assays in younger mice, as well as incorporating other behavioral tasks such as age at which pups reach developmental milestones (*i.e.*, the righting reflex) and the Morris water maze to probe for perturbations in development and intellectual ability, respectively. However, this is complicated by need to validate assays at these early ages as

well as complexity of gaining approval for some procedures in young animals. In all, however, our initial behavioral data suggest that all three models show signs of altered network excitability, making them viable candidates for evaluation.

*5.2 Differences between heterologous and synaptic data*

In addition to providing first-pass behavioral validation of the three gain-of-function mouse models presented in Chapter 2, a comparison of heterologous expression system data and synaptic data of the NMDAR-mediated waveform are presented. Each mouse model yields synaptic data that are similar to predictions using heterologous systems in that each show signs of gain-of-function via deactivation/decay measures, however, synaptic data appears to be less severe than changes in receptor response time course observed in heterologous expression systems. Functional data on NMDAR pharmacology and response time course from heterologous expression systems including *Xenopus* oocytes and transient expression in HEK cells in the Traynelis lab have become workhorse preparations for data generation. Both *Xenopus* oocytes and HEK cells are relatively inexpensive to maintain, show robust expression of recombinant NMDARs, and are relatively easy to record from making them excellent platforms for high-efficiency, first-pass screening for many ion channels, including NMDARs, in drug discovery and variant classification. These reductionist expression systems offer controls the subunit composition when investigating ion channel physiology, allowing for detailed mechanistic conclusions to be made. This functional information, along with high-resolution crystallographic and cryo-EM structures of various NMDAR assemblies have been instrumental in advancing our understanding of NMDAR biology.

Despite their numerous positive benefits, heterologous expression systems do not always accurately predict how small molecule drugs acting on synaptic NMDARs and human patient-derived NMDAR variants will behave when evaluated in native mammalian synapses. Highly

efficacious NMDAR positive allosteric modulators may only potentiate synaptic NMDAR-mediated EPSCs by less than 2-fold and predicted deleterious NMDAR variants with over 50-fold changes in glutamate potency show only modest aberrations when knocked into a mouse and recorded at native synapses. However, heterologous systems can provide baseline functional data describing effects of a variant, including peak amplitude, agonist potency, activation rise time, deactivation time, level of desensitization, overall charge transfer, level of magnesium block, sensitivity to endogenous modulators such as zinc, open-channel probability, and mean open channel time. Each of these variables are obtained to help estimate how a new small molecule drug or understudied variant may behave when moved into a more physiological-relevant model system such as a native synapse from acutely prepared mouse brain slices.

Most synaptic data is obtained at the neuron's soma where electrical stimulation of afferent excitatory fibers is used to trigger glutamate release and receptor activation in a fraction of synapses some distance from the where the signal is recorded. When data are obtained using this canonical paradigm, variables such as agonist potency, level of desensitization, zinc modulation, open channel probability, and mean open channel time all coalesce into a single macroscopic current. Open channel probability and mean channel open time can also be obtained from native receptors in fresh tissue; however, these are most easily attained from somatically expressed receptors, providing little information on synaptic receptors. This technique of using somatic receptors is not often used due to low difficulty of preparation and the experimenter recording from channels of unknown subunit composition.

Measurables from macroscopic synaptic currents include current response rise time, peak amplitude, weighted tau describing decay time, and overall charge transfer. Peak amplitude measurements from synaptic currents are complex to interpret as stimulation intensity and location

of stimulation electrode directly influence the excitatory postsynaptic current (EPSC)'s amplitude, which is not correlated with agonist potency. Furthermore, electrotonic filtering reduces all current amplitudes to varying degrees, depending both on where synapse is located relative to the soma as well as the cell size (overall capacitance). Rise time is similarly confounded by filtering. EPSC amplitude can be used to measure efficacy of a small molecule compound, however, when peak amplitude with drug on board is normalized to peak amplitude of the EPSC before drug application. This assumes the modulator can access its binding site in the absence of glutamate; compounds that only bind (or show enhanced affinity) after glutamate binding may bind so slowly that they only appreciably occupy their binding site after the peak EPSC has passed. Charge transfer is a combination of both peak amplitude and deactivation/decay time as it's simply the integral of the area under the current response/EPSC. Rise time can be measured and compared between heterologous expression systems and native synapses, however, very few small molecule compounds impact rise time. Moreover, variants impacting rise time are also relatively rare, with the only examples so far being variant that reside in the pre-M1 helix, a region that is intolerant to genetic variation (Ogden et al., 2017). Thus, deactivation time from heterologous systems and a weighted average of the multiple time constants describing most deactivation time courses are the most often compared measures.

Although deactivation/decay time is one of the best ways to compare heterologous and synaptic data of the same variant, the complexity of the mammalian CNS generally keeps these two data points from being identical. First, at native synapses, there are multiple different subunit combinations possible at any given age. In juvenile (P14-20) mouse excitatory synapses onto CA1 pyramidal cells, one can reasonably expect both GluN2A and GluN2B subunits to be expressed. In a wildtype mouse, this leads to three possible subunit combinations: diheteromeric GluN2A

NMDARs, diheteromeric GluN2B NMDARs, and triheteromeric GluN2A/GluN2B NMDARs. Although previous data obtained at this synapse suggests that triheteromeric GluN2A/GluN2B NMDARs are the majority, the possibility of diheteromeric complexes still exists. With gain-of-function GluN2A variants like GluN2A-P552R and GluN2A-S644G, six possible combinations are possible: the three mentioned above plus triheteromeric GluN2A/GluN2A-variant, diheteromeric GluN2A-variant, and triheteromeric GluN2A-variant/GluN2B. This possibility alone is sufficient to generate non-overlapping data with heterologously expressed NMDARs, even with the capability to express the triheteromeric GluN2A-variant/GluN2B construct. This same concept becomes even more complex when evaluating the GluN2D-V667I variant, as GluN2D-containing NMDARs in interneurons are hypothesized to form triheteromeric combinations of both GluN2A/GluN2D and GluN2B/GluN2D. Moreover, all current heterologous data have only been obtained with the GluN1-1a splice variant. As discussed previously, the GluN1-1b splice variant (GluN1-exon5) alters NMDAR kinetic properties, and exon5-lacking GluN1 subunits are expressed at CA1 pyramidal cell synapses and in synapses onto *stratum radiatum* interneurons (Li et al., 2021).

Synapse receptor identity, at least in terms of ionotropic glutamate receptor (iGluR) subunits, is highly heterogeneous, even within the same group of synapses onto a single neuron. iGluRs, like all proteins, are fluid with constant turnover. The activity-dependence of NMDAR subunit composition within a synapse has been extensively studied (Rao and Craig, 1997) and is thought to be one of the major driving forces responsible for the GluN2B-to-GluN2A switch that happens during early neurodevelopment (Shipton and Paulsen, 2014). Given GluN2A's ubiquitous expression in both pyramidal cells and interneurons, GluN2A variants can impact both excitatory and inhibitory cells in a given circuit. Thus, it is entirely plausible that the activity-dependent

timing of the GluN2B-to-GluN2A switch in pyramidal cells may be altered. This possibility also applies to the gain-of-function GluN2D-V667I variant as perturbations to the interneuron network are likely to change the basal activity of a given circuit and interneurons also display a similar GluN2B-to-GluN2A switch (Matta et al., 2013). Moreover, current synaptic data have only been obtained using a single afferent excitatory input, the Schaffer collaterals. NMDAR subunit composition can be input-specific (Arrigoni and Greene, 2004; Chittajallu et al., 2017) as well as spatially specific (*i.e.*, Schaffer collateral inputs on the proximal apical dendrite have different NMDAR subunit composition than Schaffer collateral inputs on the distal apical dendrite) (Major et al., 2008; Siegel et al., 1994). Variant containing NMDAR complexes may also be expressed at extrasynaptic sites that would not be activated given the single afferent stimulation paradigm used. Thus, current sampling of synaptic decay times represents only a fraction of possible synapses and locations of expression, with each likely having slightly different NMDAR subunit compositions.

Other factors which may account for differences in heterologous versus synaptic decay times include protein binding to NMDARs, post-translational modifications, temperature at which recordings were made, and co-agonist used in acute slices. NMDARs bind to and can be regulated by postsynaptic auxiliary proteins, such as neuropilin and tolloid-like (Neto) proteins (Wyeth et al., 2014) and by transsynaptic proteins, such as the leukocyte common antigen-related protein (LAR)-type receptor phosphotyrosine-phosphatases (LAR-RPTPs) (Kim et al., 2020; Sclip and Sudhof, 2020). Although supporting evidence that Neto and LAR-RPTP proteins can directly impact receptor kinetics is limited, they have been shown to influence the synaptic GluN2A:GluN2B ratio (Wyeth et al., 2014) and NMDAR receptor trafficking to the postsynaptic density (Sclip and Sudhof, 2020), respectively. NMDARs are also subjected to many post-translational modifications, including phosphorylation, glycosylation, S-nitrosylation,

ubiquitylation, and palmitoylation (Hansen et al., 2021). The functional consequences of these post-translational modifications are vast, impacting both subunit expression and function (Chen and Roche, 2007; Lussier et al., 2015). Temperature of solutions during recording has a direct influence on ion flux as described in the Goldman-Hodgkin-Katz equation and thus will impact receptor deactivation/decay time. Nearly all heterologous data are performed at ambient temperature (~22°C) whereas all synaptic data presented in this thesis were performed at 32°C. This discrepancy alone could explain why synaptically obtained decay times in our variant mouse models are faster than their corresponding deactivation time measured in HEK cells. The confounds of both glycine and D-serine being present at native synapses, combined with the possible human selection bias to choose 'healthy' cells which may inadvertently miss cells without many mutant subunits can also produce nonidentical heterologous and synaptic data. The concept of electrotonic filtering (lessen of current amplitude defined by the length constant as ions flow from dendrites to the soma) is also important to consider (Johnston and Brown, 1983).

Regardless of the limitations and caveats mentioned above, synaptic data clearly show that all three gain-of-function mouse models have a prolongation of the NMDAR-mediated decay time. This not only means an increase in cellular excitability, but also correlates to an overall increased intracellular calcium influx. Calcium is a powerful second messenger that has the ability to initiate entire genetic programs influencing neurodevelopment and synaptic plasticity. Changes in both GluN2A- and GluN2D-mediated signaling could also perturb presynaptic excitability, as both subunits have been reported presynaptically (Bouvier et al., 2015). Outside of actions on neurons, the GluN2A subunit has been reported in astrocytes. More specifically, astrocytic NMDARs are likely a triheteromeric complex of GluN2A/GluN2C subunits (Shelkar et al., 2022). GluN2A variants may alter astrocyte calcium spikes and modulate the ability of astrocytes to remove

glutamate from the synapse. GluN2D-mediated variants generate an increased excitability of inhibitory cells that then can progress into a hyperexcitable network. One possible explanation for this paradoxical finding is that excess GluN2D-mediated neurotransmission and increased calcium influx from our gain-of-function variant could produce excitotoxicity, resulting in an overall decreased interneuron cell density. Alternatively, increased excitatory neurotransmission in interneurons will push them closer to membrane potentials at which depolarization-induced block of action potential firing would occur. Here, the increased excitatory nature of the variant will serve to decrease the inhibitory output of affected interneurons. Recording spontaneous inhibitory postsynaptic currents (IPSCs) and miniature IPSCs in acutely prepared hippocampal slices, interneurons are likely not close to depolarization-induced block levels, explaining why an increased spontaneous IPSC frequency is still observed.

*5.3 GRIN2A null variants, seizure susceptibility, and parvalbumin-positive interneurons*

Careful evaluation of *GRIN2A* variants via longitudinal data from our clinical collaborators yielded a surprising finding: some *GRIN2A* null variant-harboring human patients display a transient seizure burden. That is, some *GRIN2A* null patients develop seizures as expected, but then later in life the seizures resolve. Whether this trend is not seen with *GRIN2A* missense variant-harboring human patients is currently unknown. Given this intriguing finding, we utilized *Grin2a*-knockout (*Grin2a*$^{-/-}$) mouse model to investigate potential molecular mechanisms generating this transient seizure susceptibility. Through electrophysiology and immunohistochemical staining, we show that the loss of *Grin2a* signaling causes a transient delay in the electrophysiological maturation of CA1 parvalbumin-positive (PV) interneurons. Moreover, we show that this delay is gene- and age-dependent. Both *Grin2a*$^{+/-}$ and *Grin2a*$^{-/-}$ mice show aberrant CA1 PV cell electrophysiological function at P7, however, PV cells from *Grin2a*$^{+/-}$ reach wildtype functionality

at P14 whereas PV cells from *Grin2a*$^{-/-}$ mice don't attain wildtype levels until adulthood. Given that PV cells provide essential inhibitory tone within a circuit, their dysfunction could generate epileptiform activity. While further testing is required to confirm this hypothesis, the transient nature of PV cell dysfunction suggests a possible molecular mechanism for the transient seizure burden observed in *GRIN2A* null variant-containing patients.

Caution is warranted when interpreting these clinical data, as our results represent a fraction of the total *GRIN2A* missense and *GRIN2A* null variant-harboring human patients. When viewing the totality of *GRIN2A* missense and *GRIN2A* null data, the share of missense to null variant-harboring patients is 2:1 (Benke et al., 2021; Hansen et al., 2021). In our dataset, however, the total number of null variant patients outweighs missense variant patients, with a ratio of nearly 2:1. The reasons for this aren't clear and may represent random chance. Alternatively, perhaps there is an undetermined reason why *GRIN2A* null harboring patients are more likely to be ascertained and sequenced by clinical epileptologists than *GRIN2A* missense variant-harboring patients. This seems unlikely, however, as recent data suggests that missense harboring patients report more severe clinical symptoms compared to *GRIN2A* null variant-harboring patients (Strehlow et al., 2019). More importantly, however, is that although some *GRIN2A* null variant-harboring patients may benefit from a transient seizure burden, nearly all suffer from other clinical symptoms such as developmental delay, intellectual disability, and aphasia that appear to be present throughout life (De Bernardi et al., 2022; Krey et al., 2022; Li et al., 2020; Strehlow et al., 2019; Strehlow et al., 2022).

In addition to contributions on that understanding of human *GRIN2A* variants, data presented in Chapter 3 suggest multiple potential roles for the GluN2A subunit of NMDARs in the regulation of development and maturation of γ-Aminobutyric acid (GABA) expressing

interneurons in the mouse hippocampus. While it has been reported that *Grin2a*$^{-/-}$ mice have changes in glutamatergic cells, this study provides crucial evidence that the loss of GluN2A also impacts PV interneurons (Kannangara et al., 2014). Using *Grin2a*$^{-/-}$ mice, we have demonstrated that NMDAR-mediated signaling can impact hippocampal interneuron cell density, providing supportive evidence for the potential influence of early electrical or metabotropic NMDAR-mediated signaling in interneuron survival and/or apoptosis. GABAergic interneurons mostly arise from one of two major stem cell populations – the medial ganglionic eminence (MGE) and caudal ganglionic eminence (CGE). All PV cells arise from MGE progenitors and all cholecystokinin-expressing (CCK) cells arise from CGE progenitors (Pelkey et al., 2017). As reported, the loss of *Grin2a* only impacts CA1 PV cell density, suggesting that MGE-derived interneurons may by preferentially impacted by the loss of GluN2A.

Cortical and hippocampal MGE interneuron fate is largely controlled by the early embryonic transcription factor *Nkx2.1*, which is responsible for initiating a series of determinate genetic programs (Butt et al., 2008; Sussel et al., 1999; Tricoire et al., 2010). Given that we reported no changes in cortical PV cell density (not shown), it is unlikely, although not impossible, that the loss of *Grin2a* is affecting *Nkx2.1*-derived intrinsic genetic programs and increasing the number of MGE-derived interneurons generated. However, the most parsimonious interpretation of our data is that the loss of GluN2A may be impacting interneuron survival or apoptosis, critical factors in determining overall cell density. After interneuron infiltration, nearly half of these cells will undergo apoptosis, fine-tuning the balance of pyramidal cells to interneurons (Southwell et al., 2012). The exact mechanisms of interneuron apoptosis are not fully understood but are likely controlled by a combination of internal genetic cues, external neurotropic factors, and early interneuron electrical activity (Denaxa et al., 2018; Priya et al., 2018; Southwell et al., 2012).

Recent data suggests that interneuron apoptosis, especially in CCK and PV cells, is mostly controlled by electrical activity and most importantly via calcium influx (Denaxa et al., 2018; Priya et al., 2018). However, the extent to which local electrical signaling, especially from NMDARs, within PV interneurons controls pro-apoptotic pathways has not been determined.

Why is PV cell density, but not CCK cell density, impacted by the loss of *Grin2a*? NMDARs are found on both PV and CCK cells, however, recent reports have suggested that CCK cells express less GluN2A than their PV cell counterparts (Booker et al., 2021; Matta et al., 2013; Perszyk et al., 2016). Additionally, CCK cell density has been reported to be influenced by 5-$HT_{3A}$ and AMPA receptor-mediated activity (Akgul et al., 2019; Murthy et al., 2014). Despite having a higher overall synaptic NMDA:AMPA ratio, perhaps CCK cells rely on electrical cues from non-NMDARs to control apoptotic pathways (Bortone and Polleux, 2009; Matta et al., 2013). Alternatively, since CCK cells have limited GluN2A expression, the loss of this subunit could have minimal impact on NMDAR-mediated apoptosis in this interneuron subtype. Future experiments should focus on elucidating the impact of early NMDAR signaling regarding initiating interneuron apoptosis in both MGE- and CGE-derived interneurons. Moreover, data showing no detectable change in PV cell density in *Grin2a*$^{+/-}$ mice suggests that a total loss of GluN2A-mediated signaling is needed to impact PV cell apoptosis. Given that nearly all *GRIN2A* null harboring human patients have heterozygous null variants, the interpretation of these data in the context of a molecular mechanism of human disease is limited.

We also show that NMDAR-mediated signaling may be sufficient, but not necessary, to initiate a series of genetic programs which controls cellular excitability and electrophysiological maturation of hippocampal PV cells. PV cells are known for their fast-spiking action potential characteristics, which can generate gamma oscillations that are involved in higher-order cognitive

function (Crone et al., 2006; Sohal et al., 2009). By examining both passive intrinsic properties and action potential firing characteristics, we can estimate how these cells may perform under synaptic load and how efficiently they can transduce somatic depolarizations into signal-carrying action potentials. Recordings from CA1 PV cells in juvenile (P14) *Grin2a*$^{+/-}$ and *Grin2a*$^{-/-}$ mice are associated with a host of electrophysiological changes, including an increased input resistance, a prolonged membrane time constant, longer action potential half-widths, and lower peak action-potential firing frequencies. Each of these electrophysiological measures are known to be age-dependent, with PV cells in neonatal mice displaying a higher input resistance, longer membrane time constant, and longer action potential half-widths than PV cells from preadolescent mice (Doischer et al., 2008; Goldberg et al., 2011; Miyamae et al., 2017; Okaty et al., 2009). To confirm these data are age-dependent in CA1 PV cells, we performed passive and action-potential firing properties on neonatal PV cells using the *Tac1*-Cre mouse driver line. The *Tac1* gene has recently been shown to be expressed in immature CA1 PV cells, with no expression in related MGE-derived somatostatin cells (Que et al., 2021). Indeed, we show a clear age-dependence in input resistance, membrane time constant, action-potential half-width, and action-potential firing frequency in Tac1-expressing cells.

The interpretation of these data in the context of human disease is complex. On one hand, an increased input resistance and time membrane constant, combined with an increased NMDAR-mediated EPSC decay time, in CA1 PV cells from *Grin2a*$^{+/-}$ and *Grin2a*$^{-/-}$ mice will make these PV cells *more* excitable. These same cells, however, also have a lower threshold for depolarization-induced block of action potential firing and lower peak action potential firing frequencies which makes them *less* excitable. When viewed together, an increased input resistance and a prolonged NMDAR-mediated decay time means that CA1 PV cells in *Grin2a*$^{+/-}$ and *Grin2a*$^{-}$

/- mice will reach their lower threshold for depolarization-induced block of action potential firing with less excitatory activity than age-matched wildtype mice. Thus, CA1 PV cells are fast to fire, yet fast to retire. Moreover, when they do fire, the overall firing frequency is lower than wildtype mice with the net result being decreased inhibitory tone from a major class of GABAergic interneurons. These latter electrophysiological characteristics could lead to failure of circuit level inhibition as interneuron depolarization increases, consistent with a hyperexcitable phenotype.

The exact mechanism for this transcriptional shift from immature to mature PV electrophysiological properties is not fully understood but has been shown to be activity-dependent (Dehorter et al., 2015; Miller et al., 2011). Thus, the loss of *Grin2a* in developing PV interneurons appears to slow cellular maturation, potentially stemming from a loss of GluN2A signaling on PV cell dendrites. Alternatively, this delay in electrophysiological maturation could be driven by overall changes in circuit excitability, likely driven by pyramidal cell activity. Although data showing a dependence of pyramidal cell activity on the electrophysiological profile of interneurons has not been shown, early pyramidal cell signaling can influence interneuron apoptosis (Wong et al., 2018) and interneuron protein synthesis (Bernard et al., 2022). While the overall effects of a prolonged immature electrophysiological profile in PV cells are not known, it is unlikely to be silent, as this period coincides with a critical plasticity period during preadolescence (Hensch, 2005; Takesian and Hensch, 2013). That is, the delay in maturation could have a critical impact on circuit formation in the hippocampus, and other potential regions. Specifically, larger input resistances and lower peak action potential firing frequencies will likely dampen the temporal resolution of inhibitory tone in a developing network, which may promote maladaptive plasticity and incorrect circuit wiring. Additionally, by experimentally limiting depolarization-induced block in CA1 PV cells, researchers were able to halt evoked hippocampal epileptiform activity and

restore normal pyramidal cell firing (Calin et al., 2021). Thus, disruption of PV cell function during development could have profound consequences on the excitability of mature networks.

In addition to uncovering some unique potential mechanisms of PV cell maturation, this study highlights a critical role for early GluN2A signaling in neurodevelopment. The role of NMDARs in neuronal development has been appreciated for decades, with attention mostly directed at the GluN2B and GluN2D subunits (Hanson et al., 2019; Jiang et al., 2015; Kelsch et al., 2014; Wang et al., 2011; Yamasaki et al., 2014). The GluN2A subunit, with its surge in postnatal expression at week two of rodent development, has largely been considered an adolescent and adult subunit, often associated with synapse maturation in principle cells and overall circuit refinement (Dumas, 2005; Gray et al., 2011). Incorporation of GluN2A into the postsynaptic density accelerates the NMDAR-mediated synaptic time course, generating briefer but more efficient dendritic calcium transients (Evans et al., 2012). Even though hippocampal mRNA levels of GluN2A are low before P14, interneuron survival/apoptosis is largely decided before GluN2A's increased expression around P14 (Matta et al., 2013; Tricoire et al., 2011). Additionally, wildtype CA1 PV cells in juvenile mice displayed signs of electrophysiological maturity, suggesting that GluN2A's influence on genetic programs controlling cellular excitability and action-potential firing likely happen at or before GluN2A's increased expression at P14.

These deficits are likely not due to a complete loss of NMDAR-mediated signaling in PV cells, as *Grin2a*$^{-/-}$ rodent models have similar NMDAR-mediated excitatory postsynaptic current amplitudes to age-matched WT cells (Booker et al., 2021). Although their NMDAR-mediated current amplitudes are similar, *Grin2a*$^{-/-}$ PV cells have a prolongation of NMDAR-mediated synaptic time course, as would be expected for neurons expressing only GluN2B- and GluN2D-containing NMDARs (Booker et al., 2021; Hansen et al., 2021). A prolongation of the NMDAR-

mediated synaptic time course would likely drive more calcium into the cell, impacting numerous genetic programs. Whether PV cell apoptosis and electrophysiological maturation are signaled by changes in postsynaptic calcium transients mediated by GluN2A insertion or driven by local circuit activity via pyramidal cells is currently unknown. Alternatively, second messenger signaling dependent on the C-terminal tail of GluN2A subunits may induce post translational modification of synaptic proteins sufficient for transcriptional program generation.

Regardless of the mechanism behind the delayed electrophysiological maturation of PV cells, the totality of the data presented in Chapter 3 may be useful in suggesting therapeutics for *GRIN2A* null harboring human patients. Some of the molecular findings associated with the loss of GluN2A signaling mirror those seen in mouse models of Dravet's syndrome. In Dravet's syndrome, the loss-of-function mutation in the *SCN1A* gene primarily impacts GABAergic interneurons, decreasing their overall inhibitory output (Rubinstein et al., 2015; Tai et al., 2014). Since this mutation occurs in a voltage-gated sodium channel, the use of sodium channel blockers such as Lamotrigine, Phenytoin, and Carbamazepine as anti-epileptic drugs exacerbates epileptic seizures in Dravet's syndrome patients (Genton et al., 2000; Guerrini et al., 1998; Hawkins et al., 2017). In the context of GRIN2A null variant-harboring patient treatment, it is still unclear whether delays in electrophysiological maturation of PV cells seen in null *Grin2a* mice are due to sodium channels or potassium channels. Given that previous transcriptomic data on PV cell maturation has shown that many of the changes in electrophysiological properties of PV cells are due to delays in potassium channel expression (Goldberg et al., 2011), anti-epileptic drugs that target potassium channels such as Retigabine should be avoided. Future clinical studies should evaluate whether empirical data support this hypothesis.

*5.4 GluN2C/GluN2D Subunit-Selective Modulation of Synaptic NMDARs*

Chapter 4 describes the functional evaluation of a GluN2C/GluN2D positive allosteric modulator EU1180-465. This compound is a refinement of the exemplar GluN2C/GluN2D positive allosteric modulator (+)-CIQ, with marked improvements in aqueous solubility and doubling concentration at both GluN2C- and GluN2D-containing NMDARs. By selectively targeting GluN2C/GluN2D subunits via allosteric modulation, this compound series seeks to avoid pitfalls of a failed previous set of broad-spectrum competitive antagonists and uncompetitive channel blockers. Moreover, the GluN2C and GluN2D subunits show more restricted expression profiles compared to GluN2A and GluN2B subunits. Although both GluN2C and GluN2D are expressed in midbrain nuclei such as the thalamus and Purkinje cells of the cerebellum, the appeal of this compound series comes from a desire to increase the excitability of GABAergic interneurons. GluN2C-containing interneurons have been reported in a sparse set of PV-expressing interneurons (Ravikrishnan et al., 2018), while nearly all cortical interneurons are thought to express GluN2D-containing NMDARs. A loss of GABAergic inhibition is proposed to be causal in many neuropathological disorders, including epilepsy and schizophrenia. Thus, a small molecule compound designed to enhance GABAergic interneuron output could be a welcome tool in the clinician's toolbox.

Application of 10 μM EU1180-465 (roughly 3x $EC_{50}$) increased the frequency of spontaneous IPSCs onto CA1 pyramidal cells from preadolescent (P20-26) mice. Acutely prepared brain slices will exhibit spontaneous synaptic currents in the absence of external stimuli and measuring the ability of EU1180-465 to influence these spontaneous events provides initial evidence for its ability to potentiate GABAergic interneuron output. We report that EU1180-465 potentiates spontaneous IPSC frequency 1.35-fold over baseline, whereas previously published

data show that application of 10 μM (+)-CIQ was able to potentiate spontaneous IPSC frequency 1.48-fold over baseline (Perszyk et al., 2016). Despite EU1180-465 having roughly a 16-fold improvement in the doubling concentration at GluN2D-containing NMDARs compared to (+)-CIQ, EU1180-465 appears less efficacious when comparing ability to potentiate spontaneous IPSC frequency. The reason for this discrepancy is most likely explained by the age of animal used.

Data obtained with (+)-CIQ were in neonatal (P7-14) mice, whereas data obtained with EU1180-465 were in preadolescent (P20-26) mice. GABAergic interneuron activity increases during early neurodevelopment, with peak levels attained during preadolescence. In the study using (+)-CIQ, baseline spontaneous IPSC frequency was 3.5 Hz, whereas baseline spontaneous IPSC frequency before EU1180-465 application was 7.7 Hz. Thus, perhaps an immature interneuron network with lower overall spontaneous IPSC frequencies is more amenable to potentiation than a relatively mature interneuron network at P20-26. Immature interneurons have larger input resistances, suggesting that any increase in excitatory tone would translate into an increase in action-potential firing and thus increased spontaneous IPSC frequencies. On the other hand, immature interneurons also have lower action-potential firing rates which would support an overall dampening of spontaneous IPSCs with age. GABAergic interneuron synaptic density also increases with age and during early neonatal development, GABAergic activity is depolarizing and would not necessarily be measured as spontaneous IPSC current during analysis.

Alternative explanations for the diminished efficacy of EU1180-465 in potentiation of spontaneous IPSCs center around the overall caveats with acute slice preparations. Using acutely prepared slices and relying on spontaneous activity to drive NMDAR-mediated EPSCs onto GABAergic interneurons, there will likely be less NMDAR activation than would be observed in an awake behaving mouse. The ratio of AMPAR-mediated spontaneous EPSCs to NMDAR-

mediated spontaneous EPSCs heavily weighs in the AMPAR's favor. All spontaneous IPSC data was obtained at 32°C, whereas *in vivo* activity would occur at 37°C. This temperature difference will influence circuit excitability and favor an increased glutamatergic drive onto GABAergic interneurons. Moreover, all spontaneous IPSC data was obtained at +10 mV, which will promote depolarization-induced suppression of inhibition (Wilson and Nicoll, 2001). Depolarization of pyramidal cells will drive endocannabinoid release which can act on presynaptic CB1 receptors, which are Gi-coupled G-protein coupled receptors, essentially limiting spontaneous GABA release into the synaptic cleft. Nearly all CCK-expressing interneurons have presynaptic CB1 receptors meaning that all spontaneous IPSC data obtained at +10 mV will be absent of CCK-mediated inhibitory drive. This, combined with electrotonic filtering of relatively small spontaneous IPSCs onto distal dendrites from SST-expressing interneurons and neurogliaform cells, suggests that the majority of spontaneous IPSC data recorded are derived from PV basket cells. An alternative approach would be to record spontaneous IPSCs at -60 mV in the presence of an AMPAR antagonist, but this then eliminates a major source of excitatory drive needed for the spontaneous firing of GABAergic interneurons. Future recordings should investigate ways to record spontaneous IPSCs with an internal AMPAR channel blocker so recordings can be obtained at -60 mV. Regardless of these limitations, EU1180-465 shows a clear ability to potentiate GABAergic inhibitory tone onto excitatory principal cells.

After showing EU1180-465's ability to potentiate GABAergic interneuron output, we wanted to show that this increased spontaneous IPSC frequency was due to an increase in NMDAR-mediated excitatory drive onto GABAergic interneurons themselves. Patch clamp recordings from random CA1 *stratum radiatum* interneurons showed a significant potentiation of the NMDAR-mediated EPSC after EU1180-465 application. Here, charge transfer was used as the outcome

variable as it captures the effect of both peak amplitude and decay time as described previously. Careful evaluation of these data showed that roughly half of the selected *stratum radiatum* interneurons showed clear charge transfer potentiation, whereas the other half were relatively unresponsive. A 25% (1.25-fold) increase in charge transfer over baseline was used to stratify recordings from *stratum radiatum* interneurons into 'responders' and 'nonresponders'. Currently, this 25% threshold is arbitrary, and will likely require more replicates so a histogram of potentiation values can be generated. Then, an empirically derived critical value that clearly separates the data into two groups can be ascertained. The trend of some *stratum radiatum* interneurons being unresponsive to a GluN2C/GluN2D PAM was also seen with (+)-CIQ (Perszyk et al., 2016). Thus, these data suggest that perhaps not all *stratum radiatum* interneurons express functional GluN2D-containing synaptic NMDARs. This hypothesis is confounded by findings that application of NAB-14 (GluN2C/GluN2D NAM) produced significant inhibition of NMDAR-mediated EPSCs in all *stratum radiatum* interneurons recorded (Booker et al., 2021; Swanger et al., 2018). These discrepancies are not due to differential actions of GluN1 exon5 containing or lacking NMDARs, as both (+)-CIQ (Mullasseril et al., 2010) and NAB-14 (Swanger et al., 2018) are effective on the alterative splice variant of GluN1. Additionally, both (+)-CIQ (Yi et al., 2019) and NAB-14 (Swanger et al., 2018) are efficacious at potentiation GluN2D-containing triheteromeric receptors. This discrepancy could be due to opposing effects of GluN2D-potentiation on postsynaptic NMDARs combined with potentiation of GluN2D-containing presynaptic NMDARs and/or potentiation of GluN2C-containing astrocytic receptors. Alternatively, these differences could be due to off-target effects of (+)-CIQ/EU1180-465, although if this were true, it would likely impact all interneuron recordings. Perhaps then, due to protein-protein interactions or through some unexplained mechanism, some synaptic GluN2D-

containing NMDARs are capable of measurable potentiation, whereas all are capable of measurable inhibition. An alternative hypothesis is that levels of endogenous 24(S)-hydroxy cholesterol, and/or other endogenous PAMs may be present in CNS tissue and expressed at higher levels in certain cells, which then may occlude EU1180-465 potentiation. Either way, these data set up multiple intriguing hypotheses which can be explored to further our understanding of drug action on synaptic NMDARs.

### *5.5 Conclusions*

In conclusion, the data presented in this thesis highlight many gaps in mechanistic knowledge that need to be filled. This work further illustrates promising future directions for study as well as for potential translational efforts. Most of these suggested future directions include expanding the datasets on NMDAR function to include multiple different ages, multiple different brain regions, and multiple different cell types. This then begs the question: when is enough detailed molecular and cellular data sufficient to tackle a scientist's ultimate goal – curtailing human disease? Multiple labs could spend decades obtaining look-up style data repositories for functional data similar to the Allen Brain Institute's database on gene expression. This approach would undoubtedly generate new findings, contribute to the field, and stimulate new ideas and hypotheses. However, how do each of these molecular studies then act to guide new therapeutics and advances in the treatment of human disease? Perhaps the reductionist approach is misdirected. Instead, maybe complex diseases should be approached top-down as opposed to bottom-up. After an efficacious compound is found then molecular mechanism can be established. For example, the totality of molecular mechanisms involved in ketamine's therapeutic action on rapid and long-lasting treatment-resistant depression are still unknown despite an enormous amount of effort over

the last decade. More novel small molecule compounds have been discovered via high-throughput screening than through molecular modeling and medicinal chemistry *in silico* design. However, there is promise of precision with molecular approaches, but knowing when to stop data collection and how to implement these data into novel strategies for the treatment of disease is a difficult task and one that will not be accomplished by adopting a singular (top-down or bottom-up) tactic.

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